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CARACTERIZAÇÃO FENOTÍPICA E FUNCIONAL DE CÉLULAS PRESENTES EM LESÕES DE PACIENTES COM LEISHMANIOSE CUTÂNEA E MUCOSA

Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas

Belo Horizonte, 2008

Tese apresentada ao Programa de Pós-graduação em Biologia Celular do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Biologia Celular

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Dedico está tese a minha família,
ao meu marido e principalmente
a minha linda filhinha Isabela.

AGRADECIMENTOS

Tenho tanto a agradecer...

Primeiramente, gostaria de agradecer a minha orientadora Wal pela orientação, pelo incentivo, por ter acreditado em mim e, principalmente, pela amizade e alegria contagiante. Ao meu co-orientador Ken a quem devo parte da minha orientação, por estar sempre conosco, compartilhando idéias, trabalhos e laboratório. Dos inúmeros aprendizados que levo desta etapa da minha vida, estou certa de que os mais valiosos foram vocês quem me ensinaram. Muito obrigada do fundo do meu coração.

Aos amigos do Laboratório de Biologia das Interações Celulares: Fernanda, Germano, Micena Janete, Aécio, Roberto, Cris e Paula. Aos amigos do Laboratório de Biologia dos Linfócitos: Taty, Érica, Karen, Pri e Sô, por todos os anos de convivência e amizade e principalmente pela ajuda, que foi de fundamental importância para a realização desta tese. Obrigada, muito obrigada!

À minha querida amiga Lis, que foi acima de tudo uma maravilhosa companhia neste percurso. Obrigada pela amizade verdadeira e pelas opiniões sinceras. Que nossa amizade continue por toda a nossa vida.

À Paty, que se revelou uma maravilhosa amiga. Mais do que nos momentos de descontração sua amizade foi fundamental nos momentos difíceis.

Aos amigos do laboratório de Neurofarmacologia: Cristina, Helton, Aninha, Adriane e Luciene pela amizade e por sempre estarem dispostos a nos ajudar em todos os momentos

Aos colaboradores do Serviço de Imunologia do Hospital Universitário coordenados pelo prof. Dr. Edgar Santos, UFBA em Salvador, BA, por ter gentilmente nos cedido as biópsias, pelo conhecimento adquirido e por poder me considerar integrante do grupo, um dos melhores no estudo de leishmaniose humana do Brasil.

Ao departamento de Morfologia e à Iraídes pela amizade, e por sempre nos ajudar com a parte burocrática.

Aos pacientes que gentilmente doaram as lesões para a realização deste trabalho.

À minha família pelo apoio e, principalmente, a minha mãe, à minha irmã Ana Paula e ao meu pai, que infelizmente não pode ver este nosso sonho concretizado. Eles sempre estiveram juntos comigo, me apoiando e incentivando nos momentos mais difíceis.

À família do meu marido pelo apoio e carinho.

Especialmente ao meu marido Júnior, pela tolerância e principalmente, por ser meu amigo e companheiro e por estar sempre me incentivando a buscar novos ideais, meu sincero muito obrigada.

À minha filhinha Isabela por ser a melhor coisa que aconteceu na minha vida.

À Binha por cuidar da minha filhinha com carinho de vovó.

A Deus por estar presente em todos os momentos da minha vida, e pela oportunidade de conhecer pessoas maravilhosas. Muito obrigada!

Àqueles que, porventura, eu possa não ter mencionado, mas que foram também imprescindíveis na realização deste trabalho.

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LISTA DE ABREVIATURAS

BSA	Albumina Sérica Bovina
CD	Grupo de diferenciação
CC	Citocina com cisteína adjacente
CMSP	Células mononucleares do sangue periférico
CTL	Controle de isotipo
Dapi	*4', 6-diamino - 2- phenylindole, dihydrochloride*
DN	Duplo Negativas
FITC	Isotiocianato de fluoresceína
IFN-γ	Interferon-gama
IL	Interleucina
IL-10R	Receptor de IL-10
iNOS	Óxido nítrico sintase indutível
L. braziliensis	*Leishmania braziliensis*
LC	Leishmaniose cutânea
LC-I	Leishmaniose cutânea – inicial
LC-T	Leishmaniose cutânea – Tardia
LM	Leishmaniose mucosa
MHC	Complexo principal de histocompatibilidade
MIP	Proteína de inflamação de macrófagos
μm	Micrômetro
NO	Óxido nítrico
NK	"Natural Killer"
OMS	Organização Mundial da Saúde
PBS	Tampão salina-fosfato

PCR	Reação em cadeia da polimerase
PE	Ficoeritrina
RT-PCR	Transcrição reversa seguida de reação em cadeia da polimerase
SLA	Antígeno solúvel de *Leishmania*
TCR	Receptor de células T
TGF-β	Fator de crescimento transformante-beta
TNF-α	Fator de necrose tumoral-alfa
UFMG	Universidade Federal de Minas Gerais

