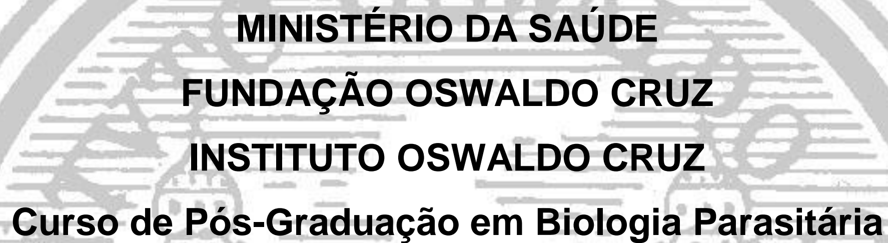

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FUNDAÇÃO OSWALDO CRUZ

INSTITUTO OSWALDO CRUZ

Curso de Pós-Graduação em Biologia Parasitária

O PAPEL DE MACRÓFAGOS EXSUDATIVOS E RESIDENTES DO FÍGADO (CÉLULAS DE KUPFFER) DURANTE A INFECÇÃO EXPERIMENTAL COM *LEISHMANIA (LEISHMANIA) INFANTUM*

Juliana Dias Costa Roque

Rio de Janeiro

Agosto de 2009

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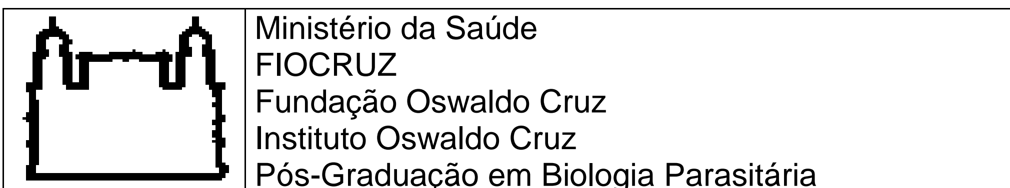

O papel de macrófagos exsudativos e residentes do fígado (células de Kupffer) durante a infecção experimental com *Leishmania (Leishmania) infantum*

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Dissertação apresentada ao Instituto Oswaldo Cruz como requisito para obtenção do título de Doutora em Ciências na área de Biologia Parasitária, e desenvolvida nos Laboratórios de Ultra-Estrutura Celular e de Pesquisa em leishmaniose (Instituto Oswaldo Cruz – FIOCRUZ).

SUPORTE FINANCEIRO: CNPq, FAPERJ e FIOCRUZ.

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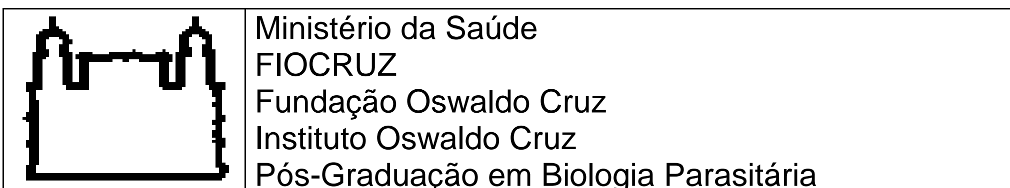
Rio de Janeiro, agosto/ 2009



Esta dissertação intitula-se:

"**O papel de macrófagos exsudativos e residentes do fígado (células de Kupffer) durante a infecção experimental com *Leishmania (Leishmania) infantum***"

apresentada por:

JULIANA DIAS COSTA ROQUE

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Local: Pavilhão Arthur Neiva **Data:** 05 de agosto de 2009 **Horário:** 14:00h

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"A alegria está na luta, na tentativa, no sofrimento envolvido. Não na vitória propriamente dita." (Mahatma Gandhi).

À DEUS, por me guiar em todos os momentos da minha existência me dando a oportunidade de novos aprendizados.

Ao meu marido André Luiz, por ser meu companheiro de todas as horas, minha cara-metade, meu melhor amigo, meu Liiiindo, meu grande e eterno amor, por ser minha VIDA. Ter você ao meu lado todos os dias me faz completamente feliz, te amo incondicionalmente! Obrigada por pensar o tempo todo em “Nós” e nunca em “Eu”.

Aos meus pais cariocas (Deise e José), meus pais paulistas (Nícia e Nivio), meus irmãos (Isabela, Vinicius e Felipe), meus avós (Clara e Durval), meus cunhados, sobrinhos, tios e primos, pela torcida e por me proporcionarem a base familiar. Amo demais vocês!

Ao meu orientador Renato Porrozzi, por ter me apoiado incessantemente em todos os momentos desta tese. Por ter confiado em mim para a realização desta, mesmo sabendo de todas as minhas escolhas. Sua participação e dedicação foram essenciais durante todo o desenvolvimento e desfecho desta tese.

A minha orientadora Maria de Nazareth Meirelles, por ter me inspirado a trabalhar com dedicação e responsabilidade, tendo me orientado nestes 10 anos de Fiocruz, desde a minha iniciação cientifica. Seu carinho maternal me confortou nos momentos mais difícies, obrigada por tudo!

A todos os meus amigos do ex-DUBC sem exceção (secretárias, corpo técnico, estudantes e pesquisadores), pelo convívio agradável em todas as ocasiões. Já sinto saudades!

Aos colegas do Laboratório de Pesquisa em leishmaniose, meus agradecimentos a todos que contribuíram de alguma forma para este momento.

Os meus queridos amigos da vida, Daniel, Kelly, Marialice e Tatiana, pela nossa amizade verdadeira que é mais forte que a distância. Obrigada pelo apoio e pelas nossas diversões dentro e fora da Fiocruz. Vocês fazem toda a diferença na minha vida!

As minhas amigas, Mariana, Elen e Luciana, pelo carinho e pelas sugestões nos trabalhos.

Ao amigo Bruno Avila, por sempre ser tão prestativo e ter tido o cuidado em ajeitar minhas imagens.

A Renata Soares e Carlos Eduardo, pelo suporte técnico na cultura de hepatócitos.

A amiga Luciane e ao Eduardo da pós-graduação e Biologia Parasitária, vocês são muito queridos e especiais. Obrigada por estarem sempre prontos a me ajudar.

A Dra. Alane Vermelho e Dra. Ana Melo (Depto de Microbiologia Médica – UFRJ), pela importante colaboração no 1º trabalho desta tese.

A Dra. Lea Cysne Finkelstein (Depto de Imunologia – IOC/FIOCRUZ), pela colaboração na padronização da amastigogênese.

A Dra Suzana Côrte-Real, pela colaboração nas fotos de microscopia eletrônica do 2º trabalho.

A Dra. Marília Seelander e seus alunos de doutorado Fábio e Daniela (Depto de Histologia/USP), meu agradecimento especial por tudo que fizeram por mim. Desde a maneira carinhosa com a qual me receberam em seu laboratório, ao auxilio na padronização da cultura de Kupffer murina.

A Dra. Marta Branquinha (Depto de Microbiologia Médica – UFRJ), por ter aceitado revisar a tese, e pelo cuidado e carinho que a fez.

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AGPI- Ácidos graxos poliinsaturados

APCs – Células apresentadoras de antígeno

cNOs - Enzima NO-sintase constitutiva

CPA- Cisteínas peptidases A

CPB – Cisteínas peptidases B

CPC – Cisteínas peptidases C

EM - Matriz extracelular

GIPLS- Glicosilinositolfosfolipídeos

GM-CSF – Fator de colonização de colônias

gp 63- Glicoproteína 63

GPI- glicosilfosfatidilinositol

IFN-γ - Interferon gama

IFN-β – Interferon beta

IgG- Imunoglobulina G

IgM- Imunoglobulina M

IL-2 – Interleucina 2

IL-3 – Interleucina 3

IL-4 – Interleucina 4

IL-5 – Interleucina 5

IL- 6 – Interleucina 6

IL-10 – Interleucina 10

IL-13 – Interleucina 13

iNOS - Enzima NO-sintase induzível

kDNA- Ácido desoxiribonucleico do cinetoplasto

KO - Camundongos "Knock-out"

L-NAME - N^G-nitro-L-arginina metil éster

L-NMMA - N^G-monometil-L-arginina

LPG- Lipofosfoglicanas

LTC- Leishmaniose Tegumentar Cutânea

LTCD- Leishmaniose Tegumentar Cutânea Difusa

LTM- Leishmaniose Tegumentar Mucosa

LV- Leishmaniose Visceral

MAFs- Fatores Ativadores de Macrófagos

MHC –complexo de histocompatibilidade

MMP- Metalopeptidase

MMP-9- Metalopeptidase 9

MSP - Principal peptidase de superfície

NFκB - Fator de transcrição nuclear do tipo κB

NOs - Enzima NO-sintase

OMS- Organização Mundial da Saúde

ON – Óxido nitrico

PCR- Reação de Polimerização em Cadeia

PKC- Proteína quinase C

PPGs- Proteofosfoglicanas

PSP - Peptidase de superfície de promastigotas

RMF- Receptor manose-fucose

RNS- Espécies reativas de nitrogênio

ROS- Espécies reativas de oxigênio

SMF- Sistema Mononuclear Fagocítico

TGF-β - Fator de crescimento tumoral beta

TGO- Aspartato aminotransferase

TGP- Alanina aminotransferase

TIMPs- Inibidores teciduais de metalopeptidases de matriz

TLR-2 – Receptor Toll like 2

TNF-α - Fator de necrose tumoral alfa

No fígado, a atividade microbicida dos macrófagos infectados por *Leishmania (Leishmania) infantum*, com a finalidade de eliminar os parasitos intracelulares, causa uma citotoxicidade extracelular nos hepatócitos. Esta citotoxicidade gera importantes alterações morfológicas e funcionais nas células parenquimais hepáticas. A produção destes mediadores ocorre por duas vias principais, gerando uma grande quantidade de mediadores no meio extracelular. A via independente de oxigênio é caracterizada pela secreção de peptidases. As três classes de peptidases apresentam suas atividades proteolíticas aumentadas no momento de maior atividade microbicida pelos macrófagos, indicando a contribuição destas peptidases ao fenomêno, particularmente as metalopeptidases. A forma ativa da metalopeptidase 9 (85kDa) foi detectada no momento de maior atividade pelos macrófagos, mostrando que ela pode contribuir para os desarranjos na matriz extracelular importante para a homeostase das células em cultura. Nenhuma atividade proteolítica das amastigotas foi relacionada com o fenômeno. Amastigotas axênicas foram obtidas a partir de uma metodologia que permite a purificação de 100% de formas puras em um curto período em cultura (4 dias). Estas amastigotas apresentaram características morfológicas e bioquímicas idênticas quando comparadas com as amastigotas intracelulares obtidas de animais experimentais. Elas apresentaram o mesmo perfil de infecção nos macrófagos, o que mimetiza as condições de infecção *in vivo*. A via dependente de oxigênio se caracteriza pela liberação de espécies reativas de nitrogênio (RNS) e oxigênio (ROS). Células de Kupffer murinas foram estabelecidas neste trabalho e constatamos que estas produziram quantidades menores de RNS em comparação aos macrófagos peritoneais durante a infecção por amastigotas de *Leishmania infantum.* Isso deve estar correlacionado com o perfil de citocinas gerado nos macrófagos residentes do fígado infectados, com grandes quantidades de IL-10 e níveis significantemente mais baixos de TNF-α em comparação aos macrófagos peritoneais. A infecção por amastigotas de *Leishmania infantum* não estimulou a produção de ROS nas co-culturas formadas de Kupffer/hepatócitos e consequentemente, as transaminases hepáticas não se apresentaram aumentadas, indicando que danos extracelulares não ocorreram. Portanto, os danos nos hepatócitos só surgiram na presença de ROS e RNS secretados pelos macrófagos infectados, pois levaram a formação de peroxinitrito. O tratamento com ácido úrico, varredor de peroxinitrito, mostrou que esta molécula é a grande responsável pela citotoxicidade extracelular encontrada nos hepatócitos. Sendo assim, os macrófagos exsudativos que chegam ao fígado apresentam suas vias microbicidas funcionantes, sendo os responsáveis pela eliminação dos parasitos mas também responsáveis pelos danos hepáticos, enquanto as células de Kupffer devem estar mais relacionadas com a apresentação antigênica e modulação da resposta imune adaptativa.

In the liver, the microbicidal activity of macrophages infected with *Leishmania (Leishmania) infantum*, in order to eliminate the intracellular parasites, causes an extracellular cytotoxicity in hepatocytes. This cytotoxicity generates significant morphological and functional changes in liver parenchymal cells. The production of these mediators occurs in two major pathways, generating a large amount of mediators in the extracellular medium. The oxygen-independent pathway is characterized by the secretion of peptidases. The three classes of peptidases, displayed increased proteolytic activities at the highest microbicidal activity by macrophages indicating the contribution of the peptidases on the phenomenon, particularly metalopeptidases. The active form of metalopeptidase 9 (85kDa) was detected at the time of highest activity by macrophages, showing that it can contribute to the disarrangement in the extracellular matrix important for the homeostasis of cells in culture. No proteolytic activity from amastigotes was correlated to the phenomenon. Axenic amastigotes were obtained from a methodology that allows the purification of 100% of pure forms in a short period in culture (4 days). These amastigotes showed similar morphological and biochemical characteristics, when compared with the intracellular amastigotes obtained from experimental animals. They showed the same profile of infection in macrophages, which mimics the conditions of infection *in vivo*. The oxygen-dependent pathway is characterized by release of reactive nitrogen species (RNS) and oxygen (ROS). Murine Kupffer cells were established in this work. They produced smaller amounts of RNS compared to peritoneal macrophages during infection with amastigotes of *Leishmania infantum*. This should be correlated to the profile of cytokines generated in the infected liver resident macrophages, with large amounts of IL-10 and lower levels of TNF-α compared to peritoneal macrophages. The infection with amastigotes of *Leishmania infantum* did not stimulate the production of ROS formed in co-cultures of Kupffer / hepatocytes and consequently, the liver transaminases are not presented increased, indicating that extracellular damages have not occurred. Therefore, the damage only occurred in hepatocytes in the presence of ROS and RNS secreted by infected macrophages, as which led to the formation of peroxynitrite. Treatment with uric acid, a scanveger of peroxynitrite, showed that this molecule is mainly responsible for extracellular cytotoxicity found in hepatocytes. Thus, the exudative macrophages that comes to the liver present functional microbicide functions, being responsible for removal of parasites but also responsible for liver damage, while Kupffer cells should be more related to antigen presentation and modulation of adaptive immune response.

1. Introdução

1.1. O gênero *Leishmania*

O gênero *Leishmania* (Ross, 1903) agrupa espécies de protozoários digenéticos que causam a doença conhecida como leishmaniose. São parasitos pertencentes à ordem *Kinetoplastida* e à família Trypanosomatidae (Lainson & Shaw, 1987). Todas as espécies de *Leishmania* são transmitidas por flebotomíneos (Diptera: Psychodidae) dos gêneros: *Lutzomyia*, vetores das leishmanioses no Novo Mundo, e *Phlebotomus*, correspondendo aos transmissores da doença no Velho Mundo (Lainson & Shaw, 1987; Grimaldi *et al*., 1991; OMS, 2009).

A leishmaniose é uma zoonose endêmica em 88 países do mundo, dos quais 72 são países em desenvolvimento, e entre eles, 13 aparecem na lista dos menos desenvolvidos (Sundar *et al*., 2003; revisto por Desjeux, 2004). Esta doença está distribuída principalmente nas regiões tropicais e subtropicais da América do Sul, América Central, África, Ásia e Mediterâneo, afetando cerca de 12 milhões de pessoas em todo o mundo, com 2 milhões de novos casos por ano e 350 milhões de pessoas sob risco de infecção (OMS, 2009). A leishmaniose pode se manifestar de forma relativamente branda (provocando lesões auto-resolutivas na pele), ou grave (atingindo mucosas e vísceras). A forma visceral, de acordo com o Ministério da Saúde (2007), resulta em óbito em aproximadamente 90% dos casos, se não tratado.

O Brasil é o único país no mundo que tem grande prevalência das três formas clínicas de leishmaniose, registrando mais de 30 mil casos/ano de leishmaniose cutânea, a forma mais prevalente no país (Ministério da Saúde, 2007). As leishmanioses no Brasil são primariamente uma zoonose que afeta diferentes espécies de vertebrados. Sua transmissão, inicialmente silvestre ou concentrada em pequenas localidades rurais, vem ocorrendo em centros urbanos de médio e grande porte devido a diversos fatores como o acelerado avanço das fronteiras agrícolas e da urbanização de áreas florestais, culminando na diminuição dos nichos, e adaptação de vetores e mamíferos sinantrópicos, roedores e marsupiais, ao ambiente domiciliar ou peridomiciliar. Há, desta forma, a inclusão de novos hospedeiros no atual ciclo do parasito, sendo assim uma endemia em franca expansão geográfica (Ministério da Saúde, 2007; Maia-Elkhoury *et al*., 2008).

1.2. Ciclo biológico das leishmânias

As leishmanioses são causadas por protozoários flagelados que são parasitos intracelulares obrigatórios e heteroxênicos, necessitando de pelo menos dois hospedeiros para completar seu ciclo biológico. Os hospedeiros vertebrados são mamíferos e os hospedeiros invertebrados são

insetos hematófagos (Lainson & Shaw, 1987; Grimaldi *et al.*, 1991). A *Leishmania* é também um parasito dimórfico, ou seja, apresenta duas formas evolutivas durante seu ciclo biológico: promastigotas e amastigotas (revisto por Sacks & Kamhawi, 2001).

Os hospedeiros vertebrados são infectados com formas promastigotas infectantes (metacíclicas) inoculadas pela fêmea do inseto vetor durante um repasto sanguíneo. Quando as promastigotas são introduzidas na pele, encontram neste local algumas células do sistema imune (linfócitos T e B, macrófagos residentes, células de Langerhans e mastócitos), que formam um compartimento bastante específico denominado sistema imune da pele. Por um mecanismo ainda não totalmente esclarecido, envolvendo receptores e ligantes em ambas as superfícies, o parasito se adere à superfície dos macrófagos e células de Langerhans passando para o meio intracelular por meio de um processo de fagocitose mediada por receptores, em que se transforma na forma amastigota, característica do parasitismo nos mamíferos.

Aqueles que se localizam dentro das células de Langerhans são levados aos linfonodos de drenagem, que ao se infectarem sofrem modificações que possibilitam sua migração. No interior das células de drenagem, as partículas antigênicas do parasito serão apresentadas às células do sistema imune. Estas, uma vez estimuladas, se dirigem ao sítio da infecção, auxiliando na formação do processo inflamatório.

Se os parasitos forem endocitados pelos macrófagos, durante este processo de endocitose do parasito, estas células hospedeiras aumentam intensamente sua atividade respiratória liberando produtos como os radicais livres de óxidos, hidroxilas, hidróxidos e superóxidos(O^-, OH^-, H+ e H_2O_2), que são conhecidos por serem altamente lesivos para as membranas celulares. Os parasitos internalizados ficam dentro de um vacúolo parasitóforo (fagolisossoma), que os separa do citoplasma celular. Embora os macrófagos sejam células fagocitárias especializadas no combate a agentes infecciosos, as leishmânias desenvolvem mecanismos de defesa capazes de subverter sua capacidade microbicida, conseguindo sobreviver neste ambiente potencialmente tóxico e multiplicar-se em amastigotas até a ruptura da célula, quando são liberadas para o meio extracelular para infectar outros macrófagos, propagando a infecção. No interstício tecidual (meio extracelular), as amastigotas serão fagocitadas ou não por outros macrófagos. As que não forem internalizadas por outros macrófagos serão destruídas no meio extracelular pela resposta inata. É provavelmente neste momento que características como intensidade e qualidade da resposta imune são definidas, influenciando assim a evolução da doença para cura espontânea, formas autolimitadas ou formas progressivas. A localização das amastigotas no interior de macrófagos faz com que o controle da infecção seja dependente da resposta imune mediada por células.

A infecção para o hospedeiro invertebrado ocorre quando os insetos vetores ingerem macrófagos parasitados de um hospedeiro vertebrado infectado durante seu repasto sanguíneo. No intestino médio do vetor, os macrófagos se rompem liberando as formas amastigotas intracelulares que imediatamente iniciam o processo de transformação para promastigotas que, em seguida, vão se diferenciar em formas promastigotas metacíclicas. Estas formas infectantes serão transmitidas ao hospedeiro vertebrado no momento de um novo repasto sanguíneo do vetor, dando continuidade ao ciclo biológico do parasito (revisto por Chappuis *et al*., 2007; Ministério da Saúde, 2007) (Figura 1.1).

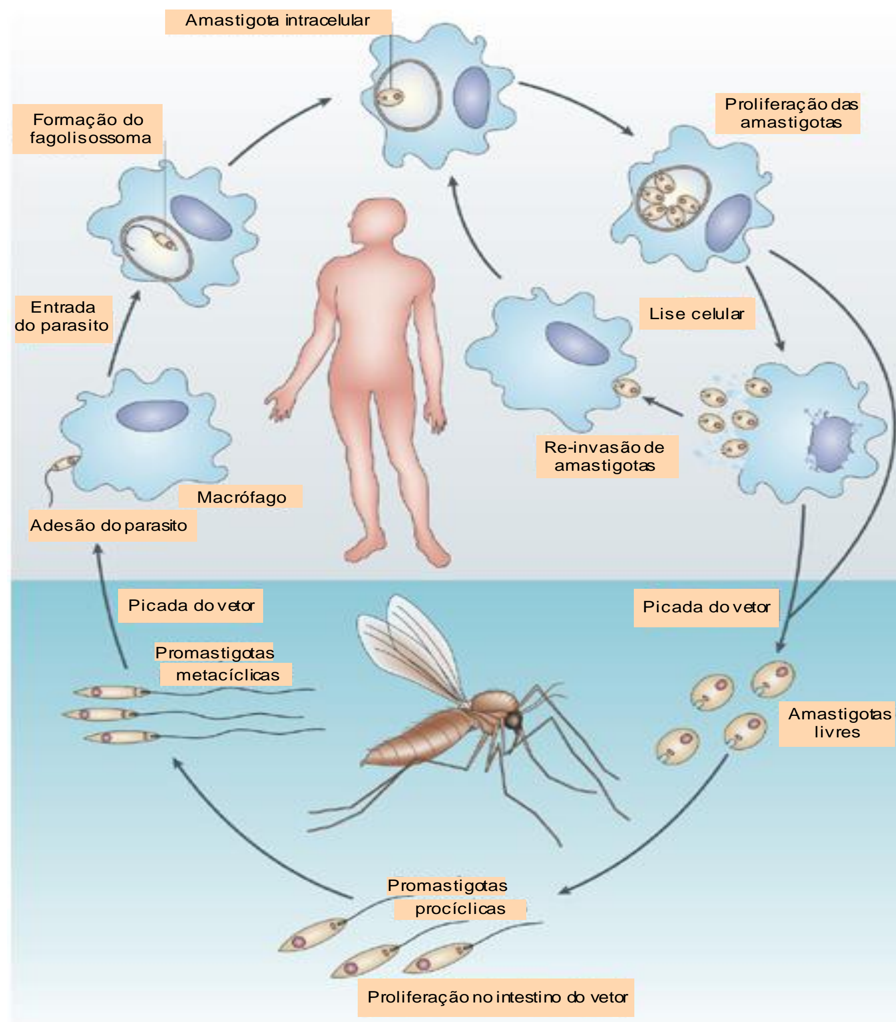

Figura 1.1. Ciclo biológico das leishmânias (adaptado de Chappuis *et al*., 2007)

1.3. A doença leishmaniose e suas formas clínicas

A patologia das leishmanioses se manifesta como um espectro de formas clínicas variando de lesões auto-resolutivas ou persistentes que acometem seja isolada ou comcomitantemente a pele e mucosas do nariz, boca, faringe e laringe, e a forma visceral, células do sistema fagocítico mononuclear (SFM), principalmente do baço e fígado. As leishmanioses podem ser divididas em quatro formas clínicas distintas: tegumentar cutânea (LTC), tegumentar mucosa (LTM), tegumentar cutânea difusa (LTCD) e visceral (LV) (Grimaldi *et al.,* 1991).

A leishmaniose tegumentar abrange diferentes formas clínicas (Pearson *et al*., 1996). A forma cutânea produz exclusivamente lesões cutâneas, ulcerosas ou não, porém limitadas, evoluindo geralmente para a cura espontânea (Costa *et al*., 1987). Esta forma se inicia geralmente como uma pápula no local da picada do inseto, progredindo depois para uma úlcera cutânea arrendondada ou ovóide com bordas bem delimitadas e eritrematosas, e fundo granuloso de fácil hemorragia. A lesão geralmente é única e localizada em partes descobertas do corpo, no local onde o parasito foi inoculado pelo inseto vetor (Marsden & Nonato, 1975).

A forma cutâneo-mucosa é secundária e metastática, e cerca de 3 a 5% dos casos de leishmaniose cutânea progridem para esta forma (Marsden *et al*., 1986; Jones *et al*., 1987). O aparecimento de lesões destrutivas nas mucosas do nariz, boca e faringe pode ou não estar associada a lesões cutâneas. A mucosa nasal, isoladamente ou associada a outras áreas, constitui a localização mais comum. Esta forma geralmente não tende à resolução espontânea e é mais resistente à terapêutica antimonial (Maresden *et al*., 1986; Oliveira-Neto *et al*., 2000). A progressão sem controle desta doença causa comprometimento das partes cartilaginosas da face com mutilações graves, podendo ainda levar a complicações como pneumonia aspirativa, sepsis e sufocamento (Cunha *et al*., 1992; Nonata *et al*., 1997).

A forma cutâneo-difusa é a forma anérgica da doença, que se caracteriza por lesões cutâneas múltiplas, não ulceradas, ricas em parasitos, estendendo-se por grande área da superfície corporal e acompanhada de severa imunodepressão celular a antígenos do parasito.

A forma visceral é caracterizada pela forma sistêmica da doença (também conhecida como calazar), em que os parasitos apresentam acentuado tropismo por células do SFM do baço, fígado, medula óssea e tecidos linfóides.

1.4. Leishmaniose Visceral

Os principais agentes etiológicos da LV são três espécies classificadas no "complexo donovani": *L. (L.) donovani*, tendo como distribuição geográfica: a Índia, a África e a China (Hoogstrall & Heyneman, 1969; Naik *et al*., 1979; Shiddo *et al*., 1995); *L.(L.) infantum*, que ocorre no Mediterrâneo, Àsia, Oriente e Novo Mundo, onde também é conhecida como *L.(L.)*

chagasi (Pampiglione *et al*., 1976; Hashemi-Nasab & Zadeh-Shirazi, 1980; Grimaldi *et al*., 1989; Mauricio *et al*., 2000); *L.(L.) archibaldi*, que ocorre somente na África (Zijlstra e El-Hassan, 2001).

A espécie de vetor envolvida na transmissão da LV nas Américas é a *Lutzomyia longipalpis* (Davidson, 1999; Herwaldt, 1999). As fêmeas são hematófagas obrigatórias, pois necessitam de sangue para o desenvolvimento dos ovos, tendo hábito alimentar noturno que se inicia cerca de 1 hora após o crepúsculo. O período de incubação varia de 10 dias a 24 meses, sendo em média de 2 a 4 meses, passando pelas fases de ovos, larvas, pupas e adultos . Em ambientes domiciliares, o vetor cria-se em abrigos de animais, em áreas sombreadas com acúmulo de matéria orgânica e decomposição (Sabroza, 1981), principalmente folhas, frutos, raízes e fezes de animais. Mamíferos silvestres (ratos silvestres, bicho preguiça, tamanduá, tatu, raposa, gambá e outros roedores) e animais domésticos (cães, ratos domésticos e equinos) são reservatórios do parasito (Maia-Elkhoury *et al.,* 2008).

A infecção por leishmânias viscerotrópicas pode resultar em diferentes tipos de infecção e manisfestações clínicas, em função da resposta do organismo infectado (Davdison, 1999; revisto por Murray *et al.,* 2005). Entre os fatores de risco associados com o desenvolvimento do calazar está a desnutrição, sendo o risco relativo nove vezes maiores em crianças desnutridas em relação àquelas bem nutridas (Ministério da Saúde, 2006).

A associação entre LV e Aids é recente e apresenta um número crescente de casos no Brasil e no mundo – sobretudo na região mediterrânea da Europa, que compreende Espanha, França, Itália e Portugal. Atualmente, no Brasil, os especialistas observam um fenômeno de sobreposição das infecções, caracterizados pela ruralização da Aids e pela urbanização das leishmanioses, que indica a emergência da doença parasitária como uma importante infecção oportunista ao HIV. O impacto epidemiológico da co-infecção é tão significativo que a OMS cogita introduzir a leishmaniose visceral como doença indicadora da Aids (Rabello *et al*., 2003; Maia-Elkhoury *et al*., 2008).

1.4.1. Sintomatologia e Patologia da LV

As infecções produzidas no homem por leishmânias viscerotrópicas do "complexo donovani" se manifestam clinicamente de maneira polimórfica (Guerin *et al*., 2002). Os indivíduos infectados com estas leishmânias geralmente não desenvolvem lesão cutânea típica no local do inóculo. Após a picada dos insetos vetores, pode haver uma reação local com formação de uma pequena lesão nódulo-papular. A infecção pode ser resolvida ou os

macrófagos infectados podem se disseminar por via sanguínea comprometendo assim, outros tecidos componentes do SFM (Desjeux, 1996).

As formas clínicas da LV são classificadas em infecções latentes assintomáticas ou oligossintomáticas, formas agudas e formas crônicas. As populações que vivem em áreas endêmicas desenvolvem geralmente a forma assintomática (Evans *et al.*, 1995; Shiddo *et al.*, 1995). Na forma oligossintomática, os pacientes apresentam alterações clínicas discretas como linfoadenomegalia ou hepatomegalia (Pampiglione *et al.*, 1976; Desjeux, 1996).

Os pacientes com formas latentes podem evoluir ou não, após um longo período de infecção, para formas agudas da doença, desenvolvendo sintomatologia de calazar clássico, ou ao contrário, apresentar melhoras do quadro clínico mesmo sem o tratamento específico (Badaró *et al.*,1986; Evans *et al.*, 1995).

As características clínicas do calazar clássico são marcantes, mesmo incidindo em diferentes regiões geográficas. Na fase aguda, têm-se formas desintéricas de evolução rápida e fatal em prazos curtos entre 20 e 40 dias e formas subagudas com evolução mais lenta, entre 5 e 12 meses, terminando pela morte por caquexia. A sintomatologia da fase aguda se caracteriza por febre alta e contínua, diarréia, anemia acentuada e hepatoesplenomegalia, podendo também ocorrer linfoadenopatias (Most & Laviestes, 1947). O parasitismo em geral é intenso no fígado e baço, sendo característica marcante o elevado nível de anticorpos IgM e IgG específicos (Kean *et al.*, 1978). Em algumas áreas endêmicas, as alterações hepáticas são mais frequentes que as esplênicas (Most & Laviestes, 1947; Prata, 1957; Alencar *et al.*,1975, Osman *et al.*, 2000). Na fase crônica, a evolução é lenta, podendo durar anos se não tratada e muitas vezes ocorrem recaídas alternando-se por períodos de semanas ou meses. A doença tende a ser mais grave em crianças apresentando um início insidioso (Prata, 1957; Alencar *et al.*, 1975, revisto por Murray *et al.*, 2005).

O quadro histopatológico é variado dependendo do estágio evolutivo da doença (Ridley, 1987; Gama *et al.*, 2004). As alterações histopatólogicas da LV mais importantes ocorrem principalmente no fígado e no baço, podendo ocorrer também no pulmão (pneumonite intersticial) e nos rins com nefrite intersticial ou glomerulonefrite (Andrade & Yaibuki, 1972; Brito *et al.*, 1975; Duarte & Cobertt, 1987). As mudanças morfológicas no fígado infectado por *L. (L.) donovani* envolvem hepatócitos (Costa *et al.*, 2007), células de Kupffer (macrófagos residentes), células de Ito, trato portal, sinusóides e veias hepáticas (El Hag *et al.*,1994).

1.4.2. Diagnóstico da LV

O diagnóstico da LV humana inclui características clínico-epidemiológicas e exames laboratoriais, uma vez que é necessário diferenciar esta doença de outras que apresentam características comuns como febre prolongada e hepatoesplenomegalia. As alterações hematológicas e bioquímicas incluem anemia, leucopenia acentuada, níveis elevados de transaminases hepáticas (alanina aminotransferase - TGP e aspartato aminotransferase - TGO), além de alterações nas proteínas plasmáticas, como na relação albumina:globulina (Veronesi, 1955). O diagnóstico definitivo da LV ainda é baseado na observação de parasitos nos tecidos (sangue, medula óssea, baço, fígado e linfonodos) empregando-se uma ou mais metodologias de pesquisa (Deane & Grimaldi, 1985), como esfregaços corados com Giemsa, histopatologia, isolamento em meios de cultura e detecção de DNA destes parasitos pela reação de polimerização em cadeia ou PCR (revisto por Chappuis *et al*., 2007).

Métodos menos invasivos de diagnóstico têm sido utilizados, destacando-se ensaios sorológicos como a técnica de ELISA, a imunofluorescência indireta, a reação de aglutinação ou de fixação de complemento que detectam anticorpos circulantes anti - *Leishmania* / antígenos recombinantes específicos como o rK26 e rK39 em pacientes infectados (Badaro *et al*., 1996; Bhatia *et al.*, 1999; Veeken *et al.*, 2003; Boelaert *et al*., 2008). Outros métodos imunológicos se baseiam em testar a imunidade celular dos indivíduos através da reação de hipersensibilidade retardada e de respostas linfoproliferativas, sendo úteis na avaliação do prognóstico da doença. Testes intradérmicos à leishmanina (reação de Montenegro) ou aos antígenos purificados são negativos em doentes com calazar, tornando-se positivos após a cura (Reed *et al*., 1986; Grimaldi & Tesh, 1993; Baleeiro *et al*., 2006), o mesmo sendo observado nas respostas *in vitro* como proliferação linfoblástica e na produção de citocinas (Boelaert *et al.*, 2004).

1.4.3. Profilaxia e Tratamento da LV

Os compostos antimoniais pentavalentes constituem as drogas de primeira escolha no tratamento da LV, mesmo apresentando toxicidade e efeitos colaterais Os pacientes com LV infectados com distintas leishmânias do “complexo donovani” respondem ao tratamento específico de forma similar e estes compostos são geralmente eficientes (revisto por Murray *et al.,* 2005). A taxa de cura com antimoniais é de aproximadamente 90%, mesmo assim muitos pacientes vêm sendo tratados com anfoterina B devido a sua grande eficácia e maior rapidez, reduzindo o tempo longo nos hospitais (revisto por Murray *et al.*, 2005).

Medidas convencionais como eliminação de vetores e reservatórios são importantes no controle das leishmanioses, porém são difíceis de serem realizadas devido às distintas

situações epidemiológicas observadas (Grimaldi, 1995; Modabber, 1995; revisto por Chappuis *et al.*, 2007).

1.5. Leishmaniose visceral experimental

Modelos experimentais *in vivo* e *in vitro* têm sido utilizados em estudos da interação *Leishmania* - hospedeiro vertebrado. Uma variedade de mamíferos é susceptível às leishmânias (Gramiccia & Gradoni, 2005). No caso de infecção experimental com leishmânias viscerotrópicas, alguns animais de laboratório como camundongos e hamsters, além do cão, reproduzem a patologia básica da LV (Preston, 1987; revisto por Pereira & Alves, 2008).

