UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

UFBA

FIOCRUZ

Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

CEPAS DE *LEISHMANIA BRAZILIENSIS* COM PERFIS GENÔMICOS DIVERSOS INDUZEM PROCESSOS INFLAMATÓRIOS DIFERENTES EM CAMUDONGOS BALB/C

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Salvador - Bahia - Brasil
2005

Ficha Catalográfica elaborada pela
Biblioteca do CPqGM/FIOCRUZ - Salvador - Bahia.

Teixeira, Maria Jania
T266c Cepas de *Leishmania braziliensis* com perfis genômicos diversos induzem processos inflamatórios diferentes em camundongos Balb/c [manuscrito] / por Maria Jania Teixeira. – 2005.
84 f. : il. ; 29 cm

Datilografado (fotocópia)
Tese (doutorado)- Universidade Federal da Bahia, Faculdade de Medicina. Centro de Pesquisas Gonçalo Moniz, 2005.
Orientador: Profª. Dra. Aldima Maria Prado Barral, Laboratório de Imunoparasitologia.

1. Leishmaniose cutânea. 2. *L. brasiliensis*. 3. Quimiocinas. 4. Balb/c. I. Título.

CDU 616.928.5:599.233

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Esta tese é dedicada *in memoriam* a meu pai, **Joaquim Américo Teixeira**, um farmacêutico competente e dedicado, com quem, ainda na infância, por trás dos velhos balcões de sua farmácia, aprendi o que era leishmaniose.

AGRADECIMENTOS

Dra. Aldina Barral, pela inestimável orientação científica que me foi dada, por sua amizade, paciência, entusiasmo, ensinamentos, sugestões sempre pertinentes e constante incentivo durante a realização deste trabalho.

Dr. Manoel Barral-Netto, pela importante contribuição na minha formação científica e na correção e sugestões dos manuscritos que se originaram deste trabalho.

Dr. João Santana da Silva, do Departamento de Imunologia da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo-USP, pela colaboração e disponibilidade em permitir que parte dos experimentos deste trabalho se realizassem no seu laboratório.

Cláudia Brodskyn, pela amizade, apoio, conselhos, inúmeras discussões científicas e valiosa colaboração na realização deste trabalho.

Camila Indiani de Oliveira, pela inestimável amizade, pelos ensinamentos de Biologia Molecular e prestimosa colaboração, principalmente pela participação como co-autora e auxílio na elaboração de um dos artigos que se originaram deste trabalho.

Marcos Welby, pelos valiosos esclarecimentos e colaboração nos experimentos de RT-PCR, principalmente por dedicar algumas horas na bancada na solução de problemas.

Clarissa Romero Teixeira, Régis Gomes, Vera Vinhas e Cecília Favali, que inúmeras vezes me acolheram em suas casas, me apoiaram, me incentivaram, me toleraram nos momentos difíceis e, acima de tudo, souberam ser amigos.

Olívia Bacellar, um agradecimento muito especial por sua amizade, incentivo, colaboração e pela alegre acolhida que sempre tive na sua casa e no seu coração.

Jorge Clarêncio e Herculano Oliveira da Cruz, amigos queridos, pelo suporte, incentivo e amizade.

Cristiane Maria Milanezi, Ana Paula Campanelli e Karen Cavassani, do Laboratório de Imunologia do Departamento de Imunologia da Faculdade de Medicina de Ribeirão Preto-USP, pela prestimosa ajuda na padronização dos experimentos de RT-PCR, imuno-histoquímica e na realização dos experimentos de citometria de fluxo.

Um especial agradecimento ao amigo **Jorge Lessa Tolentino,** pelo apoio, incentivo, amizade, conselhos e incontáveis horas de alegres e proveitosas conversas.

Carinhosamente, o meu mais sincero agradecimento a **Juliana Dumet Fernandes, Joílson Ramos de Jesus, Bruno Bezerril Andrade e Claire da Silva Santos,** os queridos estudantes de iniciação que tornaram mais fácil a realização deste trabalho.

Aos amigos e companheiros da Universidade Federal do Ceará: Dr. Ivo Castelo Branco Coêlho, Dr. Anastácio Queiroz de Sousa, Zirlane C. Branco Coelho, Érica Freitas Mota, Mércia Sindeaux Frutuoso, Lucilene Simões-Mattos e Herivaldo Costa, pelo apoio, incentivo e confiança depositados no meu trabalho.

Às funcionárias da secretaria do Curso de Pós-graduação em Patologia, **Rosália Meire**, **Iumara Evangelista e Roberta Lopes**, pela eficiência no atendimento e na resolução dos problemas.

A todos os professores, colegas e funcionários do **Centro de Pesquisa Gonçalo Moniz**, pela acolhida, pelo ambiente de cooperação, estímulo e amizade que sempre encontrei neste Centro.

Ao pessoal da secretaria do LIMI/LIP: **Jackson Lemos, Camila Mota e José Carlos Andrade Cardoso,** meus sinceros agradecimentos.

Ana Maria Fiscina e pessoal da biblioteca do CPqGM.

A todos os colegas do Curso de Pós-graduação em Patologia.

Aos demais amigos e companheiros do LIMI/LIP, cujo convívio alegre tornou extremamente prazerosa a realização deste trabalho de tese: Sílvia Andrade Cardoso (*in memoriam*), Jackson Costa, José Carlos Miranda, Johan van Weyenberg, Andréa Bomura Rosato, Fernanda Novais e Tatiana Moura (filhotas queridas), Ricardo Khouri, João Paulo de Oliveira e Rafaelle Neto (meus garotos queridos), André Báfica, Fabiano Oliveira, Viviane Boaventura, Dirceu Costa, Sebastião Neto, Deboraci Prates, Ana Cristina Bahia, Ana Paula de Souza, Maria José Menezes, Edvaldo Santos, Lílian Afonso, Theolis Barbosa, Gilvanéia Santos, Daniele Decanine, George Santana, Lourdes Farré, Adriana Almeida, Aline Báfica, Rosse Osório e Gisélia Santana.

AGRADECIMENTOS ESPECIAIS

Luiza de Sousa Teixeira, minha mãe querida, e os irmãos: **Ademir**, **Rosa Maria**, **Rosinéa**, **Adriana Luiza**, **Doris e Maria Bernadete,** que me incentivam, amam, acreditam na minha capacidade e comemoram comigo as minhas realizações.

Margarida Maria de Lima Pompeu, minha grande mentora científica, que me tem dado suporte e amizade em todos os momentos do meu aprendizado científico e que é um exemplo de abnegação, dedicação e amor pela pesquisa e ensino.

Fontes financiadoras: CNPq, CAPES, PROCAD – 0018/01-5, FAPESB.

SUMÁRIO

LISTA DE ABREVIATURAS

IFN-γ	Interferon gama
IL-1	Interleucina-1
IL-2	Interleucina-2
IL-4	Interleucina-4
IL-8	Interleucina-8
IL-10	Interleucina-10
IL-12	Interleucina-12
IL-13	Interleucina-13
TNF	Fator de necrose tumoral
TGF	Fator transformador do crescimento
LPS	Lipopolissacaridio
RAPD	"Random Amplification of Polymorphic DNA"
RT-PCR	"Reverse transcriptase – Polymerase Chain Reaction"
LC	Leishmaniose cutânea
LCM	Leishmaniose cutânea mucosa
LCD	Leishmaniose cutânea difusa
RNAm	Ácido ribonucleico mensageiro
cDNA	Ácido dexoribonucleico complementar
NK	"Natural Killer"
MHC	Complexo Principal de Histocompatibilidade
CD	"Cell Differentiation Molecule"
MCP	"Monocyte chemoattractant protein"
GRO	"Growth-regulated oncogene"
IP-10	"Interferon-inducible protein-10"
MDC	"Macrophage-derived chemokine"
MIG	"Monokine induced by interferon-γ"
MIP	"Macrophage inflammatory protein"
RANTES	"Regulated on activation, normal T-cell expressed and secreted"
KC	"Keratinocyte chemokine"
TARC	"Thymus and activation-regulated chemokine"

RESUMO

CEPAS DE *Leishmania braziliensis* COM PERFIS GENÔMICOS DIVERSOS INDUZEM PROCESSOS INFLAMATÓRIOS DIFERENTES EM CAMUNDONGOS BALB/C – Maria Jania Teixeira. Dois artigos científicos resumem o presente estudo. No primeiro, é mostrado que duas cepas de *L. braziliensis*, isoladas de pacientes de diferentes Estados (Ceará e Bahia), são geneticamente diferentes e apresentam processos patológicos distintos em camundongos BALB/c. No segundo trabalho é demonstrada uma correlação entre padrões de expressão de quimiocinas, induzidas por estas duas cepas de *L braziliensis*, com a formação de lesão e a atração de leucócitos para o sítio inflamatório. O terceiro trabalho é uma revisão sobre o papel das quimiocinas e receptores de quimiocinas nas interações parasito-hospedeiro em leishmaniose. Em particular, a revisão focaliza os estudos que contribuíram para a compreensão sobre quimiocinas na infecção por leishmania. Os principais achados desses estudos são: **1.** A *L. braziliensis* H3227 do Ceará apresentou genótipo diferente da *L. braziliensis* BA788 da Bahia, demonstrado por RAPD. **2.** A cepa H3227 levou ao desenvolvimento de lesões pequenas que se resolveram, enquanto BA788 não causou lesões em camundongos BALB/c. **3.** Em ambos os grupos de camundongos, a carga parasitária não diferiu significativamente no sítio de infecção e começou a reduzir com 30 dias de infecção. Nos linfonodos drenantes, entretanto, o número de parasitos aumentou durante o decorrer do estudo. **4.** Após a inoculação do parasito, a cepa BA788 pareceu ativar mais precocemente uma reposta mais intensa tipo Th1. Camundongos infectados com BA788 produziram níveis mais altos de IFN-γ, o que se correlacionou com um maior número de células NK nas lesões destes animais. **5.** Numa fase mais tardia, camundongos infectados com H3227 produziram mais IL-12p70 e IL-10 no sobrenadante de células recuperadas dos linfonodos. Esta regulação da produção de IL-10 coincidiu com o pico máximo do desenvolvimento da lesão e da carga parasitária nestes animais. **6.** As maiores lesões causadas por H3227, em camundongos BALB/c, foi diretamente correlacionada com o aumento progressivo da reação inflamatória, mas não com a carga parasitária. **7.** A expressão mais precoce e intensa das quimiocinas CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, CCL11/eotaxina, XCL1/linfotactina e dos seus respectivos receptores correlacionou-se com o maior influxo de leucócitos para as lesões induzidas por H3227 e com o aumento do processo inflamatório observado nos camundongos infectados com esta cepa, após 15 dias de infecção. **8.** As diferenças observadas no processo inflamatório estavam correlacionadas com a virulência do parasito, uma vez que a cepa mais patogênica H3227 mostrou-se um recrutador mais potente de todas as células observadas no sistema do bolsão inflamatório, do que a menos patogênica BA788. Em conclusão, os achados apresentados aqui indicam que duas cepas de *L. braziliensis* geneticamente diferentes, embora apresentem carga parasitária semelhante no sítio de inoculação, podem estabelecer processos patológicos distintos e induzir expressão de quimiocinas em diferentes tempos e/ou intensidades, levando ao recrutamento diverso de células e respostas inflamatórias diferenciadas que poderiam finalmente estar envolvidas em diferentes apresentações da doença.

Palavras chaves: Leishmaniose cutânea, *L. braziliensis*, BALB/c, quimiocinas.

ABSTRACT

***Leishmania braziliensis* ISOLATES DIFFERING IN THEIR GENOMIC PROFILES INDUCE DIFFERENT INFLAMMATORY PROCESSES IN BALB/C MICE – Maria Jania Teixeira.** Two scientific papers summarize the study presented herein. The first study shows that two *L. braziliensis* strains isolated in patients from different states (Ceará and Bahia) are genetically distinct and present different behavior during infection of BALB/c mice. The second study demonstrates a correlation between patterns of chemokine expression, induced by these two *L braziliensis* strains, with the lesion formation and leukocyte attraction to the inflammatory site. The third work is a review about the role of chemokines and chemokine receptors in host-parasite interactions in leishmaniasis. In particular, this review focus on reports that have contributed to our understanding of chemokine in *Leishmania* infection. The major findings of these studies are: **1.** H3227 *L. braziliensis* from Ceará was genetically different from the BA788 *L. braziliensis* from Bahia as assessed by RAPD. **2.** H3227 led to the development of small self-healing lesions, while BA788 did not cause lesions in BALB/c mice. **3.** In both groups of mice, parasite load did not differ significantly at the infection site, and it start to reduce with 30 days of infection. In draining lymph nodes, however, the number of parasites increased over the study period. **4.** BA788 strain seemed to trigger a more intense Th1-type response, early after parasite inoculation. BA788-infected mice produced higher levels of IFN-γ, a feature coupled to a higher number of NK cells. **5.** Later, H3227-infected mice produced more IL-12p70 and IL-10 in the supernatant of their lymph nodes cells. This up-regulation in IL-10 production coincided with the time point at which lesion size and parasite load detected in the footpad reached their peak. **6.** In H3227-infected BALB/c mice, larger lesions sizes were directly correlated with progressive increase of the inflammatory reaction, but not to parasite load. **7.** A higher expression of the chemokines CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, CCL11/eotaxin, XCL1/lymphotactin and their respective receptors correlated with greater leukocyte recruitment, and with the increase of inflammation observed in H3227-infected mice at 15 days post infection. **8.** Differences observed in the inflammatory processes were associated with parasite virulence, since the more pathogenic H3227 *L. braziliensis* was a more potent recruiter of all cell types than BA788 in the air pouch system. In conclusion, the findings presented here indicate that two genetically different *L. braziliensis* isolates, albeit at similar parasite load, are able to establish distinctive patterns of infections, and induce chemokine expression at different paces and/or intensities, leading to diverse cell recruitment and differential inflammatory responses which can ultimately influency in the disease outcome.

Key words: Cutaneous leishmaniasis, *L. braziliensis*, BALB/c mice, chemokines.

1. INTRODUÇÃO

1.1. Situação do problema

Leishmaniose refere-se a um grupo de doenças causadas por protozoários do gênero *Leishmania*, ordem Kinetoplastida e família *Trypanosomatidae*. O parasito ocorre sob duas formas evolutivas: a forma flagelada, extracelular, denominada promastigota, encontrada no trato digestivo dos vetores, flebotomíneos do gênero *Phlebotomus* no Velho Mundo e *Lutzomyia* no Novo Mundo (LAINSON et al., 1987); e a forma amastigota encontrada predominantemente parasitando células do sistema fagocítico mononuclear dos hospedeiros mamíferos (ALEXANDER et al., 1999).

O quadro global da leishmaniose tem permanecido estável há alguns anos. Estima-se que mundialmente 350 milhões de pessoas correm risco de contrair leishmaniose, cerca de 12 milhões apresentam alguma forma da doença e aproximadamente 1-1,5 milhões de novos casos de leishmaniose cutânea surgem por ano (WORLD HEALTH ORGANIZATION, 2004).

A doença distribui-se amplamente em todos os continentes, com exceção da Oceania. É endêmica em 88 países das Américas do Sul e Central, África e Ásia. No Brasil, a distribuição geográfica da leishmaniose tegumentar é ampla e tem sido registrada em todo o território nacional (MINISTÉRIO DA SAÚDE, 2004). As regiões Nordeste e Norte vêm contribuindo com o maior número de casos registrados no período de 1980 a 2003 - cerca de 36,9% e 36,2%, respectivamente (MINISTÉRIO DA SAÚDE, 2004). No Nordeste, os casos registrados são provenientes principalmente dos Estados do Maranhão, Bahia e Ceará (MINISTÉRIO DA SAÚDE, 2004).

A importância da leishmaniose tegumentar nas Américas reside não somente na alta incidência e ampla distribuição geográfica, mas também na possibilidade de assumir formas que podem determinar lesões desfigurantes, incapacitantes e algumas vezes fatais, o que levou a Organização Mundial de Saúde (OMS) a incluir a doença entre as onze endemias infecto-parasitárias mais importantes do mundo (TDR, 2004).

1.2. Manifestações clínicas e imunopatogênese da leishmaniose tegumentar

As leishmanias são divididas em parasitos que causam doença tegumentar e visceral. No Novo Mundo, as espécies responsáveis pela leishmaniose tegumentar são: *L. braziliensis, L.*

peruviana, L. guyanensis, L. lainsoni, L. naiffi, L. shawi, L. colombiensis, L. amazonensis e *L. mexicana* (GRIMALDI & TESH, 1993). As espécies descritas no Velho Mundo são: *L. tropica, L. major* e *L. aethiopica* (ASHFORD & BETTINI, 1987).

Clinicamente, a leishmaniose tegumentar no Novo Mundo apresenta-se de maneira variada, desde as formas inaparentes ou com ulcerações de pele, que podem ser discretas ou extensas e com evolução espontânea para cura após alguns meses, até aquelas com ulcerações múltiplas ou disseminadas, ou mesmo evoluir para uma forma mutilante que afeta as mucosas das vias aéreas superiores A manifestação clínica mais freqüente da doença é a leishmaniose cutânea (LC), que se caracteriza por lesões ulceradas, únicas, ou em pequeno número, de bordos elevados e circulares, com pouca secreção e indolores. Alguns casos podem evoluir com o controle da infecção com cura espontânea, contudo, a maioria requer tratamento prolongado com drogas leishmanicidas (MARSDEN et al., 1984; COSTA et al., 1990; CONVIT et al., 1993; HERWALDT et al., 1992).

A forma disseminada é caracterizada por úlceras típicas, associadas a inúmeras lesões papulares ou acneiformes, decorrentes possivelmente de disseminação hematogênica, podendo comprometer a mucosa (COSTA et al., 1986; CARVALHO et al., 1994). A leishmaniose cutânea difusa (LCD) é uma forma grave não responsiva ao tratamento, anérgica e muito rara. É caracterizada predominantemente por nódulos múltiplos, onde se observa extraordinária riqueza parasitária e poucos linfócitos, podendo ocorrer também lesões tuberosas, placas e raramente aparecem ulcerações (CONVIT et al., 1972; LLANOS CUENTAS et al., 1984; COSTA et al., 1992). A resposta imune nessa forma clínica é caracterizada por ausência da produção de IFN-γ e pela elevada produção de IL-10 nas lesões (TURETZ et al., 2002).

A forma mais grave do espectro clínico é a cutâneo-mucosa (LCM), ou espúndia, que apresenta significante morbidade e pode levar a um curso fatal. É caracterizada por lesão na mucosa nasal, que pode expandir e acometer palato mole e duro, úvula, faringe, gengivas e lábio superior. Paradoxalmente, essas lesões mucosas contêm um número bastante reduzido de parasitos, e o infiltrado inflamatório caracteriza-se pela presença de grande quantidade de linfócitos e macrófagos. O intenso infiltrado inflamatório resulta em um exuberante aumento do nariz e do lábio superior, conferindo à lesão um aspecto tumoral e desfigurante. A perfuração do septo nasal é observada em torno de 42% dos pacientes, podendo afetar a mucosa traqueobrônquica e esôfago (MARSDEN, 1986; WALTON, 1987; BITTENCOURT &

BARRAL, 1991; PEARSON et al., 2000). A resposta imune nessa forma clínica é caracterizada por produção elevada de IFN-γ e TNF-α aos antígenos do parasito, ainda mais intensa que na forma cutânea localizada. Assim, a presença de lesão tanto pode estar relacionada a uma ausência de resposta Th1, com proliferação do parasito, ou a uma intensa resposta Th1, sendo a lesão pobre de parasitos, com intensa resposta inflamatória e destruição tecidual (RIBEIRO DE JESUS et al., 1998).

Tem sido descrita a disseminação precoce do parasito do sítio da lesão inicial na pele para os linfonodos regionais, tanto no Velho Mundo (ARDEHALI et al, 1995; AZADEH et al, 1994) como nas Américas (PESSOA & BARRETO, 1948; MORAES et al., 1993). No Brasil, a leishmaniose tegumentar causada por *L. braziliensis* pode acarretar comprometimento dos linfonodos de drenagem da lesão (BARRAL et al., 1995; SOUSA et al., 1995), levando a um quadro de linfadenite granulomatosa (ROCHA, 1998), que geralmente antecede a lesão cutânea. Esse quadro tem sido melhor estudado nos Estados da Bahia e do Ceará, em zonas endêmicas para leishmaniose tegumentar. Na Bahia, um estudo de BARRAL et al. (1992) mostrou que 67% dos pacientes com leishmaniose tegumentar apresentam linfadenopatia. Os linfonodos acometidos apresentam em média 5 cm de diâmetro, variando de 3 a 8,0 cm, desaparecem espontaneamente e têm um curso médio de 3 semanas, podendo ser a única manifestação da doença (BARRAL et al., 1995). No Ceará, 77% dos indivíduos com leishmaniose tegumentar apresentam linfadenopatia. No entanto, na maioria dos pacientes, os linfonodos são maiores do que os vistos na Bahia, atingem até 10,5 cm e a linfadenopatia apresenta curso mais indolente, podendo persistir por vários meses, mesmo após o aparecimento das lesões (SOUSA et al., 1995). Além disso, foi observado um padrão de resposta imune diferente dos pacientes que não apresentavam linfadenopatia. A resposta imune mostrou-se mais significativa a antígenos de leishmania, quando comparada a do grupo sem linfadenopatia (SOUSA et al., 1995). Esses dados sugerem diferenças nos isolados de leishmania e/ou respostas imunes dos hospedeiros, indicando uma associação do parasito com o resultado clínico da infecção.

1.3. Eventos inicias da infecção por leishmania

Durante a picada da fêmea do flebótomo, promastigotas metacíclicas são inoculadas junto com a saliva, no lago sanguíneo produzido pelo traumatismo da probóscida do flebótomo, na

derme do hospedeiro mamífero (RIBEIRO, 1995). A quantidade de promastigotas inoculadas varia de acordo com a intensidade da infecção do flebótomo. O número de promastigotas inoculadas parece ser importante no desenvolvimento da resposta imune. Estima-se que os flebótomos podem inocular de 0 a 1000 promastigotas na derme do hospedeiro mamífero (WARBURG & SCHLEIN, 1986), o que possivelmente entra como um fator importante na variação da intensidade da infecção. Outros fatores relevantes são as substâncias presentes na saliva do flebótomo (GILLESPIE et al., 2000). Algumas delas podem induzir resposta Th2 e exacerbação da lesão, mesmo em camundongos naturalmente resistentes (MBOW et al., 1998; BELKAID et al., 1998). Entretanto, com *L. braziliensis*, o efeito da exacerbação da doença na presença de saliva parece ser dependente da composição salivar da espécie de flebótomo, uma vez que inoculação de *L. braziliensis* (10^6-10^7 promastigotas) junto com saliva de *Lutzomyia whitmani* leva ao desenvolvimento de lesões que apresentam cura espontânea (BEZERRA & TEIXEIRA, 2001), enquanto inoculação do mesmo parasito (10^6-10^7 promastigotas) com saliva de *L. longipalpis* causa lesões cutâneas nodulares progressivas, que não se curam (SAMUELSON et al., 1991; LIMA et al., 1996).

Uma vez na derme, as promastigotas podem penetrar em granulócitos, macrófagos (POMPEU et al., 1991; LIMA et al., 1998) ou em células dendríticas (MOLL et al., 1993). Os neutrófilos e eosinófilos conseguem lisar leishmania (POMPEU et al., 1991; LIMA et al., 1998). Entretanto, mais recentemente foi demonstrado que neutrófilos também podem desempenhar outras funções, tais como secretar IL-12 e TGF-β (TACCHINI-COTTIER et al., 2000) e apresentar antígenos via MHC classe II na presença de GM-CSF (GOSSELIN et al., 1993). Leishmania pode sobreviver dentro de neutrófilos nas primeiras horas ou primeiros dias após a infecção (LAUFS et al., 2002; VAN ZANDBERGEN et al., 2004) e pode atrasar, embora não evite a apoptose espontânea dos neutrófilos (AGA et al., 2002). Quando macrófagos ingerem neutrófilos apoptóticos infectados (SAVILL et al., 1989), suas funções microbicidas não são ativadas (MEAGHER et al., 1992; VAN ZANDBERGEN et al., 2004). Desta forma, acredita-se que a leishmania estaria entrando no macrófago, a sua principal célula hospedeira, de uma maneira silenciosa (LASKAY et al., 2003).

Embora a leishmania consiga diferenciar-se em amastigota e proliferar dentro de células dendríticas (PRINA et al., 2004) os macrófagos são as células hospedeiras preferenciais, onde os parasitos proliferam intensamente nos vacúolos parasitóforos (KONECNY et al., 1999). Mas

são as células dendríticas infectadas que migram para o linfonodo regional (MOLL et al., 2000), e, ainda na pele, estas células podem sofrer influências de quimiocinas ou citocinas liberadas por queratinócitos, neutrófilos, mastócitos e macrófagos presentes no sítio de inoculação. Quimiocinas e seus receptores participam na regulação da migração e das interações das células apresentadoras de antígeno (CAAg) com os linfócitos. O receptor de quimiocina CCR2 é importante na migração de células dendríticas e na localização destas células nas áreas T dos órgãos linfóides de camundongo e, conseqüentemente, no desenvolvimento da resposta celular tipo Th1 (SATO et al., 2000). CCR7 é outro receptor de quimiocina que também é requerido para a migração de células dendríticas maduras dos tecidos para as áreas T dos linfonodos (FORSTER et al., 1999).

É no linfonodo que vai ocorrer então a sensibilização dos linfócitos pelas CAAg (MOLL et al., 1993; PRINA et al., 2004). Diversas células, como as dendríticas, os macrófagos e os linfócitos B, podem apresentar antígeno às células T, dando início à sensibilização e expansão das células efetoras. A diferenciação linfocitária vai depender, entre outros fatores, do tipo de CAAg, das moléculas coestimulatórias expressas e das quimiocinas e citocinas que predominam no microambiente nestes primeiros momentos da sensibilização linfocitária.

1.4. Patogenicidade e genótipo de *Leishmania*

Após a infecção por leishmania, o curso da doença é influenciado por determinantes genéticos dos hospedeiros mamíferos, como já foi evidenciado em estudos experimentais realizados com *L. major*. O paradigma susceptibilidade versus resistência foi estabelecido usando este modelo. Algumas linhagens de camundongos apresentam resistência completa ou parcial à infecção, outras são completamente susceptíveis. Camundongos das linhagens C3H e C57BL/6 quando infectados com *L. major* apresentam lesão inicial e controlam a infecção, com cura aparente e desenvolvimento de resposta imune tipo Th1. Camundongos BALB/c são altamente susceptíveis, neles a infecção progride rapidamente, com visceralização do parasito, desenvolvimento de resposta Th2 e curso fatal (revisado em SACKS & NOBEN-TRAUTH, 2002).

Além dos determinantes genéticos dos hospedeiros, foi demonstrado que o curso da doença também pode ser influenciado pelo próprio parasito, desde que uma única espécie de *Leishmania*, como a *L. amazonensis*, ou a *L. braziliensis*, causam um amplo espectro da doença

em hospedeiros mamíferos (BARRAL et al., 1991, GONTIJO & CARVALHO, 2003). Mais tarde foi demonstrado que cepas de *L. amazonensis* isoladas de indivíduos com diferentes manifestações clínicas de leishmaniose tegumentar também apresentavam comportamentos biológicos diversos após infecção em camundongos geneticamente idênticos (ALMEIDA et al., 1996). Resultados semelhantes foram obtidos usando cepas de *L. major* (KEBAIER et al., 2001). Portanto, parece que a variação da patogenicidade do parasito, que por sua vez está relacionada com a diversidade do genótipo do parasito, é também um fator importante que controla as manifestações clínicas da leishmaniose.

Todas as espécies de *Leishmania* são morfologicamente semelhantes, exceto por diferenças discretas. Os métodos moleculares atuais nos permitem avaliar as características intrínsecas dos parasitos e a evolução destas metodologias gerou um avanço considerável no conhecimento do genótipo da *Leishmania*, permitindo muitas vezes, a caracterização de genótipos individuais. Variação no genótipo das leishmanias do subgênero *Viannia* já foi descrita (CUPOLILLO et al, 1998; SCHRIEFER et al., 2004). A associação do polimorfismo genotípico da leishmania com a origem geográfica do isolado também já foi observada em espécies de *Leishmania* do Velho Mundo (ANDRESEN et al., 1996; SCHONIAN et al., 2000; TOLEDO et al., 2002) e em isolados do Novo Mundo (GOMES et al., 1995; ISHIKAWA et al., 2002; CUPOLILLO et al., 2003). Mais recentemente, ROSATO (2004) demonstrou que existe polimorfismo genotípico entre os isolados de *L. braziliensis* dos Estados da Bahia e do Ceará.

As informações sobre os genótipos dos parasitos têm contribuído para a melhor compreensão do conceito de espécies de *Leishmania* e podem auxiliar no entendimento da epidemiologia e patogênese das leishmanioses.

1.5. Leishmaniose murina e imuno-regulação

Os modelos de leishmaniose experimental têm trazido uma grande contribuição para o esclarecimento da imuno-regulação da doença. A cura em LC é criticamente dependente do desenvolvimento de uma resposta imune do tipo Th1. Qualquer falha na montagem de uma eficiente resposta Th1 anti-leishmania resulta em doença progressiva e morte (SACKS & NOBEN-TRAUTH, 2002). As condições que desencadeiam a diferenciação Th1 ou Th2 parecem ser multifatoriais. Alguns destes fatores têm sido evidenciados, trazendo mais clareza para a compreensão da patogênese da leishmaniose, embora ainda existam muitas lacunas.

A resposta imune Th1 é caracterizada pela secreção de IFN-γ e TNF-α (SCOTT et al., 1988; SCOTT, 1991; REINER & LOCKSLEY, 1995). IFN-γ leva à ativação de mecanismos microbicidas do macrófago pela síntese de óxido nítrico (NO) (MURRAY et al., 1983; LIEW et al., 1990), de radicais livres derivados do oxigênio e síntese de enzimas lisossomais (MURRAY, 1981; CHANNON et al., 1984; PASSWELL et al., 1994). TNF-α está envolvido na indução da ativação de macrófagos em sinergismo com IFN-γ, estimulando-os a produzirem NO (LIEW et al., 1990; GREEN et al., 1990; THEODOS et al., 1991). Além disso, TNF-α participa da modulação da resposta inflamatória, induzindo apoptose de células T no sítio de infecção. Camundongos sem receptor de TNF-α p55, embora consigam eliminar o parasito, não conseguem resolver a inflamação, possivelmente decorrente do defeito na indução de apoptose (VIEIRA et al., 1996; KANALY et al, 1999).

