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TESE DE DOUTORADO

ESTUDO DE MECANISMO MOLECULARES DETERMINANTES DE DIFERENÇAS DA INTERAÇÃO DE MACRÓFAGOS DE CAMUNDONGOS CBA/J *com Leishmania major ou Leishmania amazonensis*

IVANA NUNES GOMES DE ARAÚJO

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Estudo de Mecanismos Moleculares que Determinam as Diferenças de Interação de Macrófagos de Camundongos CBA com Leishmania major ou Leishmania amazonensis

IVANA NUNES GOMES

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Ao meu Deus
por Sua infinita misericórdia e
por Seu incondicional amor.

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SUMÁRIO

LISTA DE ABREVIATURAS

GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAMP	Lisossomal associated membrane protein
LPS	Lipopolisacarid
LPG	Lipophosphoglycan
MHC	Major histocompatibility complex
NK	Natural killer
NRAMP	Natural resitance associated macrophage protein
NO	Nitric oxide
TGF	Transforming growth factor
Th	T helper
TNF	Tumoral necrosis factor
CCR	C-C chemokine receptor
CCL	C-C chemokine ligand
CXCL	CXC chemokine ligand
XCL1	Limphotaxin
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
MIF	Migration inhibitory factor

RESUMO

ESTUDO DE MECANISMOS MOLECULARES DETERMINANTES DE DIFERENÇAS DA INTERAÇÃO DE MACRÓFAGOS DE CAMUNDONGOS CBA com *Leishmania amazonensis* OU *Leishmania major*. **IVANA NUNES GOMES**

Camundongos CBA são susceptíveis à *Leishmania amazonensis* e resistentes à *L. major*. Eventos da resposta imune inata parecem cruciais na determinação da resposta à infecção por Leishmania. Macrófagos desempenham papel importante no controle da infecção, pois *in vitro* controlam a infecção por *L. major*, entretanto são permissíveis à *L. amazonensis*. Neste trabalho, pretendeu-se investigar mecanismos moleculares envolvidos na determinação dos perfis de resposta de macrófagos de CBA infectados, *in vitro*, por *L. amazonensis* ou *L. major*. Observamos que IFN-γ, apesar de induzir produção semelhante de NO, reduz a infecção causada por *L. major*, não alterando a infecção por *L. amazonensis*. Essa redução é dependente de TNF-α. Em estudos de biogênese do vacúolo parasitóforo, observou-se que há semelhança na cinética de fusão dos vacúolos contendo *L. amazonensis* ou *L. major* com lisossomas, evidenciando que a maior sobrevivência de *L. amazonensis* não está relacionada a um retardo na formação do fagolisossomo. O padrão de expressão de genes, que poderiam influenciar no desenvolvimento da resposta imune do hospedeiro à infecção por Leishmania, foi avaliado, utilizando-se a técnica de DNA*microarray*. A infecção por *L. amazonensis* ou *L. major* induz alterações no padrão de expressão de genes anteriormente relacionados à infecção por Leishmania, e outros que ainda não tinham sido associados. Genes relacionados à explosão respiratória, formação do vacúolo parasitóforo e receptores de superfície, envolvidos na ativação celular e fagocitose, estão induzidos ou suprimidos a depender da espécie de Leishmania e o tempo de infecção. Genes relacionados a receptores do tipo *scavenger* foram induzidos na infecção por *L. major*. Esse resultado está relacionado ao aumento na expressão *in vitro* do receptor *scavenger* MARCO em células infectadas por *L. major*, quando comparada à infecção por *L. amazonensis*. Estudos *in vivo* demonstraram que linfonodos de camundongos infectados por *L. major* apresentaram um aumento da expressão de MARCO em comparação à infecção por *L. amazonensis*. Observamos que *L amazonensis* induziu gene da catalase, enzima que inibe a explosão respiratória. Esse dado pode estar relacionado com a observação anterior, que na infecção *in vitro* de macrófagos com *L. amazonensis* há uma inibição da produção de H_2O_2, em comparação à célula controle. Esses dados sugerem que diferenças encontradas na infecção de macrófagos podem estar relacionadas com a determinação dos perfis de resistência ou susceptibilidade, reforçando a importância do macrófago para o estabelecimento da infecção.

Palavras-chave: Macrófago, *L. amazonensis*, *L. major*, TNF-α, Expressão gênica.

ABSTRACT

STUDY OF MOLECULAR MECHANISMS WHICH DETERMINE DIFFERENCES IN CBA MACROPHAGE INTERACTION *Leishmania amazonensis* OR *Leishmania major*. **IVANA NUNES GOMES**

CBA mice are susceptible to *Leishmania amazonensis* and resistant to *L. major* infection. Events of innate immune response are supposed to be determinants of Leishmania infection outcome. CBA macrophages control *L. major* and are permissive to *L. amazonensis* infection *in vitro*, indicating that macrophages participate in determination of host immune profile. In the present work, we intended to investigate the molecular mechanisms, which underlie these differences. We demonstrated that IFN-γ was only able to reduce *L. major* infection although induced similar NO production by both *L. amazonensis*- and *L. major*-infected cells. This reduction is a TNF-α-dependent mechanism. Furthermore, the ability of *L. amazonensis* to survive inside CBA nacrophages was not related to a delay on *L. amazonensis*-induced phagosome fusion with lysosomes as both *L. amazonensis*- and *L. major*-induced parasitophorous vacuoles present the same kinetic of fusion with lysosomes. DNAmicroarray was then performed in response to *L. amazonensis* or *L. major* infection which are the macrophages genes up or down-regulated. We showed that both parasites induced significant alterations on macrophage gene expression. Some of the expressed genes were previously related to Leishmania infection but some of them were not yet associated to Leishmania infection. Genes related to cell surface receptors, which participate both in cell activation and phagocytosis of microorganisms, as well as genes involved in respiratory burst response and in parasitophorous vacuole biogenesis had their expression modified dependending on the Leishmania species and time after infection. Interestingly, we observed that *L. major* induced higher expression of scavenger receptors. In addition, this result is related to a 20% higher expression of MARCO scavenger receptor in *L. major*-infected macrophages. We also demonstrated that *L. amazonensis* infected macrophages express genes of enzymes related to ROI scavenging such as catalase. These data is related to our previous observation that, in *L. amazonensis*-infected cells, there was similar H_2O_2 generation when compared to control non-infected cells. In summary, these data suggest that CBA macrophages are able to interact distinctly with *L. amazonensis* and *L. major*, supporting the idea that macrophage is a key cell in the determination of resistance and susceptibility in Leishmania infection.

Key words: Macrophages, *L. amazonensis*, *L. major*, TNF-α, Gene expression.

1. CAPÍTULO I - INTRODUÇÃO

1.1 O MODELO MURINO DA LEISHMANIOSE

A leishmaniose é uma doença de amplo espectro causada por diferentes espécies de Leishmania. As manifestações clínicas dessa doença dependem da espécie de Leishmania e da resposta imune do hospedeiro. *Leishmania major* é um dos principais agentes causadores da leishmaniose cutânea no Velho Mundo (Peters, 1987) e *L. amazonensis*, além de outros, no Novo Mundo (Grimaldi *et al.*, 1993).

Distintos padrões de manifestações clínicas em pacientes com leishmaniose cutânea podem ser reproduzidos em diferentes linhagens de camundongos (Behin *et al.*, 1979; Mitchell *et al.*, 1981). O modelo murino da infecção tem sido utilizado na elucidação de mecanismos de resposta imune que levam à resistência ou à susceptibilidade. A infecção de camundongos de diferentes linhagens por *L. major*, constituiu-se o primeiro modelo *in vivo* de correlação do desenvolvimento de resposta protetora com a expansão de células Th1 $CD4^+$, e de progressão da doença com o desenvolvimento da resposta Th2 $CD4^+$. Camundongos da linhagem CBA, quando infectados por *L. major*, desenvolvem resposta inflamatória característica de resistência, com infiltração linfocitária e macrofágica, formação de granulomas, necrose fibrinóide e redução do número de parasitos na lesão (Lemos de Souza *et al.*, 2000). Esses mesmos animais infectados por *L. amazonensis* apresentam perfil histopatológico semelhante ao observado nas lesões de camundongos BALB/c altamente susceptíveis (Andrade *et al.*, 1984), com extensas áreas de necrose de coagulação, infiltrado mononuclear macrofágico difuso, raros linfócitos e grande carga parasitária (Lemos de Souza *et al.*, 2000).

O padrão da resposta imune de camundongos CBA infectados por *L. major* ou *L. amazonensis* correlaciona-se com o perfil morfológico de resposta tecidual. Na infecção

por *L. major*, esses camundongos apresentam níveis elevados de IFN-γ, evidenciando uma resposta do tipo Th1 e, quando infectados por *L. amazonensis*, produzem níveis elevados de IL-4, caracterizando uma resposta do tipo Th2. Esse modelo enfatiza o papel do parasito na determinação do tipo de resposta imuno-inflamatória, pois camundongos com o mesmo perfil genético são resistentes ou susceptíveis a duas espécies distintas de Leishmania (Lemos de Souza *et al.*, 2000).

1.2 MACRÓFAGOS E A RESPOSTA IMUNE INATA

A imunidade a microrganismos pode ser agrupada em dois sistemas: imunidade inata e imunidade adquirida. A imunidade adquirida é mediada por linfócitos T e B, sendo caracterizada por especificidade e memória. A imunidade inata é definida como uma resposta imune não específica e caracterizada pelo rápido reconhecimento de antígenos, recrutamento e ativação de células inflamatórias, liberação de mediadores e moléculas microbicidas efetoras (Fearon *et al.*, 1996). Além disso, ocorre englobamento e digestão de microorganismos e de substâncias estranhas, por macrófagos e leucócitos. Estudos têm evidenciado que a imunidade inata apresenta seletividade, sendo capaz de discriminar entre o próprio e o patógeno (Aderem *et al.*, 2000; Hoffmann *et al.*, 1999; Medzhitov *et al.*, 1997). Além disso, a ativação da resposta imune inata é pré-requisito para estimular a resposta imune adquirida.

Os macrófagos desempenham papel crucial na detecção de microorganismos e apresentam tanto funções moduladoras, como efetoras da resposta imune. Durante a resposta de defesa do hospedeiro à infecção, o processo de fagocitose limita a disseminação inicial do crescimento de organismos. Conseqüentemente, os mecanismos de reconhecimento do não próprio estão ligados ao processo de fagocitose e são cruciais na indução de mecanismos imunes dependentes de macrófagos. Receptores na

superfície dessas células reconhecem moléculas do patógeno, resultando numa cascata de interações intra e intercelulares, que culminam na ativação dos macrófagos e na produção de fatores microbicidas. Diferentes tipos de células da resposta imune, tais como, célula dendrítica, células T e células NK participam desse processo.

Leishmania são protozoários que se desenvolvem e completam seu ciclo de vida predominantemente em macrófagos (Alexander *et al.*, 1992). O ciclo de vida da Leishmania compreende duas formas: promastigota móvel, que se desenvolve no intestino do inseto vetor; e amastigota, que se desenvolve no interior de fagócitos mononucleares do hospedeiro vertebrado. Ao penetrarem nos macrófagos, as promastigotas se transformam em amastigotas, que vivem e se multiplicam em compartimentos fagolisossomais (Alexander *et al.*, 1992).

Há evidências de que a resposta à infecção por Leishmania é determinada por eventos da resposta imune inata, que ocorrem nos primeiros dias de infecção, antes da diferenciação da resposta Th1 ou Th2 (Chatelain *et al.*, 1992; Sypek *et al.*, 1993). Manipulações com o objetivo de reverter a resposta imune são eficazes somente se realizadas durante a primeira semana de infecção. Tratamento com anti-IL-4 (Sadick *et al.*, 1990) ou administração de IL-12 (Sypek *et al.*, 1993) durante a primeira semana de infecção permite que camundongos BALB/c, uma linhagem altamente susceptível, desenvolvam a cura da infecção.

Macrófagos desempenham um papel importante no controle da infecção por Leishmania, atuando nas fases indutora e efetora da resposta imune. Macrófagos, além de serem as principais células hospedeiras do parasito, são células apresentadoras de antígenos para linfócitos T específicos. Além disso, podem liberar citocinas associadas à resposta Th1 (IL-12, TNF-α) ou Th2 (IL-10, TGF-β) (Wang *et al.*, 1994). O controle da infecção é dependente da capacidade dos macrófagos em destruir parasitos

intracelulares (Behin *et al.*, 1979; Nacy *et al.*, 1983) por mecanismos dependentes de NO (Green *et al.*, 1990; Liew *et al.*, 1990). Foi demonstrado que macrófagos peritoneais de camundongos resistentes apresentam maior capacidade de inibição da proliferação de amastigotas de *L. major* comparados àqueles macrófagos de camundongos susceptíveis (Handman *et al.*, 1979; Mauel *et al.*, 1987). Além disto, macrófagos de camundongos susceptíveis infectados com *L. major*, comparados a macrófagos de uma linhagem de camundongos resistentes, são menos sensíveis à ativação por citocinas derivadas de células T (Nacy *et al.*, 1983).

1.3 RECONHECIMENTO E FAGOCITOSE

Durante a resposta imune inata, o reconhecimento de diferentes patógenos é mediada, em parte, por uma série de *pattern-recognition receptors* (PRRs). Macrófagos e células dendríticas expressam uma variedade de PRRs envolvidos no reconhecimento e indução da resposta imune adquirida. Esses receptores reconhecem distintos padrões bioquímicos de moléculas de diversos parasitos invasores e compõem um grupo de receptores com especificidade geneticamente determinada, que é altamente conservado entre os diferentes organismos. As proteínas envolvidas no reconhecimento de micróbios incluem o receptor manose, os receptores *scavenger* (SRs), CD14 e receptores do tipo *toll-like* (TLRs).

Os SRs fazem parte de uma família de proteínas com múltiplos domínios transmembranares, envolvida na endocitose mediada por receptor de ligantes polianiônicos, incluindo LDL (low-density lipoproteins) (Krieger, 1997). O papel dos SRs tem sido bem estudado na patogênese da arteriosclerose, evidenciando-se que SRs classe A, MARCO (*Macrophage Receptor with Collagenous Structure*) e SRs classe B têm papel importante na imunidade inata e regulação da resposta de macrófagos.

Membros da família dos SRs estão envolvidos na ligação e internalização de micróbios. Receptores *scavengers* A são expressos na maioria dos macrófagos e interagem com bactérias e componentes da parede celular, como LPS (Dunne *et al.*, 1994; Hughes *et al.*, 1995). Macrófagos deficientes em SR-A são menos eficientes na fagocitose de bactérias mortas pelo calor (Peiser *et al.*, 2000; Thomas *et al.*, 2000). Camundongos SR-A$^{-/-}$ são mais susceptíveis a bactérias gram-positivas, tais como *Listeria monocytogenes* e *Staphylococccus aureus*, do que camundongos selvagens (Suzuki *et al.*, 1997; Thomas *et al.*, 2000). Esses dados evidenciam os papéis de SR-A como PRR e como protetor contra infecções bacterianas.

MARCO, distinto membro da classe A dos SRs, com estrutura colagenosa e domínio SRCR (*Scavenger Receptor Cystein-Rich*), participa da fagocitose de micróbios. MARCO é normalmente expresso em sub-populações de macrófagos, como os da zona marginal do baço e em macrófagos peritoneais inflamatórios (Peiser *et al.*, 2002). Esse SR interage com uma variedade de partículas, incluindo bactérias gram-positivas e gram-negativas, e partículas artificiais, como bolas de látex. Anticorpos anti-MARCO bloqueiam a internalização das partículas alvo (Palecanda *et al.*, 1999; van der Laan *et al.*, 1999).

A expressão de MARCO em macrófagos da zona marginal do baço sugere seu papel na fagocitose e na captura de antígenos provenientes da corrente sanguínea (Elomaa *et al.*, 1998). Macrófagos da zona marginal do baço estão também envolvidos na apresentação de antígenos e na indução da resposta imune adquirida. Estudos *in vivo* mostraram que a depuração inicial de *Escherichia coli* e *S. aureus* não é dependente de MARCO, pois não é afetada por anticorpo anti-MARCO (van der Laan *et al.*, 1999). Entretanto, após 30 minutos da injeção intravenosa desses anticorpos, observa-se captura de uma quantidade menor de micróbios por macrófagos da zona marginal, o que

evidencia que MARCO pode ser induzido em resposta a infecções bacterianas, sugerindo um papel importante na resposta imune inata contra patógenos.

O papel de MARCO ainda não foi estudado na resposta à infecção com Leishmania. Entretanto, outros PRRs, como o receptor TLR, parecem estar envolvidos. Em macrófagos de camundongos *knockout* para TLR2 ou TLR4, foi evidenciado o papel essencial de TLR2, porém não TLR4, na indução de IL-12, TNF-α e NO em macrófagos murinos ativados por produtos derivados de protozoários como âncora GPI (glycosylphosphatidylinositol) e GIPLs (glycoinositolphospholipids) (Campos *et al.*, 2001). Estudos recentes evidenciaram a participação de TLR4 no controle da proliferação de *L. major in vivo*, tanto na resposta imune inata, como na resposta imune adquirida. O controle da replicação do parasito se correlaciona com a indução inicial de iNOS em camundongos que expressam TLR4, enquanto que o aumento da sobrevivência do parasito em células de animais deficientes de TLR4 se correlaciona com o aumento da atividade da enzima arginase (Kropf *et al.*, 2004).

Alguns receptores e ligantes estão envolvidos na interação promastigotas e macrófagos. Receptores para o complemento aparentemente desempenham papel principal no reconhecimento de diferentes espécies de Leishmania. Moléculas de superfície de Leishmania como LPG e gp63 protegem o parasito da lise do complemento (Puentes *et al.*, 1990) e estão envolvidas na interação do parasito com o macrófago (Davies *et al.*, 1990; Kelleher *et al.*, 1995; Pimenta *et al.*, 1991). A opsonização C3b e C3bi (Brittingham *et al.*, 1995) medeia a interação de Leishmania com o macrófago por receptores do complemento CR1 e CR3, respectivamente (Blackwell *et al.*, 1985; Cooper *et al.*, 1988; Da Silva *et al.*, 1989; Mosser *et al.*, 1984; Mosser *et al.*, 1985; Mosser *et al.*, 1992). Entretanto, existem evidências de que LPG pode interagir diretamente com CR3 (Talamas-Rohana *et al.*, 1990).

Outros receptores estão envolvidos na fagocitose de Leishmania. O receptor para fibronectina pode reconhecer a molécula gp63 na superfície de Leishmania (Brittingham *et al.*, 1999; Rizvi *et al.*, 1988). Além disso, receptor manose-fucose (Channon *et al.*, 1984; Russell *et al.*, 1986; Wilson *et al.*, 1986), CR4 (Talamas-Rohana *et al.*, 1990), receptor Fc (Chang, 1981; Russell *et al.*, 1989), receptor para *advanced glycosylation end-products* (Mosser *et al.*, 1987) e receptor para proteína C reativa participam da fagocitose de promastigotas de Leishmania (Culley *et al.*, 1996). O envolvimento de vários receptores na fagocitose da forma promastigota favorece sua internalização pelo macrófago.

1.4 MECANISMOS DE DESTRUIÇÃO DE LEISHMANIA POR MACRÓFAGOS

A fagocitose é seguida por um aumento no consumo de oxigênio por células fagocíticas. Foi demonstrada uma correlação entre explosão respiratória e formação de radicais intermediários do oxigênio (ROI) (Stafford *et al.*, 2002), que são importantes moléculas microbicidas produzidas por células fagocíticas, como macrófagos e neutrófilos (Dinauer, 1993; Segal, 1996). O complexo de enzimas responsável pela indução da explosão respiratória é conhecido como oxidase da explosão respiratória ou oxidase NADPH (phox). Esse complexo enzimático se associa à superfície interna da membrana plasmática, após estimulação apropriada sendo constituído por pelo menos cinco subunidades protéicas: duas proteínas, gp91 (*glycoprotein of* 91 KDa) phox e p22 (*nonglycosylated protein of* 22 KDa) phox, que compõem o citocromo da membrana (Parkos *et al.*, 1987; Parkos *et al.*, 1988; Segal, 1987), e três proteínas citosólicas, p40 (*Protein of* 40 KDa) phox, p47 (*Phosphoprotein of* 47 KDa) phox e p67 (*Protein of* 67 KDa) phox (Nunoi *et al.*, 1988; Tsunawaki *et al.*, 1994; Tsunawaki *et al.*, 2000; Volpp

et al., 1988). As proteínas citoplasmáticas existem como um complexo citosólico (Park *et al.*, 1994; Park *et al.*, 1992) sendo recrutadas para regiões da membrana plasmática onde se encontra o citocromo após estímulo apropriado (Nauseef *et al.*, 1991; Park *et al.*, 1999; Park *et al.*, 1995; Park *et al.*, 1992)

O recrutamento dos componentes citosólicos é mediado pela fosforilação de múltiplos resíduos de tirosina e serina na p47phox e p67phox. Depois da associação com os componentes da membrana, outras cinases podem fosforilar sítios distintos de proteínas derivadas do citoplasma (Finan *et al.*, 1994; Inanami *et al.*, 1998). As cinases conhecidas como ativadoras da explosão respiratória incluem várias isoformas de PKC (*protein kinase* C), PKA (*protein kinase* A) e MAPK (*mitogen-activated protein kinases*). A proteína de 47kD citosólica é diretamente fosforilada por p38, ERK (*extracellular-signal regulated protein kinase*) e caseína cinase II. Entretanto, a fosforilação de p47phox por caseína cinase II medeia a desativação da oxidase, demonstrando que a fosforilação pode ativar ou desativar a explosão respiratória.

NADPH atua como a origem do potencial redutor para conversão do oxigênio molecular em ânion superóxido (O^{2-}). A produção de superóxido resulta em geração espontânea ou enzimática de produtos reativos do oxigênio, incluindo H_2O_2, radical hidroxila, ácido hipocloroso e peroxinitrito. Peróxido de hidrogênio pode ser formado espontaneamente ou pela ação da enzima superóxido dismutase. O ácido hipocloroso é formado em fagolisossomas ácidos pela reação de íons cloreto com peróxido de hidrogênio em decorrência da ação da mieloperoxidase. Peroxinitrito é formado pela reação espontânea de superóxido com NO produzido pela iNOS.

No modelo murino da infecção por Leishmania, a ativação de macrófagos por citocinas leva à produção de ROI e reativos intermediários do nitrogênio (RNI), que são responsáveis pela atividade leishmanicida (James, 1995). Durante a fagocitose de

promastigotas, macrófagos podem liberar radicais, incluindo superóxido, H_2O_2 e OH, que são moléculas microbicidas (Murray *et al.*, 1983; Pearson *et al.*, 1983). Uma vez estabelecida a infecção por amastigota, a produção de NO por macrófagos pode levar à destruição do parasito (Green *et al.*, 1990; James, 1995; Wei *et al.*, 1995).