O sucesso da infecção experimental por *Leishmania* em modelos murinos depende de uma variedade de fatores como a via de inoculação, tamanho do inóculo, forma do parasito (promastigotas ou amastigotas) e linhagem do camundongo (revisto por Pereira *et al.,* 2008). Estudos mostraram que algumas linhagens de camundongos são resistentes à infecção por *Leishmania donovani*, como C3H e C57Bl/6, enquanto outras como B10.D2, C57BL/10 e BALB/c são susceptíveis a esta infecção (Nickol & Bonventre, 1985; Blackwell & Plant, 1986; Wilson *et al*., 1996). Os camundongos BALB/c se apresentam inicialmente susceptíveis à infecção por *L. (L.) donovani* com carga parasitária variável e geralmente elevada no fígado e baço (Murray *et al*., 1982; Wilson *et al*., 1996; Melby *et al*., 2001). Depois de algumas semanas, eles podem controlar a carga parasitária nestes órgãos, mantendo uma infecção crônica (Murray *et al*., 1982; Blackwell *et al*., 1985; Wilson *et al*., 1996).

Os hamsters são susceptíveis às leishmânias viscerotrópicas, sendo a infecção em geral fatal (Preston, 1987; Melby *et al*., 2001). Estes animais infectados com *L. (L.) donovani* apresentam patologia semelhante à ocorrida em humanos com calazar (Duarte & Cobert, 1987; Sartori *et al*., 1991). Além disso, esta infecção reproduz dois eventos observados na LV: a supressão da resposta Th1 específica na fase ativa da infecção (Bunn-Moreno *et al*., 1985; Nickol & Bonventre, 1985) e a ativação policlonal dos linfócitos B (Campos-Neto & Bunn-Moreno, 1982; Bunn-Moreno *et al*., 1985). No fígado dos hamsters, há hiperplasia e parasitismo de células de Kupffer, assim como granulomas com macrófagos parasitados nos espaços portais (Duarte & Cobert, 1987).

O cão é um excelente modelo de estudo para o entendimento da LV, uma vez que é o principal reservatório de *L.* (*L.*) *infantum* O maior problema na utilização de cães como modelos experimentais é a falta de marcadores imunológicos e de reagente específicos importantes para determinar a base da resposta imunológica protetora destes animais (Moreno & Alvar, 2002). Já os símios representam o melhor sistema para modelar as doenças humanas,

uma vez que são os seres filogeneticamente mais próximos do homem (Kennedy *et al*., 1997). No entanto, fatores como a natureza do parasito e a dose do inóculo nestes animais influenciam o curso da doença. No caso de infecções experimentais com leishmânias viscerotrópicas em símios há manifestação clínica polimórfica desta doença (Porrozzi et al., 2006; revisto por Grimaldi, 2008).

1.6. Interação *Leishmania*-Célula Hospedeira

1.6.1. O parasito *Leishmania*

Os momentos iniciais da infecção por protozoários do gênero *Leishmania* são cruciais para determinar a evolução da doença. A interação vetor-hospedeiro, a atividade dos componentes da saliva do inseto vetor, a biologia do parasito e a resposta inicial do hospedeiro estão envolvidas nestes primeiros momentos (revisto por Almeida *et al*., 2003). Em relação à biologia do parasito, a *Leishmania* tem duas formas distintas com características próprias: promastigotas e amastigotas.

As formas promastigotas são formas alongadas, fusiformes, tendo em média 14 a 20 μm de comprimento e apresentam como características: um cinetoplasto em forma de barra anterior ao núcleo e um flagelo longo e livre que emerge da bolsa flagelar a partir da porção anterior do corpo (revisto por Vannier-Santos *et al*., 2002). As promastigotas se reproduzem por divisão binária e em cultivos axênicos, elas sofrem um desenvolvimento seqüencial até a forma infectante em várias espécies de *Leishmania* (Bates, 1993). As populações de promastigotas infectantes (metacíclicas) são diferentes das não-infectantes por um processo que foi denominado metaciclogênese por Sacks (1989). Alterações bioquímicas e antigênicas são observadas com o desenvolvimento de promastigotas infectantes (Sacks *et al*., 1995). As promastigotas metaciclícas possuem os diâmetros do corpo menores do que os apresentados pelas promastigotas em geral, e o flagelo muito longo, cerca de duas vezes o comprimento do corpo, apresenta mobilidade intensa. Estruturalmente, por microscopia eletrônica, as formas flageladas se diferenciam da forma amastigota essencialmente pelo prolongamento de flagelo, que se exterioriza para além da bolsa flagelar. Estruturas de membrana, núcleo, cinetoplasto e organelas citoplasmáticas, como veremos a seguir, são semelhantes (Sacks *et al*., 1995).

As amastigotas são formas intracelulares obrigatórias que, quando fixadas e coradas pelos métodos derivados do Romanovsky (como Giemsa e Leishman), aparecem à microscopia óptica como organismos ovais ou esféricos. No citoplasma, corado em azul-claro, são encontrados: um grande e arredondado núcleo (ocupando quase um terço do corpo do parasito) e o cinetoplasto em forma de um pequeno bastonete, ambos corados em vermelho púrpura. Vacúolos podem ou não ser visualizados. Não há flagelo livre, e a sua porção

intracitoplasmática raramente é visível; os limites micrométricos de seus diâmetros são de aproximadamente 1,5 a 3,0 x 3,0 a 6,5 µm (McConville & Blackwell, 1991).

Amastigotas examinadas ao microscópio eletrônico de transmissão apresentam microtúbulos em número variável sobre a membrana plasmática, dispostos em conformação regular e equidistantes. Nas diferentes espécies de *Leishmania*, a membrana apresenta uma invaginação na região anterior do corpo do parasito formando a bolsa flagelar, onde se localiza o flagelo. Neste local não são encontrados microtúbulos subpeculiares e as atividades excretora e de pinocitose são intensas (Killick-Kendrick, 1979). O flagelo não aparente de amastigotas apresenta microestrutura formada de nove pares de microtúbulos concêntricos e um par central, envolvido por uma matriz citoplasmática. O cinetoplasto se mostra como uma estrutura mitocondrial ligado à única mitocôndria existente na célula. No seu interior encontram-se estruturas filamentosas circulares formadas por ácido desoxiribonuclêico (kDNA). O blefaroplasto ou corpúsculo basal aparece como a continuação do flagelo. O núcleo possui configuração variada, tendendo a esférico, ora denso, ora mais frouxo, mostrando um cariossomo central ou excêntrico e a cromatina com disposição variável. São observados na matriz citoplasmática o complexo de Golgi e o retículo endoplasmático, além de vacúolos e inclusões.

A multiplicação de amastigotas ocorre por divisão binária simples, e é iniciada pela duplicação do cinetoplasto, um dos quais mantém o flagelo remanescente, enquanto o outro promove a reprodução da estrutura flagelar. A seguir, o núcleo se divide e o corpo do parasito se fende, no sentido ântero-posterior. Após sucessivas multiplicações, na ausência do controle parasitário pela célula hospedeira, esta se rompe e as amastigotas liberadas são fagocitadas por outros macrófagos (Naderer *et al*., 2004; revisto por Awasthi *et al*., 2004). O progresso nas técnicas de cultivo permitiu o crescimento de amastigotas em meio axênico. Um aumento na temperatura e um decréscimo no pH do meio de cultura mimetizam as condições encontradas no fagolisosoma onde promastigotas se transformam em amastigotas (Zilberstein & Shapira, 1994). Estas formas são conhecidas como amastigotas axênicas e apresentam características similares às amastigotas intracelulares.

Em geral, os parasitos do gênero *Leishmania* apresentam um denso glicocálix composto por várias moléculas que são presas à superfície celular através de uma âncora de glicosilfosfatidilinositol (GPI). Dentre elas estão (i) as lipofosfoglicanas (LPG) (Lang et al., 1991), (ii) as proteofosfoglicanas (PPGs) (revisto por Ilg et al., 1999), (iii) as glicoproteínas 63 (gp63) (Chaudhuri et al., 1989), e (iv) os glicosilinositolfosfolipídeos (GIPLS) (Orlandi & Turco, 1987), sendo a gp63 e a LPG as moléculas mais abundantes. Vários estudos sobre a função da LPG e gp63 revelaram que estas moléculas podem desempenhar diferentes papéis,

compreendendo desde a etapa inicial de adesão à célula hospedeira até a sobrevivência da *Leishmania* no interior dos fagolisossomos (Handman *et al*., 1986; McNeely & Turco, 1990; Brittingham *et al*., 1999, Dominguez & Toraño, 2001; Naderer *et al*., 2004). Durante os estágios iniciais da infecção a sobrevivência é garantida pelas moléculas de LPG, através da inibição da fusão fago-lisossomal (Desjardins & Descouteaux, 1997), e nos estágios mais tardios pela gp63, inibindo a ação das enzimas fagolisosomais por degradação proteolítica (Sorensen *et al*., 1994; Chaudhuri & Chang, 1988; McGwire *et al*., 2002).

A LPG é o principal glicoconjugado presente na superfície de parasitos do gênero *Leishmania* (revisto por IIg, 2001). As formas promastigotas expressam uma grande quantidade de LPG em sua superfície, estando distribuídas por toda a superfície do parasito, incluindo flagelo. Entretanto, as formas amastigotas expressam um número significativamente menor (Turco, 1984). Além da resistência ao complemento, a LPG apresenta outras funções como: participação na invasão de formas promastigotas metacíclicas – e mesmo amastigotas de *L. donovani* (Handman & Bullen, 2002) – em células hospedeiras de vertebrados via diferentes receptores. Dentre eles podem ser citados receptores como CR1 e CR3 para moléculas do complemento (Mosser & Edelson, 1987), o receptor para manose-fucose (RMF) (Hespanhol *et al*., 2005), receptores para produtos avançados de glicosilação (revisto por Vannier-Santos *et al*., 2002) e o receptor Toll Like 2 (TLR)-2 (de Veer *et al*., 2003). Além destas, outra função seria a sobrevivência de promastigotas no interior dos fagolisossomas (no hospedeiro vertebrado) pela inibição transitória da fusão fagolissomal até a sua diferenciação em amastigotas (Handman *et al*., 1986; McNeely & Turco, 1990), inibindo a expressão da iNOS com conseqüente redução da produção de óxido nítrico (ON) (Proudfoot *et al*., 1996).

A glicoproteína 63 (gp63) é uma metaloproteinase ancorada via GPI em formas promastigotas. Em amastigotas, a gp63 esta localizada majoritariamente na bolsa flagelar, diferente das promastigotas onde a localização é homogênea em toda a superfície celular (Medina-Acosta *et al*., 1989). A gp 63, assim como a LPG, apresenta um importante papel na invasão de células hospedeiras por formas promastigotas metacíclicas, utilizando diferentes receptores como CR1 e CR3 (Russell & Wright, 1988), RMF (Wilson & Pearson, 1986; Palatink-de-Souza *et al*., 1993), receptor para fibronetina (Rizvi *et al*., 1988), receptores para produtos avançados de glicosilação (Mosser & Edelson, 1987) e para β1 integrina (Brittinghan *et al*., 1999). A gp63 também participa da sobrevivência intracelular do parasito através da neutralização de enzimas lisossomais (Chauduri & Chang, 1988; Chaudhuri *et al*., 1989; McGwire & Chang, 1994; Sorensen *et al*., 1994).

As leishmânias apresentam moléculas de defesa anti-oxidativa contra as espécies reativas de oxigênio, como tióis intracelulares, lipofosfoglicanas, superóxido dismutase, tripanotiona, peroxidases e peroxidoxinas (Ariyanayagam & Fairlamb, 2001; Barr & Gedamu, 2003; Dolai et al., 2008). Estas moléculas, em sua maioria, são enzimas capazes de detoxicar ROS, principalmente peróxido de hidrogênio, produzidos pelos macrófagos, aumentando a sobrevida no interior destas células. Estes mecanismos utilizados pela *Leishmania* para escapar das atividades leishmanicidas destas células hospedeiras ainda não estão esclarecidos (Barr & Gedamu, 2003).

1.6.2. A célula hospedeira

Os macrófagos são as principais células hospedeiras da *Leishmania.* São células do SFM e têm um importante papel na detecção e eliminação dos microorganismos patogênicos. Os macrófagos surgem pelo influxo de monócitos que se diferenciam em macrófagos e pela produção de macrófagos originados das células que se dividem no local (Van Furth, 1992).

Uma vez o monócito formado, ele permanece na medula óssea por menos de 24 horas. E ao ingressar na circulação sanguínea, expressa na superfície celular proteínas heterodiméricas da subclasse β1 e β2 da superfamília das integrinas das moléculas de adesão, incluindo antígeno de função linfocitária (LFA-1; CD11a/CD18), CR3 (c3bi; CD11b/CD18), p150, 95 (CD11c/CD18) e antígeno de ativação muito tardia (VLA-4; CD49d/CD29). Estes medeiam à ligação com os respectivos ligantes expressos nas células endoteliais, promovendo a adesão dos monócitos na superfície do endotélio. Esta ligação é seguida pelo espalhamento dos monócitos na superfície das células endoteliais, migração transendotelial entre duas células endoteliais adjacentes e passagem dos monócitos para os tecidos e cavidades do organismo, onde passam a ser denominados macrófagos (Van Furth, 1992).

Estas células podem ser divididas em três grupos: macrófagos de exsudato, macrófagos residentes e células diferenciadas em células dendríticas (Takahashi *et al*., 1996; Yamate *et al*., 2001). Estes tipos se diferenciam na ontogenia, morfologia, distribuição tecidual e funções (Dijkstra *et al*., 1985; Takanashi *et al*., 1996; Valledor *et al*., 1998). Os macrófagos residentes possuem a função de quimiotaxia, fagocitose e potencial de proliferação. Recebem denominações especiais dependendo da sua localização: no sistema nervoso central são chamados micróglia; nos sinusóides vasculares do fígado são denominados células de Kupffer; nas vias respiratórias pulmonares são os macrófagos alveolares; e na cavidade peritoneal são os macrófagos peritoneais (Abbas *et al*., 1997).

Quanto às propriedades, os macrófagos podem ser divididos em macrófagos residentes e macrófagos inflamatórios. Os macrófagos inflamatórios diferenciam-se a partir de

monócitos circulantes que migram por quimiotaxia para o foco da inflamação. Estes macrófagos são incapazes de proliferar, morrendo por apoptose ao final do processo inflamatório (Takahashi *et al*., 1996). Os macrófagos residentes são encontrados em órgãos linfóides e não linfóides, podendo apresentar algumas variações morfológicas e funcionais que diferem de acordo com os tecidos onde estão localizados (Nailto *et al*., 1996). Quanto à morfologia, eles podem ser ou não esterase-positivos; e peroxidase não-específicos, expressando F4/80, Slc11a1 genes e FcRII. Quando em repouso, os macrófagos residentes apresentam baixa expressão gênica para moléculas de MHC de classe II, baixo consumo de oxigênio, pouca ou nenhuma secreção de citocinas, mas possuem atividade fagocítica e quimiotática (revisto por Noel *et al*., 2004).

As espécies de leishmânias viscerotrópicas, como *L. (L.) infantum*, são parasitos intracelulares com um acentuado tropismo por macrófagos residentes do fígado, baço, linfonodos e medula óssea. Durante a LV, há participação de duas populações de macrófagos no fígado: os imigrantes, provenientes da circulação sanguínea e os residentes, as células de Kupffer. Estas duas populações apresentam características distintas seja em sua morfologia, na capacidade de fagocitose ou eliminação parasitária (Lepay *et al*., 1985, Kausalya *et al*., 1993; Kausalya *et al*., 1996).

1.6.3. A interação *Leishmania*-macrófago

A etapa inicial da interação *Leishmania*-macrófago se caracteriza pela adesão e a entrada do patógeno no macrófago. Esta interação resulta em alterações morfológicas, aumento da quimiotaxia, fagocitose, citotoxicidade e produção de mediadores químicos (Bianco *et al*., 1975; Adams & Thomas, 1984; revisto por Naderer & McConville, 2008).

Os agentes que induzem a ativação dos macrófagos são denominados Fatores Ativadores de Macrófagos (MAFs). O MAF mais amplamente estudado é o interferon-γ (IFN-γ), mas outros como IFN-α and IFN-β, fator de necrose tumoral (TNF- α), interleucina- 2(IL-2), IL-3, IL-4 e GM-CSF também podem induzir várias funções e propriedades dos macrófagos (Adams & Tomas, 1984; Stafford *et al*., 2002). O metabolismo basal dos macrófagos é profundamente alterado pela interação receptor-ligante, ocasionando aumento no número de exposição das proteínas da membrana, arranjo do citoesqueleto, regulação da expressão de genes específicos para transcrição e modulação de sinais de transdução (MacMicking *et al*., 1997). Desta forma, a ativação consiste em alterações qualitativas e quantitativas na expressão de vários produtos gênicos que conferem ao macrófago ativado propriedades ausentes no monócito em repouso (Abbas *et al*., 1997; Engwerda *et al*., 2004). Uma vez os macrófagos ativados, eles são células secretoras potentes, produzindo mais de

100 tipos de moléculas já definidas. A ativação pode, em geral, ser regulada por dois sinais: 1) um sinal priming, ou de pré-ativação, que prepara o macrófago; e 2) um sinal desencadeante, que estimula a liberação do (s) produto (s).

O reconhecimento de microorganismos estranhos pelo macrófago resulta no processo de fagocitose e, consequentemente, na destruição eventual de microorganismos pela via microbicidas do macrófago (Lepay *et al*., 1985; Kausalya *et al*., 1996; Costa *et al*., 2007). A fagocitose desencadeia uma "explosão" respiratória caracterizada por um aumento drástico no consumo de oxigênio pelos macrófagos (Dinauer *et al*., 1993; Segal, 1996). Esta explosão oxidativa é maximizada quando da opsonização com anticorpos e de componentes do sistema complemento (Zheleznyak & Brown, 1992), sendo requerida a ativação da proteína quinase C (PKC) (Fallman *et al*., 1992; Zheleznyak & Brown, 1992; Allen & Aderem, 1996; Chan *et al*., 2001). A PKC representa uma família de serinas/tirosinas kinases que regulam diversos processos celulares. Múltiplas isoformas de PKC são encontradas nos macrófagos e diferem pela distribuição intracelular, pelos cofatores requeridos e pela especificidade do substrato (Li *et al*., 1999).

Após a endocitose, os macrófagos processam e apresentam antígenos protéicos via molécula de classe I e/ou II do complexo principal de histocompatibilidade (MHC) aos linfócitos T, atuando como importantes células apresentadoras de antígenos (APC). A apresentação de antígenos para células T por APCs pode iniciar uma ativação e/ou expansão dessas células. Porém é necessário que ocorra simultaneamente uma ligação de moléculas co-estimulatórias B7-1(CD80)/B7-2(CD86) e CD40 presentes nos macrófagos, com CD28 e CD40L presentes nas células T, respectivamente (revisto por Denkers & Butcher, 2005).

Essa cooperação induz o linfócito T a secretar citocinas variadas e com funções pleiotrópicas. As citocinas, que são mediadores químicos que atuam na comunicação celular, têm sido reconhecidas como os mais importantes sinais indutores na diferenciação das células TCD4+ Th1 e Th2 (Sacks & Noben-Trauth, 2002). A dicotomia Th1/Th2 é definida de acordo com o padrão de citocinas secretadas por estas células. As células Th1 são caracterizadas pela secreção, por exemplo, de interleucina 2 (IL-2), interferon gama (IFN-γ) e fator de necrose tumoral alfa (TNF-α), que são conhecidas por ativar as defesas do hospedeiro contra os patógenos intracelulares, enquanto as células Th2 produzem interleucina 4 (IL-4), interleucina 5 (IL-5), interleucina 10 (IL-10) e interleucina 13 (IL-13), que favorecem o desenvolvimento de respostas humorais contra patógenos extracelulares e aumento da sobrevida de parasitos intracelulares (Stanley & Engwerda, 2007). A expansão de células Th1 e, consequentemente, a produção de citocinas ativa os macrófagos gerando respostas microbicidas (Courtney *et al*., 2004).

Os efeitos das citocinas nos mecanismos imunes envolvidos na leishmaniose são essenciais na determinação das respostas protetoras do hospedeiro (Mossman & Moore, 1991; Wein *et al.*, 2004). A resistência às leishmânias está associada a um desenvolvimento da resposta imune celular Th1 resultando na ativação da atividade leishmanicida do macrófago, sendo o reconhecimento rápido e o início da resposta imune apropriada essencial para a eliminação do parasito (Reed & Scott, 2000; Awasthi *et al.*, 2004). As citocinas com perfil tipo Th2 predominam em animais susceptíveis infectados com *Leishmania* (Carvalho *et al.*, 1985; Sacks *et al.*, 1987; Reed & Scott, 2000; Kumar *et al.*, 2001) e está relacionada com a gravidade da doença (Ghalib *et al.*, 1993; Engwerda *et al.*, 2004). No caso da resposta imune com perfil Th2, as leishmânias sobrevivem e tendem a multiplicar-se nos macrófagos (Ridley, 1987).

Sendo assim, a sobrevivência destes parasitos no interior das células hospedeiras depende da resistência às respostas antimicrobicidas dos macrófagos induzidas ou presentes nos momentos da invasão (Mosser & Edelson, 1997; Stafford *et al.*, 2002). Pode-se considerar que estas respostas dos macrófagos sobre os parasitos aconteça em duas etapas: (1) no início da fagocitose da promastigota, os macrófagos podem promover uma resposta oxidativa estimulada pelo próprio processo; (2) uma vez estabelecida à infecção, o macrófago quiescente pode ser efetivamente ativado para matar o parasito intracelular (Gantt *et al.*, 2001). Portanto, a infecção por *Leishmania* depende da espécie do parasito, da predisposição genética e do estado imune do hospedeiro, sendo o estabelecimento da infecção o resultado das estratégias da invasão parasitária e das respostas antimicrobicidas do macrófago (Awasthi *et al.*, 2004; revisto por Stanley & Engwerda, 2007).

1.7. Macrófagos e seus mecanismos microbicidas

O macrófago apresenta, frente à infecção por *Leishmania*, duas vias leishmanicidas: uma independente de oxigênio e outra, dependente de oxigênio através de ROS e RNS (Lepay *et al.*, 1985; Kausalya *et al.*, 1996; Costa *et al.*, 2007).

A via independente de oxigênio se caracteriza pela produção e liberação de enzimas lisossomais/proteolíticas (as peptidases). Os macrófagos possuem um repertório de potentes moléculas microbicidas estocadas no interior de seus grânulos e lisossomas. Estas organelas contêm enzimas digestivas que são liberadas dentro do fagolisossoma depois da ingestão de organismos estranhos (Gabay *et al.*, 1993). Estas enzimas digestivas são peptidases, nucleases, fosfatases, esterases e lipases levam à destruição dos organismos fagocitados pelos macrófagos (Levy *et al.*, 1995; Ganz & Lehrer, 1997; Borelli *et al.*, 1999).

A via dependente de oxigênio se caracteriza pela produção de espécies reativas de oxigênio oriundos da explosão oxidativa, além de óxido nítrico e peroxinitrito (Kausalya *et al.*, 1996; Costa *et al.*, 2007; Beckman, 2009).

1.7.1. Proteases

A via leishmanicida independente de oxigênio é caracterizada pela liberação de enzimas hidrolíticas pelos macrófagos. Estas enzimas são chamadas de peptidases, peptídeo hidrolases ou proteases e são capazes de clivar ligações peptídicas nas proteínas e fragmentos de proteínas (Barrett, 2001). Elas podem ser divididas em relação ao tipo de reação catalisada em exopeptidases (hidrolisam aminoácidos na porção amino- ou carboxi-teminal de um polipeptídeo) e endopeptidases (clivam ligações peptídicas internas). Em relação à natureza química do sítio catalítico, as endopeptidases (que são a maioria das peptidases) se dividem em serina, cisteina, aspártico, metaloproteinases e treonina (revisto por Vermelho *et al.*, 2007).

Com o avanço dos conhecimentos da química das proteínas, elas passaram a ser agrupadas de acordo com as similaridades na estrutura molecular e origem evolutiva, o que deu origem ao sistema MEROPS, que organiza e agrupa as peptidases em famílias, e estas em clãs (Rawlings *et al.*, 2006). As peptidases têm mostrado um papel importante em várias etapas da infecção por tripanossomatídeos e interação com sua célula hospedeira como etapas como adesão, penetração, sobrevivência intracelular, replicação, diferenciação, infectividade e nutrição. Os tripanosomatídeos, como a *Leishmania*, apresentam uma grande e variada quantidade de peptidases intracelulares e extracelulares que regulam funções específicas das fases biológicas do parasito (revisto por Vermelho *et al.*, 2007).

Leishmania spp. apresentam muitas atividades de cisteína peptidases. Existem 3 tipos de cisteína nas leishmânias: cisteína peptidases A (CPA), cisteína peptidases B (CPB) e cisteína peptidases C (CPC). Em leishmânias viscerotrópicas, foi reportado uma organização genômica similar de CPB. CPBs são do grupo das catepsinas L e apresentam níveis aumentados de atividade nas formas amastigotas. As cisteína peptidases participam da nutrição do parasito, da invasão nos macrófagos e dos mecanismos de escape do sistema imune do hospedeiro (Mundodi *et al.*, 2002). Na célula hospedeira, a maioria das cisteina peptidases é de origem lisosomais, catepsinas B e L, e elas estão implicadas em processos fisiológicos. As sequências das catepsinas L e B da célula hospedeira apresentam alta similariedade comparadas com as mesmas catepsinas dos parasitos (Sakanari *et al.*, 1997).

Embora as serinas peptidases sejam amplamente investigadas, poucos membros desta classe têm sido bem caracterizados em *Leishmania* e em macrófagos. Em compensação, as

metaloproteinases são bem descritas nestes parasitos e também nas células hospedeiras. As metaloproteinases de matriz, também conhecidas como matrixinas, são enzimas envolvidas no "turnover" de proteínas da matriz extracelular, que ocorre durante o desenvolvimento embrionário, ou em determinadas condições patológicas como artrite, danos celulares, entre outros (Matrisian, 1990). Estas enzimas são dependentes de Zn^{+} e Ca^{2+} e secretadas em sua forma latente (pró-MMP). Apresentam atividade contra uma variedade de moléculas biologicamente relevantes não somente as constituintes da matriz extracelular, como colágneo fibrilar (intersticial) e não-fibrilar (Nagase & Woessner, 1999).

Na *Leishmania*, a metaloproteinase chamada gp63 ou PSP (peptidase de superfície de promastigotas) ou leishmanolisina ou MSP (principal peptidase de superfície) é a mais bem caracterizada (Yao *et al.,* 2004). Esta enzima corresponde a mais abundante gliocoproteína em promastigotas de *Leishmania* e fica ancorada via GPI. Uma significativa proporção de MSP é liberada no meio extracelular por autoproteólise ou secreção (McGwire *et al.,* 2002; Ellis *et al.,* 2002). Embora a expressão destas metaloproteinases seja reduzida nas formas amastigotas de *Leishmania*, já foi reportada a presença na surperfície e dentro do lisosoma do parasito (revisto por Vermelho *et al.,* 2007). Os macrófagos humanos expressam uma variedade de metaloproteinases, mas os macrófagos murinos produzem predominantemente metaloproteinase 2, 9 e 12 (Kuroda *et al.,* 2004; Kassim *et al.,* 2005).

1.7.2. Radicais livres

Os radicais livres são mediadores liberados pelos macrófagos durante sua atividade leishmanicida pela via dependente de oxigênio. Eles são espécies químicas que possuem um elétron não pareado, e são muito reativos. Estes incluem anion e radical oxigênio (O_2- e OH) e oxidante (H_2O_2) (Maemura *et al.,* 2005). Além disso, os radicais livres são produzidos continuamente nas células como produtos intermediários do metabolismo ou deliberadamente, por exemplo, durante a fagocitose. Estes reativos formados dentro das células podem oxidar as biomoléculas e provocar morte celular e lesão tecidual (revisto por Genestra, 2007).

Com exceção de circustâncias incomuns, como a influência da radiação ionizante, os radicais livres podem ser produzidos nas células por reações de transferência de elétrons. Estas reações podem ser mediadas pela ação de enzimas ou não-enzimaticamente, com freqüência através da química redox de íons de metais de transição. A produção de radicais livres em células animais pode ser acidental ou deliberada. Os fagócitos ativados também geram deliberadamente O_2^- como parte de seu papel bactericida. Embora os radicais livres sejam produzidos apenas na interface da membrana plasmática dos macrófagos e bactérias,

algum escape de O_2^- , H_2O_2 e de outras espécies de oxigênio reativo é inevitável (Bhusate *et al.,* 1992; Maemura *et al.,* 2005).

No homem, os radicais livres exercem papel em uma variedade de sistemas, sendo que a desregulação deles pode exercer importante papel na inflamação como por exemplo o O_2- na proliferação do fibroblasto, H_2O_2 na ativação de fatores de transcrição, como o fator de transcrição nuclear do tipo κB (NFκB) (Bax *et al*., 1992). O NFκB é um importante fator de transcrição em sistemas inflamatórios, pois controla a transição de numerosos genes de citocinas, incluindo o IL-2 e o TNF-α, assim como o gene para o receptor IL-2 e os genes MHC classe 1 (revisto por Genestra, 2007).

Macrófagos, neutrófilos e eosinófilos possuem um sistema de citocromo-b-245 $NADPH^+$ oxidase flavoproteína que parece estar diretamente relacionados na morte de patógenos intracelulares como a *Leishmania*. Durante a fagocitose, as células consomem quantidades elevadas de oxigênio (explosão respiratória). Então, a enzima NADPH+ oxidase, que possui componentes membranares e citosólicos, quando com sua conformação adequada, transfere um elétron para o O_2, produzindo O_2^- (Gantt *et al*., 2001). A ativação com a explosão oxidativa resulta em uma produção aumentada de NADPH+ pela via das pentoses e a geração de O_2^- e OH e H_2O_2**,** que são capazes de danificar membranas celulares e uma ampla variedade de biomoléculas. O H_2O_2 é formado pela dismutação do O_2^- e na presença de metais de transição (Miller, 2000).

1.7.3. Óxido nítrico (ON)

Além dos radicais livres, o ON é também liberado durante a atividade leishmanicida dos macrófagos pela via dependente de oxigênio. Ele é uma molécula gasosa simples, habitualmente encontrada no ar atmosférico em pequenas quantidades, altamente tóxica devido à presença de radical livre (elétron extra) que a torna um agente químico altamente reativo. Quando diluído, o ON tem uma meia vida de menos de 10 segundos devido a sua rápida oxidação a nitrito e nitrato (Snyder & Bredt, 1992; revisto por Brunet, 2001). Esta molécula resulta da oxidação de um dos dois nitrogênios guanidino da L-arginina, que é convertida em L-citrulina. Esta reação é catalisada pela enzima NO-sintase (NOs). A NOs pode ser constitutiva (cNOs) ou induzível (iNOS). A cNOS é dependente de cálcio e de calmodulina, que está envolvida na sinalização celular, e a iNOS é produzida por macrófagos e outras células quando ativadas por citocinas (Marletta, 1994).

A L-arginina é um aminoácido semi-essencial produzido no organismo, porém em quantidade insuficiente para todas as necessidades orgânicas. Além do ciclo da uréia, a arginina é utilizada na síntese de creatinina e fornece ornitina para a síntese de poliaminas

(Snyder & Bredt, 1992). Como há uma solicitação metabólica continuada da L-arginina, existe uma neo-síntese deste aminoácido nos túbulos proximais renais a partir da citrulina. Proteínas ingeridas são degradadas até arginina, que pode ser diretamente absorvida e utilizada no ciclo da uréia no tecido hepático, ou transformada no epitélio intestinal em ornitina que, juntamente com a glutamina secretada como glutamato, é convertida em citrulina. A citrulina também pode ser convertida diretamente em L-arginina no citoplasma de células endoteliais e de macrófagos (Dusse *et al*., 2003).

O ON é um importante mediador biológico, tendo um papel importante na sinalização celular, implicando na fisiopatologia de várias doenças e podendo ser produzido em diversas células (Nathan, 1992; Brunet *et al.,* 2001). Esta molécula é importante na regulação da resposta imune durante a infecção pela leishmânia (Kropt *et al*., 2005), sendo considerado um importante mediador citotóxico e citostático contra patógenos intracelulares (Brunet, 2001; Coleman, 2001; Vincendeau *et al*., 2003).

Uma vez os macrófagos ativados por IFN-γ, ou outros fatores, induzem a atividade de iNOS. Esta enzima cataliza a síntese de ON a partir de três isoformas de L-arginina e oxigênio molecular em todas as células de mamíferos (Lane, 1999). Os elevados níveis de ON derivado de iNOS durante os estágios iniciais da infecção por leishmânias é um pré-requisito para a sinalização de citocinas e têm um importante papel na imunidade inata (Wein *et al*., 2004).

A inibição específica da atividade de iNOS com N^G-nitro-L-arginina metil éster (L-NAME) e N^G-monometil-L-arginina (L-NMMA) (Liew & Cox, 1990; Davie *et al*., 1991) e citocinas que inibem a produção de ON como TGF-ß e IL-10 (Liew *et al*., 1991; Nelson *et al*., 1991; Barral-Neto *et al*., 1992; Vieth *et al*., 1994; Chatelain *et al*., 1999) ou o uso de camundongos "Knock-out" (KO) de iNOS têm sido utilizados na tentativa de entender o papel de ON na destruição intracelular das leishmânias (Murray et al., 2006). Estes camundongos KO de iNOS apresentaram um grande número de parasitos no interior de células de Kupffer e um granuloma ao redor do fígado, não havendo destruição parasitária (Murray et al., 2006). O tratamento com inibidores de iNOS em camundongos infectados com *L. (L.) major* resultou no aumento da carga parasitária, desenvolvendo um grande número de lesões na pele por inibir a destruição dos parasitos intracelulares (Liew *et al*., 1990; Wein *et al*., 2004). Além disso, a utilização de inibidores de iNOS resulta numa drástica redução dos danos hepáticos durante a infecção por *L. (L.) donovani* (Costa *et al*., 2007).

1.7.4. Peroxinitrito

O peroxinitrito ($ONOO^-$) é um produto formado pela reação de ON com o $O2^-$. Esta molécula é estável em solução alcalina, decaindo rapidamente quando uma solução é protonada (Beckman *et al.,* 1990). É um potente oxidante, considerado um mediador patogênico em uma variedade de doenças, e também é uma molécula efetora na resposta imune celular contra patógenos intracelulares (Hooper *et al.,* 1998).

Células do sistema imune e principalmente macrófagos, quando estimulados, podem produzir grandes quantidades de peroxinitrito devido a atividade da iNOs e da formação de NO e O_2^-. Sendo assim, ele é uma molécula efetora citotóxica derivada dos macrófagos, podendo difundir e permear membranas em células alvos. Uma vez na superfície da célula alvo, o peroxinitrito pode penetrar pela via dos canais de ânions ou pela difusão passiva na forma protonada que é resistente a reações intracelulares (revisto por Szabo *et al.,* 2007). Esta molécula é capaz de oxidar e fazer nitração em proteínas seja do parasito ou do hospedeiro (Linares *et al.,* 2008).

O efeito leishmanicida do peroxinitrito sobre o parasito parece ser mais potente que o ON (Linares *et al.,* 2008). Portanto, grande parte dos efeitos leishmanicidas bem como dos danos inflamatórios atribuídos ao ON deve, na verdade, corresponder a um efeito da molécula de peroxinitrito (Hoper *et al.,* 1998), uma vez que sua formação é rápida quando na presença de grandes quantidades de NO e O_2^-.

1.8. Danos nos hepatócitos durante a LV

O fígado é um dos sítios alvo onde acontece a proliferação das leishmânias viscerotrópicas, como a *Leishmania infantum.* Durante a leishmaniose visceral clínica e experimental são observadas condições patológicas no fígado (Goswami, 1970; Gutierrez *et al*., 1984; McElrath *et al*., 1988). Alterações na arquitetura do fígado com hipertrofia do órgão, hiperplasia de células de Kupffer, infiltração inflamatória, apoptose, necrose e esteatose de células hepáticas são frequentemente observadas (Vianna *et al.,* 2002; Duarte *et al.,* 2009).