Após os primeiros dias de ativação, em função das citocinas predominantes no microambiente, ocorre a definição da diferenciação em Th1 ou Th2 (NAKAMURA et al., 1997). Células NK e NK-T participam neste momento inicial secretando IFN-γ, que tem papel relevante na sensibilização de células Th1 (SCHARTON-KERSTEN & SCOTT, 1995; MARTIN-FONTECHA et al., 2004). IFN-γ produzido inicialmente por estas células induz a secreção de IL-12 por CCAg (células dendríticas, macrófagos, monócitos, linfócito B) e por células polimorfonucleares (PMN). A produção inicial de IL-12 pode ser desencadeada pela fagocitose do parasito (SUTTERWALA & MOSSER, 1999) e amplificada por IFN-γ. A citocina IL-12, por sua vez, amplifica a produção de IFN-γ, estabelecendo, assim, uma importante alça *feedback* positiva (BELOSEVIC et al., 1989; HEINZEL et al., 1995). A célula NK é considerada como a principal fonte de IFN-γ (SCHARTON-KERSTEN & SCOTT, 1995), mas parecem existir outras fontes alternativas, como células NK-T (LAUWERYS et al., 2000) e possivelmente células T CD8+ (SACKS & NOBEN-TRAUTH, 2002). Camundongos depletados de células NK (NK^-T^+) conseguem desenvolver resposta Th1 eficiente, com a produção de IL-12 e de IFN-γ, capazes de controlar a infecção por *L. major*, indicando que IL-12 pode induzir resposta Th1, independentemente de células NK, possivelmente pela ativação de NK-T (SATOSKAR et al., 1999).

A produção de IFN-γ nos primeiros três dias de infecção, é crítica para a diferenciação de linfócitos Th0 em Th1 e para inibir a diferenciação em Th2, e a CAAg desempenha papel

crítico neste momento (revisado em VON STEBUT & UDEY, 2004). A infecção de células dendríticas com amastigotas, na presença de IFN-γ, induz sua ativação, ocorrendo aumento da expressão de moléculas MHC classe I e II, de moléculas coestimulatórias (CD40, CD54, CD80 e CD86) e da síntese de IL-12. Por outro lado, tem sido demonstrado que a infecção de macrófagos, tanto com amastigotas como com promastigotas, não induz ativação, nem secreção importante de IL-12 (REINER et al., 1994; VON STEBUT et al., 1998; MAROVICH et al., 2000). Outras citocinas, como TNF-α, IL-1β, IL-15 e IL-18, potenciam o efeito de IL-12 na indução da produção de IFNγ por células NK (SHER et al., 1993; HUNTER et al., 1995; FEHNIGER et al., 1999; AKIRA, 2000), propiciando as condições necessárias para a proliferação das células Th1 efetoras.

Embora o fenótipo Th2, associado com a susceptibilidade à infecção, seja caracterizado pela alta produção de IL-4, IL-10, IL-13 e TGF-β (HEINZEL et al., 1989; LOCKLEY & SCOTT, 1991; BARRAL-NETTO et al., 1992; SCOTT, 1998), ainda não estão bem definidas as condições que o desencadeiam. As citocinas Th2 levam ao desenvolvimento de lesões graves em animais infectados com *L. major*, provavelmente porque desativam macrófagos. IL-4, IL-13 e TGF-β inibem a produção de intermediários reativos de oxigênio em macrófagos ativados por IFN-γ (BOGDAN et al., 2000). Diversas células podem participar na produção de IL-4 nestas primeiras horas de infecção: neutrófilos, células T $CD4^+$ e $NK1^+$ são capazes de secretar IL-4 e com isso modular a resposta para diferenciação tipo Th2 (VON DER WEID et al., 1996; BRANDT et al., 2000; NOBEN-TRAUTH et al., 2000). IL-13 é outra citocina que favorece a progressão da doença. Ela apresenta ação sinérgica com IL-4 (MATTHEWS et al., 2000), mas pode também promover a doença, independentemente da presença de IL-4 (NOBEN-TRAUTH et al., 1999). Um microambiente com predomínio de IL-4, IL-10 e IL-13 induz a diferenciação e proliferação de células Th2, levando à desativação dos macrófagos, o que propicia a multiplicação do parasito e a progressão da doença.

Uma vez diferenciados, os linfócitos sensibilizados Th1 ou Th2 migram do linfonodo regional para o sítio de inoculação da pele, dando início à resposta imuneinflamatória, que, dependendo do tipo de linfócito predominante, poderá destruir o parasito e resolver a infecção ou pode favorecer o crescimento do parasito e a doença ter um curso crônico.

Ao contrário do que ocorre com *L. major*, poucos estudos experimentais foram conduzidos com *L. braziliensis*, provavelmente porque este parasito não induz lesões em muitas linhagens

de camundongos (NEAL & HALE, 1983; CHILDS et al., 1984). Camundongos BALB/c são considerados os mais susceptíveis à *L. braziliensis*, apesar de não apresentarem lesões cutâneas muito evidentes e evoluírem para cura espontânea (CHILDS et al., 1984). A análise da resposta imune em camundongos BALB/c infectados com *L. braziliensis* mostrou que estes animais produzem menos IL-4, do que quando infectados com *L. major*. Além disso, animais infectados com *L. braziliensis*, ao serem tratados com anti-IFN-γ, aumentam significativamente o tamanho das lesões e a infecção não se resolve (DEKREY et al., 1998). Os autores sugerem que um mecanismo dependente de IFN-γ é responsável pela morte de *L. braziliensis* em camundongos BALB/c e que a fraca infectividade deste parasito nesta linhagem de camundongo pode ser causada pela incapacidade do parasito promover uma produção forte e sustentada de IL-4, diferentemente do que acontece com a infecção por *L. major*.

Recentemente nosso grupo desenvolveu um modelo experimental de infecção com *L. braziliensis* que mimetiza muito a infecção humana. Neste modelo, camundongos BALB/c foram inoculados com 10^5 de promastigotas na derme da orelha e foram capazes de reproduzir aspectos da infecção natural tais como a presença de lesão ulcerada, disseminação do parasito para os linfonodos e desenvolvimento de uma resposta imune tipo Th1 (DE MOURA et al., 2005). Este modelo poderá ser muito útil para responder questões relacionadas com a imunidade à re-infecções, persistência do parasito e desenvolvimento da forma clínica cutâneo-mucosa.

1.6. Quimiocinas

As quimiocinas são proteínas homólogas de baixo peso molecular (8 a 17 kDa), secretadas por uma variedade de células, incluindo leucócitos, células epiteliais, células endoteliais, fibroblastos e numerosos outros tipos de células. São produzidas em resposta a vários estímulos exógenos e endógenos (MOSER et al., 2004).

Geralmente as quimiocinas são produzidas durante uma resposta inflamatória, atuam em mais de um tipo celular e diferentes respostas induzidas por elas foram observadas in vitro, tais como, quimiotaxia, liberação de enzimas de estoques celulares, formação de radicais livres do oxigênio, rearranjo do citoesqueleto, geração de mediadores lipídicos e indução de adesão de células ao endotélio ou a proteínas da matriz extracelular (ROLLINS, 1997; LUSTER, 1998). As quimiocinas podem ser classificadas em 4 famílias de acordo com a configuração de quatro

resíduos de cisteína em posições altamente conservadas na porção amino terminal (NH_2) da molécula. Esses resíduos de cisteína presentes em todas as classes de quimiocinas estão envolvidos na formação de ligações dissulfeto, que participam da estabilização da estrutura da proteína (SAUNDERS & TARBY, 1999).

Dos quatro grupos de quimiocinas descritos, há duas grandes famílias que compreendem a maioria destas moléculas, as quais são chamadas de CXC quimiocinas, sendo assim denominadas por possuírem um aminoácido (X) entre as duas primeiras cisteínas próximas ao domínio NH_2-terminal, e a família das CC quimiocinas, que possuem as duas primeiras cisteínas adjacentes uma a outra e próximas ao amino terminal. As quimiocinas CC atraem principalmente monócitos e linfócitos T, apesar de que são também quimiotáticas para basófilos, eosinófilos e células NK (ROLLINS, 1997). As quimiocinas CXC são divididas em duas sub-famílias, aquelas que contêm a seqüência ácido glutâmico-leucina-arginina, chamada de seqüência ELR (ELR^+), precedendo as duas primeiras cisteínas da molécula, e aquelas que não contêm essa seqüência (ELR^-). A presença dessa seqüência está relacionada a um efeito predominante sobre a atração de neutrófilos e a ausência desta seqüência está relacionada com a migração de linfócitos (BAGGIOLINI, 2001).

Dois outros grupos menores não fazem parte destas famílias, sendo designadas como família C, que tem apenas dois resíduos de cisteína na molécula e apenas um deles na porção NH_2-terminal e família CX3C, que possui três resíduos de aminoácidos separando as duas primeiras cisteínas (ROLLINS, 1997; MACKAY, 1997). A quimiocina XCL1/linfotactina, da família C, tem sido descrita como sendo quimiotática para subpopulações específicas de linfócitos B e T, e não atrai monócitos, macrófagos ou neutrófilos (KENNEDY et al., 1995; HEDRICK et al., 1997). CX3CL1/Fractalcina, a única quimiocina CX3C, tem potente atividade quimiotática para linfócitos T, células NK e monócitos (BAZAN et al., 1997).

1.7. Receptores de Quimiocinas

As quimiocinas exercem suas funções pela ligação a receptores presentes na superfície das células. Os 21 receptores de quimiocinas descritos pertencem à família de receptores que atravessam sete vezes a membrana celular, com a porção intracelular ligada à proteína G (SAUNDERS & TARBY, 1999; MURDOCH & FINN, 2000). Sua estrutura é composta por um curto domínio NH_2-terminal, sete domínios em α-hélice transmembrânicos, com três alças

na porção extracelular e outras três na porção intracelular compostas de aminoácidos hidrofílicos, mais um domínio carboxi (COOH) terminal intracelular (MURDOCH & FINN, 2000). A maioria dos receptores de quimiocinas liga mais que uma quimiocina, além disso, diferentes tipos de células podem expressar vários destes receptores, de modo que se um ligante ou um receptor estiver defeituoso, um conjunto de quimiocinas ou receptores alternativos podem efetuar a função biológica na célula, caracterizando a família das quimiocinas e seus receptores como um sistema redundante (MANTOVANI, 1999).

O sinal intracelular induzido após ligação da quimiocina é desencadeado por uma proteína G, associada ao domínio C-terminal do receptor (BOKOCH, 1995; THELEN, 2001). A proteína G é uma molécula heterotrimérica composta por três subunidades (α, β e γ), que ficam ligadas a uma molécula de difosfato de guanosina (GDP) (MURDOCH & FINN, 2000). Após ligação da quimiocina ao receptor, o GDP é fosforilado e transformado em trifosfato de guanosina (GTP), as sub-unidades são separadas em $G\alpha\beta\gamma$ e $G\beta\gamma$, a subunidade α irá ativar a fosfolipase C (PLC) associada à membrana celular, que transforma o difosfato de fosfatidilinositol (PIP_2) em trifosfato de fosfatidilinositol (IP_3) e diacilglicerol (DAG). O IP_3 irá mobilizar cálcio intracelular e o DAG juntamente com o cálcio irão ativar várias isoformas da proteína cinase C (PKC), esta por sua vez ativa várias MAP cinases, levando assim à ativação celular, o que culmina com os efeitos que as quimiocinas exercem nas células (SAUNDERS & TARBY, 1999; MURDOCH & FINN, 2000; ROT & VON ADRIAN, 2003). Após ativação e internalização, os receptores de quimiocinas se tornam parcial ou totalmente dessensibilizados e sugere-se que este processo é mediado pela subunidade $G\alpha$ que ativa a proteína tirosina cinase (PTK), levando à ativação de MAP cinases e fosforilação de resíduos de serina e treonina na porção carboxi-terminal intracitoplasmática do receptor, causando sua inativação (MURDOCH & FINN, 2000). A velocidade da recuperação da resposta é determinada pelo destino da internalização dos receptores (degradação lisossomal versus desfosforilação e reciclagem na superfície celular) e pela rapidez da síntese de um novo receptor (THELEN, 2001).

Os receptores de quimiocinas e seus ligantes estão listados na Tabela I, de acordo com a nova nomenclatura sistemática proposta por ZLOTNIK & YOSHIE (2000).

1.8. Efeitos biológicos das quimiocinas

As quimiocinas, junto com as moléculas de adesão, são as maiores controladoras da migração de leucócitos. Adicionalmente, as quimiocinas também participam no desenvolvimento de órgãos (organogênese), na angiogênese, na angiostasia, no crescimento e metástase de células tumorais, na recirculação homeostática de leucócitos e na regulação imunológica (ROSSI & ZLOTNIK, 2000; CHRISTOPHERSON II & THROMAS, 2001).

Diferentes estudos sugeriram a participação das quimiocinas na resposta imune frente a inúmeros patógenos (BURGMANN et al., 1995; VILLALTA et al., 1998; OLSZEWSKI et al., 2001; PARK et al., 2001), possivelmente porque atuam atraindo leucócitos para os tecidos, regulando a resposta imune ou induzindo mecanismos antimicrobianos (MANNHEIMER et al., 1996; ALIBERTI et al., 1999; RITTER & MOLL, 2000). Além das atividades microbicidas, as quimiocinas e seus receptores podem exercer papel importante na polarização do padrão de resposta imune (revisto em SALLUSTO & MACKAY, 2004). Desta forma, células T polarizadas para Th1 produzem IFN-γ e co-localizam com macrófagos e neutrófilos em lesões de hipersensibilidade do tipo tardio, enquanto que células Th2 produzem IL-4, IL-5 e IL-13 e estão presentes juntamente com eosinófilos e basófilos em sítios de inflamação alérgica (SALLUSTO et al., 1998). Uma complexa rede de interação entre citocinas e quimiocinas vem sendo descrita, podendo as citocinas regular a produção das quimiocinas e vice-versa. Por exemplo, IL-4 e IL-13 estimulam a produção de CCL11/eotaxina e CCL22/MDC, um efeito que é inibido por IFN-γ (LI et al., 1998; BONECCHI et al., 1998). Por outro lado, IFN-γ induz a produção de CXCL10/IP-10 e CXCL9/MIG, efeito que é antagonizado por IL-4 (ROLLINS, 1997; FARBER, 1997). Um outro exemplo de quimiocinas e seus receptores associados com respostas polarizadas vieram de experimentos usando linfócitos T humanos. Estas células quando tratadas com IL-2 e anti-IL-4 polarizaram para Th1 e apresentaram acentuada expressão de CCR1, CCR2, CCR5 e CXCR3, este último receptor de CXCL10/IP-10 e CXCL9/MIG, ambas induzidas por IFN-γ e quimioatraentes de linfócitos (LOETSCHER et al., 1996). Além disso, linfócitos T $CD4^+$ diferenciados para Th1, migram preferencialmente em direção a um gradiente quimiotático contendo CCL3/MIP-1α, CCL4/MIP-1β ou CCL5/RANTES, o que não foi observado para células Th2, sugerindo que CCR5, receptor para estas três quimiocinas, poderia ser um marcador para linfócitos Th1 (BONECCHI et al., 1998; DORNER et al., 2002). Por outro lado, linfócitos T imaturos que foram estimulados com IL-4

e anti-IL-12, para gerar um padrão de linfócitos Th2, apresentaram alta expressão de CCR3 na sua superfície (SALLUSTO et al., 1997; SALLUSTO et al., 1998). Outros receptores de quimiocinas, como CCR4 e CCR8, foram descritos serem preferencialmente expressos em linfócitos Th2 (ZINGONI et al., 1998).

As quimiocinas estão envolvidas também em um grande número de processos fisiológicos e patológicos. A expressão individual de quimiocinas CC tem sido demonstrada em processos inflamatórios crônicos, tais como psoríase (GILLLITZER et al., 1993), reações de hipersensibilidade do tipo retardado, relacionadas com sarcoidose e tuberculose (DEVERGNE et al., 1994), inflamação gengival (YU et al., 1993) e formação de granuloma na esquistossomose e tuberculose (LUKACS et al., 1993; QIU et al., 2001). CCL3/MIP-1α, CCL4/MIP-1β e CCL5/RANTES produzidas por células T CD8+, possuem atividade supressora da replicação do vírus HIV em células T CD4+ (COCCHI et al., 1996). Além dessas funções, foram descritas outras, tais como aumento de lise mediada por células NK (TAUB et al., 1995) e indução da atividade tripanossomicida mediada por óxido nítrico (NO) em macrófagos humanos (VILLALTA et al., 1998).

1.9. Quimiocinas e leishmaniose

O possível envolvimento das quimiocinas na leishmaniose tegumentar humana foi investigado em LC e LCD causadas por *L. mexicana*. As lesões de indivíduos com LC continham altos níveis de CCL2/MCP-1, CXCL9/MIG e CXCL10/IP-10; ao contrário, lesões de indivíduos com LCD expressavam predominantemente CCL3/MIP-1α (RITTER et al., 1996). Nesse estudo, os autores sugerem que, em LC, a produção de CCL2/MCP-1 seria responsável pelo recrutamento de macrófagos mais maduros e/ou ativados, que por sua vez matariam os parasitos e controlariam a doença. Por outro lado, em LCD, CCL3/MIP-1α induziria a migração de células mais imaturas ou desativaria estas células, favorecendo a progressão da doença.

Trabalhos in vitro demonstraram que leishmania é capaz de induzir uma rápida e transitória expressão de algumas quimiocinas, tais como CCL2/MCP-1 e CXCL8/IL-8, tanto em macrófagos murinos como em humanos (BADOLATO et al., 1996; RACOOSIN & BERVERLY, 1997). Além disso, tem sido relatado que CCL2/MCP-1 pode estimular diretamente a eliminação do parasito via a geração de NO por macrófagos murinos infectados

com *L. donovani* (BHATTACHARYYA et al., 2002), ou via a indução de reativos intermediários do oxigênio em monócitos murinos infectados com *L. major* (RITTER & MOLL, 2000).

Parece haver uma associação de quimiocinas com os perfis de resistência e susceptibilidade. A infecção por *L. major*, em camundongos resistentes C57BL/6 e susceptíveis BALB/c, induz expressões significativamente diferentes de várias quimiocinas (VESTER et al., 1999). Nesse estudo, linfonodos de camundongos resistentes infectados com *L. major* induziram significativamente mais RNAm de CCL2/MCP-1, CXCL10/IP-10 e XCL1/linfotactina quando comparados com aqueles de camundongos susceptíveis, indicando que estas quimiocinas podem ter um papel importante no desenvolvimento da imunidade do hospedeiro contra *L. major*. No mesmo estudo, a administração de CXCL10/IP-10 recombinante a camundongos BALB/c aumentava a atividade das células NK e a resistência contra *L. major*. Mais recentemente foi demonstrado o envolvimento de outra quimiocina, CCL5/RANTES, na resistência à *L. major*. Camundongos resistentes infectados com *L. major* e tratados com met-RANTES, um antagonista funcional de CCR1 e CCR5 (receptores de CCL5/RANTES), tornou esses animais susceptíveis à infecção (SANTIAGO et al., 2004).

As quimiocinas foram estudadas também na leishmaniose visceral. Tentando entender o que causa a migração de linfócitos para o fígado, COTTERELL et al. (1999) estudaram o perfil de quimiocinas no fígado de camundongos SCID (animais que não têm linfócitos B e T) e BALB/c infectados com *L. donovani*. Em ambos o caso, foi encontrado uma rápida acumulação hepática das quimiocinas CCL2/MCP-1, CCL3/MIP-1α e CXCL10/IP-10. Destas, apenas CXCL10/IP-10 manteve-se elevada em camundongos BALB/c, mas não em camundongos SCID. Entretanto, quando os camundongos SCID foram reconstituídos com linfócitos T CD4+ e CD8+, os níveis das quimiocinas voltaram a subir e permaneceram elevados, sugerindo que a manutenção dos níveis hepáticos destas quimiocinas é dependente de células T. Além disso, a produção de CXCL10/IP-10, amplificada por células T, foi essencial para permitir a formação do granuloma hepático e da resposta inflamatória, conferindo resistência a esses animais. Ao contrário do fígado, entretanto, a quimiocina predominante no baço de animais com leishmaniose visceral é CCL2/MCP-1, mais que CXCL10/IP-10, levando a um influxo maior de macrófagos do que de células T para o baço (ROUSSEAU et al., 2001).

Os estudos utilizando animais deficientes de quimiocinas e/ou receptores de quimiocinas vêm se tornando uma importante ferramenta para entender o papel de várias quimiocinas na leishmaniose. Entre eles, os estudos de SATO et al. (1999) mostraram que camundongos deficientes de CCR2, CCR5 ou CCL3/MIP-1α, infectados com *L. donovani*, apresentaram uma baixa produção de IFN-γ durante a fase inicial da infecção, entretanto, durante a fase crônica da doença, as concentrações de IFN-γ aumentaram nos camundongos deficientes de CCR5 e CCL3/MIP-1α, e o aumento desta citocina se correlacionou com uma diminuição da carga parasitária. Os autores sugerem que CCR2, CCR5 e CCL3/MIP-1α têm um papel importante na geração de IFN-γ por células T. Os dados também indicam que CCL3/MIP-1α e os receptores CCR2 e CCR5 participam da defesa do hospedeiro contra *L. donovani*. Adicionalmente, outro estudo mostrou que a ausência do receptor CCR2 em camundongos infectados com *L. donovani* estava associada com uma migração diminuída de células de Langerhans para o linfonodo, resultando em uma deficiência de células dendríticas CD8α+ e deficiência na sensibilização de linfócitos Th1 (SATO et al, 2000).

Até o momento, não há nenhum trabalho mostrando o papel das quimiocinas na LC causada por *L. braziliensis*. A maioria dos estudos envolvendo quimiocinas e leishmaniose feitos em modelo murino tem utilizado *L. major* (que causa LC no Velho Mundo), *L. donovani* ou *L. infantum* (causadoras de leishmaniose visceral no Velho Mundo), e *L. amazonensis* (que causa LC no Novo Mundo). Coletivamente, os trabalhos publicados até agora sugerem que as quimiocinas desempenham importantes papéis na infecção por leishmania, incluindo funções de defesa do hospedeiro, tais como recrutamento de leucócitos e ativação celular, participação na imunidade mediada por células e atividade leishmanicida, indicando que essas proteínas podem ser relevantes para o controle e/ou progressão da doença.

Tabela I. Família das quimiocinas CXC, C, CX3C e CC e seus receptores

Nome sistemático	Ligante humano	Ligante murino	Receptor
Família CXC			
CXCL1	GROα/MGSAα	GRO/MIP2/KC?	CXCR2>CXCR1
CXCL2	GROβ/MGSAβ	GRO/MIP2/KC?	CXCR2
CXCL3	GROγ/MGSAγ	GRO/MIP2/KC?	CXCR2
CXCL4	PF4	PF4	Desconhecido
CXCL5	ENA78	GCP2/LIX?	CXCR2
CXCL6	GCP2	GCP2/LIX?	CXCR1, CXCR2
CXCL7	NAP2	Desconhecido	CXCR2
CXCL8	IL-8	Desconhecido	CXCR1, CXCR2
CXCL9	MIG	MIG	CXCR3 (CD183)
CXCL10	IP-10	IP10/CRG2	CXCR3 (CD183)
CXCL11	ITAC	ITAC	CXCR3 (CD183)
CXCL12	SDF1α/β	SDF1/PBSF	CXCR4 (CD184)
CXCL13	BCA1	BLC	CXCR5
CXCL14	BRAK/Bolecina	BRAK	Desconhecido
(CXCL15)	Desconhecido	Lungcina/WECHE	Desconhecido
CXCL16	Desconhecido	Desconhecido	CXCR6
Família C			
XCL1	Linfotactina/SCM1α/ATAC	Linfotactina	XCR1
XCL2	Linfotactina/ SCM1β	Desconhecido	XCR1
Família CX3C			
CX3CL1	Fractalcina	Neurotactina/ABCD3	CX3CR1
Família CC			
CCL1	I309	TCA3/P500	CCR3
CCL2	MCP1/MCAF/TDCF	JE	CCR2
CCL3	MIP1α/LD78α	MIP1α	CCR1, CCR5
CCL3L1	LD78β	Desconhecido	CCR1, CCR5
CCL4	MIP1β	MIP1β	CCR5 (CD 195)
CCL5	RANTES	RANTES	CCR1, CCR3, CCR5 (CD195)
(CCL6)	Desconhecido	C10/MRP1	Desconhecido
CCL7	MCP3	MARC?	CCR1, CCR2, CCR3
CCL8	MCP2	MCP2?	CCR3, CCR5 (CD195)
(CCL9/10)	Desconhecido	MRP2/CCF18/MIP-1γ	CCR1
CCL11	Eotaxina	Eotaxina	CCR3
(CCL12)	Desconhecido	MCP5	CCR3
CCL13	MCP4	Desconhecido	CCR2, CCR3
CCL14	HCC1	Desconhecido	CCR1, CCR5
CCL15	HCC/LKN1/MIP-1δ	Desconhecido	CCR1, CCR3
CCL16	HCC4/LEC/LCC1	Desconhecido	CCR1, CCR2
CCL17	TARC	TARC/ABCD2	CCR4
CCL18	DC-CK1/PARC/AMAC1	Desconhecido	Desconhecido
CCL19	MIP3β/ELC/exodus-3	MIP3β/ELC/exodus-3	CCR7 (CD197)
CCL20	MIP3α/LARC/exodus-1	MIP3α/LARC/exodus-1	CCR6
CCL21	6Ckine/SLC/exodus-2	6Ckine/SLC/exodus-2/TCA4	CCR7 (CD197)
CCL22	MDC/STCP1	ABCD1	CCR4
CCL23	MPIF1/CKβ8/CKβ8-1	Desconhecido	CCR1
CCL24	Eotaxina-2/MPIF2	MPIF2	CCR3
CCL25	TECK	TECK	CCR9
CCL26	Eotaxina-3	Desconhecido	CCR3
CCL27	CTACK/ILC	ALP/CTACK/ILC/ESkine	CCR10
CCL28	MEC	Desconhecido	CCR3/CCR10

2. JUSTIFICATIVA

A infecção por leishmania pode resultar em um amplo espectro de manifestações clínicas, sendo o curso da doença determinado pelas complexas interações entre o parasito e o hospedeiro. Os eventos iniciais dessa interação influenciam o futuro curso da doença. A *L. braziliensis* é um dos principais agentes etiológicos da leishmaniose tegumentar no Brasil, podendo causar tanto a forma cutânea localizada como a cutâneo-mucosa. Além disso, a *L. braziliensis* causa linfadenopatia, observada como um primeiro sinal da LC e algumas vezes como a única manifestação clínica da doença. No Ceará, esta linfadenopatia foi referida como "bubônica" e persiste até seis meses após o aparecimento da úlcera, enquanto na Bahia, essa manifestação clínica é mais transitória. Além disso, são observadas diversas formas de apresentação clínica, como as formas cutâneas isoladas, lesões múltiplas, leishmaniose disseminada e cutâneo-mucosa. Isso sugere que diferenças nos isolados de leishmania e/ou respostas imunes dos hospedeiros podem indicar uma associação do parasito com o resultado clínico da infecção. A composição celular no sítio inflamatório pode influenciar no tamanho da lesão cutânea na leishmaniose e as quimiocinas têm um papel fundamental nesse processo, porque atraem e estimulam subpopulações específicas para o sítio de infecção. A compreensão do envolvimento de quimiocinas na leishmaniose tegumentar pode esclarecer alguns pontos ainda desconhecidos dos eventos iniciais da resposta imune contra o parasito e da patogênese da doença, podendo contribuir para a elaboração racional de marcadores diagnósticos, bem como para o desenvolvimento de drogas adicionais ou alternativas para o tratamento da doença.

3. HIPÓTESE

A hipótese deste estudo é as diferenças entre as cepas de *Leishmania braziliensis* podem influenciar o curso clínico da doença e a resposta inflamatória.

4. OBJETIVO GERAL

Investigar se diferentes cepas de *L. braziliensis* podem apresentar comportamentos distintos e induzir diversos perfis de quimiocinas em camundongos BALB/c.

5. ARTIGO I

Título: "*Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice".

5.1. Justificativa e Objetivos do estudo:

Neste estudo, comparamos cepas de *L. braziliensis* isoladas de pacientes com leishmaniose cutânea dos Estados do Ceará e da Bahia. Ambos Estados são endêmicos para LC causada por *L. braziliensis*. No Ceará, a lesão cutânea é acompanhada e algumas vezes precedida por um expressivo aumento dos linfonodos regionais e o termo bubônico é utilizado para descrever esta manifestação. Leishmaniose bubônica é restrita à infecção por *L. braziliensis* no Ceará, entretanto, linfadenopatia também é observada nos pacientes com LC na Bahia, embora menos expressiva. Esses dados sugerem diferenças nos isolados de *Leishmania* e/ou respostas imunes dos hospedeiros, indicando uma associação do parasito com o resultado clínico da infecção. Variação na patogenicidade de isolados de *Leishmania*, que por sua vez, está relacionada com a diversidade genética dos parasitos, é um importante fator que pode estar influenciando as características clínicas na leishmaniose. A hipótese deste primeiro trabalho é que diferentes isolados de *L. braziliensis* podem induzir diversos processos patológicos em camundongos BALB/c. Nossos objetivos específicos neste estudo foram:

1. Avaliar polimorfismos genéticos em cepas de *L. braziliensis* isoladas de pacientes dos Estados do Ceará e da Bahia;
2. Comparar o comportamento biológico de duas cepas de *L. braziliensis* representativas dos Estados do Ceará e da Bahia em camundongos BALB/c;
3. Determinar a carga parasitária no sítio de inoculação do parasito e no linfonodo de drenagem da lesão;
4. Caracterizar os tipos celulares presentes no sítio de inoculação do parasito;
5. Determinar o perfil de citocinas em células recuperadas do linfonodo de drenagem da lesão.

5.2. Resumo dos Resultados: Inicialmente, investigamos o grau de polimorfismo genético entre seis cepas de *L. braziliensis*, duas do Ceará (H3227, H3456) e quatro da Bahia (BA711, BA427, BA806, BA788), utilizando a técnica de RAPD. Os perfis genômicos obtidos com as cepas do Ceará foram diferentes daqueles obtidos com as da Bahia. Nos experimentos seguintes, comparando duas cepas de *L. braziliensis*, representativas de cada Estado (H3227, do Ceará e BA788, da Bahia), encontramos que os camundongos infectados com H3227 desenvolveram lesões maiores do que aqueles infectados com BA788. Três dias após a inoculação, o número de granulócitos e macrófagos foi semelhante em ambos os grupos de camundongos, enquanto o número de células NK estava aumentado nas lesões dos animais infectados com BA788. Quinze dias após a infecção, observamos uma diminuição no número de granulócitos e células NK e um aumento no número de macrófagos e linfócitos em ambos os grupos de camundongos. A carga parasitária no sítio de inoculação não foi diferente nos dois grupos de animais. No linfonodo, foi interessante observar que o número de parasitos aumentou durante o período de infecção em ambos os grupos infectados, indicando que *Leishmania* é capaz de persistir nesse órgão linfóide secundário. As células do linfonodo dos animais infectados com BA788 produziram altos níveis de IFN-γ, enquanto nos animais infectados com H3227 houve uma produção maior de IL-4 após 3 dias de infecção. Mais tarde, 15 dias pós-infecção, ocorreu um aumento nos níveis de IL-12p70 e IL-10 no sobrenadante das células do linfonodo de animais infectados com H3227. Concluímos que cepas de *L. braziliensis* geneticamente diferentes induzem características clínicas e certos parâmetros imunológicos distintos em camundongos BALB/c

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Microbes and Infection 6 (2004) 1347
Microbes and Infection
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Erratum

Erratum to: "*Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice" [Microbes Infect. 6 (2004) 977–984]

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Available online 01 October 2004

In the list of authors for this article the name of João Santana da Silva was incorrectly spelt.