O mecanismo de destruição não é específico para cada estágio do parasito, mas é característico do processo de fagocitose. Promastigotas e amastigotas são igualmente susceptíveis a ROI (Miller *et al.*, 2000; Murray, 1982; Zarley *et al.*, 1991), mas a explosão respiratória induzida por amastigotas é significativamente menor do que a induzida na fagocitose de promastigotas (Channon *et al.*, 1984; Haidaris *et al.*, 1982). Essas diferenças estão relacionadas à interação de moléculas de superfície de Leishmania com receptores de macrófagos (Channon *et al.*, 1984). Promastigotas podem ser reconhecidas por receptor manose fucose presentes nessas células, o que levaria à indução de explosão respiratória, ao passo que a fagocitose de amastigotas, não envolve esse receptor (Channon *et al.*, 1984).

O uso de inibidores específicos da atividade de iNOS (como LNMA), de citocinas que inibem a produção de NO (como TGF-β e IL-10) (Barral-Netto *et al.*, 1992; Chatelain *et al.*, 1999; Liew *et al.*, 1991; Nelson *et al.*, 1991; Vieth *et al.*, 1994), ou, ainda, o uso de camundongos *knockout* para iNOS (Murray *et al.*, 1999; Wei *et al.*, 1995) evidenciam o papel desta molécula na destruição de Leishmania. Camundongos infectados com *L. major* e tratados com inibidores de iNOS apresentam aumento da carga parasitária, desenvolvimento de lesões e inibição da destruição intracelular dos parasitos (Liew *et al.*, 1990). Embora ROI e RNI contribuam para a destruição de Leishmania, apenas a produção de RNI é essencial para a resolução da infecção. A contribuição de ROI parece ser necessária apenas nos estágios inicias da infecção (Murray *et al.*, 1999).

Além do efeito leishmanicida do NO, esta molécula desempenha um papel regulador durante a resposta imune a Leishmania. Na ausência de NO derivado de iNOS, IL-12 foi incapaz de prevenir o crescimento de parasitos, não houve ativação de células NK para liberação de IFN-γ e não houve, também, fosforilação de STAT-4, que é indutor importante da produção de IL-12, em células NK (Diefenbach *et al.*, 1999). Durante os estágios iniciais da infecção, macrófagos ativados liberam IFN-α/β e seu efeito autócrino estimula a liberação de níveis baixos de NO (Diefenbach *et al.*, 1999). Esses níveis baixos de NO são insuficientes para a destruição do parasito, mas desempenham papel crucial na modulação da ativação de células NK mediada por IL-12. Em conseqüência, há a secreção de IFN-γ por células NK, que vai induzir a produção de iNOS por macrófagos e liberação de grandes quantidades de NO envolvido na destruição do parasito (Diefenbach *et al.*, 1999). Esses dados sugerem que a produção precoce de baixos níveis de NO é pré-requisito para a sinalização de citocinas e desenvolvimento da resposta imune inata.

Enquanto iNOS catalisa a conversão de L- arginina em NO e citrulina, a enzima arginase hidrolisa a arginina em ornitina e uréia. Em estudos anteriores, foi demonstrado que o balanço entre as duas enzimas é regulado competitivamente por citocinas secretadas por células Th1 e Th2: células Th1 induzem iNOS e Th2 induzem arginase I (Corraliza *et al.*, 1995; Modolell *et al.*, 1995). Citocinas, como IL-4, IL-10 e TGF-β, aumentam a atividade de arginase I, que induz o crescimento de *L. major* em macrófagos (Iniesta *et al.*, 2001; Iniesta *et al.*, 2002) devido à síntese, a partir de ornitina, de poliaminas essenciais à replicação de protozoários, tais como Leishmania (Iniesta *et al.*, 2002).

1.5 BIOGÊNESE DO VACÚOLO PARASITÓFORO

A morfologia e a maturação de compartimentos contendo o parasito, conhecidos como vacúolos parasitóforos (VPs), variam, dependendo da espécie de Leishmania . Os grandes vacúolos contendo *L. amazonensis* ou *L mexicana* são bem diferentes dos vacúolos menores individuais contendo *L. major* ou *L. donovani*. A despeito das diferenças morfológicas entre esses compartimentos, algumas propriedades são comuns (Antoine *et al.*, 1998). Os VPs são compartimentos ácidos e contêm enzimas lisossomais. Eles apresentam marcadores de endossoma tardio e lisossoma, incluindo rab 7, macrosialina, LAMP-1, LAMP-2 e ATPase vacuolar.

A cinética de formação do VP pode diferir de acordo com o estágio do parasito internalizado (Dermine *et al.*, 2000; Desjardins *et al.*, 1997). Foi demonstrado que, depois da fagocitose de promastigotas de *L. donovani*, os parasitos se localizam em fagossomos transitórios com pouca capacidade de fusão com endossomas tardios (Desjardins *et al.*, 1997). Em contrapartida, depois da internalização, amastigotas são encontradas em compartimentos que rapidamente se fusionam com compartimentos tardios da via endocítica (Dermine *et al.*, 2000; Lang *et al.*, 1994). Esses aspectos distintos de fagossomas primários estão relacionados com expressão estágio-específica de LPG na membrana de promastigotas, o que modifica a capacidade de fusão dessas organelas (Dermine *et al.*, 2000; Desjardins *et al.*, 1997; Schaible *et al.*, 1999). Além disso, estudos mostraram que fagossoma contendo LPG de promastigotas impede recrutamento de GTPase rab7, a qual está envolvida com a fusão homotípica de endossomas tardios ou lisossomas e com a fusão heterotípica de endossomas tardios com lisossoma (Scianimanico *et al.*, 1999). Outros estudos evidenciaram, ainda, que fagossomas primários contendo promastigotas ou amastigotas de *L. amazonensis* ou

promastigotas de *L. major* rapidamente adquirem capacidade de se fundir com endossomas tardios/lisossomas (Courret *et al.*, 2002).

1.6 SINALIZAÇÃO INTRACELULAR

Alguns estudos têm demonstrado que a infecção por Leishmania interfere em vias de sinalização intracelular do hospedeiro, facilitando sua invasão e sua sobrevivência (Reiner *et al.*, 1994). A inibição de cascatas de sinalização dependentes da fosforilação de tirosina parece uma importante estratégia do parasito para a inibição da ativação de macrófagos (Stafford *et al.*, 2002). Macrófagos infectados por *L. donovani* apresentaram redução significativa da fosforilação e da atividade de PKC (Olivier *et al.*, 1992). Além disso, em macrófagos murinos infectados por *L. donovani,* ocorre inibição da expressão de *c-fos*, induzida por PKC, embora a expressão de *c-fos* mediada por PKA não seja afetada (Moore *et al.*, 1993).

MAPK são alvos de PKC (Seger *et al.*, 1992), indicando que, provavelmente, são enzimas inibidas em células infectadas por Leishmania. MAPK1 e 2 são responsáveis pela regulação da expressão gênica em resposta a vários estímulos, tais como fatores de crescimento, citocinas e hormônios que influenciam a proliferação celular, a diferenciação e outras funções (Blenis, 1993; Pelech *et al.*, 1992). A fosforilação em resíduos de tirosina e treonina é essencial para a ativação de MAPK (Payne *et al.*, 1991). Devido ao amplo efeito das MAPK, suas atividades são controladas por uma família de fosfatases que desfosforilam resíduos de tirosina e treonina nessas moléculas inativando-as (Hunter, 1995). O domínio homólogo a Src 2 da tirosina fosfatase (SHP-1) está envolvido na desativação de MAPK via desfosforilação de resíduos de tirosina (Knutson et al, 19998). Estudos demonstraram que outra via de sinalização alterada por *L. donovani* é a ativação de fosfotirosinas

fosfatases que atenua a sinalização MAPK, *c-fos* e expressão de iNOS, e, assim, desativa macrófagos durante a infecção intracelular (Nandan *et al.*, 1999).

1.7 PRODUÇÃO DE CITOCINAS E QUIMIOCINAS

Macrófagos produzem citocinas importantes para sua autoregulação via efeito autócrino, e para modulação da resposta imune adquirida. Estudos *in vitro* mostraram que TNF-α aumenta a atividade leishmanicida de macrófagos (Mannheimer *et al.*, 1996). Similarmente, MIF desempenha um papel protetor na infecção por *L. major*, *in vitro* e *in vivo*, possivelmente, por induzir produção de NO (Juttner *et al.*, 1998; Xu *et al.*, 1998). Além disso, estudos mostraram que IFN-α/β e iNOS são reguladores da resposta imune inata na infecção por *L. major* (Diefenbach *et al.*, 1998). Outras citocinas derivadas de macrófagos, como IL-6, IL-10 e TGF-β, desativam a capacidade leishmanicida dessas células.

A indução da imunidade protetora contra leishmaniose está relacionada à produção de IL-12 (Afonso *et al.*, 1993; Heinzel *et al.*, 1993; Liew *et al.*, 1993; Scharton-Kersten *et al.*, 1995; Sypek *et al.*, 1993). IL-12 é uma citocina produzida por macrófagos que dirige a resposta Th1 $CD4^+$e induz a produção de IFN-γ por células NK e células T. Entretanto, estudos mostraram que promastigotas metacíclicas de Leishmania são importantes inibidoras de produção de IL-12 por macrófagos *in vitro* (Carrera *et al.*, 1996; Piedrafita *et al.*, 1999; Sartori *et al.*, 1997) e *in vivo* (Belkaid *et al.*, 1998; Reiner *et al.*, 1994). Estudos mostraram que LPG de *L. major* regula a síntese de IL-12 em células J774. Além disso, a infecção *in vitro* de macrófagos murinos com amastigotas de *L. mexicana* resulta na supressão prolongada da produção de IL-12. Existem evidências que células dendríticas são as principais produtoras de IL-12 (Gorak *et al.*, 1998), entretanto apenas macrófagos têm a capacidade de iniciar a resposta imune

inflamatória contra agentes infecciosos, produzindo TNF-α, IL-8 e IL-1β, além de moléculas microbicidas efetoras.

TNF-α é uma citocina importante que medeia a destruição de parasito, pois o tratamento de camundongos infectados com TNF-α recombinante leva à redução da lesão (Titus *et al.*, 1989), enquanto que a adição de anticorpo anti-TNF-α exacerba a doença (Liew *et al.*, 1990). Além disso, outros estudos sugeriram que a produção de altos níveis de NO e destruição de diferentes parasitos intracelulares por macrófagos estimulados por IFN-γ, depende de TNF-α (James, 1995).

IL-10 tem um efeito inibidor das funções de macrófagos, incluindo a redução da expressão de moléculas coestimulatórias e inibição da síntese de citocinas (Moore *et al.*, 1993). Estudos mostraram que camundongos transgênicos para IL-10 são susceptíveis à infecção por *L. major,* a despeito da produção de IFN-γ (Groux *et al.*, 1999). Esses e outros estudos apontam para um papel importante de IL-10 na regulação da resposta imune a Leishmania, enfatizando a importância desta citocina na susceptibilidade à infecção com esses parasitos. Estudos recentes mostraram que IL-10 está relacionada à persistência de Leishmania em lesões curadas de camundongos C57BL/6, já que a eliminação completa do parasito só ocorre nos animais tratados com anticorpos anti-receptor de IL-10 (Belkaid *et al.*, 2001). Por outro lado, camundongos CBA infectados por *L. major* ou *L. amazonensis* apresentam produção similar de IL-10, sugerindo que esta citocina tem um duplo papel na resposta imune contra Leishmania: no contexto da susceptibilidade, favorece uma resposta Th2; na resistência, modula a resposta Th1 (Lemos de Souza *et al.*, 2000).

TGF-β é uma citocina importante na desativação de macrófagos (Ding *et al.*, 1990; Tsunawaki *et al.*, 1988). Esse efeito inibitório está relacionado com a supressão

de IL-12 (Kima *et al.*, 1996) e com a inibição da produção de NO mediada por IFN-γ (Lopez *et al.*, 1993). Outros estudos mostraram o papel do TGF-β na exacerbação da lesão causada por *L. braziliensis* e *L. amazonensis* (Barral *et al.*, 1995).

A participação de IL-1β e GM-CSF na infecção por Leishmania ainda é controverso. Alguns estudos *in vitro* e *in vivo* indicaram que IL-1β e GM-CSF aumentam a atividade leishmanicida de macrófagos e desempenham papel protetor na leishmaniose (Al-Zamel *et al.*, 1996; Hatzigeorgiou *et al.*, 1993; Satoskar *et al.*, 1998). Foi demonstrado que o balanço da expressão de IL-1 e receptor para IL-1 está relacionado com o controle da progressão da infecção por *L. major* em camundongos resistentes (Ji *et al.*, 2003). Por outro lado, outros estudos mostraram que essas citocinas têm um papel não protetor (Liew *et al.*, 1993; Theodos *et al.*, 1994).

Muitos estudos enfatizam o papel, tanto de citocinas, como de quimiocinas, na resposta inicial à infecção por *L. major* (Scott, 1991; Vester *et al.*, 1999). É conhecido que ligantes de CCR5 (CCL3/MIP-1α, CCL4/MIP1β e CCL5/RANTES) estão envolvidos no desenvolvimento de células Th1, enquanto que ligantes de CCR2 (CCL2/MCP-1 e MCP-2), promovem diferenciação de células Th2 (Bonecchi *et al.*, 1998; Gu *et al.*, 2000).

Quimiocinas atuam, também, nas funções efetoras de células T e podem agir diretamente sobre macrófagos (Gerard *et al.*, 2001). Foi demonstrado que, na infecção por *L. major*, as quimiocinas XCL1, CXCL10 e CCL2 foram preferencialmente expressas em linfonodos de drenagem de camundongos resistentes (Zaph *et al.*, 2003). Além disso, a neutralização de IL-12 ou IFN-γ resultou no decréscimo da expressão de quimiocinas em camundongos capazes de controlar a infecção, ao passo que a administração de IL-12 em camundongos susceptíveis resultou em aumento da expressão de genes para quimiocinas (Zaph *et al.*, 2003). Esses dados mostram uma

relação entre a expressão de XCL1, CXCL10, CCL2 e mediadores da resistência à *L. major*.

Também foi demonstrado que CCL-3/MIP-1α e CCL2/MCP-1 participam na indução da atividade leishmanicida de macrófagos murinos. Esse mecanismo de destruição se dá via produção de NO (Bhattacharyya *et al.*, 2002). Paralelamente CCL2/MCP-1 atua sinergicamente com IFN-γ, ativando monócitos humanos para a destruição intracelular de *L. major* (Ritter *et al.*, 2000).

Estudos recentes mostraram que, em tecidos da pata e linfonodo de drenagem de camundongos infectados com *L. amazonensis*, houve baixa expressão de citocinas inflamatórias (IL-12, IFN-γ, IL-1α e IL-1β), CC quimiocinas (CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES e MIP-2) e receptores para quimiocinas (CCR1, CCR2, CCR5), comparada à expressão em camundongos controles infectados com *L. major* (Ji *et al.*, 2003). Esses dados indicam que *L. amazonensis* escapa da resposta imune do hospedeiro por inibir a produção de citocinas inflamatórias e quimiocinas, impedindo o desenvolvimento de uma resposta Th1.

Foi demonstrado que a infecção de macrófagos murinos com promastigotas de *L. major in vitro,* induz a expressão transitória de algumas CC quimiocinas (Matte and Olivier 2002). *L. major* também induz a produção de citocinas proinflamatórias, incluindo TNF-α por queratinócitos e IL-1β por células de Langerhans (Arnoldi *et al.*, 1998; Luster, 2002). Essas citocinas, por sua vez, induzem a produção de outros mediadores, incluindo as quimiocinas (Ohmori *et al.*, 1993). Portanto, a interação funcional entre quimiocinas e citocinas parece crítica no desenvolvimento da resposta imune efetora (Luster, 2002; Tapia *et al.*, 1994).

1.8 DNA*MICROARRAY* E O ESTUDO DA INTERAÇÃO PARASITO-CÉLULA HOSPEDEIRA

A tecnologia do DNA*microarray*, recentemente desenvolvida, vem sendo utilizada para analisar a expressão simultânea de genes em larga escala. O padrão da expressão de um gene fornece informação indireta sobre sua função e sobre as vias metabólicas com as quais o seu produto está envolvido. É uma técnica com uma grande variedade de aplicações. Uma das mais atraentes aplicações do DNA*microarray* compreende o estudo do padrão da expressão de genes associados a doenças. Essa tecnologia, associada com os resultados dos sequenciamentos dos diversos genomas, permite a análise da expressão gênica completa de um organismo durante o seu desenvolvimento natural ou no curso de uma resposta fisiológica específica. Essa metodologia é uma ferramenta prática e econômica para o estudo da expressão gênica em larga escala (Chee *et al.*, 1996; DeRisi *et al.*, 1997; Lashkari *et al.*, 1997; Lockhart *et al.*, 1996), explorando o genoma de forma sistemática e compreensiva (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997; Lockhart *et al.*, 1996; Pease *et al.*, 1994; Schena *et al.*, 1995). Além disso, a genômica comparativa abre a possibilidade de se verificar o padrão de expressão de genes conhecidos, e de se detectar seqüências desconhecidas que podem ser importantes na resposta celular.

No contexto da infecção por Leishmania, sabe-se que macrófagos murinos podem ser ativados, levando à destruição do microorganismo. Por outro lado, esses protozoários podem suprimir e escapar da resposta intracelular do hospedeiro. Esses dados refletem que parasitos intracelulares influenciam a expressão de genes relacionados à atividade microbicida de macrófagos. Matlashewski and Buates (2001) avaliaram a expressão gênica em macrófagos infectados por *L. donovani* e mostraram que cerca de 37% dos genes analisados foram inibidos em relação a macrófagos não infectados (Buates *et al.*, 2001). Entretanto, outro estudo, analisando a expressão de

12.000 genes em células dendríticas e macrófagos infectados por *L. major* ou *L. donovani*, demonstrou que houve número similar de genes inibidos ou ativados no tempo de avaliação do experimento, evidenciando que não existe uma supressão geral da expressão gênica em macrófagos humanos (Chaussabel *et al.*, 2003). Outros dados recentes mostraram inibição da expressão de genes proinflamatórios e ativação de genes envolvidos na resposta antiinflamatória em macrófagos infectados por *L. chagasi* (Rodriguez *et al.*, 2004). Todos esses estudos acima indicam que a utilização dessa técnica pode ter importantes implicações para o entendimento da interação parasito-célula hospedeira e sua repercussão no contexto da resposta imune inata e natureza da regulação gênica.

2. OBJETIVO GERAL

Investigar mecanismos celulares e moleculares envolvidos na determinação dos diferentes perfis de resposta de macrófagos de camundongos CBA infectados, *in vitro*, por *L. amazonensis* ou *L. major*.

Hipótese

L. amazonensis e *L. major* influenciam a resposta imune efetora, por mecanismos de ativação ou inibição de macrófagos.

3. OBJETIVOS ESPECÍFICOS

1- Comparar a cinética de infecção *in vitro* de macrófagos inflamatórios de camundongos CBA por *L. major* ou *L. amazonensis*.

2- Comparar o perfil de produção das citocinas e moléculas microbicidas em macrófagos inflamatórios de camundongos CBA infectados *in vitro* por *L. amazonensis* ou *L. major*.

3- Avaliar o papel do TNF-α na ativação de macrófagos infectados por *L. amazonensis* ou *L. major*.

4- Avaliar comparativamente a maturação de compartimentos contendo *L. amazonensis* ou *L. major* em macrófagos de CBA.

5- Estudar comparativamente os genes expressos ou inibidos em macrófagos de camundongos CBA não infectados ou infectados por *L. amazonensis* ou *L. major*.

6- Identificar vias metabólicas ativadas ou inibidas em resposta à infecção com diferentes espécies de Leishmania.

7- Avaliar comparativamente a expressão *in vitro* e *in vivo* do receptor MARCO na infecção por *L. amazonensis* ou *L. major*.

4. CAPÍTULO II - DIFERENTES PROPRIEDADES DE FAGÓCITOS MONONUCLEARES INFLAMATÓRIOS DE CAMUNDONGOS CBA/J SÃO ESTIMULADAS POR DUAS ESPÉCIES DE LEISHMANIA

4.1 INTRODUÇÃO

Camundongos da linhagem CBA, conhecidos por serem resistentes à *L. major* são susceptíveis à *L. amazonensis*. Sabe-se que os eventos da resposta imune inata são cruciais para a determinação da resposta à infecção por Leishmania . Os macrófagos desempenham papel central na leishmaniose (Solbach *et al.*, 1991), pois são as principais células hospedeiras de Leishmania , apresentadoras de antígeno a linfócitos T também atuam como células efetoras na destruição do parasito. No presente trabalho, avaliamos comparativamente, a infecção de macrófagos de camundongos CBA por *L. major* e *L. amazonensis*. Os resultados mostraram que entre 90 minutos e 6 horas após a adição de promastigotas em fase estacionária, a proporção de macrófagos infectados e o número de parasitos por macrófagos foi semelhante em ambos os grupos. Entretanto, após o período de caça de 24 horas o percentual de células infectadas por *L. amazonensis* foi duas vezes maior que o observado na infecção por *L. major*. Essas diferenças foram mantidas, após 48 e 72 horas de caça. Esses resultados evidenciam que os macrófagos de camundongos CBA têm maior capacidade de destruir *L. major* em relação à *L. amazonensis in vitro*. Então para estudar a contribuição de ROI na destruição de Leishmania, avaliamos comparativamente a produção de H_2O_2 nos macrófagos infectados. Essas análises evidenciaram que células infectadas por *L. amazonensis* produzem menor quantidade de H_2O_2 que células infectadas por *L. major*.

A adição de IFN-γ às culturas reduz significativamente a infecção de macrófagos por *L. major*, não modificando a infecção por *L. amazonensis* embora induza uma produção similar de NO em ambos os casos. Essa redução na infecção por *L. major* é

um mecanismo dependente de TNF-α, uma vez que macrófagos infectados por essa espécie expressam 2 vezes mais RNAm para TNF-α do que células infectadas por *L. amazonensis*, e a adição de anti-TNF-α nas culturas reverteu o efeito induzido pelo IFN-γ. Além disso, a adição de TNF-α recombinante juntamente com IFN-γ reduziu significativamente o percentual de células infectadas por *L amazonensis*, contudo numa proporção inferior à observada na infecção por *L. major*. A despeito da produção de NO em células tratadas com IFN-γ juntamente com TNF-α, a adição de AMG é capaz de reverter parcialmente a inibição da infecção por *L. major*, não tendo efeito algum sobre a infecção por *L. amazonensis*. Esse estudo demonstra que *L. amazonensis* inativa e também resiste a mecanismos de destruição inatos e induzidos por IFN-γ, indicando que a espécie do parasito e sua interação com o macrófago podem ser determinantes da polarização da resposta imune do hospedeiro.