A medida destes danos hepáticos se dá geralmente pelo acompanhamento dos níveis séricos de transaminases hepáticas. Estas enzimas produzidas pelo hepatócitos são importantes marcadores de injúria tecidual em doenças que envolvem o fígado (Kallei *et al*., 1964; Zhang *et al*., 2000; Pratt & Kaplan, 2000; Prati *et al*., 2002; Mofrad *et al*., 2003; Jadhao *et al*., 2004). O aumento dos níveis séricos das transaminases hepáticas em pacientes com LV é observado durante principalmente a fase inicial da doença (Hervas *et al*., 1991).

Evidências experimentais sugerem que os macrófagos ativados, ao liberarem grande quantidade de seus mediadores leishmanicidas (proteases, ROS e RNS), podem estar ocasionando danos no figado. Ou seja, a citotoxidade intracelular (destruição dos parasitos) produzida pela atividade leishmanicida dos macrófagos, com liberação excessiva dos mediadores, deve gerar um efeito citotóxico extracelular (Kausalya *et al*., 1993; Kausalya *et al*., 1996; Costa *et al.,* 2007). Este efeito colateral acarreta destruição do tecido adjacente, sendo uma seqüela evidente.

Existem duas populações de macrófagos (os imigrantes e as células de Kupffer) envolvidas na patogênese da LV no fígado, com características morfológicas e funcionais distintas, desempenhando atividades leishmanicidas com produção de mediadores em diferentes níveis. Estudos utilizando como modelos experimentais macrófagos residentes do fígado e macrófagos exsudativos de animais infectados com *L. (L.) donovani*, em um sistema de co-cultivo com hepatócitos, demonstraram um aumento dos níveis de TGO e TGP (Kausalya *et al*., 1993; El Hag *et al*., 1994). Estes resultados mostraram a presença de injúrias nos hepatócitos dos animais durante a infecção por *Leishmania donovani* (Kausalya *et al*., 1993). Com a infecção *in vitro* de co-culturas macrófagos-hepatócitos pela *Leishmania infantum* foi possível confimar a direta correlação entre a grande liberação dos mediadores leishmanicidas gerados pela ativação dos macrófagos e os danos nos hepatócitos (Costa *et al.,* 2007).

Sendo assim, durante o desenvolvimento desta tese desenvolvemos experimentos com a intenção de (i) obter um modelo in vitro que mimetize a interação de *Leismania*-macrófagos no contexto da infecção hepática (co-cultivo) e (ii) para tentar entender a atividade leishmanicida das duas populações de macrófagos envolvidas durante a leishmaniose visceral experimental. O corpo desta tese de doutorado compreende três trabalhos (bloco 1, bloco 2 e bloco 3), onde dois (blocos 1 e 2) já se encontram publicados e um terceiro submetido (bloco 3). Ainda como anexo (anexo 1) temos a inclusão de um artigo oriundo da dissertação de mestrado, mas que é fundamental para a apreciação desta tese.

Primeiramente (bloco 1), foi observada a participação da via independente de oxigênio (as peptidases) durante o período de maior atividade leishmanicida e consequentemente no momento dos danos nos hepatócitos. O importante envolvimento do ON pela via dependente de oxigênio na atividade leishmanicida dos macrófagos imigrantes, levando ao dano, já tinha sido reportado (Costa et al., 2007- anexo). Em nosso 2º trabalho (bloco 2), estabelecemos a cultura de Kupffer e observamos a infecção das duas populações de macrófagos por amastigotas axênicas de *Leishmania infantum*, para que os ensaios *in vitro* pudessem mimetizar as condições *in vivo*. O terceiro trabalho (bloco 3) comparou a produção dos

mediadores frente às amastigotas de *Leishmania infantum* por duas populações de macrófagos (células de Kupffer e macrófagos peritoneais) que apresentam características similares àqueles macrófagos que estão presentes durante a infecção *in vivo.* Esperamos com isso poder contribuir um pouco no entendimento de patogênese LV, uma vez que estudos desta natureza podem futuramente impactar no desenvolvimento de alvos terapêuticos que eliminem o parasito ou pelo menos limitem a expressão clinica da doença.

2. Objetivos

Objetivo Geral

Macrófagos são células fagocíticas profissionais fundamentais durante a infecção por *Leishmania*, realizando diferentes funções a depender de vários aspectos da interação parasito-hospedeiro. Nesta presente tese de doutorado tivemos com o objetivo principal estudar os mecanismos microbicidas destas células e verificar o papel de macrófagos exsudativos e residentes do fígado durante a infecção por *Leishmania* (*Leishmania*) *infantum* na geração de danos tissulares neste órgão.

Objetivos específicos

1. Analisar qualitativamente e quantitativamente os mediadores da via independente de oxigênio (peptidases) durante a infecção de macrófagos peritoneais por *Leishmania infantum*
2. Verificar se há participação das proteases nos danos nos hepatócitos pela análise dos sobrenadantes de co-culturas de macrófagos/hepatócitos infectados com formas promastigotas.
3. Estabelecer amastigogênese da *Leishmania infantum* e caracterizá-las morfologicamente e bioquimicamente
4. Estabelecer culturas de células de Kupffer obtida de camundongos e caracterizar o padrão de infecção *in vitro* no modelo de interação com *Leishmania infantum* e comparar com a infecção de macrófagos peritoneais.
5. Comparar a eficácia da atividade leishmanicida de macrófagos residentes do fígado e macrófagos peritoneais infectados por amastigotas de *Leishmania infantum*
6. Determinar os mecanismos microbicidas, assim como estabelecer as relações entre a produção dos mediadores produzidos por estes macrófagos e os dados causados ao hepatócitos em co-cultivo, como modelo de dano tissular.

3.BLOCO 1

Acta Tropica 106 (2008) 175–183

Contents lists available at ScienceDirect

Acta Tropica

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

In vitro evidence for metallopeptidase participation in hepatocyte damage induced by *Leishmania chagasi*-infected macrophages

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ARTICLE INFO

Article history:
Received 3 September 2007
Received in revised form 7 March 2008
Accepted 13 March 2008
Available online 20 March 2008

Keywords:
Macrophage
Co-culture
Leishmania chagasi
Metallopeptidases

ABSTRACT

Leishmania (*Leishmania*) *chagasi* infection activates macrophages, which release several microbicidal agents, including peptidases, to eliminate the parasite. Leishmanicidal mediators released in large amounts may cause morphological and/or functional injuries to the liver. In order to investigate the involvement of peptidases in this phenomenon, an *in vitro* co-culture model of peritoneal macrophages infected with *L. chagasi* and hepatocytes was used. High levels of released hepatic transaminases were found in supernatants from infected co-cultures at the same time point in which alterations in hepatocyte morphology and maximum proteolytic activity were observed. The largest proteolytic activity being at pH 10 as well as the greatest efficiency of treatment with 1,10-phenantroline observed in supernatants from the infected co-cultures suggests the presence of metallopeptidases during the leishmanicidal activity by infected macrophages. Furthermore, TNF-α levels and high levels of TGF-β were increased at this time point, and this can be related to the synthesis of metallopeptidases and the conversion of the latent form to the active form. Metallopeptidase activities were detected by gelatin SDS-PAGE in higher amounts in infected macrophages and co-culture supernatant; moreover, one metallopeptidase migrating at 85 kDa produced in excess (41% more) by infected macrophages was identified as MMP-9. This metallopeptidase may be participating in this phenomenon together with other leishmanicidal factors released by these host cells.

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1. Introduction

Macrophages from the liver, spleen and lymph nodes are the chosen cells for viscerotropic *Leishmania* species differentiation and replication within the mammalian host. Previous studies have shown that lysosomal hydrolases, neutral peptidases, oxygen and nitrogen reactive intermediates (ROI and RNI) released in excess by infected macrophages during its leishmanicidal activity lead to a collateral effect on adjacent hepatocytes (Takemura and Werb, 1984; Kausalya et al., 1993, 1996; Vianna et al., 2002; Costa et al., 2007). Liver tissue damage associated with pathological conditions has been reported in both human and experimental visceral leishmaniasis (Gutierrez et al., 1984; McElrath et al., 1988; El Hag et al., 1994). In *Leishmania donovani*-infected BALB/c mice, granuloma formation is the most important lesion found in the liver (Gutierrez et al., 1984; Murray, 2001; Stanley and Engwerda, 2007).

Extracellular endopeptidases produced by different liver cell populations have been implicated in the development of several hepatic pathologies (Bergers and Betz, 2000; Mohammed and Khokha, 2005). Endopeptidases are hydrolytic enzymes that cleave peptide bonds inside the protein and peptides found in different cell types, including macrophages and protozoa (Barret et al., 2001; McKerrow et al., 1993; Cuervo et al., 2006). According to the hierarchical and structure-based classification, they are subdivided into cysteine, metallo, aspartic, serine, threonine and glutamic peptidases (Rawlings et al., 2006).

Matrix metallopeptidases (MMPs) constitute a large family of Zn^{2+}- and Ca^{2+}-dependent endopeptidases, which act on extracellular matrix macromolecules and are associated with tissue

Abbreviations: MMPs, metallopeptidases; Hep, hepatocyte; MF, macrophage; PMSF, phenylmethylsulfonyl fluoride; Phen, 1,10-phenantroline; Nu, nuclei; N, non-infected; I, infected.

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doi:10.1016/j.actatropica.2008.03.006

destruction during various pathological conditions (Matrisian, 1992; Johnson et al., 1998; Nagase and Woessner, 1999). As one would predict for enzymes that may cause host damage, MMPs are tightly regulated. First, they are rarely stored and only MMP-8 and MMP-9 can be immediately released. Second, they are secreted as pro-enzymes that undergo proteolytic cleavage for activation, releasing a cysteine residue in the pro-peptide domain to form a catalytic site containing zinc (Van Wart and Birkedal-Hansen, 1990). Thirdly, MMPs are compartmentalized in close proximity to the cell (Brooks et al., 1996; Dumin et al., 2001). Finally, their activity is regulated by secretion of specific inhibitors, including tissue inhibitors of metalloproteases (TIMPs), which bind to the catalytic domain in a non-covalent manner (Brew et al., 2000).

MMPs are secreted by stromal and inflammatory cells, such as macrophages, in response to both endotoxins and cytokines, including tumour necrosis factor (TNF-α), tumour growth factor (TGF-β) and interleukin 1 (IL-1) (Unemori et al., 1991). Additionally, cell contact-dependent signaling may drive MMP up-regulation (Miltenburg et al., 1995; Lacraz et al., 1994). The synthesis and secretion of these molecules seems to be important in a large number of physiopathological processes, including the inflammatory response. Excessive inflammation following infection may cause tissue damage, and metallopeptidases are implicated in causing this immunopathology (Elkington et al., 2005). The present study was designed to provide information regarding the involvement of peptidases in hepatocyte damage in *L. chagasi*-infected macrophage–hepatocyte co-cultures. Morphological and physiological aspects of the co-cultivated cells were considered. The effect and qualitative profile of the peptidases found in the culture systems were also investigated.

2. Material and methods

2.1. Mice

Swiss Webster mice weighing about 20 g each were used to obtain primary cultures of peritoneal macrophages. Pregnant mice (18–20 days of gestation) were used to obtain hepatocyte primary cultures. The animals were obtained from the Animal Care Facility of the Oswaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil). All procedures involving animals in this study were reviewed and approved by the Oswaldo Cruz Foundation Ethics Committee (CEUA – FIOCRUZ, Resolution # 0244/05).

2.2. Parasites

Leishmania (*Leishmania*) *chagasi* (MCAN/BR/2000/CNV-FEROZ strain) was isolated from a dog in the State of Espírito Santo, Brazil, and typed by multi-locus enzyme electrophoresis at the *Leishmania* collection of the Oswaldo Cruz Institute, RJ, Brazil (CLIOC, WDCM 731). Parasites were maintained through inoculation in golden hamsters. Infective forms were obtained from hamster spleen or liver and cultured in biphase NNN blood agar medium/Schneider's *Drosophila* culture medium (Sigma–Aldrich St. Louis, MO, USA) containing 10% fetal calf serum (FCS; Sigma–Aldrich, St. Louis, MO, USA).

2.3. Macrophage isolation and infection

Mouse peritoneal macrophages were obtained from Swiss Webster mice as previously described (Araujo-Jorge and de Souza, 1986). Briefly, animals were sacrificed using CO_2 and the peritoneal cavity was washed with 10 ml of MEM/199 culture medium (Cultilab, São Paulo, SP, Brazil) at 4 °C. For each experiment, 10 mice were utilized. The cells were pooled and counted using a Neubauer chamber. The cells were seeded in tissue culture flasks (2×10^6 macrophages/flask) or on glass coverslips (2×10^5 macrophages/coverslip) and maintained in MEM/199 culture medium at 37 °C in a humidified atmosphere containing 5% CO_2.

For *in vitro* infection of macrophages, *L. chagasi* promastigotes were harvested at the stationary growth phase. Peritoneal macrophages were cultivated for 24 h and then infected at a ratio of 10 parasites per cell. The macrophages and parasites were incubated together for 1 h at 34 °C. The cultures were then washed three times with MEM/199 culture medium to remove non-adherent or non-internalized parasites.

2.4. Hepatocyte isolation

Hepatocytes were isolated from Swiss Webster mice according to previous protocols (Porrozzi et al., 1997). Briefly, 7–11 mouse embryo livers (1–2 g each) were aseptically removed and washed with HEPES buffer (Sigma–Aldrich, St. Louis, MO, USA). The livers were minced and incubated at 37 °C for 20 min with approximately 50 ml of HEPES buffer containing 0.05% Type II collagenase (Sigma–Aldrich, St. Louis, MO, USA). The cells were dispersed by pipetting and then collected by centrifugation at $200 \times g$. Viable cells were purified by sedimentation for 10 min at room temperature with MEM/199 medium containing 10% FCS.

2.5. Hepatocyte–macrophage co-culture

Hepatocytes from the same mouse strain were seeded directly onto infected-macrophage plates (Kausalya et al., 1993) after culture washing to remove all non-attached parasites. Infected peritoneal macrophages were co-cultivated with hepatocytes at a 10:1 ratio (2×10^6 macrophages/2×10^5 hepatocytes in 3 ml of MEM/199 medium plus 10% FCS in tissue culture flasks or 2×10^5 macrophages/2×10^4 hepatocytes in 300 μl of MEM/199 medium plus 10% FCS on glass coverslips). These co-cultures were maintained until 72 h post-infection (hpi) in culture medium at 37 °C in a 5% CO_2 atmosphere. Hepatocytes were co-cultured with non-infected macrophages as controls. The culture medium was maintained without changing during the entire experimentation period. After the interaction of infected macrophages and hepatocytes, coverslips were washed three times in culture medium, fixed in Bouin's solution and stained with Giemsa (Merck).

The percentage of infected macrophages and the mean number of intracellular parasites per infected macrophage in co-cultures were determined by counting at least 100 cells using light microscopy. These experiments were repeated three times to confirm that the experimental data was reproducible. All experiments were also performed in macrophage cultures alone for comparison with co-cultures. The co-culture supernatant was collected and examined by light microscopy for detached or dead cells.

2.6. Release of hepatocyte enzymes

Glutamate oxaloacetate transaminase (AST) and glutamate pyruvate transaminase (ALT) activities were assayed with commercial kits (Labtest, Lagoa Santa, MG, Brazil). Analyses were performed with both the cell lysate and the co-culture supernatant, using one Petri dish for each time point. Cell lysate was obtained by scraping culture monolayers and subjecting the scraped cells to sonication. Measurements of enzymes released from infected macrophage cultures were carried out in parallel. The results were expressed as enzyme release percentage and were calculated as

follows (Kausalya et al., 1993):

$$\frac{ES}{(ES+EL)} \times 100 = \%ER$$

ES: enzyme measured in the supernatant; EL: enzyme measured in the lysates; ER: enzyme release.

2.7. Ultrastructural examination of co-cultured cells

Co-cultures were fixed for 1 h in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer supplemented with 3.5% sucrose (Sigma–Aldrich, St. Louis, MO, USA). After that, the cells were washed with 0.1 M cacodylate buffer and post-fixed with 1% OsO_4 in the same buffer. The samples were then washed twice in buffer, dehydrated in graded acetone and embedded in Epon. Samples were observed using a Zeiss EM10C transmission electron microscope.

2.8. Culture supernatant and cell extracts

Non-infected and infected cell-free macrophage supernatant and co-culture systems were collected at 48 hpi. The samples were centrifuged for 30 min at 2000 × g at 4 °C, and then passed through a 0.22 μm filtration unit (Millipore). Samples were concentrated by dialysis (cut-off 9000 Da) against polyethyleneglycol 4000 overnight at 4 °C and SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol and 2% bromophenol blue) was added at a ratio of 7:3 (v/v) (Laemmli, 1970). The cells were scraped from the flasks and centrifuged for 30 min at 2000 × g at 4 °C, washed three times with cold phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2) and, finally, lysed by ultrasound at 4 °C for 5 min.

2.9. Quantitative proteolytic activity assay

The activities from cellular and extracellular peptidase co-cultures were spectrophotometrically measured using gelatin substrate (Merck, Darmstadt, Germany) according to the Buroker-Kilgore and Wang (1993) method. Initially, 20 μl of cell extracts and supernatant (equivalent to 100 μg of protein), at 24, 48 and 72 hpi, were incubated at room temperature for 1 h with the following peptidase inhibitors (Sigma–Aldrich, St. Louis, MO, USA): 10 μM E-64 with 50 mM sodium phosphate buffer at pH 5.5 or 10 mM 1,10-phenanthroline (Phen) with 50 mM glycine–NaOH buffer at pH 10 as well as 2 mM DTT with 50 mM sodium phosphate buffer at pH 5.5. Following incubation, gelatin substrate (0.5 mg/ml) was added and the mixtures were incubated at 37 °C for 2 h. Subsequently, three aliquots (100 μl each) of the reaction mixtures were transferred to microtiter plate wells containing 50 μl of water and 100 μl of Coomassie solution (0.025% Coomassie brilliant blue G-250, 11.75% ethanol and 21.25% phosphoric acid). The dye was allowed to bind for 10 min and the plate was read on a Thermomax Molecular Device microplate reader at an absorbance of 595 nm. One enzyme activity unit was defined as the amount of enzyme causing an increase of 0.01 absorbance units at 595 nm under standard assay conditions.

2.10. Protein determination

For protein determination, the Lowry method (Lowry et al., 1951) was employed using bovine serum albumin as standard.

2.11. Treatment of co-cultures with peptidase inhibitors

After 24 h, the co-cultures were incubated with specific peptidase inhibitors at 37 °C. A serine-peptidase inhibitor (1 mM phenylmethylsulfonyl fluoride—PMSF), a metalloproteinase inhibitor (10 mM 1,10 phenantroline—Phen) and a cysteine peptidase inhibitor (4 mM cystatin C) were employed in the cultures. Infected co-cultures without the addition of inhibitors were used as controls. Co-culture supernatants were collected after 24 h of treatment and assayed with glutamate oxaloacetate transaminase (AST) and glutamate pyruvate transaminase (ALT) commercial kits (Labtest, Lagoa Santa, MG, Brazil). The enzymes were utilized as hepatocyte damage markers. Measurement of enzyme release from non-infected co-cultures was carried out in parallel.

2.12. TNF-α and TGF-β detection in the co-culture supernatant

Supernatants from non-infected and infected co-cultures were collected after 24, 48 and 72 hpi to determine if TNF-α and TGF-β could be mediating the synthesis and/or activation of the metallopeptidases. TNF-α and TGF-β levels were detected employing a commercial sandwich ELISA (DuoSET ELISA; R&D Systems, Minneapolis, MN, USA).

2.13. Gelatin–SDS-PAGE analysis

Secreted and cellular proteolytic activities were assayed using 10% SDS-PAGE with 0.1% gelatin incorporated into the gel (Heussen and Dowdle, 1980). In brief, the gels were loaded with 40 μl of concentrated supernatant and cell extracts from macrophages and co-cultures (equivalent to 200 μg of protein) per slot. Subsequently, electrophoresis was performed at a constant voltage of 170 V at 4 °C for 2 h. The gels were then soaked for 1 h in 2.5% Triton X-100, and incubated for 24 h at 37 °C in 50 mM sodium phosphate buffer (pH 5.5) in the presence or absence of 2 mM DTT or 10 μM E-64 or in 50 mM glycine–NaOH buffer (pH 10) with or without 10 mM 1,10-phenanthroline (Phen). Gels were stained for 1 h with 0.2% Coomassie brilliant blue R-250 in methanol–acetic acid–water (50:10:40) and destained in the same solvent. Gels were then dried, scanned and digitally processed. The molecular masses of the peptidases were calculated by comparison with the mobility of Pharmacia Biotech SDS-PAGE standards. Agels strips were processed at the same time under the same conditions. The densitometry analysis was performed using the Image-Pro Plus software. These assays were accomplished independently at least three times.

2.14. Western blotting analysis

Western blotting was performed according to the method employed by Jaffe et al. (1984). Briefly, non-infected and infected co-culture supernatants were electrophoresed on 10% SDS-PAGE under reducing conditions, and were blotted to a nitrocellulose membrane. This membrane was blocked overnight with 3% non-fat dry powdered milk in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20. Then, the nitrocellulose paper was sensitized for 1 h at room temperature with a 1:500 dilution of rabbit anti-MMP-9 antibody (Sigma, St. Louis, MO, USA) in PBS containing 0.5% non-fat dry powdered milk and finally given three 10 min washes. The nitrocellulose membrane was incubated with the rabbit Extravidin Peroxidase Staining kit (Sigma, St. Louis, MO, USA) as follows: 1 h incubation with biotinylated goat anti-rabbit IgG at 1:250 dilution, three washes with PBS containing 0.05% Tween 20 and 3% non-fat dry powdered milk for 10 min, and then a 1 h incubation with Extravidin Peroxidase at a 1:250 dilution. Immunoreactive bands were developed with 10 mg diaminoben-

zidine (DAB) (Sigma, St. Louis, MO, USA) in 20 ml H_2O/1 ml 2 M Tris–HCl buffer, pH 7.6 with a final addition of 30 μl of H_2O_2. Internal Mr. Standards were identified after staining nitrocellulose membranes with 0.1% Ponceau red in 0.3% trichloroacetic acid. The densitometry analysis was performed using the Image-Pro Plus software. Western blotting assays were accomplished independently at least three times.

2.15. Statistical analysis

All experiments were carried out in triplicate. The mean values and standard deviations (S.D.) for all numerical data were calculated. The data were analyzed with the Student's *t*-test. Simple linear regression analysis was performed to calculate correlations. Differences with a P value < 0.05 were considered statistically significant.

3. Results

3.1. Hepatocyte damage evaluation

The course of *L. chagasi* infection in co-cultures was monitored from 2 h post-infection (hpi) until 72 hpi by counting the number of amastigotes and percentage of infected macrophages using light microscopy. The number of infected macrophages increased up to 24 hpi when 80% of macrophages displayed intracellular parasites (3 parasites/cell). The multiplication of these parasites was observed until 48 hpi (5 parasites/cell). Then, starting from this moment when leishmanicidal activity by macrophages was intense, the number of parasites decreased drastically (data not shown). At 72 hpi, the percent of infected macrophages (28%) and the number of amastigotes (1 parasite/cell) were statistically significantly (P < 0.05) lower than those found in the beginning of the infection.

The release of hepatic transaminases (ALT and AST) into co-culture supernatants was adopted as a hepatocyte damage marker. In the co-cultures, these enzymes were released only by hepatocytes since macrophage culture supernatants were measured and no AST or ALT activity was seen (data not shown). During the entire observation time, there was a substantial amount of released enzymes in the supernatants of *L. chagasi*-infected co-cultures, approximately 2–3 times more than the basal levels observed in non-infected co-cultures (P < 0.05). Maximum enzyme release (56% of AST release and 58% of ALT release) was observed in infected co-cultures 48 hpi (Fig. 1A), probably due to the intense leishmanicidal activity of macrophages at this time point.

Morphological analysis by transmission electron microscopy showed a preserved aspect of the non-infected co-cultures, in which macrophages and hepatocytes were found to have intact membranes and organelles during all time points of the study (Fig. 1B). In the infected co-cultures, macrophages also displayed typical morphology without any alteration. On the other hand, degenerative alterations in hepatocytes involving the entire cytoplasm, especially on mitochondria showing changes its size and shape, were detected in infected co-cultures at 48 hpi (Fig. 1C). Parasites were never found inside the hepatocytes.

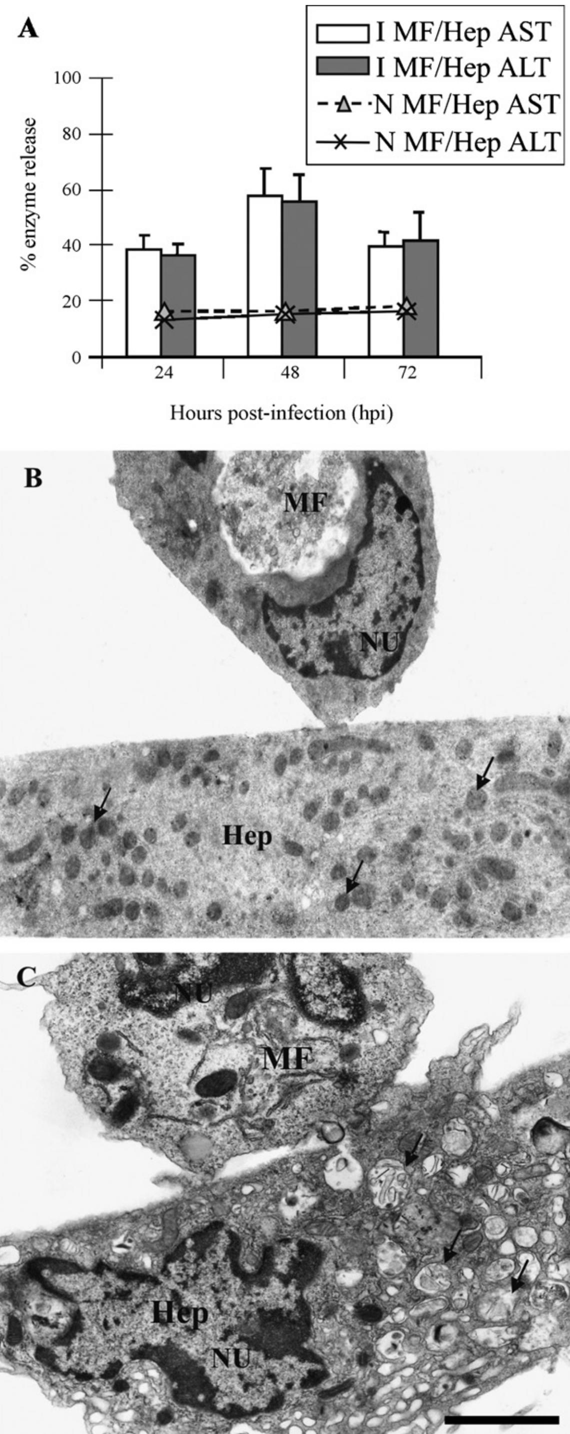

Fig. 1. Hepatocyte damage detection by AST and ALT release in infected (I) or non-infected (N) co-culture supernatants from Swiss mice (A). Bars indicate average ± standard deviation. These results represent a minimum of three experiments. B and C shows morphological analysis of macrophage (MF)–hepatocyte (Hep) co-cultures by transmission electron microscopy (TEM) at the moment of highest enzymatic release (48 hpi). The non-infected macrophages and hepatocytes displayed normal morphological characteristics with typical organelles such as mitochondria (arrow) and nuclei (NU) (B). (C) Shows infected co-cultures with cytoplasmic alterations as morphological changes in the mitochondria (arrow) in hepatocytes while the macrophages maintained normal morphology. Bar = 1 μm.

3.2. Quantitative analysis of proteolytic activities in co-cultures

The proteolytic activity present in cell extracts and cell-free supernatants from co-cultures during *L. chagasi* infection was measured at 24, 48 and 72 hpi at both pH 5.5 and 10. The results showed a statistically significant (P < 0.05) increase in the specific activity of all infected systems (supernatant and cell extract) from co-cultures at pH 5.5 (Fig. 2A) and pH 10 (Fig. 2B) during the course

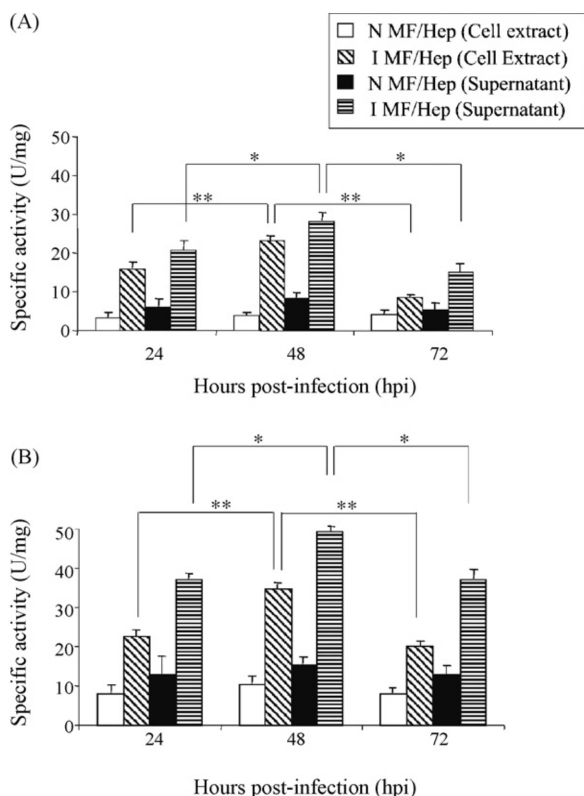

Fig. 2. Analysis of proteolytic activity from cell extracts and supernatant co-cultures during the course of infection at pH 5.5 (A) and pH 10 (B). Bars indicate average ± standard deviation. *Statistically significant differences ($P < 0.05$) between infected cell extracts obtained from co-cultures at 48 hpi and other time points. **Statistically significant differences ($P < 0.05$) between infected supernatant obtained from co-cultures at 48 hpi and other time points. This experiment was repeated at least three times.

of infection compared to non-infected co-cultures under the same conditions. The proteolytic activities were larger at pH 10 than pH 5.5 and the supernatant displayed more proteolytic activity than the cell extracts in all conditions analyzed (Fig. 2A and B). The maximum proteolytic activities, approximately 50 U/mg at pH 10 and 30 U/mg at pH 5.5, were detected in the infected supernatant at 48 hpi (Fig. 2A and B).

3.3. Treatment of co-cultures with peptidase inhibitors

To determine the involvement of the different protease classes on hepatocytes, infected co-cultures were pre-treated with three distinct peptidases inhibitors (PMSF, Phen and cystatin C), which specifically block serine, metallo- and cysteine peptidases. After the treatment, transaminase (AST and ALT) release was measured in the supernatant of co-cultures. A decrease in enzyme release was observed in the presence of all inhibitors but at different levels when compared to control system ($P < 0.05$) (Fig. 3). Infected co-cultures without treatment were utilized as controls. The metallopeptidase inhibitor was more efficient (20% and 18% of the AST and ALT release, respectively) than the serine (38% and 36% of the AST and ALT release, respectively) and cysteine (40% and 36% of the AST and ALT release, respectively) inhibitor when compared to control (Fig. 3). Non-infected co-cultures without treatment displayed lower levels of transaminase release (15% and 12% of the AST and ALT release, respectively) than the other infected systems studied (data not shown). Furthermore, there was no statistically significant difference between the levels of transaminases released in non-infected co-cultures with or without inhibitors (data not

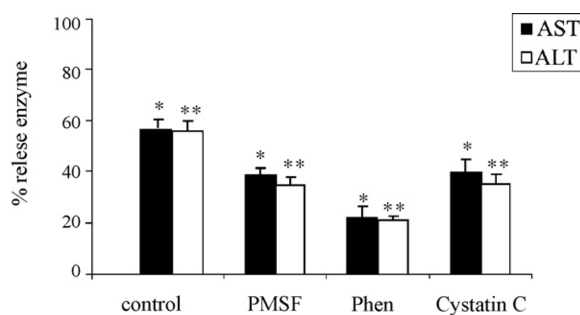

Fig. 3. Percentage of hepatic transaminases (damage markers) released in infected co-cultures treated with different peptidase inhibitors, 1 mM PMSF, 4 mM cystatin C and 10 mM Phen, at 48 hpi. Infected co-cultures without treatment were used as controls. These results represent a minimum of three experiments. *$P < 0.05$ for AST release in control compared to three inhibitors. **$P < 0.05$ for ALT release in control compared to three inhibitors.

shown), showing that the inhibitors were not toxic for the cells. The treatment of the infected co-cultures with all inhibitors combined reduced the enzymes release (22% and 20% of the AST and ALT release, respectively) to similar levels to those found in the treatment with the metallopeptidase inhibitor (data not shown).

3.4. TNF-α and TGF-β detection in co-culture supernatants

To verify whether TNF-α and TGF-β could be involved in the synthesis and/or conversion of metallopeptidase latent to active form observed in our systems, the levels of these cytokines were measured in the co-culture supernatants. Maximum levels (260 pg/ml) of TNF-α were detected in the infected co-culture supernatants at the beginning of the infection (at 24 hpi), probably when the metallopeptidases were being produced (Fig. 4A). Furthermore, results showed a statistically significant ($P < 0.05$) increase in the TGF-β levels present in infected co-culture supernatants compared to non-infected co-culture levels, which were low and remained similar during the entire course of the infection (Fig. 4B). Moreover, the maximum TGF-β level (1280 pg/ml) was detected at 48 hpi, when hepatocyte damage and the highest proteolytic activity were observed in co-cultures. The levels of TNF-α and TGF-β released by the hepatocytes were subtracted from the values obtained in the co-cultures, even though they were very low. Moreover, levels produced by macrophage cultures alone were similar (without statistically significant difference) to those levels detected in the co-cultures.

3.5. Qualitative analysis of peptidases

We performed zymograms from macrophages and co-cultures using cell extracts and culture supernatants in order to qualify the peptidases. In the macrophage cell extracts, we observed cysteine and metallopeptidase proteolytic activities (Fig. 5A). Cysteine peptidase activity, migrating at 30 kDa, was detected in non-infected and infected cell extracts from macrophages at pH 5.5 supplemented with DTT, a reducing agent that activates cysteine peptidases (Fig. 5A). This cysteine peptidase activity displayed 15% more intensity in infected cell extracts than in the non-infected when analyzed by densitometric assay (data not shown). Other cysteine peptidase activities migrating between 67 and 43 kDa were found in the infected macrophages. All cysteine peptidase activities were abrogated by E-64. Metallopeptidase activity with a molecular mass of 85 kDa was observed at pH 10 and blocked by 1,10-phenanthroline in cell extracts from infected and non-infected macrophages (Fig. 5A). The proteolytic activities detected in cell-free macrophage supernatants (Fig. 5A) were similar to macrophage

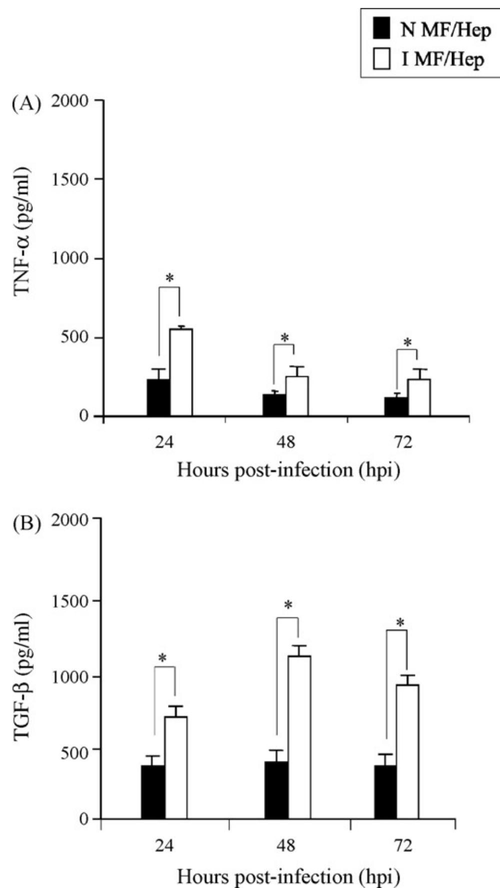

Fig. 4. TNF-α (A) and TGF-β (B) production in infected and non-infected co-cultures during the course of *L. chagasi* infection. Bars indicate average ± standard deviation. *Significant differences ($P<0.05$) between the cytokine levels produced by non-infected and infected co-cultures at all time points during the infection. These results represent a minimum of three experiments.

cell extracts (Fig. 5B), however, the densitometric analysis showed an increase of 48% in the activity for the 85 kDa metallopeptidase and approximately 16% in the activity for the 30, 43 and 67 kDa cysteine peptidase in infected supernatants compared to the same activities in the infected cell extracts. When compared to the non-infected system, we found an increase of 59% in metallopeptidase activity in the non-infected cell extracts and 42% in the non-infected supernatant (data not shown). Moreover, the metallopeptidase of 85 kDa was approximately 32% more intense in both infected cell extracts and supernatants than the 30 kDa cysteine peptidase activity, while in non-infected systems the expression levels between them were similar.

