UNIVERSIDADE FEDERAL DE MINAS GERAIS

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ASPECTOS HISTOLÓGICOS, PARASITOLÓGICOS E IMUNOLÓGICOS DA INFECÇÃO EXPERIMENTAL POR *Leishmania (Leishmania) major* NOS MODELOS MURINOS DE IMPLANTE DE PARAFINA E ESPONJA

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Tese apresentada ao Programa de Pós-Graduação em Patologia da Universidade Federal de Minas Gerais, como parte dos requisitos para a obtenção do título de doutor em Patologia.

Área de concentração: Patologia Geral

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Quando uma criatura humana desperta para um grande sonho e sobre ele lança toda a força de sua alma, todo o universo conspira a seu favor.

Goethe

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SUMÁRIO

LISTA DE ABREVIATURAS

SMF – Sistema mononuclear fagocitário

LV – Leishmaniose visceral

LT – Leishmaniose tegumentar

LCM – Leishmaniose cutâneo-mucosa

LCD – Leishmaniose cutâneo difusa

LCDA - Leishmaniose cutânea disseminada

L.major - Leishmania major

Th – linfócitos T helper

CD – cluster of differentiation

IL – Interleucina

TNF-α- fator de necrose tumoral alfa

IFN-γ– interferon gama

NO – óxido nítrico

NOS – óxido nítrico sintetase

RNAm – Ácido ribonucléico mensageiro

ELISA – Enzyme-Linked Immunosorbent Assay

DC's – Células dendríticas

qRT-PCR / RT-PCR – Reação em cadeia da polimerase quantitativo em tempo real

MCP-1 – proteína quimiotática para macrófagos/monócitos

RANTES - regulated upon activation normal T cell expressed and secreted

RESUMO

Devido à importância dos monócitos-macrófagos na infecção por *Leishmania*, investigamos nesse trabalho as principais citocinas e quimiocinas envolvidas no recrutamento dessas células, em animais susceptíveis e resistentes à infecção experimental por *Leishmania major*, em dois modelos de inflamação crônica: (1) o modelo de implante de parafina, (2) o modelo de implante de esponja. Foi investigado o perfil histológico, parasitológico e imunológico, através da análise de citocinas (IL-4, IL-10, TNF-α, IFN-γ e IL-12) e quimiocinas (MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, CXCL10/IP10, CXCL9/MIG) envolvidas no recrutamento de monócitos-macrófagos e linfócitos, tanto na resposta imunológica do tipo Th1 quanto na Th2. As avaliações foram realizadas no tempo de 7 dias após o implante de parafina concomitante com infecção por *L. major* e nos tempos de 7, 21 e 30 dias após o implante de esponja concomitante com infecção por *L. major*. Foi observado, nos dois modelos estudados, formação de tecido de granulação, constituído por grande número de macrófagos (células epitelióides e células gigantes), linfócitos e vasos sanguíneos recém-formados. Animais C57BL/6 apresentaram aumento do parasitismo tecidual em relação aos animais Balb/c. As análises de RT-PCR das citocinas e quimiocinas confirmaram o perfil de resistência e de susceptibilidade dos animais C57BL/6 e Balb/c.

ABSTRACT

Because of the importance of monocytes-macrophages in *Leishmania* infection, we investigated in this work the main cytokines and chemokines involved in recruitment of these cells in animals susceptible and resistant to experimental infection with *Leishmania major*, in two models of chronic inflammation: (1) model implantation of paraffin (2) the sponge implant model. We investigated the histological profile, parasites and immune systems, through analysis of cytokines (IL-4, IL-10, TNF-α, IFN-γ and IL-12) and chemokines (MCP-1/CCL2, MIP-1 α / CCL3, RANTES/CCL5, CXCL10/IP10, CXCL9/MIG) involved in the recruitment of monocytes-macrophages and lymphocytes in the both immune response Th1 and Th2. Evaluations were performed at the time of 7 days after implantation of paraffin concomitant infection with *L. Major* and 7, 21 and 30 days after implantation of a sponge with concomitant infection by *L. major*. Was observed in both models studies, formation of granulation tissue consisting of large numbers of macrophages (epithelioid cells and giant cells), lymphocytes and newly formed blood vessels. C57BL/6 showed an increase of tissue parasitism in the animals Balb/c. The RT-PCR analysis of cytokines and chemokines confirmed the profile of resistance and susceptibility of C57BL/6 and Balb/c.

INTRODUÇÃO

Leishmanioses

As leishmanioses apresentam ampla distribuição geográfica mundial. Dados da Organização Mundial da Saúde indicam ocorrência endêmica das leishmanioses em 88 países, incluindo-as entre as seis principais doenças tropicais do mundo. Estima-se que a prevalência global seja de 12 milhões de pessoas e que a incidência anual seja de 2 milhões de casos, sendo 1,5 milhões para a leishmaniose tegumentar e 500.000 para a leishmaniose visceral (WHO 2007).

As leishmanioses têm como agentes etiológicos mais de 20 espécies de protozoários digenéticos do gênero *Leishmania* (Ordem Kinetoplastida, Família Trypanosomatidae) que acometem a pele e/ou mucosas e vísceras do homem e de diferentes espécies de animais silvestres e domésticos das regiões quentes das Américas, Europa, África e Ásia (Grimaldi e Tesh, 1993; Salman, Rubeiz *et al.*, 1999). Na natureza, as espécies de *Leishmania* existentes são transmitidas ao homem e a outros mamíferos através da picada de fêmeas de flebotomíneos infectados (Ordem Diptera, Família Psycodidae, Subfamília Phlebotominae). Os protozoários do gênero *Leishmania* são parasitos intracelulares obrigatórios, principalmente de macrófagos e células dendríticas, embora a infecção de outros tipos celulares tenha sido descrita (Scott e Hunter, 2002).

Apesar das várias formas biológicas dos parasitos, existem dois estágios morfológicos principais da *Leishmania*: as formas promastigota e amastigota. A promastigota é extracelular, móvel, flagelada e a amastigota, intracelular obrigatória, sem flagelo aparente. A forma promastigota se desenvolve no trato digestivo dos hospedeiros invertebrados (Flebotomíneos) e adquire aspecto

morfológico e fisiológico distinto ao passar por regiões anatômicas definidas do tubo digestivo do inseto. A forma promastigota procíclica diferenciam-se no intestino anterior do flebotomíneo e não é infectante. Essa se transforma em promastigota metacíclica e migra para o aparelho bucal do inseto de onde parte, através da picada do inseto, e é inoculada no hospedeiro vertebrado. Após a inoculação, a promastigota metacíclica interage com as células do sistema mononuclear fagocitário (SMF), sobretudo o macrófago, e o invade, sendo internalizada (Chang, 1979; De Almeida, Vilhena *et al.*, 2003). No interior dos macrófagos os parasitos sofrem modificações morfológicas, bioquímicas e moleculares que resultam na transformação das formas promastigotas em amastigotas. Depois de alguns ciclos de multiplicação por divisão binária, ocorre o rompimento dos macrófagos e as formas amastigotas podem invadir novas células hospedeiras (Cunningham, 2002; Awasthi, Mathur *et al.*, 2004).

Durante o repasto sanguíneo, as fêmeas de flebotomíneos ingerem, juntamente com o sangue, macrófagos infectados. Ocorrem modificações fisiológicas, bioquímicas e morfológicas no aparelho digestivo do inseto vetor. O ciclo recomeça quando os flebotomíneos inoculam as formas promastigotas metacíclicas nos tecidos do hospedeiro mamífero (De Almeida, Vilhena *et al.*, 2003)

As formas amastigotas são responsáveis pelas diversas manifestações clínicas da leishmaniose no homem (Chang, 1979). Existem duas manifestações clínicas gerais de leishmaniose no mundo: a leishmaniose visceral (LV) ou calazar e a leishmaniose tegumentar (LT). A LV é uma infecção generalizada do SMF. A LT é causada por várias espécies de *Leishmania* e manifesta-se de várias formas

de acordo com aspectos clínicos e patológicos: a leishmaniose cutânea (LC), caracterizada por lesões localizadas e, às vezes auto-resolutivas; a leishmaniose cutâneo-mucosa (LCM), que representa um quadro hiperérgico, com lesões ulcerativas e destrutivas das mucosas; a leishmaniose cutânea difusa (LCD), associada a um quadro anérgico, sendo as lesões múltiplas, mas não ulceradas e a leishmaniose cutânea disseminada (LCDA), caracterizada por lesões múltiplas e geralmente ulceradas (Grimaldi e Tesh, 1993; Gontijo e De Carvalho, 2003; Sousa, Pompeu *et al.*, 2006).

As manifestações clínicas da leishmaniose dependem de uma complexa associação entre os fatores relacionados à virulência das espécies de *Leishmania* infectantes e à susceptibilidade genética do hospedeiro ao parasito (Salman, Rubeiz *et al.*, 1999; Sotiropoulos e Wilbur, 2001). A LV é causada por três espécies, *Leishmania donovani, L. infantum* e *L. chagasi*, sendo que as duas últimas são consideradas por alguns autores como sendo uma única espécie (Maurício, Stothard *et al.*, 2000; Cunningham, 2002). A forma cutânea difusa (LCD) está quase sempre associada à *L. (L.) amazonensis* e à *L. aethiopica*, as formas mucosa (LCM) e disseminada (LCDA) estão associadas predominantemente à *L. (Viannia) braziliensis.* A LCM é raramente associada à *L. (V.) guyanensis,* enquanto que a LCDA é também associada a outras espécies do subgênero *Viannia* (Lainson, 1983; Peters, Evans *et al.*, 1983; Silveira, Lainson *et al.*, 2004). A lesão da LC, por sua vez, é causada principalmente pela *L. major*, *L. tropica*, *L. aethiopica* na Europa, África e Ásia e *L. (V.) braziliensis, L. guyanensis* e *L. amazonensis* nas Américas (Grimaldi e Tesh, 1993; Salman, Rubeiz *et al.*, 1999).

Leishmaniose Experimental Murina

A leishmaniose experimental murina é um bom modelo para a investigação da diversidade de mecanismos regulatórios que medeiam à susceptibilidade e resistência à doença e suas possíveis implicações relacionadas a outras doenças infecciosas. Esse modelo tem sido utilizado também para estudos da leishmaniose cutânea humana com o objetivo de elucidar os mecanismos patogênicos da doença, estratégia importante na busca de novas abordagens terapêuticas (Sacks e Noben-Trauth, 2002).

O modelo murino de infecção por *Leishmania major* possibilitou a primeira correlação *in vivo* entre o desenvolvimento de uma imunidade protetora (cura da infecção e a morte dos parasitos) e a expansão de células Th1 CD4+ em camundongos resistentes C57BL/6. Ao contrário, o desenvolvimento de células Th2 CD4+ está relacionado com a progressão da doença e crescimento parasitário contínuo em camundongos susceptíveis BALB/C (Scott e Scharton, 1994)

O padrão de resposta protetora Th1, induzido pela citocina IL-12, é principalmente caracterizado pela secreção TNF-α e IFN-γ, os quais ativam as defesas contra patógenos intracelulares (Gumy, Louis *et al.*, 2004). O padrão de resposta Th2 produz interleucinas do tipo IL-4, IL-5 e IL-13, que favorecem o desenvolvimento de uma resposta contra patógenos extracelulares com uma forte resposta humoral, que no contexto da leishmaniose, está associado a uma não resolução da parasitose com ulceração e disseminação do parasito (Gumy, Louis *et al.*, 2004). Neste contexto, os macrófagos, principais células monocíticas fagocitárias envolvidas na infecção por *Leishmania,* podem se comportar como

células hospedeiras, apresentadoras de antígeno e/ou células efetoras na eliminação dos parasitos, dependendo do seu estado de ativação.

A habilidade dos macrófagos em estimular uma resposta Th2 ou Th1 pode ser aumentada ou diminuída, através da interação parasito-hospedeiro via receptor Fcγ, CR ou interação via moléculas de superfície LPG do parasito. Estas interações podem resultar, por exemplo, na inibição da produção de moléculas efetoras NO, ROS ou na indução de citocinas Th2 (Reiner e Locksley, 1995; Awasthi, Mathur *et al.*, 2004).

Mills et al. demonstraram que macrófagos das linhagens resistente (C57BL/6) e susceptível (BALB/C) respondem de maneira diferente à ativação pelos linfócitos T, frente ao mesmo estímulo. Segundo estes autores, os macrófagos poderiam ser ativados em maior ou menor proporção a produzir NO dependendo da linhagem de origem, e ter uma resposta imune (Th1/Th2) influenciada pela dominância da resposta originada por estes macrófagos. Assim, na linhagem C57BL/6 os macrófagos seriam mais facilmente ativados a produzir NO, e na BALB/C a sintetizar poliaminas, influenciando a resposta imune para caminhos opostos (Mills, Kincaid *et al.*, 2000).

Algumas das manifestações clínicas observadas em pacientes com leishmaniose cutânea podem ser reproduzidas em camundongos de diferentes linhagens inoculando experimentalmente uma das várias espécies de *Leishmania*. Na infecção experimental por *L. major*, a maioria das linhagens de camundongos, da qual a C57BL/6 é a mais representativa, apresenta fenótipo resistente à infecção. Tais camundongos desenvolvem lesões cutâneas localizadas que se curam espontaneamente, adquirindo resistência imunológica à re-infecção.

Enquanto que camundongos Balb/c, representativos do fenótipo susceptível, desenvolvem lesões graves e descontroladas, visceralização do parasito e morte (Sacks e Noben-Trauth, 2002; Awasthi, Mathur *et al.*, 2004; Pós, Müller *et al.*, 2004; Sacks e Anderson, 2004).

Citocinas e Quimiocinas

O modelo murino de infecção por *L. major* é considerado um dos melhores sistemas experimentais para estudo dos mecanismos que levam à maturação de células Th1 e Th2, sendo que a susceptibilidade à infecção foi correlacionada com o desenvolvimento de lesões associadas com a resposta imune do tipo Th2, e a cura das lesões no camundongo resistente foi correlacionada com a resposta imune do tipo Th1 (Heinzel, Sadick *et al.*, 1989; Awasthi, Mathur *et al.*, 2004; Gumy, Louis *et al.*, 2004; Sacks e Anderson, 2004). Dentre os vários fatores que influenciam o desenvolvimento celular Th, as citocinas de baixo peso molecular, (proteínas reguladoras), produzidas por uma variedade de células, são consideradas cruciais para regular este processo, sendo seu estudo de grande interesse para proporcionar informações que podem levar a medidas mais eficientes para prevenir ou controlar infecções por patógenos (Gumy, Louis *et al.*, 2004; Sommer e Kress, 2004).

As células Th1 são caracterizadas por secretarem interferon-γ (IFN-γ), interleucina 2 (IL-2) e fator de necrose tumoral α (TNF-α), que ativam as defesas do hospedeiro contra os patógenos intracelulares, enquanto que a subpopulação Th2 produz IL-4, IL-5, IL-10 e IL-13, que favorecem o desenvolvimento de resposta humoral contra os patógenos extracelulares (Sacks e Noben-Trauth, 2002; Gumy, Louis *et al.*, 2004). Dependendo das citocinas

produzidas, expressam-se as ações reguladoras das duas subpopulações de linfócitos T. IFN-γ inibe a proliferação de células Th2 e a produção de IL-4 e IL-10, enquanto estas últimas inibem a proliferação de células Th1 e a produção de IFN-γ (Sacks e Noben-Trauth, 2002). Neste contexto, foi sugerido que, em camundongos susceptíveis Balb/c, IL-4 é necessária e suficiente para iniciar os eventos moleculares que direcionam a uma maturação de células Th2 e susceptibilidade à *L. major* (Himmelrich, Launois *et al.*, 2000), enquanto outro trabalho sugeriu que IL-10 é tão importante quanto IL-4/IL-13 na susceptibilidade à *L. major* (Noben-Trauth, Lira *et al*., 2003).