Figs. 3–5 had been transposed; however, the legends were placed in the correct order. Fig. 5 should have been with the legend to Fig. 3; Fig. 3 should have been with the legend to Fig. 4; and Fig. 4 should have been with the legend to Fig. 5.

doi of original article 10.1016/j.micinf.2004.05.003.
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doi:10.1016/j.micinf.2004.09.003

Available online at www.sciencedirect.com
SCIENCE DIRECT
Microbes and Infection 6 (2004) 977–984
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Leishmania braziliensis isolates differing at the genome level display distinctive features in BALB/c mice

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Received 5 January 2004; accepted 13 May 2004

Available online 23 July 2004

Abstract

Leishmania braziliensis is the species responsible for the majority of cases of human cutaneous leishmaniasis in Brazil. In the present study, *L. braziliensis* isolates from two different geographic areas in Brazil were studied by RAPD, using arbitrary primers. We also evaluated other biological features of these two isolates. We compared (a) the clinical features they initiate or not once delivered subcutaneously as stationary-phase promastigotes in the footpad of BALB/c mice; (b) the parasite load in both the footpad and the draining lymph node; (c) the cytokines present in the supernatant of cultures of the cell suspensions from the draining lymph nodes; and (d) the cell types present at the site of parasite delivery. The results show that the *L. braziliensis* strain from Ceará (H3227) is genotypically different from the *L. braziliensis* strain from Bahia (BA788). H3227-parasitized mice developed detectable lesions, whereas BA788-parasitized mice did not. Fifteen days post parasite inoculation there was an increase in the numbers of macrophages and lymphocytes in the footpads, whatever the parasite inoculum. Parasite load at the inoculation site—namely the footpad—did not differ significantly; in draining lymph nodes, however, it increased over the period under study. Early after parasite inoculation, the cells recovered from the draining lymph nodes of BA788-parasitized mice produced higher levels of IFN-γ, a feature coupled to a higher number of NK cells. Later, after the parasite inoculation, there was an increased content of IL-12p70 and IL-10 in the supernatant of cells recovered from the lymph nodes of H3227-parasitized mice. This comparative analysis points out that *L. braziliensis* isolates differing in their genomic profiles do establish different parasitic processes in BALB/c mice.

Keywords: *Leishmania braziliensis*; Random amplification of polymorphic DNA; Interferon gamma; Interleukin-12p70; Interleukin-10

1. Introduction

Leishmania is the etiological agent of leishmaniasis, a parasitic disease with diverse clinical manifestations in human beings and other mammals. The mammalian organism becomes a parasitized host when the sand fly probes the skin for a blood meal, injecting metacyclic promastigotes together with saliva. *Leishmania braziliensis* parasites usually cause a self-healing ulcer at the site of parasite delivery; however, parasites may also metastasize to the nasopharyngeal tract—a process that is thought to be the starting event leading to mucocutaneous leishmaniasis [1]. More rarely, parasite invasion of the bloodstream results in disseminated skin lesion [2].

Extensive studies using *L. major* have been interpreted according to the following frame. The resistance or the susceptibility to disease, in different mouse strains, is bound by genetic determinants of the host [3]: resistance is mediated by a Th1-type cellular immune response, characterized by the presence of high levels of IFN-γ and low levels of IL-4; susceptibility is mediated by a Th2-type cellular immune

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1286-4579/$ - see front matter
doi:10.1016/j.micinf.2004.05.009

response, characterized by low levels of IFN-γ and high levels of IL-4. Not only do these cytokines act on parasite-loaded cells or further host cells by killing the parasites or preventing their replication, but they also contribute to the inflammatory processes occurring in the parasite-loaded tissue. In contrast to *L. major*, considerably less experimental work has been conducted with *L. braziliensis*, probably because most mouse strains do not display lesions at the site of inoculation of *L. braziliensis* [4,5]. This phenotype (absence of lesion) has been associated with the inability to sustain a strong type 2 immune response [6]. Nonetheless, it has been shown that skin biopsies from patients with cutaneous leishmaniasis caused by *L. braziliensis* displayed IFN-γ, TNF-α [7] and iNOS[8]. These cytokines play a key role in the control of the parasite load and, in parallel, contribute to the development of intense inflammatory processes at the parasite inoculation site.

Alternatively, it has been shown that the parasite also plays a role in determining the parasitic process outcome, i.e. disease or long-term asymptomatic parasitism. For example, once located in the skin, the development of *L. amazonensis* is known to lead to many different clinical presentations, including localized cutaneous lesions, mucocutaneous and visceral leishmaniasis, in humans [9], some of these features being reproduced in mice [10]. More recently, it was shown that genotypic characteristics of *L. mexicana* could be correlated with features of the clinical disease [11] and that *L. major* isolates from the field show differences in pathogenicity upon inoculation into BALB/c mice [12]. Therefore, variation in the pathogenicity of the parasite, which is related to its genetic diversity, is also an important factor influencing the clinical features of leishmaniasis. Although extensive genetic diversity has been documented in *L. braziliensis* [13], it was correlated with geographical origin [14,15] and not with clinical manifestations of the disease.

In the present study, we used molecular techniques to examine the genetic polymorphism of *L. braziliensis* strains isolated from two states in Brazil, namely Ceará and Bahia, located in northeastern Brazil. Both Ceará and Bahia are endemic for cutaneous leishmaniasis caused by *L. braziliensis*. In Ceará, however, the cutaneous lesion is accompanied and sometimes preceded by an impressive enlargement of regional lymph nodes, whereas in Bahia, such findings have not been documented. We have also examined (a) the cell types present at the site of parasite delivery and (b) the cytokine production after in vitro re-stimulation of the cells recovered from the draining lymph nodes and the cell types present at the site of parasite delivery.

2. Materials and methods

2.1. Mice

Male 4–6-wk-old BALB/c mice were obtained from CPqGM/FIOCRUZ Animal Facility, where they were maintained under pathogen-free conditions. The Animal Care and Utilization Committee from CPqGM/FIOCRUZ approved all experimental procedures.

2.2. Parasite culture

The *L. braziliensis* strains MHOM/BR/94/H3227 and MHOM/BR/01/BA788 used were isolated from cutaneous ulcers from patients with cutaneous leishmaniasis, from the states of Ceará and Bahia (northeastern Brazil), respectively, after brief (2–4) passages in culture medium. Both isolates were identified as *L. braziliensis* by PCR [16] and monoclonal antibodies [17]. Promastigotes were grown in 199 medium (Gibco, Grand Island, NY) at 25 °C supplemented with 10% heat-inactivated fetal calf serum, 20 mM Hepes, 4 mM $NaHCO_3$, 100 U/ml of penicillin and 100 μg/ml streptomycin (all from Gibco).

2.3. The readout assay for typing some signatures of polymorphism of the parasites at the nuclear genomic level

Genomic DNA extraction, RAPD (Random amplification of polymorphic DNA) was done as described [18]. Briefly, 150 ng of parasite DNA was amplified in a final reaction volume of 25 μl containing 10 mM Tris–HCl pH 8.5, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP, 1.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 1.25 μM of either primer 3302 (5′-CTGATGCTAC-3′); 3303 (5′-TCACGATGCA-3′) or 3304 (5′-GCACTGTCA-3′). The amplification cycles consisted of an initial denaturation step at 95 °C for 5 min, two cycles with denaturation at 95 °C for 30 s, annealing at 30 °C for 2 min and extension at 72 °C for 1 min, followed by 33 cycles in which annealing was increased to 40 °C. Ten-microliter samples were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

2.4. Inoculation of parasites into mice

Stationary-phase promastigotes were inoculated subcutaneously into the hind left footpad of BALB/c mice at a dose of 10^6 parasites in 20 μl of saline. Observation of lesion development was made at weekly intervals and footpad swelling was measured in millimeters by a dial gauge caliper. Lesion size was defined as the increase in footpad thickness after subtraction of the size of the controlateral uninfected footpad.

2.5. Parasite load estimate

Parasite load was determined using the quantitative limiting dilution assay as described [19]. Briefly, popliteal lymph nodes draining the infected footpad were aseptically excised and homogenized with a tissue glass grinder in 2 ml of Schneider's medium (Sigma, St. Louis, MO). The homoge-

nates were serially diluted in Schneider's medium supplemented with 100 U of penicillin/ml, 100 μg/ml of streptomycin/ml, 10% heat inactivated fetal calf serum (Gibco) and 2% sterile human urine in 96-well plates containing biphasic blood agar medium. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown out after up to 3 weeks of incubation at 25 °C.

2.6. Culture of lymph node cells and quantitation of cytokine in the culture supernatants

For measurement of in vitro cytokine production, single-cell suspensions of infected footpad draining popliteal lymph nodes were prepared aseptically at 3 and 15 days post infection. The cells were diluted 5×10^6cells/ml and dispensed into 96-well plates with *L. braziliensis* H3227 or BA788 live promastigotes (stationary phase), at a ratio of five parasites to one cell, or without parasites, in RPMI-1640 medium (Gibco) containing 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg/ml of streptomycin/ml, 10% heat inactivated fetal calf serum (Gibco) and 0.05 mM β-mercaptoethanol. Cell culture supernatants were harvested after 48 h (for IL-4, IL-10 and IL-12p70 measurement) or 72 h (for IFN-γ measurement) of culture at 37 °C in 5% CO_2. Levels of IFN-γ, IL-4, IL-10 and IL-12p70 were determined by ELISA using commercial kits (BD Biosciences, San Diego, CA). The cytokine production of lymph node cells from non-infected animals, upon stimulation with live *L. braziliensis* promastigotes, was below the detection level of the kits used for cytokine detection.

2.7. Flow cytometric analysis

To characterize leukocytes present in the inoculation site, infected foot tissue was collected at 3 and 15 days post infection and incubated 1 h at 37 °C, in RPMI-1640 medium containing 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg/ml of streptomycin/ml, 10% heat inactivated fetal calf serum (all from Gibco), 0.28 units/ml of Liberase CI (Roche, São Paulo, SP). Foot tissues were processed in the presence of 0.05% DNAse (Sigma-Aldrich, St. Louis, MO) using Medimachine (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. After processing, cell viability was assessed by trypan blue exclusion and cells were filtered trough a 50-μm filter and washed before flow cytometry analysis. Viable leukocytes were incubated with antibodies specific to GR-1, Mac-1, NK 1.1, CD3 or MHC class II molecules or with isotype control antibodies (all from BD Biosciences, San Diego, CA) for 30 min at 4 °C in the dark. Cells were washed and resuspended in PBS, 1% formaldehyde. For each sample, 10,000 cells were examined. Data were acquired using a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

2.8. Statistical analysis

The data are presented as mean ± standard error of the mean. The significance of the results was calculated by Student's *t*-test, except for the parasite load analysis, over time, within the BA788- and H337-infected mice groups where ANOVA was used. All analyses were performed using Prism (Graph Pad Software, San Diego, CA) software and a *P*-value <0.05 was considered significant.

3. Results

3.1. The BA788 and H3227 L. braziliensis strains display genomic polymorphisms: a study with RAPD

In preliminary experiments, we investigated the degree of genetic polymorphism by RAPD between six *L. braziliensis* isolates, two from Ceará (MHOM/BR/94/H3227, MHOM/BR/94/H3456) and four from Bahia (MHOM/BR/00/BA711, MHOM/BR/02/BA427, MHOM/BR/02/806 and MHOM/BR/01/BA788). In these experiments, the genomic profiles obtained with the isolates from Ceará were different from those obtained with the isolates from Bahia (data not shown); data obtained with two representative isolates, namely BA788 (Bahia) and H3227 (Ceará), are shown (Fig. 1). RAPD generated 43 amplified bands, varying from 100–2000 bp in length, of which six are common to the two strains. The most diverse genomic profiles were obtained with primers 3303 and 3304, in which 26 of the 35 amplified fragments were polymorphic. The distinct genomic profiles obtained for strains BA788 and H3227, with the three primers tested here, assess DNA polymorphism between these *L. braziliensis* strains.

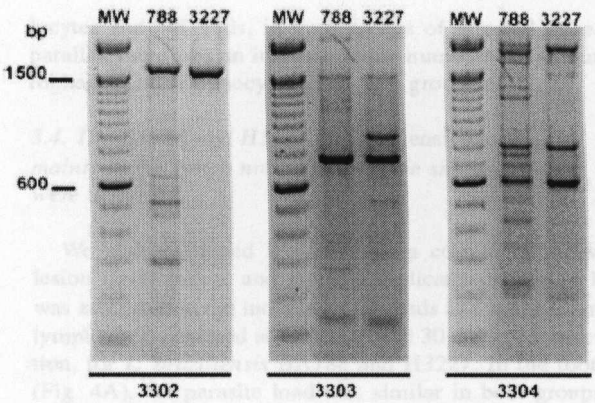

Fig. 1. Genomic profiles of *L. braziliensis* strains BA788 and H3227. Genomic DNA of each *L. braziliensis* strain was subjected to RAPD using primers 3302, 3303 and 3304. MW: 100 bp ladder DNA size marker.

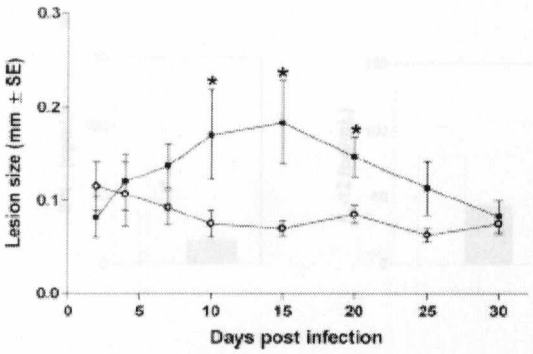

Fig. 2. Course of lesion development in BALB/c mice inoculated with *L. braziliensis* strains. BALB/c mice were infected with 10^6 of either H3227 (black square) or BA788 (white circle) promastigotes in the left hind footpad, and lesion size was measured for 30 days. The footpads of 3–5 mice per group were measured. The data shown, reported as the mean ± standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values at the indicated time point as determined by Student's *t*-test (* $P < 0.05$).

3.2. The amplitude of the transient clinical features occurring at the site of L. braziliensis *delivery differs according to whether the isolate is BA788 or H3227*

The genomic polymorphism observed between BA788 and H3227 prompted us to investigate whether these strains might display differences in pathogenicity upon inoculation into mice of an inbred strain. BALB/c mice received, subcutaneously in the footpad, 10^6 promastigotes of either BA788 or H3227 (Fig. 2). Small increases in footpad thickness were observed early after infection (6 h to 5 days). Mice infected with H3227 developed lesions detectable at 7 days post inoculation. Lesions progressed steadily, peaked at 15 days post parasite inoculation and gradually resolved 30 days post infection. At this time, we observed the presence of a small nodular lesion associated with cutaneous fibrosis (data not shown). All lesions caused by H3227 *L. braziliensis* were unulcerated. Even at maximum size, lesions remained non-ulcerative and non-necrotic. In mice infected with BA788, in contrast, no similar transient pathogenic processes were noticed. The differences in footpad thickness, between both groups of mice, were significant ($P < 0.05$) in the period ranging from 10–20 days post inoculation.

3.3. The phenotypic composition of leukocytes present at the site of BA788 or H3227 L. braziliensis *delivery differs according to the isolate*

In order to characterize the leukocyte lineages present at the site of parasite delivery, the footpads were excised at 3 and 15 days post parasite inoculation and analyzed by flow cytometry. At 3 days post inoculation, the number of granulocytes, macrophages and lymphocytes is similar in both groups of mice, whereas the number of NK cells is higher in BA788-parasitized mice (Fig. 3). Fifteen days post parasite inoculation, we observed a decrease in the numbers of granulocytes and NK cells, in both groups of infected mice. In parallel, there was an increase in the numbers of both macrophages and lymphocytes in the two groups of mice.

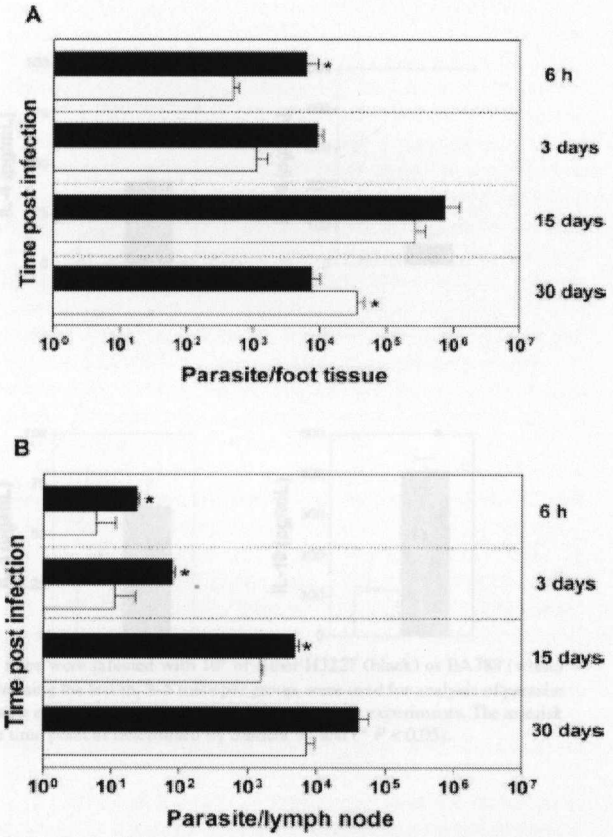

Fig. 3. Phenotypic composition of leukocyte population recovered from the footpad of mice inoculated with *L. braziliensis* strains. BALB/c mice were inoculated with 10^6 promastigotes of either H3227 (black) or BA788 (white) in the left hind footpad of BALB/c mice. Three (A) and 15 days (B) post inoculation. Leukocytes from inoculated footpads were analyzed by flow cytometry for the presence of lymphocytes, granulocytes, macrophages and NK cells. The data shown are from a single experiment representative of two separate experiments.

3.4. The BA788 and H3227 L. braziliensis *loads differ mainly in the lymph node draining the site where they were delivered*

We also examined if there was a correlation between lesion development and parasite replication. Parasite load was estimated in the inoculated footpads and in the draining lymph nodes, excised at 6 h, 3, 15 and 30 days post inoculation, for *L. braziliensis* BA788 and H3227. In the footpad (Fig. 4A), the parasite load was similar in both groups of mice, but statistically significant differences were observed at 6 h and 30 days post inoculation. In mice inoculated with H3227 parasites, the parasite load peaked at 15 days, coin-

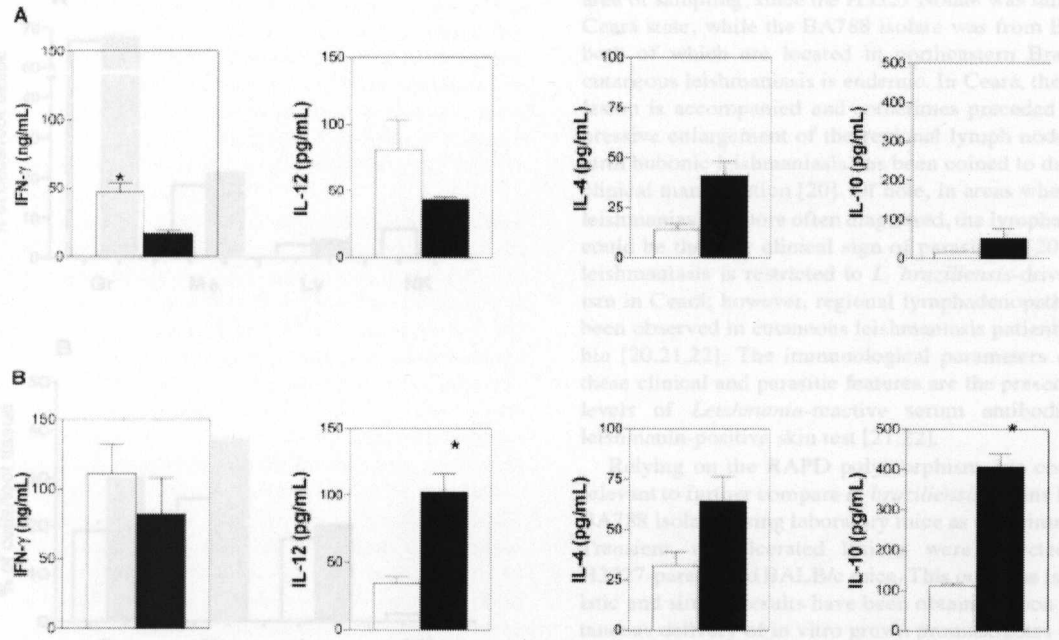

Fig. 4. Parasite load estimate in mice infected with *L. braziliensis* strains. BALB/c mice were infected with 10^6 of either H3227 (black) or BA788 (white) promastigotes in the left hind footpad. Footpads (A) and popliteal lymph nodes (B) draining the lesion, 3–5 mice per group, were used for analysis of parasite load. The data shown, reported as the mean ± standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values from H3227 and BA788 at the same time point as determined by Student's *t*-test (* $P < 0.05$).

ciding with lesion development. However, at this time point, no clinical lesion was observed in mice inoculated with BA788, despite a similar parasite load estimate, indicating dissociation between parasite replication and lesion development. In the draining lymph nodes (Fig. 4B), the parasite load was significantly higher in H3227-parasitized mice, from 6 h to 15 days post parasite inoculation. Interestingly, in the draining lymph nodes, the parasite load showed a consistent increase in both groups of parasitized mice, indicating that parasites are able to multiply in these secondary lymphoid tissues, regardless of lesion resolution at the upstream site where parasites were inoculated and developed, such as that seen in H3227-parasitized mice.

When comparing the parasite load estimate within the BA788 and H3227 groups, separately, over time, we did not find any significant differences in the footpads inoculated with H3227. In the footpads inoculated with BA788, however, there were statistically significant differences between 6 h and 15 days ($P < 0.01$), 3 and 15 days ($P < 0.01$) and 15 and 30 days post inoculation ($P < 0.05$). Therefore, in the footpad, the parasite load estimate found at 15 days post inoculation is higher than that found at 6 h, 3 days and 30 days post inoculation only in mice receiving the BA788 isolates. In the draining lymph nodes, we did not find any statistically significant differences, between the BA788 and H3227 isolates when comparing the parasite load estimate.

3.5. At the later time point studied, there is a difference between the ex vivo cytokine profiles displayed by the leukocytes recovered from lymph nodes draining the site of delivery of BA788 or H3227

The observations that H3227-infected mice controlled the cutaneous lesion and that BA788-infected mice did not develop clinical lesions suggest that, in both cases, a Th1-type cellular immune response had developed. At 3 and 15 days post infection, cells prepared from the lymph nodes draining the infection site were incubated with live BA788 or H3227 promastigotes and were monitored for the production of Th1- and Th2-type cytokines. Three days post infection (Fig. 5A), cells from BA788-infected mice produced higher levels of both IFN-γ ($P < 0.05$) and IL-12, than cells from H3227-infected mice which, on the contrary, produced higher amounts of both IL-4 and IL-10. Fifteen days post-infection (Fig. 5B), Th1-type cytokine production was markedly up-regulated in H3227-infected mice as shown by the significant increase in IL-12 production, in parallel with IFN-γ. Cells from H3227-infected mice also produced higher amounts of both IL-4 and IL-10, the latter showing a significant up-regulation at this time point. This up-regulation in IL-10 production coincides with the time point at which lesion size and parasite load detected in the footpad reached their peak. Moreover, lymph node cell counts were consistently higher in H3227 *L. braziliensis*-infected mice than in BA788-

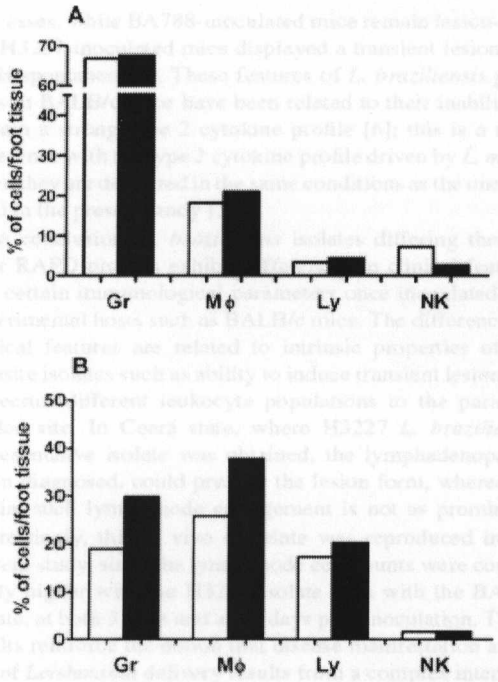

Fig. 5. Cytokine production by mononuclear cells from mice infected with *L. braziliensis* strains. BALB/c mice were infected with 10^6 promastigotes of either H3227 (black) or BA788 (white) in the left hind footpad. Three (A) and 15 days (B) post infection, mononuclear cells (3–5 mice per group) from popliteal lymph nodes draining the lesion were incubated with live *Leishmania* promastigotes and the supernatants were assayed for IL-4, IL-10, IL-12p70 and IFN-γ production. The data shown, reported as the mean ± standard error of the mean, are from a single experiment representative of three separate experiments. Cytokine production of lymph node cells from non-infected animals, upon stimulation with live *L. braziliensis* promastigotes, was below the detection level of the ELISA kits used: IFN-γ (55 pg/ml), IL-4 (5.5 pg/ml), IL-10 (3 pg/ml) and IL-12p70 (5 pg/ml). The asterisk indicates a significant difference between values as determined by Student's *t*-test (*$P < 0.05$).

infected mice, at both 3 days post infection ($14.8 \times 10^6 \pm 1.6 \times 10^6$ vs. $8.1 \times 10^6 \pm 0.3 \times 10^6$, respectively) and at 15 days post infection ($24.0 \times 10^6 \pm 2.8 \times 10^6$ vs. $14.7 \times 10^6 \pm 1.3 \times 10^6$, respectively).

4. Discussion

L. braziliensis parasite populations are extremely diverse, as assessed by RAPD-based readout assays [13]. However, this diversity is less pronounced among parasite populations obtained from areas of geographical proximity [14]. Random amplification of *L. braziliensis* H3227 and BA788 DNA generated different genomic profiles, including the presence of amplification fragments not shared between strains. These fragments may constitute nuclear genomic markers for strains from these areas. As far as the BA788 and H3227 *L. braziliensis* isolates are concerned, the RAPD-based polymorphism can therefore be correlated with their geographical area of sampling, since the H3327 isolate was sampled from Ceará state, while the BA788 isolate was from Bahia state, both of which are located in northeastern Brazil, where cutaneous leishmaniasis is endemic. In Ceará, the cutaneous lesion is accompanied and sometimes preceded by an impressive enlargement of the regional lymph nodes, and the term bubonic leishmaniasis has been coined to describe this clinical manifestation [20]. Of note, in areas where bubonic leishmaniasis is more often diagnosed, the lymphadenopathy could be the only clinical sign of parasitism [20]. Bubonic leishmaniasis is restricted to *L. braziliensis*-driven parasitism in Ceará; however, regional lymphadenopathy has also been observed in cutaneous leishmaniasis patients from Bahia [20,21,22]. The immunological parameters coupled to these clinical and parasitic features are the presence of high levels of *Leishmania*-reactive serum antibodies and a leishmanin-positive skin test [21,22].

Relying on the RAPD polymorphism, we considered it relevant to further compare *L. braziliensis* strains H3227 and BA788 isolates using laboratory mice as experimental hosts. Transient, non-ulcerated lesions were detected only in H3227-parasitized BALB/c mice. This outcome is characteristic and similar results have been obtained upon the subcutaneous delivery of in vitro grown promastigotes of other *L. braziliensis* isolates to BALB/c mice [23–25]. We did not find any correlation between lesion development and parasite load: indeed the parasite load in the footpad was not significantly different between BA788- and H3227-parasitized mice.

H3227-loaded footpads were processed for histological analysis at 3 and 15 days post inoculation: an inflammatory infiltrate consisting mainly of polymorphonuclear leucocytes and macrophages was noticed. The BA788-loaded footpad sections exhibited a less intense and more transient leukocyte infiltrate (data not shown). Similarly to other *L. braziliensis* strains, the presence of granulocytes and lymphocytes was noticed during the first week post inoculation; later, many more macrophages and lymphocytes were observed [26].Three days post inoculation, when compared to footpads of H3227-inoculated mice, more NK leukocytes were observed on footpad samples prepared from BA788-inoculated mice. NK leucocytes are known to produce IFN-γ and may contribute to resistance to *L. braziliensis* as previously shown for *L. major* [27]. Moreover, NK cells are detectable as soon as 24 h post inoculation at the site of *L. major* delivery in resistant mice [28]. Therefore, it is possible that the early and higher IFN-γ production observed at the site of BA788 parasite delivery is due to the presence of a higher number of NK cells.

Following the in vitro stimulation of lymph node cells, the ratio of IFN-γ to IL-10 was higher when the cells were recovered from BA788-parasitized mice than when the cells were recovered from H3227-parasitized mice, at both 3 days post inoculation (2.28 vs. 0.24), respectively, and at 15 days post inoculation (0.77 vs. 0.15), respectively. These immunological features are coupled to the outcome of the parasitic

processes while BA788-inoculated mice remain lesion-free, the H3227-inoculated mice displayed a transient lesion that heals spontaneously. These features of *L. braziliensis* parasites in BALB/c mice have been related to their inability to sustain a strong type 2 cytokine profile [6]; this is a main difference with the type 2 cytokine profile driven by *L. major* when they are delivered in the same conditions as the ones we used in the present study [3].

In conclusion, *L. braziliensis* isolates differing through their RAPD profiles exhibit differences in clinical features and certain immunological parameters once inoculated into experimental hosts such as BALB/c mice. The differences in clinical features are related to intrinsic properties of the parasite isolates such as ability to induce transient lesion and to recruit different leukocyte populations to the parasite-loaded site. In Ceara state, where H3227 *L. braziliensis* representative isolate was obtained, the lymphadenopathy, when diagnosed, could precede the lesion form, whereas in Bahia, such lymph node enlargement is not as prominent. Interestingly, this in vivo correlate was reproduced in the present study, since the lymph node cell counts were consistently higher with the H3227 isolate than with the BA788 isolate, at both 3 days and at 15 days post inoculation. These results reinforce the notion that disease manifestation at the site of *Leishmania* delivery results from a complex interplay between genetically determined parasite as well as host traits. Studies are now in progress to monitor more relevant parasite genetic polymorphism [29].