In the cell extracts obtained from non-infected co-cultures, cysteine peptidases migrating at 30 and 20 kDa at pH 5.5 supplemented with DTT (blocked by E-64) and one metallopeptidase activity migrating at 85 kDa at pH 10 (blocked by Phen) were observed (Fig. 6A). Metallopeptidase activities migrating at 67 and 43 kDa were detected in the cell extracts from infected co-cultures, active at both pH 5.5 (supplemented with DTT or E-64) and pH 10 (Fig. 6A). These metallopeptidase activities were inhibited in the presence of Phen at pH 5.5 and 10. Furthermore, two metallopeptidase activities migrating at 85 and 102 kDa were also observed in the cell extracts from infected co-cultures (Fig. 6A), but these activities were detected only at pH 10 and were blocked by Phen.

In the supernatant of non-infected and infected co-cultures, only one cysteine peptidase was observed migrating at 30 kDa at pH 5.5 supplemented with DTT (Fig. 6B). This activity was blocked by E-64. Metallopeptidase activities migrating at 102, 85 and 67 kDa, active at pH 5.5 with E-64 and at pH 10, were also observed in the supernatant of infected and non-infected co-cultures (Fig. 6B). These activities were blocked at pH 10 in the presence of Phen (Fig. 6B). Although they were inhibited in the presence of DTT at pH 5.5 (Fig. 6B), they were detected at pH 5.5 without DTT (data not shown). The 85 kDa metalloprotease activity was 36% more intense in the infected supernatant than in the infected cell extracts, 43% more intense in the non-infected cell extracts and 35% in the non-infected supernatant as also observed in the macrophage cultures (data not shown).

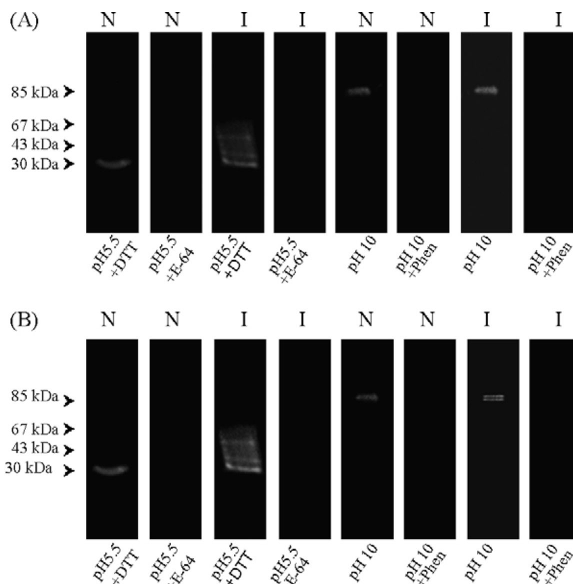

Fig. 5. Gelatin–SDS-PAGE analysis of cellular extract (A) and extracellular (B) peptidase expression in non-infected (N) and *L. chagasi*-infected (I) macrophage cultures at 48 hpi. Gel strips were incubated at pH 5.5 (supplemented with DTT or E-64) and pH 10 (with or without Phen). Numbers on the left indicate relative molecular mass markers (kDa).

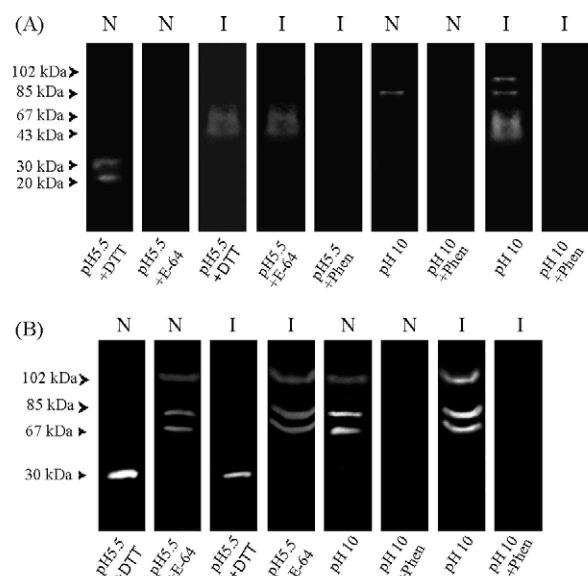

Fig. 6. Gelatin–SDS-PAGE analysis showing the expression of cellular (A) and extracellular (B) peptidases in non-infected (N) and *L. chagasi*-infected (I) co-cultures at 48 hpi. Gel strips were incubated at pH 5, pH 5.5 (supplemented with DTT or E-64) and pH 10 (with or without Phen). Numbers on the left indicate relative molecular mass markers (kDa).

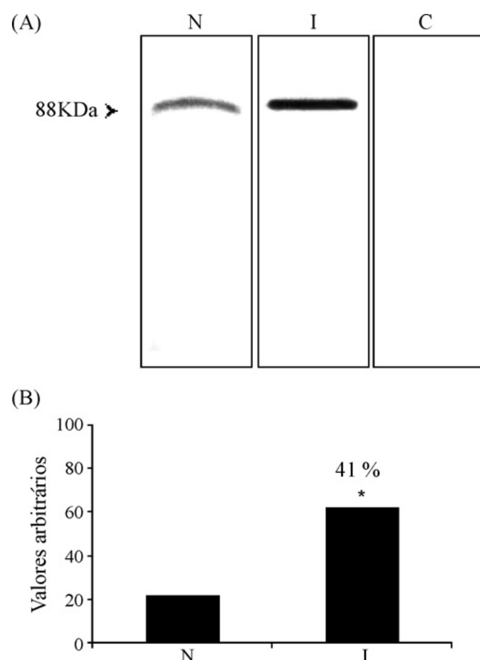

Fig. 7. Detection of MMP-9 in non-infected (N) and infected (I) co-culture supernatants. Protein bands from systems reacted positively with rabbit anti-MMP-9 antibody (A). The control (C) strip shown without the antibody (A). The molecular mass marker (kDa) is indicated on the left side of this figure. (B) Shows the analysis of bands obtained in the Western blot by optical densitometry (Image-Pro Plus program).

The proteolytic activities of intracellular amastigotes were analyzed separately, but no metallopeptidase activity migrating at 85 kDa was detected at pH 5.5 and 10. On the other hand, cysteine protease activities were observed migrating at 59 and 43 kDa (data not shown).

3.6. *Western blotting analysis*

Using Western blotting analysis with a rabbit anti-MMP-9 antibody, protein bands with a molecular mass of 88 kDa were recognized in the co-culture supernatants. The extracellular metallopeptidase was identified as matrix metalloproteinase-9 (MMP-9) in the non-infected and infected co-culture supernatants. This 88 kDa MMP-9 corresponds to the active form of this matrix metallopeptidase (Fig. 7A). The densitometry analysis of this 88 kDa metalloprotease also showed it to be augmented in the infected co-cultures compared to non-infected (Fig. 7B).

4. Discussion

Activated macrophages produce different proteolytic enzymes such as serine, cysteine and metallopeptidases for intracellular protein degradation. When these enzymes are in excess, they are released into the extracellular environment (Russell et al., 2002). Besides the peptidases, macrophages can release other mediators such as intermediary reactive oxygen and nitrogen (Takemura and Werb, 1984; Kausalya et al., 1996; Antoine et al., 2004). Together, all of these products account for intracellular cytotoxicity leading to the control of *Leishmania* growth. However, this may result in an undesirable extracellular cytotoxic effect on the adjacent tissue, as has been reported in murine models of viscerotropic *Leishmania* species infection (Kausalya et al., 1993, 1996; Costa et al., 2007). Other authors (Martin et al., 1999) have pointed to a significant impact of peptidases on the development of hepatic pathologies.

In the present work, the peptidases participating as mediators of hepatocyte damage in infected co-cultures was investigated. During the course of *L. chagasi* infection inside macrophages, there was an enhancement of transaminase release, followed by intracellular degenerative damage in co-cultivated hepatocytes involving several cytoplasmic organelles, mainly the mitochondria. The time correlated with such observations was 48 hpi, probably due to the intense parasiticidal activity at this time point. Since previous studies have shown that peptidases might play a role in the hepatocyte damage induced by macrophages (Kausalya et al., 1993, 1996; Costa et al., 2007), we decided to measure the peptidase activity in the cells as well as the supernatant of *L. chagasi*-infected macrophage co-cultures, and qualify whether the enzymes are potential inducers of hepatocyte damage.

Peptidase activity was higher in supernatants from infected co-cultures than in cell extracts, showing an increase of extracellular proteolysis in this model. This excess of secreted peptidases may mediate degradation of extracellular matrix proteins, hepatocyte ligands and/or other membrane associated proteins (McGwire et al., 2003). On the other hand, extracellular peptidases released by liver macrophages regulate signaling for cell division and extracellular matrix remodeling (Mohammed and Khokha, 2005). The interaction of cells with the extracellular matrix (ECM) is critical for the normal development and function of organisms. ECM plays a central role in maintaining the structural integrity of the organism's tissue (Chakraborti et al., 2003; Bedossa and Paradis, 2003).

Our results demonstrate that all three peptidase classes – cysteine, serine and metallopeptidase – are involved in the observed phenomenon, but they are not alone in this process; other mediators, such as ROS and RNI, produced by macrophages during the leishmanicidal activity have been implicated as well (Costa et al., 2007). The treatment of co-cultures with inhibitors specific to each peptidase class decreased the release of damage markers compared to cultures without treatment. Furthermore, metallopeptidase activities at pH 10 seem to play an important role in extracellular damage since its specific inhibitor most efficiently reduced hepatic transaminase release. Overexpression of several matrix-degrading metallopeptidases (MMPs), including MMP-1, MMP-3 and MMP-9, has been demonstrated in pathological processes and is particularly associated with macrophage-secreted enzymes (Galis et al., 1994; Rajavashisth et al., 1999). The evidence outlined above suggests that excess MMP activity may contribute to host damage during infectious diseases; therefore, the question arises whether modulating MMP activity can improve outcomes (Elkington et al., 2005).

Macrophages are a primary source of MMPs. The secretion of metallopeptidases from these cells depends largely on cell-to-cell and cell-to-matrix interactions, mediated by the engagement of integrins rather than inflammatory mediators (Saren et al., 1996; Galt et al., 2001). Despite this, metallopeptidase overexpression by macrophages can be induced by pro-inflammatory stimuli including cytokines (such as TNF-α), chemokines and bacterial wall components (Opdenakker et al., 2001; Dubois et al., 2002). The high levels of TNF-α detected at the beginning of the infection in our system could result in increased metallopeptidase synthesis. In addition, TGF-β is also a well-known mediator of matrix deposition and acts a stopping signal for hepatocyte cell division (Mohammed and Khokha, 2005). We observed an increase in TGF-β production in co-culture supernatants during the period of highest leishmanicidal activity by macrophages, the same time point at which the highest enzymatic activity was registered, suggesting that this cytokine could be participating in metallopeptidase activation/conversion and probably in matrix deposition by hepatocytes in culture.

Zymograms analysis of the macrophage cultures showed proteolytic activities (one metallopeptidase migrating at 85 kDa and some cysteine activities migrating between 67 and 30 kDa) in the

cell extracts and supernatants. Such activities were most intense in infected supernatants. The double band migrating at 85 and 82 kDa observed in the infected macrophage supernatants may be a result of the degradation of the 85 kDa band generating a smaller product by *Leishmania*'s proteolytic activities present in this system as described elsewhere (Ramu et al., 2007). Three new metallopeptidase activities were detected in cell extracts and supernatants obtained from co-cultures, most of which were active within a pH range of 5.5–10. The presence of DTT inhibited these metallopeptidase activities, even though they were active at pH 5.5. Previous work showed that metallopepetidase activities could be inhibited by DTT, which binds to disulfide bonds important for their activity (Xu et al., 2006).

Other proteolytic activities were detected in the co-cultures, which were not found in the macrophage cultures. They may have been produced and secreted by hepatocytes and/or amastigotes of *L. chagasi.* Two cysteine peptidase activities were detected from isolated intracellular amastigotes. One of these detected enzymes migrated at 59 kDa and could be related to those cysteine peptidase activities that migrated between 67 and 43 kDa observed in the infected cell extracts and supernatants from macrophage cultures. We could not be detected leishmanolysin, a 63 kDa metallopeptidase, in amastigote forms since the expression of this enzyme is much reduced in this parasite stage (Vermelho et al., 2007). Metallopeptidase activities migrating at 85 kDa were never detected in amastigotes or hepatocyte cultures. Such observation was restricted to macrophage cultures alone and when co-cultivated with hepatocytes; therefore such activity was probably due to macrophages.

Different proteolytic activities appeared in co-cultures when compared to macrophages. One hypothesis is that these proteolytic activities are products of a complex interaction between different cell types, resulting in modulation of their synthesis and secretion. Metallopeptidase activities showed greater band intensities in infected co-culture supernatants compared to all the other systems, consistent with our quantitative results, which detected the biggest proteolytic activity in the infected cell supernatants. Furthermore, the greatest proteolytic activity at 48 hpi at pH 10, at which time we verified metallopeptidase activities, indicates an increase in the secretion of this class of proteases. The 85 kDa metallopeptidase activity produced by macrophages and identified as MMP-9 was produced and secreted in large amount by *L. chagasi*-infected macrophages during their microbicidal activity and it may be co-participating in the hepatocyte damage.

The present study pointed to the release of peptidases during the leishmanicidal activity of macrophages, which may synergize with other mediators such as RNI and ROS leading to the hepatocyte damage observed *in vitro* in experimentally infected macrophages co-cultivated with hepatocytes. Despite the involvement of cysteine and serine in this phenomenon, metallopeptidases seem to make the largest contribution, which can be directly related to the breakdown of matrix compounds. The ECM architectural support is essential for the growth and survival of hepatocytes (Guguen-Guillouzo and Guillouzo, 1992). Further studies are necessary to clarify the role of these metallopeptidases during LV pathogenesis.

Acknowledgements

The authors would like to thank Dr. José Batista de Jesus for critical review of the manuscript. We also thank Bruno Ávila for his skillful computer imaging support. This work was supported by grants from Fundação Oswaldo Cruz (Fiocruz), Conselho de Ensino para Graduados- Universidade federal do Rio de Janeiro (CEPG-UFRJ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (MCT-CNPq).

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4.BLOCO 2

Parasitol Res (2009) 105:227–236
DOI 10.1007/s00436-009-1390-4

ORIGINAL PAPER

Fast high yield of pure *Leishmania* (*Leishmania*) *infantum* axenic amastigotes and their infectivity to mouse macrophages

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Received: 16 December 2008 / Accepted: 27 February 2009 / Published online: 18 March 2009
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Abstract *Leishmania* (*L.*) *infantum* (syn. *Leishmania chagasi*) is a dimorphic protozoan parasite that lives in promastigote and amastigote form in its sandfly vector and mammalian hosts, respectively. Here, we describe an in vitro culture system for the generation of a pure population of *L. infantum* axenic amastigotes after only 4 days incubation in culture medium supplemented with fetal calf serum, human urine, L-glutamine, and HEPES at 37ºC (pH 5.5). Ultrastrutural analysis and infection assays in two macrophage populations (Kupffer cells (KUP) and peritoneal macrophages (PM)) infected with axenic amastigotes demonstrated that they maintained morphological and biochemical (A2 expression) features and a similar infection pattern to tissue-derived *L. infantum* amastigotes. The susceptibility of the macrophage lines to axenic or tissue-derived amastigotes and promastigotes was investigated. We found a completely different susceptibility profile for KUP and PM. Liver macrophages, both KUP and immigrant macrophages, are intimately involved in the response to *L. infantum* infection; this difference in susceptibility is probably related to their capacity to eliminate these parasites. Our in vitro system was thus able to generate axenic amastigotes that resemble tissue-derived amastigotes both in morphology and infectivity pattern; this will help in further investigation of the biological characteristics of the host–parasite relationship as well as the process of pathogenesis.

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Abbreviations
PM Peritoneal macrophage
KUP Kupffer cells

Introduction

New World visceral leishmaniasis (VL) is a zoonotic disease that affects humans, dogs, and several wild animal species, with geographic distribution in tropical and subtropical areas. VL results from infection of the mammalian host with *Leishmania* (*L.*) *infantum* (syn. *Leishmania chagasi*; Mauricio et al. 2000) and is usually fatal if left untreated following clinical diagnosis (Handman 2001). Dogs constitute the main domestic reservoir of the parasite

and play a central role in the transmission cycle (Moreno and Alvar 2002).

L. infantum is an obligatory intracellular parasite that preferentially infects macrophages throughout the viscera and is readily found in the liver, spleen, and bone marrow (Bryceson 1996). The liver is the site of acute infection, whereas the spleen becomes a site of persistent infection. Control of hepatic infection requires a coordinated host response involving the development of cellular infiltrates and granulomas, with infected resident macrophages surrounded by immigrant macrophages and lymphocytes (Stanley and Engwerda 2007). In the liver, the two macrophage populations involved in infection are immigrant macrophages recruited from the blood and the resident macrophages, the Kupffer cells. These cells are the tissue macrophage in the liver and can be found lining the sinusoids, where they are a major target for *Leishmania* infection (Crocker et al. 1984). During infection, resident liver macrophages initially produce chemokines that recruit monocytes and neutrophils, important cells for effective control of parasite growth (Cotterell et al. 1999; Kaye et al. 2004). Later, CD4+ T cells and CD8+ T cells are attracted to the infection site and produce inflammatory cytokines that lead to the activation of infected cells for parasite elimination (Stanley and Engwerda 2007).

Several factors are involved in the macrophage–*Leishmania* interactions and the outcome of the disease (Nadere and McConville 2008), including the *Leishmania* species, the form of the parasite, and the type of macrophage population. *Leishmania* is a dimorphic organism that lives and replicates in the gut of sandflies as a promastigote or in mammalian cells as a rounded amastigote form.

Both the promastigote and amastigote forms can initiate infections (Kima 2007). When infections are initiated by promastigotes, the parasites transform into amastigotes within parasitophorous vacuoles, and the infections are then sustained by amastigote forms. The host cell response to the promastigote and amastigote forms can vary (Pearson et al. 1983). Such differences are expected since these parasite forms express stage-specific molecules such as lipophosphoglycan (LPG) and A2 gene. LPG is expressed on the surface of the promastigote form, but is minimally expressed by the amastigote form (Ilgoutz and McConville 2001) while that of the A2 gene is only express in amastigotes. A2 protein is expressed during protein tyrosine phosphorylation in amastigotes in determinate conditions of temperature and pH (Nascimento et al. 2003). Historically, promastigotes have been readily cultured in cell-free media, whereas amastigotes began to be axenically cultivated only a few years ago (Bates et al. 1992; Bates 1994; Zilberstein and Shapira 1994). Previous studies have shown that acidic pH and elevated temperature induce developmentally regulated changes in the shape and gene expression of promastigotes, generating amastigote forms that resemble animal tissue-derived amastigotes (Doyle and Dwyer 1993; Zilberstein and Shapira 1994; Debradant et al. 2004).

This paper describes a fast method to obtain large quantities of in vitro amastigotes from *L. infantum* promastigotes. Moreover, we verified the susceptibility of liver resident macrophages, the Kupffer cells (KUP) and peritoneal macrophages (PM), to infection by these *L. infantum* axenic amastigotes and also promastigotes and tissue amastigotes.

Materials and methods

Animals

BALB/c mice weighing about 20 g each were used to obtain primary cultures (peritoneal macrophages and Kupffer cells), and golden hamsters were utilized for maintaining and isolating the parasites. The animals were obtained from the animal care facility of the Oswaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil). All procedures involving animals in this study were reviewed and approved by the Oswaldo Cruz Foundation Ethics Committee (CEUA-FIOCRUZ).

Parasites

L. (*Leishmania*) *infantum* (strain MCAN/BR/2000/CNV-FEROZ) was isolated from a dog in the State of Espírito Santo, Brazil, and typed by multilocus enzyme electrophoresis at the *Leishmania* Collection at the Oswaldo Cruz Institute, RJ, Brazil (CLIOC, WDCM 731). Parasite infectivity was maintained through inoculation in golden hamsters.

Intracellular amastigotes were obtained from infected hamster spleens 3 to 6 months after infection. All procedures were performed aseptically at 4ºC. Each infected spleen was excised and ground with 40 ml Hank's balanced salt solution (HBSS) in a tissue grinder. The suspension was centrifuged at 150×*g* for 5 min to remove tissue debris. Then, the supernatant was centrifuged at 1,000×*g* for 20 min, and the amastigote pellet was suspended in buffer and centrifuged again at 1,000×*g* for 20 min. The pellet with the amastigotes was suspended in medium for experiments.

To obtain promastigotes, spleens removed from infected hamsters were chopped and cultured in biphase NNN blood agar medium/Schneider's *Drosophila* culture medium (Sigma-Aldrich St. Louis, MO, USA) containing 10% fetal calf serum (FCS; Sigma-Aldrich) at pH 7.2 and 25ºC. After a few days, promastigotes were observed in the culture medium.

Axenic amastigotes were obtained from stationary phase promastigotes in the first stage after adaptation at pH 5.5

and 37ºC. These were only utilized to analyze interactions with host cells after establishment of growth kinetics.

Growth kinetics

Promastigote cultures at pH 5.5 and 37ºC were used for growth kinetics analysis. Cultures were initiated with 1×10^6 cells/ml in 25 cm^2 tissue culture flasks containing 5 ml of medium. The culture medium was Schneider's *Drosophila* supplemented with 20% FCS, 2% human urine, 0.5% L-glutamine (Sigma-Aldrich), and 0.5% HEPES (Sigma-Aldrich). Promastigote cultures were analyzed from the moment of the culture initiation (time 0), when the temperature was increased from 25ºC to 37ºC and the pH of the culture medium decreased, until the tenth day. The curve obtained was used to choose the time for harvesting of axenic parasites and subsequent use in interaction studies.

Together with the growth curve, the parasite forms were observed by light microscopy and classified as promastigotes, an intermediate form, and a round form (amastigotes) by counting in a Neubauer chamber during the same period.

Ultrastructural examination

Amastigotes located inside the spleen cells, intracellular amastigotes removed from the spleen, promastigotes obtained from infected spleens in culture medium, and axenic amastigotes were analyzed by transmission electron microscopy for analysis of morphology and macrophage–parasite interactions. These samples were fixed for 1 h in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer supplemented with 3.5% sucrose (Sigma-Aldrich). They were then washed with 0.1 M cacodylate buffer and post-fixed with 1% OsO_4 in the same buffer. Samples were then washed twice in buffer, dehydrated in graded acetone, and embedded in Epon. These samples were observed using a Zeiss EM10C transmission electron microscope.

Macrophage isolation

Mouse PM were obtained from mice as previously described (Araujo-Jorge and de Souza 1986). Briefly, animals were killed using CO_2, and the peritoneal cavity was washed with 10 ml of RPMI culture medium (Cultilab, São Paulo, SP, Brazil) at 4ºC. For each experiment, ten mice were utilized. Cells were pooled and counted using a Neubauer chamber. Cells were seeded in tissue culture flasks (2×10^6 macrophages/flask) or on glass coverslips (2×10^5 macrophages/coverslip) and maintained in RPMI culture medium with FCS (Sigma-Aldrich) at 37ºC in a humidified atmosphere containing 5% CO_2.

Analysis of axenic amastigote infectivity

To determine the infectivity of axenic amastigotes in their stationary phase (4 days), peritoneal macrophages were used. The percentage of infected peritoneal macrophages and the number of intracellular parasites were analyzed after 2 and 24 h of co-incubation at a ratio of one, five, and ten parasites per peritoneal macrophage. After co-incubation, coverslips were washed three times in culture medium, fixed in Bouin's solution, and stained with Giemsa (Merck).

To verify if axenic amastigotes obtained on the fourth day of culture maintain their infectivity after one, two, three, four, and five passages in axenic culture (Schneider's *Drosophila* supplemented with 20% FCS, 2% human urine, 0.5% L-glutamine, and 0.5% HEPES medium), they were utilized after each passage to infect peritoneal macrophages (ratio of 10 amastigotes:1 peritoneal macrophage). After co-incubation at 24 h, coverslips were washed three times in culture medium, fixed in Bouin's solution, and stained with Giemsa (Merck).

Values obtained in these experiments were expressed as the percentage of macrophages infected by parasites and also the total number of amastigotes within 100 peritoneal macrophages. Experiments were performed in triplicate chambers, and a minimum of 300 macrophages were counted from each coverslip.

Kupffer cell isolation

Kupffer cells were isolated using a slightly modified version of the pronase–collagenase perfusion method (Seelaender et al. 1999). Briefly, after intraperitoneal anesthesia with Zoletil (50 mg/kg IM), the liver was perfused through the portal vein with Ca^{2+}-free HBSS to completely remove blood. The solution was then exchanged with 0.4% pronase (Calbiochem, USA) in HBSS at 10 ml/min. After this, a new solution containing HBSS with 0.02% collagenase type 1 (Sigma-Aldrich) was perfused at 10 ml/min. The organ was then detached from the perfusion device and incubated at 37ºC for 5 min with 0.1% pronase and 0.01% DNase I (Sigma-Aldrich) in HBSS. The tissue was minced into small pieces, forming the liver homogenate. The liver homogenate was filtered through a 125-μm nylon mesh to remove undigested tissue, and the cell suspension was centrifuged three times at 400×*g* for 7 min at 4ºC. Kupffer cells were separated by 28.7% Nycodenz differential gradient centrifugation (Sigma-Aldrich). The cells were growth on culture slides in RPMI medium containing 10% FCS and fixed for immunofluorescence. The F4/80 antibody (Sigma-Aldrich), a resident macrophage marker, was used to confirm that the cells were Kupffer cells.

Immunoblotting

Axenic amastigote and promastigote forms of *L. infantum* were washed three times with PBS and resuspended with 150 µl of lysis buffer [2 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM $NaHCO_3$, 10% Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA)] containing 1% Triton x-100. Samples were frozen in −80°C until use, and protein concentration was measured using the BCA protein quantification kit (Pierce, Rockford, IL, USA). Protein (20 µg) was loaded and resolved in 10% SDS-polyacrylamide gels. After resolving, proteins were transferred to nitrocellulose membranes (BioRad). The membranes were blocked with 5% skim milk in TBST (TBS and 0.5% Tween 20) for 30 min and then incubated with serum from rhesus monkeys immunized with recombinant A2 protein diluted in TBST with 5% skim milk overnight at 4°C. The rA2 protein was used as positive control. Both immune serum and rA2 was kindly provided by Dr. Gabriel Grimaldi from Oswaldo Cruz Institute, Rio de Janeiro, Brazil. Membranes were washed with TBST and incubated with secondary rabbit anti-monkey IgG HRP-conjugated antibody (Sigma-Aldrich) for 1 h at 25°C followed by washes with TBST and incubation with chemoluminescent kit ECL (Pierce) and exposed to X-ray film.

Susceptibility of *L. infantum* to peritoneal macrophages and liver resident macrophages

For in vitro infection of macrophages (peritoneal and Kupffer cells), *L. infantum* promastigotes and intracellular and axenic amastigotes were utilized. Peritoneal macrophages and Kupffer cells were cultivated for 24 h and then infected at a ratio of ten parasites per macrophage. Macrophages and parasites were incubated together in RPMI medium for 1 h at 25ºC. Cultures were then washed three times with RPMI culture medium to remove non-adherent and non-internalized parasites. These cultures were maintained until 120 h post-infection (hpi) in culture medium with 10% FCS at 37ºC in a 5% CO_2 atmosphere. The culture medium was maintained without changes during the entire experimentation period. After incubation of infected macrophages and parasites, coverslips were fixed in Bouin's solution and stained with Giemsa (Merck). The percentage of infected macrophages in cultures was determined by counting at least 300 cells using light microscopy. These interactions were also observed by transmission electron microscopy. Experiments were repeated three times to confirm that experimental data were reproducible.

Statistical analysis

All experiments were carried out in triplicate and repeated at least three times. The mean values and standard deviations for all numerical data were calculated. The data were analyzed using Student's *t* test. Simple linear regression analysis was performed to calculate correlations. Differences with $P<0.05$ were considered statistically significant.

Results

Development of *L. infantum* axenic amastigotes

During the adaptation period when *L. infantum* promastigotes differentiated into axenic amastigotes and grew, they exhibited a variety of morphological forms as observed by phase-contrast microcopy. This adaptation process was initiated (time 0) when promastigotes (1×10^6) were incubated in culture medium at pH5.5 and 37ºC (Fig. 1a). At time 0, 100% of cells displayed typical promastigote morphology with elongated ellipsoidal bodies and apically disposed flagella (Fig. 1b). After 24 h of adaptation in acid conditions (day 1), they displayed different morphologies, including shortened forms (10%), intermediate forms (63%), and round forms (27%). This mixed population of amastigotes and micromastigote/spheromastigote-like forms appeared to last

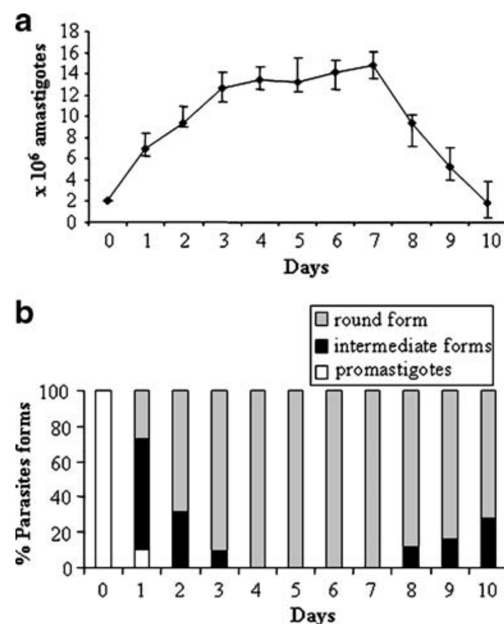


Fig. 1 Generation and growth of *L. infantum* axenic amastigotes in vitro. **a** Growth curve of *L. infantum* in triplicate cultures initiated with 1×10^6 cells/ml of *L. infantum* promastigotes. Samples were taken at the given times for cell counting. Promastigotes were grown and proliferated at 37°C in pH5.5 buffered medium supplemented with FCS, HEPES, L-glutamine, and human urine. *Bars* indicate mean± standard deviation. **b** Amount of different parasitic forms (promastigotes, intermediate, and round forms) was calculated by counting in a Neubauer chamber. Values represent the means of three separate determinations for each of three cultures per time point

until the third day. On the second and third days, 32% and 9% of parasites displayed intermediate forms and 68% and 91% showed round forms, respectively. None displayed shapes similar to the promastigote forms at these times (Fig. 1b). The growth curve showed a log phase until the fourth day when the stationary phase was initiated (Fig. 1a).

On the fourth day, the parasites were approximately 100% amastigotes. This phase lasted until the seventh day, when 100% of the parasites were round amastigotes (Fig. 1b). No other shapes were observed. After the seventh day, the number of parasites decreased, marking the start of a new phase (Fig. 1a).

Ultrastrutural analysis of different forms of parasites

The morphology of intracellular amastigotes, promastigotes, and axenic amastigotes was analyzed by electron microscopy to verify the success of in vitro amastigogenesis. Intracellular amastigotes, as shown in Fig. 2a, were found inside narrow parasitolophorous vacuoles and displayed oval bodies, small flagella, kinetoplasts, and nuclei with peripherally condensed chromatin. After isolation, parasites displayed oval bodies, kinetoplasts, and small flagella inside a large flagellar pocket (Fig. 2b).

Promastigotes obtained after isolation from infected spleen fragments, the same organ utilized to isolate intracellular amastigotes, showed elongated bodies, nuclei with peripheral chromatin condensation, rough endoplasmic reticula with normal profile, long flagella emerging from the flagellar pocket, kinetoplasts, and only one mitochondrion (Fig. 2c).

Axenic amastigotes obtained from promastigotes on the fourth day after the change in the conditions (pH5.5 and 37ºC), displayed oval bodies with two nuclei, indicating cellular division. As in the intracellular form, kinetoplasts and small flagella inside the large flagellar pocket were also present (Fig. 2d).

Infectivity of axenic amastigotes and their continuous propagation

The infectivity of parasites obtained on the fourth, fifth, sixth, and seventh days of the stationary phase when 100% of these parasites displayed round forms was verified to infect peritoneal macrophages (ratio 1:1, 1:5, and 1:10 macrophage/parasite). There was no statistically significant difference between the number of parasites after 2 versus 24 h of incubation with macrophages in all rations

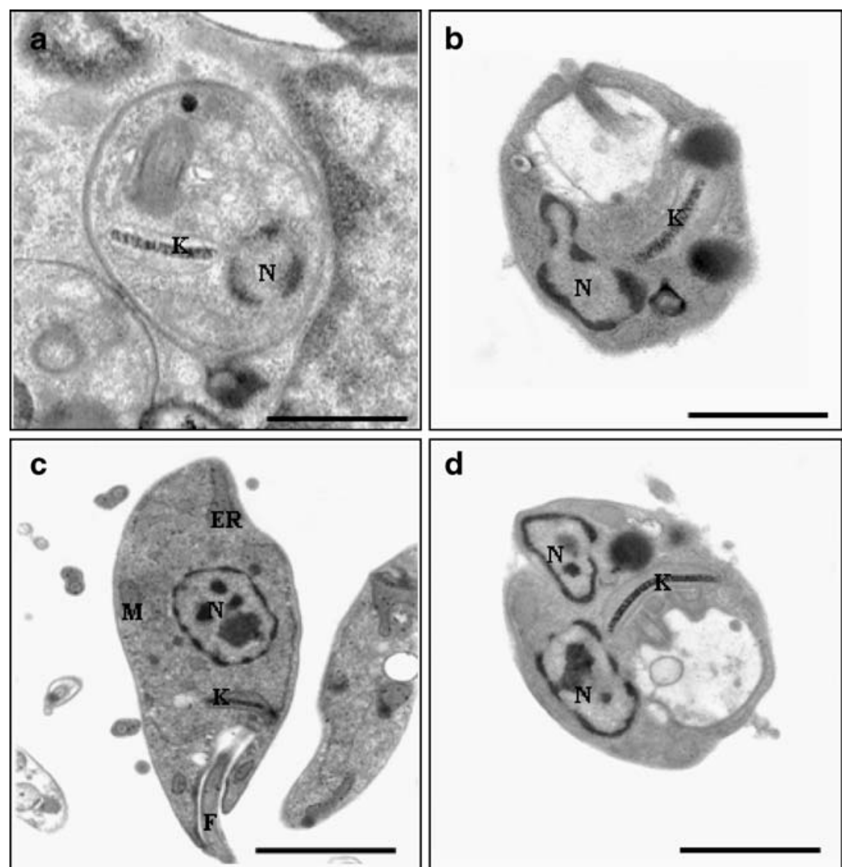


Fig. 2 Comparative analysis of different forms of *L. infantum*. **a** Amastigotes inside spleen cells displaying small flagella (*F*), kinetoplasts (*K*), and nuclei (*N*). **b** Amastigotes obtained from spleen cells displaying kinetoplasts (*K*), small flagella (*F*), and large flagellar pockets. **c** Promastigotes displaying nuclei (*N*) with peripheral chromatin condensation, endoplasmatic reticula (*ER*), kinetoplasts (*K*), and mitochondrion (*M*). **d** Axenic amastigotes obtained from promastigotes at fourth day at 37ºC in pH5.5 buffered medium supplemented with FCS, HEPES, L-glutamine, and human urine displaying two nuclei (*N*), kinetoplasts, and small flagella (*F*) inside the large flagellar pocket. Scale bar=1 µm

macrophage/parasite and also between the different ratio of macrophage/parasite utilized. After 2 h of interaction, approximately 98% of peritoneal macrophages were infected, and the number of intracellular parasites was approximately nine parasites/cell, regardless of the parasite used. The percent of infected macrophages remained high (approximately 95%) after 24 h of co-incubation but the number of parasite/macrophages decreased (six parasites/cell; Fig. 4c). These data are present at Fig. 3b together with other analyzed moments.