A dicotomia Th1/Th2 não é absoluta. Dentre as linhagens resistentes existe uma controvérsia em relação ao camundongo Balb/c deficiente de IL-4 (IL-4-/-). Experimentos no modelo clássico de infecção por *L. major* utilizando o camundongo IL-4-/-, apresentaram resultados conflitantes em relação à importância do IL-4 no processo da infecção. Estudos descreveram que o camundongo Balb/c IL-4-/-, permaneceu susceptível à *L. major* (Noben-Trauth, Kropf *et al.*, 1996; Kropf, Schopf *et al.*, 1999), enquanto outros descreveram resistência da mesma linhagem à infecção (Kopf, Brombacher *et al.*, 1996; Mohrs, Ledermann *et al.*, 1999). Estudos sugeriram ainda que TNF-α e IFN-γ não são fatores decisivos no fenótipo resistente x susceptível na infecção por *Leishmania* (Anderson, Mendez *et al.*, 2005). Deve-se ressaltar que a produção simultânea de citocinas Th1 e Th2 em camundongos infectados demonstra a importância da regulação do balanço entre as duas respostas para o controle da doença (Lohoff, Gessner *et al.*, 1998).

Apesar da variedade de experimentos envolvendo a resposta imune na leishmaniose experimental, as interações moleculares Th1/Th2 na leishmaniose cutânea experimental ainda não foram totalmente esclarecidas (Pós, Müller *et al.*, 2004). Este fato torna relevante a investigação de células alternativas e citocinas envolvidas na susceptibilidade e/ou resistência neste modelo de infecção.

Tem-se demonstrado que os variados quadros clínicos das leishmanioses cutâneas estão intimamente relacionados à forma de expressão das quimiocinas e consequentemente na composição do infiltrado inflamatório no sítio da lesão. Essa correlação sugere que elas exercem importante participação na resposta imune aos protozoários parasitas. Por exemplo, nas leishmanioses cutâneas localizadas, com cura espontânea, verifica-se o predomínio de resposta Th1 associada às quimiocinas CCL2/MCP-1, CXCL9/MIG e CXCL10/IP-10 e pequenas quantidades de CCL3/MIP-1α. Em quadros crônicos, associados às leishmanioses cutâneas difusas, ocorre o oposto com a expressão de CCL3/MIP-1α dominando em relação a CCL2/MCP-1, CXCL9/MIG e CXCL10/IP (Ritter e Körner, 2002). Citocinas estão diretamente envolvidas com a produção de quimiocinas. Nas leishmanioses, TNF-α e IL-1β estimulam a produção de CCL3 (MIP-1-α) enquanto IL-12 induz a secreção de quimiocinas relacionadas ao recrutamento de macrófagos, como CXCL-10 (IP-10) e CCL2 (MCP-1); IFN-γ induz CXCL9 (MIG) e também CXCL-10. A atuação sinérgica entre citocinas e quimiocinas tem fator decisivo na eliminação de parasitos dos sítios de lesão. Infecções com *L. major* induzem aumento de CCL5, CCL2, CCL3 e CXCL10

em peles e linfonodos regionais de camundongos resistentes à leishmaniose cutânea (Teixeira, Teixeira *et al.*, 2006).

O papel dos monócitos-macrófagos, na patogenia da infecção por protozoários do gênero *Leishmania* nos hospedeiros vertebrados, tem sido estudado, mais ainda não é completamente compreendido (Teixeira, Teixeira *et al.*, 2006; Lima, Oliveira *et al.*, 2007; Reis, Martins-Filho *et al.*, 2009). Usando o modelo murino para a leishmaniose visceral, Murray, tem sugerido o papel efetor dos macrófagos em eliminar esse agente etiológico (Murray, 1994; 2001). Contrariamente, Soong e colaboradores, trabalhando com o modelo cutâneo com *L. amazonensis*, sugere que monócitos-macrófagos exercem mais a função de células hospedeiras perpetuando a infecção(Soong, Chang *et al.*, 1997) .

Modelos de Implante de Parafina e Esponja

O implante de um agente inerte, tablete de parafina, no subcutâneo de camundongos induz um quadro de inflamação crônica (Raso, 1976), tanto em camundongos Balb/c quanto em C57BL/6 (Tafuri, Melo *et al.*, 2000). Além disso, há formação de uma cápsula fibrosa ao redor da parafina constituída por grande número de macrófagos (células epitelióides e células gigantes), linfócitos e vasos sanguíneos recém-formados e hiperemiados, caracterizando o tecido de granulação. Tafuri e colaboradores associaram o implante de parafina à infecção experimental com *L. major* e observaram intenso parasitismo tecidual nos animais C57BL/6, geneticamente resistentes à infecção, em comparação aos Balb/c, suceptíveis (Tafuri, Melo *et al.*, 2000).

Apesar de ser um ótimo modelo de inflamação crônica, o modelo de implante de parafina apresenta algumas limitações, como:

1. O deslocamento da parafina no subcutâneo dos animais, afastando do local da infecção;
2. Dificuldade de esterilização da parafina;
3. O tecido de granulação formado, cápsula inflamatório, é muito pequeno, o que dificulta a realização de mais de uma análise na mesma amostra.

Outro modelo de inflamação crônica "in vivo" é o de implantação subcutânea de matriz esponjosa de poliéster-poliuretano. Esse modelo de implantação em animais foi descrito inicialmente por Grindlay & Waugh e modificado por Andrade et al., em 1987 (Grindlay e Waugh, 1951; Andrade, Fan *et al.*, 1987).

O modelo permite o estudo temporal do infiltrado inflamatório, análise bioquímica dos fluidos coletados, efeitos de drogas sobre o processo, além de estudos histológicos e morfométricos (Andrade, Fan *et al.*, 1987; Bailey, 1988; Barcelos, Talvani *et al.*, 2004).

No sentido de evitar repetições, maiores justificativas do uso desses modelos no estudo da infecção murina experimental por *L. major* serão explicitadas nos artigos científicos que serão apresentados adiante nesse trabalho.

OBJETIVOS

Objetivo Geral

Comparar aspectos histológicos, parasitológicos e imunológicos envolvidos em camundongos das linhagens C57BL/6 e Balb/c em resposta aos implantes de parafina e esponja, associados ou não à infecção por *Leishmania major*.

Objetivos Específicos

A) Avaliar as alterações histológicas (inflamação crônica) nos fragmentos de pele e cápsula no modelo da parafina durante a infecção experimental com *L. major*.

B) Avaliar o parasitismo tecidual nos fragmentos de cápsula no modelo da parafina durante a infecção experimental com *L. major*

C) Avaliar o perfil imunológico, a partir da análise da expressão quantitativa de algumas quimiocinas (MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, CXCL10/IP10, CXCL9/MIG) e citocinas (IL-4, IL-10, TNF-α, IFN-γ e IL-12) relacionadas principalmente à migração de macrófagos e linfócitos, nos fragmentos de cápsula no modelo da parafina durante a infecção experimental com *L. major*.

D) Avaliar as alterações histológicas (inflamação crônica) na esponja no modelo de implante de esponja durante a infecção experimental com *L. major*.

E) Avaliar a cinética imunológica, a partir da análise da expressão quantitativa de algumas quimiocinas (CCL2/MCP-1, CCL5/RANTES) e citocinas (IL-4, IL-10, TNF-α, IFN-γ) relacionadas principalmente à migração de macrófagos e

linfócitos, na esponja no modelo de implante de esponja durante a infecção experimental com *L. major*.

MATERIAL E MÉTODOS, RESULTADOS E DISCUSSÃO

Estes tópicos serão apresentados na forma de dois artigos científicos. O artigo 1 é referente aos objetivos A, B e C e o artigo 2 refere-se aos objetivos D e E.

Artigo 1 - Murine immune response induced by *Leishmania major* during the implantation of paraffin tablets

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ORIGINAL ARTICLE

Murine immune response induced by *Leishmania major* during the implantation of paraffin tablets

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Abstract We carried out a model of chronic inflammation using a subcutaneous paraffin tablet in mice experimentally infected with *Leishmania major*. It was previously reported that the parasite load following paraffin implantation occurred at a peak of 21 days in both BALB/c and C57BL/6 mice. At the present study, we have investigated what cytokines and chemokines are directly related to the parasite load in C57BL/6 mice. All mice were divided in four groups: mice implanted with paraffin tablets; mice experimentally infected with *L. major;* mice implanted with paraffin tablets and experimentally infected with *L. major*; and mice submitted only to the surgery were used for the Real-Time Polymerase Chain Reaction (RT-PCR) controls. Fragments of skin tissue and the tissue surrounding the paraffin tablets (inflammatory capsule) were collected for histopathology and RT-PCR studies. By 21 days, a diffuse chronic inflammatory reaction was mainly observed in the deep dermis where macrophages parasitized with *Leishmania* amastigotes were also found. RT-PCR analysis has shown that BALB/c mice showed strong IL-4 and IL-10 mRNA expression than controls with very little expression of IFN-γ. In contrast, both IFN-γ and IL-10 mRNA was found in higher levels in C57BL/6 animals. Moreover, in C57BL/6 mice the expression of chemokines mRNA of CCL3/MIP-1α was more highly expressed than CCL2/MCP-1. We conclude that the Th1 immune response C57BL/6 did not change to a Th2 response, even though C57BL/6 animals presented higher parasitism than BALB/c mice 21 days after infection and paraffin implantation.

Keywords *Leishmania major* · Paraffin tablets · Chronic inflammation · Cytokines · C57BL/6 and BALB/c mice

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Introduction

Leishmania parasites are obligatory intracellular pathogens that replicate primarily within macrophages, and also in dendritic cells (DCs) and fibroblasts [1, 2]. The initial step in protective host defense against *Leishmania* is confinement of the parasite to the site of infection. Infected macrophages are stimulated to produce antimicrobial molecules by interferons [3]. Twenty-four hours after infection, migrating NK cells at the site of the infection up-regulate IFN-γ, which amplifies the host immune defense [4, 5]. Immature DCs in the skin (*Langerhans* cells) then take up *Leishmania* promastigotes and migrate to the regional lymph nodes where they differentiate into mature antigen-presenting DCs [6]. These DCs have two main functions: they present antigens to naïve T cells and they produce interleukin (IL)-12, the cytokine most crucial for the development of a Th1 adaptive immune response [7]. IFN-γ is produced in large amounts by activated Th1 cells, enhancing anti-*Leishmania* activity at the infection site [8]. In consequence, resolution of the infection depends on the early presence of macrophages and the activation of these cells by IFN-γ. Activated Th1 cells enhance parasite killing by macrophages, when they release IFN-γ [9]. However, these immunological processes differ in effectiveness among human individuals. In fact, *Leishmania* parasites can cause a wide spectrum of cutaneous diseases, including: (1) localized cutaneous leishmaniasis (LCL); (2) mucocutaneous leishmaniasis involving erosive lesions of the oral and nasal mucosa; (3) diffuse cutaneous leishmaniasis (DCL) characterized by multiple non-healing lesions; and (4) a distinct clinical form called disseminated cutaneous leishmaniasis [10–12].

In recent years, the regulation of cell migration to the site of inflammation has been intensively investigated. Chemokines, small chemotactic cytokines, are responsible for coordinating leukocyte trafficking with immune cell differentiation and effector functions [13, 14]. LCL lesions show strong expression of Th1-associated chemokines such as CCL2/MCP-1, CXCL9/MIG, and CXCL10/IP10. In contrast, chronic DCL entails lower expression of Th1-associated chemokines and higher levels of CCL3/MIP-1α [9].

The mouse is generally considered a valuable model for human disease. For cutaneous leishmaniasis, it provides an excellent model for identifying the factors involved in developing T helper subsets, since Th1 cells confer protection in resistant strains of mice (C57BL/6), whereas Th2 cells are associated with a fatal outcome in susceptible mice (BALB/c) [15]. Despite technological advances in characterizing the influx of different monocyte–macrophage subpopulations at the molecular level [16, 17], the role of inflammatory macrophages in leishmaniasis remains relatively poorly defined. In 2000, Tafuri et al. [18] described a model of monocytic inflammation using a subcutaneous paraffin tablet. This inert irritant induces the recruitment of monocytes to the site of inflammation and the localized immobilization of macrophages around the tablet. Monocyte infiltration and macrophage accumulation following paraffin implantation was similar in BALB/c and C57BL/6 mice. When paraffin implantation was coupled with *Leishmania major* infection, both strains of mice had large numbers of parasites associated with these inflammatory macrophages at 21 days post-infection. Thus, the early accumulation of parasites in macrophages occurs in both genetically susceptible (BALB/c) and resistant (C57BL/6) strains of mice. Despite these heavy early parasite burdens, the C57BL/6 mouse is able to resolve infection, whereas the BALB/c mouse does not. In that time, based on Murray et al. [19], we discussed that the heavy parasitism that we observe is dependent on the type of macrophages recruited into lesions, and that paraffin implantation can recruit a population of permissive macrophages even in the genetically resistant C57BL/6 mouse. However, in the previous histological examination, the nature of cytokines and chemokines that were produced by these inflammatory cells was not determined [18].

Considering that C57BL/6 mice presented a parasite load similar to BALB/c mice 21 days after infection during the implantation of paraffin tablets, the aim of this study was to determine the chemokines that are produced in this tissue. We hypothesized that the chemokines could be directly related to the parasite load increasing in C57BL/6 mice after paraffin implantation associated to the *Leishmania* infection. We show that over the first 3 weeks post-implantation, there was a remarkable similarity in the histological features and the degree of parasitism in these two mouse strains. However, unlike the BALB/c mouse that is unable to clear infection, the parasite infection was transient in C57BL/6 mice, which had begun to clear the infection by 30 days. Real-time PCR analysis has shown that both IFN-γ and IL-10 mRNA was found in higher levels in the skin and inflammatory capsule of C57BL/6 animals that were infected with *L. major* when compared to paraffin implant alone. In addition, in C57BL/6 mice the expression of chemokines mRNA of CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, CXCL9/MIG, and CXCL10/IP-10 were found in the infected tissues. Particularly, CCL3/MIP-1α was more highly expressed than CCL2/MCP-1, suggesting a differential and important role for them in the recruitment and activation of different immune cells that can define a permissive profile at the site of infection with *L. major*.

Materials and methods

Subcutaneous implantation of paraffin tablets

Paraffin tablets (220.0 mg;Queel-Indústrias Químicas Ltda, São Paulo, Brazil) with the dimensions - 1.0x0.5 cm (HXL) were prepared as described previously. These tablets have a

smooth surface and are maintained under aseptic conditions. To implant them, mice were anesthetized and shaved, and then a skin incision (1.5 cm) was made on the dorsum using blunt point scissors. Tablets were inserted into a small subcutaneous cavity, and the wound was stitched [18].

Mice

Section "Mice"

One hundred mice, 4–6 weeks old, obtained from our animal facility at ICB/UFMG, were divided into two groups: 50 C57BL/6 and 50 BALB/C mice where they were subdivided in four groups.

Group 1 (P) This group comprised mice implanted with paraffin tablets (n=30; 15 of each mice strain);

Group 2 (L) This group comprised mice experimentally infected with *L. major* promastigotes (n=30; 15 of each mice strain)

Group 3 (P+L) This group comprised mice implanted with paraffin tablets and infected with promastigotes immediately thereafter (n=30; 15 of each mice strain);

Group 4 (Group sham–"Wound Healing Surgery Group") This group comprised of ten mice that were submitted only to the surgery (skin incision) where the wound was stitched (wound healing). We collected the skin samples from dorsal area. (n=10; 5 of each mice strain)

These animals were sacrificed at 7, 21 and 30 days.