Acknowledgments

The authors thank Alexandra R.V. Dias for technical assistance. This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB). C. Brodskyn, M. Barral-Netto and A. Barral are senior investigators from CNPq and Instituto de Investigação em Imunologia.

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6. ARTIGO II

Título: "Distinct *Leishmania braziliensis* isolates induce different paces of chemokine expression patterns"

6.1. Justificativa e Objetivos do estudo:

Infecções por leishmania podem resultar em um amplo espectro de manifestações clínicas. O desenvolvimento da doença é determinado por interações complexas entre o parasito e o hospedeiro. Acredita-se que os eventos iniciais da interação parasito-hospedeiro influenciam o futuro curso da doença. Após a inoculação de leishmania na pele, uma reação inflamatória local é iniciada e envolve o influxo de muitos leucócitos para o sítio de infecção. O tamanho da lesão é dependente da resposta imune do hospedeiro e altamente influenciado pela composição celular no local inflamatório. Portanto, a relativa composição da população celular recrutada na fase inicial da infecção parece ser essencial para definir o curso da doença, e neste processo, as quimiocinas têm um papel importante porque podem atrair e estimular subpopulações específicas de leucócitos para o sítio da infecção. Neste trabalho, investigamos a correlação entre padrões de expressão de quimiocinas, a formação da lesão e a atração de leucócitos para o sítio de infecção, comparando duas cepas de *L. braziliensis* (H3227 e BA788) geneticamente diferentes e que apresentavam distintos comportamentos em camundongos BALB/c. A hipótese deste estudo é que cepas de *L. braziliensis* geneticamente diferentes induzem distintas quimiocinas e respostas inflamatórias diferenciadas. Os objetivos específicos deste estudo foram:

1. Avaliar a composição celular nas lesões de camundongos BALB/c causadas por cepas de *L. braziliensis* com perfis genômicos diferentes;

2. Avaliar a migração de leucócitos induzida por essas cepas de *L. braziliensis* no modelo do bolsão de ar inflamatório;

3. Avaliar os padrões de expressão e a cinética "in situ" de quimiocinas e seus respectivos receptores induzidos por essas cepas de *L. braziliensis*.

6.2. Resumo dos Resultados: Os resultados obtidos inicialmente, utilizando a técnica de RAPD, demonstraram que as cepas de *L. braziliensis* H3227 e BA788 apresentavam genótipos diferentes. Grupos de camundongos BALB/c foram infectados com essas cepas e acompanhadas por 30 dias. Na análise histopatológica do sítio inflamatório, observamos que enquanto as lesões dos animais infectados com BA788 não sofreram nenhuma mudança qualitativa nem quantitativa no infiltrado inflamatório nos períodos avaliados, as maiores lesões induzidas por H3227 estavam correlacionadas com uma maior reação inflamatória, com a presença de muitos granulócitos nos primeiros três dias após a infecção e mais macrófagos a partir do 15° dia de infecção. Os resultados do bolsão de ar inflamatório mostraram que o recrutamento de leucócitos foi rapidamente induzido pelas duas cepas de *Leishmania*, atingindo um pico máximo de 12 h após a inoculação dos estímulos dentro do bolsão inflamatório, declinando após o período de 24 h. A resposta induzida por H3227 foi três vezes maior que aquela induzida por BA788, sugerindo que a cepa H3227 foi a mais potente recrutadora de todos os tipos celulares, atraindo significantemente mais neutrófilos e macrófagos do que a cepa BA788. Em relação as quimiocinas, foi encontrada uma precoce e alta expressão de CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, CCL11/eotaxina, XCL1/linfotactina e seus respectivos receptores nos camundongos infectados com H3227, o que se correlacionou com um maior recrutamento de leucócitos para a lesão e com o aumento da inflamação observada nestes camundongos após quinze dias de infecção. Ao contrário, animais infectados com BA788 apresentaram uma expressão tardia e menos forte das quimiocinas e receptores de quimiocinas estudados, quando comparados com aqueles infectados com H3227. Estes dados estavam associados com a ausência de uma significante resposta inflamatória na lesão destes animais. Concluímos que cepas de *L. braziliensis* com genótipos diversos, apesar de carga parasitária semelhante, induzem expressão de quimiocinas em diferentes tempos e/ou intensidades, levando a um recrutamento celular distinto e respostas inflamatórias diferenciadas.

INFECTION AND IMMUNITY, Feb. 2005, p. 1191–1195 Vol. 73, No. 2
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.2.1191–1195.2005

Distinct *Leishmania braziliensis* Isolates Induce Different Paces of Chemokine Expression Patterns

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Received 30 May 2004/Returned for modification 24 September 2004/Accepted 18 October 2004

Inflammatory events during *Leishmania braziliensis* infection in mice were investigated. Large lesions were directly correlated with the inflammatory reaction but not with parasite burden. Different *L. braziliensis* strains induce different paces of chemokine expression patterns, leading to diverse cell recruitment and differential inflammatory responses.

Chemokines have been implicated in inflammatory responses against numerous infectious agents, including *Leishmania* (5, 17, 18, 20). *Leishmania braziliensis* is the main agent of cutaneous leishmaniasis (CL) in Brazil; it causes single self-limited cutaneous ulcers and highly destructive mucosal leishmaniasis (10). In this study, using a murine model, we compared *L. braziliensis* strains isolated from two states in Brazil, namely, Ceará and Bahia, located in northeastern Brazil. CL caused by *L. braziliensis* is endemic in both states. In Ceará, the cutaneous lesion is accompanied and sometimes

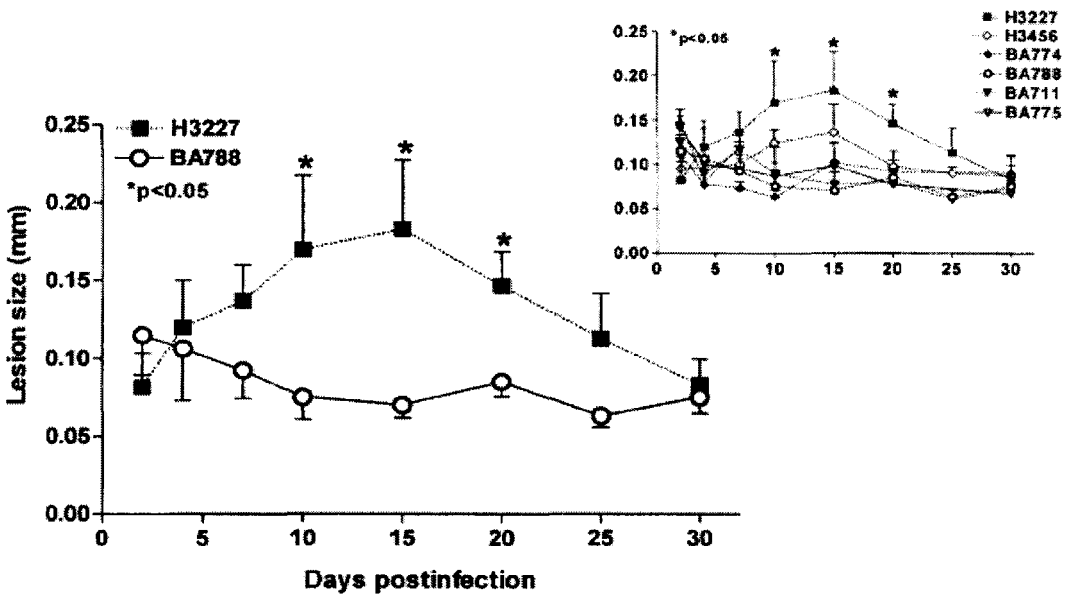

FIG. 1. Course of infection with *L. braziliensis* in BALB/c mice. The inset shows kinetics of lesion development in BALB/c mice during the course of infection with six *L. braziliensis* isolates. The main figure illustrates the time course of infection with the two isolates used here, showing a polar pattern of infection. Mice were inoculated in the hind footpads with 10^6 stationary-phase *L. braziliensis* promastigotes, and lesions were measured weekly for 30 days p.i. The footpads of three to five animals per group were measured. The data shown, reported as the mean and standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values at the indicated time point, as determined by Student's *t* test ($P < 0.05$). Experiments with all *L. braziliensis* isolates were repeated three times, with similar results.

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preceded by an impressive enlargement of the regional lymph nodes; the term "bubonic leishmaniasis" has been coined to describe this manifestation (23). Bubonic leishmaniasis is restricted to *L. braziliensis* infection in Ceará; however, localized lymphadenopathy has been observed in CL patients from Bahia (2, 3).

Mice were infected with 10^6 stationary-phase forms of *L. braziliensis* (6). In preliminary experiments, the isolates obtained from CL patients from Ceará (MHOM/BR/94/H3227 [H3227]and MHOM/BR/94/H3456) and from Bahia (MHOM/BR/00/BA711, MHOM/BR/00/BA774, MHOM/BR/00/BA775, and MHOM/BR/01/BA788 [BA788]) showed significant differences in pathogenicity (Fig. 1, inset). Further experiments were performed with two of these *L. braziliensis* isolates, H3227 and BA788. The lesions caused by H3227 were larger and persisted longer than those caused by BA788 (Fig. 1). Lesion size differences did not appear to be due to diverse parasite loads, since parasite numbers were not significantly different between H3227- and BA788-infected mice at 15 days postinfection (p.i.) (mean and standard error of the mean, $7.29 \times 10^5 \pm 5.28 \times 10^5$ and $2.64 \times 10^5 \pm 1.20 \times 10^5$, respectively), when lesion sizes were different. Lesions from H3227-infected mice exhibited an inflammatory infiltrate consisting mainly of polymorphonuclear leukocytes and macrophages at 3 days p.i., and these histopathological features persisted at 15 days p.i. Sections from BA788-infected mice showed a less intense and more transient leukocyte infiltrate.

In order to explore the role of the parasite in the histopathological differences observed, we evaluated cell recruitment induced by H3227 and BA788 by using the air pouch model (14, 15). Responses induced by H3227 were three times higher than those induced by BA788 (Fig. 2A); these responses were correlated with a more intense exudate of leukocytes observed in the lesions of H3227-infected mice. H3227 was able to induce more influx of all cell types, attracting mainly more neutrophils and macrophages than BA788 (Fig. 2B). These data reinforce a role of the parasite in the differences observed in the inflammatory processes induced by the two *L. braziliensis* isolates used here.

RNA was extracted from lesions for reverse transcription-PCR analysis of chemokine expression at 6 h, 3 days, and 15 days p.i (12, 16). The sequences of the primers used are shown in Table 1. The expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC was upregulated at 6 h p.i. on H3227-induced lesions and only at 3 days p.i. in BA788-infected mice (Fig. 3A). In addition, CCL2/MCP-1, CCL3/MIP-1α, XCL1/lymphotactin-1, CXCL1/KC, and CCL11/eotaxin expression was more strongly induced by H3227 than by BA788. CXCL10/IP-10 was the only chemokine that appeared to be more strongly expressed by BA788 than by H3227. Regarding chemokine receptor expression, H3227 showed significantly higher expression of all chemokine receptors studied here than did BA788 (Fig. 3B). CCR5 was slightly upregulated in BA788-infected mice. Immunohistochemical analysis for the presence of CCL2/MCP-1 and CXCL10/IP-10 proteins in lesions induced by H3227 and BA788 confirmed the results obtained by mRNA expression analysis (Fig. 3C).

Lesions from patients with CL show a significant increase in the expression of CCL2/MCP-1 and CCL3/MIP-1α (20), and in vitro infection with *Leishmania* induces CCL2/MCP-1 and

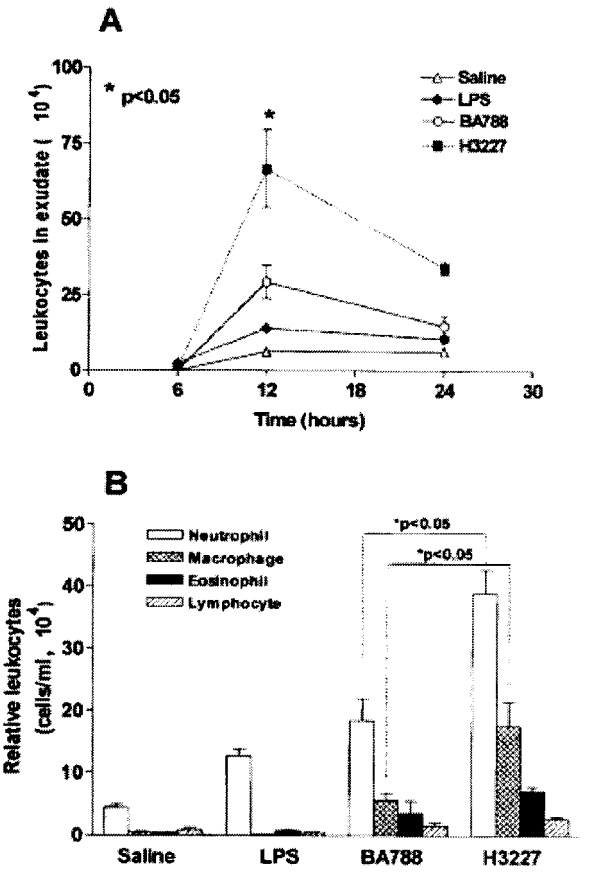
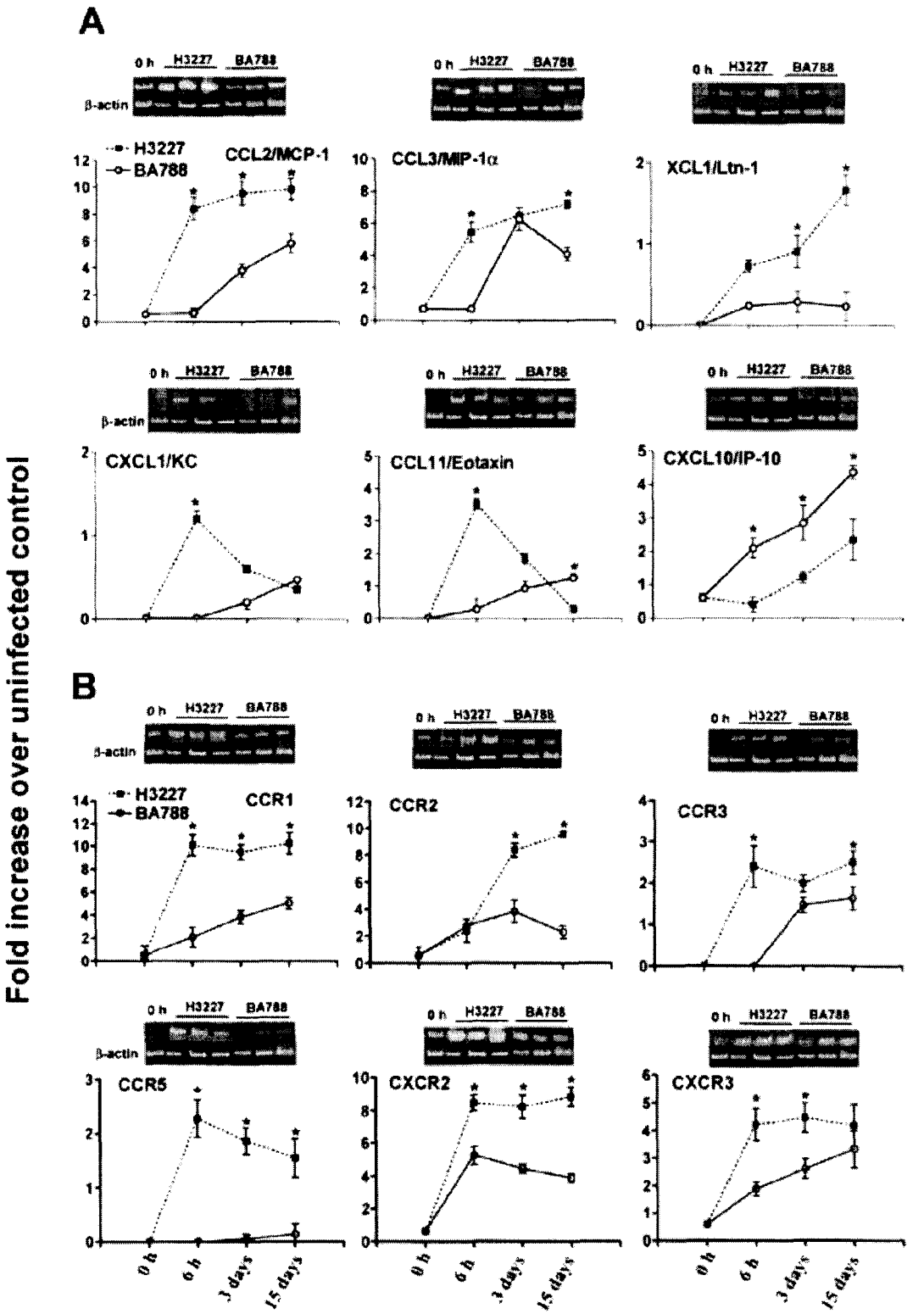


FIG. 2. Kinetics of leukocyte recruitment (A), expressed as total numbers of neutrophils, macrophages, eosinophils, and lymphocytes, in pouch exudates in response to lipopolysaccharide (LPS) or *L. braziliensis* (BA788 and H3227) and comparison of results at 12 h after inoculation (B). Air pouches were prepared by injecting 3 ml of air into the dorsal surface of mice under light anesthesia. Stationary-phase *L. braziliensis* promastigotes (10^7) were injected immediately following the air injection. Control mice were injected with endotoxin-free saline (negative control) and LPS (20 μg/ml; positive control). Mice were killed at the indicated time points, and the pouch contents were washed several time with saline. Exudate cells were centrifuged and stained; proportions of neutrophils, macrophages, eosinophils, and lymphocytes/200 cells were enumerated; and relative cell numbers were calculated from the total number of exudate leukocytes. Data represent the mean and standard error of the mean for three to five mice. The asterisk indicates a significant difference between values at the indicated time point, as determined by Student's *t* test or one-way analysis of variance ($P < 0.05$). Results are representative of two independent experiments.

CXCL1/KC/GRO-α expression in mouse and human macrophages (1, 19). CCL2/MCP-1 and CCL3/MIP-1α are potent chemoattractants for monocytes (9, 13). CXCL1/KC recruits neutrophils and is a dominant chemokine in murine inflammatory responses (4). The earlier expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC in more severe lesions may explain the significant and early accumulation of neutrophils and macrophages at the H3227 infection site and suggests that these chemokines can be factors regulating the differential


A
B
0 h
H3227
BA788
β-actin
H3227
BA788
CCL2/MCP-1
CCL3/MIP-1α
XCL1/Ltn-1
CXCL1/KC
CCL11/Eotaxin
CXCL10/IP-10
CCR1
CCR2
CCR3
CCR5
CXCR2
CXCR3
Fold increase over uninfected control
0 h
6 h
3 days
15 days

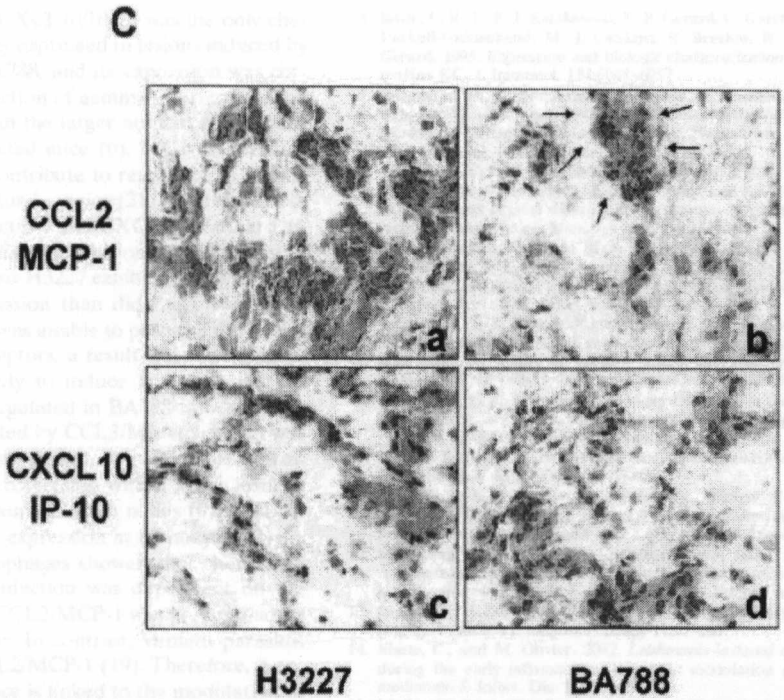


FIG. 3. Chemokine (A) and chemokine receptor (B) mRNA expression and protein production, determined by immunohistochemical analysis (C), in lesions of *L. braziliensis*-infected BALB/c mice. (A and B) Mice were infected with 10^6 H3227 or BA788 promastigotes and killed at 6 h, 3 days, and 15 days p.i. The infected hind footpads were used in assays of mRNA expression by reverse transcription-PCR. Densitometric analysis was performed, and quantification was normalized to the levels of β-actin expression. Results are expressed as *n*-fold increases over results obtained with uninfected control animals (0 h). Upper and lower rows in the gels show the expression of chemokines and β-actin, respectively, at 0 h, 6 h, 3 days, and 15 days p.i. (lanes from left to right). The profiles are representative of at least three independent experiments. In each experiment, mRNA was prepared from pools of three or four mice per time point. Each point in the graphs represents the mean and standard error of the mean for a pool of three or four mice per time point in three experiments. CCL5/RANTES, CXCL9/MIG, and CCL22/MDC expression did not show significant modulation in this model of *L. braziliensis* infection (data not shown). (C) Frozen 5-μm sections of infected and uninfected foot tissues were used to perform immunohistochemical analysis for chemokines. Immunoperoxidase staining clearly showed at 3 days p.i. strong expression of CCL2/MCP-1 in H3227-infected sections (a) and weak expression in BA788-infected sections (b). Strong anti-CXCL10/IP-10 immunoreactivity was seen in sections of BA788-infected mice (d) but not in sections of H3227-infected mice (c). Magnification, ×34.

inflammatory responses which develop upon infection with the two *L. braziliensis* isolates used here.

H3227 induced XCL1/lymphotactin-1 and, to a lesser extent, CXCL10/IP-10. XCL1/lymphotactin-1 is chemotactic for NK, $CD4^+$, and $CD8^+$ T cells in vitro and in vivo (8, 11), and CXCL10/IP-10 activates NK cells in vivo (25). Furthermore, XCL1/lymphotactin-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES are associated with a Th1 immune re-

TABLE 1. Primer sequences and sizes of PCR products

Oligonucleotide	Sense primer (5′-3′)	Antisense primer (3′-5′)	Product size (bp)
β-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C	TAA AAA GCA GCT CAG TAA CAG TCC G	349
CXCL1/KC/GRO-α	CC TTG ACC CTG AAG CTC CCT TGG TTC	CGT GCG TGT TGA CCA TAC AAT ATG	422
CXCL9/MIG	GAT CAA ACC TGC CTA GAT CC	GGC TGT GTA GAA CAC AGA GT	399
CXCL10/IP-10	TCG CAC CTC CAC ATA GCT TAC AG	TCA GCA GAG ATG TCT GAA TC	310
CCL11/eotaxin	AGT CCT TGG GCG ACT GGT GC	GCA GAG CTC CAC AGC GCT TC	243
CCL2/MCP-1/JE	CTA AGG ACC ACT TGC CAT GGA	CTG GTA GCT CTC TGC CCT GTT T	445
CCL3/MIP-1α	C CGG AAG ATT CCA CGC CAA TTC	T GAG GAA CGT GTC CTG AAG	427
CCL5/RANTES	C CCA CGT CAA GCA GTA TTT C	CTG GTT TCT TGG GTT TGC TGT G	506
CCL22/MDC	GTG GCT CTC GTC CTT CTT GC	GGA CAG TTT ATG GAG TAG CTT	249
XCL1/lymphotactin-1	CAA GAC CTC AGC CAT GAG AC	TGC AAT GGG TTT GGG AAC TG	397
CCR1	TCT CTG ATC TGG TCT TCC TTT T	CCC AGG TGA TAA TAC TGG TGA T	295
CCR2	CTA CGA TGA TGG TGA GCC TTG T	ACC AAT GTG ATA GAG CCC TGT G	368
CCR3	CAA CTT GGC ATT TTC TGA CCT G	TTT CCA GCT GTC TTC TTC ACC T	334
CCR5	CTC TTC CTG CTC ACA CTA CCA T	TGT GTA GAA AAT GAG GAC TGC A	322
CXCR2	GAG AAC CTG GAA ATC AAC AGT T	GTA CTT GTG GCA TGT ACA ATG G	339
CXCR3	ATC TAC CTA TCA GCC AAC TAC G	ACA TCC ACA TTT GCT CTC TGA A	433

sponse (7, 22). Interestingly, CXCL10/IP-10 was the only chemokine that was more strongly expressed in lesions induced by the less pathogenic strain BA788, and its expression was correlated with the earlier production of gamma interferon in the draining lymph nodes and with the larger number of NK cells in the lesions of BA788-infected mice (6). NK cells produce gamma interferon and may contribute to resistance to *L. braziliensis*, as previously shown for *L. major* (21). Therefore, it is possible that XCL1/lymphotactin-1 and CXCL10/IP-10 are involved in resistance to *L. braziliensis* infection in BALB/c mice. Lesions caused by *L. braziliensis* H3227 exhibited a higher level of chemokine receptor expression than did those caused by *L. braziliensis* BA788. BA788 was unable to promote the strong expression of chemokine receptors, a result which was correlated with its reduced capacity to induce leukocyte recruitment. CCR5 was slightly upregulated in BA788-infected mice at 3 days p.i. and was stimulated by CCL3/MIP-1α, which was expressed during the same time period. A low level of expression of CCR5 in lesions was correlated with a lower level of expression of IL-10 in the draining lymph nodes (6), as IL-10 selectively upregulates CCR5 expression in monocytes (24).

Studies with murine macrophages showed that chemokine induction after *Leishmania* infection was dependent on the parasite strain used. Indeed, CCL2/MCP-1 was predominantly induced by avirulent *L. major*. In contrast, virulent parasites induced considerably less CCL2/MCP-1 (19). Therefore, it appears that *Leishmania* virulence is linked to the modulation of chemokine expression by macrophages. The kinetics of chemokine induction seem to be more important than parasite multiplication, and this fact may be related to structural differences between the two isolates used here. Of note, results from an analysis by random amplification of polymorphic DNA showed that strains H3227 and BA788 of *L. braziliensis* are genetically diverse (6).

Collectively, the findings presented here indicate that two *L. braziliensis* isolates, albeit at similar parasite burdens, induced chemokine expression patterns at different paces and/or intensities, leading to diverse cell recruitment and differential inflammatory responses; these features might ultimately be implicated in disease presentations.

We thank Cristiane M. Milanezi and Jorge L. Tolentino for technical assistance.

This work was supported by grants from FAPESB, CAPES (PROCAD 0103/03-5), and CNPq. M.J.T. and C.R.T. received fellowships from CAPES. J.S.e.S., C.I.B., M.B.-N., and A.B. are senior investigators from CNPq. J.D.F. and B.B.A. received scientific initiation fellowships from CNPq.

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Editor: A. D. O'Brien

7. ARTIGO III - REVISÃO

Título: "Chemokines in host-parasite interactions in leishmaniasis"

7.1. Comentários sobre o artigo:

Nesta revisão são abordados os potenciais papéis das quimiocinas e seus receptores nas interações entre leishmania e hospedeiro, incluindo recrutamento de leucócitos e ativação celular, participação na imunidade mediada por células e atividade leishmanicida. Nesta revisão, são discutidos os trabalhos mais recentes que têm contribuído para uma melhor compreensão do papel das quimiocinas nas interações parasito-hospedeiro em leishmaniose. Além disso, como as quimiocinas parecem ser relativamente seletivas em suas ações, comparadas com muitos outros mediadores inflamatórios, são abordados também seus papéis potenciais como alvos terapêuticos.

Chemokines in host-parasite interactions in leishmaniasis

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Critical to the defense against *Leishmania* infection is the host ability to mount a cell-mediated immune response capable of controlling and/or eliminating the parasite. Cell recruitment to the site of infection is essential to the development of host cellular immune response. The process is controlled by chemokines, which are chemotactic cytokines produced by leukocytes and tissue cells. We review here the role of chemokines and chemokine receptors in host-parasite interactions in leishmaniasis, with emphasis on the steps immediately after infection by *Leishmania*.

Introduction

Leishmania are protozoan parasites that cause a wide spectrum of clinical manifestations, either in tegument or viscera. In tegument it ranges from localized cutaneous and mucocutaneous leishmaniases (CL and MCL) to diffuse cutaneous leishmaniasis (DCL), whereas in viscera it ranges from subclinical to potentially fatal disease [1]. The most severe forms are associated either to high parasite numbers and absence of an effective Th1 type immune response (IFN-γ and TNF-α), as seen in VL patients, or to a high inflammatory response with few parasites but also a lot of tissue damage, as seen in MCL [1]. Fifteen million people worldwide are infected with this parasite, and more than 400,000 new cases are reported annually (http://www.who.int/emc/diseases/leish/leisdis1.html). The leishmaniases are found in at least 88 countries but more than 90% of cases are observed in underdeveloped or developing countries such as Brazil, Bangladesh, India and Sudan. Parasites that cause New World CL are grouped under the *L. braziliensis* and *L. mexicana* complexes, whereas those that cause VL are grouped under the *L. donovani* complex [1]. The etiological agents of Old World CL are represented by *L. tropica*, *L. aethiopica* and *L. major*. *Leishmania* parasites are obligatory

intracellular pathogens that preferentially invade macrophages, or dendritic cells for replication. Early events on the host-parasite interactions are likely to influence future course of the disease. After infection with *Leishmania* in the skin, a local inflammatory process is initiated, which involves the accumulation of leukocytes at the site of parasite delivery [2]. The relative composition of cell populations recruited in this early phase of the infection seems to be essential to define the outcome of the disease, and during this process, members of the chemokine family play a fundamental role by attracting and stimulating specific subsets of leukocytes to the site of infection [3]. The potential roles of chemokines in *Leishmania* infection include host defense functions such as leukocyte recruitment, participation in cell-mediated immunity, cell activation and antileishmanial activity. Here we review the role of chemokines and chemokine receptors on different stages of the leishmanial infection.

Box 1. Chemokines and chemokine receptors

- Chemokines are a superfamily of low molecular weight (6-17 kDa) cytokines that recruit distinct subsets of leukocytes along a chemotactic gradient and active these cells through increased adhesion, degranulation and respiratory burst [3]. To date, over 44 different chemokines have been described and there are 21 known chemokine receptors.