Continuous propagation of axenic amastigotes obtained on the fourth day was observed after the first, second, third, fourth, and fifth passages (Table 1). There were no statistically significant differences among the axenic amastigotes obtained from different passages in the percentage of infected peritoneal macrophages (96–91% after five passages) or in the number of intracellular parasites (six to seven parasites/macrophage after five passages) at 24 hpi (Table 1).

Expression of A2 protein in axenic amastigotes

Immunoblots demonstrated that axenic amastigotes (line 2) expressed two bands that migrated at 75 and 60 kDa (Fig. 3). The band that migrated at 75 kDa was also detected in the positive control (line 3), using a recombinant A2 protein. The extracts of promastigotes (line 1) showed no reaction for this immune serum (Fig. 3).

Susceptibility of two macrophage populations to parasites

Three forms of parasites (promastigotes, Fig. 2c; intracellular amastigotes, Fig. 2b; and axenic amastigotes, Fig. 2d) as observed by electron microscopy were used to infect peritoneal and liver resident (Kupffer cells) macrophages.

The highest percentage of peritoneal macrophages (98%) and Kupffer cells (86%) infected with *L. infantum* promastigotes occurred at the same time (48 hpi), when the highest amount of intracellular parasites was also

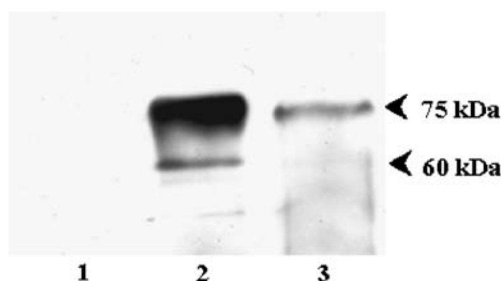


Fig. 3 Immunoblotting analysis of promastigotes and axenic amastigotes of *L. infantum* revealed by immune serum of rhesus monkey immunized with rA2 protein. *Line 1* promastigotes. *Line 2* axenic amastigotes. *Line 3* rA2 protein. *Numbers on the left* indicate relative molecular mass markers (kDa)

Table 1 Axenic amastigotes' infectivity in peritoneal macrophages after passages in axenic culture

	1st Passage	2nd Passage	3rd Passage	4th Passage	5th Passage
% Infected macrophages	96±1.3	94±0.8	95±1.7	92±1.5	92±0.4
Parasite/ macrophage	7±0.5	7±0.2	6±0.7	6±0.5	6±0.8

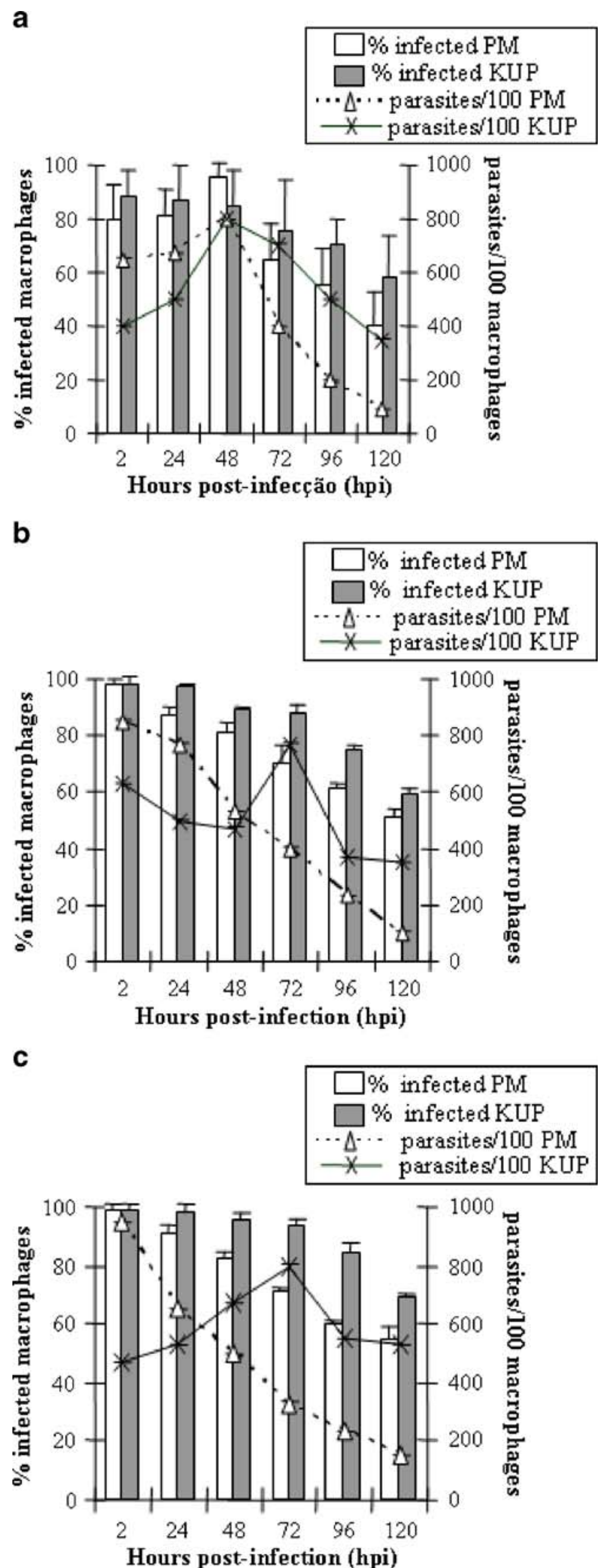
The percentage of infection and the number of parasite/macrophage were determined by quantification by light microscopy in triplicate experiments

observed in peritoneal macrophages (800 parasites/100 macrophages) and Kupffer cells (780 parasites/100 macrophages; Fig. 4a). There were statistically significant differences ($P<0.05$) in the percentage of infected peritoneal macrophages at 48 hpi versus later times (72 hpi, 66%; 96 hpi, 58%; and 120 hpi, 41%), whereas the percentage of infected Kupffer cells was only significantly different ($P<0.05$) between 48 hpi and the last time point (120 hpi) observed in this study (59%; Fig. 4a). After 48 hpi, the number of intracellular parasites decreased inside Kupffer cells and peritoneal macrophages (Fig. 4a). At 120 hpi, there were four times as many parasites inside Kupffer cells (four parasites per cell) as there were in peritoneal macrophages (one parasite per cell; $P<0.05$). Parasitic growth curves were similar in the two populations of macrophages.

The two populations of macrophages were also infected with intracellular amastigotes (Fig. 4b), and the percentage of infected peritoneal macrophages showed a significant difference ($P<0.05$) between the highest percentage at 2 hpi (98.5%) and the values at 96 hpi (62%) and 120 hpi (51%; Fig. 4b). The percentage of infected Kupffer cells was only significantly different ($P<0.05$) between 2 hpi (98%), the time with the highest percentage, and 120 hpi (60%; Fig. 4b). The greatest amount of intracellular parasites was detected at 2 hpi in peritoneal macrophages (840 parasites/100 cells) and 72 hpi in Kupffer cells (780 parasites/cells). The course of the axenic amastigote infection in peritoneal macrophages was completely different from that in Kupffer cells. The number of parasites in peritoneal macrophages decreased during the period studied while the number of parasites in Kupffer cells increased to 48 hpi and only decreased after this time (Fig. 4b). At 120 hpi, there were approximately four parasites per Kupffer cell, whereas there was only one parasite per macrophage in peritoneal macrophages ($P<0.05$; Fig. 4b).

The course of axenic amastigote infection in peritoneal macrophages and Kupffer cells (Fig. 4c) was similar to the infection course for intracellular amastigotes (Fig. 4b). The maximum number of intracellular parasites (980 parasites/100 macrophages) and percentage of infected macrophages

Fig. 4 Susceptibilities of peritoneal macrophages (*PM*) and Kupffer cells (*KUP*) to different forms of *L. infantum*. The infection patterns of promastigotes (**a**), intracellular amastigotes (**b**), and axenic amastigotes (**c**) were observed in both macrophages populations. The percentage of infected macrophages is depicted with *open bars* (*PM*) or *shaded bars* (*KUP*), and the values that represent the number of parasites/100 macrophages are shown as *straight* (*PM*) and *dashed* (*KUP*) lines. *Bars* indicate average±standard deviation. These results represent the minimum of three experiments



(99%) occurred at the same time point (2 hpi) in peritoneal macrophages (Figs. 4c, 5a and c). There was a statistically significant ($P<0.05$) decrease between the first three time points analyzed (2, 24, and 48 hpi) and the last two time points (96 and 120 hpi). The highest number of intracellular parasites in Kupffer cells (810 parasites/100 macrophages) and the percentage of infected macrophages (98%) were also simultaneously observed at 72 hpi (Figs. 4c, 5b and d). There were no significant differences among the percentage of infected macrophages observed at 2, 24, 48, and 72 hpi in Kupffer cells, but a statistically significant decrease ($P<0.05$) was observed at 120 hpi. At 120 hpi, there was also a statistically significant difference ($P<0.05$) in the number of intracellular parasites in peritoneal macrophages (approximately one parasite per cell) as compared to the number of parasites inside Kupffer cells (approximately five parasites per cell).

Discussion

The first successful long-term propagation of amastigote-like *Leishmania*, *Leishmania pifanoi*, in a cell-free medium was reported in the early 1980s (Pan 1984). Since then, attempts to cultivate Old and New World *Leishmania* amastigotes in cell-free media have been carried out by many groups (Doyle et al. 1991; Zilberstein et al. 1991; Bates 1993; Pan et al. 1993; Zilberstein and Shapira 1994; Hodgkinson et al. 1996; Teixeira et al. 2002; Tavares et al. 2005; Walker et al. 2006). Some have reported viscerotropic amastigote transformation in axenic cultures of *Leishmania donovani* (Al-Bashir et al. 1992; Debrabant et al. 2004; Saar et al. 1998); however, few studies have examined differentiation of New World *L. infantum*, another visceral species, to axenic amastigotes (Sereno and Lesmere 1997; Teixeira et al. 2002).

Teixeira et al. (2002) reported that the maximum rate of *L. chagasi* axenic amastigote transformation was approximately 90% after 13 days of culture at pH5.5 and 37ºC, whereas in this work, we showed 100% generation of axenic amastigotes after 4 days in our supplemented culture medium. Supplements such as human urine, HEPES, and L-glutamine have not been used in any other previous studies but may have contributed to our success in generating pure

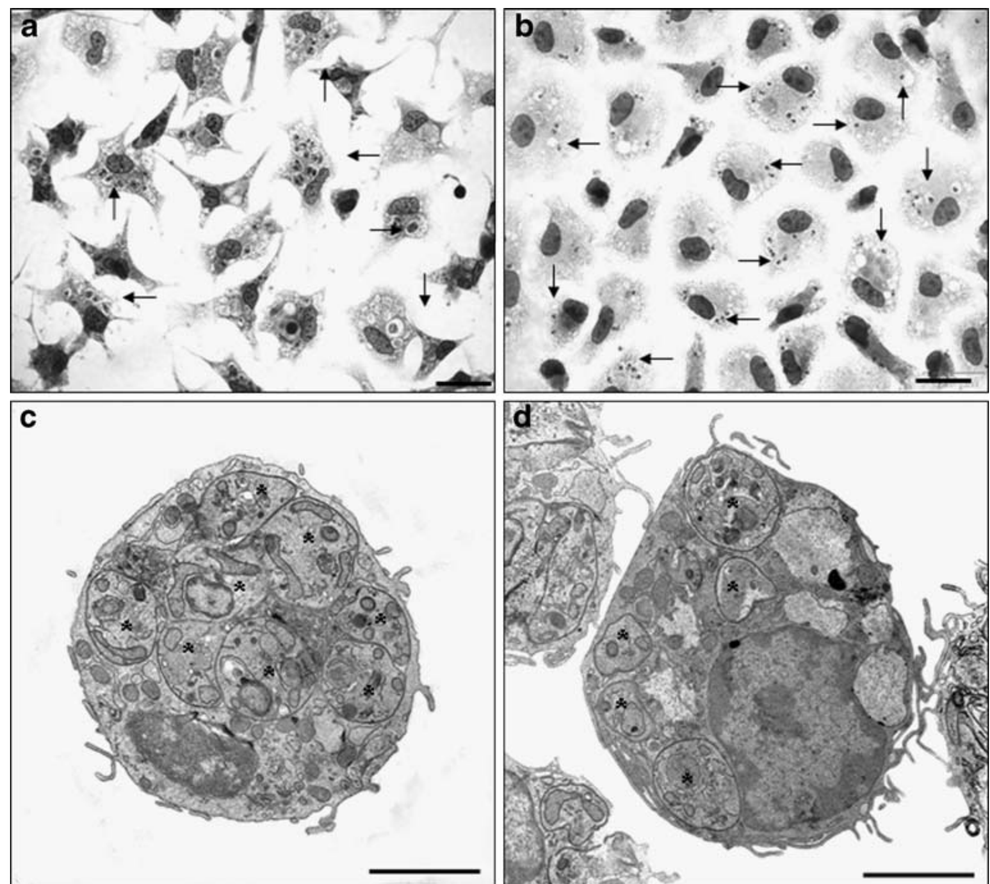


Fig. 5 *L. infantum* axenic amastigote infection in peritoneal macrophages and Kupffer cells. Light and transmission electron microscopic analysis of peritoneal macrophages (**a** and **c**) and Kupffer cells (**b** and **d**) show the highest number of intracellular parasites (*arrow* and *asterisk*) and the highest percentage of infected macrophages at 2 h post-infection (hpi) and 72 hpi, respectively. **a** and **b** have scale bars that represent 10 µm, while **c** and **d** have scale bars that represent 1 µm. Depicted results represent at least three experiments

L. infantum amastigotes in such a short period of time. A previous study showed that human urine is a strong promoter of *Leishmania* growth in vitro (Iqbal et al. 2006). The other two supplements, HEPES and L-glutamine, probably also contributed direct or indirectly to amastigote differentiation and growth as constituents of the culture medium. L-Glutamine is a non-essential amino acid that regulates protein synthesis and degradation, thereby exerting an important role in the control of catabolism and anabolism. Moreover, glutamine can be utilized as energy by parasites, thereby promoting their growth (Foster et al. 1989). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is an organic chemical buffering agent that helps to maintain pH (O`Daly and Rodriguez 1988). In our study, the addition of HEPES to the culture medium was important for maintaining the pH at 5.5, a condition that is essential for amastigote differentiation and growth.

Acidic pH and elevated temperature are critical for triggering differentiation of *Leishmania* from promastigotes to amastigotes (Saar et al. 1998), but other factors such as FCS have also been observed to be important for differentiation and growth of different *Leishmania* species (Teixeira et al. 2002). Our in vitro culture system was designed to mimic some of the environmental conditions that intracellular *L. infantum* amastigotes would encounter within the phago-lysosomal system of host cells in vivo (e.g., acidic pH, elevated CO_2, physiological temperature, and high potassium/low sodium). We detected a stationary phase with a duration of 4 days after *L. infantum* differentiation during the growth curve. All amastigotes obtained during this period were viable as determined by light microscopy. Transmission electron microscopy confirmed the viability of in vitro-grown amastigotes and confirmed morphological features of *L. infantum* in in vivo/tissue-derived amastigotes. The bodies, nuclei, cytoplasmic organelles, flagellar pockets, and kinetoplasts were similar to those observed in intracellular amastigotes found in host spleen cells and isolated intracellular amastigotes freshly removed from host cells. A previous study showed that *L. donovani* amastigotes generated in vitro maintained their biological and biochemical properties (Debrabant et al. 2004).

In the present study, we also showed that axenic amastigotes obtained in the stationary phase had significant infectivity in mouse peritoneal macrophages in vitro. These axenic amastigotes were also infectious in hamsters (data not shown). Infectivity analysis is essential for complete characterization of axenic culture-derived *Leishmania* amastigotes. We performed a comparative analysis of the infectivity of axenic amastigotes obtained on the first day of

the stationary phase and also of intracellular amastigotes and promastigotes in two important populations of macrophages involved in LV.

L. infantum is a visceralizing *Leishmania* species whose promastigotes enter vertebrate hosts through the bite of infected phlebotomine sandflies, after which they are converted to the amastigote form. Amastigotes divide within macrophages, primarily in the liver and spleen, until the host carries an enormous parasite burden in the late stage of disseminated leishmaniasis (Haidaris and Bonventre 1981). The mechanism involved in the dissemination and visceralization of the parasites remains unclear (Engwerda et al. 2004). Two distinct macrophage populations are involved in liver infections: immigrant macrophages and Kupffer cells (Kausalya et al. 1993). Immigrant macrophages respond to *Leishmania* infection by activating both oxygen dependent and independent microbicidal pathways, as do peritoneal macrophages. In the liver, the acute site of infection, Kupffer cells are primarily responsible for storing the parasite, inducing a controlled and coordinated local response, promoting parasite clearance, and generating resistance to infection (Engwerda et al. 2004).

In this work, we analyzed the pattern of *L. infantum* infection of peritoneal macrophage and Kupffer cells. Promastigote infection in both macrophage populations was similar, but peritoneal macrophages eliminated parasites more efficiently than Kupffer cells, in which a large number of parasites still remained at the end of the study. On the other hand, the pattern of infection by amastigotes (intracellular and axenic) was completely different from that by promastigotes in both macrophage populations. The observed difference was probably due to the distinct metabolic pathways and virulence mechanism at each stage within mammalian hosts (Naderer and McConville 2008).

The molecular basis for regulating differentiation from promastigotes to amastigotes is still not understood; however, previous studies (Santora et al. 2000; Nascimento et al. 2003) showed that the tyrosine phosphorylation plays a significant role in *Leishmania* differentiation. The signal transduction pathways involving protein tyrosine phosphorylation regulate directly or indirectly the expression of A2 protein. The expression of A2 is dependent on both an increase in temperature and a decrease in pH resulting in an increasing stability of A2 mRNA in amastigotes (Charest and Metlashewski 1994). In our study, we detected A2 protein by immunoblotting, using serum from rhesus monkeys immunized with rA2 protein in *L. infantum* axenic amastigotes, confirming that these parasites generated with our methodology were amastigotes and not amastigotes-like. The band that was also observed at 60 kDa should be a proteolytic activity recognized by monkey serum utilized in these experiments.

Axenic and intracellular amastigote infections displayed a different pattern between peritoneal macrophages and Kupffer cells. At the end of the experiment, we always observed a larger number of parasites inside Kupffer cells than in peritoneal macrophages. The efficiency of elimination by peritoneal macrophages is probably related to their ability to trigger leishmanicidal activity associated with multiple pathways including reactive oxygen intermediates, reactive nitrogen intermediates, and lysosomal enzymes. This is particularly characteristic of bloodstream-derived macrophages. On the other hand, Kupffer cells cannot respond to *Leishmania* infection with the release of oxygen metabolites (Kausalya et al. 1996). It is thus likely that Kupffer cells follow an oxygen-independent pathway to limit infection.

Intracellular amastigote infection and axenic amastigote infection showed similar patterns and virulence in both macrophages populations. The susceptibilities of the two macrophages populations were also similar. In conclusion, the in vitro culture system utilized in this work provides a useful tool for generating uniform populations of *L. infantum* axenic amastigotes in large quantities in a short period of time. Axenic amastigotes kept their virulence in both tested macrophage populations. The interaction of *L. infantum* axenic amastigotes with host cells, especially Kupffer cells, constitutes an important research model for further investigation of the biological properties of this lethal human pathogen and its pathogenesis in the liver.

Acknowledgments The authors would like to thank Bruno Ávila for his skillful computer imaging support and Daniel Adesse for his support in the immunoblotting assay. This work was supported by grants from Fundação Oswaldo Cruz (Fiocruz), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (MCT-CNPq).

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5.BLOCO 3

Parasite Immunology

Impaired peroxynitrite formation in Leishmania infantum-infected Kupffer cells prevents hepatocyte damage

Journal:	*Parasite Immunology*
Manuscript ID:	draft
Manuscript Type:	Original Paper
Date Submitted by the Author:	
Complete List of Authors:	Costa, Juliana; Instituto Oswaldo Cruz, Laboratório de Pesquisas em Leishmaniose Soares, Renata; Instituto Oswaldo Cruz, Laboratório de Ultra-Estrutura Celular Meirelles, Maria de Nazareth; Instituto Oswaldo Cruz, Laboratório de Ultra-Estrutura Celular Porrozzi, Renato; Instituto Oswaldo Cruz, Laboratório de Pesquisas em Leishmaniose
Key Words:	Leishmania, Macrophage

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Abstract

Leishmania infantum must survive the antimicrobial activity of host macrophages to establish infection. In the liver, two macrophage populations, immigrant macrophages (IM) and resident macrophages (RM), respond to this infection by producing reactive nitrogen species (RNS), reactive oxygen species (ROS) and lysosomal enzymes. The mediators released by IM can have a cytotoxic effect on hepatocytes. However, the effect of these mediators in *Leishmania* infection on RM cells has not been explored. Hepatic transaminases were present at similar levels in the supernatants of infected and uninfected Kupffer cell (KC) co-cultures. Hepatocye damage should be directly related to a reduction in ROS production by infected KC. In addition, KC displayed a Th2 immune response pattern, with increased IL-10 levels at the beginning of the infection. Treatment of co-cultures with molecules involved in the activation of leishmanicidal pathways demonstrated that hepatocyte damage only occurs in the presence of ROS and RNS, with the formation of peroxynitrite. These results were confirmed by utilizing mixed co-cultures with two macrophage populations on the same tissue culture plate. Our data suggest that KC infected by axenic *L. infantum* amastigotes do not cause hepatocyte damage because they have an impairment in ROS production, and consequently, in peroxynitrite formation.

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Keywords: Kuppfer cells, Nitric oxide, Peroxynitrite; *Leishmania Leishmania infantum*;

Abbreviations: Peritoneal macrophage (PM); Kupffer cells (KC); Hepatocytes (HEP); Peritoneal macrophage and hepatocyte co-culture (PM/HEP); Kupffer cell and hepatocyte co-culture (KC/HEP); Immigrant macrophages (IM).

1. Introduction

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Visceral leishmaniasis with zoonotic characteristics is caused by *Leishmania infantum (syn. L. chagasi)*. The disease is transmitted to man, dog and other animals by a sandfly vector. The liver is one of the target organs where parasitic proliferation occurs (1). *L. infantum* infection can cause liver injury in mammalian hosts (2,3) and experimental models (4), compromising organ structure and function. Some alterations found in the liver during visceral leishmaniasis include changes in organ architecture with hypertrophy and hyperplasia of Kupffer cells, inflammatory infiltration, apoptosis, necroses and steatosis of hepatic cells (3,5).

Macrophages play a central role in the mammalian host defense system against *Leishmania* (6,7). In the liver, two macrophage populations are involved in the infection process: resident macrophages (the Kupffer cells) and immigrant macrophages recruited from the bloodstream. Peripheral blood monocytes seem to play an important role in parasite elimination, while Kupffer cells are essential for the physiological and pathological aspects of the immune response (8,9). Both cell types are involved in the pathogenesis of the hepatic injury (10,11). During *Leishmania* infection, the control of parasite multiplication and leishmanicidal activity is associated with activated macrophages that exit from the bloodstream and kill the intracellular parasites by producing both reactive oxygen species (ROS) and reactive nitrogen species (RNS). Tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) expression can enhance the anti-leishmanial effects of the macrophages (12) .However, the overexpression and release of such mediators commonly leads to undesired cytotoxic effects on parenchymal liver cells, and degenerative damage to hepatocytes (4, 13). Cytokine-activated macrophages can generate large amounts of nitric oxide (NO) during *Leishmania* infection. NO can have both cytoprotective and cytotoxic effects (14) depending on its concentration, and multiple factors are important in determining this dual role of NO. The local synthesis of high concentrations of NO is an essential

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determinant of hepatocyte cytotoxicity during *L. infantum/chagasi* infection of macrophages (4). While NO is known to be a major cytotoxic effector molecule derived from infected macrophages, there is another molecule, peroxynitrite, that is a potent oxidant and pathogenic mediator in a variety of disease conditions, and may have also a role in this system (15). Peroxynitrite is a product created, under appropriate conditions, by the reaction of NO and superoxide radicals. Most cells, and notably macrophages, can produce substantial amounts of peroxynitrite (16), but the role of this molecule in hepatic cytotoxicity is still unknown.

Bloodstream-derived macrophages have the ability to trigger leishmanicidal activity by different pathways, including proteolytic enzymes (17), ROS and RNS. However, *Leishmania*-infected Kupffer cells have a deficiency in the oxygen–dependent pathway (18). Although studies have shown that Kupffer cells display a deficient oxidative response to *Leishmania* infection, an increase in hepatic transaminase release was also observed in co-cultures of hepatocytes and *L. donovani* infected- Kupffer cells (18,19). These results demonstrate that several molecules are secreted by Kupffer cells in response to *Leishmania* infection, and either individually or combined they can damage the adjacent tissue. Previous studies (4) also demonstrated that scavengers of reactive oxygen species (such as superoxide dismutase, SOD), together with L-NAME (NO inhibitor), were able to protect hepatocytes from infected macrophage-induced cytotoxicity. The inhibition of NO totally protected hepatocytes from damage associated with macrophage infection; however, other molecules could also been implicated in the liver cell injury. Such a phenomenon must be the result of an additive or synergistic effect of many molecules secreted in an attempt to kill the parasite, creating an extremely toxic milieu in the micro-environment around these cells. Moreover, *Leishmania* can affect the post-transcriptional stages of NO synthesis during macrophage infection (20). In this study, to investigate the role of macrophage-derived leishmanicidal mediators, we adopted a co-culture model of *L. infantum*

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axenic amastigotes with *in vitro*-infected adherent peritoneal macrophages or Kupffer cells and hepatocytes.

2. Materials and methods

2.1. Animals

BALB/c mice weighing about 20 g each were used to obtain primary cultures of peritoneal macrophages and Kupffer cells. Pregnant mice (18-29 days of gestation) were utilized for hepatocyte primary cultures. Golden hamsters were utilized to maintain the parasite infectivity. The animals were obtained from the animal care facility of the Oswaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil). All procedures involving animals in this study were reviewed and approved by the Oswaldo Cruz Foundation Ethics Committee (CEUA - FIOCRUZ).

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2.2. Parasites

Leishmania (Leishmania) infantum (strain MCAN/BR/2000/CNV-FEROZ) was isolated from a dog in the State of Espírito Santo, Brazil, and typed by multilocus enzyme electrophoresis at the *Leishmania* Collection at the Oswaldo Cruz Institute, RJ, Brazil (CLIOC, WDCM 731). Parasite infectivity was maintained through inoculation in golden hamsters. Axenic amastigotes were obtained from 4th day of stationary phase growth of first stage promastigotes after adaptation at pH 5.5 and 37ºC. In our previous work, we demonstrated the establishment of axenic amastigote growth kinetics and your infectivity in peritoneal macrophage and Kupffer cell cultures (17).

2.3. Macrophage isolation

Mouse peritoneal macrophages (PM) were obtained from mice as previously described [21] Briefly, animals were sacrificed using CO_2 and the peritoneal cavity was washed with 10 ml of RPMI culture medium (Cultilab, São Paulo, SP, Brazil) at 4ºC. For each experiment, ten mice were utilized. Cells were pooled and counted using a Neubauer chamber. Cells were seeded in

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tissue culture flasks (2×10^6 macrophages/flask) or on glass coverslips (2×10^5 macrophages/coverslip) and maintained in RPMI culture medium with fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA) at 37ºC in a humidified atmosphere containing 5% CO_2.

2.4. Kupffer cell isolation

Kupffer cells were isolated using a slightly modified version of the pronase-collagenase perfusion method (22). Briefly, after intraperitoneal anesthesia with Zoletil (50 mg/kg IM), the liver was perfused through the portal vein with Ca^{2+}-free Hank´s balanced salt solution (HBSS) to completely remove the blood. The solution was then exchanged with 0.4% pronase (Calbiochem, USA) in HBSS at 10 ml/min. After this, a new solution containing HBSS with 0.02% collagenase type 1 (Sigma-Aldrich) was perfused at 10 ml/min. The organ was then detached from the perfusion device and incubated at 37ºC for 5 minutes with 0.1% pronase and 0.01% DNase I (Sigma-Aldrich) in HBSS. The tissue was minced into small pieces, forming the liver homogenate. The liver homogenate was filtered through a 125 µm nylon mesh to remove undigested tissue, and the cell suspension was centrifuged three times at 400 x *g* for 7 minutes at 4ºC. Kupffer cells were separated by 28.7% Nycodenz differential gradient centrifugation (Sigma-Aldrich). The cells were growth on culture slides in RPMI medium containing 10% FCS and fixed for immunofluorescence. The F4/80 antibody (Sigma-Aldrich), a resident macrophage marker, was used to confirm that the cells were Kupffer cells.

2.5. Macrophage infection

For *in vitro* infection of peritoneal macrophages and Kupffer cells, the *L. infantum* axenic amastigotes were harvested at the stationary growth phase (4th day). Peritoneal macrophages and Kupffer cells were cultivated for 24 hours with or without 1 µg/ml LPS (Sigma-Aldrich). After that, they were infected at a ratio of 10 parasites per cell. The macrophages and parasites were

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allowed to interact for 1 hour at 34ºC. The cultures were then washed three times with MEM/199 culture medium to remove non-adherent or non-internalized parasites. Experiments were repeated three times to confirm that experimental data were reproducible.

2.6. Hepatocyte isolation

Hepatocytes were isolated according to (23). Briefly, 7-11 mouse embryo livers (1-2 g each) were aseptically removed and washed with Hepes buffer (Sigma-Aldrich). The livers were minced and then incubated at 37ºC for 20 minutes in about 50 ml of Hepes buffer containing 0.05% Type II collagenase (Sigma-Aldrich). The cells were dispersed by pipetting and then collected by centrifugation at 200 x g. Viable cells were purified by sedimentation for 10 min at room temperature with MEM/199 medium containing 10% fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA).

2.7. Hepatocyte-infected macrophage co-culture

Hepatocytes were seeded directly onto the infected-macrophage plates, as described previously (13). Infected peritoneal macrophages or Kupffer cells were co-cultivated with hepatocytes at a ratio of 10 (peritoneal macrophages or Kupffer cells):1 (hepatocyte) in 3 ml of MEM/199 medium plus 10% FCS in tissue culture plates or glass coverslips. These co-cultures were maintained in culture medium at 37ºC in a 5% CO_2 atmosphere. Hepatocytes were co-cultured with uninfected macrophages as controls. The culture medium was maintained without exchanging during the entire experimentation period. After the interaction of infected macrophages and hepatocytes, the coverslips were washed three times in culture medium, fixed in Bouin`s solution, and stained with Giemsa (Merck, Darmstadt, Germany). The percentage of infected macrophages and the mean number of intracellular parasites per infected macrophage were determined by counting at least 100 cells. All experiments were also performed in macrophage cultures alone for comparison with the co-cultures. The co-culture supernatant was

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collected and examined by light microscopy for detached or dead cells. No infected hepatocytes were found. These experiments were repeated three times to confirm that the experimental data were reproducible.

2.8. Release of hepatocyte enzymes

Glutamate oxaloacetate transaminase (AST) and glutamate pyruvate transaminase (ALT) activities were assayed with commercial kits (Labtest, Lagoa Santa, MG, Brazil). Lactate dehydrogenase (LDH) activity was measured with the Merck kit (Merck, Darmstadt, Germany). Enzymatic activity assays were conducted according to the manufacturer's instructions and adopted as hepatocyte damage markers. Analyses were performed with both the cell lysates and the co-culture supernatants. The results were expressed as enzyme release percentage and calculated as follows:

$$\frac{ES}{(ES + EL)} \times 100 = \%\ ER$$

Where: ES = Enzyme measured in the supernatant;

EL = Enzyme measured in the lysates;

ER = Enzyme release;

2.9. Nitrite (NO^{-2}) accumulation

The ability of the co-cultures to produce NO was evaluated by measuring the accumulated nitrite, a stable breakdown product of NO, in the co-culture supernatants. Cells were cultured up to 120 hours on culture plates. The supernatants were collected, and 100 µL was mixed with an equal volume of Griess reagent (50 µL of 1% sulfanilamide plus 50 µL of 0.1% *N*-1-naphthylethylenediamine in 5% phosphoric acid solution). The absorbance was measured at 550

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nm. Nitrite concentration was calculated by employing a standard curve constructed with different sodium nitrite solutions of known concentrations.

2.10. ROS production

The production of reactive oxygen species (ROS) was observed by measuring the hydrogen peroxide in the co-culture supernatants by QuantiChrom™ Peroxide Assay Kit (BioAssay Systems) at 2, 24, 48, 72 and 120 hours.

2.11. Phagocytosis of zymosan particles

To address the endocytic ability of ingesting large particles, peritoneal macrophages and Kupffer cells were incubated with 100 µl of a suspension (2 mg/ml) of *Saccharomyces cerevisae* zymosan (Sigma-Aldrich) in phosphate buffered saline (PBS). The cultures were incubated at 37ºC for 30, 45, 60 and 120 minutes at a ratio of 10 particles per cell. The cultures were then washed three times with MEM/199 culture medium to remove non-adherent or non-internalized particles. After that, the supernatants of co-cultures were collected and used in ROS analysis. Experiments were repeated three times to confirm that experimental data were reproducible.

2.12. Detection of cytokines in the co-culture supernatant

Supernatants obtained from control and infected co-cultures were collected after 2, 24, 48, 72, 96 and 120 hours. Tumor necrosis factor (TNF-α) and interleukin 10 (IL-10) were detected employing a commercial sandwich ELISA (DuoSET ELISA; R&D Systems, Minneapolis, MN, USA).