Day 7–20 mice were distributed in groups 1, 2, 3 and 4 (five mice in each group)

Day 21–15 mice were distributed in groups 1, 2 and 3 (five mice in each group)

Day 30–15 mice were distributed in groups 1, 2 and 3 (five mice in each group)

Experimental infection

C57BL/6 mice were injected with 1×10^6 *L. major* promastigotes in 100 μl (subcutaneous) in the dorsum nearest the paraffin tablet, but not inside of the paraffin tablets and immediately after implantation. Control mice (without paraffin) were infected at the same time. BALB/c mouse experiments followed the same protocol.

Parasites

The Friendlin strain of *L. major* clone IV (MHOM/IL/80/Friedlin) was used in all experiments. Promastigotes were grown at 25°C in Grace's insect cell culture medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin G (100 U/ml) and streptomycin (100 μg/ml). Stationary-phase promastigotes, obtained from 7- to 9-day cultures, were used for all experimental infections.

Disease progression and collection of tissue samples for histopathology

Five animals from each group were sacrificed at 7, 21 and 30 days after the experimental infection. At each of the designated days, fragments of skin tissue and the tissue surrounding the paraffin tablets (inflammatory capsule) were collected for histopathology [18]. Tissues were fixed in 10% buffered formalin, dehydrated, cleared, embedded in paraffin, cut (3–4 μm thick) and stained with Hematoxylin and Eosin (HE).

Microtitration

Parasites were quantified by limiting dilution, as previously [20]. The inflammatory capsule formed by the paraffin implantation was homogenized using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at 150×g and the cells were concentrated by centrifugation at 2,000×g. Pellets were resuspended in 400 ml supplemented Grace's culture medium (see above). Samples were plated on to culture plates and serially diluted 1:10 in supplemented Grace's insect tissue culture medium. Each sample was plated in duplicate and read 5 days after the start of culture. Pipette tips were discarded after each dilution to avoid carrying adhering parasites from one well to another. Results are expressed as the negative log of the titer (i.e., the dilution corresponding to the last positive well).

Tissue extraction and determination of *N*-acetyl-β-D-glucosaminidase activity

Fragments of the inflammatory capsule were collected, weighed and homogenized (vortex) in 1.9 ml of 0.1 M NaCl, 0.02 M NaH_2PO_4 0.015 M sodium EDTA, pH 4.7, 4°C, per 100 mg tissue. Infiltration of mononuclear cells was quantified by measuring the levels of the lysosomal enzyme *N*-acetylglucosaminidase (NAG), which is present at high levels in activated macrophages [21]. Part of the pellet remaining after hemoglobin measurement was retained for this assay. These pellets were weighed, homogenized in 0.9% w/v NaCl containing 0.1% v/v Triton X-100 (Promega, Madison, WI, USA) and centrifuged (10,000×g, 10 min, 4°C). Samples of the resulting

supernatant (100 μl) were incubated for 10 min with 100 μl of 2.24 mM *p*-nitrophenyl-*N*-acetyl-h-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA), prepared in 0.1 M citrate/phosphate buffer (pH 4.5). The reaction was terminated by adding 100 μl 0.2 M glycine buffer, pH 10.6. Hydrolysis of the substrate was determined by measuring the absorption at 405 nm. NAG activity was expressed as the change in OD per gram wet tissue.

Quantitative real-time polymerase chain reaction

Fragments of the inflammatory capsule were collected and Total RNA was extracted using a single-step phenol/chloroform procedure (TRIZOL®) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, the tissue was soaked with TRIZOL solution and homogenized using a PowerGen Generator (Fisher Scientifics, Franklin, MA,USA). Chloroform (0.25 ml) was added to 1 ml of TRIZOL homogenate and mixed thoroughly. After centrifugation, the upper aqueous phase was transferred to a fresh tube and 0.7 ml of isopropyl alcohol was added to precipitate the RNA. The RNA pellet was washed once with 70% ethanol and air-dried for no more than 10 min at room temperature. Contaminating genomic DNA was eliminated by dissolving the RNA pellet in 10 mM Tris-HCl, pH 7.5, containing RNase-free DNase and 1 mM $MgCl_2$ and incubating for 30 min at room temperature. TRIZOL® solution was then added to stop the reaction and the total RNA was further purified as described above. The final RNA pellet was dissolved in RNase-free water and the A260/280 ratio was measured to evaluate quality [22].

First-strand cDNA synthesis by reverse-transcription reaction: First strand cDNA was generated using ThermoScript® Reverse Transcriptase (Invitrogen,). A 5 μg of genomic DNA-free total RNA and oligo$(dT)_{20}$ primer were used; the detailed procedure is described in the manufacturer's instructions (Invitrogen).

Quantitative-PCR: This was performed using a LightCycler®480 Real-time PCR System (Roche Applied Science, Indianapolis, IN, USA) with SYBR Green PCR reagents purchased from BioRad. Melting curve analyses were carried out to ensure that a single product with the expected melting curve characteristics was obtained. The relative differences among samples were analyzed using the ΔΔCt method described previously. A ΔCt value was determined for each sample using the Ct value for GAPDH to normalize loading differences. A ΔΔCt value was then obtained by subtracting the ΔCt value for the mice submitted only to the surgery (group sham-without paraffin and Leishmania infection) from the corresponding experimental ΔCt. The fold difference over the value for the sham mice was calculated as $2^{\Delta\Delta Ct}$.

Statistical analysis: GraphPad Prism software was used. Data were analyzed statistically by the Student *t* test. Data are expressed as mean±SD. The level of significance was set at $p<0.05$.

Results

Histology

C57BL/6 and BALB/c mice implanted with paraffin alone (Group P)

Sterile paraffin tablets were implanted subcutaneously in the back of C57BL/6 and BALB/c mice. According to Tafuri et al. (2000) [18], both strains of mice had an inflammatory capsule of tissue formed around the paraffin tablets as a result of granulomatous tissue formation.

C57BL/6 and BALB/c mice experimentally infected with Leishmania alone (Group L)

By 21 days, the inflammatory reaction in the BALB/c mice was intense and large numbers of parasitized macrophages were present throughout the lesion. In C57BL/6 mice, a discrete chronic inflammatory process in the deep dermis was observed. Only a few macrophages parasitized with *L. major* were observed (Fig. 1a, b).

C57BL/6 and BALB/c mice experimentally infected with Leishmania and implanted with paraffin (Group P+L)

By 21 days, a diffuse chronic inflammatory reaction was mainly observed in the deep dermis and hypodermis (Fig. 1c, d, and e). Macrophages parasitized with *Leishmania* amastigotes were also found in the dermis (Fig. 1e). Moreover, a new formation of blood vessels and fibroblast proliferation with mature collagen deposition were observed mainly in the capsules (Fig. 1f). However, collagen deposition was observed in both strains of mice, and the degree of deposition was not substantially different between the two strains. Some formation of giant cells was observed, and some of these cells had parasites within their cytoplasm (Fig. 1g, h).

C57BL/6 and BALB/c mice submitted only to the surgery without Leishmania and paraffin implanted (sham group)

Macroscopical lesions were not observed in the dorsum of all mice as an extension of the wound healing. In fact, microscopically skin sections of all mice did not show any histological alteration in the epidermis or dermis of all the samples at 21 days post-surgery assay.

Tissue parasite load

According to Tafuri et al. [18], higher parasite load was observed at 21 days post-infection in BALB/c mice and C57BL/6 when compared to the other groups, at 7 and 21 days. Interestingly, C57BL/6 mice showed higher parasitism than BALB/c mice in the tissue capsules ($p \leq 0.01$). By 30 days, the parasite load had decreased in C57BL/6 but not in BALB/c (Fig. 2).

N-acetyl-β-D-glucosaminidase activity

We had shown by histological examination that the capsule tissue obtained from C57BL/6 presented an intense chronic

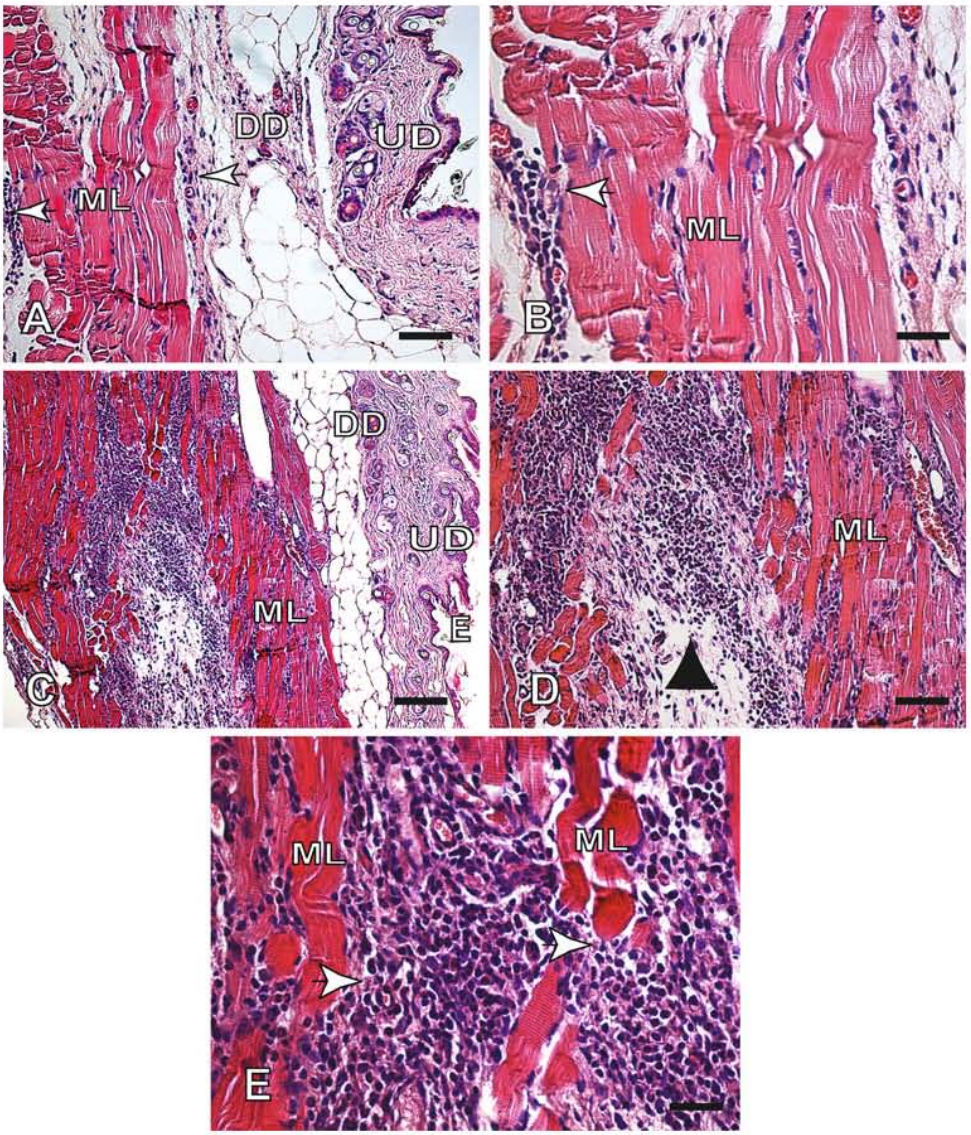

Fig. 1 **a–e**: **a, b** Skin fragment of C57BL/6 mice infected with *L. major* (21 days). **a** Note the presence of a discrete chronic inflammatory exudate in the deep dermis (*DD; white arrows*). Hematoxylin and eosin (HE) stain. *Bar*=32 μm. *E* Epidermis; *UD* Upper dermis; *DD* Deep dermis; *ML* Muscular layer. **b** High magnification showing mononuclear cells and some macrophages with amastigotes forms of *Leishmania* (*white arrow*). HE stain. *Bar*= 16 μm. **c–e** Skin fragment of C57BL/6 implanted with paraffin tablets and infected with *L. major* (21 days). **c** There is an intense chronic inflammatory exudate dissecting the muscular layer (*ML*) in the deep dermis. HE stain. *Bar*=62 μm. **d, e** Higher magnification showing inflammatory mononuclear cells also provoking tissue damage (*black triangle*). Amastigotes forms of *Leishmania* can be observed inside macrophages (*white arrows*). HE stain. *Bar*=32 and 16 μm, respectively. **f–h** Inflammatory capsule of C57BL/6 implanted with paraffin tablets and infected with *L. major* (21 days). **f** Granulomatous tissue formation with blood vessels (*arrowheads*), collagen and mononuclear exudate can be noted. HE stain. *Bar*=16 μm. **g** Giant cell formation (*white arrowheads*) and macrophages parasitized with *Leishmania* amastigotes were readily observed (*white arrows*). HE stain. *Bar*= 16 μm. **h** Granuloma formation with giant cells (*white arrowhead*) and epithelioid cells (*white arrows*). HE stain. *Bar*=16 μm

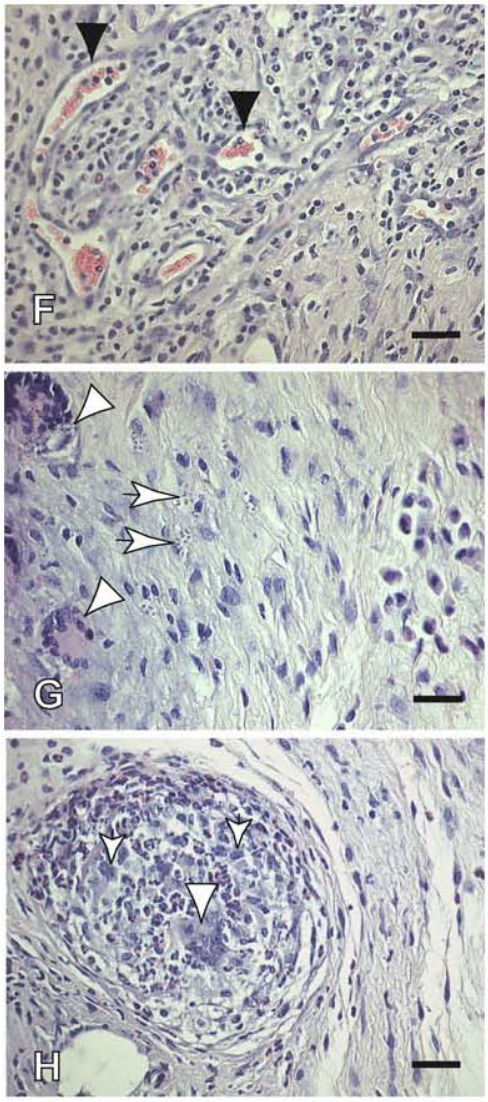

Fig. 1 (continued)

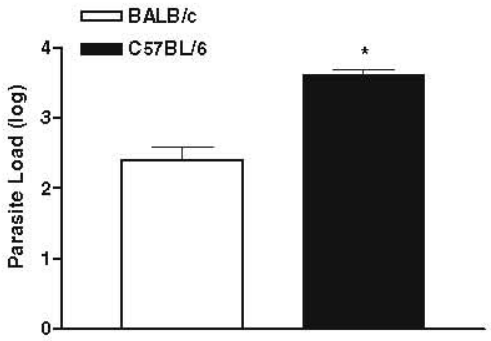

Fig. 2 Parasite load of inflammatory capsule of C57BL/6 and BALB/c implanted with paraffin tablets and infected with *L. major* (21 days). Statistical difference was noted by 21 days (*Bars*, mean±SD; *asterisk*, $p<0.01$ compared to BALB/c group)

inflammatory reaction by 21 days. We therefore investigated the accumulation of activated inflammatory macrophages by an indirect method, NAG activity. At the site of the lesion (capsule tissue) induced by the paraffin implantation associated with *L. major*, NAG activity was significantly increased ($p \leq 0.05$) at 21 days after experimental infection, compared to paraffin-implantation group (Fig. 3).