4.2 ARTIGO 1

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Original article

Differential properties of CBA/J mononuclear phagocytes recovered from an inflammatory site and probed with two different species of *Leishmania*

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Abstract

While CBA/J mice fail to be permissive to *Leishmania amazonensis*-driven pathogenic processes, they heal easily following *Leishmania major* infection. The early-phase events are crucial to the outcome of *Leishmania* infection and it is known that macrophages (Mϕ) are important in infection control. In the present study we investigated the role of Mϕ in driving CBA/J susceptibility to *L. amazonensis*. We performed kinetic studies and compared the capacity of *L. amazonensis* and *L. major* to infect Mϕ. There was no difference in percentages of infection or parasite burden for 6 h between the two groups. In contrast, after 12 h we observed that infection was about twice as high in *L. amazonensis*- than in *L. major*-infected Mϕ. In addition, rIFN-γ added to the cultures induced nitric oxide (NO) production, and did not modify *L. amazonensis* infection, although the percentage of *L. major* infection was significantly reduced. This reduction in *L. major* infection is a TNF-α dependent mechanism as *L. major*-infected Mϕ expressed twice as much TNF-α mRNA as *L. amazonensis*-infected cells, and anti-TNF-α reversed the IFN-γ effect. Moreover, rTNF-α plus IFN-γ were able to significantly reduce the percentage of *L. amazonensis*-infected cells but not to the same extent as in *L. major* infection. Despite having higher NO production than IFN-γ-treated cells, AMG addition to IFN-γ-plus TNF-α-treated cells only partially reversed the inhibition in *L. major*, but not in *L. amazonensis* infection. Thus, in this study, we demonstrated that *L. amazonensis* both inactivated and resisted innate and IFN-γ-induced Mϕ killing mechanisms, indicating that the nature of the parasite and its interaction with Mϕ could determine immune response polarization.

Keywords: Macrophages; CBA/J mice; *L. amazonensis*

1. Introduction

Leishmaniases are a group of diseases caused by the trypanosomatidae *Leishmania* sp. affecting about 2 million people every year in Africa, Asia, South America and the Mediterranean area. In humans its development manifests either as visceral or cutaneous diseases, as well as asymptomatic processes. Cutaneous leishmaniasis is caused mainly by *L. major* in the Old World [1] and *L. amazonensis* and *L.* (*Viannia*) *braziliensis* in the New World [2].

Experimental leishmaniasis has been widely used to characterize the immune response against *Leishmania* parasites. Most studies have been performed comparing strains of mice of different genetic backgrounds that are susceptible or resistant to *L. major* infection [3–5]. Resistance and susceptibility to infection depend on the development of specific CD4+ T helper (Th)-1 or Th-2 responses, respectively [6]. We have recently developed an in vivo mouse model and demonstrated that while CBA/J mice fail to acquire *L. amazonensis* infection, they recover following infection with *L. major* [7]. These results emphasize the importance of parasites in driving the host immune response. *L. amazonensis* induced lesions showing a monomorphic infiltrate of highly parasitised and large vacuolated macrophages (Mϕ) [7]. Lymph node (LN) cells from CBA/J mice infected by *L. major* produced large amounts of gamma interferon (IFN-γ) when restimulated in vitro with *Leishmania* promastigotes, whereas infec-

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tion by *L. amazonensis* led to interleukin (IL)-4 production by LN cells from infected mice [7]. The first cellular events, which could elucidate these differences, are unknown.

There is considerable evidence of the importance of the innate immune response in the outcome of *Leishmania* infection [8–13]. Mϕ play an important role against *Leishmania*, since they are the principal host cell and they present antigens to specific Th cells [14,15]. In addition, they produce cytokines which drive Th-1 and Th-2 responses, modulating the immune responses and influencing intracellular parasite survival [16]. Mϕ produce nitric oxide (NO) and/or oxygen radicals, which are toxic to intracellular parasites [17–21]. The importance of Mϕ in the fate of *Leishmania* infection has been demonstrated by studies in vitro using Mϕ from mice with different susceptibilities to infection by *Leishmania*. Thus, Mϕ from resistant mice inhibit the proliferation of *L. major* amastigotes [22,23] and are easily activated by T cell-derived cytokines [24,25]. Furthermore, Mϕ from susceptible mice have an impaired response to signal activation after *L. major* infection [26]. In this report we investigated the role of CBA/J Mϕ on the determination of the differences observed between in vivo *L. amazonensis* and *L. major* infection [7]. We tested whether CBA/J susceptibility to *L. amazonensis* infection is related to parasite resistance to Mϕ killing mechanisms, or whether *L. amazonensis* was unable to induce Mϕ activation to kill intracellular amastigotes. We established an in vitro model to compare the ability of *L. amazonensis* and *L. major* to infect and activate CBA/J Mϕ in vitro. We also presented evidence of whether Mϕ from this mouse strain were activated or not in the presence of IFN-γ and whether they were able to kill *L. major*, but not *L. amazonensis*.

2. Materials and methods

2.1. Mice

Male and female CBA/J mice, 6–12 weeks old, were provided by the Central Animal Facility of the IOC/FIOCRUZ and from the Animal Facility of CPqGM/FIOCRUZ. These animals were housed under specific-pathogen-free conditions and fed on commercial ration and water ad libitum.

2.2. Parasites

Promastigotes in stationary phase used in this work were *L. amazonensis* (MHOM/Br88/Ba-125) and *L. major* (MHOM/RI/-/WR-173) provided by Dr. Aldina Barral, from the Laboratory of Immunopathology of CPqGM/FIOCRUZ. Fresh *L. amazonensis* or *L. major* promastigotes were derived from isolated amastigotes from LNs of C57BL/6 resistant mice, resuspended in Novy-Nicolle-MacNeal blood agar and then transferred to total LIT plus 10% fetal bovine serum (FBS) (Cultilab, Campinas, Brazil) for a maximum of six passages. For Mϕ experiments, the promastigotes were expanded for 3–5 d until reaching the stationary phase, washed with saline, and then adjusted to the desired concentrations, indicated in the results. According to procyclic form susceptibility to be lysed by complement present in fresh serum, the percentage of metacyclic forms was determined by incubating the promastigotes at stationary phase with different concentrations of fresh serum for 30 min at 37 °C [27]. Logarithmic phase cultures were used as control, and we observed that all promastigotes were lysed in fresh serum-containing medium. The number of live parasites in stationary-phase cultures was counted by optical microscopy, and we obtained similar amounts of complement-resistant promastigotes in levels of 50% for both *L. amazonensis* and *L. major* stationary promastigotes.

2.3. Mϕ culture

Thioglycolate-induced peritoneal exudate cells (PEC Mϕ) were prepared from the peritoneal cavity of CBA/J mice 3–4 d after intraperitoneal injection of 2.5 ml 3% thioglycollate medium (Sigma Chemical, St. Louis, MO). PEC Mϕ were obtained by flushing the peritoneal cavity twice with 10 ml ice-cold saline plus heparin (20 UI/ml) and centrifuged at 300 × *g* for 10 min. The cells were resuspended in DMEM supplemented with 25 mM HEPES (*N*–2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) pH 7.4, 2 mM glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin, 2.0 g/l sodium bicarbonate plus 10% FBS (Gibco Laboratories) (complete medium). Mϕ (2×10^5 or 1×10^6 per ml) in complete medium were plated in 24-well culture plates (Costar, Cambridge, MI) containing or not 13-mm diameter glass coverslips (Glasstécnica Imp. São Paulo, BR) at 37 °C in 5% CO_2/95% humidified air. After 4 h, non-adherent cells were washed three times with RPMI-1640 supplemented with 25 mM HEPES. Afterwards, Mϕ were incubated in fresh medium with different stimuli.

2.4. Mϕ infection

Mϕ were infected with *L. amazonensis* or *L. major* promastigotes in stationary phase, at ratio 10:1. After 90 min, 3, 6 or 12 h, cells were washed and fixed. In some experiments, cells were washed to remove non-internalized parasites after 12 h of infection, at time zero (0), and reincubated for an additional 24, 48 and 72 h after infection. The cells were fixed with methanol or 99.3% ethanol for 10 min and stained with hematoxilin and eosin. The percentage of infected cells and the *Leishmania* number per Mϕ were determined by light microscopy observations (magnification, ×1000), and at least 200 Mϕ were counted per coverslip.

2.5. Hydrogen peroxide determination

H_2O_2 was measured by the peroxidase-dependent oxidation of phenol red [28]. The 1×10^5 Mϕ were plated onto 96-well plates and infected with *Leishmania* for 30 min as described above. Extracellular parasites were removed by

washing and reincubated for an additional 1 h in PBS containing 129 mM $CaCl_2$, 109 mM $MgCl_2$, 555 mM D-glucose, 100 U/ml of peroxidase (Sigma) and 0.56 mM of phenol red (Sigma). After 1 h at 37 °C in a 5%-CO_2 atmosphere, the absorbance of the supernatants was estimated at OD_{650} in a Molecular Device 96-well microplate reader after mixing with 10 μl of 1 N NaOH per well. H_2O_2 was quantified by comparison with a standard curve prepared with known concentrations of H_2O_2. Non-infected cells and cells treated with 100 ng/ml phorbol myristate acetate (PMA) served as control.

2.6. Determination and inhibition of nitrite accumulation

NO_2^- was determined in culture supernatants. Equal volumes of cell culture medium were mixed with Griess reagents (1% sulfanilamide, 0.1% naphtylethylenedamine, and 2.5% H_3PO_4). This mixture was distributed in a 96-well plate and estimated at OD_{570} in a Molecular Device 96-well microplate reader. The standard curve used $NaNO_2$ as a reference, in concentrations from 1 to 200 μM, and the results were expressed in μM per number of cells in culture. To inhibit NO production 100 mM of AMG (aminoguanidine), a competitive inducible NO synthase (iNOS) inhibitor, was added to the cultures at the same time as IFN-γ ± TNF-α.

2.7. NO-releasing agents

NO-releasing agent, *S*-nitroso-*N*-acetylpenicillamine (SNAP), was purchased from Calbiochem (La Jolla, CA). SNAP was dissolved in dimethyl sulfoxide (Sigma) at a concentration of 100 mM, stored at –20 °C. Cells were pre-treated every 4 h for 48 h with 100 μM of SNAP in culture medium.

2.8. Cytokine treatment of Mϕ

Mϕ adhered to glass coverslips were incubated for 12–18 h with 100 UI/ml of recombinant (r)IFN-γ (Pharmingen, San Diego, CA or R&D system, Minneapolis, MN), in combination or not with 100 UI/ml of r tumor necrosis factor (TNF)-α, kindly provided by Dr. Jeanne Wietzerbin, or 10 ng/ml of r transforming growth factor (TGF)-β (R&D System) and subsequently infected with *L. amazonensis* or *L. major* stationary-phase promastigotes. In parallel, cells were infected and treated with rTNF-α or rTGF-β alone. After 12 h of infection, cells were washed to remove free parasites and fresh cytokines were replaced using similar concentrations. Non-infected Mϕ were used as control.

2.9. Neutralization of cytokines produced by infected Mϕ

Mϕ were pre-treated with a combination of 100 UI/ml of rIFN-γ and 10 μg/ml of anti-TNF-α (R&D System) or normal goat serum (Sigma), as control, for 12–18 h. Subsequently, Mϕ were infected with *L. amazonensis* or *L. major* and, at time 0, they were washed to remove non-internalized parasites, and antibodies were replaced at similar concentrations. After 72 h of infection, the percentage of infected cells was estimated.

2.10. Cytokine and iNOS mRNA detection

Total mRNA was extracted using Trizol LS reagent. Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Gibco laboratories) at 42 °C for 55 min. Expression of mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) for TNF-α, TGF-β, IL-10 and iNOS. PCRs were performed on a PT-100 thermal cycler (Perkin-Elmer) in a reaction containing 2.5 mM PCR nucleotide mix (Roche Molecular Biochemicals), forward and reverse primers (0.5 pmol/μl each), 1× PCR buffer with 1.7 mM $MgCl_2$, 2 μg/μl cDNA and 0.025 U/μl *Taq* polimerase (Roche Molecular Biochemicals) made up to 20–30 μl with distilled water. The same master mix containing all reagents was used for each sample. Reaction conditions were as follows: 94 °C for 5 min, then 35 cycles of denaturation condition at 95 °C for 1 min, annealing at 60 °C (TGF-β, TNF-α) or 58 °C (iNOS) or 55 °C (IL-10) for 2 min, and polymerization at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were resolved by agarose gel electrophoresis (1.6% gel) and stained with ethidium bromide.

Primer sequences were as follows: TGF-β sense, 5'-CCAGATCCTGTCCAAACTAAGG-3'; TGF-β antisense, 5'-GAATCGAAAGCCCTGTATTCC-3'; TNF-α sense, 5'-ATGCACCACCATCAAGGACT-3'; TNF-α antisense, 5'-GCAAAAGAGGAGGCAACAAG-3'; IL-10 sense 5'-AGAAAAGAGAGCTCCATCATGC-3'; IL-10 antisense, 5'-AATCACTCTTCACCTGCTCCA-3'; iNOS sense, 5'-GTTCCAGAATCCCTGGACAA-3'; iNOS antisense, 5'-AACATTTCCTGTGCTGTGCTACA-3'; hypoxanthine guanine phosphoribosyl transferase (HPRT) sense, 5'-AGCTTGCTGGTGAAAAGGAC-3'; HPRT anti-sense, 5'-TTATAGTCAAGGGCATATCC-3'.

2.11. Statistical analysis

All experiments were done in triplicate and independently repeated at least two times. Most of the results are expressed as mean ± S.E.M. of three or more experiments or, when indicated, one is representative of similar experiments. Statistical significance between experimental groups and controls was analyzed by Student's *t*-test or by one-way ANOVA to compare three or more groups. A P value of <0.05 was considered statistically significant.

3. Results

3.1. L. amazonensis *and* L. major *induce different infection patterns in CBA/J Mϕ*

CBA/J PEC Mϕ were cultivated in complete DMEM medium for at least 24 h at 37 °C and then were infected with *L.*

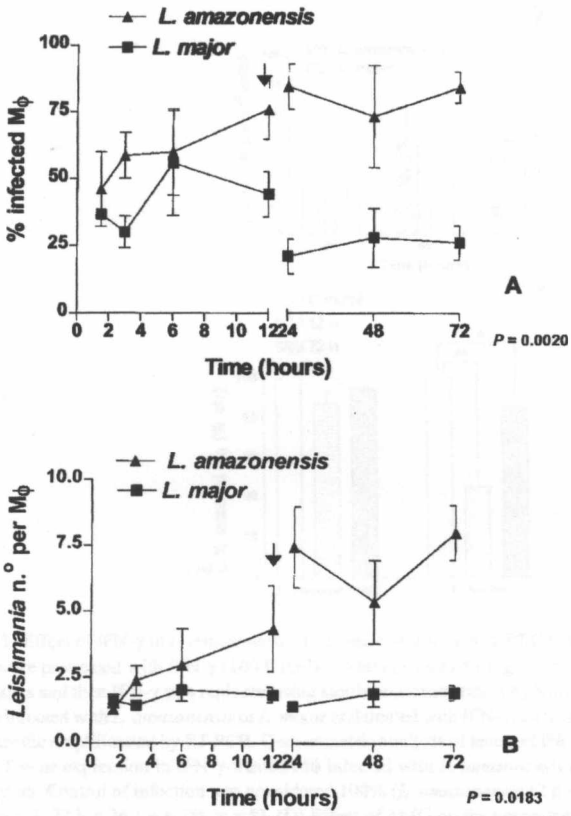

Fig. 1. Kinetics of CBA/J Mϕ infection by *L. amazonensis* or *L. major*. (A) Percentage of *L. amazonensis*- or *L. major*-infected Mϕ; (B) Parasite burden of *L. amazonensis*- or *L. major*-infected Mϕ. PEC Mϕ were infected with stationary-phase promastigotes at a ratio of 10:1. The percentages of infection and parasite number per Mϕ were determined. These cells were fixed at 1.5, 3.0, 6.0 and 12 h after infection. Twelve hours later, the time 0 (arrows), cells were washed and re-incubated for an additional 24, 48 and 72 h. This data represents the mean ± S.E.M. of five experiments; *P* values are indicated in each figure (paired Student's *t*-test).

amazonensis or *L. major* stationary-phase promastigotes. Kinetic studies were performed to compare the capacity of each parasite to infect CBA/J mouse Mϕ. We observed no differences between the percentages of infected Mϕ or parasite numbers per Mϕ until 6 h after parasite addition to the cultures (Fig. 1A, B). However, after 12 h of infection, we detected significant differences between the two groups. In order to analyze the effect of long-term infection, after 12 h of infection, at time 0, Mϕ were washed to remove non-internalized parasites and were cultivated for an additional 24, 48 and 72 h. After each of these periods, cells were fixed and analyzed according to the parameters described above. At time 0, the percentage of *L. amazonensis*-infected cells was 76.1 ± 11.2% ($n = 3$) and increased to 84.8 ± 5.8% ($n = 8$) 72 h postinfection. On the other hand, the percentage of *L. major*-infected cells decreased from 44.3 ± 8.4 % ($n = 3$) at time 0 to 26.1 ± 6.3% ($n = 8$) after 72 h of infection ($P = 0.002$) (Fig. 1A). Parasite burden was then determined and an enhancement was observed from 3.0 to 8.0 parasites per *L. amazonensis*-infected cell from 6 to 72 h postinfection. On the other hand, 2.0 parasites per *L. major*-infected Mϕ were detected, and there was no variation in this number for all periods analyzed ($P = 0.0183$) (Fig. 1B). Similar results were observed by infecting Mϕ at 35 °C (not shown), indicating that the *L. amazonensis* strain used in our system is adapted to infect cells at 37 °C. Together these results suggest that the first contact between CBA/J Mϕ and *L. major* activates specific host cell responses, which are sufficient to contain parasite infection, in contrast to Mϕ response to *L. amazonensis* infection.

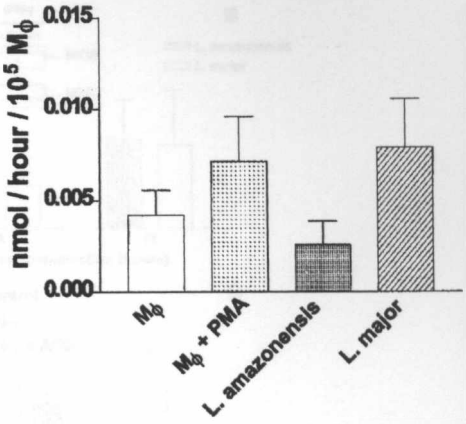

Fig. 2. Release of hydrogen peroxide by *L. amazonensis*- or *L. major*-infected Mϕ. PEC Mϕ were plated and infected with *L. amazonensis* or *L. major* stationary-phase promastigotes at a ratio of 10:1 for 30 min, and extracellular parasites were removed by washing. Non-infected cells and cells treated with 100 ng/ml PMA served as control. The amount of H_2O_2 was measured after 1 h incubation at 37 °C according to the peroxide-mediated H_2O_2-dependent oxidation as indicated in Material and methods. Results are reported as mean ± S.E.M. of two experiments done in triplicate.

3.2. L. amazonensis *inhibited H_2O_2 accumulation in Mϕ cultures*

To study the contribution of radical oxygen intermediates (ROI) in *Leishmania* killing, we compared H_2O_2 production by control non-infected, PMA-treated cells and *L. amazonensis*- or *L. major*-infected Mϕ. Fig. 2 shows that *L. amazonensis*-infected Mϕ produced half of the H_2O_2 production as that of *L. major*-infected Mϕ, although this difference was not statistically significant. In addition, the microbicidal molecule production by *L. major*-infected cells was similar to that of the positive control cells. This data suggests that the first contact of the parasite with Mϕ induced differential ROI production, and this may be responsible, at least in part, for the differences in CBA/J Mϕ capacity to destroy *L. amazonensis* and *L. major* parasites.

3.3. *IFN-γ activation of infected Mϕ impaired* L. major *but not* L. amazonensis *infection*

IFN-γ was added to the cultures to evaluate its role in the control of *L. amazonensis* infection. PEC Mϕ were pretreated with rIFN-γ and then cells were infected with *L.*

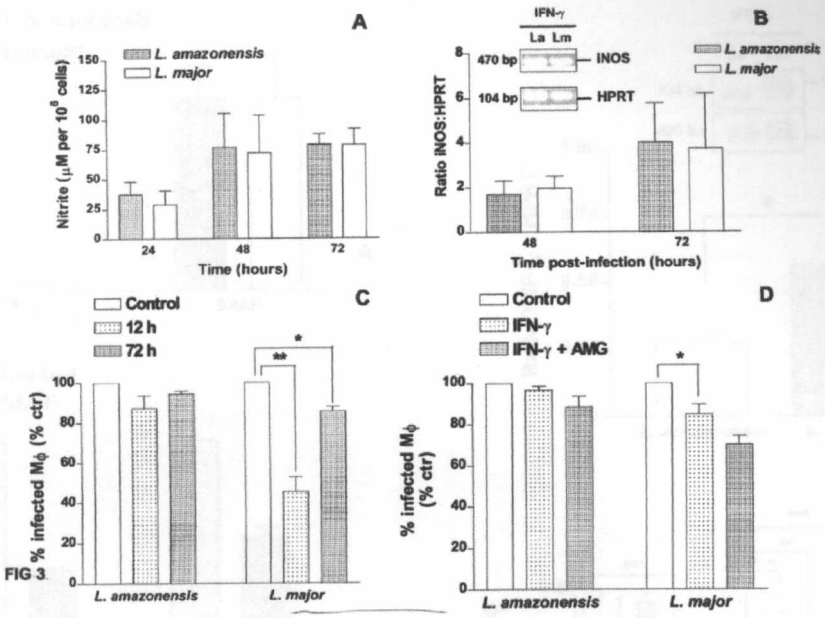

Fig. 3. Effect of IFN-γ in *L. amazonensis*- or *L. major*-infected Mϕ. PEC Mϕ were cultured and infected as described in Fig. 1 and served as control. In parallel, Mϕ were pretreated with IFN-γ (100 UI/ml) ± AMG (1 mM) during 12–18 h. After this time, cells were infected; 12 h later they were washed to remove free parasites and then IFN-γ was replaced using similar concentration. (A) Nitrite levels were measured by Griess reaction in supernatants from cultures of 1×10^6 cells infected with *L. amazonensis* or *L. major* and treated with IFN-γ. Data are means of three experiments ± S.E. ($P > 0.05$). (B) iNOS and HPRT gene products of specific amplification by RT-PCR. Densitometric analysis of levels of iNOS-specific mRNA were determined by RT-PCR after standardization of cDNAs for HPRT gene expression in IFN-γ-treated Mϕ infected with *L. amazonensis* or *L. major*. ($P > 0.05$, Student's *t*-test). (C) Effect of IFN-γ on the percentage of infection. Control of infection was considered 100% (*L. amazonensis* 12 h = 76.1 ± 11.2%, $n = 3$ and 72 h = 84.3 ± 5.8%, $n = 8$; *L. major* 12 h = 44.3 ± 8.4% and $n = 3$, 72 h = 26.1 ± 6.3%, $n = 8$). (D) Effect of AMG on the percentage of infected cells treated with IFN-γ 72 h postinfection. Control of infection was considered 100% (86.3 ± 3.9% in *L. amazonensis* infection and 30.5 ± 6.1% in *L. major* infection, $n = 8$). In C and D the results represent the percentage of infection related to control cells. Data are means of (n) experiments ± S.E. (ANOVA).

amazonensis or *L. major* promastigotes. Non-internalized promastigotes were washed out at time 0, and cells were reincubated for an additional 24, 48 and 72 h in the absence or in the presence of IFN-γ. NO was measured in cell supernatants to evaluate Mϕ activation. Non-infected cells and infected cells without IFN-γ pretreatment served as controls, and produced small amounts of NO. As expected, non-infected Mϕ treated with IFN-γ produced high levels of NO after 72 h of infection (47.5 ± 11.3 μM per 10^6 cells). Surprisingly, there was no difference between NO produced by *L. amazonensis*- and *L. major*-infected PEC Mϕ during the whole period ($P = 0.1931$) (Fig. 3A). To confirm similar NO production by these cells, we evaluated the iNOS mRNA expression by RT-PCR. After 12 h (not shown) until 72 h postinfection, iNOS mRNA expression was detected at a similar level by IFN-γ-pre-stimulated *L. amazonensis*- and *L. major*-infected Mϕ (Fig. 3B).