2.13. Treatment with different inhibitors and molecules involved in the activation of microbicidal pathways in the co-cultures

The co-cultures received different treatments to inhibit and activate microbicidal pathways after the parasite-cell interaction (1 hour post-infection). The inhibitors utilized were

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catalase (100 μg/ml) (Sigma-Aldrich), uric acid (100 μg/ml) (Sigma-Aldrich), and N^G-nitro-L-arginine-methyl ester (L-NAME, 4mM) (Sigma-Aldrich). To stimulate the activation of ROS and RNS pathways, hydrogen peroxide or H_2O_2 (400 μM) (Merck, Darmstadt, Germany) and DETA/NONOoate (1 μM) (Sigma-Aldrich) were added to the co-cultures. Infected co-cultures without treatments were used as controls. Supernatants from the co-cultures were collected after 24 and 48 hours of treatment. The samples were then analyzed with kits for the measurement of hepatic transaminases (AST and ALT). The inhibition percentage of enzymes released in the co-cultures was calculated as follows:

$$100 - \frac{\text{Value found of enzyme release x 100}}{\text{Control}}$$

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2.14. Co-cultures with different macrophage populations

In all previous assays, the co-cultures consisted of Kupffer cells and hepatocytes or peritoneal macrophages and hepatocytes. In this assay, the two macrophage populations (peritoneal macrophages and Kupffer cells) were seeded in tissue culture plates containing a Cell Culture Insert with 0.4 μm of PET track-etched membrane (Falcon). The peritoneal macrophages were cultured for four hours prior to the Kupffer cell isolation, and were then seeded under part of the tissue culture plates. Kupffer cells were later seeded on the top of the Cell Culture Insert membrane, in the same tissue culture plates that were already seeded with peritoneal macrophages. There was not direct contact between the two macrophage populations, but there was contact among molecules or mediators released by them. The mixed co-cultures were incubated at a ratio of 1 (5×10^5 peritoneal macrophages) :1 (5×10^5 Kupffer cells), 2 (6.6×10^5 peritoneal macrophages) :1 (3.3×10^5 Kupffer cells), and 3 (7.5×10^5) peritoneal macrophages:1

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(2.5 x 10^5 Kupffer cells). After 24 hours of peritoneal macrophage-Kupffer cell interaction, axenic amastigotes were utilized to infect these cultures (ratio 1:10 macrophages:amastigotes). The infection was performed in two separate ways: either the peritoneal macrophages localized under the membrane were infected, or the Kupffer cells were infected on the top of the membrane. These infections occurred at the same time in different tissue culture plates. Only one population of macrophage was infected in the same tissue culture plate. After 1 hour of macrophage infection, hepatocytes were added to the membrane or directly under part of the tissue culture at a ratio of 10 (peritoneal macrophages or Kupffer cells):1 (hepatocyte). Hepatocytes were seeded together with the uninfected macrophage population, and the infected macrophage population was left without hepatocytes. The co-culture supernatants were collected after 24 hours to observe the release of AST and ALT and production of IL-10. Experiments were repeated three times to confirm that experimental data were reproducible.

2.15. Statistical analysis

All experiments were carried out in triplicate and performed at least 3 times. The mean values and standard deviations (SD) for all numerical data were calculated. The data were analyzed with the Student's *t*-test. Simple linear regression analysis was performed to calculate correlations. Differences with a p-value < 0.05 were considered statically significant.

3. Results

3.1. Analysis of hepatic damage markers in co-cultures

The release of hepatic transaminases (AST and ALT) into co-culture supernatants was adopted as a hepatocyte damage marker. The enzymes were released only by hepatocytes co-cultivated with infected peritoneal macrophages; the levels of enzyme release found in uninfected co-cultures represent basal levels. Kupffer cell co-cultures did not show any enzyme activity in the supernatants (data not shown). There was no statistically significant difference in hepatic

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transaminase release throughout the entire observation period among uninfected peritoneal macrophage co-cultures or Kupffer cell co-cultures (Fig. 1A and B). Moreover, both uninfected co-cultures displayed no statistically significant difference when compared with one another during the period analyzed (Fig. 1 A and B).

There was a statistically significant increase ($P<0.05$), by approximately three fold, in hepatic transaminase release detected in the supernatants from infected peritoneal macrophage co-cultures at 24 hpi (66% of AST release and 60% of ALT release) and 48 hpi (60% of AST release and 58% of ALT release) compared to uninfected peritoneal macrophage co-cultures (18% of AST release and 16% of ALT release at 24 hpi; 20% of AST release and 17% of ALT release at 48 hpi) (Fig 1A and B). A three-fold increase was also observed in the same period (24 and 48 hpi) when comparing the infected peritoneal macrophage co-cultures (approximately 63% of AST release and 60% of ALT release) and infected Kupffer cell co-cultures (approximately 19% of AST release and 20% of ALT release) (Fig 1A and B). The values detected in the supernatant from infected Kupffer cell co-cultures were similar to those found in the uninfected peritoneal macrophage co-cultures. There was no statistically significant difference in enzyme release between the uninfected and infected Kupffer cell co-cultures throughout the course of infection (Fig 1A and B). The values of AST and ALT detected in all conditions were similar and without a statistically significant difference (Fig 1A and B).

3.2. Nitrite production by co-cultures

NO release by peritoneal macrophage and Kupffer cell co-cultures during experimental infection with *L. infantum* axenic amastigotes was measured by nitrite accumulation in the co-culture supernatant. Nitrite levels were measured in hepatocytes cultured alone in the same amount used to seed the co-cultures (data not shown), and these values were subtracted from the total value found in the nitrite analysis of peritoneal macrophage and Kupffer cell co-cultures.

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There was no statistically significant difference between the basal nitrite production of uninfected peritoneal macrophage and uninfected Kupffer cell co-cultures during the observation period (Fig 2). Overall, there was a statistically significant increase ($P<0.05$) in nitrite accumulation in the supernatants between uninfected and infected peritoneal macrophage co-cultures. The highest nitrite production in the infected peritoneal macrophage co-cultures was detected at 48 hpi (5.9 mM nitrite), and it was approximately twice that observed in the uninfected peritoneal macrophage co-cultures (2.6 mM nitrite) (Fig 2).

In the infected Kupffer cell co-cultures, a statistically significant increase ($P<0.05$) was observed from 48 hours (4 mM nitrite) to 120 hpi as compared to uninfected Kupffer cell co-cultures (2 mM nitrite) (Fig 2). Prior to 72 hpi, infected Kupffer cell co-cultures produced a lower amount of nitrite ($P<0.05$) than infected peritoneal macrophages. At 96 and 120 hours, the nitrite production was similar for both infected peritoneal macrophages and Kupffer cell co-cultures (approximately 4 mM nitrite) (Fig 2).

3.3. ROS accumulation in the co-culture supernatants

ROS production by co-cultures was estimated by measuring the levels of hydrogen peroxide. Throughout the observation period, ROS production increased ($P<0.05$) in the supernatants of infected peritoneal macrophage co-cultures as compared to uninfected ones (Fig 3A). In addition, there was a significantly higher ROS release ($P<0.05$) in the infected peritoneal macrophages than in the supernatants from infected Kupffer cells (Fig 3A and B). The presence of the parasite did not alter ROS accumulation in the supernatants from infected Kupffer cell co-cultures (Fig 3B).

Lipopolysaccharide (LPS), a potent macrophage activator, was added to the co-cultures in order to investigate the potential of these cells to produce ROS. In all analyses, regardless of the type of macrophage population, the presence of LPS activated the macrophages, increasing

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($P<0.05$) the ROS production compared to uninfected macrophages without LPS (Fig 3A and B). Moreover, the ROS production detected in peritoneal macrophage co-cultures infected with *L. infantum* axenic amastigotes was higher ($P<0.05$) than in the same co-cultures with LPS and without infection (Fig 3A). On the other hand, ROS production in the uninfected Kupffer cells co-culture by LPS activation was larger than in those activated by amastigote infection (Fig 3B). At the beginning of the analysis (2 and 24 hours), ROS accumulation in the supernatant of infected Kupffer cell co-cultures was two times less (1.4 mM H_2O_2) than in uninfected Kupffer cell co-cultures without LPS (Fig 3B). In the infected peritoneal macrophage co-cultures, the addition of LPS had a synergistic effect on ROS accumulation in the supernatants. This accumulation was greater than that found in infected co-cultures without LPS (data not shown). Nevertheless, the presence of LPS in the infected Kupffer cell co-cultures did not augment the ROS production, and the ROS values were similar to these detected in the infected co-cultures without LPS (data not shown).

Zymosan was also added to the peritoneal macrophage and Kupffer cell co-cultures for ROS measurement. As with the addition of LPS, the infected peritoneal macrophage co-cultures produced higher ($P<0.05$) amounts of ROS than uninfected peritoneal macrophage co-cultures with Zymosan during the entire observed period (Fig 3A). However, uninfected Kupffer cell co-cultures that ingested Zymosan displayed a higher ROS accumulation than those infected without Zymosan (Fig 3B). The increase was statistically significant ($P<0.05$) at 2 hours (1.5 mM H_2O_2 in infected Kupffer cells and 4.6 mM H_2O_2 in uninfected Kupffer cells plus Zymosan), 24 hours (1.6 mM H_2O_2 in infected Kupffer cells and 4.2 mM H_2O_2 in uninfected Kupffer cells plus Zymosan) and 48 hours (1.9 mM H_2O_2 in infected Kupffer cells and 3.2 mM H_2O_2 in uninfected Kupffer cells plus Zymosan) (Fig 3B).

3.4. Cytokine production

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Cytokine production (TNF-α and IL-10) by the *L. infantum*-infected co-cultures was assessed by ELISA. The level of TNF-α in the supernatants from infected peritoneal macrophage and infected Kupffer cell co-cultures was higher ($P<0.05$) than the basal levels found in the uninfected co-cultures (Fig 4A). At the beginning of infection (until 48 hours), there was a statistically significant increase ($P<0.05$), by approximately two-fold, in the TNF-α level observed in the infected peritoneal macrophage co-culture (9.8 pg/ml) compared to the infected Kupffer cell co-culture (480 pg/ml) (Fig 4A). At the end of infection, the TNF-α levels were similar in both co-cultures. No statistically significant increase or decrease was detected among the infected Kupffer cell co-cultures until 96 hours (Fig 4A).

As observed in the assays for TNF-α detection, the IL-10 levels found in the infected supernatants of peritoneal macrophage and Kupffer cell co-cultures were significantly higher ($P<0.05$) than in uninfected co-cultures (Fig 4C). The maximum IL-10 levels in the infected peritoneal macrophage co-cultures were observed after 72 hpi, while the maximum levels of this cytokine in the infected Kupffer cell co-cultures were detected as early as 2 hpi (Fig 4C). At 48 hpi, there was no statistically significant difference in IL-10 production between the infected Kupffer cell co-cultures and infected peritoneal macrophage co-cultures, although at all other times during the infection there was a statistically significant difference ($P<0.05$) between the infected co-cultures (Fig 4C).

3.5. Co-culture treatments

To investigate the importance of reactive nitrogen and oxygen intermediates (RNS and ROS) in the hepatocyte damage detected during *L. infantum in vitro* infection, infected cultures were treated with L-NAME, catalase, uric acid, DETA/NONOate and H_2O_2 separately and in combination. These analyses were performed at 24 and 48 hpi for the measurement of hepatic

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3 transaminases (AST and ALT). In all systems, there was no statistically significant difference (P<0.05) between the results observed at 24 and 48 hpi, nor between those obtained with AST and ALT measurement. For this reason, the values shown in Table 1 are AST measurements at 48 hpi. Uninfected co-cultures were analyzed with the same treatments to investigate their toxicity. No statistically significant increase was detected in hepatic transaminase release in treated uninfected co-cultures compared to untreated uninfected co-cultures (data not shown). The infected co-cultures without treatment were used as controls for this assay.

The presence of DETA/NONOate (NO donor) in the infected supernatant of the peritoneal macrophage co-cultures increased the release of hepatic transaminases compared to the control, while the addition of L-NAME (NO synthase inhibitor) led to a statistically significant decrease (P<0.05) in transaminase levels (Table 1). Moreover, the treatment with L-NAME inhibited about half (49.8%) of the AST/ALT release in peritoneal macrophage co-cultures. On the other hand, there was no alteration in enzyme release when infected Kupffer cell co-cultures were treated with the same drug dose. The addition of H_2O_2, a molecule involved in oxidative metabolism, caused an increase (P<0.05) in hepatic transaminase release in the infected Kupffer cell co-cultures, although this was not observed in the infected peritoneal macrophage co-cultures. The presence of catalase (ROS scavenger) only decreased AST/ALT release in the infected peritoneal macrophage co-cultures (Table 1). The inhibition index was 32.5% in this system, while no alteration in enzyme release was detected in the infected Kupffer cell co-cultures treated with catalase. Uric acid, a natural scavenger of peroxynitrite, led to a drastic reduction (P<0.05) in enzyme release by both infected co-cultures compared to the controls (Table 1), with an enzyme release inhibition of 67.1% in peritoneal macrophage co-cultures and 34.6% in Kupffer cell co-cultures. In addition, this treatment fully inhibited the release of hepatic transaminases in the infected co-cultures compared to uninfected ones. The AST/ALT release in

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3 both infected co-cultures (19.6% in the peritoneal macrophage co-cultures and 16.8% in the Kupffer cell co-cultures) was similar to that found in the uninfected co-cultures (18.9% in the peritoneal macrophage co-cultures and 16.3% in the Kupffer cell co-cultures) (Table 1).

H_2O_2 and DETA/NONOate were also added together to the co-cultures. This treatment led to an increase ($P<0.05$) in enzyme release in both infected co-cultures compared to control (Table 1). On the other hand, the use of inhibitors (catalase and L-NAME) at the same time significantly reduced ($P<0.05$) the AST/ALT release in comparison to the control, with 55.2% inhibition in peritoneal macrophage co-cultures and 24.9% in Kupffer cell co-cultures (Table 1). There was no statistically significant difference in enzyme release in both infected co-cultures in the treatments utilizing one of the inhibitors (catalase or L-NAME) plus one of the molecules that can activate the pathways (H_2O_2 or DETA/NONOate).

3.6. Inhibition of macrophage-induced hepatocyte damage by Kupffer cells

To mimic the more likely relationship between the two predominant macrophage populations found in the infected liver and determine if the molecules released by these populations have an influence on the release of hepatic transaminases, peritoneal macrophages and Kupffer cells were seeded in trans-well tissue culture plates.

The supernatants collected at 24 hpi were analyzed for hepatic transaminases and IL-10 levels. There was no statistically significant difference between uninfected co-cultures in all different ratios of macrophages (Fig 5). When peritoneal macrophages were infected, our results showed that the amount ($P<0.05$) of hepatic transaminase release was directly proportional to the increase in the ratio of macrophages to Kupffer cells (Fig 5A). Co-cultures with a ratio of 3:1 displayed approximately 92% enzyme release, while cultures with a ratio of 1:1 had 64% enzyme release. On the other hand, for the assays with infected Kupffer cells, there was no statistically significant difference in AST/ALT release in the infected co-cultures in all cell proportions (Fig

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5B). IL-10 levels did not significantly change in the co-cultures with infected peritoneal macrophages compared to uninfected co-cultures and with different ratios of macrophages (Fig 5C). On the other hand, infected Kupffer cells had higher IL-10 levels in the co-cultures compared to uninfected co-cultures (Fig 5D). There was no statistically significant difference ($P<0.05$) in IL-10 production with the reduction of the number of Kupffer cells present in the co-cultures.

4. Discussion

In diseases that affect the liver, enzymes produced by hepatocytes, called hepatic transaminases, are utilized as important markers of tissue injury (24-28). An increase in the levels of hepatic transaminases in the serum of patients with VL has been observed during the course of this disease (29). Studies utilizing experimental co-culture systems, composed of liver resident macrophages or immigrant macrophages with hepatocytes, obtained from mice infected with promastigotes of *Leishmania donovani,* showed an increase in AST and ALT levels (13). In a previous study, we also observed an increase in hepatic transaminase levels in co-cultures of adherent peritoneal macrophages infected *in vitro* with *L. chagasi* promastigotes (4). In the present work, we utilized co-cultures of hepatocytes with Kupffer cells or adherent peritoneal macrophages infected *in vitro* with *L. infantum* axenic amastigotes. The infection by amastigotes is the model that mimics the infection in the liver. Our results showed that there was no injury to the hepatocytes during the infection of Kupffer cells by axenic amastigotes, while the infection of peritoneal macrophages led to hepatocyte damage until 48 hpi, when the number of parasites was high and leishmanicidal defenses were augmented.

Mammalian phagocytes generate peptidases, superoxide, hydrogen peroxide, and nitric oxide as a part of their antimicrobial armamentarium (30, 31). Previous studies showed that adherent peritoneal macrophages are able to trigger leishmanicidal activity via several pathways

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(peptidases, ROS and RNS), which is a characteristic shared by bloodstream-derived macrophages (4, 17). Kupffer cells show impairment in the oxygen-dependent-pathway (13). RNS and ROS were assayed in this study to investigate the leishmanicidal activity of the Kupffer cells and the participation of such mediators in the extracellular damage. Our results showed that infected Kupffer cells produced higher amounts of NO than uninfected Kupffer cells, but not compared to that observed for peritoneal macrophages. NO is considered one of the most important cytotoxic agents in protozoan infections (32), and it must be directly involved in hepatocyte damage (4). NO is formed after macrophages are stimulated to express iNOS II, which catalyses the synthesis of NO from L-arginine and molecular oxygen (33). In addition, such stimuli can trigger a respiratory burst with ROS production. ROS include oxygen anions and radicals (O_2^- and OH), as well as milder oxidants such as hydrogen peroxide (H_2O_2) (34).

In this study, we observed that peritoneal macrophages, when stimulated by *Leishmania infantum* axenic amastigote infection, responded with an increase in ROS production. In contrast, the production of ROS by infected Kupffer cells was low or absent during infection, with levels comparable to uninfected Kupffer cells. Previous studies have shown that macrophages containing intracellular *Leishmania donovani* amastigotes have impaired antimicrobial responses, signaling, and cell surface marker expression (35, 36). Multiple factors could be responsible for such phenomena in macrophages in general, and specifically in Kupffer cells (37) .The resistance of macrophages to *Leishmania* may be due to Slc11a1 genes (solute carrier family 11a member 1; formerly NRAMP1), which lead to *Leishmania* susceptibility in macrophages and consequently inhibition of their leishmanicidal pathways (37-40).; The Slc11a1 gene product acts as a proton/divalent cation antiporter that controls the replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagosome (37).

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In the present work, a stimulus independent of *Leishmania* infection, lipopolysaccharide (LPS), was utilized. The intracellular signal transduction cascade that takes place during macrophage activation by LPS requires the binding of specific cellular receptors, including toll-like receptors, and results in the activation and stimulation of a wide spectrum of host-defensive systems (41). The presence of LPS increased ROS production in uninfected peritoneal macrophages, but the *Leishmania infantum* axenic amastigote infection proved more potent. However, Kupffer cells activated by LPS did not show diminished ROS production; in fact, the amount of ROS measured was higher ($P<0.05$) than that observed in Kupffer cells infected by *L. infantum*. These results demonstrate that the parasite is responsible for inhibiting ROS production.

Zymosan, inert particles of *Saccharomyces cerevisae*, was added to these analyzed systems to compare ROS release by macrophage populations with these particles and with axenic amastigotes. Zymosan ingestion led to an increase in ROS release in the supernatants of uninfected Kupffer cells as compared to infected Kupffer cells (42, 43) have already reported that the impairment of macrophage activation by intracellular *Leishmania* contributes to their survival in the toxic environment of the host. ROS production in uninfected peritoneal macrophages with Zymosan was lower than in peritoneal macrophages infected with *L. infantum* axenic amastigotes, once these macrophages display a full activation of leishmanicidal activity.

Macrophages, when stimulated, modulate the immune reaction by secreting various cytokines, and the control of parasite multiplication and leishmanicidal activity is dependent on this process (44, 45). Both IFN-γ and TNF-α cytokines are known to activate monocytes and macrophages to express enhanced anti-leishmanial defenses. Herein, we observed the production of TNF-α in the infected peritoneal macrophages at the beginning of infection, indicating early

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macrophage activation leading to parasite elimination. However the TNF-α levels detected in infected Kupffer cells were similar throughout the infection, but augmented when compared to uninfected ones.

IL-10 is secreted by several cells, including macrophages and T lymphocytes, and ultimately leads to macrophage deactivation, resulting in intracellular parasite proliferation and leading to disease progression (46). Kupffer cells are not able to completely eliminate the intracellular parasites, while peritoneal macrophages quickly eliminate the *Leishmania* due to a potent leishmanicidal activity. Our results suggest that this discrepancy results from the early production of IL-10 by infected Kupffer cells. Although IL-10 can favor the progression of parasitic proliferation in the early phase of leishmaniasis, a cure can be achieved due to its effector functions on parasitized macrophages (47, 48), limiting tissue damage.

Our results confirmed that NO plays an important role in hepatocyte damage, since the presence of an NO donor increased the hepatic transaminase release in peritoneal macrophages. But in this model (peritoneal macrophage co-cultures), the addition of ROS and RNS inhibitors separately or together decreased ($P<0.05$) the enzyme's release, showing that the presence of ROS plus RNS may be necessary to cause hepatic damage. The treatments with different molecules able to inhibit or activate the microbicidal pathway in Kupffer cells infected by *L. infantum* confirmed this direct additive effect on hepatocyte damage. The presence of an ROS scavenger did not alter the AST/ALT release in Kupffer cells due to the lack of ROS, but when H_2O_2 was added in this system, hepatic transaminase release increased. The addition of uric acid to the co-cultures was most potent treatment, corroborating our previous results. Uric acid, which naturally occurs as product of purine metabolism, is a strong peroxynitrite scavenger, as demonstrated in a previous study (49). It has the ability to bind scavenger peroxynitrite, but not

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NO. Peroxynitrite formation depends of the presence of free NO radicals and ROS. This molecule has been implicated in the pathogenesis of inflammatory disorders (50), inducing both cellular apoptosis and necrosis, depending on production rates (16). Uric acid, when added to the cultures, fully inhibited hepatocyte damage by scavenging peroxynitrite from the culture medium. This was confirmed by measuring the transaminases levels in both macrophage populations, which were similar to those observed in uninfected co-cultures. Thus, in the peritoneal macrophages, high levels of peroxynitrate likely caused hepatocyte damage, while in the Kupffer cells, peroxynitrite was absent or in low levels due to the absence of ROS.

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ROS and RNS produced by macrophage populations during *Leishmania* infection seem to be responsible for hepatocyte damage by acting as peptidases (4, 31). Herein, when we co-cultivated both macrophage populations together with hepatocytes, we found similar results to those observed with peritoneal macrophages alone. Notably, in this assay, there was no direct contact between infected macrophages and hepatocytes, so that mediators released into the culture medium are responsible for the observed effects. The values of enzymes release were higher in the co-cultures with the two macrophage populations than observed for infected peritoneal macrophages and uninfected Kupffer cells (basal levels). However, when the ratio used was 1:1, transaminase release was lower than that observed for infected peritoneal macrophages. The levels of IL-10 in these co-cultures was high, probably due to the infected Kupffer cells, and was able to inhibit the extracellular damage induced by the excessive production of microbicidal compounds.

In summary, our data demonstrate that macrophage populations vary in the quality and quantity of their leishmanicidal activity, and this difference can influence the outcome of the disease depending on the species of *Leishmania* involved. Hepatocyte damage is induced by infected macrophages only when there is cooperation between ROS and RNS that might lead to

the formation of peroxynitrite. The impairment of the oxygen-dependent pathway leads to a decrease in NO production and undetectable levels of ROS, and peroxynitrite is not formed. This phenomenon is a consequence of the parasitic infection, but also reflects the involvement of the host cell. Moreover, the high IL-10 production and secretion by Kupffer cells observed during *L. infantum* infection highlights the important role of this molecule in limiting tissue damage caused by an influx of bloodstream monocytes/macrophages. It may also have a role in the nature of the adaptive immune response. These findings are important for a full understanding of the mechanisms of visceral leishmaniasis pathogenesis.

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Acknowledgements

The authors would like to thank Dr. Marilia Seelaender from Departament of Histology and Embryology, University of São Paulo for his support in the Kupffer cell culture. This work was supported by grants from Fundação Oswaldo Cruz (Fiocruz), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (MCT-CNPq).

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Figure legends

Figure 1. Hepatocyte damage markers released into the supernatant from *L. infantum*-infected (I) or uninfected peritoneal macrophage co-cultures (PM/HEP) or Kupffer cell co-cultures (KC/HEP). A. Detection of AST release. B. Detection of ALT release. Bars indicate mean ± standard deviation. * Statistically significant difference (P<0.05).Values represent the means of three separate measurements for each of three cultures per time point.

Figure 2. Nitrite levels detected in the supernatant from uninfected and *L. infantum*-infected (I) peritoneal macrophage co-cultures (PM/HEP) or Kupffer cells co-culture (KC/HEP). Bars indicate mean ± standard deviation. * Statistically significant difference (P<0.05) between uninfected Kupffer cells co-cultures and infected ones. ** Statistically significant difference (P<0.05) between uninfected peritoneal macrophages co-cultures and infected ones. These results represent a minimum of three experiments.

Figure 3. ROS production measured by hydrogen peroxide detection in the supernatant from uninfected and *L. infantum*-infected (I) co-cultures with the presence of LPS or Zymosan particles. A. Analysis of peritoneal macrophage co-cultures (PM/HEP). B. Analysis of Kupffer cell co-cultures (KC/HEP). Bars indicate mean ± standard deviation. * Statistically significant difference (P<0.05). This experiment was repeated at least three times.

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Figure 4. Cytokine detection in the supernatant from *L. infantum*-infected (I) or uninfected peritoneal macrophage co-cultures (PM/HEP) or Kupffer cell co-cultures (KC/HEP). A. TNF- α levels. B. IL-10 levels. Bars indicate mean ± standard deviation. * Statistically significant difference ($P<0.05$). Depicted results represent at least three experiments.

Figure 5. The interaction of two macrophage populations with hepatocytes in the same tissue culture plate was analyzed by hepatic transaminase and IL-10 measurements. A. AST and ALT release in the supernatants of co-cultures composed of uninfected or infected peritoneal macrophages (I) and hepatocytes with uninfected Kupffer cells. B. AST and ALT release in the supernatants of co-cultures composed of uninfected peritoneal macrophages with uninfected or infected Kupffer cells (I) and hepatocytes. C. IL-10 production in the supernatants of co-cultures composed of uninfected or infected peritoneal macrophages (I) and hepatocytes with uninfected Kupffer cells. D. IL-10 production in the supernatants of co-cultures composed of uninfected peritoneal macrophages with uninfected or infected Kupffer cells (I) and hepatocytes. Bars indicate mean ± standard deviation. * Statistically significant difference ($P<0.05$). These results represent a minimum of three experiments.

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Table legend

Table 1. Effect of inhibitors (L-NAME, catalase and uric acid) and/or activators (DETA/NONOate and H_2O_2) in the supernatant from *L. infantum*-infected peritoneal macrophage co-cultures (PM/HEP I) or Kupffer cell co-cultures (KC/HEP I). The co-cultures were treated with different inhibitors and/or activators after macrophage infection, and hepatic transaminase (AST and ALT) release was measured. Untreated infected co-cultures were used as controls. This table shows the values of AST release after 48 hpi of the treatments in the co-cultures; the values obtained by ALT release were similar to AST release. * Statistically significant difference

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Figure 1.

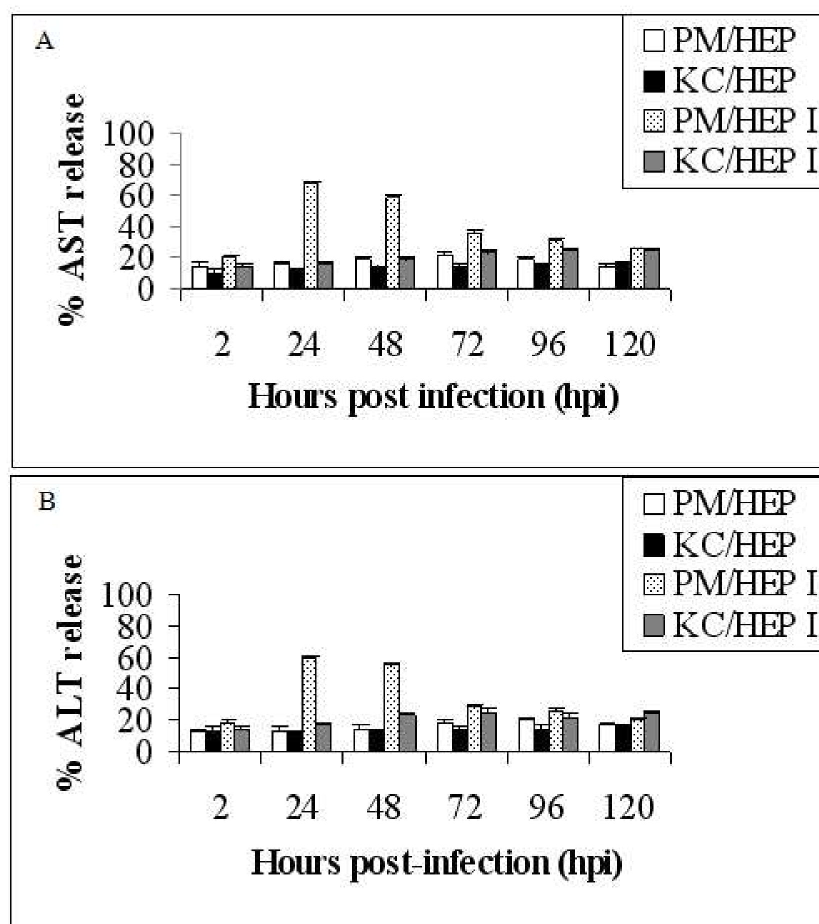

Figure 1. Hepatocyte damage markers released into the supernatant from L. infantum-infected (I) or uninfected peritoneal macrophage co-cultures (PM/HEP) or Kupffer cell co-cultures (KC/HEP). A. Detection of AST release. B. Detection of ALT release. Bars indicate mean ± standard deviation. * Statistically significant difference ($P<0.05$).Values represent the means of three separate measurements for each of three cultures per time point.
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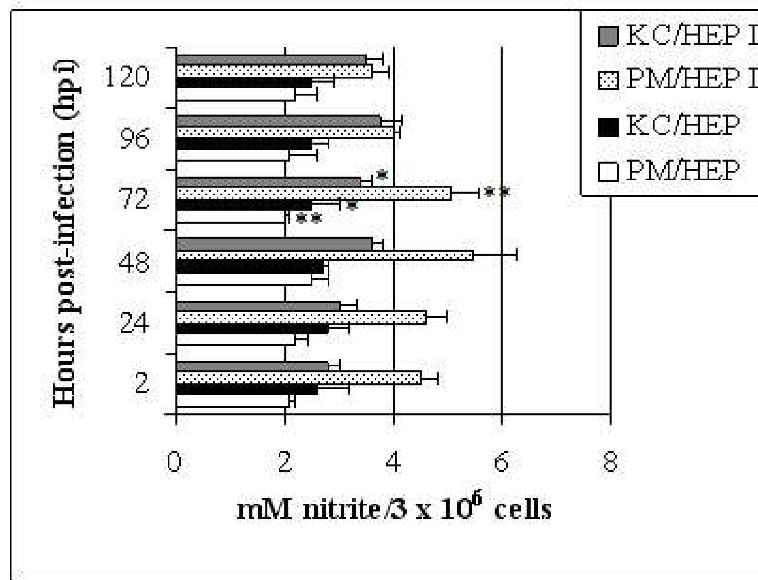

Figure 2. Nitrite levels detected in the supernatant from uninfected and L. infantum-infected (I) peritoneal macrophage co-cultures (PM/HEP) or Kupffer cells co-culture (KC/HEP). Bars indicate mean ± standard deviation. * Statistically significant difference ($P<0.05$) between uninfected Kupffer cells co-cultures and infected ones. ** Statistically significant difference ($P<0.05$) between uninfected peritoneal macrophages co-cultures and infected ones. These results represent a minimum of three experiments.
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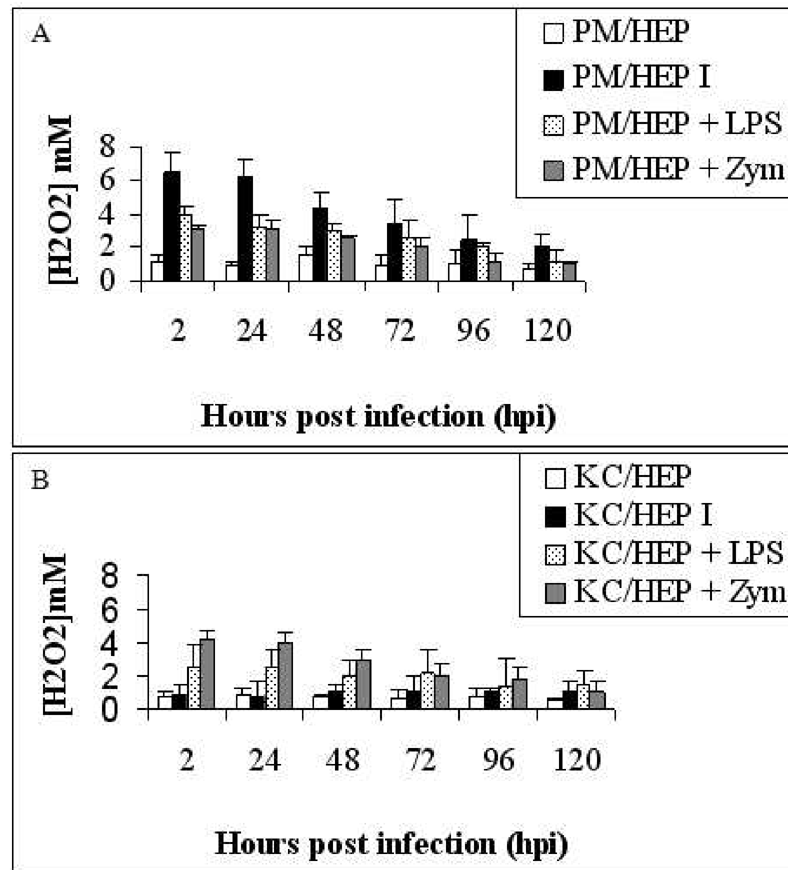

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Figure 4.

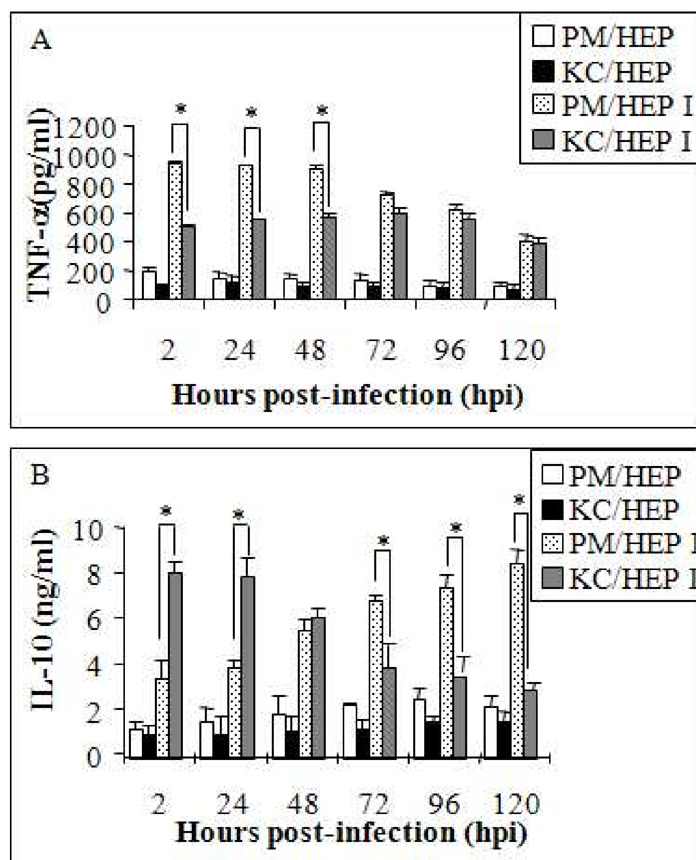

Figure 4. Cytokine detection in the supernatant from L. infantum-infected (I) or uninfected peritoneal macrophage co-cultures (PM/HEP) or Kupffer cell co-cultures (KC/HEP). A. TNF- α levels. B. IL-10 levels. Bars indicate mean ± standard deviation. * Statistically significant difference (P<0.05). Depicted results represent at least three experiments.
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Figure 5.