- Most chemokines are secreted proteins of 67 to 127 amino acids; only CXCL16 and CX_3CL1 are membrane-bound molecules. Their production is stimulated by a variety of agents, including LPS, mitogens, proinflammatory cytokines and several pathogens [4]. The two major structural subfamilies are distinguished by the arrangement of the two NH_2-terminal cysteine (Cys) residues, which are either separated by a single amino acid (CXC) or are in adjacent (CC) positions. C (which lack two out or four canonical Cys) and CX_3C (with three intervening amino acids between the first two Cys) chemokines are minor structural subfamilies [5].

- Chemokine actions are mediated via specific cell-surface receptors, which are members of the seven-transmembrane domain, G-protein coupled receptor family. These receptors are named according to their ligands (CCR1-10, CXCR1-6, XCR1-2 and CX3CR1). The chemokine-receptor interactions is characterized by considerable promiscuity: one receptor interacts with several chemokines and one chemokine binds to several receptors [4]

- Chemokines have two main sites of interactions with their receptors, one in the NH_2-terminal region and the other within an exposed loop of the backbone that extends between the second and third Cys. The NH_2-terminal binding site is essential for triggering of the receptor. Modification of the NH_2-terminal region by truncation or amino acid substitution markedly influences chemokine activity [4].

- All chemokine receptors identified are membrane-bound molecules composed of 7-transmembrane domains and coupled to heterotrimeric G proteins. They have a glycosylated extracellular NH_2-terminal region that is involved in chemokine binding, while the intracellular COOH-terminal region is involved in G-protein linking and is subject to regulatory phosphorylation [6].

- Phosphorylation and internalization of chemokine receptors results in the transient interruption of responsiveness to chemokines, a process termed cellular desensitization. The speed of response recovery is determined by the fate of internalized receptors (lysosomal degradation versus dephosphorylation and cell surface recycling) and by the rate of *de novo* chemokine receptor synthesis [7].

Cytokine-chemokine networks

Cytokines are directly involved with chemokines production and can also precede the expression of some chemokines that, in turn, induce the production of additional inflammatory mediators. Cytokines exert a secondary effect on leukocyte recruitment by inducing the expression of several chemokines genes [8]. TNF-α and IL-1β released from activated neutrophils and macrophages, have been implicated in chemokine synthesis in a number of cell types, including neutrophils, fibroblasts, endothelial and epithelial cells [4].

In leishmaniasis, cytokines seem to synergize with leishmanial elements to regulate chemokine production. TNF-α and IL-1β through CCL3/MIP-1α expression were reported to be involved with Langerhans cell (LC) migration in murine cutaneous leishmaniasis [9]. These cytokines as well as CCL3/MIP-1α, CCL4/MIP-1β, MIP-2, CCL2/MCP-1, and CCL1/TCA-3 were also produced in the pouch exudate of mice injected with *L. major,* in contrast, *L. donovani* was much less stimulatory [10]. IL-12 has been shown as required to the induction of chemokines such as XCL1/lymphotactin, CXCL10/IP-10, and CCL2/MCP-1 in draining lymph nodes (LN) of mice infected with *L. major* [11]. Interestingly, Th1- and Th2-derived cytokines can have antagonistic effects on chemokines. For example, some chemokines such as CXCL9/MIG and CXCL10/IP-10 are more selectively induced by IFN-γ [12]. The Th2-related cytokines IL-4 and IL-13 induce CCL22/MDC and CCL6/C10 production in macrophages and this production is inhibited by IFN-γ [13,14]. Under circumstances of infection, cytokines can also act synergistically with chemokines. Accordingly, IFN-γ acts with CCL2/MCP-1 to eliminate *L. major* from infected macrophages that were previously recruited by CCL2/MCP-1, while IL-4 antagonizes the production of this chemokine by *Leishmania*-infected macrophages [15]. Cytokines regulation of chemokines also appears to be cell specific, as illustrated by the observation that IL-4 and IL-13 strongly induce CCL2/MCP-1 in endothelial cells but inhibit production in epithelial cells [16]. Therefore, many microbial products can directly evoke chemokines, but the spectrum and degree of chemokines produced in response to infectious agents will be modified in part by cytokines produced during host innate and adaptive immune responses.

The potential roles of chemokines in *Leishmania* infection

Leukocyte recruitment (innate immunity)

Chemokines play different roles after *Leishmania* infection, and the most obvious one is the recruitment of immune cells to the site of parasite delivery. The immune response is initiated at the site of pathogen entry by sentinel cells, including dendritic cells (DC), macrophages and γδ T cells. Such cells are well equipped with Toll-like and phagocytic receptors allowing for sensing pathogen-associated molecular patterns (PAMPs) and uptake of pathogens and opsonized particles [17]. Sentinel cells also express various receptors for proinflammatory cytokines and together with tissue cells produce numerous chemokines initiating a cascade of innate responses [18].

The infection by *Leishmania* begins when an infected female sand fly takes a blood meal from a human host. The sand fly injects the mammalian host with *Leishmania* in the presence of its saliva. Sand fly saliva contains uncharacterized molecules that attract polymorphonuclear neutrophils (PMN) as well as monocytes [19]. The parasite itself also produce a chemoattractant protein called *Leishmania* chemotactic factor (LCF) that can attract neutrophils [20]. More recently we have shown that after 2 h of saliva injection, an intense and diffuse inflammatory infiltrate, comprised of neutrophils, eosinhophils, and macrophages is induced in pre-exposed mice [21]. PMN are the first cells to arrive at the site of *Leishmania* infection [2]. PMN containing *Leishmania* start secreting chemokines, such as CXCL8/IL-8 [22], essential in attracting more neutrophils at the site of infection. Upon infection with *L. major*, MIP-2 and KC (the functional murine homologues of human GRO) are rapidly produced in the skin [2]. In vitro studies have also shown that *L. major* promastigotes induce rapid and transient KC expression by murine macrophages [23] and CXCL8/IL-8 by human macrophages [24]. All these chemokines are chemoattractants for PMN [5]. PMN can function as phagocytic cells, taking up and killing *Leishmania* [25], and they have been implicated in early parasite control. The role of PMN in the context of early response to *Leishmania* has experienced a major change in the last few years. Early influx of PMN has been demonstrated to be beneficial for *Leishmania* survival in the tissues [26]. *Leishmania* extends the life span of neutrophils [27] and can survive intracellularly in these cells within the first hours or days after infection [26]. The parasites delay but do not prevent the spontaneous apoptosis of neutrophils, since infected cells become apoptotic after 2-3 days [27]. After being ingested by neutrophils, *Leishmania*

induce the release of CCL4/MIP-1β, recruiting monocytes/macrophages to the site of infection [26]. Infected neutrophils are taken up by macrophages does not activate macrophage microbicidal function [26,28]. After ingesting apoptotic cells macrophages have their proinflammatory cytokine production inhibited though mechanisms involving TGF-β, PGE_2 and PAF [29,30]. In this context, these events contribute to a silent entry of *Leishmania* to macrophages, its main host cells [31].

Natural killer (NK) cells come to the site of infection as early as 24h after *Leishmania* infection [2]. *L. major* infection leads to migration of NK cells both to the infected skin and into the draining LN [32]. NK cells are also detectable very early in the lesions of *L. braziliensis*-infected mice [33]. The migration of NK cells correlates with the expression of the NK cell-activating chemokine CXCL10/IP-10 in resistant mice [34]. Treatment of susceptible BALB/c mice with recombinant CXCL10/IP-10 resulted in significantly increased NK cell cytotoxic activity in the draining LN [2,34]. NK cells are known to produce IFN-γ and early NK-cell activity might influence the kinetics of the Th1 response. Moreover, NK cells have been shown to be important [35] for overall resistance to *L. major* infection although not essential, as immune deficient T-cell reconstituted mice, which selectively lack NK cells, have efficient IL-12-dependent IFN-γ production by CD4+ T-cells and heal their lesions [36].

Monocytes/macrophages are the second wave of cells that enter the site of *Leishmania* infection. These accessory cells display multiple functions; they serve as host cells for parasite replication, as antigen-presenting cells, and as source of cytokines modulating the T cell-mediated immune response. And later, after appropriate activation by T cells, they serve as effector cells for intracellular killing of the organisms. Monocytes/macrophages are early attracted by products of sand fly saliva [19,21], and two to three days later by chemokines, such as CCL4/MIP-1β [26]. *Leishmania* can also induce other monocyte-attractant chemokines. Accordingly, *L. major* promastigotes induce rapid and transient expression of JE in murine macrophages [23] and of its homologue CCL2/MCP-1 in human macrophages [24]. Besides attracting monocytes/macrophages, CCL2/MCP-1 can attract other CCR2+ cells, such as NK cells and DC [15,37]. Other monocyte-attractant chemokines such as CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-β, and CCL2/MCP-1 may also play a role as evidenced by *L. major* injection in an air pouch model [10]. In human leishmaniasis, CCL2/MCP-1 and CCL3/MIP-1α seem to be responsible for macrophage activation in the skin lesions. Biopsy samples from

patients with *L. mexicana* LCL exhibited high CCL2/MCP-1 expression and moderate levels of CCL3/MIP-1α, in contrast, low levels of CCL2/MCP-1 and high levels of CCL3/MIP-1α were present in the nonhealing DCL lesions [38]. The authors suggest that macrophages stimulated by the synergistic action of CCL2/MCP-1 and IFN-γ kill parasites in LCL, whereas the presence of IL-4 in DCL lesions may suppress CCL2/MCP-1 expression and progression of disease.

Skin DC, which are potent antigen presenting cells, have a decisive role as the bridge from innate to adaptive immune responses by priming naive T cells. DC take up *Leishmania* parasites, acquire a mature phenotype by upregulation of class I and II major histocompatibility complex (MHC) surface antigens, increased expression of costimulatory molecules (CD40, CD54, CD80, and CD86), release IL-12 p40, and transport the parasites from the infected skin to the draining LN for presentation to antigen-specific T cells [39]. In *Leishmania*-infected mice, DC ability to transport the parasites to the draining LN seems to rely on the expression of CCR2 and CCR7. CCR2-/- mice are defective in DC migration from the LN marginal zone to T cell area and they are markedly impaired in antigen specific T cell activation [40]. CCR7 is also required for the migration of mature DC from tissues to T cell areas of draining LN [41]. It has been shown that *L. donovani*-induced down regulation of CCR7 impaired DC migration contributing to disease progression [42].

***Cell-mediated immunity* (adaptive immunity)**

Chemokines and chemokine receptors have a role in the development Th1 response, as their deletion influence IFN-γ production by T cells. Accordingly, *L. donovani*-infected mice lacking CCR2, CCR5, or CCL3/MIP-1α (a ligand for CCR5) present antigen-specific IFN-γ response lower in the early phases of infection following ligation of the TCR. However, this defective response is transient as during chronic infection it is restored and correlated with an enhanced control of parasite replication [43].

Chemokines also seem to be implicated in T-cell amplification of the inflammatory response, an important step for a protective host defense in leishmaniasis. In *L. donovani*-infected mice, a rapid hepatic accumulation of CCL3/MIP-1α, CCL2/MCP-1, and CXCL10/IP-10 was observed after infection [44]. However, only CXCL10/IP-10 expression, amplified by T cells, remained high at the late phase. CXCL10/IP-10 expression was essential to allow liver

granuloma formation and inflammatory response. Monocytic cells are attracted by CCL3/MIP-1α and CCL2/MCP-1 and after IFN-γ stimulation they can be the source of Th1-mobilizing chemokines such as CXCL10/IP-10 [12]. Unlike the liver, spleen cells from *L. infantum*-infected mice produced both, Th1- and Th2-type cytokines with Th2-type predominance response was dominant, compatible with the consistent expression of CCL2/MCP-1 rather than CXCL10/IP-10 [45].

Chemokines and chemokine receptors have also a role in Th1-Th2 polarization upon *Leishmania* infection. In CCR2-deficient mice, which are susceptible to *L. major* infection, DC migration to the draining LN and spleen was markedly impaired, especially for the CD8α+ Th1-inducing DC subset [40]. These CCR2-deficient mice had a dominant Th2 phenotype but the implication of CCR2 in a Th1 phenotype is not so clear. Indeed, mice lacking CCL2/MCP-1 (a major ligand of CCR2) have impaired Th2 responses but secrete normal amounts of IFN-γ and are resistant to *L. major* infection [46]. This discrepancy might not be surprising given the fact that CCR2 has at least two additional high-affinity ligands in the mouse (CCL7/MCP-3 and CCL12/MCP-5), which, in an appropriate context, might induce Th1 polarization in CCL2/MCP-1-deficient mice. In summary, it is clear that the CCR2-CCL2/MCP-1 axis participates in innate immunity to *Leishmania* infection such as cell recruitment, but also takes part in adaptive immunity through control of Th1-Th2 balance. However, CCL2/MCP-1 involvement in preferential Th cell polarization is not yet firmly established. In this same context, other chemokine receptors were also studied. CCR1-deficient C57BL/6 mice infected by *L. major* showed that CCR1 (preferentially expressed on CD4+ Th1 cells) plays a role in pathogenesis of cutaneous *L. major* infection [47]. CCR1 modulates Th1/Th2 response by upregulating production of Th2-type cytokines such as IL-4 and IL-10 in the early course of disease. However, CCR1 is not essential for leukocyte trafficking to the site of infection nor to the LN following *L. major* infection [47].

Of note, some *Leishmania* strains may evade host immune responses by preventing early production of inflammatory cytokines, chemokines and chemokine receptors and thus impairing antigen-specific Th1 cells development. *L. amazonensis* leads to delayed and reduced CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, MIP-2, CCR1, CCR2, and CCR5 expression in the early stages of infection compared to *L. major*-infected mice. These alterations were

accompanied by reduced T-cell responsiveness, which could be overcamed by direct transfer of antigen-specific Th1 cells [48].

Cell activation and parasite killing

In leishmaniasis, CCL2/MCP-1 activates macrophages and might participate in reduction of parasite numbers [15,38]. *L. infantum*-infected human macrophages treated with CCL2/MCP-1 or CCL3/MIP-1α significantly enhanced nitric oxide production and leishmanicidal ability in vitro [49]. CCL2/MCP-1 has also been shown to induce antileishmanial activity in either *L. donovani*-infected or *L. major* infected human macrophages [15,50]. CCL3/MIP-1α and CCL2/MCP-1 control the intracellular growth and multiplication of *L. donovani* via NO-mediated regulatory mechanism [51].

Chemokines and parasite virulence

Besides other factors *Leishmania* virulence seems to be linked to the early modulation of chemokines expression in the host. Recently, we have shown that lesions from BALB/c mice caused by a more pathogenic *L. braziliensis* exhibited a higher expression of CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, CCL11/eotaxin, XCL1/lymphotactin, and their respective receptors when compared to lesions caused by a less pathogenic *L. braziliensis* [52]. This higher expression of chemokines correlated with a greater leukocyte recruitment at the infection site, resulting in the increase of inflammation observed in pathogenic *L. braziliensis*-infected mice. Furthermore, studies performed with murine macrophages showed that the induction of chemokines upon infection with *Leishmania* was dependent on the parasite virulence. The magnitude of CCL2/MCP-1 and KC expression was higher with avirulent than with virulent *L. major* strains induction [23]. The parasite lipophosphoglycan (LPG), a major surface molecule which binds to macrophage surface receptors, seems to be involved in modulating the signal for chemokine induction. It has been shown that *L. donovani* LPG alters the migration of inflammatory cells by reducting the expression of specific adhesion molecules [53]. Indeed, chemokine gene expression of CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, CXCL10/IP-10, and CCL2/MCP-1 was more strongly up-regulated in air pouch lining of viscerotropic *L. donovani*-infected animals than in that of dermotropic *L. major*-infected animals [10], suggesting that leukocyte transendothelial migration could have been blocked by *L. donovani*

LPG [54]. Infective *Leishmania* promastigotes also express on their surface gp63 protease and an abundant class of small glycolipids termed GIPLs [55]. Any of these parasite molecules, alone or in combination, could provide the signal required for chemokine induction. Therefore, if chemokines have a role in *Leishmania* virulence, these immune mediators will be more important in the parasite-host equilibrium than is currently believed.

Chemokines as targets for therapy in leishmaniasis

Since chemokines appear relatively selective in their actions compared to many other inflammatory mediators, they have been regarded as promising targets for development of anti-inflammatory therapies [56]. However, a potential problem to the success of anti-chemokine strategies is that the chemokine family has a large number of members, many of which have overlapping functions. Consequently, it may be difficult to control inflammation with an agent designed to neutralize the activity of only one chemokine. Despite this possible problem, it has already been shown that treatment with antibody directed against a single chemokine can be effective in diminishing inflammation and tissue damage glomerulonephritis and lung reperfusion injury [57,58] or increase resistance to *Toxoplasma gondii* infection [59]. Another potential problem is that some of the chemokines have activities in addition to attracting and activating leukocytes as already cited above. Moreover, chemokines operate as part of a delicately balanced network of cytokines and other inflammatory and immune mediators. Thus, inhibiting the functions of chemokines in vivo may lead to unwanted and even unanticipated secondary effects. Development of additional strains of transgenic mice that lack one or more chemokines may help in determining whether undesirable consequences of abolishing activity of these mediators will preclude the use of anti-chemokine therapies.

Up to now, only two studies using chemokine blockade or recombinant chemokine treatment have been reported in leishmaniasis. The administration of recombinant mouse CXCL10/IP-10 to susceptible BALB/c mice enhanced significantly NK cell cytotoxic activity and resistance against *L. major*, indicating that CXCL10/IP-10 might contribute to promote the development of a protective immune response [34]. Treatment with Met-RANTES (functional antagonist of CCR1 and CCR5) or anti-CCL5/RANTES rendered C57BL/6 mice more susceptible to *L. major*, skewing the immune response towards Th2 [60].

Our data also reinforce the notion of members of the chemokine family contributing to the development of inflammation in cutaneous leishmaniasis [52]. We have shown that large lesions in BALB/c infected by *L. braziliensis* were correlated with the inflammatory reaction and higher expression of CCL2/MCP-1, CCL3/MIP-1α, CXCL1/KC, CCL11/eotaxin, and XCL1/lymphotactin, but not with parasite load [52]. Whether chemokines can be exploited therapeutically to limit the extent of inflammation and whether this would be beneficial in leishmaniasis is not certain. The feasibility of these mediators for treatment of inflammatory diseases could be determined as a more complete understanding of the biology of chemokines emerges.

Closing remarks

The diversity of chemokines' roles in the course of infectious diseases such as leishmaniasis probably reflects some characteristics of this family: redundancy in their action on targets cells, promiscuity in receptor use and multiple chemokines produced in an redundant way by a single cell. Nevertheless, two effects on the roles of the molecules on the physiopathology of leishmaniasis can be envisaged. As most intracellular protozoan parasites *Leishmania* can invade many types of cells (macrophages, dendritic cells and fibroblasts). Chemokines attract immune cells, source of potential host cells, and might participate in the virulence of parasites. The recruitment of these cells such as macrophages, which are major contributors to defense, mediating innate resistance to intracellular pathogens, as *Leishmania*, can participate in the regulation of their own number and ensure both host and parasite survival. However, even if our knowledge about involvement of chemokines in protozoan diseases is more limited than in viral diseases, chemokines appear to be of paramount importance in the pathophysiology of leishmaniasis. It is likely that the concerted and timely actions of several chemokines and chemokine receptors are necessary to control *Leishmania* infection and their roles are just beginning to be understood.

Acknowledgements

The work in the authors' laboratory is supported by grants from NIH, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do

Estado da Bahia (FAPESB). A.B. and M.B-N are senior investigators from CNPq and Instituto de Investigação em Imunologia.

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Table 1. Chemokines and chemokine receptors in *Leishmania* infection

Cutaneous leishmaniasis

Chemokines and receptors	regulation	effector	Species	References
		Initial phase		
CXCL1/KC, CXCL8/IL-8, MIP-2	↑	attract neutrophils	*L.major*	2, 22, 23, 24
CCL3/MIP-a, CCL4/MIP-1b CCL5/RANTES	↑	attracts monocytes/macrophages	*L.major*	10, 26
CXCL10/IP-10	↑	activate and recruit NK cells to lesion and lymph nodes	*L.major* *L. braziliensis*	2, 32, 34, 52
CCL2/MCP-1	↑	recruit macrophages, NK and DC	*L. major*	23, 24, 37
CCR2 CCR7	↑	DC migration to lymph nodes and colocalization in T-cell areas	*L.major*	40, 41
CCL2/MCP-1, CCL3/MIP1a, CXCL1/KC XCL1/lymphotactin	↑	increase inflammation in the lesion	*L. braziliensis*	52
		Late Phase		
CCR2-CCL2/MCP-1	↑	control of Th1/Th2 polarization (?)	*L.major*	40, 46
CCR1	↑	upregulation of Th2 type cytokines	*L.major*	47
MIP-2, CCL3/MIP-1a, CCL4/MIP-1b, CCL5/RANTES, CCR1, CCR2 and CCR5	↓	impairment of antigen-specific Th1 cell response	*L. amazonenesis*	48

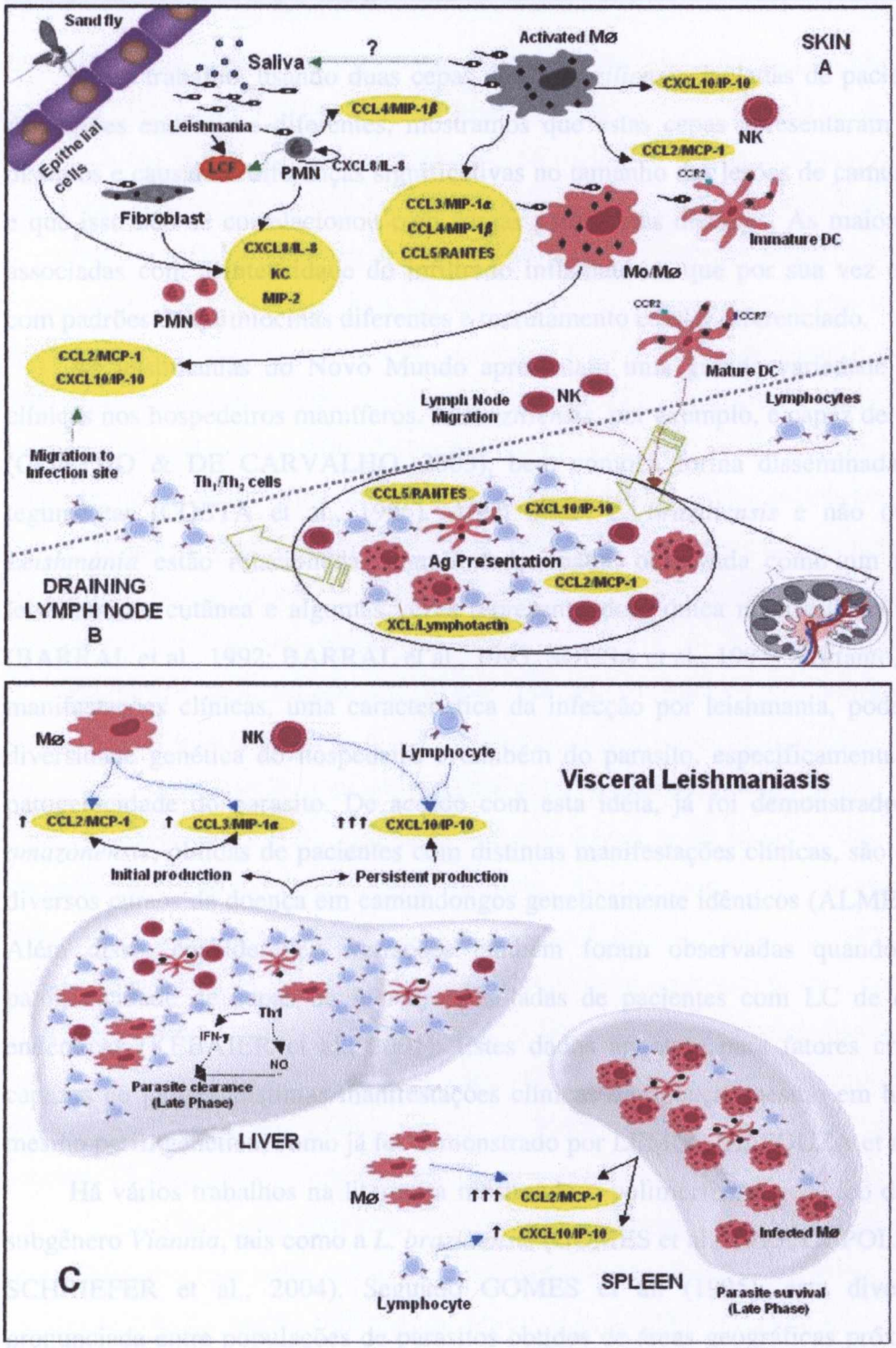
Visceral leishmaniasis

Chemokines and receptors	regulation	effector	Species	References
		Initial Phase		
CCL3/MIP-1a CCL2/MCP-1	↑	attract monocytic cells source for Th1-mobilizing chemokines	*L.donovani*	44
CCR7	↓	impaired DC migration to lymph nodes	*L.donovani*	42
		Late Phase		
CXCL10/IP-10	↑	participates in granuloma formation and attract lymphocytes (liver)	*L.donovani*	44
CCR2, CCR5 CCL3/ MIP-1a	↑	role in generation of IFN-g by T cells	*L.donovani*	43
CCL2/MCP-1 CXCL10/IP-10	↑ ↓	influx of macrophages and sustained parasite persistence (spleen)	*L.donovani*	45

Legends

Figure 1. Host chemokine interactions in cutaneous and visceral leishmaniasis. (A) Role of chemokines in innate immune response against *Leishmania*. *Leishmania* promastigotes together with saliva induce CXCL8/IL-8, MIP-2, and KC production by resident cells. Attracted neutrophils ingest parasites and produce CCL4/MIP-1β and CXCL8/IL-8 that in turn recruit macrophages and more neutrophils, respectively. Recruited macrophages after infection secrete CCL2/MCP-1 that attract CCR2+ cells, such as NK, Dendritic cells DC) and macrophages. Infected macrophages also secrete other chemoattractants for monocytes such as CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES. **(B) Interaction between innate and adaptative immune response.** DC respond to *Leishmania* by altering their migratory properties through the expression of CCR2, CCR5 and CCR7. Mature DC, NK cells and lymphocytes migrate to the draining lymph nodes. DC present antigen to naïve T cells and induce antigen-specific clonal expansion. Mature Th1 or Th2 cells migrate back to the site of infection attracted by CXCL10/IP-10 or CCL2/MCP-1, respectively. **(C) Role of chemokines in visceral leishmaniasis.** After infection with *Leishmania* occurs a rapid hepatic accumulation of CCL2/MCP-1, CCL3/MIP-1α, and CXCL10/IP-10. The CXCL10/IP-10 expression remains high and this attacts more Th1 cells than macrophages to liver, resulting in control of the infection in this organ. Unlike the liver, in the spleen occurs a consistent expression of CCL2/MCP-1 rather than CXCL10/IP-10, and there is an influx of macrophages rather than T-cells with a dominance of Th2 cytokines, and sustained parasite persistence.

Figure 1


Sand fly
Saliva
?
Activated MØ
SKIN
A
CXCL10/IP-10
CCL4/MIP-1β
Leishmania
NK
CCL2/MCP-1
Epithelial cells
LCF
PMN
CXCL8/IL-8
Fibroblast
CCL3/MIP-1α
CCL4/MIP-1β
CCL5/RANTES
Immature DC
CXCL8/IL-8
KC
MIP-2
Mo/MØ
PMN
CCR2
CCR7
CCL2/MCP-1
CXCL10/IP-10
Mature DC
Lymph Node Migration
NK
Lymphocytes
Migration to Infection site
Th1/Th2 cells
CCL5/RANTES
CXCL10/IP-10
Ag Presentation
CCL2/MCP-1
XCL/Lymphotactin
DRAINING LYMPH NODE
B
MØ
NK
Lymphocyte
Visceral Leishmaniasis
↑ CCL2/MCP-1
↑ CCL3/MIP-1α
↑↑↑ CXCL10/IP-10
Initial production
Persistent production
Th1
IFN-γ
NO
Parasite clearance (Late Phase)
LIVER
MØ
↑↑↑ CCL2/MCP-1
↑ CXCL10/IP-10
Infected MØ
C
SPLEEN
Lymphocyte
Parasite survival (Late Phase)

8. DISCUSSÃO GERAL

Neste trabalho, usando duas cepas de *L. braziliensis*, isoladas de pacientes provenientes de regiões endêmicas diferentes, mostramos que estas cepas apresentaram perfis genômicos distintos e causaram diferenças significativas no tamanho das lesões de camundongos BALB/c, e que isso não se correlacionou com cargas parasitárias distintas. As maiores lesões estavam associadas com a intensidade do infiltrado inflamatório, que por sua vez estava relacionado com padrões de quimiocinas diferentes e recrutamento celular diferenciado.

As leishmanias do Novo Mundo apresentam uma grande variedade de manifestações clínicas nos hospedeiros mamíferos. *L. braziliensis*, por exemplo, é capaz de causar LC e LCM (GONTIJO & DE CARVALHO, 2003), bem como a forma disseminada da leishmaniose tegumentar (COSTA et al., 1986). Além disso, *L. braziliensis* e não outras espécies de *Leishmania* estão relacionadas com linfadenopatia, observada como um primeiro sinal de leishmaniose cutânea e algumas vezes representando a única manifestação clínica da doença (BARRAL et al., 1992; BARRAL et al., 1995; SOUSA et al., 1995). Portanto, a diversidade das manifestações clínicas, uma característica da infecção por leishmania, pode ser resultado da diversidade genética do hospedeiro e também do parasito, especificamente, variabilidade na patogenicidade do parasito. De acordo com esta idéia, já foi demonstrado que cepas de *L. amazonensis*, obtidas de pacientes com distintas manifestações clínicas, são capazes de causar diversos cursos da doença em camundongos geneticamente idênticos (ALMEIDA et al., 1996). Além disso, consideráveis variações também foram observadas quando se comparou a patogenicidade de cepas de *L. major*, isoladas de pacientes com LC de diferentes regiões endêmicas (KÉBAIER et al., 2001). Estes dados apontam para fatores críticos do parasito capazes de induzir distintas manifestações clínicas da doença, mesmo em hospedeiros com o mesmo perfil genético, como já foi demonstrado por LEMOS DE SOUZA et al. (2000).

Há vários trabalhos na literatura mostrando o polimorfismo genético das leishmanias do subgênero *Viannia*, tais como a *L. braziliensis* (GOMES et al., 1995; CUPOLILLO et al., 1998; SCHRIEFER et al., 2004). Segundo GOMES et al. (1995), esta diversidade é menos pronunciada entre populações de parasitos obtidos de áreas geográficas próximas. Os isolados de *L. braziliensis* H3227 e BA788 foram geneticamente diferentes e embora H3227 tenha sido isolado no Estado do Ceará e BA788 tenha sido isolado no Estado da Bahia, ambos Estados

endêmicos para leishmaniose tegumentar, não podemos correlacionar este polimorfismo com a origem geográfica das cepas, uma vez que trabalhamos com apenas uma cepa de cada Estado. Estes dados não foram uma coincidência, uma vez que em experimentos anteriores, encontrou-se significante polimorfismo entre as cepas de *L. braziliensis* da Bahia e do Ceará (ROSATO, 2004). Além disso, mais recentemente, SCHRIEFER et al. (2004) mostraram também diferenças genéticas entre isolados de *L. brazilinesis* uma mesma região endêmica.