Although similar amounts of NO were induced by IFN-γ in *L. amazonensis*- and *L. major*- infected cells (Fig. 3A), IFN-γ induced a not very significant reduction in *L. amazonensis* infection, related to control cells at time 0 and 72 h postinfection (Fig. 3C). In contrast, IFN-γ significantly reduced the percentage of *L. major* infection at time 0 ($P < 0.001$) (Fig. 3C). After 72 h of infection, the IFN-γ effect on the percentage of *L. major* infection was smaller than at time 0, but was still significant ($P < 0.05$) (Fig. 3C). This data indicates that, in contrast to *L. major*, *L. amazonensis* is resistant to IFN-γ-induced NO production by CBA/J Mϕ.

To evaluate the role of NO in our system, AMG was added to the cultures. AMG completely blocked NO production induced by IFN-γ in infected cells (not shown). Surprisingly, its addition to CBA/J Mϕ neither modified *L. amazonensis* infection ($P > 0.05$) nor reversed IFN-γ inhibition of *L. major*-infected cells ($P > 0.05$) (Fig. 3D). The NO donor SNAP was then added to the cultures to test whether very high NO concentrations have any effect on the control of *Leishmania* infection. Indeed, NO concentration detected in SNAP-treated cells was higher than in IFN-γ-treated Mϕ, and the percentage of infection was reduced in both *L. amazonensis*-and in *L. major*-infected Mϕ compared to levels observed in non-treated infected cells (Fig. 4A, B).

3.4. Role of TNF-α in Leishmania infection

As the role of TNF-α in *L. amazonensis* infection has not been evaluated until now, and the control of *L. major* infection is still controversial [29–31], we tested whether IFN-γ-activated Mϕ inhibited *Leishmania* infection by a mechanism dependent on TNF-α. We first determined TNF-α mRNA levels at periods before and after time 0 and we were only able to detect expression in IFN-γ-activated cells at time 0. In all experiments ($n = 5$), TNF-α mRNA levels in IFN-γ-

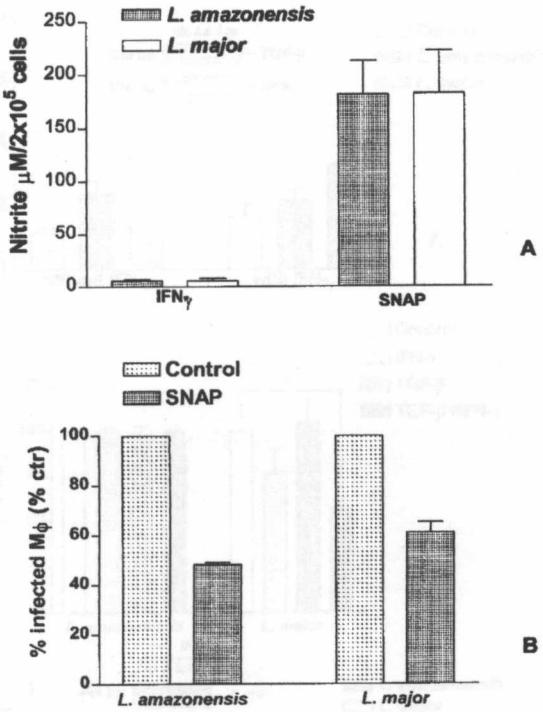

Fig. 4. High NO concentrations inhibit Mϕ infection by *L. amazonensis* and *L. major*. (A) Nitrite levels after SNAP addition. (B) Percentage of Mϕ infection with *L. amazonensis* or *L. major* in SNAP-treated cells. Control of infection was considered 100% (*L. amazonensis* 73.10 ± 8.8 and *L. major* 33.37 ± 6.7). PEC Mϕ were cultured and infected as described in Fig. 1 and served as control. Cells were infected and 12 h later were washed to remove free parasites. After this time cells were re-incubated and treated every 4 h for 48 h with 100 μM of SNAP. Results are reported as mean ± S.E.M. of two experiments done in triplicate, $P < 0.0001$ (ANOVA).

activated *L. amazonensis*-infected Mϕ were half of those expressed by *L. major*-infected cells ($P = 0.022$) (Fig. 5A). As we were only able to detect a very low expression in *L. amazonensis*-infected cells, anti-TNF-α was only added to IFN-γ-activated *L. major*-infected cells. Anti-TNF-α was able to completely reverse the reduction in *L. major* infection induced by IFN-γ (data not shown). Throughout the time postinfection we were not able to detect TNF-α in cell supernatants.

The effect of TNF-α added exogenously was then tested. Recombinant TNF-α was added to the cultures alone or at the same time as rIFN-γ. As expected, after 72 h of infection, rTNF-α alone significantly reduced the percentage of *L. major*-infected cells (Fig. 5B), and associated with rIFN-γ was able to significantly reduce the percentage of both *L. amazonensis*- ($n = 5$; $P < 0.001$) and *L. major*-infected cells ($n = 5$; $P < 0.001$). As we have observed in IFN-γ-treated cells, NO production was similar in *L. amazonensis*- or *L. major*-infected cells treated with both IFN-γ and TNF-α. Despite the synergic effect on NO production by TNF-α added to IFN-γ-treated cells, three times more than in IFN-γ-treated cells, AMG did not reverse the IFN-γ plus TNF-α effect on *L. amazonensis* infection ($n = 3$; $P = 0.504$). Instead, the iNOS inhibitor partially reversed the IFN-γ plus

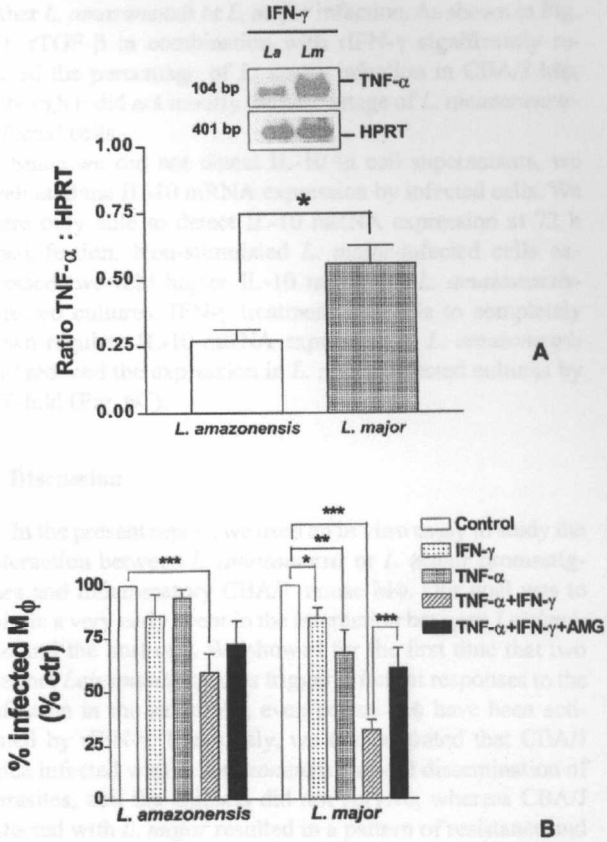

Fig. 5. Role of TNF-α in Mϕ infection by *Leishmania*. (A) TNF-α and HPRT gene products of specific amplification by RT-PCR 12 h postinfection. Densitometric analysis of levels of TNF-α-specific mRNA were determined by RT-PCR after standardization of cDNAs for HPRT gene expression in IFN-γ-treated Mϕ infected with *L. amazonensis* or *L. major*. This data represent the means of five experiments ± S.E. (B) AMG effect on the percentage of infected cells treated with TNF-α plus IFN-γ 72 h postinfection. Control of infection was considered 100% (*L. amazonensis* = 83.6 ± 3.8% $n = 12$; *L. major* = 30.5 ± 6.1%, $n = 12$). Cells were cultured as described in Fig. 1 and pretreated with IFN-γ (100 UI/ml) ($n = 9$) or IFN-γ (100 UI/ml) plus TNF-α (100 UI/ml) ($n = 6$) ± AMG (1 mM) ($n = 3$) during 12–18 h, after 12 h MΦ were washed to remove free parasites and then cytokines plus AMG were replaced using similar concentrations. This data represents the means of experiments (n) ± S.E. Significant differences are indicated in the figure, $P < 0.05$ *, $P < 0.01$** and $P < 0.001$ or $P < 0.0001$ *** (ANOVA).

TNF-α inhibition of *L. major* infection ($n = 3$, $P = 0.037$) (Fig. 5B).

3.5. *Effect of TGF-β and IL-10 on* L. amazonensis- *or* L. major-*infected Mϕ*

We also investigated the role of TGF-β and IL-10, Mϕ-produced cytokines implicated in driving the Th-2 response. We first compared mRNA levels of expressed cytokines by *L. amazonensis*- or *L. major*-infected cells, then measured cytokines in infected cell supernatants. TGF-β mRNA expression increased according to time of infection (not shown), reaching its maximum at 72 h postinfection. As shown in Fig. 6A, after infection, TGF-β mRNA expression is slightly

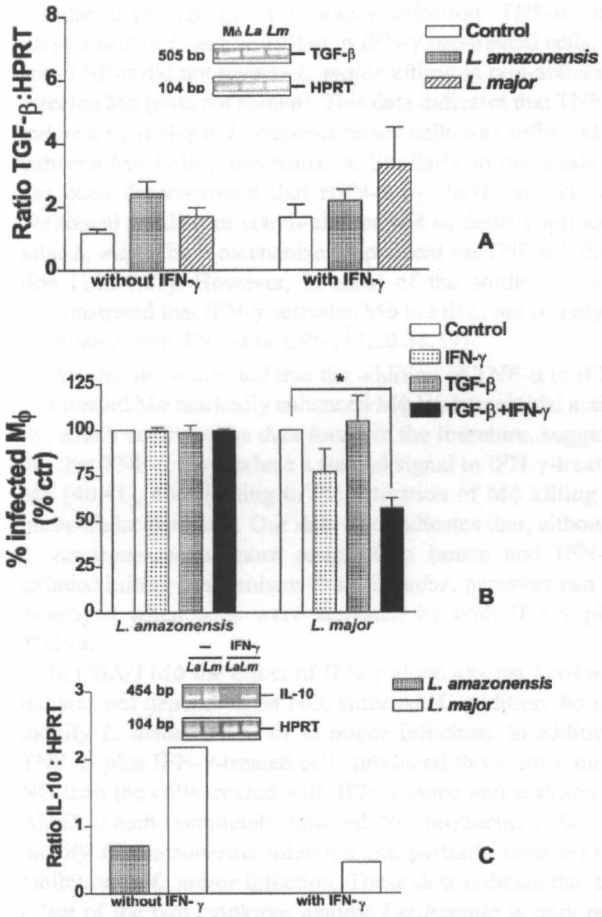

Fig. 6. Expression of cytokines and TGF-β effect on *L. major*- or *L. amazonensis*-infected Mϕ. (A) TGF-β and HPRT gene products of specific amplification by RT-PCR 72 h postinfection. Densitometric analysis of levels of TGF-β-specific mRNA were determined by RT-PCR after standardization of cDNAs for HPRT gene. PEC Mϕ (1×10^6 per ml) were cultured and infected as indicated in Fig. 3. This data represents the means of five experiments ± S.E. ($P > 0.05$, one-way ANOVA). (B) Effect of TGF-β ± IFN on *L. amazonensis* or *L. major* infection at 72 h. Cells were cultured as described in Fig. 1 and Mϕ were pre-treated with IFN-γ (100 UI/ml) ± TGF-β (10 ng/ml) during 12–18 h. After 12 h of infection, cells were washed to remove free parasites and cytokines were replaced using similar concentration. Control of infection was considered 100% (*L. amazonensis* = 93.8 ± 1.7%, $n = 3$, $P = 0.9094$; *L. major* 35.8 ± 8.6%, $n = 3$, $P = 0.0149$, ANOVA). Results represent the percentage of infection related to control cells. (C) IL-10 and HPRT gene products of specific amplification by RT-PCR 72 h post infection. Densitometric analysis of levels of IL-10-specific mRNA was determined by RT-PCR after standardization of cDNAs for HPRT gene expression. PEC Mϕ (1×10^6 per ml) were cultured and infected as indicated in Fig. 3. This data is one representative of two similar experiments ± S.E.

higher in the remaining cells ($P = 0.4061$) and upon IFN-γ activation ($P = 0.7352$) than in control non-infected Mϕ. Similarly, levels of TGF-β in supernatants of *L. amazonensis*- and *L. major*-infected cultures showed no differences, and were slightly higher than in control non-infected cells (not shown). In the next step, cells were treated before and during infection with only rTGF-β, or both rIFN-γ and rTGF-β. Addition of rTGF-β did not modify either *L. amazonensis* or *L. major* infection. As shown in Fig. 6B, rTGF-β in combination with rIFN-γ significantly reduced the percentage of *L. major* infection in CBA/J Mϕ, although it did not modify the percentage of *L. amazonensis*-infected cells.

Since we did not detect IL-10 in cell supernatants, we evaluated the IL-10 mRNA expression by infected cells. We were only able to detect IL-10 mRNA expression at 72 h postinfection. Non-stimulated *L. major*-infected cells expressed two-fold higher IL-10 mRNA as *L. amazonensis*-infected cultures. IFN-γ treatment was able to completely down-regulate IL-10 mRNA expression in *L. amazonensis* and reduced the expression in *L. major*-infected cultures by 4.7-fold (Fig. 6C).

4. Discussion

In the present report, we used an in vitro assay to study the interaction between *L. amazonensis* or *L. major* promastigotes and inflammatory CBA/J mouse Mϕ. Our goal was to mimic a very early event in the interaction between *Leishmania* and the host cell. We showed for the first time that two distinct *Leishmania* species trigger different responses to the infection in the same Mϕ, even before Mϕ have been activated by rIFN-γ. Previously, we demonstrated that CBA/J mice infected with *L. amazonensis* showed dissemination of parasites, and the animals did not survive, whereas CBA/J infected with *L. major* resulted in a pattern of resistance and cure [7].

A possible bias in our system is the use of stationary-phase promastigotes instead of purified metacyclic forms. However, the limitations of this are minimized by the fact that we are comparing the infection of the same Mϕ in two different species of *Leishmania*, and the percentage of complement-resistant promastigotes was similar in *L. amazonensis* and *L. major* stationary-phase promastigotes.

We showed that *L. amazonensis*-infected Mϕ generated half the H_2O_2 of that produced by *L. major*-infected Mϕ. Our data suggest that the incapacity of CBA/J Mϕ to destroy *L. amazonensis* depends, at least in part, on the deficiency of ROI production, emphasizing the role of innate immune elements on the determination of *Leishmania* infection outcome. Although we did not address this question, it has been demonstrated that *Leishmania*'s ability to survive inside cells may depend on the interaction of parasite surface molecules with Mϕ receptors [32–35]. Recently, it was demonstrated that one mechanism used by *L. amazonensis* to survive in the host cell is the very early acquisition of amastigote features inside the parasitophorous vacuole [36]. This interaction might influence activation of host signaling pathways and parasite intracellular fate inside CBA/J Mϕ.

Twelve hours postinfection, in IFN-γ activated Mϕ, TNF-α expression was lower in *L. amazonensis*- than in *L. major*-infected Mϕ. In addition, neutralization of TNF-α led to a higher parasite burden in *L. major*-infected Mϕ pretreated with IFN-γ, indicating that TNF-α expression lead to

a reduced percentage of *L. major* infection. TNF-α only plays a role in *L. major* killing in IFN-γ pre-treated cells, as anti-TNF-α did not reverse *L. major* killing in non-activated infected Mϕ (data not shown). This data indicates that TNF-α induced by IFN-γ in *L. major*-infected cells was sufficient to enhance Mϕ killing mechanisms. Similarly to our study, it has been demonstrated that rIFN-γ by itself can activate peritoneal resident or starch-elicited Mϕ to destroy intracellular *L. major* by a mechanism dependent on TNF-α induction [18,31,37]. However, in most of the studies, it was demonstrated that IFN-γ activates Mϕ to kill *L. major* only if associated with TNF-α or LPS [17,20,38,39].

We also demonstrated that the addition of TNF-α to IFN-γ-activated Mϕ markedly enhanced Mϕ leishmanicidal activity, which confirms the data found in the literature, suggesting that TNF-α may induce a second signal to IFN-γ-treated Mϕ [40,41], contributing to the activation of Mϕ killing of intracellular parasites. Our data also indicates that, although *L. amazonensis* is more resistant to innate and IFN-γ-induced killing mechanisms than *L. major*, parasites can be destroyed when cells were activated by both IFN-γ plus TNF-α.

In CBA/J Mϕ the effect of IFN-γ alone against *Leishmania* was not dependent on NO, since AMG addition did not modify *L. amazonensis* or *L. major* infection. In addition, TNF-α- plus IFN-γ-treated cells produced three times more NO than the cells treated with IFN-γ alone and addition of AMG, which completely blocked NO production, did not modify *L. amazonensis* infection and partially reversed the inhibition in *L. major* infection. These data indicate that the effect of the two cytokines against *Leishmania* is only partially dependent on NO. Previously, we have shown that in vivo establishment and dissemination of *L. amazonensis* infection are not related to an inability of Mϕ to produce NO [7], but that, in comparison to *L. major*, *L. amazonensis* is more resistant to being killed by the levels of NO or other host killing mechanisms produced in the early phases of the infection. These data is in accordance with another study where it was demonstrated that control of *L. amazonensis* infection by inflammatory mediators such as platelet activating factor is independent of NO [42]. Recently, it was demonstrated that in vivo as well as in vitro TNF-α induces *L. major* killing by a mechanism partially independent on NO. TNF-α knockout C57BL/6 mice cannot control *L. major* infection, although iNOS protein is readily detectable in skin lesions and draining LNs [30]. Furthermore, we showed that SNAP, a chemical NO donor, added to CBA/J Mϕ highly enhanced NO in the cultures, but did not sterilize infected Mϕ. Together, this data support the idea that NO can kill intracellular parasites, but its effect is potentially enhanced, depending on whether or not NO is associated with other host toxic molecules, such as oxygen reactives [19,43], similarly to the model of in vitro control of *Salmonella typhimurium* infection [44].

In CBA/J Mϕ expressed TGF-β mRNA and TGF-β in cell supernatants were produced in the same levels by *L. amazonensis*- and *L. major*-infected cells, slightly higher than in non-infected cells. In addition, we showed that TGF-β alone had no effect on parasite infection, but the addition of this cytokine in the presence of IFN-γ significantly reduced the percentage of *L. major*-infected cells, demonstrating that in vitro TGF-β potentialized the IFN-γ effect on CBA/J Mϕ activation to destroy *L. major*. In contrast to our work, data found in the literature [45,46] have clearly demonstrated that in vivo TGF-β suppresses resistance to *L. major* infection, and that treatment with TGF-β promotes disease in resistant C57BL/6 mice infected with either *L. amazonensis* or *L. braziliensis*. On the other hand, treatment of susceptible BALB/c mice with anti-TGF-β antibody enhanced resistance to *L. amazonensis* infection [46]. It has been shown that TGF-β influences CD4+ T-cell maturation, although contradictory effects on T-cell differentiation have been reported in various in vitro studies showing that TGF-β can either promote or suppress Th-1 development [47–50].

IL-10 mRNA expression was higher in non-stimulated infected cells than in IFN-γ-treated Mϕ. This result is in accordance with data in the literature, which demonstrates the IFN-γ role on modulation of IL-10 production [51]. Furthermore, we showed that *L. major* induced 3-fold higher expression of IL-10 mRNA than *L. amazonensis*-infected Mϕ. In our previous work we demonstrated a 4-fold higher production of IL-10 by LN cells of *L. major*-infected mice over those produced by LN cells of *L. amazonensis*-infected animals [7]. These data support the idea that in CBA/J mice, IL-10 can exert a dual role in the immune response against *Leishmania*. In a context of Th-2 response as observed in *L. amazonensis*-infected CBA/J mice, IL-10 can exacerbate the infection. On the other hand, IL-10 can possibly modulate a Th-1 type of immune response in *L. major*-infected CBA/J mice.

In summary, we showed that in vitro *L. amazonensis* inactivates and is resistant to innate and IFN-γ-induced Mϕ killing mechanisms. We also demonstrated that TNF-α induced in response to IFN-γ activation is involved in the control of *L. major* infection by a mechanism partially dependent on NO, suggesting that the NO effect is associated with the effect of other host toxic molecules. TGF-β and IL-10 are seemingly not determinants of the infection of CBA/J mouse Mϕ by *L. amazonensis* or *L. major*. In conclusion, the results presented here emphasize the role of the parasites in determining the outcome of *Leishmania* infection, and indicate that the first contact of parasites with Mϕ can translate information, regarding the nature of the pathogen, into differences in produced microbicidal molecules and cytokines by host cells, which play a role in the polarization of the acquired immune response.

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5. CAPÍTULO III - ANÁLISE COMPARATIVA DA BIOGÊNESE DO VACÚOLO PARASITÓFORO CONTENDO *L. amazonenis* E *L. major*

5.1 INTRODUÇÃO

Macrófagos são importantes células no controle da infecção por Leishmania, pois estão presentes na resposta imune inata e participam da fase efetora da resposta imunológica a Leishmania. *L. amazonensis, in vitro,* inativa e resiste aos mecanismos de destruição de macrófagos, embora *L. major* seja destruída por essas células. Esses dados evidenciam que o primeiro contato de diferentes espécies de Leishmania com a célula hospedeira é acompanhado de distintos padrões de resposta (Gomes *et al.*, 2003).

Algumas moléculas foram descritas como participantes da interação do macrófago com a Leishmania (receptores para moléculas do complemento, Mac-1, receptores manose/fucose, entre outros). Essas moléculas, além de promoverem a ligação entre o parasito e a célula hospedeira, desencadeiam eventos de transdução de sinal que influenciam a resposta contra o parasito. É possível que a diferença na ligação e ativação desses receptores por diferentes espécies de Leishmania seja, em parte, responsável pelos perfis distintos de resposta do macrófago (Da Silva *et al.*, 1989; Rosenthal *et al.*, 1996)

Após serem fagocitadas, os parasitos do gênero Leishmania se localizam em fagossomas denominados vacúolos parasitóforos (VPs). Os VPs amadurecem fusionando com vesículas da via endocítica, endossomas tardios e/ou lisossomas. A maioria dos estudos sobre a biogênese dos VPs tem utilizado como modelo a infecção *in vitro* de macrófagos murinos por *L. amazonensis* ou *L. mexicana*. Esses estudos demonstraram que os vacúolos induzidos por Leishmania do complexo *mexicana* apresentam características de fagolisossoma. Eles apresentam pH baixo, em torno de 4,7-5,2 (Antoine *et al.*, 1990), hidrolases lisossomais, como catepsinas D, B, H, e L (Prina *et al.*, 1990) e marcadores de membrana lisossomais, como o receptor manose

independente de cátion, LAMP1, LAMP2, rab7, macrosialina e a próton ATPase, além de moléculas de MHC classe II (Lang *et al.*, 1994; Russell, 1994; Russell *et al.*, 1992). Foi também demonstrado que o vacúolo maduro tem capacidade aumentada de se fusionar com endossoma primário. Por outro lado, embora VP de células infectadas com *L. amazonensis* adquiram partículas de zimosan, nem bolas de látex, nem eritrócitos fagocitados acessam esse compartimento (Veras *et al.*, 1992). Portanto, nem todo material endocitado alcança o VP induzido por *L. amazonensis*, sugerindo que este fagossoma fusiona seletivamente com outras vesículas de endocitose.