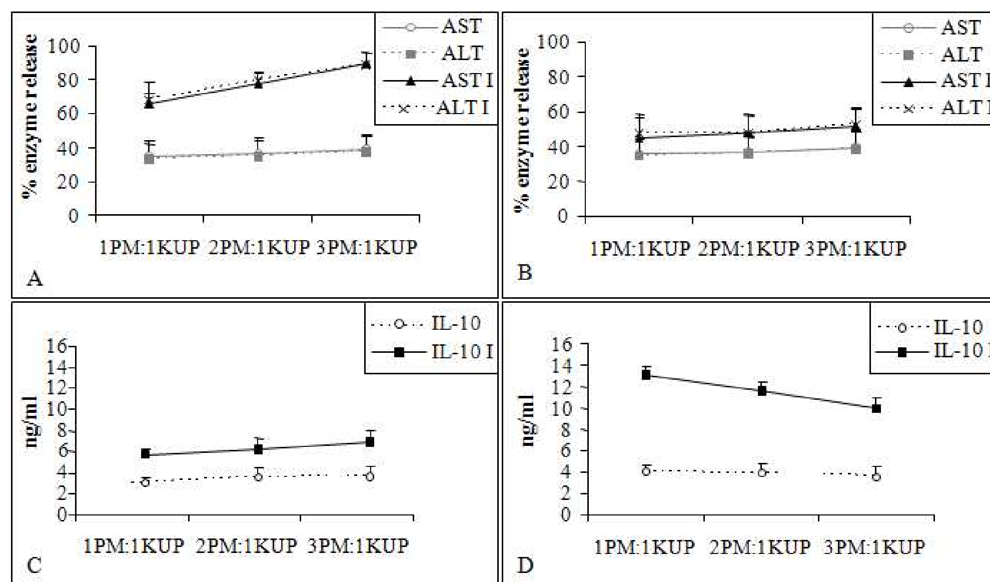

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Table 1.

	MP/HEP I (% Inhibition)	KC/HEP I (% Inhibition)
Control	**59.6 ± 0.5**	**25.7 ± 0.9**
TREATMENTS:		
DETA/NONOate	68.2 ± 0.6*	29.9 ± 0.4
L-NAME	29.9 ± 0.8* (49.8%)	21.6 ± 0.8
H2O2	62.6 ± 1.2	35.3 ± 1.6 *
Catalase	40.2 ± 1.5* (32.5%)	23.9 ± 0.2
Uric acid	19.6 ± 0.9* (67.1%)	16.8 ± 0.5 * (34.6%)
H2O2 + DETA/NONOate	76.3 ± 0.3*	49.1 ± 1.2*
Catalase + L-NAME	26.7 ± 0.7* (55.2%)	19.3 ± 0.5* (24.9%)
Catalase+ DETA/NONOate	53.7 ± 0.5	30.2 ± 1.0
H2O2+ L-NAME	52.8 ± 0.5	21.4 ± 0.3

Table 1. Effect of inhibitors (L-NAME, catalase and uric acid) and/or activators (DETA/NONOate and H2O2) in the supernatant from L. infantum-infected peritoneal macrophage co-cultures (PM/HEP I) or Kupffer cell co-cultures (KC/HEP I). The co-cultures were treated with different inhibitors and/or activators after macrophage infection, and hepatic transaminase (AST and ALT) release was measured. Untreated infected co-cultures were used as controls. This table shows the values of AST release after 48 hpi of the treatments in the co-cultures; the values obtained by ALT release were similar to AST release. * Statistically significant difference ($P<0.05$). Values represent the means of three separate determinations for each of three cultures per time point.

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6. Discussão

A *Leishmania (Leishmania) infantum* é o agente causal da LV no Novo Mundo e possui tropismo por macrófagos do baço, fígado e medula óssea. O fígado é um dos principais órgãos acometidos pela infecção causada por este protozoário (revisto por Stanley & Engwerda, 2007). Os mecanismos e as moléculas envolvidas no processo de visceralização deste parasito, e os processos que determinam a chegada deste parasito aos órgãos alvo, ainda não estão completamente esclarecidos (Wilson *et al*., 2005). Este parasito pode chegar ao fígado através de macrófagos provenientes da circulação sanguínea, chamados de macrófagos imigrantes (Kausalya *et al*., 1993). Caso os parasitos não sejam eliminados por estes macrófagos, as células podem se romper, e uma vez os parasitos presentes no meio extracelular, eles podem ser fagocitados pelos macrófagos residentes do local, chamados células de Kupffer. O modelo murino vem sendo utilizado em estudos para investigar a infecção pelas leishmânias viscerotrópicas no fígado, por ser um modelo que permite controlar as características da infecção do hospedeiro e do parasito (Kausalya *et al*., 1993; 1996; Costa *et al*., 2007), permitindo esclarecer importantes aspectos da interação parasito-hospedeiro, assim como os fenômenos relacionados com a resposta imunológica (Pereira & Alves, 2008).

Para tentar entender a interação parasito-célula hospedeira, é preciso conhecer alguns fatores envolvidos, como: fatores genéticos e imunológicos do hospedeiro, a espécie de *Leishmania* envolvida, a quantidade e a forma do parasito inoculado (Courret *et al*., 2003). São as combinações destes fatores que determinarão a susceptibilidade ou resistência do hospedeiro à infecção por uma determinada espécie de *Leishmania* (Rosas *et al*., 2005). A susceptibilidade/resistência à infecção por *Leishmania major* para linhagens inbread de camundongos é bem conhecida (revisto por Gumy *et al.,* 2004). Os camundongos da linhagem BALB/c representam o pólo susceptível, apresentando uma resposta Th2 que resulta no aumento da lesão e do número de parasitos, culminando na exacerbação da doença. Já os

camundongos da linhagem C57Bl/6 apresentam o pólo resistente da doença, onde ocorre uma resposta de perfil Th1, com inibição da multiplicação dos parasitos e a cura da lesão (revisto por Gumy *et al.,* 2004). Utilizando estas duas linhagens de camundongos, avaliamos a susceptibilidade/resistência *in vitro* (infecção de macrófagos) frente à *Leishmania infantum,* denominada *L. chagasi* neste artigo (Costa *et al.*, 2007). Macrófagos peritoneais obtidos de camundongos da linhagem BALB/c foram mais susceptíveis à infecção do que as mesmas células infectadas obtidas de camundongos C57Bl/6 com a mesma cepa/dose de parasitos (Costa *et al.*, 2007). Porém, as células dos camundongos C57Bl/6 foram parcialmente resistentes à infecção por *L. infantum* (Costa *et al.*, 2007), assim como já reproduzido frente à infecção por *L. amazonensis* (Pereira & Alves*,* 2008). Uma multiplicação dos parasitos no interior destas células dos camundongos C57Bl/6, produzindo citocinas de perfil Th1 em níveis similares às células dos camundongos BALB/c, foi observada, juntamente com a produção aumentada de citocinas de perfil Th2 (Costa *et al.*, 2007). Concluindo, para o modelo de infecção com *L infantum*, os macrófagos obtidos da linhagem C57Bl/6 não se mostraram completamente resistentes, não sendo capazes de eliminar completamente os parasitos intracelulares, mas produzindo grandes quantidades de ON.

A produção de citocinas pró-inflamatórias, TNF-α e IFN-γ, ativa os macrófagos e está relacionada com uma resposta imune de perfil Th1, levando ao controle da carga parasitária nos períodos iniciais da infecção por *Leishmania* (revisto por Gumy et al., 2004). Estudos relataram que os níveis de TNF-α estão aumentados concomitantemente ao período de proliferação parasitária no fígado durante uma infecção com leishmânia viscerotrópica, e os macrófagos então ativados disparam seus mecanismos leishmanicidas (Reiner *et al.*, 1990; Costa *et al.*, 2007). Por outro lado, citocinas com perfil Th2 como IL-10 têm um papel supressor e desativador de macrófagos, tanto em modelos *in vivo* quanto *in vitro*, e consequentemente inibem os mecanismos leishmanicidas (Gazzinelli *et al.*, 1992; Murray *et al.*, 2003; Gantt *et al.*, 2001). A IL-10 é predominantemente secretada em animais

susceptíveis infectados por leishmânias viscerotrópicas (Heinzel *et al*., 1991; revisto por Wilson *et al.,* 2005) e é responsável pela diminuição na apresentação de antígeno por macrófagos e pela inibição da produção de citocinas por células Th1 (Fiorentino *et al*., 1991; Brodskyn *et al*., 1997). A produção aumentada de IL-10 foi observada em macrófagos peritoneais infectados por *L. chagasi* nos momentos mais tardios, o que nos leva acreditar que após a resposta leishmanicida ocorra um disparo dos mecanismos de desativação dos macrófagos (Costa *et al.,* 2007).

A ativação dos macrófagos pela infecção por *Leishmania* dispara uma variedade de mecanismos microbicidas que envolvem ROS, RNS, bem como enzimas hidrolíticas (Murray, 1988; Kausalya *et al.,* 1993). Durante a infecção por *Leishmania chagasi* em macrófagos peritoneais, verificou-se uma produção exacerbada destes mediadores pelos macrófagos infectados na tentativa de eliminar os parasitos intracelulares (Costa *et al*., 2007). O conjunto destes mediadores leva a uma citotoxicidade intracelular caracterizada pela eliminação do parasito, e concomitantemente a esta atividade intracelular, há uma atividade citotóxica extracelular produzindo um efeito colateral que leva danos às células adjacentes, no caso, os hepatócitos (Kausalya *et al.,* 1993; Costa *et al.,* 2007). Os hepatócitos representam a maior população celular do fígado, preenchendo todo parênquima do órgão (Malik *et al.*, 2002). São células de extrema importância na manutenção homeostática dos organismos, e por isso a ação citotóxica nos hepatócitos observada durante a infecção por *Leishmania* pode acarretar sérios problemas para o paciente ou animal com leishmaniose (El Hag *et al*., 1994; Kausalya *et al*., 1993).

Em estudos com modelos murinos foi observado que, durante a infecção por *L. (L.) donovani*, mudanças ultra-estruturais nos hepatócitos como no sistema endomembranar, na distribuição de glicogênio hepático e no compartimento peroxissomal, levam a destruição das organelas citoplasmáticas, principalmente mitocôndrias, com alto grau de degeneração incluindo ruptura das cristas de suas membranas e desaparecimento de grânulos mitocondriais

(Vianna *et al*., 2002). Também observamos, durante a infecção experimental por *Leishmania infantum* em macrófagos co-cultivados com hepatócitos de camundongos, mudanças intracelulares degenerativas, com alterações nas organelas, principalmente nas mitocôndrias (Costa *et al.,* 2007). El Hag *et al*. (1994) relataram distúrbios morfológicos e funcionais no fígado de pacientes com leishmaniose visceral, como hipertrofia de células de Kupffer, dilatação do retículo endoplasmático de hepatócitos e de células de Ito, além de alterações no trato portal, sinusóides e veias hepáticas. Neste mesmo trabalho, foram detectados hepatócitos apresentando degeneração com sinais de necrose na periferia dos lóbulos. Estudos mais recentes envolvendo cães com leishmaniose visceral também mostraram dano hepático, com alterações na cápsula de Disse e no espaço portal, formação de granuloma, hipertrofia/hiperplasia de células de Kupffer e degeneração dos hepatócitos (Giunchetti *et al*., 2004). Além das alterações morfológicas encontradas principalmente nos hepatócitos durante uma infecção por uma leishmânia viscerotrópica, marcadores enzimáticos demonstram danos nas células parenquimais do fígado, as transaminases hepáticas como aspartato aminotransferase (TGO) e a alanina aminotransferase (TGP) produzidas nos hepatócitos com origem citoplasmática e mitocondrial, respectivamente (Dos Santos, 1999). Durante a leishmaniose visceral, há um aumento das transaminases hepáticas no soro ou no sobrenadante das culturas, indicando a presença de lesão nos hepatócitos (Hervas *et al*., 1991; Kausalya *et al*., 1993; El Hag *et al*., 1994; Costa *et al.,* 2007). Este aumento na liberação das transaminases hepáticas pelos hepatócitos parece estar relacionado com o momento de maior atividade leishmanicida pelos macrófagos (Kausalya *et al*., 1993; Costa *et al*., 2007).

A interação entre as células hospedeiras da *Leishmania* (macrófagos) e a principal célula do fígado (hepatócitos) é importante para o entendimento das doenças que acometem este órgão, como a leishmaniose visceral (Kausalya *et al*., 1993). Co-cultivando macrófagos/hepatócitos é possível investigar os danos a estas células hepáticas durante a infecção por *Leishmania infantum*, uma vez que as co-culturas apresentam as mesmas

características morfológicas de macrófagos e hepatócitos cultivados isoladamente, sendo possível observar a manutenção da capacidade de diferenciação dos hepatócitos e de sua organização característica (Guguen-Guillouzo & Guillouzo, 1992; Porrozzi *et al.* 1997; Costa *et al.*, 2007).

Tratamentos realizados nas co-culturas hepatócitos/macrófagos infectados por *Leishmania infantum*, com diferentes inibidores/competidores dos mediadores liberados durante a atividade leishmanicida dos macrófagos, mostraram que há uma correlação direta entre o excesso dos mediadores produzidos e o dano nos hepatócitos (Costa *et al.,* 2007). Enzimas hidrolíticas ou peptidases liberadas em excesso pelos macrófagos infectados por *Leishmania infantum* parecem contribuir para os danos nos hepatócitos (Costa *et al.*, 2007). Peptidases de diferentes classes (serina, cisteína e metalopeptidase) vêm sendo implicadas no desenvolvimento de patologias hepáticas (Elkington et al., 2005; Altadill *et al.,* 2009). Estas enzimas têm se mostrado importantes na interação parasito-célula hospedeira, pois elas estão envolvidas em muitos processos celulares, como a inativação de proteínas do hospedeiro envolvidas nos mecanismos de defesa (McKerrow *et al.*, 1993; Melo *et al.*, 2004). Além disso, o excesso de peptidases liberadas pelos macrófagos pode mediar a degradação de proteínas da matriz extracelular, receptores dos hepatócitos, proteínas associadas à membrana e remodelação da matriz extracelular (McGwire *et al.*, 2003; Mohammed & Khokha, 2005). As três classes de peptidases produzidas em excesso pelos macrófagos durante sua atividade leishmanicida parecem estar envolvidas nos danos nos hepatócitos, porém as metalopeptidases apresentam um papel importante nesta citotoxicidade extracelular (Costa *et al.*, 2007; Costa *et al.*, 2008).

O aumento da expressão de metalopeptidases 1, 2 e 9 pelos macrófagos tem sido demonstrado em diferentes processos patológicos (Galis *et al.*, 1995; Rajavashisth *et al.,* 1999). Nos danos nos hepatócitos também foi observado um aumento significativo na produção de metalopeptidases, através das análises quantitativas e qualitativas das atividades

proteolíticas nas co-culturas formadas por hepatócitos e macrófagos infectados por *Leishmania chagasi* durante o momento de maior atividade leishmanicida pelos macrófagos (Costa *et al.*, 2008). As metalopeptidases provavelmente participam dos danos nos hepatócitos atuando diretamente sobre os componentes de matriz extracelular (EM), levando a um desarranjo na arquitetura da EM, que é essencial para o crescimento e a sobrevivência dos hepatócitos (Guguen-Guillouzo & Guillouzo, 1992).

Os macrófagos são grandes produtores de diversas metalopeptidases. No momento em que sua atividade microbicida frente à *Leishmania chagasi* está mais intensa e os danos nos hepatócitos são observados, uma forma ativa de metalopeptidase 9 foi detectada em grande quantidade (Costa *et al.*, 2008). Por outro lado, durante alguns processos patológicos hepáticos como cirrose e hepatite C, a atividade proteolítica da MMP-9 pode estar reduzida (Sujaku *et al.*, 1998; Lichtinghagen *et al.*, 1999) devido à diminuição do número de células que secretam esta peptidase (Tomita *et al.*, 1994; Petermann *et al.*, 1996). A redução de MMP-9 também foi observada em hepatócitos infectados com *Trypanosoma cruzi*, provavelmente devido à presença de TIMPs, que são proteínas extracelulares capazes de se ligarem às formas ativa e latente de todos os membros da família das MMPs, bloqueando e regulando suas atividades (Gomez *et al.*, 1997; Mottram *et al.*, 2004). Além disso, várias citocinas, tais como TGF-β, e outros mediadores celulares podem afetar a expressão de MMP-9 pelas células (Opdenakker et al., 2001). Durante o período em que foi detectado este fenômeno, os níveis de TGF-β estavam aumentados nos macrófagos infectados por *Leishmania chagasi* (Costa *et al.*, 2008).

As atividades proteolíticas que parecem estar contribuindo para os danos nos hepatócitos são todas proveniente da liberação extracelular pelos macrófagos, pois nenhuma atividade proteolítica oriunda da *Leishmania chagasi* foi observada nas condições estudadas (Costa *et al.*, 2008). A atividade proteolítica do parasito pode não ter sido detectada ou estar em baixa expressão, uma vez que no momento dos danos hepáticos, os macrófagos infectados

possuem em seu interior formas amastigotas. As formas amastigotas apresentam uma expressão bastante reduzida de sua principal metalopeptidase, a gp63, que é bastante importante na virulência e na patogenicidade do parasito. Somente nas formas promastigotas de *Leishmania* há abundância de gp63 (revisto por Vermelho *et al*., 2007).

A atividade microbicida dos macrófagos pode variar dependendo da forma do parasito em que o processo de adesão e interação aconteça (Nadere & McConville, 2008). A utilização de formas amastigotas de *Leishmania* nos estudos de infecção *in vitro* de macrófagos mimetiza a infecção no fígado, pois são estas formas que entram em contato com as células Kupffer. Para realizar este tipo de ensaios de interação, amastigotas-macrófagos, podem-se utilizar amastigotas intracelulares ou axênicas. O uso de formas amastigotas intracelulares requer a extração do fígado ou baço de animais que mantenham o parasito, como o hamster, o que torna todo o processo oneroso e trabalhoso, sem contar com as implicações éticas. Para evitar a utilização de animais para obtenção de amastigotas em cada de ensaio de interação amastigota-macrófagos, padronizamos uma metodologia chamada amastigogênese, onde amastigotas axênicas são produzidas. As formas axênicas são obtidas pela transformação de promastigotas de 1ª passagem em meio de cultura em pH 5.5 a 37ºC. Desde o inicio dos anos 80, a amastigogênese vem sendo realizada em diferentes espécies de *Leishmania* (Pan, 1984). Em leishmânias viscerotrópicas do Novo Mundo, há poucos estudos na tentativa de estabelecer o melhor protocolo de amastigogênese (Sereno & Lesmere, 1997; Teixeira *et al*., 2002; Costa *et al*., 2008). O protocolo que padronizamos mostrou-se mais eficaz, gerando em menos tempo grandes quantidades de amastigotas axênicas (100% de parasitos em 4 dias). Utilizamos, para isso, meio Schneider suplementado com 20% de soro fetal bovino, 2% de urina humana, 0,5% de L-glutamina e 0,5% de HEPES em pH 5.5 a 37ºC. Os suplementos adicionados mostraram-se eficazes para uma produção rápida e pura destes parasitos. As amastigotas axênicas produzidas por esta metodologia são semelhantes morfologicamente e fisiologicamente às amastigotas intracelulares e apresentam o mesmo perfil da infecção por

amastigotas intracelulares em macrófagos peritoneais e células de Kuppfer. As duas amastigotas testadas apresentam um percentual de infecção significativamente maior nos macrófagos comparado com as promastigotas, além de apresentarem uma curva de infecção com perfil distinto das promastigotas (Costa *et al*., 2008).

As células de Kupffer são o principal alvo das leishmanias viscerotrópicas no fígado. A infecção destas células ocorre seja pela entrada de formas amastigotas livres no interstício, seja pela fagocitose de células infectadas (Stanley *et al.*, 2007). Entretanto, a interação de formas amastigotas com estas células é de suma importância para o estabelecimento da infecção e deflagração da resposta imune adaptativa (Kima, 2007). Com a intenção de estudar esta interação, culturas de células de Kupffer foram padronizadas para compará-la com o típico padrão de infecção dado pelos macrófagos peritoneais que servem de modelo, pelo menos referente aos mecanismos leishmanicidas, aos macrófagos exsudativos ou imigrantes. As células de Kupffer cultivadas *in vitro* apresentaram semelhanças morfológicas e bioquímicas comparadas ás células *in vivo* (Seelaender *et al*, 1999; Costa *et al.,* 2008). Os macrófagos exsudativos são os macrófagos oriundos do influxo sanguíneo que chegam ao fígado e as células de Kupffer, os macrófagos residentes locais. A comparação destas duas populações de macrófagos se faz necessária por serem as células que estão diretamente envolvidas na patogênese da leishmaniose visceral no fígado.

Em geral, existem duas vias leishmanicidas utilizadas pelos macrófagos: via independente de oxigênio e a dependente de oxigênio (Lepay *et al*., 1985; Kausalya *et al*., 1993; Costa *et al*., 2007). A via independente de oxigênio se caracteriza pela secreção de enzimas hidrolíticas, enquanto a via dependente de oxigênio está relacionada com a liberação de espécies reativas de oxigênio (ROS) e nitrogênio (RNI) .

Assim como as proteases, que contribuem para o dano nos hepatócitos, o ROS e RNS também estão envolvidos. ROS incluem ânion ($O2^-$), radical de oxigênio (OH) e peróxido de hidrogênio (H_2O_2), e têm a capacidade de mediar à destruição celular, isolados ou em

associação com as proteases (Halliwell *et al.*, 1992). Além disso, a geração dos radicais livres domina as defesas oxidativas no fígado e resulta na destruição oxidativa de membranas celulares e levando a lesões teciduais (Blake *et al.,* 1989). A membrana celular é primariamente composta de ácidos graxos poliinsaturados (AGPI), que são particularmente susceptíveis ao ataque por radicais oxidantes. O efeito global da peroxidação lipídica é a diminuição da fluidez da membrana, desestabilizando receptores da mesma. Produtos da peroxidação lipídica e particularmente os derivados aldeídos podem inibir a síntese protéica, bloquear a ação dos macrófagos, provocar alterações na quimiotaxia e alterar diversas atividades enzimáticas (Sciandra & Subjeck 1984; Boumpas *et al.*, 1986, Floyd *et al.*, 1986; Donati *et al.*, 1990, Greenwald, 1991, Fairburn *et al.*, 1992).

As células de Kupffer apresentam um impedimento na produção de ROS quando infectadas por amastigotas de *Leishmania infantum*, enquanto que os macrófagos peritoneais produzem grandes quantidades de ROS na presença deste parasito. O impedimento nas células de Kupffer é específico ao estímulo deste parasito, uma vez que estas células produzem grandes quantidades de ROS quando estimuladas com partículas inertes de Zymozan (Costa *et al.*, 2009b). Os mecanismos que levam a este impedimento nas células de Kupffer ainda não foram esclarecidos. Acreditamos que este impedimento pode ter relação direta com o tipo de receptor que a amastigota utiliza para invadir este macrófago residente, diferente daquele em que ela usa para invadir macrófagos peritoneais. Porém, não podemos afirmar, já que não há relatos na Literatura fazendo uma comparação entre os receptores que são usados nos dois tipos de macrófagos estudados (peritoneais e células de Kupffer) durante a infecção por *Leishmania chagasi*. O impedimento também pode ter ligação com mecanismos de escape gerados pelo próprio parasito. Existem vários mecanismos de escape utilizados pelas formas amastigotas para impedir sua destruição frente aos mecanismos leishmanicidas dos macrófagos, permitindo sua sobrevivência. O aumento da expressão, nas formas amastigotas de *Leishmania*, de peroxidoxinas, que são enzimas que detoxificam H_2O_2, impede a ação anti-

oxidativa, permitindo sua multiplicação e aumentando sua sobrevida (Barr & Gedamu, 2003). Porém, a expressão destas peroxidoxinas nos diferentes macrófagos analisados infectados por *Leishmania chagasi* é desconhecida.

Além da ausência da secreção de ROS pelas células de Kupffer e do perfil da infecção por *Leishmania infantum* observados nestas células (Costa *et al.,* 2009a), existem outras diferenças nas células de Kupffer em relação aos macrófagos peritoneais. Enquanto as células de Kupffer produzem grandes níveis de citocinas como IL-10, quando infectadas por amastigotas de *Leishmania infantum*, os macrófagos peritoneais apresentam níveis aumentados de citocinas envolvidas na ativação como TNF-α. De fato, observamos grande quantidade de IL-10 sendo prematuramente secretado, o que pode estar diretamente relacionado com um possível mecanismo de "desligamento" das células de Kupffer (Costa *et al*., 2009a). Isso pode explicar a persistência parasitária encontrada nos tempos mais tardios nas culturas de Kupffer e a eliminação quase que completa dos parasitos nas culturas de macrófagos peritoneais (Costa *et al.,* 2009a). A presença de quantidades aumentadas de IL-10 leva a uma desativação dos macrófagos, que resulta na proliferação do parasito intracelular levando a progressão da infecção (Iniesta et al., 2002). A produção de citocinas pró-inflamatórias (como TNF-α) nas células de Kupffer em níveis mais baixos que nos macrófagos peritoneais provavelmente está relacionada a uma menor produção de óxido nítrico pelas células Kupffer (Costa et al., 2009b), uma vez que macrófagos ativados expressam iNOS por uma variedade de estímulos como IFN-γ, TNF-α e LPS, catalisando síntese de altas concentrações de óxido nítrico a partir de L-arginina e oxigênio molecular (Stuehr & Marletta, 1987; Proudfoot *et al*., 1996). A capacidade de produção de óxido nítrico, assim como de iNOs, em camundongos BALB/c infectados por *L.* (*L.*) *donovani* ocorre paralelamente ao aumento da síntese de TNF-α (Mukherjee *et al.,* 2003).

O óxido nítrico tem um importante papel nos vários tipos de danos observados no fígado (Kane *et al*., 1997; Rockey & Chung, 1997; Clemens, 1999; Kausalya et al., 1993;

Costa *et al.,* 2007). Os efeitos do óxido nítrico no tecido hepático são complexos, e as propriedades pro-oxidativas e anti-oxidativas têm sido observadas (Billiar, 1997; Cottard *et al.*, 1999). A via dependente de oxigênio nas células de Kupffer é caracterizada somente pela liberação de óxido nitrico, já que a secreção de ROS está inibida.

Para formação do dano nos hepatócitos é necessário a secreção de todos os mediadores liberados pelas duas vias: peptidases, ROS e RNS. Portanto, as células de Kupffer infectadas por amastigotas de *Leishmania infantum* não contribuem para este fenômeno. Estas células infectadas apresentam níveis baixos de transaminases hepáticas, similares aos níveis destes marcadores de dano nas células sem infecção (Costa *et al.*, 2009b). O tratamento das co-culturas (formadas por macrófagos peritoneais infectados com amastigotas de *Leishmania infantum* e hepatócitos ou formadas por células de Kupffer infectadas com amastigotas de *Leishmania infantum* e hepatócitos) realizado com diferentes inibidores ou ativadores das vias leishmanicidas confirmou que ambos os mediadores, ROS e RNS, precisam estar sendo produzidos para que haja um efeito citotóxico extracelular nos hepatócitos (Costa *et al.*, 2009b).

A produção conjunta de ROS e RNS leva a formação de uma molécula mais citotóxica do que o óxido nítrico, chamada peroxinitrito (Scott & Hooper, 2001). A formação do peroxinitrito depende da presença de óxido nítrico e superóxido (O2-) e esta molécula vem sendo implicada em diversos processos inflamatórios, induzindo lesões celulares (Szabo *et al.,* 2007). A utilização do varredor desta molécula no tratamento inibiu completamente os danos nos hepatócitos nas co-culturas formadas de macrófagos peritoneais, mostrando que o grande responsável por este fenômeno é o peroxinitrito, e não o óxido nítrico sozinho. O impedimento na produção de ROS nas células de Kupffer infectadas não leva a formação de peroxinitrito, e consequentemente não há dano para as células adjacentes. Sendo assim, o fígado apresenta duas populações de macrófagos participando da infecção por leishmânias viscerotrópicas, cada uma apresentando uma função diferente. Os macrófagos exsudativos

parecem ser os responsáveis pela eliminação parasitária com uma potente atividade leishmanicida que pode gerar dano nos hepatócitos, enquanto as células de Kupffer são responsáveis pela apresentação de antígenos e recrutamento de células para combater a infecção. E também possível que o perfil Th2 que leva a persistência parasitária e conseqüente piora do quadro clínico encontrada na leishmaniose visceral seja modulado por estas células, já que estas produzem grandes quantidades de IL-10. A presença desta citocina no momento da apresentação antigênica pode direcionar a resposta para o perfil Th2. De fato, um mecanismo semelhante já foi relatado no modelo de infecção murina pelo *Schistossoma mansoni* (Hayashi *et al*., 1999).

7. Conclusões

- As três classes de peptidases (serina, cisteína e metalopeptidase) contribuem em diferentes níveis para os danos nos hepatócitos, uma vez que a maior atividade proteolítica produzida pelos macrófagos infectados por *Leishmania infantum* foi observada no momento de maior secreção de transaminases hepáticas;

- As metalopeptidases foram mais expressas nas co-culturas de hepatócitos/macrofágos infectados que as outras classes de peptidases. A maior atividade proteolítica foi detectada no pH 10 e o tratamento com o inibidor de metalopeptidase mostrou uma proteção mais eficaz contra os danos observados;

- A detecção da forma ativa da metalopeptidase 9 nos sistemas estudados mostrou que esta enzima é um dos mediadores liberados em excesso pelos macrófagos durante sua atividade leishmanicida, podendo gerar desarranjo na matriz extracelular dos hepatócitos, o que afeta diretamente a sobrevida destas células;

- A metodologia utilizada para a amastigogênese gerou a produção de amastigotas axênicas puras de *Leishmania infantum* em um curto período de tempo de cultivo. As análises morfológicas e bioquímicas das amastigotas axênicas e amastigotas intracelulares de *Leishmania infantum* comprovaram as similaridades entre elas.

- O perfil de infecção de amastigotas intracelulares e axênicas em macrófagos mostrou-se semelhante, diferindo do perfil encontrado com a infecção de formas promastigotas;

- A metodologia utilizada para a obtenção de células de Kupffer gerou células *in vitro* com característica similares àquelas observada *in vivo.*

- As culturas de células de Kupffer murina estabelecidas apresentaram um perfil de secreção de citocinas durante a infecção por *Leishmania infantum* diferente do perfil de citocinas encontrado nos macrófagos peritoneais. As células de Kupffer apresentaram uma elevada produção de IL-10 no início da infecção, concomitantemente com uma baixa produção de TNF-α. Isso deve explicar uma produção menor de óxido nítrico e H_2O_2 nos macrófagos residentes comparados com os exsudativos.

- Os danos nos hepatócitos gerados durante a infecção por amastigotas de *Leishmania infantum* não foram observados nas co-culturas formadas por células de Kupffer devido ao impedimento na produção de ROS nestas células de Kupffer infectadas, e consequente bloqueio na geração de peroxinitrito.

- O peroxinitrito é o grande responsável pelos danos nos hepatócitos, sendo este somente formado pela produção de ROS e RNS concomitantemente e por isso só encontrado nos macrófagos peritoneais infectados por *Leishmania infantum*.

- As duas populações de macrófagos estudadas apresentam características distintas no fígado durante a infecção por *Leishmania infantum,* sendo os macrófagos exsudativos responsáveis pela eliminação parasitária e as células de Kupffer, pela apresentação antigênica e modulação da resposta imune adaptativa, o que deve determinar a evolução da infecção no hospedeiro vertebrado.

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9.ANEXO

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Experimental Parasitology 117 (2007) 390–398
Experimental Parasitology
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Leishmania chagasi: Cytotoxic effect of infected macrophages on parenchymal liver cells

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Received 2 August 2006; received in revised form 7 May 2007; accepted 14 May 2007
Available online 20 July 2007

Abstract

Leishmania (*Leishmania*) *chagasi*, the ethiological agent of New World visceral leishmaniasis, causes morphological and functional injury to the liver. To investigate the role of macrophage-released leishmanicidal factors in hepatocyte damage, we used a co-culture model of hepatocytes and *L. chagasi* promastigote-infected peritoneal macrophages obtained from C57BL/6 or BALB/c mice. C57BL/6 macrophages killed intracellular parasites more efficiently than BALB/c macrophages, leading to higher number of intracellular amastigotes in the BALB/c culture during the entire course of infection. Early TNF-α production led to macrophages activation resulting in parasite growth control. Hepatic transaminases and lactate dehydrogenase were present at high levels in the supernatants of both co-cultures; concurrently, parasites were eliminated from infected macrophages. Nitric oxide production was higher in C57BL/6 co-cultures than in BALB/c co-cultures. Inhibitors of the oxidative burst and secreted proteinases protected hepatocytes against toxicity, and treatment with an inducible nitric oxide synthase inhibitor fully impeded the enzyme release. Our data suggest that the intracellular cytotoxic effects elicited by macrophages for parasite destruction are directly associated with hepatocyte damage, and that nitric oxide plays a pivotal role in this phenomenon.
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Index Descriptors and Abbreviations: ALT, glutamate pyruvate transaminase; AST, glutamate oxaloacetate transaminase; iNOS, inducible nitric oxide synthase; IL-10, interleukin 10; Hep, hepatocytes; LDH, lactate dehydrogenase; MF, macrophages; NO, nitric oxide; ROI, reactive oxygen intermediates; RNI, reactive nitrogen intermediates; TNF-α, tumor necrosis factor-alpha; SOD, superoxide dismute; PMSF, phenylmethylsulfonyl fluoride; 1-10 PHEN, 1,10-phenanthroline; *Leishmania chagasi*; Macrophage; Hepatocyte; Co-culture

1. Introduction

Trypanosomatid protozoa of the *Leishmania* genus are intracellular parasites that cause a large spectrum of clinical symptoms in humans, ranging from self-healing cutaneous infections, to mucocutaneous disfigurement, to visceral leishmaniasis, which can be fatal when not treated. The outcome of infection in humans depends on both the parasite species involved and on the host immune response (Goto and Lindoso, 2004).

Natural transmission of *Leishmania* occurs during the blood meal of phlebotomine sandflies, which introduces metacyclic infective promastigotes into the vertebrate host dermis. These forms are resistant to complement attack and quickly invade local phagocytes, subsequently transforming into amastigotes inside the host cells. The mechanism involved in the dissemination or visceralization of the parasites is not clear. Infected macrophages may migrate from the initial site of infection to the

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doi:10.1016/j.exppara.2007.05.015

spleen, liver and bone marrow (Murray et al., 2003); alternatively, free promastigotes may directly enter the bloodstream as a result of the pool-feeding behavior of the sandfly (Engwerda et al., 2004a). Why *Leishmania* species of the *L. donovani* complex are viscerotropic while other species are exclusively cutaneous is a key question that still remains unanswered (Engwerda et al., 2004b).

In order to survive, intracellular organisms must escape the anti-microbial mechanisms of macrophages. During *Leishmania* promastigote infection, macrophages respond by producing reactive oxygen intermediates (ROI), together with reactive nitrogen intermediates (RNI), lysosomal hydrolases, and neutral peptidases (Murray and Nathan, 1999). Nitric oxide (NO) is a regulatory molecule that is important in host protective responses, acting not only as an anti-microbial effector molecule (Brunet, 2001), but also as a potential host-destructive mediator in several pathologies, mainly in infectious diseases (Holzmuller et al., 2006). In murine models of *Leishmania* infection, it has been well established that cytokine activation (e.g. IFN-γ and TNF-α) of macrophages leads to ROI and RNI production, which is ultimately responsible for leishmanicidal activity (Bogdan et al., 2000). NO production is the main microbicidal mechanism in murine macrophages (Dey et al., 2005).