Cytokines and chemokines at the site of infection

mRNA expression of the cytokines IL-12, TNF-α, and IFN-γ, which are associated with the resistant phenotype to *L. major* infection (C57BL/6 mice), and IL-4 and IL-10, which are associated with the susceptible phenotype (BALB/c), were determined by real-time polymerase chain reaction (RT-PCR). Expression of chemokines related to monocyte-macrophage (CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES) and lymphocyte (CXCL9/MIG and CXCL10/IP-10) recruitment was also investigated. For these studies, all capsule samples from BALB/c and C57BL/6 mice were obtained 21 days after paraffin implantation concurrent with *Leishmania* infection. This time point was chosen because the kinetics of the inflammatory response peaked and the parasite load was prominent in both BALB/c and C57BL/6 mice. Skin tissue samples of sham group were used as controls to compare the cytokine levels. Figure 4a shows that C57BL/6 mice have a resistant phenotype marked by the higher expression of IFN-γ and IL-12 mRNA. Both of these expressions were increased in mice receiving parasites plus paraffin (gray bars), relative to mice receiving paraffin implantation alone (black bars). TNF-α and IL-10 mRNA were detected in lower levels in these mice, and there was no increase relative to paraffin alone. BALB/c mice, on the other hand, expression higher levels of IL-10 and IL-4 mRNA than C57BL/6 mice, and these levels were higher in mice receiving both parasites and paraffin implantation (Fig. 4b).

C57BL/6 mice from the P+L group showed higher levels of CCL3/MIP-1α, CCL5/RANTES, CXCL9/MIG and CXCL10/IP-10 mRNA when compared to the paraffin alone group (Fig. 5a). In particular, CXCL9/MIG level in the P+L group was dramatically increased. BALB/c mice, in contrast, expressed higher levels of CCL2/MCP-1 and CCL3/MIP-1α than C57BL/6 (Fig. 5b).

Discussion

In these paraffin implantation model, both C57BL/6 and BALB/c mouse strains showed an early increase in the numbers of parasites in the lesions accompanies the inflammation. This increase in parasite numbers was

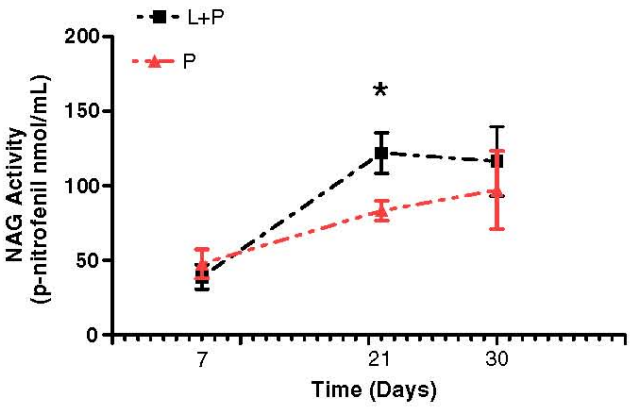

Fig. 3 *N*-acetyl-β-D-glucosaminidase activity in C57BL/6 mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 7, 21, and 30 days after the infection and implantation. The NAG activity was determined individually and each *symbol* represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only.). Statistical difference was noted by 21 days (*Bars*, mean±SD; *asterisk*, $p<0.05$ compared to paraffin group)

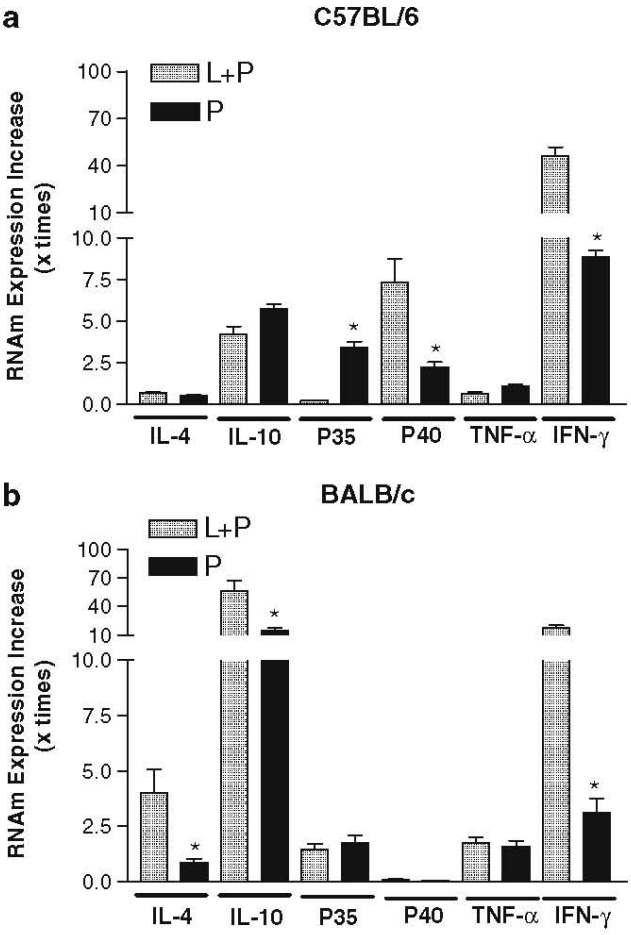

Fig. 4 **a** mRNA expression by RT-PCR of cytokines in C57BL/6 mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each *bar* represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only.). Statistical difference was noted by 21 days (*Bars*, mean±SD; *asterisk* $p<0.05$ compared to L+P group). **b** mRNA expression by RT-PCR of cytokines in BALB/c mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each *bar* represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only. Statistical difference was noted by 21 days (*Bars*, mean±SD, *asterisk*, $p<0.05$ compared to L+P group)

observed over the first 3 weeks of infection with a peak at 21 days. In fact, these results were the same observed by Tafuri et al. (2000) [18] following the same experimental protocol. The increase in parasite burden due to paraffin implantation was associated with an intense and diffuse chronic inflammatory reaction, with macrophages heavily loaded with parasites. Moreover, NAG activity was higher at 21 days in the inflammatory capsule of C57BL/6 mice than BALB/c. However, this increase in parasites was transient and these mice subsequently began to resolve their infections. In fact, 30 days after infection, the number of parasites in their lesions begun to be reduced relative to those in the BALB/c mice.

C57BL/6 mice produce IFN-γ, TNF-α and IL-12 in response to *Leishmania* infection, and are able to resolve their infection and develop long-lasting immunity. BALB/c mice, in contrast, produce IL-4 and IL-10 rather than IFN-γ and fail to resolve their lesions, ultimately succumbing to fulminating infection [15, 23]. In this work, C57BL/6 implanted with paraffin concurrently with *L. major* infection showed a marked expression of IFN-γ mRNA by 25 times greater than controls. Small amounts of IL10 (10 ng/ml) inhibit IL-12, TNF-α, and IFN-γ production by macrophages and also render the macrophages refractory to the activating effect of IFN-γ have shown by Kane and Mosser [24]. In consequence, IL-10 prevents macrophages from eliminating intracellular parasites. IL-10 mRNA was also expressed concomitantly with IFN-γ expression. In addition to macrophages, one subset of CD4+CD25-Foxp3-Th1 cells with IFN-γ production produces high amounts of IL-10. IL-10 derived from these Th1 cells and macrophages play a pathologic role in the pathogenesis of *Leishmania* infection, as reported by Anderson [25]. Thus, our data are consistent with these earlier studies and could explain the increased tissue parasite load at 21 days (transient susceptibility), related to higher IL-10 mRNA expression at the site of

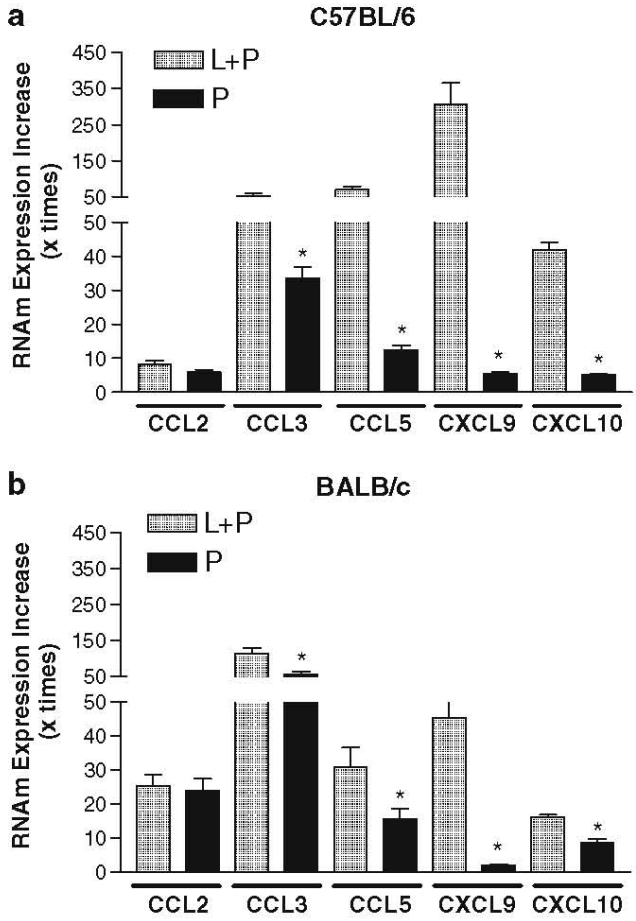

Fig. 5 **a** mRNA expression by RT-PCR of chemokines in C57BL/6 mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each *bar* represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only. Statistical difference was noted by 21 days (*Bars*, mean±SD; *asterisk*, $p<0.05$ compared to L+P group). **b** mRNA expression by RT-PCR of chemokines in BALB/c mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each *bar* represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only. Statistical difference was noted by 21 days (*Bars*, mean±SD; *asterisk*, $p<0.05$ compared to L+P group

infection, probably without changing the systemic Th1 profile in C57BL/6. In contrast, BALB/c mice showed strong IL-10 mRNA expression by 51 times higher than controls with very little expression of IFN-γ; IL-4 message was also mainly detected in BALB/c mice. IL-4 production induces Th2 differentiation and progressive leishmaniasis in susceptible mice, but the persistence of infection in these mice could be related to an overproduction of IL-10, which inhibits Th1 cells, and not by exacerbation of the Th2 response [26]. In addition to the Th2 cell-polarizing conditions that underlie the susceptibility of BALB/c mice to cutaneous *Leishmania* infection, overproduction of endogenous IL-10 by the host has been well recognized as a central factor that compromises Th1 response to efficiently clean parasites, not only in a variety of mouse models but also in human beings.

On the other hand, IL-12 is essential for an effective polarization of naïve Th cells to the Th1 phenotype characterized by the expression of IFN-γ. IL-12 is composed of two subunit, p35 and p40. IL-12p40 expression is readily detectable in C57BL/6 strain with higher expression in paraffin-*Leishmania* group than paraffin one. However, IL-12p40 expression is hardly detected in BALB/c strain. Scharton-Kersten and Scott found that BALB/c mice lose the capacity to produce IL-12 in the initial phases of *L. major* infection owing to genetic defect [27]. Furthermore, IL-12p40 reduction can also be due to the inhibitory effect of Fcγ-mediated signaling induced by interaction of opsonized parasites on the macrophages [22].

Matte and Olivier found that *L. major* is a strong inducer of an early inflammatory response, and this immunological scenario could potentially restrain the parasite to the inoculation site, favoring the development of local swelling and cutaneous lesions [28]. In our paraffin model, we found increased expression of all chemokines investigated in the skin inflammatory capsule tissue (CCL2, CCL3, CCL5, CXCL9 and CXCL10), especially those induced by IFN-γ and directly involved in the Th1 response, such as CCL3/MIP1-α, CXCL9/MIG and CXCL10/IP-10. These last two chemokines are chemoattractants for CXCR3 T lymphocytes [13]. The onset of *Leishmania donovani*-induced hepatic inflammation and granuloma formation was found to be delayed in CXCR3-/-C57BL/6 mice [29]. The increased expressions of CXCL9/MIG and CXCL10/IP-10 may attract regulatory T cell populations with subsequent IL-10 production at the site of inflammation [30, 31].

CCL5/RANTES up-regulates IL-12, IFN-γ and migration of Th1 cells, particularly memory T cells to the site of infection [32, 33]. Here, we observed significantly greater production of the chemokine CCL5/RANTES in the skin capsules of C57BL/6 mice implanted with paraffin concurrently with experimental infection with *L. major* promastigotes compared to mice that had received only paraffin implantation. ELISA assays (data not shown) revealed a notably greater production of CCL5/RANTES in the initial phases of experimental infection (7 days), especially in mice with both paraffin implantation and *L. major* infection. Consistently with the kinetics of this experimen-

tal protocol (LP) in C57BL/6 mice, the CCL5/RANTES mRNA levels decreased by 21 days and fell to the levels found in non-infected mice by 30 days. Thus, the decrease in CCL5/RANTES might be directly correlated with the inflammatory response at the site of experimental *Leishmania* infection in C57BL/6 mice. Santiago and co-authors have demonstrated that treatment with Met-RANTES or anti-CCL5/RANTES rendered C57BL/6 animals more susceptible to *L. major* and skewed the immune response from type 1 to type 2 by diminishing IFN-γ production by draining lymph node cells and increasing IL-4 mRNA expression in the lesions [33].

CCL2/MCP-1 is known to attract monocytes, DCs, natural killer cells, and memory T lymphocytes [32]. It has been suggested that CC chemokine receptor 2 (CCR2), the receptor that binds CCL2/MCP-1, increases monocyte recruitment, whereas CCL2/MCP-1 plays a variety of roles in host defense against *Leishmania* [34–36]. It has been reported that high doses of CCL2/MCP-1 activate anti-*Leishmania* macrophage killing mechanisms, either directly by inducing reactive oxygen intermediates [6], or indirectly via nitric oxide production [37]. Recently, a subset of CCR2-positive monocytes has been identified and shown to migrate into inflammatory sites. This population of cells may play a role in pathogen clearance [16]. Here, C57BL/6 mice showed low levels of CCL2/MCP-1 at 21 days after infection. Thus, the lower levels of CCL2/MCP-1 detected in C57BL/6 mice may be consistent with the intense tissue parasite load in the skin capsules. In fact, Conrad and others, working with CCL2/MCP-1 transgenic *L. major* promastigotes, considered that CCL2/MCP-1 is the main chemoattractant recruiting a restrictive CCR2-positive macrophage subset to the site of infection in resistant mouse strains [38]. These macrophages may assist in resolving the lesions when coactivated with CCL2/MCP-1, whereas the lack of CCL2/MCP-1 production may preclude any such recruitment of CCR2-positive monocytes/macrophages in susceptible BALB/c mice. In our work, BALB/c mice showed no statistical difference from controls in CCL2/MCP-1 production. It has been reported in humans that CCL2/MCP-1 is present in self-healing lesions but absent in non-healing diffuse cutaneous leishmaniasis [9].