Os vacúolos induzidos por outras espécies de Leishmania possuem características comuns com os induzidos por *L. amazonensis* ou *L. mexicana*, porém apresentam diferenças morfológicas e funcionais. Por exemplo, os vacúolos induzidos por *L. amazonensis* são grandes, ao passo que os induzidos por *L. major* e *L. donovani*, que são vacúolos pequenos, sugerindo que os processos que controlam a fusão ou fissão destes vacúolos são diferentes (Antoine *et al.*, 1998).

A inibição da fusão fagossoma-lisossoma é um mecanismo de sobrevivência utilizado por alguns microorganismos intracelulares (Joiner, 1997; Small *et al.*, 1994). Foi demonstrado que VPs de *L. donovani* apresentam bloqueio temporário da fusão do fagossoma com lisossomas, e que isso pode favorecer o tempo de diferenciação para o estágio de amastigota, melhor adaptado à vida intracelular (Desjardins *et al.*, 1997).

Para explicar um dos mecanismos pelos quais macrófagos de camundongos CBA controlam a infecção por *L. major* e são permissivos à *L. amazonensis*, nossa hipótese é que esses parasitos de espécies distintas interagiriam com receptores distintos no macrófago, o que levaria à formação de fagossomas com cinéticas diferentes de fusão com lisossomas. Assim, os fagossomas contendo promastigotas de *L. major* se fusionariam rapidamente com lisossomas, antes que o parasito adquirisse características

de amastigotas, levando à morte do parasito nos primeiros momentos após a fagocitose. Por outro lado, a fusão dos vacúolos contendo *L. amazonensis* seria mais tardia, favorecendo a sobrevivência maior deste parasito, em comparação com *L. major*.

5.2 MATERIAL E MÉTODOS

Cultura de células

Macrófagos peritoneais do exsudato inflamatório foram obtidos pela lavagem com salina gelada da cavidade peritoneal dos camundongos da linhagem CBA. Foram distribuídos 2 x 10^6 macrófagos por poço em placas de 6 poços em duplicata, em meio DMEM completo. Após 24 horas de incubação a 37° C e 5% de CO_2, as culturas foram lavadas com meio RPMI para a remoção das células não aderentes.

Infecção de macrófagos e processamento para microscopia eletrônica

Para marcar lisossomos, as células foram incubadas, antes da infecção, com BSA (bovine serum albumin) conjugada a partículas de ouro coloidal (Ouro-BSA) de 15 nm por 3 horas, em seguida foram lavadas e reincubadas, por mais 18h, em meio DMEM completo a 37°C e 5% de CO_2. Logo após esse período de incubação, as células foram infectadas com promastigotas de *L. amazonensis* ou *L. major* em fase estacionária, na proporção de 10 parasitos por macrófago. Após 15, 30, 60 e 90min de infecção, as células foram fixadas e processadas para microscopia eletrônica, como descrito previamente (Frehel *et al.*, 1997). As células infectadas foram fixadas a 4°C em glutaraldeído a 2.5% (Polyscience, Warrington, PA) em 0.1 mM tampão cacodilato (pH 7.2), suplementado com 0.1 M sucrose, 5 mM $CaCl_2$ e 5 mM $MgCl_2$. Após 1h de lavagem com tampão de lavagem, as células foram pós-fixadas por mais 1h à temperatura ambiente em tetróxido de ósmio a 1% em 0.1 mM tampão cacodilato na ausência de sucrose. As células foram, então, raspadas, concentradas em ágar a 2% em tampão cacodilato, e tratadas por 1h à temperatura ambiente com acetado de uranila a 1% e tampão veronal. As amostras foram desidratadas em uma série de soluções

graduadas de acetona e, por fim, incluídas em Epon. Cortes ultrafinos foram contrastados com acetato de uranila e citrato de chumbo. Foram feitas observações quantitativas no microscópio eletrônico para determinação do percentual de fagossomas que fusionaram com lisossomas. Foi considerado que houve fusão de fagossomas com lisossomas quando pelo menos uma partícula de ouro-BSA era encontrada em VPs.

5.3 RESULTADOS E DISCUSSÃO

Macrófagos inflamatórios de camundongos CBA, previamente incubados com ouro-BSA para marcação de lisossomas, foram infectados com *L. amazonensis* ou *L. major* por 30, 60, 90 e 120min para avaliar, comparativamente, a cinética de fusão dos fagossomas com lisossomas. Foi observado que há um percentual de fusão de cerca de 60-70% dos fagossomas induzidos por *L. amazonensis* ou *L. major* com lisossomas, desde os tempos mais curtos após a infecção (Figura 1). Os dados mostram que, a despeito de *L. amazonensis* e *L. major* serem parasitos de espécies distintas e induzirem respostas diferentes em macrófagos infectados de camundongos CBA, os VPs de *L. amazonensis* ou *L. major* apresentam cinéticas semelhantes de fusão com lisossomas.

Anteriormente, foi descrito que a cinética da formação do VP depende do estágio do parasito internalizado (Dermine *et al.*, 2000; Desjardins *et al.*, 1997). Desjardins e colaboradores demonstraram que LPG de promastigotas de *L. donovani* é a molécula responsável pela inibição transitória da fusão dos fagossomas de *L. donovani* com lisossomas (Desjardins *et al.*, 1997; Scianimanico *et al.*, 1999). De acordo com esses autores, o bloqueio temporário da maturação do fagossoma permitiria promastigotas iniciarem sua transformação em amastigotas. Esses dados são consistentes com estudos que mostraram uma diminuição da sobrevivência de promastigotas de *L. major* mutantes para LPG (Spath *et al.*, 2000). Entretanto, mutante

de LPG de *L. mexicana* se replica normalmente em macrófago, mantendo sua virulência em camundongos (Ilg, 2000; Ilg *et al.*, 2001; Turco *et al.*, 2001). Isso indica que LPG é um importante fator de virulência para *L. major*, enquanto que outros glicoconjugados parecem exercer esse papel para a *L. mexicana*. Nossos dados evidenciam que a despeito do possível papel desses glicoconjugados na sobrevida de *L. amazonensis* e de *L. major*, seus mecanismos de ação não interferem na cinética de fusão dos VPs.

As diferenças morfológicas observadas entre os VPs de *L. amazonensis* e *L. major* não refletem diferenças na capacidade de fusão com lisossomas, como mostrado na figura 2. Em concordância com os nossos dados, foi demonstrado que fagossomas contendo promastigotas ou amastigotas de *L. amazonensis* bem como promastigotas de *L. major*, rapidamente adquirem capacidade de se fundir com endossomas tardios/lisossomas, sugerindo que outros mecanismos estejam modulando a composição desses vacúolos (Courret *et al.*, 2001).

Sabe-se que mudanças na morfologia dos VPs de Leishmania são acompanhadas por modificações bioquímicas, incluindo mudanças na composição da membrana limitante, devido, em parte, à fusão do fagossoma com compartimentos endocíticos (Antoine *et al.*, 1998). Estudos demonstraram que a expansão dos compartimentos induzidos por *L. amazonensis* ocorre entre 5 e 12h de infecção, enquanto que a fusão dos VPs com organelas de macrófago ocorre entre 12-18h após a infecção (Courret *et al.*, 2001). Foi também demonstrado que grandes vacúolos de *L. amazonensis* são formados pela fusão dos VPs entre si (Pouchelet *et al.*, 1995) e destes com compartimentos da via endocítica (Veras *et al.*, 1996). Pouco se sabe sobre as mudanças morfológicas e bioquímicas que ocorrem durante a formação dos compartimentos induzidos por *L. major*.

É possível que a formação e a maturação dos VPs contendo *L. amazonensis* ou *L. major* sejam diferentemente moduladas após a fagocitose, pela liberação de moléculas específicas por promastigotas, ou haja diferenças no balanço da fusão e fissão em tempos tardios. De fato, foi sugerido que proteofosfoglicana secretada por amastigotas de *L. mexicana* no lúmen do VP pode estar envolvida na sua expansão (Peters *et al.*, 1997). Sabe-se que, além da alta capacidade de fusão dos VPs induzidos por Leishmania do complexo *mexicana*, os vacúolos induzidos por esses parasitos podem adquirir moléculas aniônicas provenientes do citosol por transporte ativo, através de transportadores na membrana vacuolar (Schaible *et al.*, 1999).

A formação do ambiente do VP pode ter importantes efeitos no resultado da doença. Por exemplo, Gruenheid e colaboradores (Gruenheid *et al.*, 1997) demonstraram que NRAMP1, associada à resistência contra microorganismos intracelulares, é expressa em membranas de endosssomas tardios, apresenta uma seqüência putativa de uma proteína transportadora de íons e colocaliza em compartimentos de macrófagos contendo LAMP1. Esses dados sugerem que NRAMP poderia controlar a replicação de parasitos intracelulares alterando o ambiente vacuolar do fagossoma (Gruenheid *et al.*, 1997). É possível que os compartimentos de *L. amazonensis* e *L. major* apresentem outras diferenças que ainda não foram investigadas, como a expressão de NRAMP.

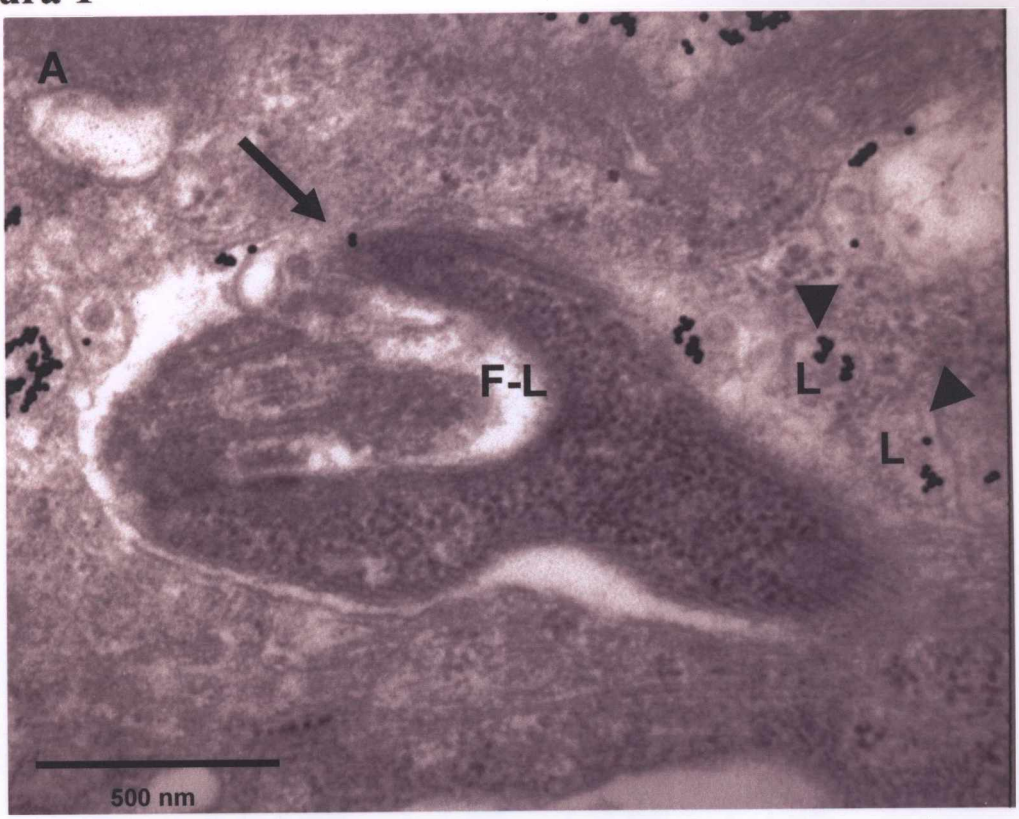
Foi demonstrado que VP contendo *L. amazonensis* seqüestra moléculas de MHC II, que são degradadas por cisteínas proteases do hospedeiro e do parasito (De Souza Leao *et al.*, 1995; Lang *et al.*, 1994), mecanismo que favorece sua sobrevivência indiretamente, por influenciar a resposta imunológica. Ainda não foi investigada a distribuição de moléculas de MHCII em células infectadas por *L. major*.

Nossos dados mostram que os VPs contendo *L. amazonensis* ou *L. major*, conhecidos por serem ambientes ácidos e ricos em enzimas lisossomais (Antoine *et al.*, 1990), apresentam semelhanças na cinética de fusão com lisossomas. *L. amazonensis* é capaz de viver e se multiplicar dentro desse ambiente hostil, enquanto a maioria dos parasitos *L. major* é destruída pelos macrófagos após algumas horas. No contexto da infecção por *L. amazonensis*, é possível que a concentração das hidrolases esteja diluída nos grandes vacúolos, diminuindo sua atividade. Estudos utilizando coinfecção de *L. amazonensis* e *L. major* talvez evidenciem se o ambiente do VP é capaz de influenciar o perfil de infecção dos macrófagos.

Finalmente, esse artigo sugere que outras diferenças, além da fusão desses compartimentos com lisossomas, estão relacionadas a modificações bioquímicas distintas, tanto da membrana, como do conteúdo destes VPs, não investigados nesse trabalho. Novos estudos devem ser realizados com o objetivo de melhor caracterizar a natureza desses compartimentos.

5.4 FIGURAS

Figura 1



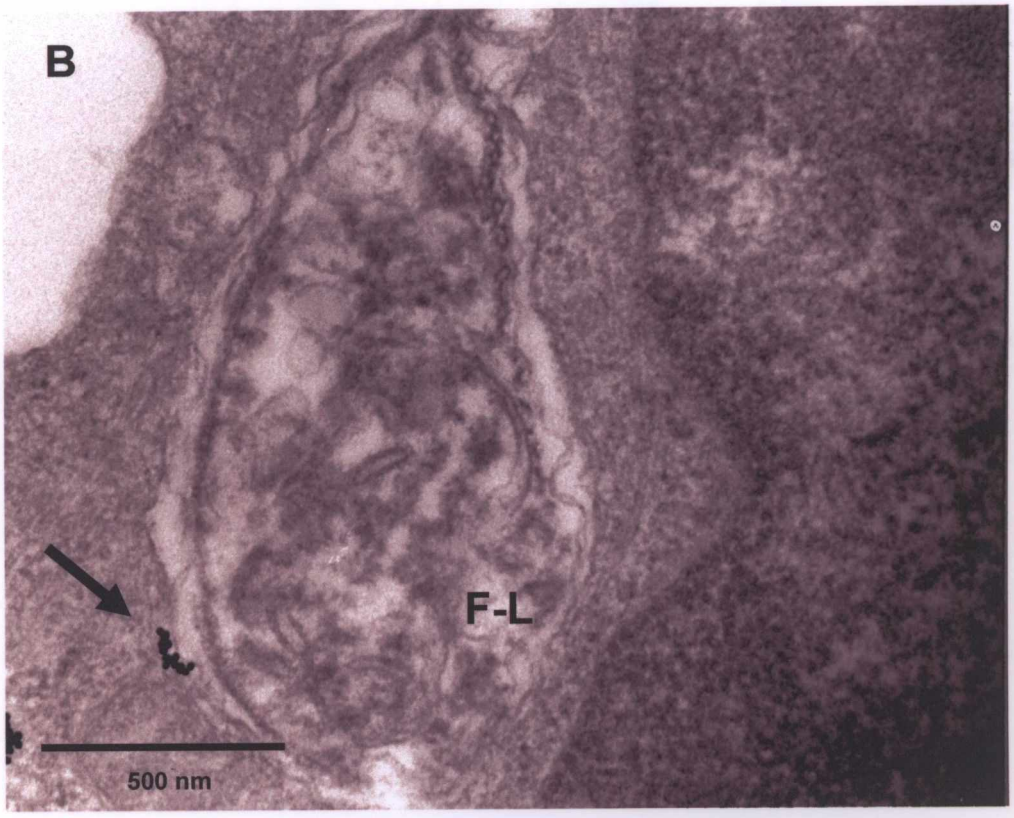
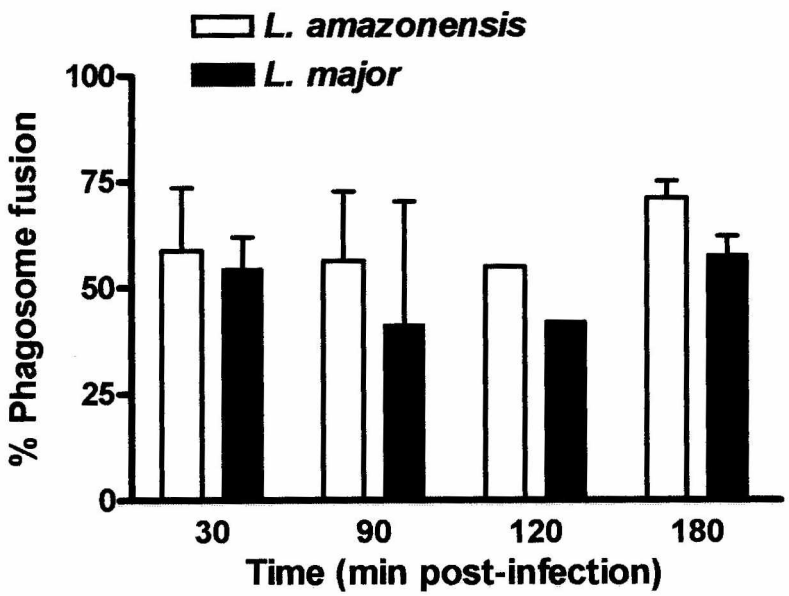


Figura 2


L. amazonensis
L. major
100
75
50
25
0
% Phagosome fusion
30
90
120
180
Time (min post-infection)

5.5 LEGENDAS

Figura 1. Ultra-estrutura do fagossoma induzido por *L. amazonensis* ou *L. major*

Para marcar lisossomos, macrófagos de camundongos CBA foram incubados com partículas de ouro-BSA de 15nm por 3h, depois foram lavados e infectados com promastigotas de *L. amazonensis* ou *L. major* na proporção de 10:1. Após 30, 90, 120 e 180min de infecção, as células foram fixadas e processadas para microscopia eletrônica como descrito em material e métodos. Macrófagos de camundongos CBA infectados *in vitro* por *L. amazonensis* (A) ou *L. major* (B) em fagossomas que fusionam com lisossomas marcados com ouro-BSA. Setas indicam partículas de ouro-BSA dentro de fagolissomas e cabeça de setas indicam partículas de ouro-BSA apenas em lisossomas. F = fagossoma, F-L = fagolisossoma e L = lisossoma (50.000 X).

Figura 2. *L. amazonensis* and *L. major* induzem fagossomas com capacidade similar de fusão em macrófagos de camundongos CBA. Macrófagos de camundongos foram cultivados, infectados e processados como indicado na figura 1. Para cada amostra, foram feitos 2 cortes com 10 μm de distância e, no mínimo, 150 fagossomas foram contados. A fusão de fagossomas com lisossomas foi considerada quando pelo menos uma partícula de ouro-BSA era encontrada no vacúolo. O percentual de fagossoma que fusionava com lisossoma foi quantificado. O gráfico representa a média de 2 experimentos ± SEM.

6. CAPÍTULO IV – O PROGRAMA TRANSCRICIONAL DE MACRÓFAGOS DE CAMUNDONGOS CBA/J É ALTERADO EM RESPOSTA À INFECÇÃO POR LEISHMANIA

6.1 INTRODUÇÃO

Os eventos que ocorrem nas fases iniciais da infecção por Leishmania são crucias na determinação do curso da doença. Macrófagos são células importantes no controle da infecção, participando da resposta imune inata e da fase efetora da resposta imune a Leishmania. *L amazonensis*, *in vitro*, inativa e resiste aos mecanismos de destruição do macrófago, entretanto *L. major* é destruída pelos macrófagos, evidenciando que o primeiro contato de diferentes espécies de Leishmania com a célula hospedeira induzem um perfil distinto de resposta (Gomes *et al.*, 2003).

Para investigar em larga escala os mecanismos envolvidos na resposta de macrófagos infectados, utilizamos DNA*microarray* e caracterizamos o perfil de expressão gênica de mais de 12.000 genes de macrófagos, após a infecção por *L. major* ou *L. amazonensis*. Demonstramos que a infecção por *L. amazonensis* ou *L. major* induz alterações significativas no perfil de expressão de numerosos genes relacionados ao metabolismo celular, resposta imuno-inflamatória, apoptose, fagocitose, sinalização e citoesqueleto. Alguns desses genes estão relacionados com a resposta do hospedeiro à Leishmania enquanto que outros ainda não foram associados à infecção.

Genes relacionados à explosão respiratória, formação do vacúolo parasitóforo e receptores da superfície celular envolvidos na fagocitose, estão induzidos ou suprimidos a depender da espécie de Leishmania e o tempo de infecção. Observamos que na infecção por *L. amazonensis* houve um aumento na expressão de genes que codificam enzimas que inibem a explosão respiratória. Genes relacionados a receptores do tipo *scavenger*, que podem ser importantes nos diferentes perfis de resposta do macrófago, foram ativados na infecção por *L. major*, o que pode estar associado à expressão 20%

maior de MARCO na superfície de células infectadas por esse parasito, quando comparada à infecção por *L. amazonensis*. Finalmente, estudos *in vivo* mostraram que linfonodos de camundongos CBA infectados por *L. major* apresentaram um aumento da expressão de MARCO em comparação à infecção por *L. amazonensis*.

Os dados sugerem que características específicas de *L. major* ou *L. amazonensis* induzem alterações no programa de transcrição gênica em macrófagos levando a um padrão distinto de resposta à infecção. Esses dados contribuem para o estudo de diferentes mecanismos de sobrevivência do parasito e suas possíveis conseqüências para a determinação de uma resposta protetora ou não protetora em camundongos CBA.

6.2 ARTIGO 2

TITLE

The Transcriptional Program of CBA/J Mice Macrophages Is Altered In Response to Infection by Leishmania

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ABSTRACT

CBA mice are resistant to *L. major* and susceptible to *L. amazonensis*. The early events are crucial to *Leishmania* infection outcome and macrophage is an important cell in infection control. *L. amazonensis, in vitro,* inactivates and resists to innate macrophage killing mechanisms, whereas *L. major* is destroyed by macrophages. To identify molecules involved in macrophage response to *Leishmania*, DNAmicroarray was used to characterize the mRNA expression profile of more than 12,000 mouse genes after macrophage infection with *L. amazonensis* or *L. major*. Some of the expressed genes were previously related to *Leishmania* infection but some of them were not yet associated to *Leishmania* infection. Genes related to cell surface receptors which participate both in cell activation and phagocytosis of microorganisms, genes involved in respiratory burst response and in parasitophorous vacuole biogenesis have their expression modified dependent on *Leishmania* species and time of infection. There is an up-regulation of genes that code to enzymes which participate in scavenging of hydrogen peroxide and superoxide anions in *L. amazonensis* infection. Genes that code for pattern-recognition receptors such as CD14 and scavenger receptor, which could be involved in cell activation, are up regulated in response to *L. major* infection. This result is correlated with 20% more expression of MARCO scavenger receptor in *L. major* infected macrophages when compared to *L. amazonensis* infected cells *in vitro*. Furthermore, *in vivo* studies demonstrated that there was higher expression of MARCO receptor in lymph nodes from *L. major*- in comparison to *L. amazonensis*-infected CBA mice. Results herein strongly suggest that factors related to parasite species drive differential transcriptional program in early infected macrophages that are related to different infection outcome. These data may contribute to understanding strategy of intracellular survival and its possible consequences in immune response to *Leishmania* that determine protection or susceptibility in CBA mice.