O polimorfismo genético entre as cepas de *L. braziliensis* H3227 e BA788 e o fato que estas cepas foram isoladas de áreas que apresentavam particularidades na apresentação da leishmaniose cutânea (linfadenopatia expressiva no Ceará), levou-nos a pesquisar possíveis diferenças na patogenicidade destes dois isolados. Os resultados mostraram que apenas os animais infectados com H3227 desenvolveram lesões. A carga parasitária nos linfonodos drenantes mostrou um consistente aumento em ambos os grupos infectados (ANEXO 11.1A), indicando que os parasitos são capazes de se multiplicar nesses tecidos linfóides secundários, apesar da resolução da lesão nos camundongos infectados com H3227 e nenhuma lesão ter sido detectada nos animais infectados com BA788.

Entretanto, um achado importante foi que o número de parasitos na lesão dos camundongos infectados com H3227 e BA788 com 15 dias de infecção, período em que se observou a maior diferença no tamanho das patas infectadas, não se mostrou significativamente diferente (ANEXO 11.1B). Isto sugeriu que a diferença no tamanho das lesões poderia ser causada por reações inflamatórias distintas. Corroborando esta hipótese, a análise da composição celular no sítio inflamatório, com 15 dias após a infecção, revelou que as maiores lesões causadas por H3227 estavam diretamente correlacionadas com um infiltrado inflamatório mais intenso (ANEXO 11.2C e D). Especificamente, 3 dias após a infecção, as lesões dos camundongos infectados com H3227 e BA788 exibiram um discreto infiltrado inflamatório, consistindo principalmente de PMN e macrófagos (dados não mostrados). No entanto, 15 dias após a infecção, enquanto a reação inflamatória observada nas lesões dos camundongos infectados com H3227 mostrou-se mais intensa, com macrófagos predominando sobre PMN; nas lesões dos camundongos infectados com BA788 não houve nenhuma alteração na intensidade da reação inflamatória observada (ANEXO 11.2A e C). Nossos achados da composição celular das lesões, visto na histopatologia (dados não mostrados) e citometria de fluxo, foram semelhantes a outros encontrados na literatura, onde a presença de muitos PMN e

alguns macrófagos foi notada durante as primeiras semanas após a infecção, e mais tarde, muito mais macrófagos e linfócitos passaram a predominar no infiltrado inflamatório (POMPEU et al., 1991; DONNELLY et al., 1998).

A reação inflamatória diferenciada observada nas lesões de BALB/c infectados por H3227 e BA788 levou-nos a avaliar os padrões de expressão e a cinética de algumas quimiocinas e receptores de quimiocinas no sítio de inoculação do parasito, uma vez que estas moléculas exercem um papel crucial na migração de leucócitos para as áreas de infecção durante os processos inflamatórios. Além disso, as quimiocinas podem ser secretadas em resposta a vários agentes infecciosos, incluindo leishmania (BRENIER-PINCHART et al., 2001; CHENSUE, 2001; ROYCHOUDHURY & ROY, 2004). As duas cepas de *L. braziliensis* do nosso estudo foram capazes de induzir a expressão de várias quimiocinas, no entanto, em tempos e intensidades diferentes. H3227 induziu a expressão de todas as quimiocinas estudadas mais intensamente e tão cedo quanto 6h após a infecção, quando comparada com a cepa BA788. Este achado se correlacionou com o maior número de células encontradas no infiltrado inflamatório das lesões dos camundongos infectados com H3227, e conseqüente amplificação da reação inflamatória. Ao contrário, BA788 não se mostrou uma forte indutora de quimiocinas, só passando a induzir a maioria delas no 3^0 dia pós-infecção e sempre de maneira menos intensa que H3227. Uma significante associação com a expressão do RNAm e a produção da proteína dessas quimiocinas também foi observada, como visto nos ensaios de imunohistoquímica.

A cinética das quimiocinas induzidas pelos dois isolados de *L. braziliensis*, H3227 e BA788 relacionou-se com a cinética dos diferentes tipos celulares encontrados nas análises histopatológica e da composição fenotípica feita por citometria de fluxo. As quimiocinas recrutadoras de PMN, tais como CXCL1/KC e CCL1/eotaxina, foram expressas mais cedo e de uma forma decrescente nas lesões dos animais infectados com H3227, relacionando-se com o predomínio de granulócitos na fase inicial da infecção, e diminuição destas células na fase mais tardia. CCL2/MCP-1 e CCL3/MIP-1α, quimiocinas que atraem monócitos/macrófagos, apresentaram expressão constante nas lesões de ambos os grupos de camundongos, sugerindo que o estímulo para a migração destas células para o foco inflamatório foi contínuo até pelo menos 15 dias após a infecção, período máximo em que estas quimiocinas foram avaliadas. CXCL10/IP-10 e XCL1/linfotactina, que atraem preferencialmente linfócitos para os focos

inflamatórios, foram induzidas de maneira crescente nas lesões de ambos os grupos, se associado com o achado de poucos linfócitos na fase inicial da infecção e um significante aumento destas células a partir do 15^0 dia de infecção, período que coincide com o começo da resolução das lesões nos camundongos infectados com H3227 e diminuição da carga parasitária nas lesões de ambos os grupos de animais (ANEXO 11.1B).

Alguns estudos in vitro mostraram que leishmania é capaz de induz a expressão de CCL2/MCP-1 e CXCL1/KC em macrófagos murinos e humanos (BADOLATO et al., 1996; RACOOSIN & BEVERLEY, 1997). Além disso, foi demonstrado que lesões de pacientes com leishmaniose cutânea apresentam um aumento significativo na expressão de CCL2/MCP-1 e CCL3/MIP-1α (RITTER et al., 1996), duas quimiocinas quimioatraentes para monócitos (LEONARD & YOSHIMURA, 1990; FAHEY et al., 1992). CXCL1/KC recruta neutrófilos e é uma quimiocina importante nas respostas inflamatórias causadas por parasitos em camundongos (BOZIC et al., 1995). CXCL1/KC não foi encontrada nas lesões dos camundongos infectados com BA788 nas primeiras 6 horas de infecção e foi fracamente regulada no 3^0 e 15^0 dias após a infecção. Apesar disso, no 3^0 dia após a infecção, neutrófilos também foram encontrados em grande quantidade entre as células recuperadas da lesão dos animais infectados com BA788, quando se utilizou citometria de fluxo, sugerindo que outra quimiocina, possivelmente CCL3/MIP-1α estivesse atraindo neutrófilos nestes animais, uma vez que esta quimiocina pode atrair e ativar neutrófilos em camundongos (HAELENS et al., 1996). A expressão de CCL2/MCP-1, CCL3/MIP-1α e CXCL1/KC foi observada mais precocemente e mais intensamente nas lesões dos animais infectados com H3227 do que nas lesões dos camundongos infectados com a cepa BA788, o que sugere que essas quimiocinas podem ser um dos fatores que regulam inicialmente a resposta inflamatória diferenciada que se desenvolve após a infecção com estas cepas de *L. braziliensis*. Provavelmente, no 3^0 dia após a infecção as células comecem a chegar mais intensamente nas lesões dos animais infectados com H3227 do que naquelas causadas por BA788, e este quadro é amplificado com a constante e intensa produção de mais quimiocinas, levando a um influxo maior de células e culminando com a intensa reação inflamatória observada nestes animais no 15^0 dia após a infecção.

Neutrófilos e macrófagos foram as principais células observadas nas lesões dos animais de ambos os grupos. Macrófagos não servem apenas como células hospedeiras preferenciais, onde os parasitos se dividem e proliferam, mas podem atuar também como células

apresentadoras de antígeno que modulam a resposta imune específica, e como células reguladoras da infecção, por sua habilidade de fagocitar e matar o parasito (revisado por ALMEIDA et al., 2003). Recentemente, foi demonstrado que neutrófilos também podem albergar leishmania nas primeiras horas ou dias após a infecção (LAUFS et al., 2002; VAN ZANDGERGEN et al., 2004). Leishmania é capaz de retardar a apoptose de neutrófilos, um mecanismo que envolve a inibição de caspase-3, que é conhecida como indutora de apoptose em PMN (AGA et al., 2002). Os parasitos que são ingeridos, mas não mortos pelos PMN, nesta fase inicial, podem se beneficiar desta acumulação precoce de neutrófilos para o sítio de infecção, como já foi demonstrado para *L. major* (TACCHINI-COTTIER et al., 2000). Além disso, foi relatado também que promastigotas de *Leishmania* podem induzir a migração de PMN liberando um fator chamado fator quimiotático para leishmania (LCF), que tem potente atividade quimioatraente para neutrófilos, mas não para outros leucócitos, como monócitos ou células NK (VAN ZANDGERGEN et al., 2002). No nosso estudo, não podemos afirmar que o recrutamento destas células para o sítio de inoculação do parasito favoreceu a sobrevivência da do parasito, podemos apenas especular. Por outro lado, neutrófilos atuam como células fagocíticas efetoras em leishmaniose, matando leishmania (PEARSON et al., 1981; CHANG, 1981; LIMA et al., 1998). Estudos mais recentes têm demonstrado que neutrófilos interagem com macrófagos pela via CD28-CD80/CD86, resultando em secreção de IFN-γ, que por sua vez controlaria o crescimento de leishmania dentro de macrófagos (VENUPRASAD et al., 2002). No nosso estudo, é provável que as quimiocinas CCL2/MCP-1 e CCL3/MIP-1α, que ainda aparecem fortemente expressas nas lesões dos dois grupos de camundongos, 15 dias após a infecção, podem estar participando, junto com IFN-γ, da indução da atividade leishmanicida em macrófagos, via a geração de óxido nítrico ou superóxido como previamente demonstrado para *L. infantum*, *L. donovani e L. major* (BHATTACHARYYA et al., 2002; BRANDONISIO et al., 2002; RITTER & MOLL, 2000).

A quimiocina CCL11/eotaxina também foi fortemente expressa nas lesões dos camundongos infectados com H3227 e apresentou menor expressão nos animais infectados com BA788. CCL11/eotaxina atrai células CCR3+ tais como eosinófilos, basófilos e linfócitos Th2 (PONATH et al., 1996; UGUCCIONI et al., 1997; SALLUSTO et al., 1997). Na análise histopatológica das lesões de ambos os grupos de camundongos foi visualizada a presença de eosinófilos, detectada principalmente nos 3 primeiros dias após a infecção e alguns poucos após

15 dias de infecção (dados não mostrados). Além disso, os dois isolados de *L. braziliensis* também foram capazes de induzir o influxo de muitos eosinófilos para os exsudatos dos bolsões inflamatórios. Foi demonstrado que outras espécies de *Leishmania*, como *L. major* e *L. donovani* também são capazes de induzir a migração de eosinófilos (MATTE & OLIVIER, 2002). O real papel e a importância destas células na leishmaniose cutânea ainda não foi esclarecido. Sabe-se apenas que assim como os neutrófilos, os eosinófilos também são capazes de albergar e matar leishmania (POMPEU et al., 1991; LIMA et al., 1998).

Um interessante achado do nosso estudo foi o fato que a quimiocina CXCL10/IP-10 foi a única expressa mais fortemente nas lesões causadas por BA788, do que naquelas induzidas por H3227, em todos os tempos examinados. Esta alta expressão de CXCL10/IP-10 foi encontrada estar associada com a produção precoce de IFN-γ no linfonodo drenante e um maior número de células NK nas lesões dos camundongos infectados com BA788. Corroborando com nossos achados, alguns estudos mostraram que células NK migram para a pele infectada de camundongos infectados com *L. major* 24 horas após a infecção (LASKAY et al., 1995). Foi demonstrado também que a migração de células NK em camundongos resistentes infectados com *L. major* está associada com a expressão de CXCL10/IP-10 (VESTER t al, 1999). In vivo, células NK são as principais fontes de IFN-γ junto com linfócitos Th1 e uma atividade precoce destas células pode influenciar a cinética da resposta Th1, como foi relatado mais recentemente por MARTIN-FONTECHA et al. (2004). Os autores observaram que células NK também são capazes de migrar para linfonodos estimulados, mediadas pelo receptor CXCR3 (receptor para CXCL10/IP-10 e CXCL9/MIG) e que nestes sítios estas células são a fonte inicial de IFN-γ, uma citocina necessária para a polarização de Th1. Além disso, foi demonstrado que as células NK são importantes, embora não parecem ser essenciais, para a resistência na leishmaniose cutânea causada por *L. major* (SCHARTON & SCOTT, 1993). Camundongos imunodeficientes e reconstituídos com células T, que seletivamente perderam células NK, apresentaram eficiente produção de IFN-γ por células T CD4+, que por sua vez é dependente de IL-12, e são capazes de curar suas lesões (SATOSKAR et al., 1999). Além disso, CXCL10/IP-10 também recruta células Th1 CXCR3+ que são capazes de secretar maior quantidade de IFN-γ no sítio inflamatório (RITTER & KORNER, 2002). Portanto, é possível que a quimiocina CXCL10/IP-10 esteja envolvida na resistência mais precoce à infecção pela cepa BA788 em camundongos

BALB/c, induzindo uma resposta inata inicial mais efetiva, com a presença de muitas células NK e, conseqüentemente, alta produção de IFN-γ.

Em relação aos receptores de quimiocinas, foi de interesse a observação que CCR5 foi ausente ou fracamente regulado nos camundongos infectados com BA788 nos tempos avaliados. CCR5 é estimulado por CCL3/MIP-1α, que só foi expresso nas lesões desses camundongos no 3^0 dia de infecção e começou a declinar 15 dias após a infecção. A inexpressiva ou quase ausente expressão de CCR5 nas lesões dos camundongos infectados com BA788 associou-se à baixa produção de IL-10 no linfonodo drenante desses animais. Estudos prévios mostraram que IL-10 aumenta de uma forma seletiva a expressão de CCR5 em monócitos (SOZZANI et al., 1998). Outro ligante preferencial de CCR5, CCL5/RANTES, não apresentou expressão diferente daquela encontrada nos controles não infectados, não exibindo modulação significante neste modelo de infecção com *L. braziliensis* (ANEXO 11.3).

Os resultados no modelo do bolsão inflamatório mostraram que o isolado H3227 foi mais potente recrutador de todos os tipos celulares do que o isolado BA788, assemelhando-se, portanto, aos resultados da infecção em BALB/c. Ao contrário, BA788, que neste sistema recrutou três vezes menos leucócitos, não gerou resposta inflamatória significante em camundongos. Estes dados reforçam o papel do parasito nas diferenças observadas nos processos inflamatórios induzidos por esses dois isolados de *L. braziliensis*. Estudos com macrófagos murinos mostraram que após infecção por leishmania, a indução de quimiocinas foi dependente do grau de virulência da cepa utilizada (RANCOOSIN & BEVERLEY, 1997). Nesse estudo, CCL2/MCP-1 foi induzido predominantemente por uma cepa avirulenta de *L. major*, e ao contrário, a cepa virulenta induziu consideravelmente menos CCL2/MCP-1. Os autores sugerem que LPG, a principal molécula de superfície da leishmania que se liga na membrana do macrófago, estaria envolvida na modulação do sinal para a indução das quimiocinas. Corroborando esses achados, MATE & OLIVIER (2002) demonstraram que a expressão de quimiocinas foi muito mais intensa na parede do bolsão inflamatório dos animais injetados com a cepa viscerotrópica *L. donovani* do que na dos animais injetados com a cepa dermotrópica *L. major*. As células foram recrutadas, mas não conseguiram migrar para o exsudato inflamatório do bolsão injetado com *L. donovani*, sugerindo que na infecção por esta espécie de leishamania, a migração transendotelial das células pode ter sido bloqueada pelo LPG dos parasitos, como já demonstrado anteriormente (LO et al., 1998). Promastigotas de

Leishmania também expressam em sua superfície a protease gp63 e uma abundante classe de pequenos glicolipídeos chamados GILPs (CHANG et al., 1990; ILG et al., 1999). Qualquer uma dessas moléculas, sozinhas ou em combinação, poderia providenciar o sinal requerido para a indução de quimiocinas.

A observação que os camundongos infectados com H3227 controlaram a lesão cutânea e que os camundongos infectados com BA788 não desenvolveram lesões sugere que, em ambos os casos, uma resposta imune celular tipo Th1 se desenvolveu, embora em tempos diferentes. A análise da produção de citocinas nos linfonodos drenantes mostrou que no 3^0 dia após a infecção os camundongos infectados com BA788 produziram altos níveis de IFN-γ e IL-12. Ao contrário, animais infectados com H3227 produziram concentrações mais altas de IL-4 e IL-10. Nos animais infectados com H3227, a produção das citocinas tipo Th1, tais como IFN-γ e IL-12 só aumentou significativamente 15 dias após a infecção. Estes dados estão de acordo com os achados de que a fraca infectividade de *L. braziliensis* em camundongos BALB/c está relacionada com a capacidade destes animais desenvolverem uma forte resposta Th1 (DeKREY et al., 1998), ao contrário da infecção por *L. major*, na qual a resposta imune tipo Th2 é responsável pela susceptibilidade à doença (SACKS & NOBEN-TRAUTH, 2002). Camundongos BALB/c infectados com *L. braziliensis*, quando tratados com anti-IFN-γ apresentaram lesões grandes que não regrediram e altos níveis de IL-4, indicando, portanto que a resolução das lesões e cura da doença nestes animais é dependente de IFN-γ (DeKREY et al., 1998). Os camundongos infectados com a cepa BA788 apresentaram uma resposta do tipo Th1 mais precocemente, e isso pode explicar porque esses animais não desenvolveram lesões. Ao contrário, nos animais infectados com H3227, a alta produção de IL-4, IL-10 e TGF-β (ANEXO 11.4A) na fase inicial da doença possivelmente inibiu o desenvolvimento de uma resposta Th1, o que levou a progressão da doença até o 15^0 dia, quando então os níveis de IFN-γ subiram e as lesões começaram a regredir. TGF-β correlaciona-se com susceptibilidade à infecção tanto por *L. braziliensis* (BARRAL-NETTO et al., 1992) como por *L. major* (STENGER et al., 1994). A produção de TGF-β também foi induzida por BA788, no entanto, em níveis mais baixos. BARRAL et al. (1993) já tinham demonstrado que diferentes isolados de *L. braziliensis* podem variar em sua habilidade de induzir a produção de TGF-β.

Outra citocina que pode inibir a resposta tipo Th1 é IL-10, uma vez que esta citocina é capaz de causar a supressão de muitas funções efetoras dos macrófagos (FIORENTINO et al.,

1991). A produção de IL-10 nos camundongos infectados com H3227 aumentou significativamente após 15 dias de infecção, quando comparado com aqueles infectados com BA788. Esse aumento de IL-10 estava associado com o pico máximo da carga parasitária e com o maior tamanho da lesão. Além disso, mesmo com 30 dias após a infecção, a produção de IL-10 continuou aumentada no linfonodo de ambos os grupos infectados (ANEXO 11.4B), o que se correlacionou com a persistência dos parasitos neste órgão linfóide secundário. Outros estudos já documentaram que leishmania pode persistir indefinidamente dentro de macrófagos e/ou células dendríticas nos linfonodos drenantes do sítio de inoculação do parasito, facilitando a manutenção da memória imunológica (MOLL et al., 1995). Leishmania também é capaz de persistir dentro de fibroblastos nos linfonodos, uma vez que estas células apresentam baixa capacidade de produzir NO e, portanto controlar o crescimento do parasito, mesmo na presença de IFN-γ (BOGDAN et al., 2000). As células dendríticas infectadas podem apresentar os antígenos de leishmania para as células T de memória por longos períodos, mantendo, desta forma, a polarização de Th1 através da produção de IL-12 (STEBUT & UDEY, 2004). Também foi demonstrado que a produção de IL-10 é crítica para a persistência do parasito em camundongos resistentes (BELKAID et al., 2001). Nesse trabalho, os autores mostram que a cura e esterilização do parasito pode acontecer em camundongos deficientes de IL-10, mas esses animais falham em manter uma imunidade duradoura contra leishmania. Estes dados sugerem que IL-10 tem um papel importante na manutenção da resposta de memória imunológica. Mais recentemente, demonstrou-se que células T CD4+CD25+ regulatórias são capazes de controlar a resposta Th1 protetora, permitindo a sobrevida do parasito e manutenção da resposta de memória imunológica em camundongos resistentes (BELKAID et al., 2002). Tanto no homem como no camundongo, as células T regulatórias CD25+ secretam altos níveis de IL-10 e TGF-β, que são pelo menos parcialmente responsáveis pela capacidade destas células suprimir certas respostas patológica ou imune protetoras in vivo (O'GARRA & VIEIRA, 2004).

Em conclusão, no presente estudo mostramos que dois isolados de *L. braziliensis* com perfis genômicos diferentes exibiram diferenças no curso clínico da doença e em alguns parâmetros imunológicos, quando inoculados em camundongos BALB/c. Além disso, esses isolados também induziram a expressão de quimiocinas em diferentes tempos e/ou intensidades, o que levou a um recrutamento celular diverso e respostas inflamatórias diferenciadas. Nossos

resultados também mostraram que a cinética da indução das quimiocinas parece ser mais importante que a multiplicação do parasito, e este fato pode estar relacionado com as diferenças estruturais desses dois isolados. Considerando que existe um número de parâmetros não controlados que determinam a infecção humana por leishmania, é especulativo tentar achar uma ligação entre a manifestação clínica na infecção humana e a patogenicidade do parasito no modelo experimental. No entanto, vale salientar que esses dois isolados de *L. braziliensis* vieram de regiões endêmicas com peculiaridades na apresentação clínica da leishmaniose tegumentar, como já discutido acima. Mais interessante é que essa correlação in vivo foi reproduzida no presente estudo, uma vez que a quantidade de células nos linfonodos dos animais infectados com H3227 foi consistentemente mais alta do que a encontrada nos linfonodos dos camundongos infectados com BA788, tanto no 3^0 como no 15^0 dia de infecção. Estes resultados reforçam a noção que as manifestações da doença no sítio de inoculação de *Leishmania* também podem resultar de uma complexa interação entre determinantes genéticos dos parasitos e dos hospedeiros, entre outros fatores. Seria interessante determinar se o polimorfismo genético entre leishmanias pode estar associado com a expressão de conhecidos fatores de virulência do parasito (LPG, gp63, GILPs, etc) e se tais expressões se correlacionam com a patogênese da doença.

9. CONCLUSÕES FINAIS

Com base nos resultados obtidos nos dois artigos:

1. Cepas de *L. braziliensis* com genótipos diferentes induzem processos patológicos diversos em camundongos BALB/c, apesar das cargas parasitárias semelhantes;

2. As maiores lesões causadas pelo isolado H3227 em camundongos BALB/c foram correlacionadas com o processo inflamatório;

3. A cepa menos patogênica BA788 induziu mais precocemente a produção de níveis mais altos de IFN-γ, e isto se correlacionou com a presença de um número maior de células NK nas lesões dos animais infectados com esta cepa;

4. Os altos níveis de IL-10 e TGF-β nos animais infectados com a cepa mais patogênica H3227 coincidiram com o pico máximo do desenvolvimento da lesão e da carga parasitária nestes animais;

5. A resposta inflamatória e o recrutamento celular diferenciados correlacionaram-se com a indução da expressão de quimiocinas e receptores de quimiocinas em diferentes tempos e/ou intensidades.

Concluímos:

Cepas de *L. braziliensis* com genótipos distintos causam diferentes processos inflamatórios induzindo recrutamento celular e quimiocinas diversos.

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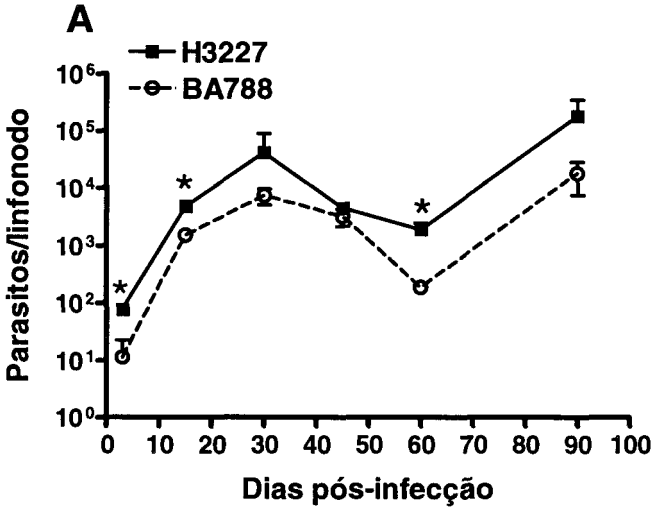
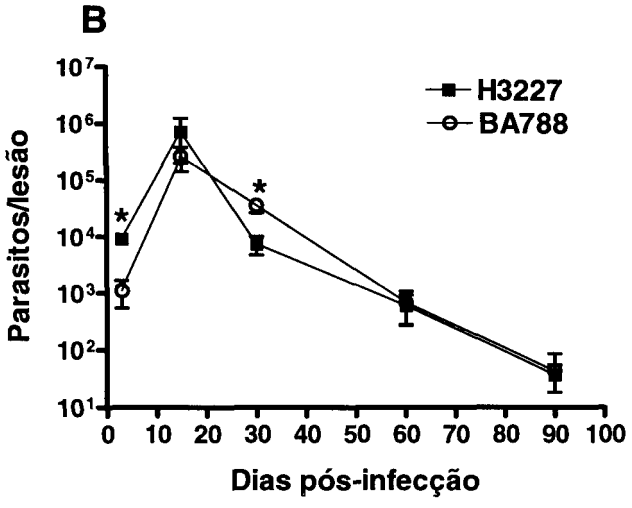
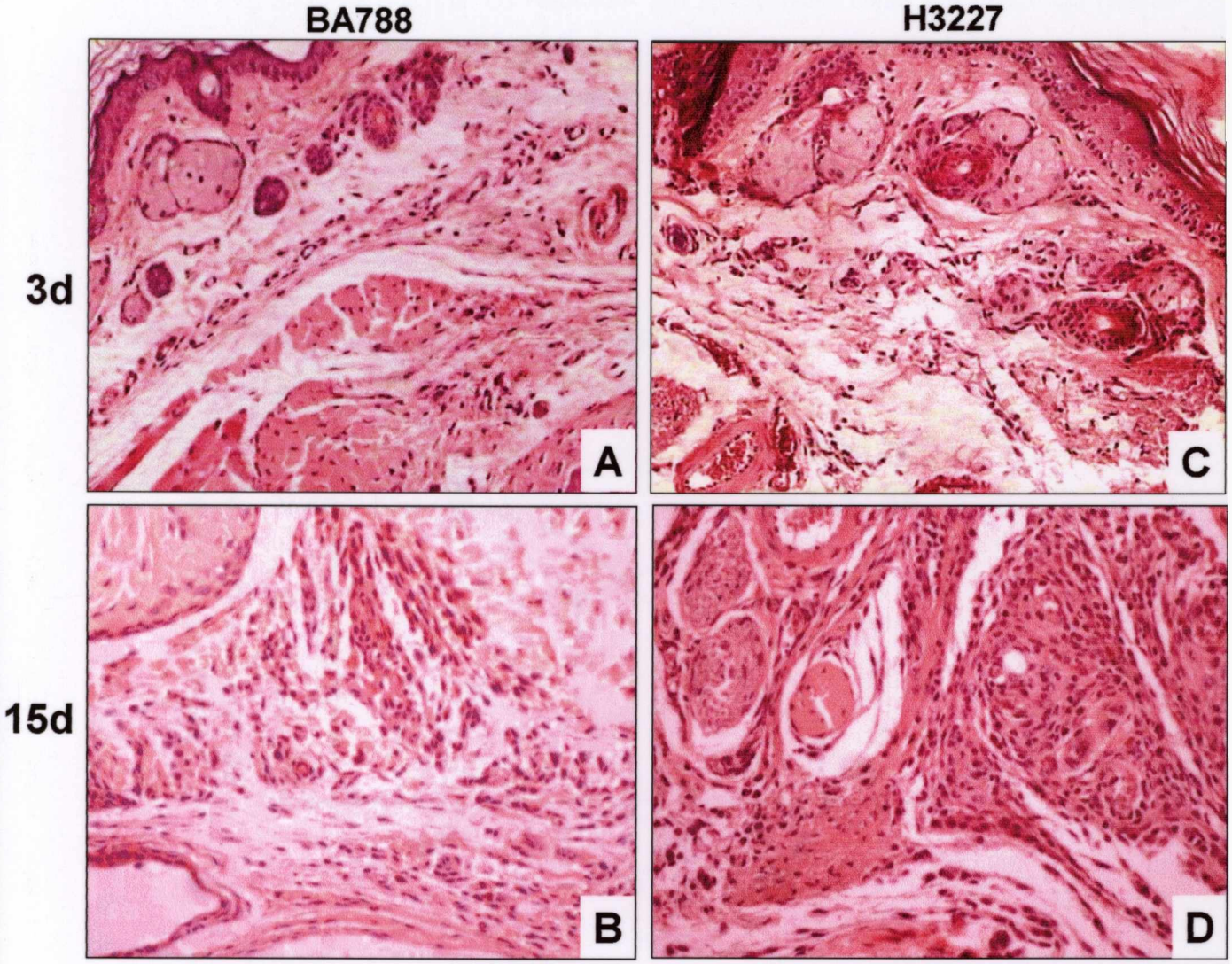
11. ANEXOS

Anexo 11.1. Carga parasitária em camundongos infectados com isolados de *L. braziliensis*. Camundongos BALB/c foram infectados com 10^6 promastigotas de H3227 (■) ou BA788 (○) na pata posterior esquerda. Linfonodos (A) ou patas (B) de 3-5 camundongos por grupo foram usados para a análise da carga parasitária. Os dados representam a média ± SEM. Diferença significante entre os valores é indicada quando $*p<0,05$.