Ritter and Heinrich-Korner described divergent expression of inflammatory dermal chemokines in self-healing localized cutaneous leishmaniasis and progressive diffuse cutaneous leishmaniasis [9]. LCL is characterized by strong expression of Th1-associated chemokines such as CCL2/MCP-1, CXCL9/MIG and CXCL10/IP-10, and small amounts of CCL3/MIP-1α. Chronic DCL is associated with the opposite expression pattern: CCL3/MIP-1α is dominant and CCL2/MCP-1, CXCL9/MIG and CXCL10/IP-10 are expressed at low levels. In our model, C57BL/6 mice concurrently implanted with paraffin and infected with *Leishmania* dominantly expressed CXCL9/MIG whereas in BALB/c mice, CCL3/MIP-1 is the highest expressed chemokines. In parallel with Th1-phenotype in C57BL/6 and Th2-phenotype in BALB/c as demonstrated by the expression pattern of cytokines and chemokines, the lesion in C57BL/6 is transient and healed after 30 days whereas BALB/c mice have a persistent and non-healing lesion. It has been suggested that immigrating monocytes–macrophages act more like host cells that are permissive for *Leishmania* growth than as effector cells that restrict parasite replication [17]. This permissiveness exists even in the face of the intense inflammatory reaction that occurs in response to paraffin. In fact, Tafuri et al. (2000) [18], in a morphological study, showed that *L. major* lesions were more intense when induced along with the inert inflammatory agent. In both the BALB/c and C57BL/6 strains of mice, an early increase in the numbers of parasites in lesions accompanied this inflammation. This increase in parasite numbers in the lesion was observed over the first 3 weeks of infection. In this present study, we have attempted to determine the immunological response in C57BL/6 during this transiently susceptibility picture related to the highest tissue parasite load. We conclude that the Th1 immune response C57BL/6 did not change to a Th2 response, even though C57BL/6 animals presented higher parasitism than BALB/c mice 21 days after infection and paraffin implantation. Importantly, our models of C57BL/6 mice implanted with paraffin and infected with *Leishmania* vs. BALB/c strain with same manipulation will be useful models to mimic self-healing localized cutaneous leishmaniasis (LCL) vs. progressive diffuse cutaneous leishmaniasis (DCL) in human beings.

Conflict of interest statement We declare that we have no conflict of interest.

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Artigo 2 - Profile of histological and immunological chronic inflammation stimulated by Leishmania major in a murine sponge model.

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Profile of histological and immunological chronic inflammation stimulated by Leishmania major in a murine sponge model

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Second page:

Summary

A model of chronic inflammation consisting of subcutaneous implantation of polyether-polyurethane sponge in mice experimentally infected with *Leishmania major* was developed. Given the importance of cytokines as mediators of inflammation processes, the aim was to evaluate the recruitment of monocytes and macrophages in a sponge implantation model in mice susceptible and resistant to *L. major* infection. The mice were divided into three groups: mice implanted with sponge (S group)*;* mice implanted with sponge and experimentally infected with *L. major* (S+L group); mice subjected to surgery to be used as controls for Real-Time Polymerase Chain Reaction (RT-PCR). Sponges were collected for histopathological and RT-PCR studies. Expression of cytokines (IFN-γ, TNF-α, IL-4 and IL-10) and chemokines (CCL2 and CCL5) were analyzed after 7, 21 and 30 days. Both experimental groups produced an intense chronic inflammatory reaction characterized by a diffuse cellular exudate of macrophages, lymphocytes, neutrophils and eosinophils. In C57BL6 mice, the mononuclear cells organized into nodules, demonstrating granulomatous formation. The S+L group had higher levels of CCL5/RANTES mRNA in C57BL/6 mice and higher levels of CCL2/MCP-1 mRNA in Balb/c mice than the S group. IFN-γ mRNA was increased in both strains of mice after sponge implantation concurrent with *Leishmania* infection. C57BL/6 mice have a resistant immunological phenotype characterized by higher expression levels of IFN-γ than in Balb/c mice. It is concluded that this immunologically resistant phenotype was confirmed by the histological results presented herein and the presence of granulomas formation in C57BL/6 mice.

Keywords

Leishmania major, Sponge Implantation, Chronic Inflammation, Cytokines, C57BL/6 and BALB/c mice

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Introduction:

Leishmania is an intracellular protozoan parasite introduced into host tissues by infected phlebotomine sand flies. It is subsequently phagocytosed by tissue macrophages stimulated to produce antimicrobial molecules by interferons (Diefenbach, *et al.*, 1998). IFN-γ is produced in large amounts by activated Th1 cells, enhancing anti-*Leishmania* activity at the site of infection (Liew, *et al.*, 1990).

Under laboratory conditions, the majority of mouse genotypes including C57BL/6 mice control *L. major* infection, but certain strains such as Balb/c mice fail to control infection and develop progressive lesions and systemic disease. The susceptibility or resistance to *L. major* infection in mice correlates with the dominance of an IL-4 and IL-10 driven Th2 response that causes disease, or an IL-12, IFN- γ and TNF-α dominated Th1 response that promotes healing and parasite clearance (Sacks and Noben-Trauth, 2002, Scott, 1991).

Leishmania sp. infection induces the expression of various cytokine and chemokine genes (Brenier-Pinchart, *et al.*, 2001, Racoosin and Beverley, 1997, Ritter and Körner, 2002). Chemokines are small chemotactic cytokines responsible for coordinating leukocyte trafficking with immune cell differentiation and effector functions (Farber, 1997). The chemokine RANTES (CCL5) is a chemoattractant for monocytes and T cells, and is expressed by several cell types including T cells, macrophages, fibroblasts and endothelial cells (Danoff, *et al.*, 1994, Marfaing-Koka, *et al.*, 1995, Rathanaswami, *et al.*, 1993, Schall, *et al.*, 1988) CCL5 up-regulates IL-12 (Aliberti, *et al.*, 2000) and IFN-gamma (Makino, *et al.*, 2002). CCL2 is induced after infection with *L. major* (Racoosin and Beverley, 1997, Vester, *et al.*, 1999) and can stimulate the killing of *L. major* by human monocytes (Ritter and Moll, 2000). Localized cutaneous leishmaniasis lesions have high expression levels of Th-1-associated chemokines such as CCL-2.

Recently, using a model of monocytic inflammation subcutaneous paraffin tablet implantation after *L. major* infection described by Tafuri et al., our group demonstrated that C57BL/6 and Balb/c strains of mice had an intense inflammatory reaction associated with high macrophage infiltration and high parasite load (Letícia Costa Reis, *et al.*, 2010, Tafuri, *et al.*, 2000).

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A subcutaneous sponge matrix model has been used to facilitate the assessment of angiogenesis, cellular proliferation (Andrade*, et al.*, 1987), cytokine production kinetics and other components of inflammation. This inert irritant induces the recruitment of monocytes to the site of inflammation and the localized immobilization of macrophages in the sponge matrix.

Given the importance of cytokines as mediators of inflammatory processes, the aim of this study was to evaluate the recruitment of monocytes and macrophages using the sponge implantation model in mice susceptible and resistant to *L. major* infection. Cytokine (IFN-γ, TNF-α, IL-4 and IL-10) and chemokine (CCL2 and CCL5) expression kinetics were analyzed in C57BL/6 and Balb/c mice.

Methods:

Subcutaneous implantation of sponge disks

The implanted material was 5 mm thick x 8 mm diameter disk-shaped polyether-polyurethane sponge (Vitafoam Ltd., Manchester, UK) that had been incubated overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 20 min before implantation. Mice were anesthetized by intra-peritoneal injection of 4 µl g^{-1} of a mixture containing ketamine (150 mg kg^{-1}) and xylazine (10 mg kg^{-1}), and their dorsal hair was shaved and their skin wiped with 70% ethanol in preparation for implantation. The sponge disks were aseptically implanted into a subcutaneous pouch, made with curved artery forceps through a one cm long dorsal mid-line incision. Post-operatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress.

Mice

Eighty-four mice, 4-6 weeks old, obtained from the animal facility at ICB/UFMG, were divided into two groups according to strain: forty-two C57BL/6 and forty-two BALB/C mice. These were further subdivided in three groups:

Group 1 (S): comprised mice implanted with sponge (n=36; 18 of each mouse strain);

Group 2 (S+L): comprised mice implanted with sponge and infected with promastigotes immediately thereafter (n=36; 18 of each mouse strain);

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Group 3 (Group Sham – "Wound Healing Surgery Group"): comprised twelve mice that were subjected to surgery (skin incision) and the wound was stitched (wound healing). (n=12; six of each mouse strain

The animals were sacrificed at 7, 21 and 30 days.

Day 7 – 18 mice were distributed between groups 1, 2 and 3 (six mice in each group)

Day 21 – 12 mice were distributed between groups 1 and 2 (six mice in each group)

Day 30 – 12 mice were distributed between groups 1 and 2 (six mice in each group)

Experimental Infection

C57BL/6 mice were injected with 1 x 10^6 *L. major* promastigotes in 100µl (subcutaneous) into the sponge implant immediately after implantation. BALB/c mouse experiments followed the same protocol.

Parasites

The Friendlin strain of *Leishmania major* clone IV (MHOM/IL/80/Friedlin) was used in all experiments. Promastigotes were grown at 25ºC in Grace's insect cell culture medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum, glutamine (2mM), penicillin G (100 U/ml) and streptomycin (100 µg/ml). Stationary-phase promastigotes, obtained from 7-9 day cultures, were used for all experimental infections.

Histopathology

Six animals from each group were sacrificed and the sponge implants carefully excised, dissected free of adherent tissue, fixed in 10% buffered formalin, dehydrated, cleared, embedded in paraffin, cut (5µm thick) and stained with Hematoxylin and Eosin (H&E).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Sponges were collected and total RNA was extracted using a single-step phenol/chloroform procedure (TRIZOL®) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, tissue was soaked with TRIZOL solution and homogenized using a PowerGen Generator (Fisher Scientifics, Franklin, MA, USA). Chloroform (0.25 ml) was added to 1 ml of TRIZOL homogenate and mixed thoroughly. After centrifugation, the upper aqueous phase was

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transferred to a fresh tube and 0.7 ml of isopropyl alcohol was added to precipitate the RNA. The RNA pellet was washed with 70% ethanol and air-dried for a maximum of 10 min at room temperature. Contaminating genomic DNA was eliminated by dissolving the RNA pellet in 10 mM Tris-HCl, pH 7.5 containing RNase-free DNase and 1 mM $MgCl_2$ and incubating for 30 min at room temperature. TRIZOL® solution was added to stop the reaction and the total RNA was further purified as described above. The final RNA pellet was dissolved in RNase-free water and the A260/280 ratio was measured to evaluate quality (Yang, *et al.*, 2007).

First-strand cDNA Synthesis by Reverse-transcription Reaction: First strand cDNA was generated using ThermoScript® Reverse Transcriptase (Invitrogen,). Five micrograms of genomic DNA-free total RNA and oligo (dT) 20 primers were used; the detailed procedure is available in the manufacturer's instructions (Invitrogen).

Quantitative-PCR: Performed using a LightCycler®480 Real-time PCR System (Roche Applied Science, Indianapolis, IN, USA) with SYBR Green PCR reagents purchased from BioRad. Melting curve analyses were carried out to ensure that a single product with the expected melting curve characteristics was obtained. The relative differences among samples were analyzed using the ΔΔCt method described previously. A ΔCt value was determined for each sample using the Ct value for GAPDH to normalize loading differences. A ΔΔCt value was obtained by subtracting the ΔCt value for the mice subjected only to the surgery (group sham - without sponge and *Leishmania* infection) from the corresponding experimental ΔCt. The fold difference over the value for the sham mice was calculated as $2^{\Delta\Delta Ct}$.

Statistical Analysis

ANOVA (Tuckey) tests were carried out for all differences in the responses of the groups. Student's *t* test was carried out for paired groups ($p < 0.05$ was considered statistically significant).

Results

Histology

Group I (S): Mice implanted with sponge

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Both strains of mice presented with a moderate chronic inflammatory reaction around the sponge matrix after seven days, characterized by a cellular exudate composed of macrophages, lymphocytes and plasma cells, and some neutrophils. Discrete granulation tissue characterized by the formation of small capillaries was also evident. However, after 21 and 30 days an intense and diffuse chronic inflammatory reaction was observed in both strains of mice. Prominent formation of new blood vessels and fibroblast proliferation with mature collagen deposition was observed at 30 days (Figs. A, C, E, G)

Group II (S+L): Mice implanted with sponge and infected with Leishmania

The sponge was implanted and the mice immediately inoculated with parasites adjacent to the implantation site. After 7, 21 and 30 days, both strains of mice demonstrated an intense chronic inflammatory reaction with a diffuse cellular exudate containing macrophages, lymphocytes, and some neutrophils and eosinophils. Macrophages loaded with amastigotes of *Leishmania* could be observed in all animals. New blood vessel formation, edema and loose collagen tissue with discrete fibroblast proliferation were formed as granulation tissue, particularly evident after 21 and 30 days. Moreover, giant cell formation was observed in both strains of mice (Figs B, D, F, H). However, there were marked differences in the lesions from the two strains of mice by 21 and 30 days after implantation. In C57BL6 mice the mononuclear cells were organized into nodules, a characteristic of granuloma formation (Figure 2A-H). These were composed of macrophages and lymphocytes, and some macrophages appeared with peculiar epithelioid cells with an ellipsoid morphology, hypochromatic nucleus (fine granular chromatin) and eosinophilic cytoplasm.

Cytokine and Chemokine mRNA Expression

Expression of cytokines (IFN-γ and TNF-α) associated with the resistant phenotype to *L. major* infection (C57BL/6 mice) and those associated with the susceptible phenotype (IL-4 and IL-10; BALB/c mice) were determined.

The relationship between chemokine expression and monocyte-macrophage recruitment (CCL2/MCP-1 and CCL5/RANTES) was investigated. For these studies, samples from BALB/c

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and C57BL/6 mice were obtained 7, 21 and 30 days after implantation concurrent with *Leishmania* infection.

The S+L group had higher levels of CCL5/RANTES mRNA than the S group at seven and 21 days after implantation in C57BL/6 mice, and at 7, 21 and 30 days in Balb/c mice. However, C57BL/6 mice expressed higher levels of CCL5/RANTES than Balb/c mice (Figure 3 A, B).

The S+L group had higher levels of CCL2/MCP-1 mRNA than the S group seven and 21 days after implantation in Balb/c mice, and seven and 30 days in C57BL/6 mice. Balb/c mice presented with higher levels of CCL2/MCP-1 than C57BL/6 mice (Figure 3C, D).

Figure 4 demonstrates that IFN-γ mRNA was increased in the two mouse strains 7, 21 and 30 days after implantation concurrent with *Leishmania* infection. C57BL/6 mice have a resistant phenotype marked by higher expression levels of IFN-γ than in Balb/c mice (Figure 4A, B). TNF-α mRNA expression was comparable in all groups (Figure 4C, D).

IL10 and IL-4 mRNA were detected at low levels relative to sponge implantation alone (group S) and were increased relative to the S+L group after 7, 21 and 30 days in Balb/c mice (Figure 5A, C). C57BL/6 mice produced lower levels of IL-10 and IL-4 than Balb/c mice, irrespective of whether the mice received both parasites and sponge implantation or sponge implantation alone (Figure 5B, D).