KEY WORDS

Macrophages; *Leishmania amazonensis*; *Leishmania major*; DNAmicroarray; MARCO receptor

INTRODUCTION

Experimental murine cutaneous leishmaniasis has been used to investigate factors involved in the generation of protective or non protective to intracellular pathogens. CBA mice have been used as a model to study resistance and susceptibility to *Leishmania*, since they control *L. major*- while are permissive to *L. amazonensis*- infection. *L. amazonensis*-driven pathogenic processes induce a Th2-type of response, whereas CBA mice heal following *L. major* infection induces a Th1-type of response (1). A critical question concerns whether the initial events dictate the differentiation of Th1 versus Th2 cells *in vivo* setting of *Leishmania* infection. We have recently demonstrated that *L amazonensis* both inactivates and resists innate macrophage killing mechanisms, whilst this same macrophage is capable to destroy *L. major* (2). These data indicate that the nature of parasite and its interaction with macrophages could determine immune response.

The macrophages participate in recognition, capture and processing of potential antigens which activate specific T and B lymphocytes effector mechanisms. In addition, macrophages secrete molecules that induce and regulate local inflammatory and immune responses. During *Leishmania* infection, macrophages not only serve as host cells for parasites, but are also important effector's cells for parasites killing. The internalization of the promastigote forms has been shown to be mediated by different receptors on the surface of macrophages (3) and depending on the receptor involved in the recognition of the parasites the promastigotes fail to trigger the macrophage respiratory burst (4). *Leishmania* lives and multiplies within organelles known as parasitophorus vacuoles (PVs) of phagolysossomal origin (5). However, surface molecules of *Leishmania* transiently inhibited phagossome-endosome fusion (6) facilitating its survival. Thus, *Leishmania* recognition by plasma-membrane receptor of

macrophages results in the phagocytosis, intracellular signaling and complex changes in gene activation and repression, which determine its intracellular fate.

Macrophages contain a set of receptors that can directly recognize pathogen-associated common structures, important in innate immune response. These receptors, known as pattern recognition receptors (PRRs), include mannose receptor, CD14, toll-like receptors, and scavenger receptors (SRs). Participation of scavenger receptor in pathogen recognition is not well known. MAcrophage Receptor with COllagenous structure (MARCO) is a distinct type-A SR with collagenous and Scavenger Receptor Cystein-Rich (SRCR) domains. This mouse molecule is able to bind Gram-positive and Gram-negative bacteria, but is normally expressed by only a subpopulation of macrophages – for example in spleen marginal zone and freshly harvested peritoneal populations (7). There is very little knowledge of the consequences of receptor ligation on SR biosynthesis and functions. One example is the rapid up-regulation of MARCO expression after LPS or microbial uptake.

Genome-wide expression profiling by DNAmicroarrays is a powerful technique for monitor changes in gene expression on a large scale in different organisms, tissues and temporal or spatial arrangements (8, 9). The goal of the present study was to use the DNAmicroarray to analyze the global changes in CBA mouse macrophages gene expression in response to *L. amazonensis* or *L. major* infection.

To improve understanding of the macrophage role on the protective and non protective immune responses against *Leishmania*, we undertook a detailed, comparative examination of the CBA/J mouse infected macrophage transcriptional program. This experimental approach allowed us to expand the themes that define the complexity of phagocytic interaction of *L. amazonensis* versus *L. major* with macrophages and its consequences for the immune response. These studies allowed us to identify already

known as well as novel genes such as MARCO SR which may be involved in *Leishmania* infection.

MATERIAL AND METHODS

Mice

Male and female CBA/J mice, 6-12 weeks old, were provided by the Central Animal Facility of IOC/FIOCRUZ and from the Animal Facility of CPqGM/FIOCRUZ. These animals were housed under specific pathogen-free conditions and fed on commercial ration and water *ad libitum*.

Parasites

Promastigotes in stationary phase used in this work were *L. amazonensis* (MHOM/Br88/Ba-125) and *L. major* (MHOM/RI/–/WR-173) provided by Dr. Aldina Barral, from the Laboratory of Immunoparasitology of CPqGM/FIOCRUZ. Fresh *L. amazonensis* or *L. major* promastigotes were derived from isolated amastigotes from lymph nodes (LN) of C57BL/6 resistant mice, resuspended in Novy-Nicolle-MacNeal blood agar and then transferred to total LIT plus 10 % Fetal Bovine Serum (Cultilab, Campinas, Brazil) for a maximum of six passages. For macrophage experiments, the promastigotes were expanded for 3-5 days until reaching the stationary phase, washed with saline, and then adjusted to the desired concentrations indicated in the results.

Macrophage culture

Thioglycolate-induced peritoneal exudate cells (PEC Macrophages) were prepared from the peritoneal cavity of CBA/J mice 3-4 days after intraperitoneal injection of 2.5 mL of 3 % thioglycollate medium (Sigma chemical, St. Louis, MO). PEC Mϕ were obtained by flushing the peritoneal cavity twice with 10 mL ice-cold saline plus heparin (20 UI/mL) and centrifuged at 300x*g* for 10 min. The cells were resuspended in DMEM supplemented with 25 mM HEPES (N-2-

Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.4, 2 mM glutamine, 100 UI/mL penicillin, 100 μg/mL streptomycin, 2.0 g/L sodium bicarbonate plus 10% fetal bovine serum (Gibco Laboratories) (complete medium). Macrophages (1 X 10^7) in complete medium were cultured in 6 well-plates at 37 °C in 5% CO_2/95% humidified air. After 4 h, non-adherent cells were washed three times with RPMI 1640 supplemented with 25 mM HEPES.

Macrophage infection

Macrophages were infected with *L. amazonensis* or *L. major* promastigotes in stationary phase, at ratio 10:1. After 3, 6 or 12 h non-internalized parasites were washed out. In some experiments, cells were washed to remove non-internalized parasites after 12 h of infection, at time zero (0), and reincubated for additional 24 and 72 h after infection.

RNA preparation

Total RNA from non-infected macrophages or infected with *L. major* or *L. amazonensis* was harvested using Qiagen RNeasy mini-prep columns (Qiagen, Valencia, CA). The integrity of each RNA preparation was checked on a 1.2% agarose gel electrophoresis.

The RNA was reverse transcribed (Superscript II, Invitrogen) by using oligo(dT) linked to a T7 RNA polymerase promoter sequence (Proligo, La Jolla, CA) to prime cDNA synthesis. After second-strand synthesis, biotinylated cRNA was produced by *in vitro* transcription using biotinylated UTP and CTP (Bioarray high-yield RNA transcript labeling kit, Enzo Diagnostics), and purified with RNeasy mini columns (Qiagen). The

biotinylated cRNA was heated at 94 °C 30 min in MgOAc/KOAc buffer to produce 35–200 base fragments.

Probe arrays hybridization and scanning

Briefly, 16 μg of fragmented labeled cRNA was hybridized to Murine Genome U74v2 Genechip® (Affymetrix, Santa Clara, CA), which contains nearly 400,000 probes covering approximately 12,000 different murine genes. Washing and staining with streptavidin-phycoerythrin was done using a GeneChip Fluidics station 400 (Affymetrix). Scanning was performed with an Affymetrix GeneArray. Microarray Analysis Software (Affymetrix) was used to scan and analyze the image from the scanned array.

Bioinformatics analysis

The images obtained were processed by the Affy package of Bioconductor software project (http://www.bioconductor.org). Briefly, the images were submitted to three steps, namely background correction, normalization aiming to reduce intersample bias and extraction of a numerical quantity directly related to the mRNA concentration in the original sample of each gene. These steps were done according to the RMA (robust multi-array analysis) method (10).

Differentially expressed genes in early (3, 6 and 12h) and late (24 and 72h) infection time were accessed by using the SAM (Significance Analysis of Microarrays) software (11). This software performs a statistical analysis of differential expression, with a method based in a modification of Student's *t* test, and allows strong control over multiple testing, a very important statistical issue in genomic analysis.

Clustering analysis of patterns of gene expression through both *L. amazonensis* and *L. major* infection were performed by the EXPANDER software (12), using the SOM (self-organizing maps) method (13). Clustering was performed to visualize patterns of differential expression that pass the stringent statistical criteria described above.

Flow Cytometry assay

Cells were cultured in 6-well plates (10^6 cells per well) in DMEM medium containing glutamine, penicillin/streptomycin, and 10% of FBS. Cells were infected by *L. amazonensis* or *L. major*, and untreated cells or those treated with IFN-γ and/or TNF-α were used as control. After stimulation for 24 h, cells were detached from the culture plates using a cell scraper, and MARCO expression was determined by flow cytometry using a hybridoma supernatant contaning a rat anti-mouse MARCO mAb. The secondary antibody was a phycoerythrin-conjugated anti-rat IgG, and its binding was detected using a FACScan flow cytometer (Becton Dickinson).

Immunohistochemistry

In order to study the induction of MARCO expression *in vivo* during *Leishmania* infection, immunohistochemistry was performed in 5-μm cryostat sections of infected and non-infected CBA mouse lymph nodes. Sections were fixed with acetone for 30 min, air dried, and endogenous peroxidase activity was quenched using sodium azide and hydrogen peroxide (H_2O_2) diluted in PBS by incubation for 30 min at 37°C. Avidin-biotin blocking agents were used (DAKO Corporation, USA). Sections were incubated with MARCO mAb, diluted 1:3 in PBS/BSA 1%. After 90 min incubation at room temperature, sections were washed twice with PBS and incubated with biotinylated rabbit anti-rat IgG (DAKO Corporation, USA) diluted in PBS/BSA 1% containing 5%

rabbit serum and 5% mouse serum, for 30 min at 37°C, washed twice with PBS and followed by incubation with streptavidin-peroxidase for 30 min. Peroxidase activity was demonstrated using the DAKO EnVision System™ HRP (DAB). Sections were counterstained with hematoxylin.

RESULTS

Transcriptional response of *L. amazonensis*- or *L. major*-infected macrophages

To determine in large scale which are the molecules involved in initiating and modulating distinct host response to infection with *L. amazonensis* or *L. major*, DNAmicrorray was used. mRNA expression profile of more than 12,000 mouse genes was characterized following *Leishmania* infection. We demonstrated that *L. amazonensis* and *L. major* infection caused significant changes in the expression of numerous specific genes related to immune-inflammatory response, apoptosis, cell signaling, cell metabolism, DNA/RNA processing and binding, vesicular trafficking and fusion and cytoskeleton. Several of these genes have not been previously implicated in the host responses to *L. amazonensis* and *L. major* infection, while other genes have been previously shown to be regulated by these infections.

We investigated the early events (3, 6 and 12h) and the late events (24 and 72h). Table I lists the up regulated and down regulated genes in the early events in response to *L. amazonensis* related to *L. major* infection. We have observed marked differences between *L. amazonensis* infection in the number of genes that were repressed in comparison to *L. major*. The majority of differences in gene suppression was represented in the early events. In contrast, many genes were induced in the late events. Genes related to regulatory factors of immune-inflammatory response, such as TNF receptor (TNFR), IFN-α/β receptor, chemokine ligand (CCL) 1, CD47 antigen, MHCII

were repressed in the *L. amazonensis* infection. On the other hand, in the late events (Table II) Interleukin (IL) 10 receptor, integrin beta 1, C-C chemokine receptor 5 (CCR5), SRs, TRAF, MAD genes were induced.

Genes related to respiratory burst such as catalase (early events) was induced, besides genes of antioxidant enzymes such as thioredoxin, glutathione-S-transferase and casein kinase (late events) were induced (Table I and II) in response to *L amazonensis* infection. Some genes related to intracellular signal transduction were expressed. Genes related to the family of the mitogen-activated protein kinases (MAPKs) were repressed in the early events, whereas there was induction of genes related to phosphatases suggesting a possible deactivation of macrophages. However, protein kinase C (PKC), an important enzyme known to be suppressed in response to *L. donovani*, was induced in the late events in response to *L. amazonensis*. Genes related to vesicular trafficking and fusion, such as proteins of Rab family, vesicle-associated membrane proteins (VAMPs), syntaxin and coat protein (COP) were induced in the late events of *L. amazonensis* infection. In addition, *L. amazonensis* also induced in late events beclin I gene, related to autophagic pathway (14).

Functional assignments of coordinately expressed gene profile

To investigate the relationships among expressed genes with respect to patterns of differential expression we selected two main clusters. Cluster I is represented by a group of genes related to innate immune response such as, phospholipase A2 enzyme that is involved in the Prostanglandin E_2 synthesis, Interferon-regulatory factor (IRF) 7, IL-1 receptor antagonist, macrophage SR 1, CD14, CCL5 which were induced in response to *L. major*. Of interest in cluster II some genes were also induced in response to *L. major*, TNFR, TRAF, leukotriene B4, cytocrome c oxidase and cytocrome b-5.

L. major infection induces *in vitro* and *in vivo* MARCO receptor expression

In DNAmicroarray analyses we observed up regulation of the macrophage SR 1 gene in response to *L. major* and SR class B in response to *L. amazonensis*. Since MARCO is known to be an important receptor involved in innate immune response and until now its role has not been investigated in response to *Leishmania* infection, we compared its expression on the surface of *L. amazonensis*- or *L. major*-infected macrophages. Figure 2 shows a 20% increase in surface of MARCO receptor in *L. major*-infected macrophages when compared to *L. amazonensis* infection.

To determine whether *in vivo* MARCO expression was also enhanced in response to *L. major*, we investigated MARCO distribution in draining lymph nodes from infected mice. Saline-injected mice were used as control. After 7 days, in lymph nodes from *L. amazonensis*-infected mice (Figure 3B) we observed weaker MARCO expression when compared to those from control mice (Figure 3A). In contrast in *L. major*-infected mice we observed a strong MARCO expression (Figure 3C,D). In both saline-injected and *L. major*-infected mice MARCO was predominantly distributed in lymph node lymphoid follicles.

DISCUSSION

To better understand the differences in CBA mouse macrophage responses to *L. amazonensis* or *L. major* infection, we performed DNAmicroarray analyses and determined macrophage transcriptional program change at different time points of infection. Our study showed that *L. amazonensis* and *L. major* induced and suppressed specific genes that can be determinant of CBA macrophage response to infection.

Previously, Matlashewsky and Buates (15) demonstrated that *L. donovani* induced a general macrophage deactivation. In agreement with our present work, it has been recently shown that macrophage response to *Leishmania* is more complex (plastic) and in response to *L. chagasi* there was a down-modulation of macrophage genes encoding Th1-type response, whereas up-regulation genes implicated in anti-inflammatory or Th2-type responses. We showed that phagocytosis of *Leishmania* did not induce an initial overall state of dormancy but instead depending on parasites species they can induce up or down regulation of genes involved in early and late events of infection.

Macrophages play an important role in clearance of apoptotic cells and microorganisms. Phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens. Several receptors have been implicated in promastigote binding to macrophages. The complement receptor apparently plays a major role in taking of different *Leishmania* species. It is known that the type of receptor involved on *Leishmania* phagocytosis, influence the parasite fate. Different microbe-recognition receptors induce different signaling pathways, and these signals interact cooperatively to mediate ultimate responses to particles. We found that there was a down regulation in response to *L. amazonensis* of genes encoding for surface receptors such as Fc receptor, already known to be involved in parasite recognition. In contrast, other receptors were up regulated in response to *L. amazonensis* but were not previously related to *Leishmania* infection such as CD14 and SR.

Of particular interest, we observed for the first time an involvement of SR in *Leishmania* infection. We showed *in vitro* as well as *in vivo* that in response to *L. major* infection there is an up regulation of MARCO SR, providing indirect evidence that MARCO plays a role in parasite binding or internalization. In mice, MARCO is

expressed constitutively in macrophage of the spleen and lymph nodes, but is up regulated in bacterial infections in macrophage of most tissues (16-18). Similarly, we observed a higher expression of MARCO in *L. major*-infected lymph nodes. This expression pattern suggests a role for MARCO in resistant response to *L. major*. The presence of MARCO may be related not only to the clearance of apoptotic cells, as other scavenger receptors, but it may also contribute to *L. major* recognition and killing. Indeed, recent studies have shown that MARCO expressed in alveolar macrophages plays a role not restricted to environmental particle binding, but also in antimicrobial host-defense (19).

Whereas it is clear that other SRs participate in the phagocytosis of microbes, it is likely that MARCO may contribute to *Leishmania* binding, while other co-receptors generate the internalization signals. Studies have demonstrated that the simultaneous binding by different phagocytic receptors might produce synergistic effects. Macrophage from mice lacking β2 integrin (and therefore CR3) respond normally to LPS with respect to induction of some genes such as TNF-α and IP-10, but are deficient in LPS-induced activation of genes such as COX-2, IL-12 p40 and IL-12 p35 (20). Thus, these two receptors cooperate in generating the ultimate inflammatory response and suggest that signaling by phagocytic receptors may influence or modify pro-inflammatory signaling through other receptors.

During receptor-mediated phagocytosis of the promastigote-stage of the parasite, macrophages elicit a respiratory burst (21, 22). Our data demonstrated that enzyme such as catalase which eliminates H_2O_2 and others antioxidant enzymes are induced in *L. amazonensis* infection. Consistent with this result, previously we showed that *L. amazonensis* inhibits the H_2O_2 production in CBA mouse macrophages (2). Thus it is possible that H_2O_2 inhibition will be related to up-regulation of antioxidant enzymes and

enzymatic mechanisms to detoxify reactive oxygen species, known to be important in host defense during the early stages of *Leishmania* infection (23, 24).

It has been demonstrated that, during the early stages of infection, activated macrophages release IFN-α/β that functions in an autocrine manner to stimulate the release of low levels of NO (25). These low levels of NO are insufficient for parasite killing but play a key role in modulating IL-12 activation of NK cells. Release of IFN-γ by NK cells then activates macrophages for induction of iNOS and release of large amounts of NO for killing of intracellular parasites (25). These findings suggested that low levels of iNOS-derived NO released by autocrine production of IFN-α/β during early stages of infection is a prerequisite for cytokine signaling and function in innate immunity. We observed the inhibition of IFN-α/β receptor expression in *L. amazonensis* infection. The inhibition of IFN-α/β receptor by *L. amazonensis* is one of the possible mechanisms used by *L. amazonensis* to avoid macrophage destruction. It is possible that in *L. major*-infected CBA macrophages with low levels of released NO in response to IFN-α/β production will interact with superoxide ions leading peroxinitrite production that is an important microbicidal molecule (26-28).

Cell function depends on multiple signaling pathways that control proliferation, differentiation or apoptosis (29). It is known that the activation of tyrosine phosphatases plays a major role in the deactivation of MAPKs in *Leishmania*-infected cells (30, 31). Impaired tyrosine phosphorilation and activation of MAPK could be potentially explained by the action of a tyrosine phosphatase. Indeed, incubation of cells prior to infection with sodium orthovanadate, a phosphatase inhibitor, restored MAPK activation as well as the expression of both *c-fos* and iNOS. These findings are consistent with a model in which infection induces the activation of cellular phosphotyrosine phopsphatases, leading to cell deactivation (32). In our data we found

that in response to *L. amazonensis* infection it was observed a down regulation of genes related to groups of protein kinases as well as an up regulation of phosphatase genes. Perhaps, in *L. amazonensis*-infected macrophages phosphatase induction is responsible to macrophage desactivation. Furthermore, it is possible that a *Leishmania*-derived phophatase with activity towards MAPK will also participate in macrophage deactivation.

Activation of gene transcription and in consequence cytokine and chemokine production during phagocytosis is a critical step in the development of an effective immune response. TNF-α has been shown to be involved in parasite killing in a process that depends on NO production, and endogenously produced TNF-α has been reported to be necessary for *in vitro* NO production by macrophages (33-36). Our data demonstrated that genes related to TNFR, TRAF are up-regulated in *L. major* and not in *L. amazonensis* infection, reinforcing the protective role of this cytokine. In previous publication we demonstrated that *L. major*-infected cells showed a higher TNF-α expression in comparison to *L. amazonensis* infection (2). Furthermore, TNF-α added to the culture enhanced both *L. amazonensis*- and *L. major*-killing by macrophages (2).

On the other hand, genes related with cytokine which function as macrophage deactivators, such as IL-10 receptor and MAD are induced in *L. amazonensis* infection. These data suggest that *L. amazonensis* induces macrophage deactivation by inhibition of inflammatory cytokines and induction of anti-inflammatory cytokine. IL-10 has been previously implicated in disease progression and long-term persistence of *Leishmania* in both human and experimental animal infection (37-40). It is also known to be synthesized by a variety of cells, including macrophage. IL-10 can inhibit the production of several cytokines, including TNF-α and IL-1β, as well as NO in macrophages (38, 41, 42). There is limited information about the role of IL-10 in *L.*

amazonensis infection (43, 44). Recently, was demonstrated that saliva enhanced infectivity of *L. amazonensis* parasite by stimulating IL-10 production in macrophages and T cells (45). In *Leishmania*-infected CBA macrophage, the early production or inhibition of TNF-α or IL-10 may influence parasite survival.

Results herein strongly suggest that factors related to parasite species drive differential transcriptional program in early infected macrophages that are related to different infection outcome. These data may contribute to understanding strategy of intracellular survival and its possible consequences in immune response to *Leishmania* that determine protection or susceptibility in CBA mice.

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LEGENDS

Table I - Differentially expressed genes in early events (3, 6 and 12h) of infection were accessed by using the SAM software (11). Listed genes were selected based on statistical analysis of differential expression in *L. amazonensis*- related *L. major*-infected cells, with a method based in a modification of Student's *t* test.

Table II - Differentially expressed genes in late events (24 and 72h) of infection were accessed by using the SAM software (11). Listed genes were selected based on statistical analysis of differential expression in *L. amazonensis*- related *L. major*-infected cells, with a method based in a modification of Student's *t* test.

Figure 1 - SOM clustering of genes regulated in *L amazonensis*- or *L. major*-infected macrophages. Clustering analyses of gene expression through both *L. amazonensis* and *L. major* infection were performed to visualize patterns of differential expression which were not selected by the stringent statistical criteria previously described. These two selected clusters contain genes with increased expression in *L. major* infection. The yellow and blue cells identifies the up and down regulation of gene expression in the infection process, according to the scale in the upper left area, which represents the $\log_2$ transformation of the ratio between infected and control mRNA hybridization level.