Although ROI, RNI, and lysosomal enzymes are crucial for eliminating intracellular parasites, there are numerous clinical situations in which macrophage-secreted mediators cause tissue injury as a collateral effect of microbicidal activity (Clemens, 1999). Liver tissue damage associated with pathological conditions has been reported in both clinical and experimental visceral leishmaniasis (Gutierrez et al., 1984; McElrath et al., 1988; El Hag et al., 1994). For example, hepatocyte damage in experimental *Leishmania* infection has been associated with an increase in serum transaminases and lipid peroxidation in liver tissue (Kausalya et al., 1996; Vianna et al., 2002), and granuloma formation has been reported to be the most prevalent lesion found in *L. donovani*-infected BALB/c mice (Gutierrez et al., 1984). Interestingly, experimental data indicate that liver resident macrophages (Kupffer cells) fail to produce toxic oxygen intermediates, a key anti-microbicidal mechanism (Kausalya et al., 1996), whereas competent blood-recruited monocytes and monocyte-derived macrophages are the major cells involved in killing and degrading ingested intracellular parasites.

In this study, we adopted an adherent peritoneal macrophage–hepatocyte co-culture model to study *L. chagasi* infection. Peritoneal macrophages from two mouse strains with different patterns of susceptibility to *Leishmania* infection were used; these cells generate microbicidal products via both oxygen-dependent and -independent pathways. We evaluated hepatocyte damage and the role of microbicidal mediators secreted by infected macrophages in this process.

2. Materials and methods

2.1. Mice

C57BL/6 and BALB/c mice weighing about 20 g each were used to obtain primary cultures of peritoneal macrophages. Pregnant mice (18–20 days of gestation) were utilized for hepatocyte primary cultures. The animals were obtained from the Animal Care Facility of the Oswaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil). All procedures involving animals in this study were reviewed and approved by the Oswaldo Cruz Foundation Ethics Committee (CEUA-FIOCRUZ).

2.2. Parasites

Leishmania (*Leishmania*) *chagasi* (strain MCAN/BR/2000/CNV-FEROZ) was isolated from a dog in the State of Espírito Santo, Brazil, and typed by multilocus enzyme electrophoresis at the *Leishmania* Collection at the Oswaldo Cruz Institute, RJ, Brazil (CLIOC, WDCM 731). The parasites were maintained through inoculation in golden hamsters. Infective forms were obtained from hamster spleen or liver and cultured at 25 °C in biphase NNN blood agar medium/Schneider's Drosophila culture medium (Sigma–Aldrich St. Louis, MO, USA) containing 10% FCS.

2.3. Macrophage isolation and infection

Mouse peritoneal macrophages were obtained from both mouse strains as previously described (Araujo-Jorge and de Souza, 1986). Briefly, the animals were sacrificed using CO_2 and the peritoneal cavity was washed with 10 ml of MEM/199 culture medium (Cultilab, São Paulo, SP, Brazil) at 4 °C. For each experiment, ten mice from each strain were utilized. The cells were pooled and counted using a Neubauer chamber. The cells were seeded in tissue culture flasks (2×10^6 macrophages/flask) or on glass coverslips (2×10^5 macrophages/coverslip) and maintained in MEM/199 culture medium at 37 °C in a humidified atmosphere containing 5% CO_2.

For *in vitro* infection of the macrophages, the *L. chagasi* promastigotes were harvested at the stationary growth phase. Peritoneal macrophages were cultivated for 24 h and then infected at a ratio of 10 parasites per cell with or without 1 µg/ml LPS (Sigma Chemical Co., St Louis, MO, USA). The macrophages and parasites were incubated together for 1 h at 34 °C. The cultures were then washed three times with MEM/199 culture medium to remove non-adherent or non-internalized parasites.

2.4. Hepatocyte isolation

Hepatocytes from both mouse strains were isolated according to Porrozzi et al., 1997. Briefly, 7–11 mouse embryo livers (1–2 g each) were aseptically removed and

washed with Hepes buffer (Sigma–Aldrich, St. Louis, MO, USA). The livers were minced and then incubated at 37 °C for 20 min with about 50 ml of Hepes buffer containing 0.05% Type II collagenase (Sigma–Aldrich, St. Louis, MO, USA). The cells were dispersed by pipetting and then collected by centrifugation at 200*g*. Viable cells were purified by sedimentation for 10 min at room temperature with MEM/199 medium containing 10% fetal calf serum (FCS, Sigma–Aldrich, St. Louis, MO, USA).

2.5. Hepatocyte-infected macrophage co-culture

Hepatocytes were seeded directly onto the infected-macrophage plates, as reported previously (Kausalya et al., 1993). Infected peritoneal macrophages were co-cultivated with hepatocytes from the same mouse strain at a 10:1 ratio (2×10^6 macrophages/2×10^5 hepatocytes in 3 ml of MEM/199 medium plus 10% FCS in tissue culture flasks or 2×10^5 macrophages/2×10^4 hepatocytes in 300 μl of MEM/199 medium plus 10% FCS on glass coverslips). These co-cultures were maintained in culture medium at 37 °C in a 5% CO_2 atmosphere. Hepatocytes were co-cultured with uninfected macrophages as controls. The culture medium was maintained without changing during the entire experimentation period. After the interaction of infected macrophages and hepatocytes, the coverslips were washed three times in culture medium, fixed in Bouin's solution, and stained with Giemsa (Merck). The percentage of infected macrophages and the mean number of intracellular parasites per infected macrophage were determined by counting at least 100 cells. These experiments were repeated three times to confirm that the experimental data were reproducible. All experiments were also performed in macrophage cultures alone for comparison with the co-cultures. The co-culture supernatant was collected and examined by light microscopy for detached or dead cells.

2.6. Detection of cytokines in the co-culture supernatant

Aliquots of culture supernatant were collected from control and infected co-cultures after 24, 48, and 72 h. Tumor necrosis factor (TNF-α) and interleukin-10 (IL-10) were detected using a commercial ELISA kit (DuoSET ELISA; R&D Systems, Minneapolis, MN, USA).

2.7. Release of hepatocyte enzymes

Glutamate oxaloacetate transaminase (AST) and glutamate pyruvate transaminase (ALT) activities were assayed with commercial kits (Labtest, Lagoa Santa, MG, Brazil). Lactate dehydrogenase (LDH) activity was measured using a Merck activity assay kit (Merck, Darmstadt, Germany). Enzymatic activity assays were performed according to the manufacturer's instructions and adapted for detecting hepatocyte damage markers. Analyses were performed with both the cell lysate and the co-culture supernatant, using one Petri dish for each time point. Cell lysate was obtained by scraping the culture monolayers and then subjecting the scraped cells to sonication. Measurements of enzymes released from infected macrophage cultures were carried out in parallel. The results were expressed as enzyme release percentage and calculated as follows:

$$\frac{\text{ES}}{(\text{ES} + \text{EL})} \times 100 = \%\ \text{ER}$$

ES, enzyme measured in the supernatant; EL, enzyme measured in the lysates; ER, enzyme release.

2.8. Ultrastructural examination of co-cultured cells

The co-cultured cells were fixed for 1 h in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer supplemented with 3.5% sucrose (Sigma–Aldrich, St. Louis, MO, USA). The cells were then washed with 0.1 M cacodylate buffer and post-fixed with 1% OsO_4 in the same buffer. The samples were then washed twice in buffer, dehydrated in graded acetone, and embedded in Epon. Samples were observed using a Zeiss EM10C transmission electron microscope.

2.9. Treatment of co-cultures with microbicidal inhibitors

Infected and uninfected co-cultures were incubated with different microbicidal mediator inhibitors: superoxide dismutase (SOD, 30 U); mannitol (20 μM); N^G-nitro-L-arginine-methyl ester (L-NAME, 4 mM); phenylmethylsulfonyl fluoride (PMSF, 1 mM); 1,10-phenantroline (1-10 PHEN, 10 mM); and cystatin C (4 mM). Untreated infected co-cultures were treated with the same inhibitors as controls. Samples of supernatant from C57BL/6 and BALB/c hepatocyte–macrophage co-cultures were collected after 24 and 48 h of treatment, respectively. The inhibition results indicate the difference between infected and uninfected treated co-cultures. Samples were then analyzed for hepatic transaminases and LDH as described above.

2.10. Nitrite (NO^{2-}) accumulation

The production of NO in the co-cultures was evaluated by measuring nitrite, a stable breakdown product of NO, in the culture supernatant. Cells were cultured for up to 72 h on culture plates, and then culture supernatant was collected. A 100 μL aliquot was mixed with an equal volume of Griess reagent (50 μL of 1% sulfanilamide plus 50 μL of 0.1% N-1-naphthylethylenediamine in 5% phosphoric acid solution), and absorbance was measured at 550 nm. Nitrite concentration was calculated using a standard curve.

2.11. Statistical analysis

All experiments were carried out in triplicate, and repeated at least three times. The mean values and standard

deviations (SD) for all numerical data were calculated. The data were analyzed with the Student's *t*-test. Simple linear regression analysis was performed to calculate correlations. Differences with a *P* value <0.05 were considered statistically significant.

3. Results

3.1. Infection of co-cultured cells with *L. chagasi*

The C57BL/6 and BALB/c macrophage–hepatocyte co-cultured cells grew in a distinct morphological pattern: groups of closely associated cells that appeared to be differentiated hepatocytes were surrounded by spread-out, separated macrophages (Fig. 1A and B). The progression of *L. chagasi* infection in co-cultures was monitored by counting the number of amastigotes and infected macrophages using light microscopy (Fig. 1C). Hepatocytes were not infected, so that amastigotes were not observed inside these cells in any of the co-cultures. The course of *L. chagasi* infection in the C57BL/6 co-cultures exhibited a similar pattern to that in the BALB/c co-cultures, but had different kinetics. In cultures of infected macrophages from both mouse lineages, infection followed a similar course as in the co-cultures (data not shown). During the course of infection, C57BL/6 co-cultures had fewer parasites inside the macrophages than in the BALB/c co-cultures. In addition, the macrophages from C57BL/6 mice reduced the number of intracellular amastigotes faster than the BALB/c cells (Fig. 1C). The number of infected BALB/c mouse macrophages increased up to 48 h post-infection (hpi), accompanied by intracellular parasite multiplication, while the number of infected C57BL/6 mouse macrophages and parasites had already declined at this time point (Fig. 1C). However, despite the reduction in the number of parasitized cells at 72 hpi, neither BALB/c nor C57BL/6 co-cultures were able to control the infection completely (Fig. 1C). The co-cultures showed no significant differences in the total number of cells during the course of the experiment. We also counted cells in the supernatant of the co-cultures and very few cells were found (data not shown).

3.2. Cytokine production

Cytokine production by the *L. chagasi*-infected co-cultures was assessed by ELISA. Following the infection, the C57BL/6 and BALB/c hepatocyte–macrophage co-cultures displayed differences in the level and kinetics of cytokine production. Production of the cytokine TNF-α was observed for both mouse strain co-cultures, demonstrating

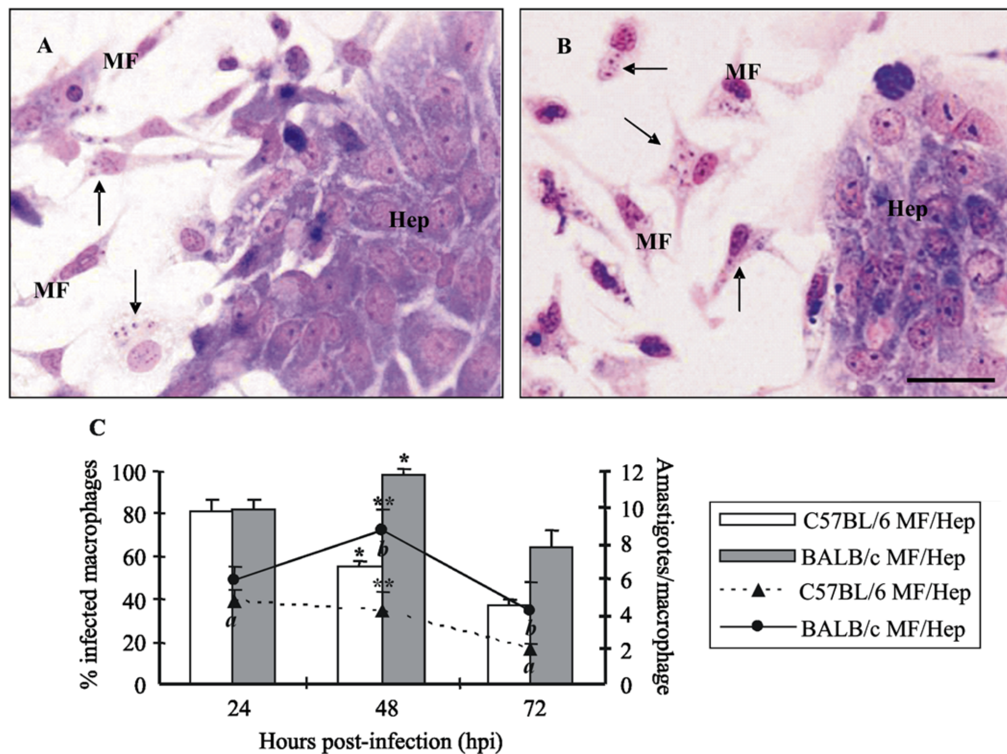

Fig. 1. *L. chagasi* infection in macrophage (MF)-hepatocyte (Hep) co-cultures. A and B show C57BL/6 and BALB/c co-cultures, respectively, at 48 h post infection (hpi). The amastigote forms of *L. chagasi* (arrows) were located only inside macrophages. Scale bar = 10 μm. In (C), the percentage of infected macrophages is represented as open bars (C57BL/6) or shaded bars (BALB/c), and intracellular parasites in C57BL/6 and BALB/c cells are shown as straight or dashed lines, respectively. Bars indicate mean ± standard deviation. *Statistically significant difference ($P < 0.05$) between the percentage of infected BALB/c and C57BL/6 macrophages at 48 hpi. **Statistically significant difference ($P < 0.05$) between the number of amastigotes at 48 hpi in BALB/c and C57BL/6 macrophages. (A) $P < 0.05$ for the number of intracellular parasites inside C57BL/6 macrophages at 24 hpi compared to 72 hpi. (B) $P < 0.05$ for the number of intracellular parasites inside BALB/c macrophages at 48 hpi compared to 72 hpi.

macrophage activation (Fig. 2A). TNF-α levels peaked at 24 hpi for C57BL/6 derived co-cultures, while the secretion by BALB/c co-cultures peaked at 48 hpi (Fig. 2A). At 72 hpi, there was a statistically significant ($P < 0.05$) decrease in the production of this cytokine by the infected co-cultures from both strains, compared to the levels produced at the beginning of the infection (Fig. 2A).

The level of the cytokine IL-10 increased significantly ($P < 0.05$) from 24 to 48 hpi in co-cultures derived from C57BL/6 mice. For the BALB/c system, the production of this cytokine increased ($P < 0.05$) from 48 to 72 hpi (Fig. 2B). These time points coincided with the reduction of intracellular parasites in each co-culture (Fig. 2B).

3.3. Evaluation of hepatocyte damage

The release of hepatic transaminases and LDH in the co-culture supernatants served as markers of hepatocyte damage. There was a substantial amount of these two enzymes in the supernatants of *L. chagasi*-infected co-cultures compared to basal levels in uninfected co-cultures. Infected macrophages were assayed for enzymes released during the course of infection, and there were none detected in the supernatant (data not shown). Maximum enzyme release was observed in the infected C57BL/6 and BALB/c co-cultures at 48 and 72 hpi, respectively, probably due to the intense leishmanicidal activity by macrophages at these times (Fig. 3A–C). Moreover, the increase in enzyme release was statistically significant ($P < 0.05$) when compared with the enzyme levels observed at the same time points in the supernatant of uninfected systems (Fig. 3A–C).

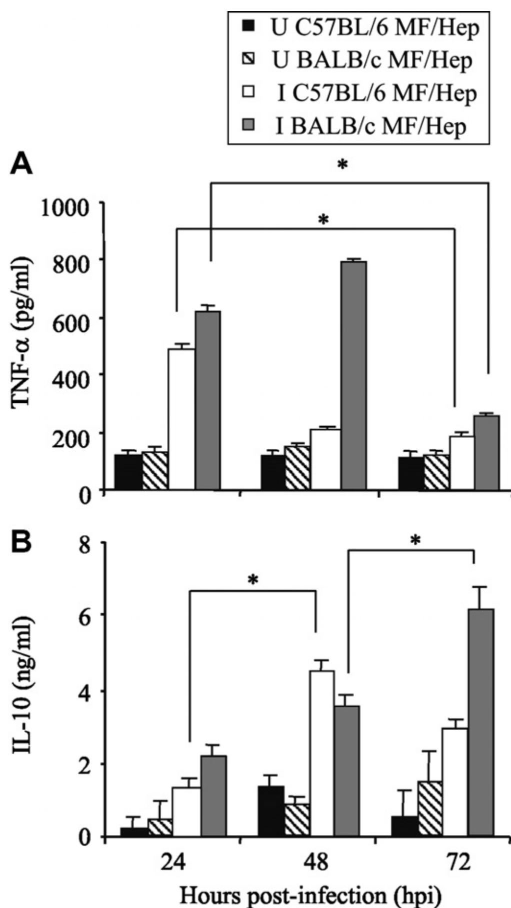

Fig. 2. Macrophage activation status is revealed by the presence of cytokines in the supernatant of *L. chagasi*-infected (I) or uninfected (U) C57BL/6 and BALB/c co-cultures. The levels of the cytokine TNF-α in the C57BL/6 and BALB/c co-cultures are shown in (A), while (B) shows the production of IL-10 in each co-culture. Bars indicate mean ± standard deviation. *Statistically significant difference ($P < 0.05$).

The addition of LPS to the co-cultures prior to infection caused macrophage activation, leading to the release of hepatic transaminases and LDH in the supernatants earlier and in higher amounts than in co-cultures without LPS treatment (data not shown). The LPS treatment also activated the macrophages from uninfected co-cultures; however, uninfected cells produced lower levels of the enzymes studied than did the infected co-cultures (data not shown).

The co-cultured cells were analyzed by transmission electron microscopy to confirm hepatocyte damage. Parasites were only observed inside macrophages. Well-preserved macrophages and hepatocytes were seen in uninfected C57BL/6 and BALB/c co-cultures throughout the experiment (Fig. 4A and B). However, degenerative alterations in the hepatocytes were detected in infected co-cultures, with severe damage visible starting at 48 hpi in C57BL/6 co-cultures and at 72 hpi in BALB/c co-cultures (Fig. 4C and D). Mitochondrial degeneration was observed at the beginning of the infection. Later on, degeneration throughout the cytoplasmic compartment was observed in both infected co-cultures (Fig. 4C and D).

3.4. Inhibition of hepatocyte damage

To investigate the relevance of the leishmanicidal molecules secreted by infected macrophages on hepatocyte damage, we treated the co-cultures with mediator-specific inhibitors and measured the release of two hepatic transaminase (AST and ALT) and LDH. Treatment with ROS scavengers, which suppress the oxidative burst, SOD and mannitol, led to a reduction in enzyme release, particularly in infected BALB/c co-culture supernatant ($P < 0.05$) compared with the untreated co-cultures at the same time point (Table 1). We observed a decrease in enzyme release in both C57BL/6 and BALB/c co-cultures when they were treated with the protease inhibitors PMSF, cystatin C, and 1-10 PHEN. Moreover, treatment with L-NAME, an iNOS inhibitor, fully inhibited the release of hepatic transaminases and LDH in the infected C57BL/6 and BALB/c co-cultures, the enzyme levels in both co-cultures were similar to those found in the uninfected controls (Table 1).

3.5. Nitrite production

NO release by macrophages during experimental infection with *L. chagasi* promastigotes was measured by nitrite

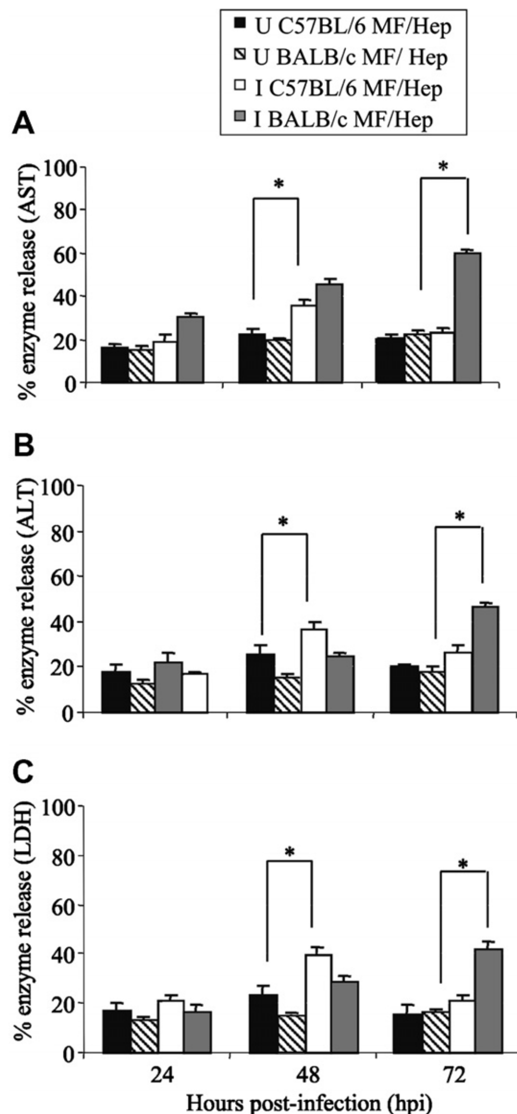

Fig. 3. Hepatocyte damage detected by AST (A), ALT (B), and LDH (C) release in infected (I) or uninfected (U) co-culture supernatants from C57BL/6 and BALB/c mice. Bars indicate mean ± standard deviation. *Statistically significant difference ($P < 0.05$).

accumulation in the co-culture supernatant. During the course of infection in the BALB/c and C57BL/6 systems, nitrite levels in the infected co-culture supernatants were higher than in the uninfected co-culture supernatants (Fig. 5). The maximum nitrite level was observed in infected C57BL/6 co-cultures at 48 hpi, a period in which the number of amastigotes inside macrophages was also high. The nitrite level detected in the infected BALB/c co-culture supernatant was consistently and significantly lower than levels detected in the infected C57BL/6 co-culture supernatants ($P < 0.05$; Fig. 5). Treatment of macrophages with LPS resulted in increased NO production in both co-cultures (data not shown). NO production in hepatocyte cultures was also evaluated, and a low level of NO was detected (data not shown). This suggests that macrophages are the major source of NO in the co-culture supernatant.

4. Discussion

Interestingly, macrophages are the vertebrate host cells for *Leishmania*, as well as cells that are essential for host defense (Basu and Ray, 2005). Even though anti-microbial activity is directly associated with parasite load decrease, this activity can cause tissue damage. As of yet, the role of macrophages in liver injury during *Leishmania* spp. infection has not been explored in depth. The principal goal of this study was to determine the relationship of parasite elimination by macrophages to extracellular cytotoxicity, assessed by measuring hepatocyte damage in a co-culture model system.

A co-culture system of macrophages and hepatocytes was used to investigate the hepatocyte damage induced by *L. chagasi* infection. Previous studies have demonstrated that macrophages obtained from distinct mouse strains show similar patterns of *L. donovani* infection *in vitro*, although different macrophage populations show a remarkable difference in their phagocytic activity (Olivier and Tanner, 1987). In our study, peritoneal macrophages were chosen for the co-culture system because of their ability to trigger leishmanicidal activity via several pathways (i.e. ROI, RNI, and lysosomal enzymes), which is a characteristic they have in common with bloodstream-derived macrophages. *Leishmania* parasites can inhibit the respiratory burst, which helps the parasites survive. Kausalya et al., 1996 highlight the different pathways adopted by liver resident and recruited macrophages, in which Kupffer cells displayed impairment in their oxidative metabolism. Since that study was performed with cells isolated from infected animals, this impairment might have been related to the infection.

Our goal was to correlate hepatocyte damage with specific leishmanicidal compounds secreted by infected macrophages. In this study, peritoneal macrophages from different mouse strains reduced *L. chagasi* infection, as did exudative macrophages recruited from peripheral circulation, but strain-specific differences in the course of the infection were observed. Our results demonstrated that macrophages from C57BL/6 and BALB/c mice have differences in susceptibility and in the kinetics of infection by *L. chagasi*. Specifically, C57BL/6 macrophages kill intracellular amastigotes faster and more efficiently than BALB/c macrophages.

High levels of TNF-α were detected at the beginning of the infection in the co-cultures of both strains indicating early macrophage activation and consequent parasite elimination. An increase in the Th1 cytokine levels was previously found at the beginning of liver infection by *Leishmania* parasites, which helped control parasite growth (Gumy et al., 2004). TNF-α plays an important role in protection against *Leishmania* infection by activating macrophage oxidative mediator release (Engwerda et al., 2004a). After the peak of macrophage activation, there was a significant reduction in intracellular amastigotes in the co-cultures; in addition, the reduction in parasite load

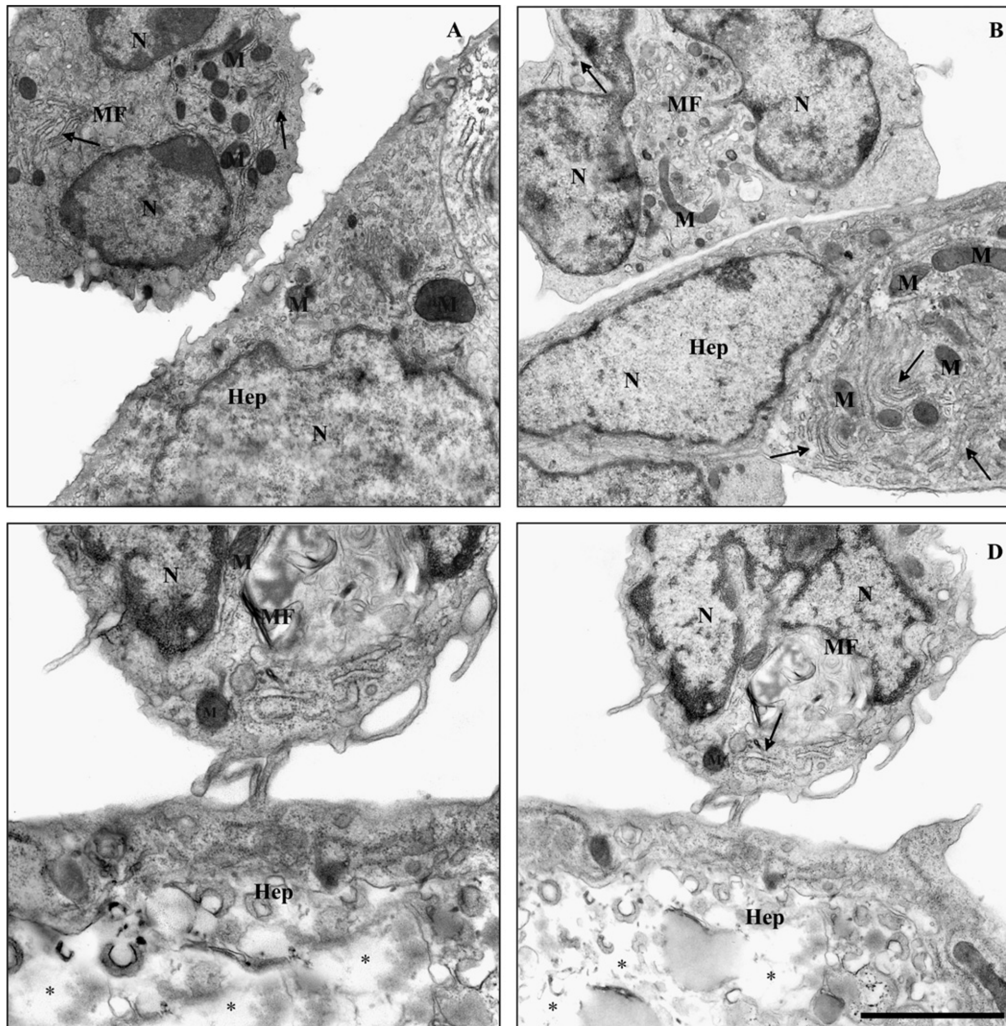

Fig. 4. Transmission electron microscopy of the macrophage–hepatocyte co-cultures. Ultrastructural analysis shows that in uninfected macrophages and hepatocytes from C57BL/6 (A) and BALB/c (B) mice, organelles such as mitochondria (M), endoplasmic reticulum (arrow), and nuclei (N) show normal morphological characteristics, while infected C57BL/6 (C) and BALB/c (D) co-cultured cells show organelle degeneration (*) in the hepatocytes at 48 and 72 hpi, respectively. Scale bar = 1 μm.

Table 1
Effect of inhibitors during the leishmamcidal activity of macrophages in infected C57BL/6 and BALB/c co-cultures

Inhibitors	C57BL/6			BALB/c		
	ALT	AST	LDH	ALT	AST	LDH
Percentage of enzyme release						
Infected	40.2 ± 1.2	30.6 ± 1.9	32.3 ± 1.6	57.9 ± 0.9	45.1 ± 1.7	41.6 ± 0.8
Uninfected	14.5 ± 1.8	15.6 ± 1.3	12.6 ± 1.8	18.9 ± 1.2	17.4 ± 1	13.7 ± 1.5
SOD	23.3 ± 1.3*	18.9 ± 1.5*	25.8 ± 1	25.5 ± 0.9*	21.2 ± 1.9*	21.8 ± 1.7*
Mannitol	31.4 ± 1.5*	29.9 ± 0,9	31.9 ± 1.8	28.1 ± 0.6*	19.4 ± 1.5*	21.6 ± 1.4*
PMSF	24.1 ± 1.3**	20.1 ± 1.4**	22.8 ± 1.2**	43.2 ± 1.4**	33.6 ± 1**	32.3 ± 0.8**
10-PHEN	22.6 ± 1.9**	20.9 ± 1.5**	21.3 ± 1.3**	36.5 ± 0.5**	31.1 ± 1.2**	29.5 ± 1.1**
Cystatin C	30.8 ± 1.5**	24.6 ± 1.5**	23.9 ± 1.3**	38.5 ± 0.8**	40.2 ± 1.8**	35.8 ± 1.3**
L-NAME	15.6 ± 13***	15.9 ± 1.5***	14 ± 1.7***	19.9 ± 1.6***	16.6 ± 1.8***	16.3 ± 2***

The C57BL/6 and BALB/c co-cultures were treated with different inhibitors after macrophage infection. Untreated infected co-cultures were used as controls. *Statistically significant difference ($P < 0.05$) between the BALB/c and C57BL/6 controls and co-cultures treated with SOD or mannitol. **Statistically significant difference ($P < 0.05$) between the BALB/c and C57BL/6 controls and co-cultures treated with proteases inhibitors. ***Statistically significant difference ($P < 0.05$) between the BALB/c and C57BL/6 controls and co-cultures treated with iNOS inhibitor.

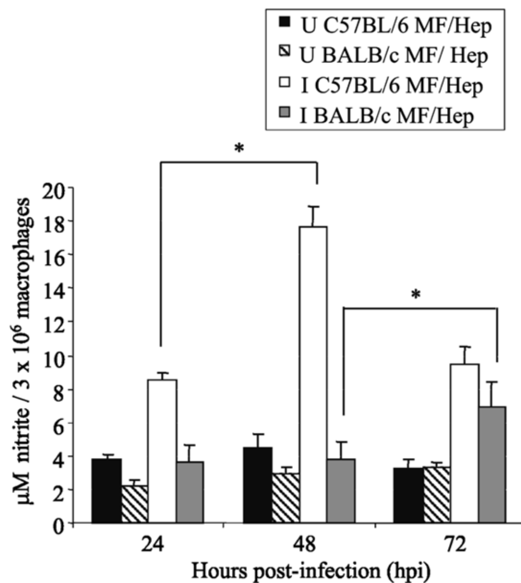

Fig. 5. Nitrite levels in uninfected (U) and *L. chagasi*-infected (I) C57BL/6 and BALB/c co-culture supernatants. Bars indicate mean ± standard deviation. *Statistically significant difference ($P < 0.05$).

was followed by an increase in IL-10. The increase in these cytokines accounted for macrophage deactivation and might be associated with parasite persistence. Studies in murine models *in vitro* and *in vivo* found that high levels of IL-10 later during the course of *Leishmania* infection inhibited the leishmanicidal mechanisms (Gazzinelli et al., 1992; Alexander and Bryson, 2005).

Although macrophage activation is responsible for parasite control, the activated cells can also be implicated in hepatocyte injury (Wang et al., 1998). Since most physiological liver activities are due to hepatocyte cells, any disturbance in the number of healthy cells may be pivotal to organ homeostasis and function. During human or experimental visceral leishmaniasis, there is an increase in the production of hepatic transaminases, indicating hepatocyte lesions (Kausalya et al., 1993; El Hag et al., 1994). Furthermore, the LDH enzyme is considered to be a specific marker for tissue damage (Kikkawa et al., 2005). We followed *L. chagasi* infection in BALB/c mice and found an increase in serum transaminases between the third and fourth weeks post-infection along with an increase in parasite load in the liver (data not shown). In this study, hepatic transaminases and LDH release increased in the macrophage/hepatocyte co-cultures during the *L. chagasi* infection, indicating hepatocyte damage induced by infected macrophages. The enzyme release was associated with the parasite destruction inside the macrophages, where enzyme detection was related to intense parasite destruction. The activation of infected BALB/c co-cultured macrophages by LPS resulted in enzyme release earlier than in uninfected cultures (data not shown). Hepatocyte damage occurred at the same time point in infected C57BL/6 co-cultures without the addition of LPS, confirming that C57BL/6 macrophages are naturally more efficient at eliminating parasites than are BALB/c macrophages.

The damage of hepatocytes in infected co-cultures from both mice strains was confirmed by morphological observation. Ultrastructural alterations were mostly associated with mitochondria, but extended to other cytoplasmic structures. This intracellular degeneration has been reported in both human and experimental visceral leishmaniasis (El Hag et al., 1994; Vianna et al., 2002). Our results clearly indicate that infected macrophages induce hepatocyte damage as a non-desirable side effect. During *in vivo Leishmania* infection, degeneration with signs of necrosis has been noted in the lobe periphery, but the hepatic changes were related neither to the presence of parasites nor to the inflammatory reaction (El Hag et al., 1994).

The results presented in the present study demonstrated that inhibitors of different microbicidal agents reduced hepatocyte damage. However, only the NO inhibitor was able to completely abrogate enzyme release. Several stimuli promote macrophage iNOS expression, which catalyzes the synthesis of NO from L-arginine and molecular oxygen (Vouldoukis et al., 1997). NO is considered to be one of the most important cytostatic and cytotoxic agents in protozoan infection (Coleman, 2001), and is involved in several types of hepatocyte injury (Abramson et al., 2001).

In spite of macrophage activation, as established by the high TNF-α production, the infected BALB/c co-cultures produced low NO levels during the course of the infection resulting in parasite persistence. These results corroborated previous studies by Stenger et al. (1994) and Chen et al. (2005). However, infected C57BL/6 co-cultures displayed high levels of NO production from the beginning of the infection and, consequently, were able to kill the amastigotes earlier than the BALB/c macrophages. Moreover, our results also established a correlation between the levels of NO and hepatocyte damage marker release (LDH and transaminases). Thus, the highest levels of NO production and the greatest enzyme release were observed at the same time point. This suggests that NO production by the macrophages has a direct role in hepatocyte damage.

In conclusion, the results of this study indicate that hepatocyte damage induced by *L. chagasi*-infected macrophages is mediated mainly by NO production. The NO production level correlated with the production of hepatic transaminases and LDH, both cell damage markers, which also indicates the pivotal role of NO in cellular damage. Our inhibition studies indicated that other mediators also play a role in liver damage. The inhibitors tested protected hepatocytes to different extents, indicating that different mediators might work synergistically to destroy intracellular parasites, while causing incidental damage at the cellular and tissue levels. Defining the roles of each mediator in hepatocyte damage will be important for a full understanding of the mechanisms of the visceral leishmaniasis pathogenesis.

Acknowledgments

The authors thank Dr. Maurílio José Soares and Dr. Elisa Cupolillo for critical review of the manuscript, Bruno Ávila for computer imaging support. This work was supported by grants from Fundação Oswaldo Cruz (Fiocruz), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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