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Figure 3

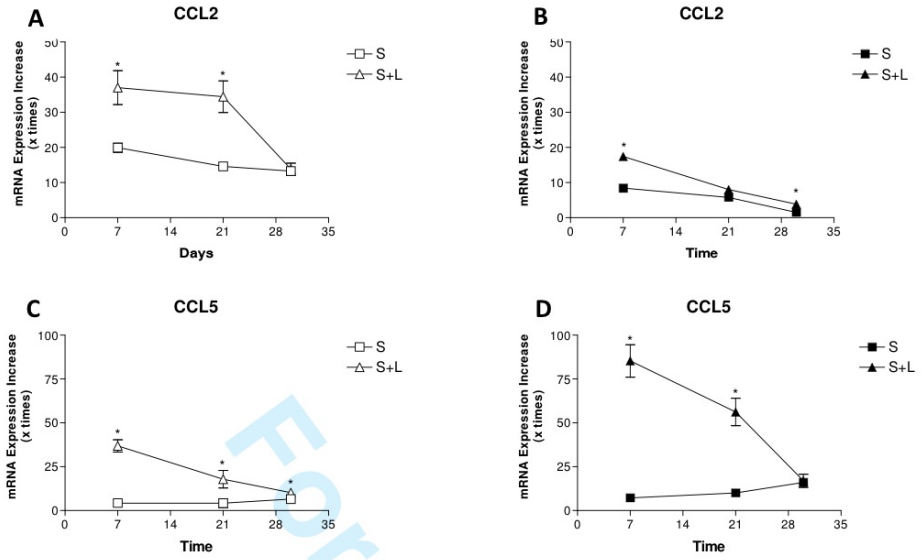

Figure 4

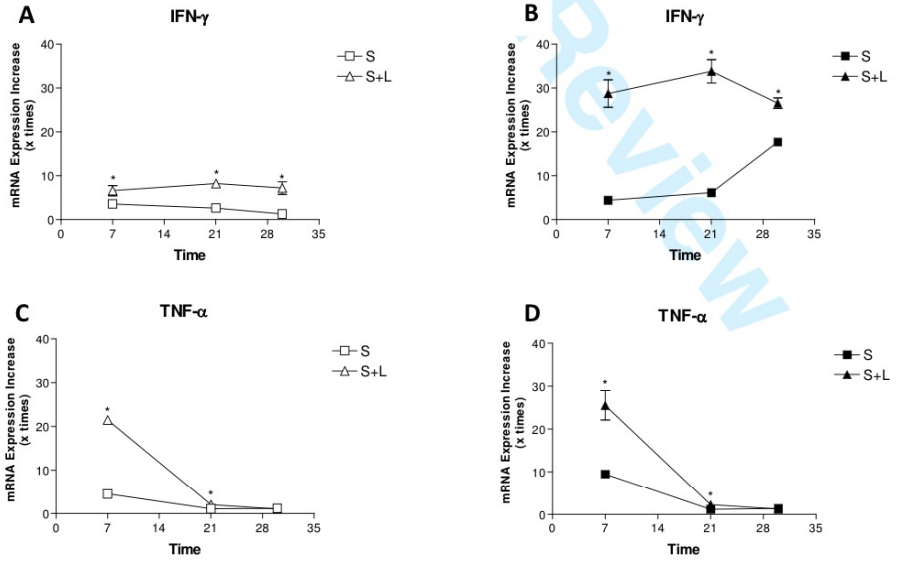

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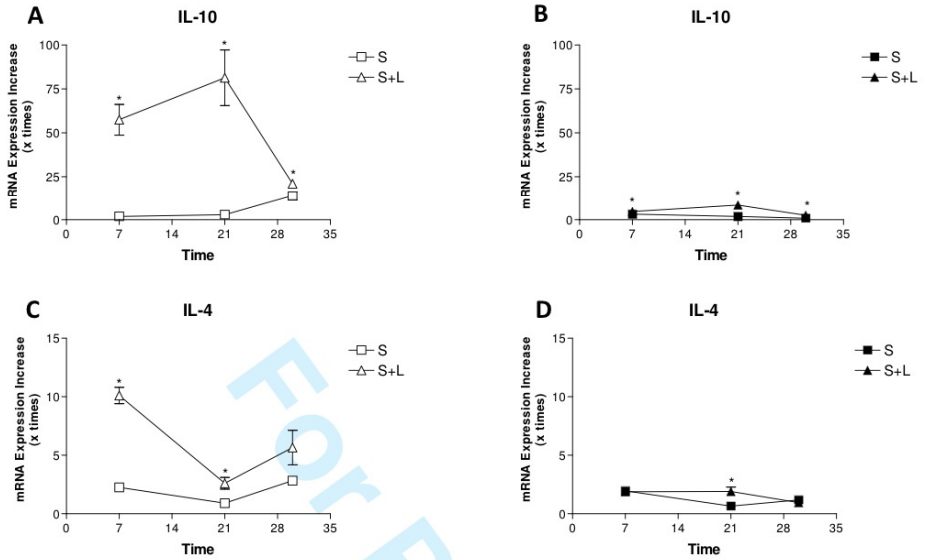
Figure 5



Discussion

Monocytes and macrophages were recruited in response to *Leishmania major*, and may be associated with the pattern of chemokine gene expression. This study demonstrated the kinetics of expression of CCL2/ MCP-1, CCL5 /RANTES, IFN-γ, TNF-α, IL-10 and IL-4 in response to *Leishmania major* infection.

CCL2/MCP-1 attracts monocytes, dendritic cells (DCs), natural killer cells and memory T lymphocytes (Aliberti, Reis e Sousa, Schito, Hieny, Wells, Huffnagle and Sher, 2000). It has been suggested that CC chemokine receptor 2 (CCR2), which binds CCL2/MCP-1, increases monocytes recruitment, whereas CCL2/MCP-1 plays a variety of roles in the host defense against *Leishmania* (Conrad*, et al.*, 2007, Murdoch and Finn, 2000, Sato*, et al.*, 2000). Balb/c and C57BL/6 mice had high levels of CCL2/MCP-1 after infection. Therefore, the higher levels of CCL2/MCP-1 detected in the present study may be consistent with the increase in accumulated macrophages observed during histological examination. It has been reported that high doses of CCL2/MCP-1 activate anti-*Leishmania* macrophage killing mechanisms, either directly by inducing reactive oxygen intermediates (Moll, 2000) or indirectly via nitric oxide

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production (Ritter and Moll, 2000). Recently, a subset of CCR2-positive monocytes that migrate into inflammatory sites has been identified. This population of cells may play a role in pathogen clearance (Gordon and Taylor, 2005). Conrad et al., researching CCL2/MCP-1 transgenic *L. major* promastigotes, considered CCL2/MCP-1 as the main chemoattractant, recruiting a restrictive CCR2-positive macrophage subset to the site of infection in resistant mouse strains (Conrad, Strauss-Ayali, Field, Mack and Mosser, 2007). These macrophages may assist in resolving the lesions when co-activated with CCL2/MCP-1, whereas the lack of CCL2/MCP-1 production may preclude such recruitment of CCR2-positive monocytes/ macrophages in susceptible BALB/c mice. In the present study, BALB/c infected mice were statistically different from controls in terms of CCL2/MCP-1 production at all time periods analyzed, and produced high levels compared with C57BL/6 mice. In humans it had been reported that CCL2/MCP-1 is present in self-healing lesions but absent in non-healing diffuse cutaneous leishmaniasis (Ritter and Körner, 2002).

Resistance to *Leishmania* is highly dependent on a Th1 response (Sacks and Noben-Trauth, 2002). CCL5 up-regulates IFN-γ and migration of Th1 cells, particularly memory T cells, to the site of infection (Makino, Cook, Smithies, Hwang, Neilson, Turka, Sato, Wells and Danoff, 2002). Treatment with Met-RANTES or anti-CCL5 rendered C57BL/6 mice more susceptible to *L. major* and skewed the immune response from a type one to a type two by diminishing IFN-γ production by increasing IL-4 mRNA expression in lesions (Santiago*, et al.*, 2004). Herein, significantly greater production of CCL5/RANTES and IFN-γ was evident in C57BL/6 mice implanted with sponge concurrently with experimental infection with *L. major* promastigotes, compared with mice that had received only the sponge implant.

Mosser and colleagues reported that macrophages activated by IFN-γ, classically activated macrophages, have microbicidal activity (Mosser, 2003). Thus, C57BL/6 mice produce IFN-γ and TNF-α in response to *Leishmania* infection, and resolve the infection and develop long-lasting immunity. In contrast, BALB/c mice produce IL-4 and IL-10 rather than IFN-γ and fail to resolve the lesions, ultimately succumbing to infection (Sacks and Noben-Trauth, 2002,

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Scott, 1991). In this work, C57BL/6 and Balb/c mice implanted with sponge concurrently with *L. major* infection presented with a statistically different expression of IFN-γ mRNA from controls where C57BL/6 mice had marked expression of IFN-γ mRNA compared with Balb/c mice. This immunological result must be directly correlated with granulomatous formation observed during histological examination. In fact, under histological analysis C57BL/c infected mice implanted with sponge showed notable granulomas formation in comparison to Balb/c mice. In literature we can found that IFN-γ treatment induces a granulomatous tissue reaction in localized cutaneous Leishmaniasis (Haas, *et al.*, 2002).

Low levels of IL-10 (10 ng/ml) inhibit TNF-α and IFN-γ production by macrophages and also render the macrophages refractory to the activating effect of IFN-γ (Kane and Mosser, 2001). Therefore, IL-10 prevents macrophages from eliminating intracellular parasites. The data presented herein demonstrate an increased expression of IL-10 in infected mice at all time points analyzed, and Balb/c infected mice presented with higher expression than C57BL/6 mice. IL-4 production induces Th2 differentiation and progressive Leishmaniasis in susceptible mice, but the persistence of infection could be related to an overproduction of IL-10, which inhibits Th1 cells, and not due to exacerbation of the Th2 response (Anderson, et al., 2005). The data demonstrate increased IL-4 expression in Balb/c infected mice compared with C57BL/6 mice. However, C57BL/6 mice had increased IL-4 expression 21 days post infection.

Mosser and colleagues reported that macrophages activated by IL-4, alternatively activated macrophages, exhibit activities in the tissue repair process. IL-4 stimulates the activity of arginase in these macrophages, inducing the conversion of arginine into ornithine, the precursor of collagen, contributing to the production of extracellular matrix (Mosser, 2003, Mosser and Edwards, 2008). In addition, macrophages activated by an alternative pathway enhance fibrogenesis by regulating fibroblast proliferation and activation (Song, *et al.*, 2000). As we mentioned, we have found that IL-4 expression was higher in Balb/c infected mice than C57BL/6. Thus, we have start investigating possible correlation to the collagen deposition and this immunological pattern. In addition, Kodelja, et al. (1997) previously revealed that

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Legends

Figure 1A-H BALB/C mice with sponge implantation (A,C,E,G) and BALB/C mice implanted with sponge and infected with *Leishmania major*(B,D,F,H). (A) 7 days after implantation note the presence of a moderate chronic inflammatory reaction. Hematoxylin and Eosin, (Bar = 32 µm); (C,E) 21 and 30 days after implantation, respectively. Same magnification showing an intense chronic inflammatory reaction, Hematoxylin and Eosin. (Bar = 32 µm). (G) Higher magnification of (E) showing cellular exudate composed by macrophages and lymphocytes. Hematoxylin and Eosin. (Bar = 16 µm). (B,D,F) 7, 21 and 30 days after implantation and experimental *Leishmania* infection. In all time points note a higher intensity of the chronic inflammatory reaction than observed in A, C and E. Hematoxylin and Eosin, (Bar = 32 µm). (H) Observe macrophages parasitized with intracellular amastigotes of *L. major* (*arrows*). Giant cell formation nearby sponge fragment matrix could be seen (arrowhead). Hematoxylin and Eosin, (Bar = 16 µm). SM (Sponge Matrix); BV (Blood Vessels)

Figure 2A-H C57BL/6 mice implanted with sponge and infected with *Leishmania*. (A) 7 days after implantation and the experimental infection. Panoramic picture has shown an intense chronic inflammatory reaction. Hematoxylin and Eosin (Bar = 64µm); (B) Higher magnification showing a tendency of the cellular exudate forming a nodular arrangement (arrowheads). Hematoxylin and Eosin (Bar = 32µm); (C-F) 21 and 30 days after implantation and experimental infection. Higher magnification showing a chronic inflammatory reaction where the mononuclear exudate showed a tendency of nodular or ellipsoid arrangement (arrowheads) characterizing granulomas formation. Hematoxylin and Eosin. C,E (Bar = 64 µm); F (Bar = 32 µm); D,G,H (Bar = 16 µm). SM (Sponge Matrix); BV (Blood Vessels)

Figure 3 Kinetics of chemokine mRNA expression by RT-PCR of CCL2 (3A-3B) and CCL5 (3C-3D) in Balb/c (3A-3C) and C57BL/6 (3B-3D) mice. Sponges implanted were obtained from 7, 21 and 30 days after the infection. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group Sham (considered zero - axis graph). S+L represents mice with the sponge implantation and infection with *L. major*; *S* represents mice with sponge implantation only. Values shown are the means (±SEM) from groups of 6 animals. * $p < 0.05$ versus sponge implantation only.

Figure 4 Kinetics of cytokine mRNA expression by RT-PCR of IFN-γ (4A-4B) and TNF-α (4C-4D) in Balb/c (4A-4C) and C57BL/6 (4B-4D) mice. Sponges implanted were obtained from 7, 21 and 30 days after the infection. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group Sham (considered zero - axis graph). S+L represents mice with the sponge implantation and infection with *L. major*; *S* represents mice with sponge implantation only. Values shown are the means (±SEM) from groups of 6 animals. * $p < 0.05$ versus sponge implantation only.

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Figure 5 Kinetics of cytokine mRNA expression by RT-PCR of IL-10 (5A-5B) and IL-4 (5C-5D) in Balb/c (5A-5C) and C57BL/6 (5B-5D) mice. Sponges implanted were obtained from 7, 21 and 30 days after the infection. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group Sham (considered zero - axis graph). S+L represents mice with the sponge implantation and infection with *L. major*; *S* represents mice with sponge implantation only. Values shown are the means (±SEM) from groups of 6 animals. * $p < 0.05$ versus sponge implantation only.