Figure 2 - *L. major* infection *in vitro*, induce higher MARCO receptor expression in comparison to *L. amazonensis* infection. MARCO expression was determined in PEC macrophages infected with stationary phase promastigotes at a ratio 10:1. After twenty four hours of infection these cells were stained with monoclonal antibody anti-MARCO and its binding was detected by flow cytometry. Each symbol represents the percentage of MARCO expression in *L. amazonensis*- or *L. major*-infected cells related to expression in control uninfected macrophages and the horizontal bars the mean expression of five experiments. MARCO expression in control uninfected macrophages was considered 100% (13.70±4.62; n=5). Difference indicated in the figure was considered significant when $p<0.05$ (Student's *t* test).

Figure 3 – *In vivo* induction of MARCO expression by *L. major*. After 7 days of *L. major* or *L amazonensis* infection lymph nodes were collected and analyzed by immunohystochemstry with antibody against MARCO. Saline injected CBA mice were used as negative control infected mice. Strong expression of MARCO was found in lymphoid follicles from *L. major*-infected mice (arrows). Lymph nodes from control non-infected mice (A); *L. amazonensis*-infected mice (B); *L. major*-infected mice (C,D).

TABLE I – Early expression of genes in *L. amazonensis*- or *L. major*-infected MΦ

Genes/Fuction	*L. amazonensis* vs. *L. major*	
	Induced	**Repressed**
Imunne inflammatory response	lymphocyte antigen 6 complex; locus E platelet factor 4 zinc finger protein 36; C3H type-like 2	apolipoprotein E cysteine rich intestinal protein glucose phosphate isomerase 1 tumor necrosis factor receptor superfamily histocompatibility 2; T region locus 10 SAM domain and HD domain; 1 tumor rejection antigen gp96 serum amyloid A 3 Fc receptor; IgG; low affinity IIb CD47 antigen arginase 1; liver histocompatibility 2; T region locus 17 interferon (alpha and beta) receptor 2 chemokine (C-X-C motif) ligand 1 ninjurin 1 basigin CD52 antigen metallothionein 1
Apoptosis		programmed cell death 5 Bcl2-associated athanogene 3
Signal transduction	G protein-coupled receptor 44 activin receptor IIB hypothetical protein MNCb-4137 adrenergic receptor; alpha 2a	N-myc downstream regulated Protein kinase inhib. mitogen activated protein kinase kinase kinase 1 tumor protein D52 pleckstrin homology; Sec7 and coiled/coil domains MAP/microtubule affinity-regulating kinase 2 inositol 1;4;5-triphosphate receptor 5 mitogen-activated protein kinase kinase kinase kinase 4 annexin A2 protein tyrosine phosphatase; non-receptor type 1 RAS-like; family 2; locus 9 RIKEN cDNA 2900073G15 gene RIKEN cDNA 1110038P24 gene transmembrane 4 superfamily member 7 RIKEN cDNA 1810033K10 gene phospholipase A2; group VII (PAF acetylhydrolase) SH3 domain protein 3 split hand/foot deleted gene 1 RIKEN cDNA 1110008B24 gene RNA binding motif protein RNA binding protein regulatory subunit
Protein synthesis folding and degrad.	seven in absentia 1B	chaperonin subunit 5 (epsilon) proteasome (prosome; macropain) subunit; alpha type 7 ubiquitin-like 5 proteasome (prosome; macropain) subunit; beta type 3 ubiquitin-activating enzyme E1; Chr X ubiquitin-like 5 proteasome (prosome; macrop.) subunit; beta type 1 proteasome (prosome; macrop.) 26S subunit; ATPase 2 proteasome (prosome; macrop.) subunit; beta type 4 proteasome (prosome; macrop.) 26S subunit; non-ATPase; 8 proteasome (prosome; macrop.) subunit; beta type 5 RIKEN cDNA 1600023E10 gene RIKEN cDNA 2300001E01 gene hypothetical protein MGC6696 praja1; RING-H2 motif containing
Metabolism	catalase prolyl 4-hydroxylase; beta polypeptide hemoglobin alpha; adult chain 1 malate dehydrogenase; soluble glutamate oxaloacetate transaminase 2; mitoc. glutamate-ammonia ligase (glutamine synthase) loricrin transcription factor AP-2; gamma apolipoprotein B editing complex 2	CDP-diacylglycerol synthase 2 RIKEN cDNA 9530090G24 gene ATP citrate lyase aminolevulinic acid synthase 1 ornithine decarboxylase antizyme adenylosuccinate synthetase 1; muscle triosephosphate isomerase ATPase inhibitor hexokinase 2 solute carrier family 2 (facilitated glucose transporter) chloride intracellular channel 1 solute carrier family 11 (proton-coupled dival. metal ion transp ATPase; Cu++ transporting; alpha polypeptide hypoxanthine guanine phosphoribosyl transferase ribophorin II
DNAbinding/ RNAprocessing	bromodomain containing 3 Wilms tumor homolog NK6 transcription factor related even skipped homeotic gene 2 homolog ribonucleic acid binding protein S1	small nuclear ribonucleoprotein D2 lymphoblastomic leukemia RIKEN cDNA 0910001B06 gene matrin 3 origin recognition complex; subunit 3-like (S. cerevisiae) small nuclear ribonucleoprotein polypeptide G X-box binding protein 1 RIKEN cDNA 0610009D07 gene U6 snRNA-associated SM-like protein 4 SWI/SNF related; matrix assoc.; actin dep. regulator of chrom cleavage stimulation factor; 3' pre-RNA subunit 2; tau splicing factor 3b; subunit 1; 155 kDa RIKEN cDNA 6230400O18 gene DNA methyltransferase 3A nuclear fragile X mental retardation protein interacting protein RIKEN cDNA 0610040H15 gene RIKEN cDNA 2310003F16 gene similar to high-glucose-regulated protein 8
		schlafen 2 integral membrane protein 2C RIKEN cDNA 1810010L20 gene S100 calcium binding protein A6 (calcyclin) RAN; member RAS oncogene family schlafen 2 S100 calcium binding protein A1 S100 calcium binding protein A10 (calpactin) cyclin-dependent kinase inhibitor 1C (P57) absent in melanoma 1 arginine-rich; mutated in early stage tumors hypothetical protein MGC30562 upregulated during skeletal muscle growth 5
Biogenesis vacuolar/ Vesicular traffick	coated vesicle membrane protein	
Cytoskeleton	tubulin; alpha 3 RIKEN cDNA 2410002J21 gene	

TABLE II – Late expression of genes in *L. amazonensis*- or *L. major*- infected MΦ

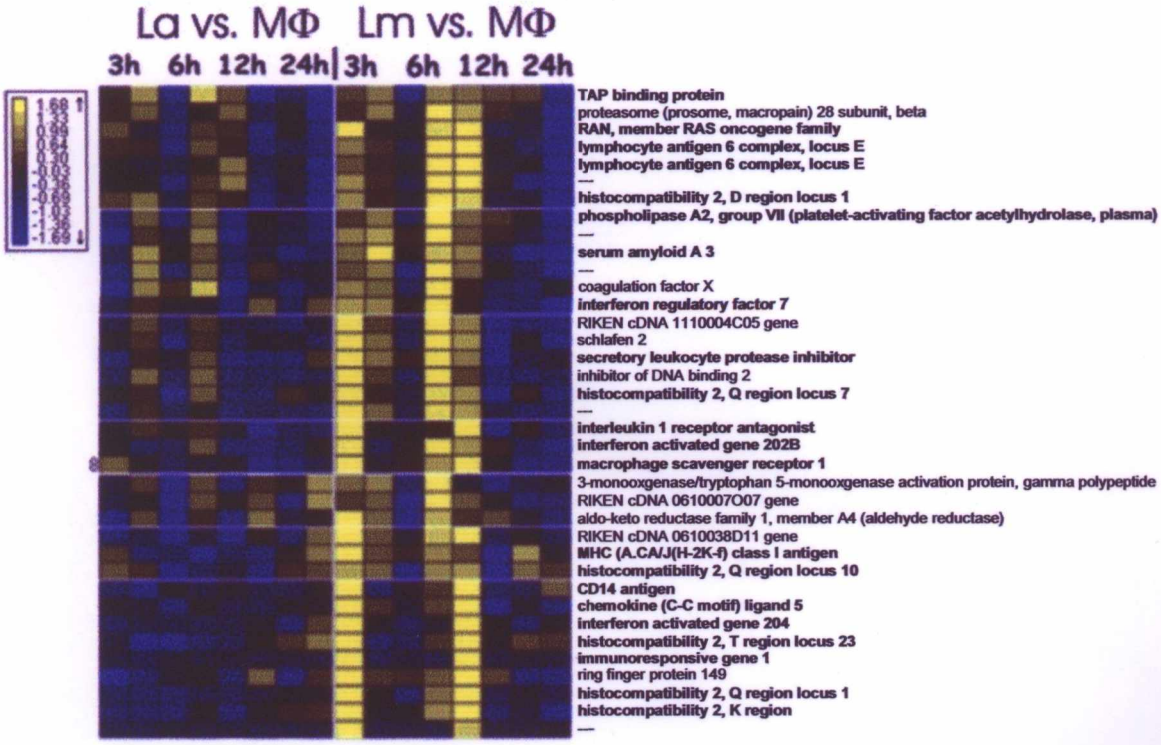
Genes/Fuction	*L. amazonensis* vs. *L. major*
	Induced
Imunne inflammatory response	interleukin 10 receptor; beta
	integrin beta 1 (fibronectin receptor beta)
	chemokine (C-C motif) receptor 5
	B-cell linker
	histocompatibility 2; D region locus 1
	scavenger receptor class B; member 2
Apoptosis	fibroblast growth factor inducible 14
	SH3-domain GRB2-like B1 (endophilin)
	integral membrane protein 2B
Signal transduction	striatin; calmodulin binding protein 3
	TRAF family member-associated Nf-kappa B activ.
	protein phosphatase 1;
	MAD homolog 2 (Drosophila)
	protein kinase C; delta
	mitogen activated protein kinase 14
	similar to Ras GTPase-activating protein 1; (RasGAP)
	guanine nucleotide binding protein (G protein)
	RAS-like; family 2; locus 9
	casein kinase 1; alpha 1
	inosine 5'-phosphate dehydrogenase 2
	inosine 5'-phosphate dehydrogenase 2
	reticulocalbin 2
	protein tyrosine phosphatase; receptor type; E
	protein tyrosine phosphatase 4a2
	annexin A1
	RIKEN cDNA 2810425K19 gene
	RAN binding protein 1
	RIKEN cDNA E430034L04 gene
	tumor protein D52
	postsynaptic protein Cript
	tumor differentially expressed 1
	tyrosine 3-monooxygenase; eta polypeptide
	platelet-activating factor acetylhydrolase
Protein synthesis folding and degrad.	ribosomal protein L30
	ribosomal protein L18
	ribosomal protein L10A
	RIKEN cDNA 2610011N19 gene
	RIKEN cDNA 2700079K05 gene
	ribosomal protein L8
	heat shock protein 1 (chaperonin 10)
	heat shock protein 1 (chaperonin)
	t-complex protein 1
	DnaJ (Hsp40) homolog
	chaperonin subunit 4 (delta)
	chaperonin subunit 8 (theta)
	proteasome (prosome; macropain)
	RIKEN cDNA 2700059C12 gene
	ring finger protein 11
	ubiquitin specific protease 14
	proteasome (prosome; macropain) subunit
	ubiquitin-like 3
	praja1; RING-H2 motif containing
	SMT3 (supressor of mif two; 3)
	Nedd4 family interacting protein 1
	RIKEN cDNA 2300001E01 gene
	RIKEN cDNA 2400006A19 gene
Metabolism	isocitrate dehydrogenase 1 (NADP+); soluble
	alcohol dehydrogenase 5 (class III); chi polypeptide
	thioredoxin domain containing
	dihydrolipoamide dehydrogenase
	pyruvate dehydrogenase (lipoamide) beta
	RIKEN cDNA 0610038L10 gene
	hypoxia inducible factor 1; alpha subunit
	NADPH cytochrome B5 oxidoreductase
	transaldolase 1
	inosine 5'-phosphate dehydrogenase 2
	translocase of inner mitochondrial membrane
	malate dehydrogenase; mitochondrial
	voltage-dependent anion channel 2
	syntaxin binding protein 3
	hippocampus abundant gene transcript 1
	mitochondrial carrier homolog 2
DNAbinding/ RNAprocessing	transcription elongation factor A (SII) 1
	transcription elongation factor B (SIII)
	eukaryotic translation initiation factor 3
	similar to Transcription factor BTF3
	retinoblastoma binding protein 7
	trans-acting transcription factor 3
	transcription factor 12
	RIKEN cDNA 1500010B24 gene
	von Hippel-Lindau binding protein 1
	p300/CBP-associated factor
	prothymosin alpha
	kidney cell line derived transcript 1
	upregulated during skeletal muscle growth 5
	core binding factor beta
	heterogeneous nuclear ribonucleoprotein K
	heterogeneous nuclear ribonucleoprotein D-like
	small nuclear ribonucleoprotein E
	H3 histone; family 3A
	high mobility group nucleosomal binding domain 1
	RNA binding motif protein
	zinc finger RNA binding protein
	metallothionein-I activator
	nucleophosmin 1
	polymerase (RNA) II (DNA directed)
	chromodomain helicase DNA binding protein
	heterogeneous nuclear ribonucleoprotein H1
	H2A histone family; member Z
	nuclease sensitive element binding protein 1
	zinc finger protein 207
	S-adenosylhomocysteine hydrolase
	step II splicing factor SLU7
	splicing factor; arginine/serine-rich 2 (SC-35)
	H2A histone family; member X
	budding uninhibited by benzimidazoles 3 homolog
	hypothetical protein MGC37309
	RIKEN cDNA 1810080D16 gene
	valosin containing protein
	matrin 3
	quaking
	RIKEN cDNA 1810003N24 gene
	embryonic ectoderm development

TABLE II – Late expression of genes in *L. amazonensis*- or *L. major*- infected MΦ-continued

Genes/Fuction	*L. amazonensis* vs. *L. major*
	Induced
Biogenesis vacuolar/ Vesicular traffick	RIKEN cDNA 2610002D06 gene
	RIKEN cDNA 1700025B18 gene
	syntaxin 12
	beclin 1
	ATPase; H+ transporting; lysosomal interacting protein 1
	RAB18; member RAS oncogene family
	vacuolar protein sorting 29 (S. pombe)
	RAB12; member RAS oncogene family
	coatomer protein complex
	vacuolar protein sorting 35
	COP9 (constitutive photomorphogenic) homolog
	golgi reassembly stacking protein 2
	vesicle-associated membrane protein 8
	vacuolar protein sorting 4b (yeast)
	gamma-glutamyl hydrolase
	lysophospholipase 1
	RAB2; member RAS oncogene family
	vesicle-associated membrane protein 4
	vacuolar protein sorting 35
	Down syndrome critical region gene 3
	beta-glucuronidase
	RIKEN cDNA 5730403E06 gene
	golgi phosphoprotein 3
	RIKEN cDNA 5830493P14 gene
	EGF-like module containing;hormone receptor-like sequence 1
	HIV-1 Rev binding protein
Cytoskeleton	cysteine rich protein 1
	dynein; cytoplasmic; intermediate chain 2
	microtubule-associated protein
	ARP10 actin-related protein 10 homolog
Hormone	nuclear receptor subfamily 1
	RIKEN cDNA 2010200I23 gene

Figure 1

Cluster I



Cluster II

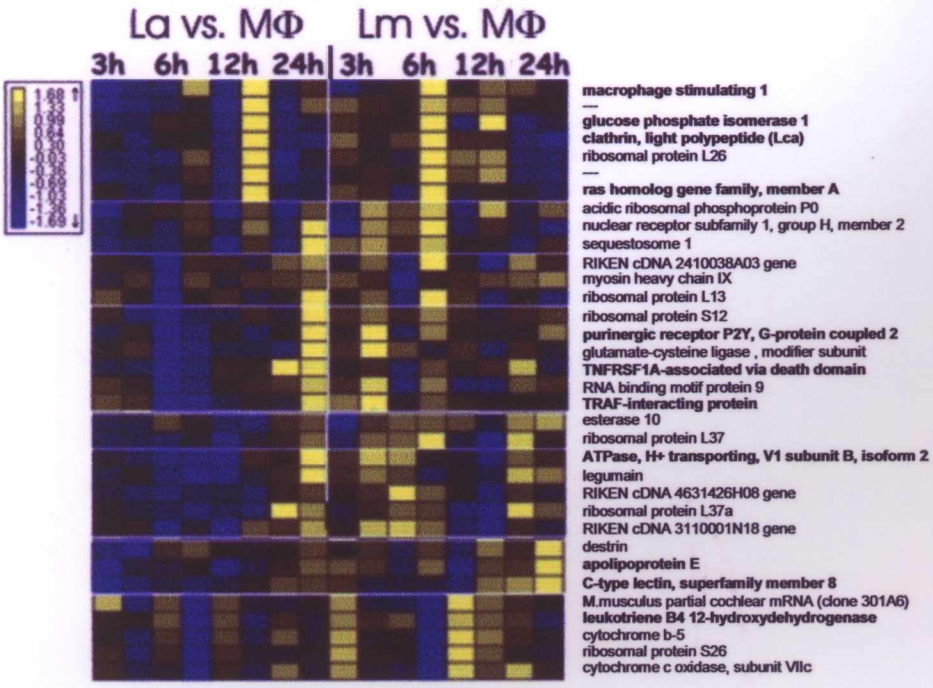


Figure 2

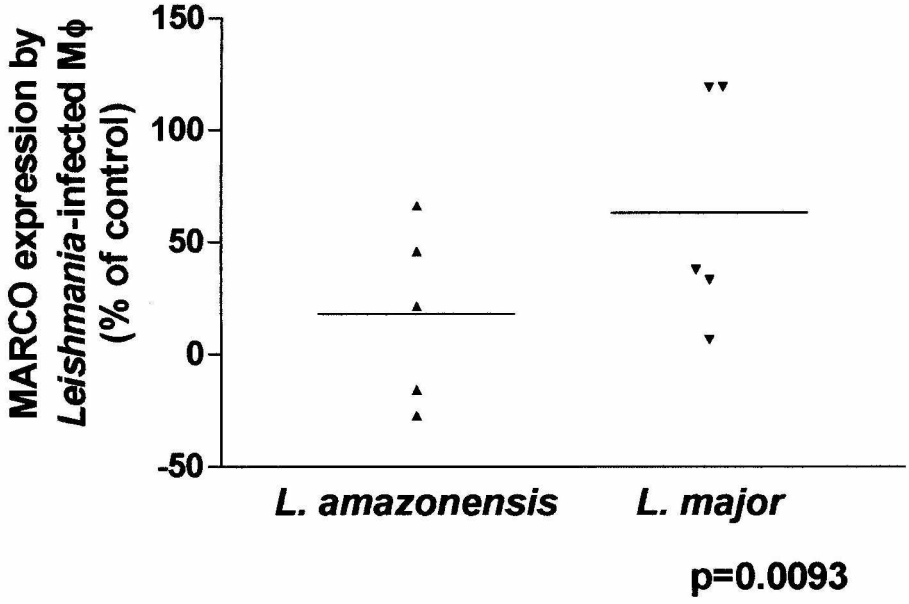
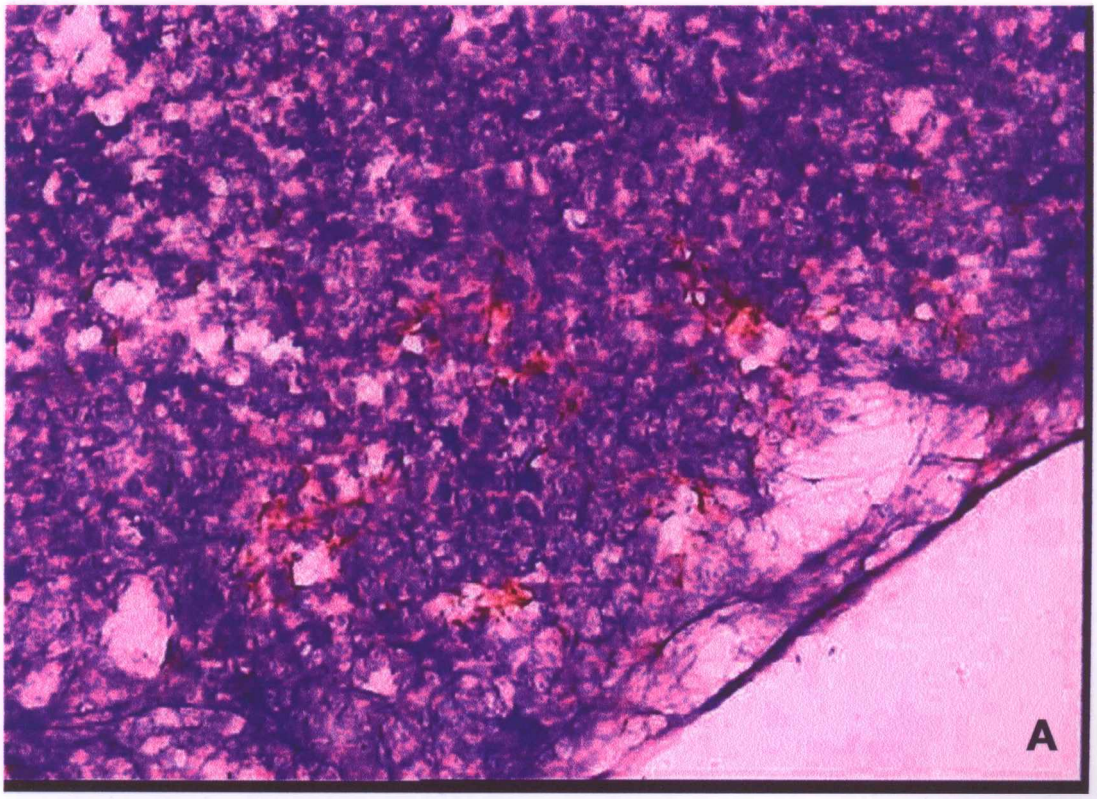

MARCO expression by
Leishmania-infected Mϕ
(% of control)
150
100
50
0
-50
L. amazonensis
L. major
p=0.0093