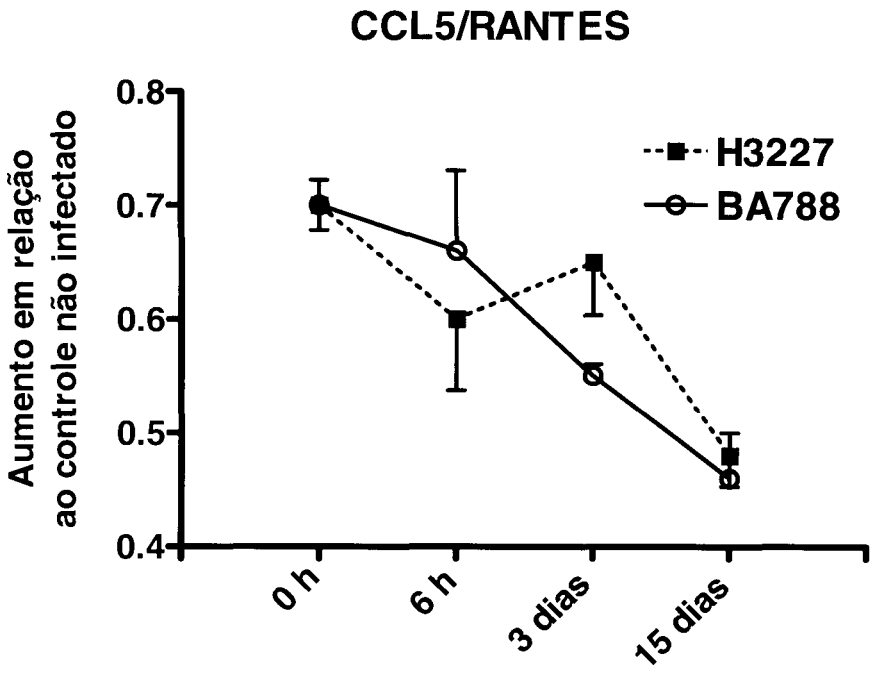




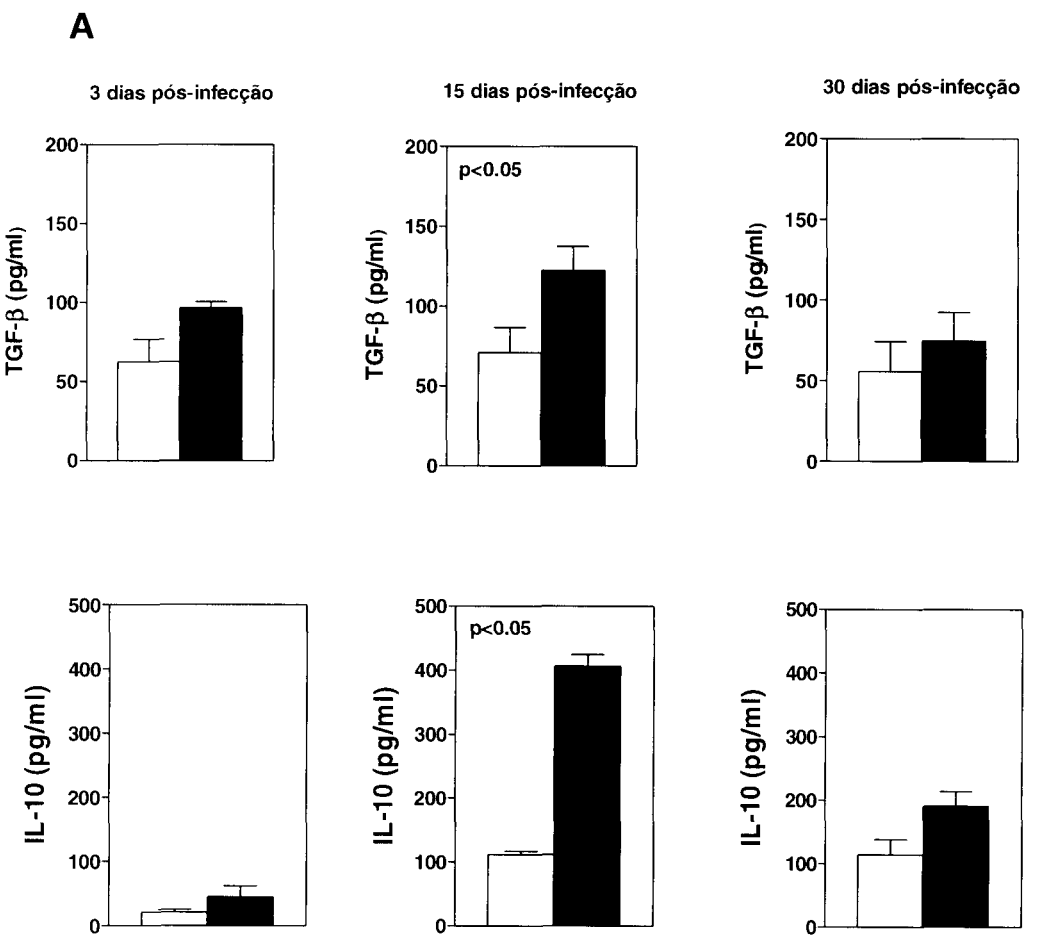
Anexo 11.2. Aspectos histopatológicos das lesões causadas por *L. braziliensis* em camundongos BALB/c. As lesões dos camundongos infectados com a cepa BA788 no 3^0 dia (A) ou no 15^0 dia (B) após a infecção exibiram discreto infiltrado inflamatório. Camundongos infectados com a cepa H3227 no 3^0 dia (C) ou no 15^0 dia (D) após a infecção apresentaram um significante aumento na intensidade da reação inflamatória. Original, x40 (HE).



Anexo 11.3. Expressão de CCL5/RANTES em lesões de camundongos BALB/c infectados com *L. braziliensis*. Camundongos foram infectados com 10^6 promastigotas de H3227 (■) ou BA788 (○) na pata posterior esquerda. Foram sacrificados com 6 h, 3 dias e 15 dias pós-infecção, e as patas infectadas foram usadas para a detecção da expressão do RNAm, utilizando RT-PCR. Análise densitométrica foi realizada e a quantificação das bandas normalizada em relação aos níveis de expressão de β-actina. Os resultados são apresentados como n-aumento em relação aos níveis de expressão nos animais controles não infectados (0 hora). Cada ponto representa a média ± SEM de 3-4 animais de 1 experimento de 3 realizados.



Anexo 11.4. Produção de citocinas por células do linfonodo drenante de camundongos infectados com isolados de *L. braziliensis*. Camundongos BALB/c foram infectados com 10^6 promastigotas de BA788 (□) ou H3227 (■) na pata posterior esquerda. Células do linfonodo drenante da lesão de 3-5 animais por grupo foram incubadas com promastigotas de leishmania e os sobrenadantes analisados com 3, 15 e 30 dias pós-infecção para a produção de TGF-β (A) e IL-10 (B). Os dados representam a média ± SEM de um experimento representativo de 3 experimentos realizados. Diferença significante entre os valores é indicada quando $*p<0,05$.



INFECTION AND IMMUNITY, Feb. 2005, p. 1191–1195 Vol. 73, No. 2
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.2.1191–1195.2005

Distinct *Leishmania braziliensis* Isolates Induce Different Paces of Chemokine Expression Patterns

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Received 30 May 2004/Returned for modification 24 September 2004/Accepted 18 October 2004

Inflammatory events during *Leishmania braziliensis* infection in mice were investigated. Large lesions were directly correlated with the inflammatory reaction but not with parasite burden. Different *L. braziliensis* strains induce different paces of chemokine expression patterns, leading to diverse cell recruitment and differential inflammatory responses.

Chemokines have been implicated in inflammatory responses against numerous infectious agents, including *Leishmania* (5, 17, 18, 20). *Leishmania braziliensis* is the main agent of cutaneous leishmaniasis (CL) in Brazil; it causes single self-limited cutaneous ulcers and highly destructive mucosal leishmaniasis (10). In this study, using a murine model, we compared *L. braziliensis* strains isolated from two states in Brazil, namely, Ceará and Bahia, located in northeastern Brazil. CL caused by *L. braziliensis* is endemic in both states. In Ceará, the cutaneous lesion is accompanied and sometimes

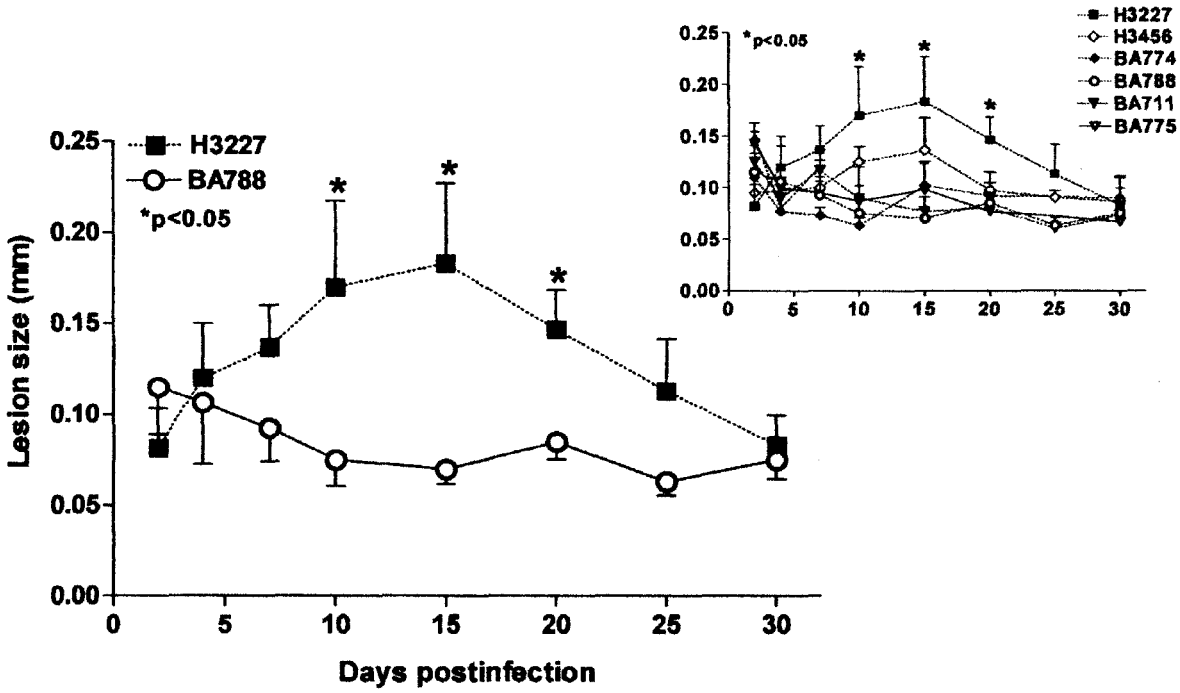

FIG. 1. Course of infection with *L. braziliensis* in BALB/c mice. The inset shows kinetics of lesion development in BALB/c mice during the course of infection with six *L. braziliensis* isolates. The main figure illustrates the time course of infection with the two isolates used here, showing a polar pattern of infection. Mice were inoculated in the hind footpads with 10^6 stationary-phase *L. braziliensis* promastigotes, and lesions were measured weekly for 30 days p.i. The footpads of three to five animals per group were measured. The data shown, reported as the mean and standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values at the indicated time point, as determined by Student's *t* test ($P < 0.05$). Experiments with all *L. braziliensis* isolates were repeated three times, with similar results.

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preceded by an impressive enlargement of the regional lymph nodes; the term "bubonic leishmaniasis" has been coined to describe this manifestation (23). Bubonic leishmaniasis is restricted to *L. braziliensis* infection in Ceará; however, localized lymphadenopathy has been observed in CL patients from Bahia (2, 3).

Mice were infected with 10^6 stationary-phase forms of *L. braziliensis* (6). In preliminary experiments, the isolates obtained from CL patients from Ceará (MHOM/BR/94/H3227 [H3227]and MHOM/BR/94/H3456) and from Bahia (MHOM/BR/00/BA711, MHOM/BR/00/BA774, MHOM/BR/00/BA775, and MHOM/BR/01/BA788 [BA788]) showed significant differences in pathogenicity (Fig. 1, inset). Further experiments were performed with two of these *L. braziliensis* isolates, H3227 and BA788. The lesions caused by H3227 were larger and persisted longer than those caused by BA788 (Fig. 1). Lesion size differences did not appear to be due to diverse parasite loads, since parasite numbers were not significantly different between H3227- and BA788-infected mice at 15 days postinfection (p.i.) (mean and standard error of the mean, $7.29 \times 10^5 \pm 5.28 \times 10^5$ and $2.64 \times 10^5 \pm 1.20 \times 10^5$, respectively), when lesion sizes were different. Lesions from H3227-infected mice exhibited an inflammatory infiltrate consisting mainly of polymorphonuclear leukocytes and macrophages at 3 days p.i., and these histopathological features persisted at 15 days p.i. Sections from BA788-infected mice showed a less intense and more transient leukocyte infiltrate.

In order to explore the role of the parasite in the histopathological differences observed, we evaluated cell recruitment induced by H3227 and BA788 by using the air pouch model (14, 15). Responses induced by H3227 were three times higher than those induced by BA788 (Fig. 2A); these responses were correlated with a more intense exudate of leukocytes observed in the lesions of H3227-infected mice. H3227 was able to induce more influx of all cell types, attracting mainly more neutrophils and macrophages than BA788 (Fig. 2B). These data reinforce a role of the parasite in the differences observed in the inflammatory processes induced by the two *L. braziliensis* isolates used here.

RNA was extracted from lesions for reverse transcription-PCR analysis of chemokine expression at 6 h, 3 days, and 15 days p.i (12, 16). The sequences of the primers used are shown in Table 1. The expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC was upregulated at 6 h p.i. on H3227-induced lesions and only at 3 days p.i. in BA788-infected mice (Fig. 3A). In addition, CCL2/MCP-1, CCL3/MIP-1α, XCL1/lymphotactin-1, CXCL1/KC, and CCL11/eotaxin expression was more strongly induced by H3227 than by BA788. CXCL10/IP-10 was the only chemokine that appeared to be more strongly expressed by BA788 than by H3227. Regarding chemokine receptor expression, H3227 showed significantly higher expression of all chemokine receptors studied here than did BA788 (Fig. 3B). CCR5 was slightly upregulated in BA788-infected mice. Immunohistochemical analysis for the presence of CCL2/MCP-1 and CXCL10/IP-10 proteins in lesions induced by H3227 and BA788 confirmed the results obtained by mRNA expression analysis (Fig. 3C).

Lesions from patients with CL show a significant increase in the expression of CCL2/MCP-1 and CCL3/MIP-1α (20), and in vitro infection with *Leishmania* induces CCL2/MCP-1 and

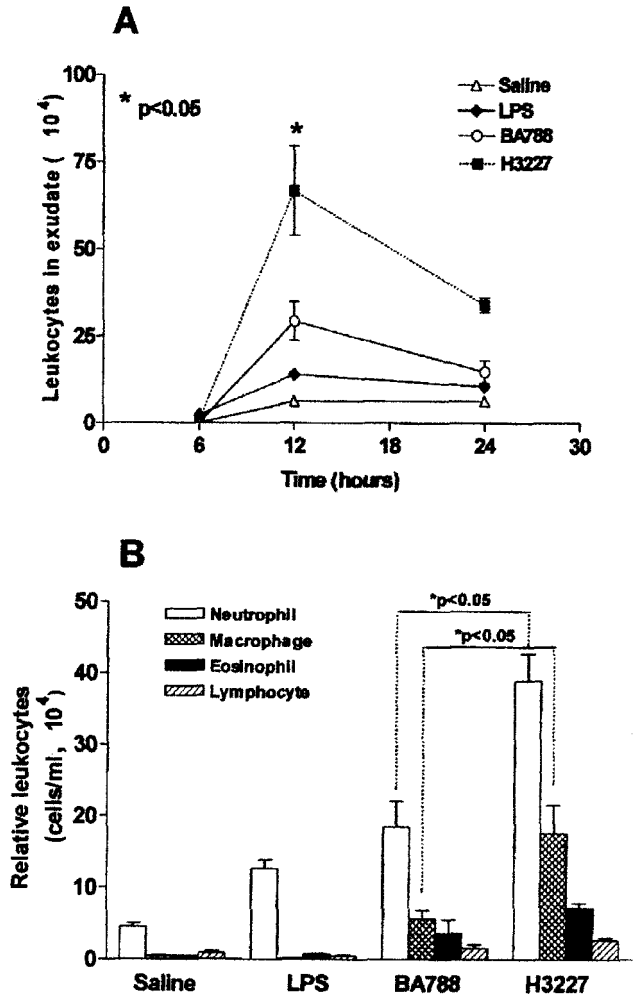
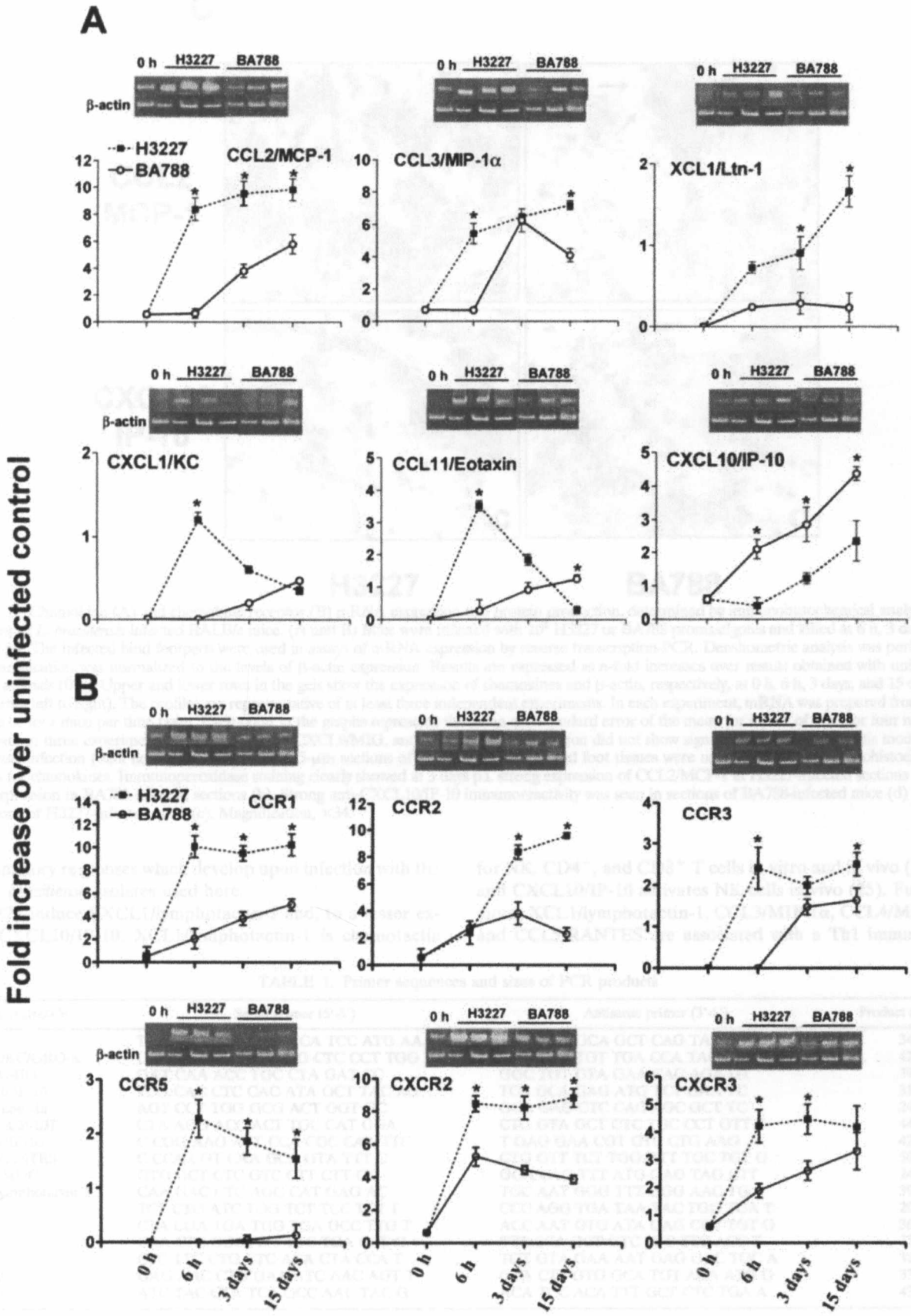


FIG. 2. Kinetics of leukocyte recruitment (A), expressed as total numbers of neutrophils, macrophages, eosinophils, and lymphocytes, in pouch exudates in response to lipopolysaccharide (LPS) or *L. braziliensis* (BA788 and H3227) and comparison of results at 12 h after inoculation (B). Air pouches were prepared by injecting 3 ml of air into the dorsal surface of mice under light anesthesia. Stationary-phase *L. braziliensis* promastigotes (10^7) were injected immediately following the air injection. Control mice were injected with endotoxin-free saline (negative control) and LPS (20 μg/ml; positive control). Mice were killed at the indicated time points, and the pouch contents were washed several time with saline. Exudate cells were centrifuged and stained; proportions of neutrophils, macrophages, eosinophils, and lymphocytes/200 cells were enumerated; and relative cell numbers were calculated from the total number of exudate leukocytes. Data represent the mean and standard error of the mean for three to five mice. The asterisk indicates a significant difference between values at the indicated time point, as determined by Student's *t* test or one-way analysis of variance ($P < 0.05$). Results are representative of two independent experiments.

CXCL1/KC/GRO-α expression in mouse and human macrophages (1, 19). CCL2/MCP-1 and CCL3/MIP-1α are potent chemoattractants for monocytes (9, 13). CXCL1/KC recruits neutrophils and is a dominant chemokine in murine inflammatory responses (4). The earlier expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC in more severe lesions may explain the significant and early accumulation of neutrophils and macrophages at the H3227 infection site and suggests that these chemokines can be factors regulating the differential


A
0 h H3227 BA788
β-actin
H3227
BA788
CCL2/MCP-1
CCL3/MIP-1α
XCL1/Ltn-1
CXCL1/KC
CCL11/Eotaxin
CXCL10/IP-10
B
CCR1
CCR2
CCR3
CCR5
CXCR2
CXCR3
Fold increase over uninfected control
0 h
6 h
3 days
15 days

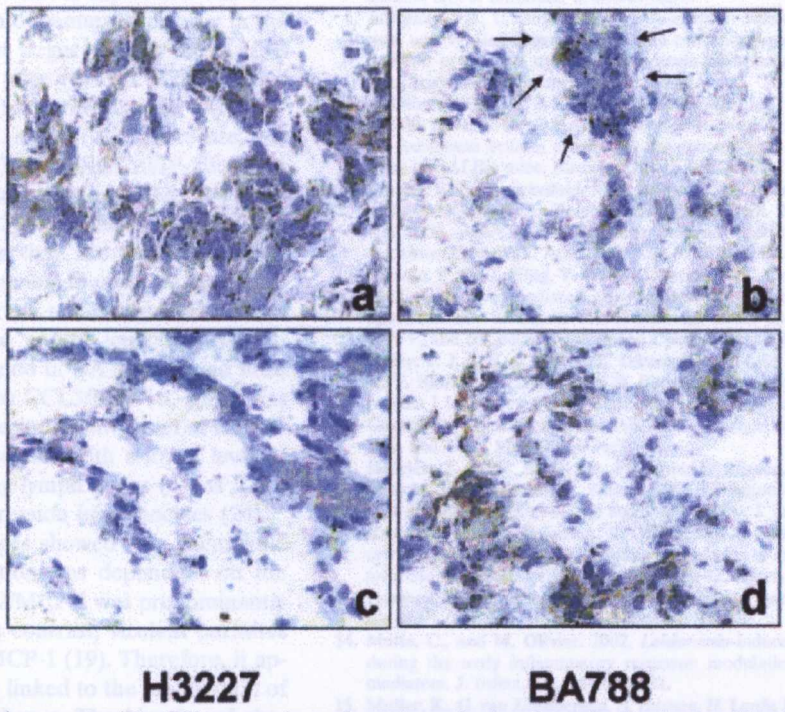


FIG. 3. Chemokine (A) and chemokine receptor (B) mRNA expression and protein production, determined by immunohistochemical analysis (C), in lesions of *L. braziliensis*-infected BALB/c mice. (A and B) Mice were infected with 10^6 H3227 or BA788 promastigotes and killed at 6 h, 3 days, and 15 days p.i. The infected hind footpads were used in assays of mRNA expression by reverse transcription-PCR. Densitometric analysis was performed, and quantification was normalized to the levels of β-actin expression. Results are expressed as *n*-fold increases over results obtained with uninfected control animals (0 h). Upper and lower rows in the gels show the expression of chemokines and β-actin, respectively, at 0 h, 6 h, 3 days, and 15 days p.i. (lanes from left to right). The profiles are representative of at least three independent experiments. In each experiment, mRNA was prepared from pools of three or four mice per time point. Each point in the graphs represents the mean and standard error of the mean for a pool of three or four mice per time point in three experiments. CCL5/RANTES, CXCL9/MIG, and CCL22/MDC expression did not show significant modulation in this model of *L. braziliensis* infection (data not shown). (C) Frozen 5-μm sections of infected and uninfected foot tissues were used to perform immunohistochemical analysis for chemokines. Immunoperoxidase staining clearly showed at 3 days p.i. strong expression of CCL2/MCP-1 in H3227-infected sections (a) and weak expression in BA788-infected sections (b). Strong anti-CXCL10/IP-10 immunoreactivity was seen in sections of BA788-infected mice (d) but not in sections of H3227-infected mice (c). Magnification, ×34.

inflammatory responses which develop upon infection with the two *L. braziliensis* isolates used here.

H3227 induced XCL1/lymphotactin-1 and, to a lesser extent, CXCL10/IP-10. XCL1/lymphotactin-1 is chemotactic for NK, $CD4^+$, and $CD8^+$ T cells in vitro and in vivo (8, 11), and CXCL10/IP-10 activates NK cells in vivo (25). Furthermore, XCL1/lymphotactin-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES are associated with a Th1 immune re-

TABLE 1. Primer sequences and sizes of PCR products

Oligonucleotide	Sense primer (5′-3′)	Antisense primer (3′-5′)	Product size (bp)
β-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C	TAA AAA GCA GCT CAG TAA CAG TCC G	349
CXCL1/KC/GRO-α	CC TTG ACC CTG AAG CTC CCT TGG TTC	CGT GCG TGT TGA CCA TAC AAT ATG	422
CXCL9/MIG	GAT CAA ACC TGC CTA GAT CC	GGC TGT GTA GAA CAC AGA GT	399
CXCL10/IP-10	TCG CAC CTC CAC ATA GCT TAC AG	TCA GCA GAG ATG TCT GAA TC	310
CCL11/eotaxin	AGT CCT TGG GCG ACT GGT GC	GCA GAG CTC CAC AGC GCT TC	243
CCL2/MCP-1/JE	CTA AGG ACC ACT TGC CAT GGA	CTG GTA GCT CTC TGC CCT GTT T	445
CCL3/MIP-1α	C CGG AAG ATT CCA CGC CAA TTC	T GAG GAA CGT GTC CTG AAG	427
CCL5/RANTES	C CCA CGT CAA GCA GTA TTT C	CTG GTT TCT TGG GTT TGC TGT G	506
CCL22/MDC	GTG GCT CTC GTC CTT CTT GC	GGA CAG TTT ATG GAG TAG CTT	249
XCL1/lymphotactin-1	CAA GAC CTC AGC CAT GAG AC	TGC AAT GGG TTT GGG AAC TG	397
CCR1	TCT CTG ATC TGG TCT TCC TTT T	CCC AGG TGA TAA TAC TGG TGA T	295
CCR2	CTA CGA TGA TGG TGA GCC TTG T	ACC AAT GTG ATA GAG CCC TGT G	368
CCR3	CAA CTT GGC ATT TTC TGA CCT G	TTT CCA GCT GTC TTC TTC ACC T	334
CCR5	CTC TTC CTG CTC ACA CTA CCA T	TGT GTA GAA AAT GAG GAC TGC A	322
CXCR2	GAG AAC CTG GAA ATC AAC AGT T	GTA CTT GTG GCA TGT ACA ATG G	339
CXCR3	ATC TAC CTA TCA GCC AAC TAC G	ACA TCC ACA TTT GCT CTC TGA A	433

sponse (7, 22). Interestingly, CXCL10/IP-10 was the only chemokine that was more strongly expressed in lesions induced by the less pathogenic strain BA788, and its expression was correlated with the earlier production of gamma interferon in the draining lymph nodes and with the larger number of NK cells in the lesions of BA788-infected mice (6). NK cells produce gamma interferon and may contribute to resistance to *L. braziliensis*, as previously shown for *L. major* (21). Therefore, it is possible that XCL1/lymphotactin-1 and CXCL10/IP-10 are involved in resistance to *L. braziliensis* infection in BALB/c mice. Lesions caused by *L. braziliensis* H3227 exhibited a higher level of chemokine receptor expression than did those caused by *L. braziliensis* BA788. BA788 was unable to promote the strong expression of chemokine receptors, a result which was correlated with its reduced capacity to induce leukocyte recruitment. CCR5 was slightly upregulated in BA788-infected mice at 3 days p.i. and was stimulated by CCL3/MIP-1α, which was expressed during the same time period. A low level of expression of CCR5 in lesions was correlated with a lower level of expression of IL-10 in the draining lymph nodes (6), as IL-10 selectively upregulates CCR5 expression in monocytes (24).

Studies with murine macrophages showed that chemokine induction after *Leishmania* infection was dependent on the parasite strain used. Indeed, CCL2/MCP-1 was predominantly induced by avirulent *L. major*. In contrast, virulent parasites induced considerably less CCL2/MCP-1 (19). Therefore, it appears that *Leishmania* virulence is linked to the modulation of chemokine expression by macrophages. The kinetics of chemokine induction seem to be more important than parasite multiplication, and this fact may be related to structural differences between the two isolates used here. Of note, results from an analysis by random amplification of polymorphic DNA showed that strains H3227 and BA788 of *L. braziliensis* are genetically diverse (6).

Collectively, the findings presented here indicate that two *L. braziliensis* isolates, albeit at similar parasite burdens, induced chemokine expression patterns at different paces and/or intensities, leading to diverse cell recruitment and differential inflammatory responses; these features might ultimately be implicated in disease presentations.

We thank Cristiane M. Milanezi and Jorge L. Tolentino for technical assistance.

This work was supported by grants from FAPESB, CAPES (PROCAD 0018/01–5), and CNPq. M.J.T. and C.R.T. received fellowships from CAPES. J.S.d.S., C.I.B., M.B.-N., and A.B. are senior investigators from CNPq. J.D.F. and B.B.A. received scientific initiation fellowships from CNPq.

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Editor: A. D. O'Brien

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Microbes and Infection 6 (2004) 1347
Microbes and Infection
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Erratum

Erratum to: "*Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice" [Microbes Infect. 6 (2004) 977–984]

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Available online 01 October 2004

In the list of authors for this article the name of João Santana da Silva was incorrectly spelt.

Figs. 3–5 had been transposed; however, the legends were placed in the correct order. Fig. 5 should have been with the legend to Fig. 3; Fig. 3 should have been with the legend to Fig. 4; and Fig. 4 should have been with the legend to Fig. 5.