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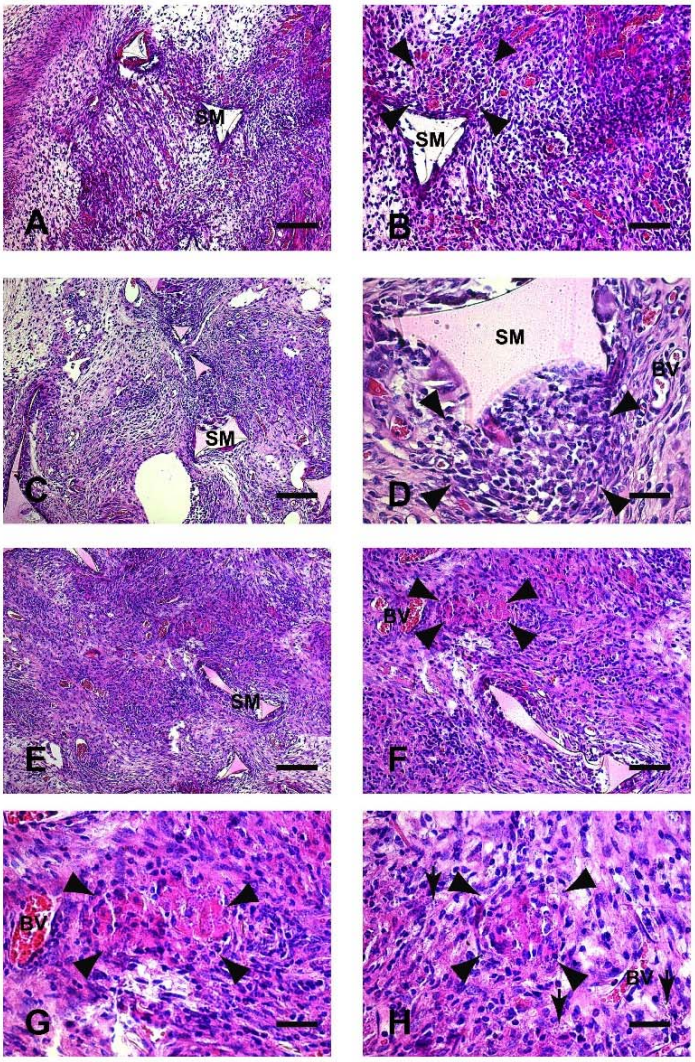

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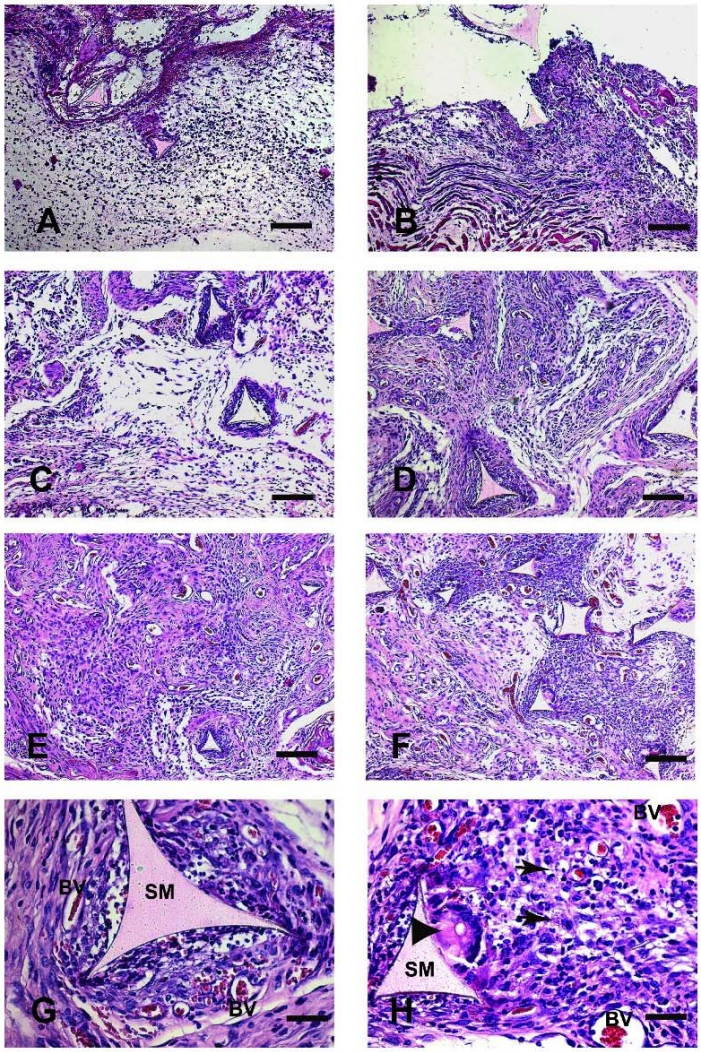

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DISCUSSÃO GLOBAL

Devido à importância dos monócitos-macrófagos na infecção por *Leishmania*, investigamos nesse trabalho as principais citocinas e quimiocinas envolvidas no recrutamento dessas células, em animais susceptíveis e resistentes à infecção experimental por *Leishmania major*, em dois modelos de inflamação crônica: (1) o modelo de implante de parafina (artigo 1); (2) o modelo de implante de esponja (artigo 2).

Tafuri e colaboradores, em um estudo morfológico, mostraram que as lesões induzidas por *L. major* foram mais intensas quando associadas a um agente inerte inflamatório: a parafina. Assim, baseado nesse modelo de inflamação crônica induzido pelo implante de um tablete de parafina, descrito por Raso (1976), foi observado que animais resistentes à infecção, C57BL/6, apresentaram maior parasitismo tecidual que animais susceptíveis, Balb/c. (Raso, 1976; Tafuri, Melo et al., 2000). Esses estudos foram também observados por Ferreira (2008), que avaliou o perfil imunológico desses animais no pico do parasitismo observado por Tafuri et al. (2000) aos 21 dias após o implante e infecção experimental por *L. major*. De fato, Ferreira (2008) relatou um aumento significativo de IL-10 nas cápsulas inflamatórias induzidas pelo implante de parafina nos animais C57BL/6, que coincidiu com o pico de parasitismo tecidual (Ferreira, 2008).

Como publicado no artigo 1 foi investigado o perfil histológico, parasitológico e imunológico, através da análise de citocinas (IL-4, IL-10, TNF-α, IFN-γ e IL-12) e quimiocinas (MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, CXCL10/IP10, CXCL9/MIG) envolvidas no recrutamento de monócitos-

macrófagos e linfócitos, tanto na resposta imunológica do tipo Th1 quanto na Th2. As avaliações foram realizadas no tempo de 21 dias após o implante de parafina concomitante com infecção por *L. major*. No artigo enviado para publicação (artigo 2) foi investigado o perfil histológico e imunológico, através da análise de citocinas (IL-4, IL-10, TNF-α e IFN-γ) e quimiocinas (MCP-1/CCL2 e RANTES/CCL5) envolvidas no recrutamento de monócitos-macrófagos e linfócitos, tanto na resposta imunológica do tipo Th1 quanto na Th2. As avaliações foram realizadas nos tempos de 7, 21 e 30 dias após o implante de esponja concomitante com infecção por *L. major*. O perfil parasitológico observado no modelo de implante de esponja foi semelhante ao modelo de implante de parafina, ou seja, animais C57BL/6 apresentaram maior parasitismo tecidual nos tempos de 21 e 30 dias (dados não mostrados).

Como é sabido da literatura animais C57BL/6 produzem IFN-γ, TNF-α e IL-12 em resposta a infecção por *Leishmania* e são capazes de resolver a infecção e desenvolver imunidade duradoura. Em contraste, animais Balb/c produzem IL-4 e IL-10 e falham na resolução das lesões sucumbindo à infecção fulminate (Scott, 1991; Sacks e Noben-Trauth, 2002). Mosser e colaboradores relataram que macrófagos ativados por IFN-γ, ou macrófagos ativados classicamente, apresentam atividade microbicida (Mosser, 2003) No artigo 1, C57BL/6 implantado com parafina e infectado por *L. major* mostrou marcante expressão RNAm de IFN-γ. No modelo de esponja, animais infectados de ambas as linhagens mostraram aumento da expressão de RNAm de IFN-γ nos três tempos analisados, sendo que, os animais C57BL/6 mostraram aumento expressivo dessa citocina em comparação aos Balb/c. Esse aumento na expressão de RNAm

de IFN-γ, pode ser correlacionado com a formação dos granulomas observados durante a análise histológica.

Ao contrário do papel protetor do IFN-γ na infecção experimental murina, é sabido da literatura, que a citocina IL-10 está relacionada à susceptibilidade no mesmo modelo. Pequenas quantidades de IL-10 inibem a produção de IL-12, TNF-α e do próprio IFN-γ, como mostrado por Kane e Mosser (Kane e Mosser, 2001). Em consequência, IL-10 impede a eliminação dos parasitos pelos macrófagos. Em ambos os modelos, RNAm de IL-10 também foi expresso concomitantemente com IFN-γ. Além dos macrófagos, um subconjunto de células Th1, CD4 + CD25-Foxp3, com produção de IFN-γ, produzem grandes quantidades de IL-10. IL-10 derivada dessas células Th1 e macrófagos apresentam papel patológico na infecção por *Leishmania* como relatado por Anderson (Anderson, Oukka et al., 2007) Assim, os dados observados no modelo de parafina são consistentes com os estudos anteriores e poderiam explicar o aumento da carga parasitária tecidual aos 21 dias (susceptibilidade transitória), relacionado com a maior expressão de RNAm de IL-10 no local da infecção, provavelmente sem alterar o perfil sistêmico Th1 em camundongos C57BL/6. Em contraste, camundongos Balb/c mostraram forte expressão de RNAm de IL-10, várias vezes maior que os controles, e muito pouca expressão de IFN-γ no modelo da parafina. A cinética da expressão de IL-10, observada no artigo 2, mostrou-se crescente após infecção nas duas linhagens de camundongos avaliadas, sendo que os animais Balb/c apresentaram maior expressão quando comparado aos C57BL/6.

RNAm de IL-4 também foi detectado, principalmente em camundongos Balb/c nos dois modelos utilizados. A produção de IL-4 induz diferenciação Th2 e leishmaniose progressiva em camundongos susceptíveis. Mosser e colaboradores relataram que macrófagos ativados por IL-4, ou seja, macrófagos ativados pela via alternativa, apresentam atividades no processo de reparo tecidual. IL-4 estimula a atividade da arginase nesses macrófagos, induzindo a conversão de arginina em ornitina, precursor de colágeno, contribuindo para a produção de matriz extracelular (Mosser, 2003; Mosser e Edwards, 2008). Além disso, macrófagos ativados pela via alternativa regulam a proliferação e ativação de fibroblastos (Song, Ouyang et al., 2000). Kodelja e colaboradores relataram que macrófagos ativados pela via alternativa induzem, in vitro, maior proliferação de células endoteliais, favorecendo assim a angiogênese, do que macrófagos ativados pela via clássica (Kodelja, Müller et al., 1997).

Além da diferenciação de células Th2, que fundamentam a susceptibilidade de camundongos Balb/c à infecção por *Leishmania*, excesso de produção endógena de IL-10, pelo hospedeiro, tem sido apontado como um fator central que compromete a resposta Th1 na eliminação de parasitos, não somente em modelos animais, como também em seres humanos (Anderson, Mendez et al., 2005).

Por outro lado, IL-12 é essencial para o direcionamento da diferenciação Th1, caracterizada pela expressão de IFN-γ. A IL-12 é composta de duas subunidades, p35 e p40. Expressão de IL-12p40 é facilmente detectável na linhagem C57BL/6 com maior expressão no grupo parafina e *Leishmania*. No entanto, a expressão de IL-12p40 dificilmente é detectada em Balb/c. Scharton-Kersten e Scott

descobriram que camundongos Balb/c perdem a capacidade de produzir IL-12 nas fases iniciais da infecção por *L. major* devido a defeito genético (Scharton-Kersten e Scott, 1995). Matte & Olivier descobriram que *L. major* é um forte indutor de resposta inflamatória precoce, e esse cenário imunológico poderia conter o parasita no local de inoculação, favorecendo o desenvolvimento local de lesões cutâneas (Matte e Olivier, 2002). Em ambos os modelos de inflamação estudados, encontramos aumento da expressão de todas as quimiocinas investigadas na cápsula inflamatória (CCL2, CCL3, CCL5, CXCL9 e CXCL10), principalmente aquelas induzidas por IFN-γ e diretamente envolvidas na resposta Th1, como CCL3 / MIP1-α CXCL9/MIG e CXCL10/IP-10. Estas duas últimas são quimiocinas atrativas de linfócitos T CXCR3. Uma maior expressão de CXCL9/MIG e CXCL10/IP-10 pode atrair populações de células T reguladoras, com subsequente produção de IL-10 no local da inflamação (Daly e Rollins, 2003; Peters e Sacks, 2006).

CCL5/RANTES regula positivamente IL-12, IFN-γ e a migração de células Th1, particularmente as células T de memória, para o local de infecção (Aliberti, Reis E Sousa et al., 2000; Santiago, Oliveira et al., 2004). No modelo de implante de parafina, observamos uma produção significativamente maior da quimiocina CCL5/RANTES nas cápsulas dos camundongos C57BL/6 implantados e infectados com formas promastigotas de *L. major*, em comparação aos camundongos que receberam apenas implante de parafina. Ensaio de ELISA (dados não apresentados) revelou uma produção maior de CCL5/RANTES nas fases iniciais da infecção experimental (7 dias), especialmente em camundongos com implante de parafina e infectados por *L. major*. No modelo de implante de

esponja observamos maior expressão de CCL5/RANTES nos animais infectados das duas linhagens, porém a expressão nos animais C57BL/6 foi mais exacerbada. Santiago e colaboradores demonstraram que o tratamento com Met-RANTES ou anti-CCL5/RANTES em C57BL/6 torna esses animais susceptíveis a *L. major* e altera a resposta imune do tipo 1 para tipo 2, diminuindo a produção de IFN-γ e aumentando a expressão de RNAm de IL-4 nas lesões (Santiago, Oliveira et al., 2004).

A CCL2/MCP-1 é conhecida por atrair monócitos, células dendríticas (DCs), células natural killer e linfócitos de memória (Aliberti, Reis E Sousa et al., 2000). Tem sido sugerido que o receptor da quimiocina CCL2 (CCR2), receptor que se liga CCL2/MCP-1, aumenta o recrutamento de monócitos, enquanto CCL2/MCP-1 desempenha uma variedade de papéis na defesa do hospedeiro contra *Leishmania* (Murdoch e Finn, 2000; Sato, Ahuja et al., 2000; Conrad, Strauss-Ayali et al., 2007). É relatado que altas doses de CCL2/MCP-1 ativam mecanismos de defesa anti-*Leishmania* dos macrófagos, quer por induzir diretamente espécies reativas de oxigênio (Moll, 2000), ou indiretamente através da produção de óxido nítrico (Ritter e Moll, 2000). Recentemente, um subconjunto de monócitos CCR2-positivo foi identificado e parece migrar para locais de inflamação. Esta população de células pode desempenhar um papel importante na eliminação de patógenos (Gordon e Taylor, 2005). No modelo de implante de parafina, camundongos C57BL/6 mostraram baixos níveis de CCL2/MCP-1, 21 dias após a infecção. Assim, os níveis mais baixos de CCL2/MCP-1 detectada em camundongos C57BL/6 pode ser consistente com a carga parasitária tecidual nas cápsulas. Na verdade, Conrad e outros, consideram

que CCL2/MCP-1 é o principal quimioatrativo no recrutamento de um subconjunto de macrófagos CCR2 positivo para o local da infecção nas linhagens mais resistentes (Brandonisio, Panaro et al., 2002). Estes macrófagos podem ajudar na resolução das lesões quando co-ativados com CCL2/MCP-1, enquanto a falta de produção de CCL2/MCP-1 pode impedir o recrutamento de monócitos/macrófagos, tais como CCR2-positivo, em animais susceptíveis, Balb/c. No artigo 1, camundongos Balb/c não apresentaram diferença estatística na expressão CCL2/MCP-1. No modelo de implante de esponja, observamos aumento da expressão de CCL2/MCP-1 principalmente durante a infecção aos 7 e 21 dias. Dados da literatura revelam em humanos, que CCL2/MCP-1 está presente nas lesões auto-resolutivas e ausente na leishmaniose cutânea não resolutiva (Ritter e Körner, 2002). Ritter & Heinrich-Korner descreveram divergências na expressão de quimiocinas inflamatórias na derme com lesão auto-resolutiva na leishmaniose cutânea localizada (LCL) e na leishmaniose cutânea difusa progressiva (DCL) (Ritter e Körner, 2002). LCL é caracterizada pela forte expressão de quimiocinas associadas à resposta Th1, tais como CCL2/MCP-1, CXCL9/MIG e CXCL10/IP-10, e pequenas quantidades de CCL3/MIP-1α. DCL crônica é associada a um padrão de expressão oposta: CCL3/MIP-1α é dominante e CCL2/MCP-1, CXCL9/MIG e CXCL10/IP-10 são expressos em níveis baixos. No modelo da parafina, camundongos C57BL/6 implantados e infectados com *Leishmania* expressam maior quantidade de CXCL9/MIG, enquanto que nos camundongos Balb/c, CCL3/MIP-1 é a quimiocina mais expressa. Em paralelo com fenótipo Th1 em camundongos C57BL/6 e fenótipo Th2 em camundongos Balb/c, como demonstrado pelo padrão de expressão de citocinas e quimiocinas, a lesão em camundongos

C57BL/6 é transitória e curada, depois de 30 dias, enquanto camundongos Balb/c apresentam lesões persistentes que não cicatrizam.