Figure 3



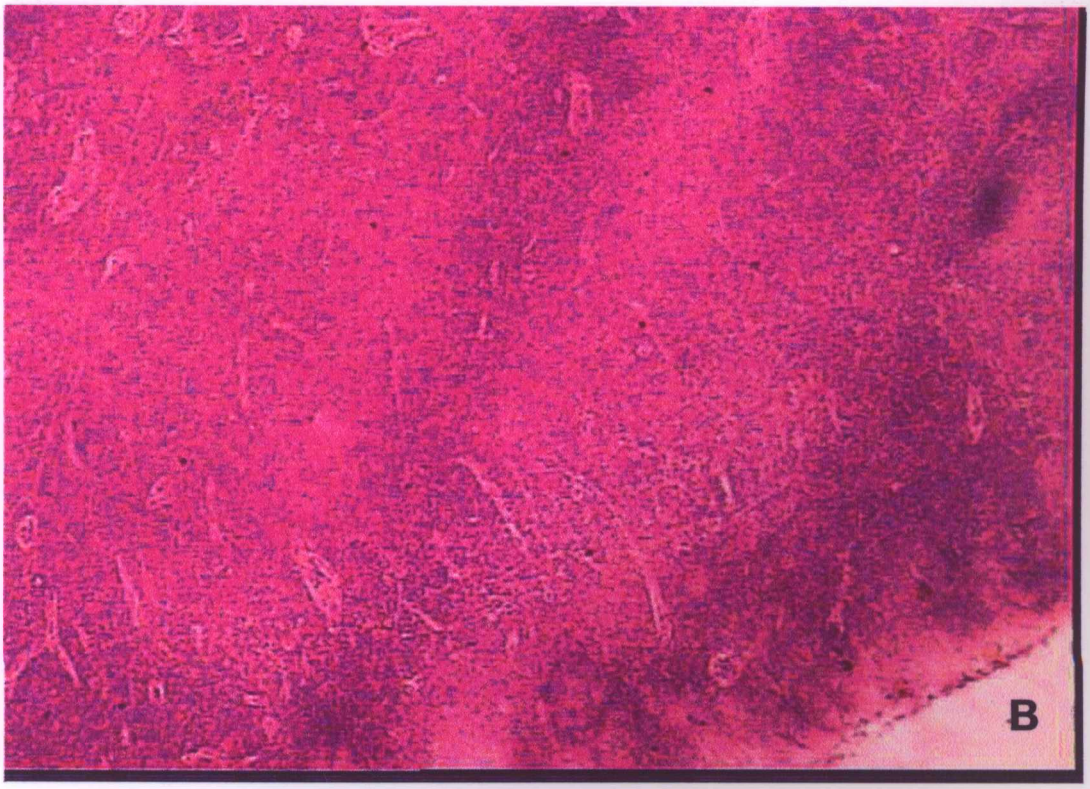
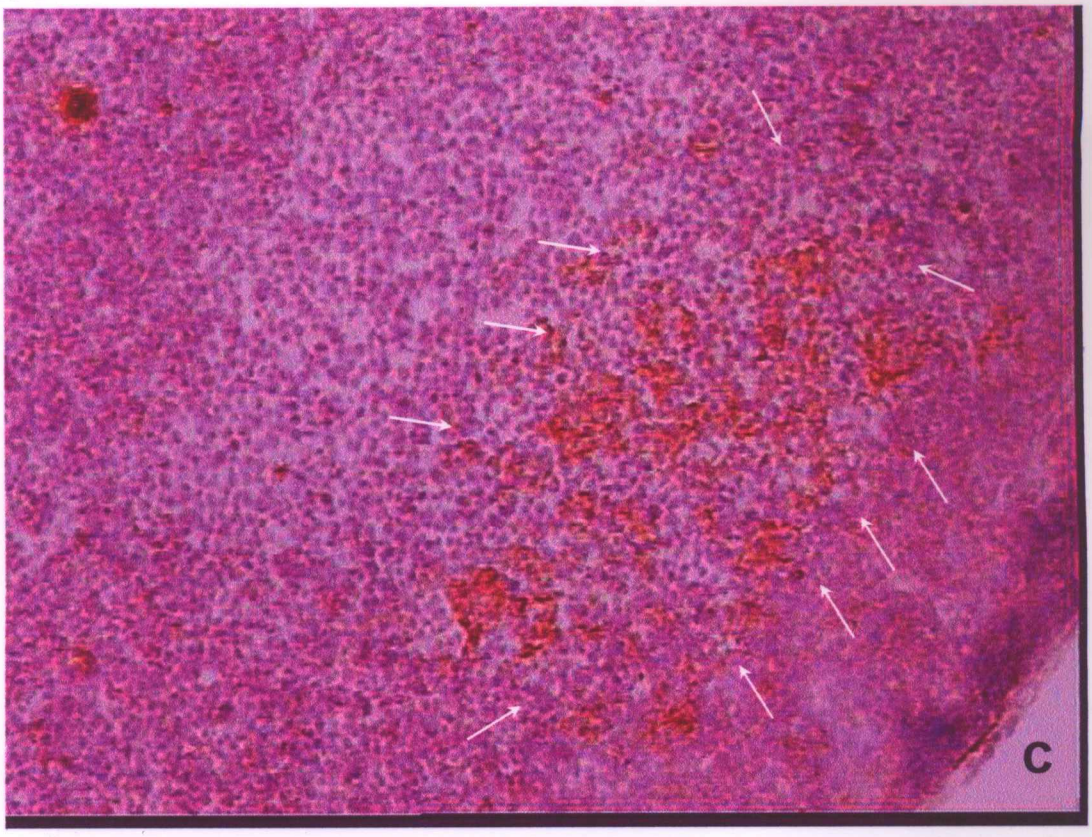
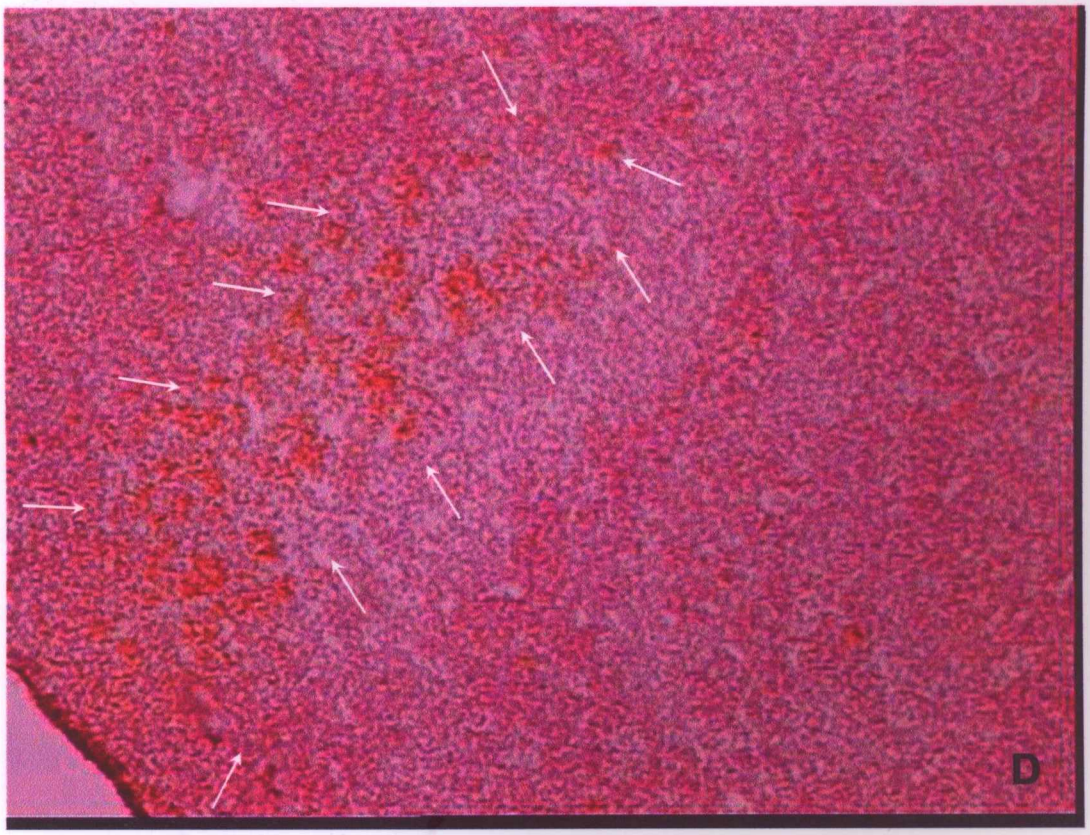


Figure 3





7. CAPÍTULO V – DISCUSSÃO GERAL

Para investigar os mecanismos envolvidos na determinação dos perfis de resistência ou susceptibilidade à infecção por Leishmania em camundongos CBA, utilizamos macrófagos peritoneais inflamatórios infectados *in vitro* por *L. amazonensis* ou *L. major*. Na primeira etapa do trabalho, demonstramos que não existem diferenças no percentual de infecção e na carga parasitária, até 6h após a adição de *L. amazonensis* ou *L. major*. Entretanto, após 12h, observou-se que a infecção por *L. amazonensis* foi duas vezes maior que por *L. major*, evidenciando que essas células, mesmo sem estimulação prévia, apresentam capacidade de destruir parasitos. Os dados confirmam observações anteriores de que há seletividade nos eventos envolvidos na resposta imune inata e sugerem que os macrófagos exercem papel central na determinação do curso da infecção.

Sabe-se que os eventos da resposta imune inata são determinantes da resposta imune adquirida e que macrófagos são células importantes na defesa contra Leishmania, pois são células envolvidas tanto no início da infecção, fagocitando parasitos (resposta inata), como são células efetoras na destruição de Leishmania, por mecanismos dependentes de NO (resposta adquirida) (Alexander *et al.*, 1992). Assim, estudar os mecanismos inatos envolvidos na resposta a Leishmania é de importância crucial para o entendimento da patogênese da doença. Portanto, a proposta desse trabalho foi avaliar o papel de macrófagos de camundongos CBA na determinação da resposta imune contra Leishmania, investigando quais os mecanismos moleculares e celulares envolvidos na interação de *L. amazonensis* e *L. major* com essas células e as possíveis conseqüências dessa interação para o desenvolvimento da resposta imune.

A infecção de camundongos CBA por *L. amazonensis* ou *L. major* constitui um modelo interessante no estudo da leishmaniose, pois, permite a investigação de

fenômenos de susceptibilidade e resistência em animais com o mesmo perfil genético. Desta forma, a avaliação das alterações no programa transcricional de macrófagos infectados nesse modelo é potencialmente mais elucidativo que o uso de células de camundongos de diferentes *backgrounds* genéticos explorado na maioria dos trabalhos sobre resistência e susceptibilidade.

Os dados obtidos nos ensaios de DNA*microarray* evidenciam que conjuntos de genes específicos são inibidos ou ativados a depender da espécie de Leishmania envolvida. A tecnologia de DNA*microarray* nos permitiu a avaliação da cinética da expressão gênica simultânea em larga escala de macrófagos infectados. De fato, observamos que essa técnica é uma ferramenta importante para análise do estado de ativação ou desativação de macrófagos infectados, o que permite formular inúmeras hipóteses e abre perspectivas para diferentes estudos. Além disso, permite-nos entender os mecanismos de interação entre macrófago e Leishmania, o que possibilita a investigação do papel do parasito em regular a expressão de genes e modular a resposta da célula hospedeira contra esses patógenos.

Os dados indicam que as diferenças observadas na infecção de macrófagos provavelmente estejam associadas ao reconhecimento dos parasitos. Sabe-se que receptores fagocíticos podem induzir resposta inflamatória além de influenciarem o destino intracelular do parasito. Foi evidenciado que receptores para fatores do complemento são os principais receptores envolvidos na fagocitose de promastigotas de Leishmania. Em nosso estudo, foi observado um aumento na expressão gênica de receptores *scavengers* (Capítulo VI), cujo papel não foi ainda estabelecido na infecção por Leishmania. Além disso, observamos aumento da expressão do SR MARCO, tanto *in vitro,* como *in vivo* na infecção por *L. major* (Capítulo VI). Esses dados sugerem que,

além dos receptores do complemento, outros receptores podem estar envolvidos no reconhecimento de Leishmania e na ativação da célula hospedeira.

Os SRs têm papel importante na resposta imune inata contra outros patógenos. Alguns estudos demonstraram a participação de MARCO no reconhecimento de bactérias (Brannstrom *et al.*, 2002; Elomaa *et al.*, 1995; Elomaa *et al.*, 1998). Outros dados evidenciaram a participação de SR na indução de processo inflamatório e na ativação de sinais intracelulares (Matsuno *et al.*, 1997; Schackelford *et al.*, 1995; Yang *et al.*, 1996). Os nossos dados mostraram que a resposta inicial à infecção por *L. major* parece induzir forte explosão respiratória com aumento dos níveis de H_2O_2 (Gomes *et al.*, 2003), sugerindo que, além do receptor para o complemento, outros receptores estejam envolvidos no reconhecimento desse parasito, uma vez que a fagocitose via receptores do complemento não induz explosão respiratória (Mosser *et al.*, 1997). Por outro lado, na infecção por *L. amazonensis,* há inibição da produção de H_2O_2 e aumento da expressão de enzimas antioxidantes. É possível que, no contexto da infecção por *L. major,* a ligação com o receptor MARCO esteja relacionada à indução de sinais que ativam macrófagos, como a indução de H_2O_2. O papel preciso de MARCO na resposta imune inata durante a infecção por Leishmania precisa ser mais bem estabelecido.

Alguns receptores de macrófagos estão envolvidos no reconhecimento de ligantes na superfície de promastigotas. Em alguns casos, esses receptores atuam cooperativamente, promovendo a fagocitose do microorganismo. Foi demonstrada a cooperação entre CR3 e receptor manose-fucose na ligação e ingestão de *L. donovani* (Blackwell, 1996; Wilson *et al.*, 1988). Além disso, LPG atua como ligante do complemento e também de receptores como manose fucose (Chakraborty *et al.*, 1998). É possível que ligantes distintos na superfície de *L. amazonensis* e *L. major* sejam reconhecidos por receptores distintos e promovam, em cooperação, a ativação de sinais

intracelulares, influenciando a destruição de *L. major* e favorecendo a sobrevivência de *L. amazonensis*.

Nós observamos um aumento da expressão de MAPKs na infecção por *L. major*, sugerindo que esse parasito induz ativação celular. Cinases são importantes ativadoras da explosão respiratória, incluindo isoformas de PKC (el Benna *et al.*, 1994; El Benna *et al.*, 1996; El Benna *et al.*, 1996; el-Benna *et al.*, 1995; Nixon *et al.*, 1999), PKA (Chanock *et al.*, 1994; De Leo *et al.*, 1996; El Benna *et al.*, 1996) e MAPK (Chanock *et al.*, 1994; El Benna *et al.*, 1996). Estudos que utilizaram inibidores farmacológicos de MAPKs evidenciaram o papel modulador dessas cinases na explosão respiratória (El Benna *et al.*, 1996; Rose *et al.*, 1997; Zu *et al.*, 1996; Zu *et al.*, 1998). p47phox citosólico é diretamente fosforilada pela p38, ERK e caseína cinase II (El Benna *et al.*, 1996; Park *et al.*, 2001). A fosforilação por caseína cinase II de p47phox parece mediar a desativação dessa oxidase (Park *et al.*, 2001).

A inibição de H_2O_2 e a indução de genes que codificam enzimas fosfatases, observadas na infecção por *L amazonensis*, evidenciam que esse parasito induz desativação de macrófagos. Outros estudos reforçam esses dados, evidenciando que Leishmania desativa macrófagos. Assim, foi demonstrado que fatores de virulência de *L. donovani* podem levar à desativação de macrófagos por meio da ligação do fator de elongação-1alpha de Leishmania com o domínio homólogo a Src 2 contido na proteína tirosina fosfatase 1 (SHP1) (Nandan *et al.*, 2002). A ativação e translocação de SHP1 torna a célula refratária à ativação de sinais. A ativação de MAPK medeia uma variedade de eventos intracelulares, como a ativação do gene *c-fos*, que é dependente da translocação de componentes de MAPK, ERK1 e ERK2. Proteínas fosfatases podem inativar ERK por desfosforilação de um simples resíduo. É possível que as amastigotas de Leishmania atenuem a fosforilação de tirosina de ERK1 por inibição MAPK

(Nandan *et al.*, 1999). Finalmente, baixas concentrações de NO podem facilitar a produção de superóxido por ativar MAPK (Lee *et al.*, 2000).

A explosão respiratória não tem papel aparente na produção de NO (Iyengar *et al.*, 1987), entretanto a reação do NO com superóxido forma peroxinitrito, importante molécula microbicida (Augusto *et al.*, 1996; Denicola *et al.*, 1993; Zhu *et al.*, 1992). Em camundongos resistentes foi evidenciado que o mecanismo leishmanicida de peroxinitrito e radicais derivados pode estar associado a nitração/oxidação da membrana do parasito (Linares *et al.*, 2001). Os dados evidenciam que *L amazonensis* é um microorganismo altamente resistente aos mecanismos de destruição dos macrófagos de CBA, pois, mesmo em macrófagos ativados por IFN-γ, esses parasitos resistem ao efeito microbicida do NO (Gomes *et al.*, 2003). Além disso, observamos que os VPs induzidos por *L. amazonensis* não retardam a fusão com lisossomas (Capítulo III), reforçando que essa espécie, mesmo na sua forma promastigota, resiste a mecanismos inatos, como o ambiente fagolisossomal.

Embora todas as espécies de Leishmania sobrevivam em compartimentos lisossomais, apenas membros do complexo Mexicana (*L. mexicana* e *L. amazonensis*) induzem grandes vacúolos em suas células hospedeiras. Alguns dados sugerem que a indução de grandes vacúolos está relacionada à liberação de proteofosglicana do parasito (Ilg *et al.*, 1995). Outros estudos mostraram que *L. mexicana* pode adquirir vacúolos autofágicos (Schaible *et al.*, 1999). No presente estudo, observamos que, na infecção por *L amazonensis*, há aumento da expressão de beclina, uma proteína essencial no processo autofágico (Yue *et al.*, 2003). É possível que VPs induzidos por *L. amazonensis* fusionem também com vacúolos autofágicos além de fusionarem com vesículas da via endo/fagocítica (Veras *et al.*, 1994; Veras *et al.*, 1992; Veras *et al.*,

1995). Estudos em andamento em nosso laboratório têm como objetivo determinar a relação entre os VPs induzidos por *L. amazonensis* e a via autofágica.

Embora tenha sido observado que VPs induzidos por *L. major* e *L. amazonensis* apresentem a mesma cinética de fusão com lisossomos, é possível que eles se fundam de forma distinta a outras vesículas intracelulares ou apresentem retardo na recirculação de membranas (Capítulo III). Nos estudos de DNA*microarray*, observamos que a infecção por *L. amazonensis* é acompanhada de um aumento na expressão de genes que codificam proteínas participantes no tráfego e na fusão de vesículas, tais como Rabs e VAMPs (Capítulo IV). Uma variedade de proteínas Rab e (*soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor*) SNAREs foi identificada em fagossomas, incluindo Rab4, Rab5, Rab7 e Rab11 (Cox *et al.*, 2000; Desjardins *et al.*, 1994; Mosleh *et al.*, 1998), synaptobrevinas I e II e (*N*-ethylmaleimide sensitive factor) NSF (Desjardins *et al.*, 1997), bem como sintaxinas 2, 3 e 4 (Hackan et al, 1996). Todas essas proteínas estão também presentes em endossomas, sugerindo que os mecanismos que dirigem a interação entre membranas ao longo da via endocítica estão relacionadas com o tráfego na via fagocítica. Griffts e colaboradores (Jahraus *et al.*, 1998) demonstraram o envolvimento de pequenas GTPases e moléculas SNARE na fusão entre endossomas e fagossomas. A identificação da expressão gênica de proteínas envolvidas no tráfego e na fusão de vesículas durante a infecção por *L. amazonensis* permite-nos especular sobre a participação dessas proteínas na formação dos grandes VPs associados a esses parasitos. Estudos devem ser feitos para avaliar o papel dessas proteínas, cuja expressão gênica foi detectada no nosso modelo.

A modulação da expressão de citocinas, quimiocinas e seus respectivos receptores pode ter importantes efeitos reguladores sobre a resposta imuno-inflamatória, por afetar a polaridade de células T e a reatividade de macrófagos a sinais inflamatórios.

A expressão de TNF- α por células infectadas com *L. major* e o aumento da destruição desse parasito, após a adição dessa citocina, reforçam o seu papel protetor. Além disso, observamos, nos estudos de *microarray,* um aumento na expressão do gene do TNFR, em células infectadas por *L. major*. É possível que, na infecção por *L. major,* o incremento na produção de TNF- α (Gomes *et al.*, 2003) e TNFR (Capítulo IV) esteja relacionado com a ativação da explosão respiratória detectada nos estudos de produção de H_2O_2 (Gomes *et al.*, 2003). Dados evidenciam que TNF- α estimula a explosão respiratória por induzir o aumento da transcrição de p47phox e p67phox em macrófagos (Green *et al.*, 1994). A produção de ROI também estimula o incremento da produção de TNF- α por macrófagos.

IL-10 e TGF-β são citocinas produzidas após a infecção por Leishmania. Essas citocinas promovem a replicação do parasito e são importantes fatores na determinação da susceptibilidade (Barral *et al.*, 1993; Kane *et al.*, 2001). No nosso modelo, a expressão de TGF-β, IL-10 (Gomes *et al.*, 2003) e receptor para IL-10 (Capítulo IV) em macrófagos infectados por *L. amazonensis* evidencia, mais uma vez, a desativação de macrófagos, induzida por esse parasito, e reforça o papel dessas citocinas na determinação da susceptibilidade à infecção por Leishmania.

Quimiocinas são importantes reguladores do recrutamento apropriado de células efetoras para os sítios de inflamação. Nossos dados mostraram que há um aumento da expressão de CCR5 em células infectadas por *L. amazonensis*. É conhecido que ligantes de CCR5 estão envolvidos no desenvolvimento de célula Th1 (Bonecchi *et al.*, 1998). Entretanto, foi demonstrado que camundongos deficientes em CCR5 e MIP-1α, são capazes de controlar a replicação de *L. donovani*, indicando um possível papel deletério dessas moléculas na infecção por esse parasito (Bhattacharyya *et al.*, 2002). No nosso modelo, o papel de CCR5 precisa ser mais bem investigado.

A interação de *L. amazonensis* com macrófagos de CBA evidencia a eficiência dos mecanismos de evasão desse parasito; em contrapartida, a interação com *L. major* demonstra a capacidade do macrófago em controlar a infecção por esse patógeno. Os resultados do nosso trabalho indicam que *L. major* é indutora de uma resposta mais potente do hospedeiro que *L. amazonensis*. Alguns estudos têm sugerido que essas duas espécies de Leishmania utilizam diferentes fatores de virulência na invasão de macrófagos (Colmenares *et al.*, 2002; Turco *et al.*, 2001) e ativam diferentes fatores derivados do hospedeiro (Charlab *et al.*, 1990) para seu crescimento e progressão da doença (Kima *et al.*, 2000; Soong *et al.*, 1997). Dados de proteômica comparativa, ainda não publicados por Ji and Song, indicam que existem diferenças importantes entre os padrões de expressão de proteínas de promastigotas de *L. amazonensis* e *L. major*.

Embora nosso trabalho não tenha investigado a interação de macrófagos com o desenvolvimento de células Th, os dados indicam que a deficiência de uma imunidade protetora para *L. amazonensis* ocorre em vários níveis, desde a interação inicial, passando pelo estabelecimento do compartimento intracelular, até a inibição de mediadores inatos. É possível que o conjunto destes eventos iniciais resultem no estabelecimento da infecção por *L. amazonensis*. Alternativamente, a interação de *L. major* com o macrófago de CBA resulta na ativação e na inibição de um conjunto de moléculas que induzem controle do número de parasitos e cura da doença.

8- CONCLUSÕES

1- Macrófagos de camundongos CBA/J são resistentes à *L. major*, entretanto eles são permissivos à *L. amazonensis*.

2- *L. amazonensis* resiste e desativa mecanismos leishmanicidas do macrófago, elementos importantes no desenvolvimento da resposta imune adquirida.

3- *L. major* é susceptível ao ambiente fagolisossomal de macrófagos de camundongos CBA/J, enquanto *L. amazonensis* resiste a esse microambiente.

4- *L. major* e *L. amazonensis* alteram diferentemente a expressão gênica de macrófagos de camundongos CBA/J.

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