☆ doi of original article 10.1016/j.micinf.2004.05.003.
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doi:10.1016/j.micinf.2004.09.003

Available online at www.sciencedirect.com
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Microbes and Infection 6 (2004) 977–984
Microbes and Infection
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Original article

Leishmania braziliensis isolates differing at the genome level display distinctive features in BALB/c mice

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Received 5 January 2004; accepted 13 May 2004

Available online 23 July 2004

Abstract

Leishmania braziliensis is the species responsible for the majority of cases of human cutaneous leishmaniasis in Brazil. In the present study, *L. braziliensis* isolates from two different geographic areas in Brazil were studied by RAPD, using arbitrary primers. We also evaluated other biological features of these two isolates. We compared (a) the clinical features they initiate or not once delivered subcutaneously as stationary-phase promastigotes in the footpad of BALB/c mice; (b) the parasite load in both the footpad and the draining lymph node; (c) the cytokines present in the supernatant of cultures of the cell suspensions from the draining lymph nodes; and (d) the cell types present at the site of parasite delivery. The results show that the *L. braziliensis* strain from Ceará (H3227) is genotypically different from the *L. braziliensis* strain from Bahia (BA788). H3227-parasitized mice developed detectable lesions, whereas BA788-parasitized mice did not. Fifteen days post parasite inoculation there was an increase in the numbers of macrophages and lymphocytes in the footpads, whatever the parasite inoculum. Parasite load at the inoculation site—namely the footpad—did not differ significantly; in draining lymph nodes, however, it increased over the period under study. Early after parasite inoculation, the cells recovered from the draining lymph nodes of BA788-parasitized mice produced higher levels of IFN-γ, a feature coupled to a higher number of NK cells. Later, after the parasite inoculation, there was an increased content of IL-12p70 and IL-10 in the supernatant of cells recovered from the lymph nodes of H3227-parasitized mice. This comparative analysis points out that *L. braziliensis* isolates differing in their genomic profiles do establish different parasitic processes in BALB/c mice.

Keywords: *Leishmania braziliensis*; Random amplification of polymorphic DNA; Interferon gamma; Interleukin-12p70; Interleukin-10

1. Introduction

Leishmania is the etiological agent of leishmaniasis, a parasitic disease with diverse clinical manifestations in human beings and other mammals. The mammalian organism becomes a parasitized host when the sand fly probes the skin for a blood meal, injecting metacyclic promastigotes together with saliva. *Leishmania braziliensis* parasites usually cause a self-healing ulcer at the site of parasite delivery; however, parasites may also metastasize to the nasopharyngeal tract—a process that is thought to be the starting event leading to mucocutaneous leishmaniasis [1]. More rarely, parasite invasion of the bloodstream results in disseminated skin lesion [2].

Extensive studies using *L. major* have been interpreted according to the following frame. The resistance or the susceptibility to disease, in different mouse strains, is bound by genetic determinants of the host [3]: resistance is mediated by a Th1-type cellular immune response, characterized by the presence of high levels of IFN-γ and low levels of IL-4; susceptibility is mediated by a Th2-type cellular immune

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doi:10.1016/j.micinf.2004.05.009

response, characterized by low levels of IFN-γ and high levels of IL-4. Not only do these cytokines act on parasite-loaded cells or further host cells by killing the parasites or preventing their replication, but they also contribute to the inflammatory processes occurring in the parasite-loaded tissue. In contrast to *L. major*, considerably less experimental work has been conducted with *L. braziliensis*, probably because most mouse strains do not display lesions at the site of inoculation of *L. braziliensis* [4,5]. This phenotype (absence of lesion) has been associated with the inability to sustain a strong type 2 immune response [6]. Nonetheless, it has been shown that skin biopsies from patients with cutaneous leishmaniasis caused by *L. braziliensis* displayed IFN-γ, TNF-α [7] and iNOS[8]. These cytokines play a key role in the control of the parasite load and, in parallel, contribute to the development of intense inflammatory processes at the parasite inoculation site.

Alternatively, it has been shown that the parasite also plays a role in determining the parasitic process outcome, i.e. disease or long-term asymptomatic parasitism. For example, once located in the skin, the development of *L. amazonensis* is known to lead to many different clinical presentations, including localized cutaneous lesions, mucocutaneous and visceral leishmaniasis, in humans [9], some of these features being reproduced in mice [10]. More recently, it was shown that genotypic characteristics of *L. mexicana* could be correlated with features of the clinical disease [11] and that *L. major* isolates from the field show differences in pathogenicity upon inoculation into BALB/c mice [12]. Therefore, variation in the pathogenicity of the parasite, which is related to its genetic diversity, is also an important factor influencing the clinical features of leishmaniasis. Although extensive genetic diversity has been documented in *L. braziliensis* [13], it was correlated with geographical origin [14,15] and not with clinical manifestations of the disease.

In the present study, we used molecular techniques to examine the genetic polymorphism of *L. braziliensis* strains isolated from two states in Brazil, namely Ceará and Bahia, located in northeastern Brazil. Both Ceará and Bahia are endemic for cutaneous leishmaniasis caused by *L. braziliensis*. In Ceará, however, the cutaneous lesion is accompanied and sometimes preceded by an impressive enlargement of regional lymph nodes, whereas in Bahia, such findings have not been documented. We have also examined (a) the cell types present at the site of parasite delivery and (b) the cytokine production after in vitro re-stimulation of the cells recovered from the draining lymph nodes and the cell types present at the site of parasite delivery.

2. Materials and methods

2.1. Mice

Male 4–6-wk-old BALB/c mice were obtained from CPqGM/FIOCRUZ Animal Facility, where they were maintained under pathogen-free conditions. The Animal Care and Utilization Committee from CPqGM/FIOCRUZ approved all experimental procedures.

2.2. Parasite culture

The *L. braziliensis* strains MHOM/BR/94/H3227 and MHOM/BR/01/BA788 used were isolated from cutaneous ulcers from patients with cutaneous leishmaniasis, from the states of Ceará and Bahia (northeastern Brazil), respectively, after brief (2–4) passages in culture medium. Both isolates were identified as *L. braziliensis* by PCR [16] and monoclonal antibodies [17]. Promastigotes were grown in 199 medium (Gibco, Grand Island, NY) at 25 °C supplemented with 10% heat-inactivated fetal calf serum, 20 mM Hepes, 4 mM $NaHCO_3$, 100 U/ml of penicillin and 100 μg/ml streptomycin (all from Gibco).

2.3. The readout assay for typing some signatures of polymorphism of the parasites at the nuclear genomic level

Genomic DNA extraction, RAPD (Random amplification of polymorphic DNA) was done as described [18]. Briefly, 150 ng of parasite DNA was amplified in a final reaction volume of 25 μl containing 10 mM Tris–HCl pH 8.5, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP, 1.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 1.25 μM of either primer 3302 (5′-CTGATGCTAC-3′); 3303 (5′-TCACGATGCA-3′) or 3304 (5′-GCACTGTCA-3′). The amplification cycles consisted of an initial denaturation step at 95 °C for 5 min, two cycles with denaturation at 95 °C for 30 s, annealing at 30 °C for 2 min and extension at 72 °C for 1 min, followed by 33 cycles in which annealing was increased to 40 °C. Ten-microliter samples were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

2.4. Inoculation of parasites into mice

Stationary-phase promastigotes were inoculated subcutaneously into the hind left footpad of BALB/c mice at a dose of 10^6 parasites in 20 μl of saline. Observation of lesion development was made at weekly intervals and footpad swelling was measured in millimeters by a dial gauge caliper. Lesion size was defined as the increase in footpad thickness after subtraction of the size of the controlateral uninfected footpad.

2.5. Parasite load estimate

Parasite load was determined using the quantitative limiting dilution assay as described [19]. Briefly, popliteal lymph nodes draining the infected footpad were aseptically excised and homogenized with a tissue glass grinder in 2 ml of Schneider's medium (Sigma, St. Louis, MO). The homoge-

nates were serially diluted in Schneider's medium supplemented with 100 U of penicillin/ml, 100 μg/ml of streptomycin/ml, 10% heat inactivated fetal calf serum (Gibco) and 2% sterile human urine in 96-well plates containing biphasic blood agar medium. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown out after up to 3 weeks of incubation at 25 °C.

2.6. Culture of lymph node cells and quantitation of cytokine in the culture supernatants

For measurement of in vitro cytokine production, single-cell suspensions of infected footpad draining popliteal lymph nodes were prepared aseptically at 3 and 15 days post infection. The cells were diluted 5×10^6cells/ml and dispensed into 96-well plates with *L. braziliensis* H3227 or BA788 live promastigotes (stationary phase), at a ratio of five parasites to one cell, or without parasites, in RPMI-1640 medium (Gibco) containing 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg/ml of streptomycin/ml, 10% heat inactivated fetal calf serum (Gibco) and 0.05 mM β-mercaptoethanol. Cell culture supernatants were harvested after 48 h (for IL-4, IL-10 and IL-12p70 measurement) or 72 h (for IFN-γ measurement) of culture at 37 °C in 5% CO_2. Levels of IFN-γ, IL-4, IL-10 and IL-12p70 were determined by ELISA using commercial kits (BD Biosciences, San Diego, CA). The cytokine production of lymph node cells from non-infected animals, upon stimulation with live *L. braziliensis* promastigotes, was below the detection level of the kits used for cytokine detection.

2.7. Flow cytometric analysis

To characterize leukocytes present in the inoculation site, infected foot tissue was collected at 3 and 15 days post infection and incubated 1 h at 37 °C, in RPMI-1640 medium containing 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg/ml of streptomycin/ml, 10% heat inactivated fetal calf serum (all from Gibco), 0.28 units/ml of Liberase CI (Roche, São Paulo, SP). Foot tissues were processed in the presence of 0.05% DNAse (Sigma-Aldrich, St. Louis, MO) using Medimachine (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. After processing, cell viability was assessed by trypan blue exclusion and cells were filtered trough a 50-μm filter and washed before flow cytometry analysis. Viable leukocytes were incubated with antibodies specific to GR-1, Mac-1, NK 1.1, CD3 or MHC class II molecules or with isotype control antibodies (all from BD Biosciences, San Diego, CA) for 30 min at 4 °C in the dark. Cells were washed and resuspended in PBS, 1% formaldehyde. For each sample, 10,000 cells were examined. Data were acquired using a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

2.8. Statistical analysis

The data are presented as mean ± standard error of the mean. The significance of the results was calculated by Student's *t*-test, except for the parasite load analysis, over time, within the BA788- and H337-infected mice groups where ANOVA was used. All analyses were performed using Prism (Graph Pad Software, San Diego, CA) software and a *P*-value <0.05 was considered significant.

3. Results

3.1. The BA788 and H3227 L. braziliensis strains display genomic polymorphisms: a study with RAPD

In preliminary experiments, we investigated the degree of genetic polymorphism by RAPD between six *L. braziliensis* isolates, two from Ceará (MHOM/BR/94/H3227, MHOM/BR/94/H3456) and four from Bahia (MHOM/BR/00/BA711, MHOM/BR/02/BA427, MHOM/BR/02/806 and MHOM/BR/01/BA788). In these experiments, the genomic profiles obtained with the isolates from Ceará were different from those obtained with the isolates from Bahia (data not shown); data obtained with two representative isolates, namely BA788 (Bahia) and H3227 (Ceará), are shown (Fig. 1). RAPD generated 43 amplified bands, varying from 100–2000 bp in length, of which six are common to the two strains. The most diverse genomic profiles were obtained with primers 3303 and 3304, in which 26 of the 35 amplified fragments were polymorphic. The distinct genomic profiles obtained for strains BA788 and H3227, with the three primers tested here, assess DNA polymorphism between these *L. braziliensis* strains.

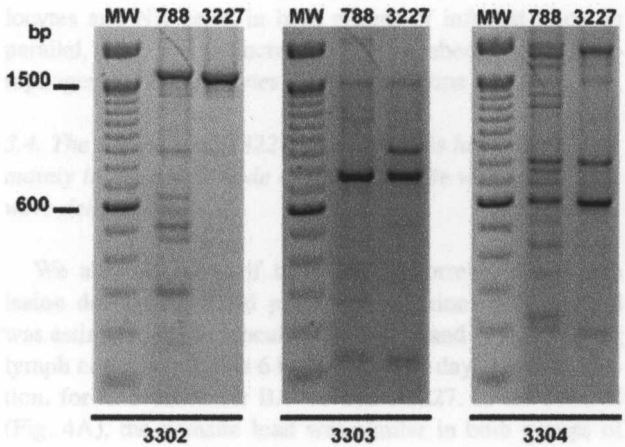

Fig. 1. Genomic profiles of *L. braziliensis* strains BA788 and H3227. Genomic DNA of each *L. braziliensis* strain was subjected to RAPD using primers 3302, 3303 and 3304. MW; 100 bp ladder DNA size marker.

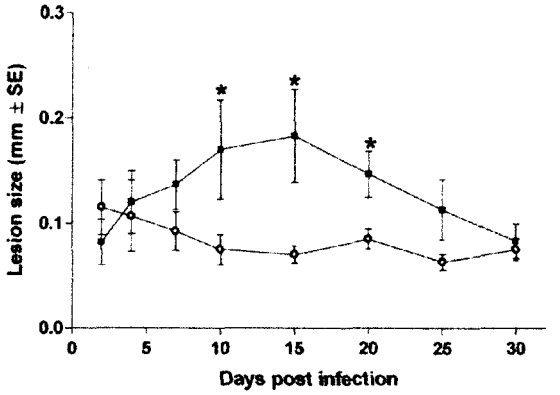

Fig. 2. Course of lesion development in BALB/c mice inoculated with *L. braziliensis* strains. BALB/c mice were infected with 10^6 of either H3227 (black square) or BA788 (white circle) promastigotes in the left hind footpad, and lesion size was measured for 30 days. The footpads of 3–5 mice per group were measured. The data shown, reported as the mean ± standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values at the indicated time point as determined by Student's *t*-test (* $P < 0.05$).

3.2. The amplitude of the transient clinical features occurring at the site of L. braziliensis *delivery differs according to whether the isolate is BA788 or H3227*

The genomic polymorphism observed between BA788 and H3227 prompted us to investigate whether these strains might display differences in pathogenicity upon inoculation into mice of an inbred strain. BALB/c mice received, subcutaneously in the footpad, 10^6 promastigotes of either BA788 or H3227 (Fig. 2). Small increases in footpad thickness were observed early after infection (6 h to 5 days). Mice infected with H3227 developed lesions detectable at 7 days post inoculation. Lesions progressed steadily, peaked at 15 days post parasite inoculation and gradually resolved 30 days post infection. At this time, we observed the presence of a small nodular lesion associated with cutaneous fibrosis (data not shown). All lesions caused by H3227 *L. braziliensis* were unulcerated. Even at maximum size, lesions remained non-ulcerative and non-necrotic. In mice infected with BA788, in contrast, no similar transient pathogenic processes were noticed. The differences in footpad thickness, between both groups of mice, were significant ($P < 0.05$) in the period ranging from 10–20 days post inoculation.

3.3. The phenotypic composition of leukocytes present at the site of BA788 or H3227 L. braziliensis *delivery differs according to the isolate*

In order to characterize the leukocyte lineages present at the site of parasite delivery, the footpads were excised at 3 and 15 days post parasite inoculation and analyzed by flow cytometry. At 3 days post inoculation, the number of granulocytes, macrophages and lymphocytes is similar in both groups of mice, whereas the number of NK cells is higher in BA788-parasitized mice (Fig. 3). Fifteen days post parasite inoculation, we observed a decrease in the numbers of granulocytes and NK cells, in both groups of infected mice. In parallel, there was an increase in the numbers of both macrophages and lymphocytes in the two groups of mice.

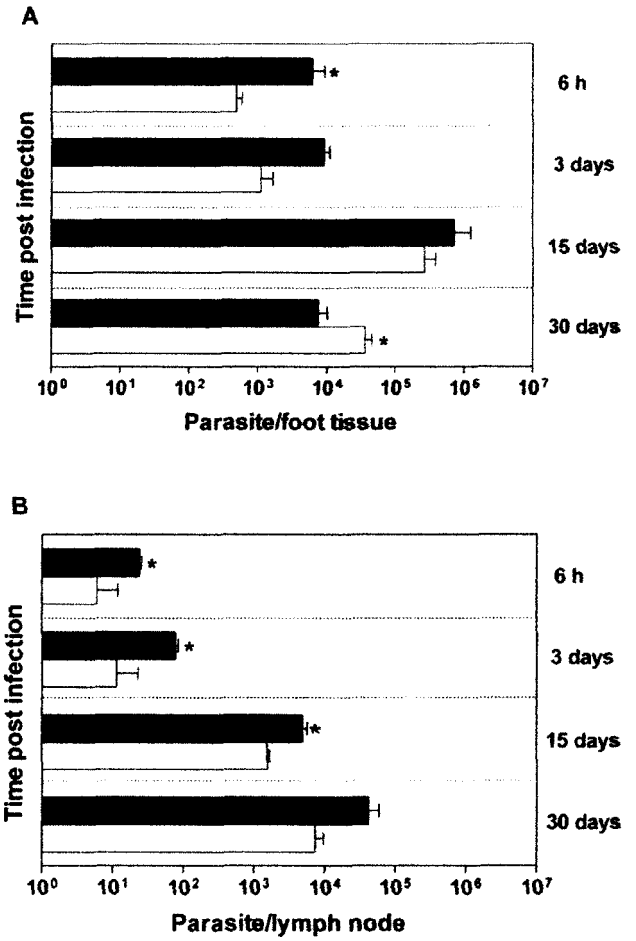

Fig. 3. Phenotypic composition of leukocyte population recovered from the footpad of mice inoculated with *L. braziliensis* strains. BALB/c mice were inoculated with 10^6 promastigotes of either H3227 (black) or BA788 (white) in the left hind footpad of BALB/c mice. Three (A) and 15 days (B) post inoculation. Leukocytes from inoculated footpads were analyzed by flow cytometry for the presence of lymphocytes, granulocytes, macrophages and NK cells. The data shown are from a single experiment representative of two separate experiments.

3.4. The BA788 and H3227 L. braziliensis *loads differ mainly in the lymph node draining the site where they were delivered*

We also examined if there was a correlation between lesion development and parasite replication. Parasite load was estimated in the inoculated footpads and in the draining lymph nodes, excised at 6 h, 3, 15 and 30 days post inoculation, for *L. braziliensis* BA788 and H3227. In the footpad (Fig. 4A), the parasite load was similar in both groups of mice, but statistically significant differences were observed at 6 h and 30 days post inoculation. In mice inoculated with H3227 parasites, the parasite load peaked at 15 days, coin-

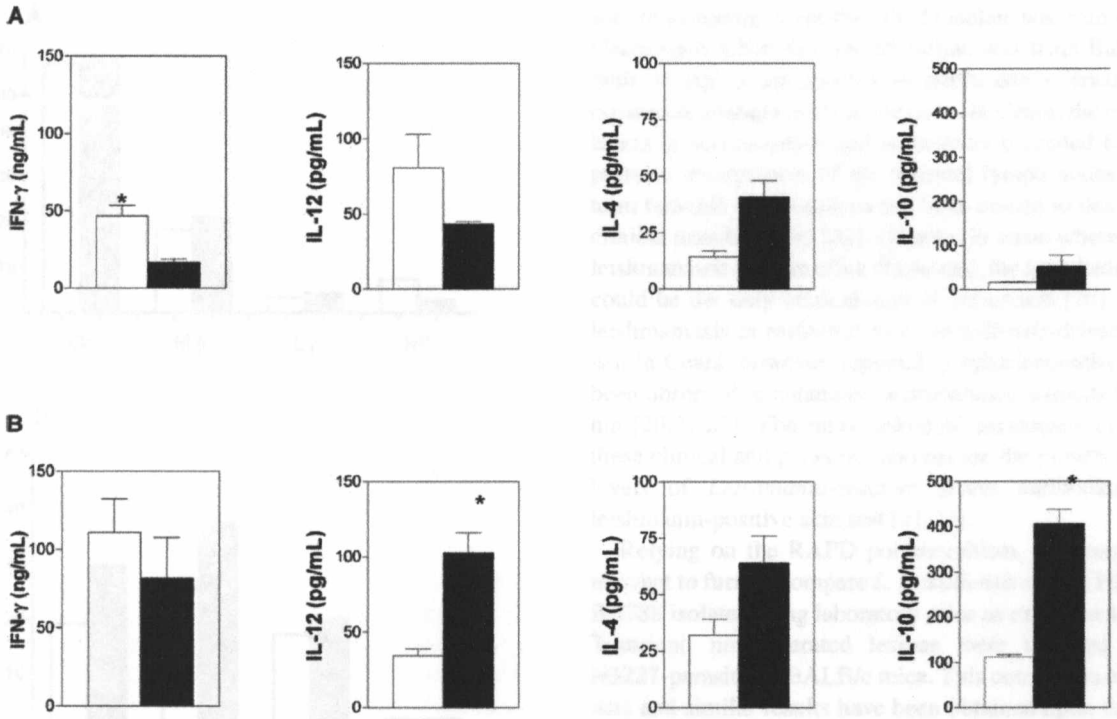

Fig. 4. Parasite load estimate in mice infected with *L. braziliensis* strains. BALB/c mice were infected with 10^6 of either H3227 (black) or BA788 (white) promastigotes in the left hind footpad. Footpads (A) and popliteal lymph nodes (B) draining the lesion, 3–5 mice per group, were used for analysis of parasite load. The data shown, reported as the mean ± standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values from H3227 and BA788 at the same time point as determined by Student's *t*-test (* $P < 0.05$).

ciding with lesion development. However, at this time point, no clinical lesion was observed in mice inoculated with BA788, despite a similar parasite load estimate, indicating dissociation between parasite replication and lesion development. In the draining lymph nodes (Fig. 4B), the parasite load was significantly higher in H3227-parasitized mice, from 6 h to 15 days post parasite inoculation. Interestingly, in the draining lymph nodes, the parasite load showed a consistent increase in both groups of parasitized mice, indicating that parasites are able to multiply in these secondary lymphoid tissues, regardless of lesion resolution at the upstream site where parasites were inoculated and developed, such as that seen in H3227-parasitized mice.

When comparing the parasite load estimate within the BA788 and H3227 groups, separately, over time, we did not find any significant differences in the footpads inoculated with H3227. In the footpads inoculated with BA788, however, there were statistically significant differences between 6 h and 15 days ($P < 0.01$), 3 and 15 days ($P < 0.01$) and 15 and 30 days post inoculation ($P < 0.05$). Therefore, in the footpad, the parasite load estimate found at 15 days post inoculation is higher than that found at 6 h, 3 days and 30 days post inoculation only in mice receiving the BA788 isolates. In the draining lymph nodes, we did not find any statistically significant differences, between the BA788 and H3227 isolates when comparing the parasite load estimate.

3.5. At the later time point studied, there is a difference between the ex vivo cytokine profiles displayed by the leukocytes recovered from lymph nodes draining the site of delivery of BA788 or H3227

The observations that H3227-infected mice controlled the cutaneous lesion and that BA788-infected mice did not develop clinical lesions suggest that, in both cases, a Th1-type cellular immune response had developed. At 3 and 15 days post infection, cells prepared from the lymph nodes draining the infection site were incubated with live BA788 or H3227 promastigotes and were monitored for the production of Th1- and Th2-type cytokines. Three days post infection (Fig. 5A), cells from BA788-infected mice produced higher levels of both IFN-γ ($P < 0.05$) and IL-12, than cells from H3227-infected mice which, on the contrary, produced higher amounts of both IL-4 and IL-10. Fifteen days post-infection (Fig. 5B), Th1-type cytokine production was markedly up-regulated in H3227-infected mice as shown by the significant increase in IL-12 production, in parallel with IFN-γ. Cells from H3227-infected mice also produced higher amounts of both IL-4 and IL-10, the latter showing a significant up-regulation at this time point. This up-regulation in IL-10 production coincides with the time point at which lesion size and parasite load detected in the footpad reached their peak. Moreover, lymph node cell counts were consistently higher in H3227 *L. braziliensis*-infected mice than in BA788-

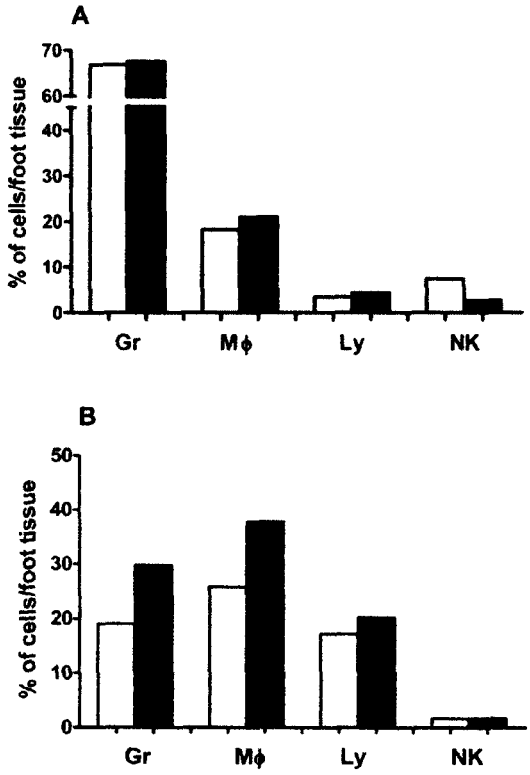

Fig. 5. Cytokine production by mononuclear cells from mice infected with *L. braziliensis* strains. BALB/c mice were infected with 10^6 promastigotes of either H3227 (black) or BA788 (white) in the left hind footpad. Three (A) and 15 days (B) post infection, mononuclear cells (3–5 mice per group) from popliteal lymph nodes draining the lesion were incubated with live *Leishmania* promastigotes and the supernatants were assayed for IL-4, IL-10, IL-12p70 and IFN-γ production. The data shown, reported as the mean ± standard error of the mean, are from a single experiment representative of three separate experiments. Cytokine production of lymph node cells from non-infected animals, upon stimulation with live *L. braziliensis* promastigotes, was below the detection level of the ELISA kits used: IFN-γ (55 pg/ml), IL-4 (5.5 pg/ml), IL-10 (3 pg/ml) and IL-12p70 (5 pg/ml). The asterisk indicates a significant difference between values as determined by Student's *t*-test (*$P < 0.05$).

infected mice, at both 3 days post infection ($14.8 \times 10^6 \pm 1.6 \times 10^6$ vs. $8.1 \times 10^6 \pm 0.3 \times 10^6$, respectively) and at 15 days post infection ($24.0 \times 10^6 \pm 2.8 \times 10^6$ vs. $14.7 \times 10^6 \pm 1.3 \times 10^6$, respectively).

4. Discussion

L. braziliensis parasite populations are extremely diverse, as assessed by RAPD-based readout assays [13]. However, this diversity is less pronounced among parasite populations obtained from areas of geographical proximity [14]. Random amplification of *L. braziliensis* H3227 and BA788 DNA generated different genomic profiles, including the presence of amplification fragments not shared between strains. These fragments may constitute nuclear genomic markers for strains from these areas. As far as the BA788 and H3227 *L. braziliensis* isolates are concerned, the RAPD-based polymorphism can therefore be correlated with their geographical area of sampling, since the H3327 isolate was sampled from Ceará state, while the BA788 isolate was from Bahia state, both of which are located in northeastern Brazil, where cutaneous leishmaniasis is endemic. In Ceará, the cutaneous lesion is accompanied and sometimes preceded by an impressive enlargement of the regional lymph nodes, and the term bubonic leishmaniasis has been coined to describe this clinical manifestation [20]. Of note, in areas where bubonic leishmaniasis is more often diagnosed, the lymphadenopathy could be the only clinical sign of parasitism [20]. Bubonic leishmaniasis is restricted to *L. braziliensis*-driven parasitism in Ceará; however, regional lymphadenopathy has also been observed in cutaneous leishmaniasis patients from Bahia [20,21,22]. The immunological parameters coupled to these clinical and parasitic features are the presence of high levels of *Leishmania*-reactive serum antibodies and a leishmanin-positive skin test [21,22].

Relying on the RAPD polymorphism, we considered it relevant to further compare *L. braziliensis* strains H3227 and BA788 isolates using laboratory mice as experimental hosts. Transient, non-ulcerated lesions were detected only in H3227-parasitized BALB/c mice. This outcome is characteristic and similar results have been obtained upon the subcutaneous delivery of in vitro grown promastigotes of other *L. braziliensis* isolates to BALB/c mice [23–25]. We did not find any correlation between lesion development and parasite load: indeed the parasite load in the footpad was not significantly different between BA788- and H3227-parasitized mice.

H3227-loaded footpads were processed for histological analysis at 3 and 15 days post inoculation: an inflammatory infiltrate consisting mainly of polymorphonuclear leucocytes and macrophages was noticed. The BA788-loaded footpad sections exhibited a less intense and more transient leukocyte infiltrate (data not shown). Similarly to other *L. braziliensis* strains, the presence of granulocytes and lymphocytes was noticed during the first week post inoculation; later, many more macrophages and lymphocytes were observed [26].Three days post inoculation, when compared to footpads of H3227-inoculated mice, more NK leukocytes were observed on footpad samples prepared from BA788-inoculated mice. NK leucocytes are known to produce IFN-γ and may contribute to resistance to *L. braziliensis* as previously shown for *L. major* [27]. Moreover, NK cells are detectable as soon as 24 h post inoculation at the site of *L. major* delivery in resistant mice [28]. Therefore, it is possible that the early and higher IFN-γ production observed at the site of BA788 parasite delivery is due to the presence of a higher number of NK cells.

Following the in vitro stimulation of lymph node cells, the ratio of IFN-γ to IL-10 was higher when the cells were recovered from BA788-parasitized mice than when the cells were recovered from H3227-parasitized mice, at both 3 days post inoculation (2.28 vs. 0.24), respectively, and at 15 days post inoculation (0.77 vs. 0.15), respectively. These immunological features are coupled to the outcome of the parasitic

processes: while BA788-inoculated mice remain lesion-free, the H3227-inoculated mice displayed a transient lesion that heals spontaneously. These features of *L. braziliensis* parasites in BALB/c mice have been related to their inability to sustain a strong type 2 cytokine profile [6]; this is a main difference with the type 2 cytokine profile driven by *L. major* when they are delivered in the same conditions as the ones we used in the present study [3].

In conclusion, *L. braziliensis* isolates differing through their RAPD profiles exhibit differences in clinical features and certain immunological parameters once inoculated into experimental hosts such as BALB/c mice. The differences in clinical features are related to intrinsic properties of the parasite isolates such as ability to induce transient lesion and to recruit different leukocyte populations to the parasite-loaded site. In Ceará state, where H3227 *L. braziliensis* representative isolate was obtained, the lymphadenopathy, when diagnosed, could precede the lesion form, whereas in Bahia, such lymph node enlargement is not as prominent. Interestingly, this in vivo correlate was reproduced in the present study, since the lymph node cell counts were consistently higher with the H3227 isolate than with the BA788 isolate, at both 3 days and at 15 days post inoculation. These results reinforce the notion that disease manifestation at the site of *Leishmania* delivery results from a complex interplay between genetically determined parasite as well as host traits. Studies are now in progress to monitor more relevant parasite genetic polymorphism [29].

Acknowledgments

The authors thank Alexandra R.V. Dias for technical assistance. This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB). C. Brodskyn, M. Barral-Netto and A. Barral are senior investigators from CNPq and Instituto de Investigação em Imunologia.

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