Tem sido sugerido que monócitos-macrófagos exerçam o papel principal de células hospedeiras, que permitem o crescimento da *Leishmania*, do que células efetoras, que restringem a replicação do parasito (Mosser e Edwards, 2008).

A introdução de um novo modelo de estudo, o modelo de implante de esponja, em nosso laboratório, veio sanar algumas dificuldades metodológicas enfrentadas no modelo de implante de parafina, como:

1. A esponja de poliéster-poliuretano não se desloca após o implante no subcutâneo dos animais, permanecendo no local da infecção durante todo o tempo de experimento;
2. A esponja de poliéster-poliuretano permite a esterilização em autoclave antes da utilização em animais;
3. O tecido de granulação formado dentro da esponja fornece maior quantidade de tecido, permitindo diferentes análises da mesma amostra, em comparação com a cápsula formada ao redor do tablete de parafina.

CONCLUÇÕES FINAIS

Com esse trabalho, concluímos que:

A utilização de um modelo de inflamação crônica, seja ele implante de parafina ou implante de esponja, juntamente com a infecção experimental por *Leishmania (Leishmania) major*, aumenta a expressão das principais citocinas e quimiocinas que fazem o recrutamento de monócitos-macrófagos e linfócitos para o sítio da infecção.

A presença de um agente inerte é capaz de modificar o parasitismo tecidual na infecção experimental por *Leishmania (Leishmania) major* em animais resistentes (C57BL/6), alterando a resistência nesses animais.

A perda da resistência temporária dos animais C57BL/6 parece não estar relacionada a modificações na expressão de RNAm das citocinas e quimiocinas estudadas, dos perfis Th1/Th2.

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ANEXOS

ANEXO 1 - Modelos de formato para Teses e Dissertações

1) Formato da Tese

A tese poderá ser apresentada sob dois formatos:

Formato 1 (clássico):

Texto redigido em português segundo as normas da ABNT contendo os seguintes itens:

- folha de rosto: título, nome do pós-graduando e orientador, especificação do nível e área de concentração, local e data
- ficha catalográfica
- agradecimentos e dedicatória – a critério dos autores
- sumário
- listas de tabelas, quadros, gráficos, figuras e abreviaturas e siglas
- resumo em português – uma página, espaço simples, sem parágrafos
- Introdução breve indicando a motivação do trabalho / Objetivos do trabalho / revisão da literatura pertinente ou Introdução a respeito da matéria incluindo revisão da literatura / Objetivos
- Material e Métodos
- Resultados
- Discussão
- Conclusões
- abstract – mesmos moldes do resumo
- Referências Bibliográficas
- Anexos – artigos já publicados ou enviados para publicação; planilhas de dados

quando couber; documento de aprovação no Comitê de Ética em Pesquisa da área. Será obrigatório: pelo menos um artigo publicado em periódico Qualis A Nacional (mínimo) e um artigo submetido para publicação com a carta de recebimento da revista. Os trabalhos que atendem às exigências mínimas não poderão ser utilizados para atender exigências de outras teses, nem podem ser relativos a trabalho anterior de mestrado.

Formato 2 (alternativo):

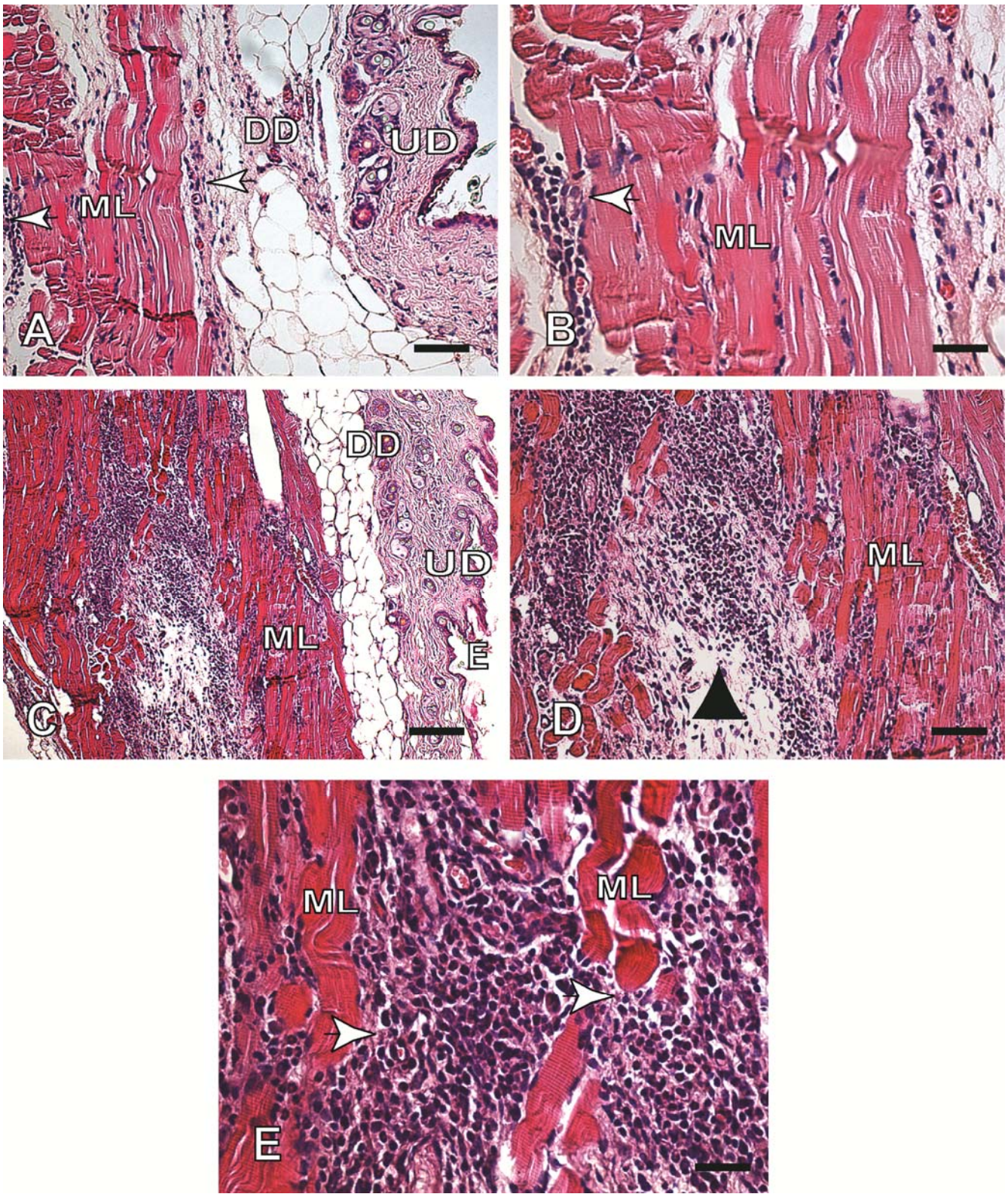
A tese poderá ainda ter um formato alternativo, com um mínimo de dois trabalhos, um aceite para publicação (carta de aceite) e um outro encaminhado para publicação (com carta de recebimento da revista), em que o estudante seja o primeiro autor. O Periódico deve ser pelo menos Qualis A Nacional. Estes trabalhos não poderão ser utilizados para atender exigências de outras teses, nem podem ser relativos a trabalho anterior de mestrado. O conjunto de trabalhos deverá ser precedido de uma introdução, objetivos do trabalho e revisão de literatura (abrangente) e seguido de uma discussão e conclusão globais, todas redigidas em português.

2) Formato da Dissertação

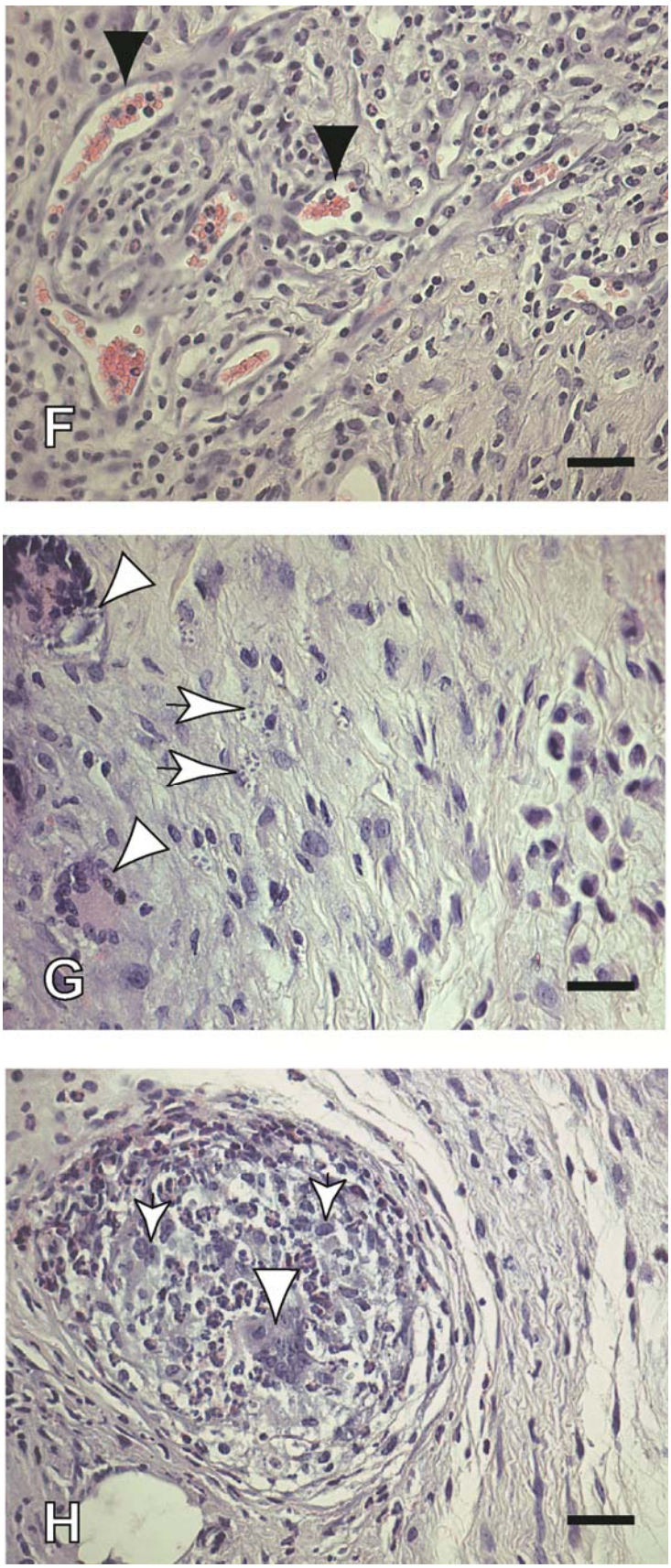
Texto redigido em português segundo as normas da ABNT nos mesmos moldes da Tese. O(s) anexo(s) deverão conter artigo em periódico pelo menos Qualis A nacional com carta de recebimento do periódico.

Formato alternativo: Facultado, nos moldes do formato alternativo para a tese, na eventualidade do pós-graduando ter publicado o trabalho.

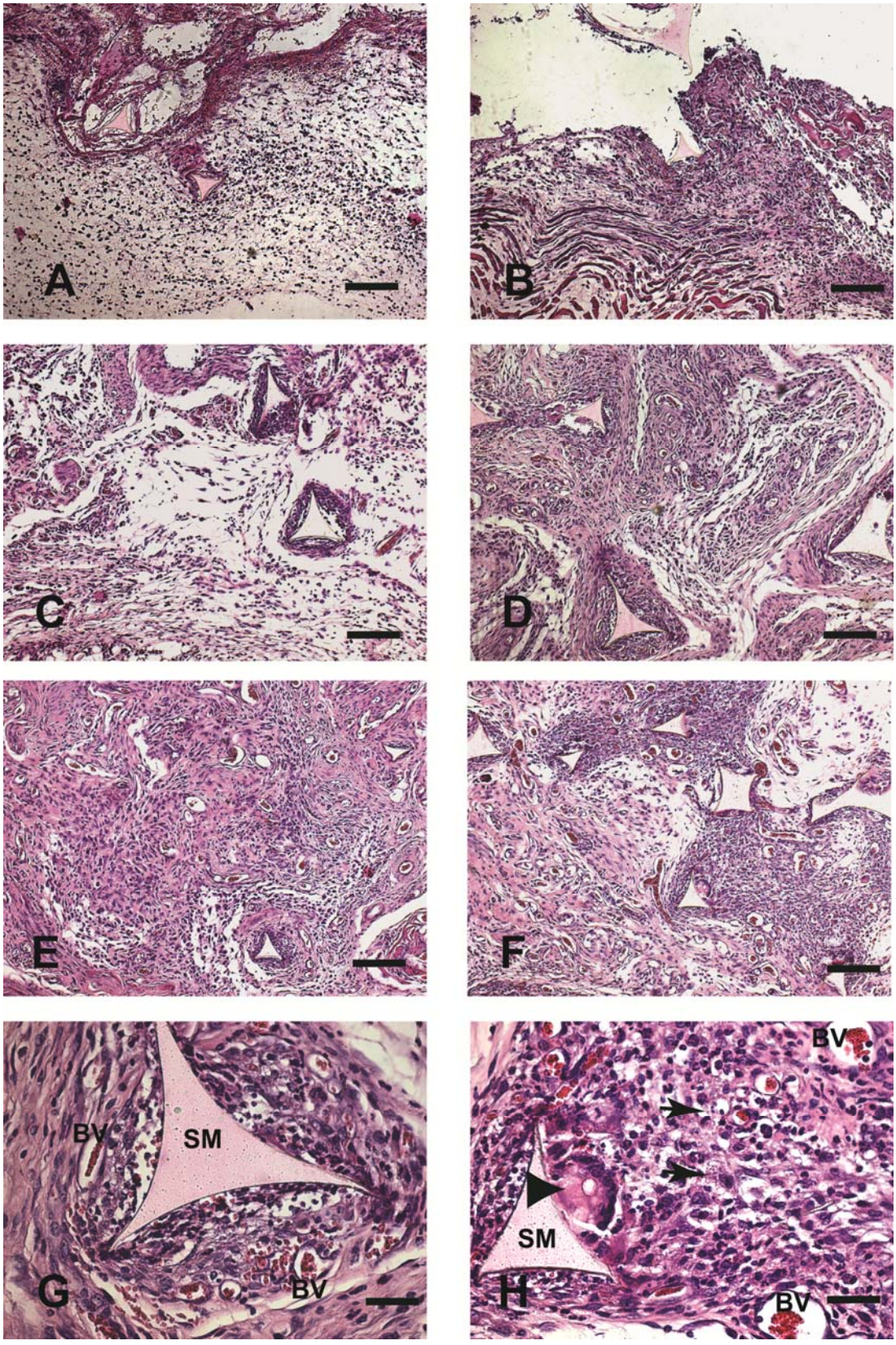
ANEXO 2 – Prancha 1 – Artigo 1



ANEXO 3 – Prancha 2 – Artigo 1



ANEXO 4 – Prancha 1 – Artigo 2



ANEXO 5 – Prancha 2 – Artigo2