RESUMO

A infecção humana por *Leishmania braziliensis* pode levar ao desenvolvimento das formas clínicas cutânea (LC) e mucosa (LM). Independentemente da forma clínica, as lesões representam a conseqüência patológica principal na leishmaniose tegumentar. Neste trabalho, compararam-se lesões de pacientes com LC e LM, com os objetivos de determinar a expressão de citocinas imunoregulatórias (IFN-γ, TNF-α, IL-10), suas fontes produtoras e a expressão de moléculas possivelmente envolvidas com processos de citotoxicidade celular (iNOS e Granzima A). Observou-se um infiltrado inflamatório mais intenso e um maior número de células expressando IFN-γ e granzima A em lesões de pacientes com LM, comparando-se com LC. Além disso, observou-se uma baixa expressão do receptor de IL-10 (IL-10R) em lesões de pacientes com LM. Esses dados suportam a idéia da existência de uma resposta inflamatória exacerbada nas lesões desses pacientes, comparando-se às lesões de pacientes com LC. Estudou-se ainda a composição celular e a expressão de citocinas em lesões de pacientes com LC em diferentes estágios da infecção: com 15 dias de infecção (LC-I) e 60 dias de infecção (LC-T). Os resultados mostraram um elevado número de células polimorfonucleares nas lesões de pacientes com LC-I e o recrutamento preferencial de células T $CD8^+$ em LC-T. Além disso, observou-se uma maior expressão de granzima A em lesões de LC-T, que pode estar associada com a destruição mais elevada do tecido nestes pacientes. Foram também realizadas análises comparativas em lesões de pacientes com leishmaniose cutânea, grávidas ou não, submetidas ou não ao tratamento com antimônio. Os resultados mostram intenso infiltrado inflamatório, com um maior número de neutrófilos em lesões de pacientes grávidas quando comparadas às não grávidas. De forma interessante, a resposta ao tratamento com antimônio foi satisfatória, já que 85% das pacientes grávidas apresentam cura com apenas um curso de tratamento. Tomados em conjunto, nossos resultados mostram a importância da resposta imunológica na imunopatologia da leishmaniose, indicando que (1) a LM está associada a uma hiper-atividade imunológica, caracterizada pela abundância de citocinas inflamatórias e mediadores de destruição tecidual, associada à baixa expressão do receptor de IL-10, (2) a evolução da lesão cutânea associa-se ao recrutamento de células T $CD8^+$ e ao possível aumento de citotoxicidade (3) A LC durante a gravidez está correlacionada surgimento de lesões mais exuberantes e com possíveis complicações gestacionais. Esperamos que esse estudo possa contribuir para uma melhor compreensão dos mecanismos envolvidos na imunopatologia da leishmaniose e para o futuro desenvolvimento de intervenções imunoterápicas.

ABSTRACT

Human infection with *Leishmania braziliensis* leads to a group of diseases known as American tegumentar leishmaniasis, that may appears as cutaneous (CL) and mucosal (ML) clinical forms. Despite the clinical form, lesions are the main pathological consequence of tegumentary leishmaniasis. In this study, we performed a comparative analysis of lesions from patients CL and ML to determine the *in situ* expression of immunomodulatory cytokines (IFN-γ, TNF-α, IL10), their cellular sources and the expression of molecules possibly involved with cytotoxic activity (iNOS, Granzyme A). A more intense inflammatory infiltrate, as well as great number of IFN-γ and granzyme A producing cells were observed in ML, when compared with CL lesions. Moreover, the expression of IL-10 receptor was lower in ML than in CL lesions. These data confirm the hypothesis that an exacerbated immune response is associated with ML. We also evaluated the cellular composition and cytokines expression in lesions from CL patients with distinct time of infection: approximately 15 days of illness ("early" CL – E-CL) and approximately 60 days of illness ("late" CL – L-CL). Histopathological analysis showed that lesions from L-CL had a more exuberant inflammatory infiltrate as compared to E-CL. Although both E-CL and L-CL lesions were predominantly composed of mononuclear cells, lesions from patients with E-CL presented higher neutrophil and eosinophil counts, as compared to L-CL. Confocal microscopy analysis showed that, although the absolute numbers of CD4+ and CD8+ T cells were higher in L-CL, the percentage of CD4+ T cells was similar between groups, while a higher proportion of $CD8^+$ cells was observed in L-CL, as compared to E-CL. Moreover, CD8+ from L-CL expressed significantly higher levels of granzyme A than E-CL. The percentages of IFN-γ^+ and IL-10+ cells was similar in both groups, with CD4+ T cells and CD68+ monocytes as the main sources of these cytokines, respectively. These results suggest that CD8+ T cells are involved in tissue destruction and lesion progression in human CL. Finally, we performed comparative analysis between lesions from pregnant and non-pregnant CL, submitted or not with antimonial treatment. Pregnant patients displayed much larger lesions, with an intense inflammatory infiltrate and a large number of neutrophils when compared in non-pregnant patients. Interestingly, treatment was satisfactory, with 85% of cure rate after 1 course of treatment with pentavalent antimony therapy. Together, our results show the important role of immune response on leishmaniasis immunopathology, suggesting that: (1) ML is associated with immunological hyper-activity, characterized by abundance of inflammatory cytokines and tissue destruction mediators, and to low expression of IL-10-receptor; (2) development of CL lesions is associated with the recruitment of $CD8^+$ T cells expressing granzyme A (3) during pregnancy, CL is correlated with appearance of larger lesions and more intense inflammation and adverse fetal effects. We hope that our study may contribute to a better understanding of immunopathology mechanisms associated to American tegumentary leishmaniasis, enlightening the development of new therapeutic approaches.

CAPÍTULO I - INTRODUÇÃO

Considerações gerais sobre a leishmaniose

A leishmaniose é uma doença parasitária causada por protozoários da família Trypanosomatidae, do gênero *Leishmania*. Estima-se que aproximadamente 350 milhões de pessoas estejam ameaçadas de adquirir a doença em 88 países e que 1,5 milhões desenvolvam a forma cutânea da doença (World Health Organization, 2005; Desjeux, 2004). Além da forma cutânea, que pode ser localizada ou disseminada, a patologia também pode apresentar-se sob formas mais graves, como a mucosa e a visceral. No Brasil, o aumento do número de casos de leishmaniose, associado às altas taxas de morbidade e à difusão da doença para novas áreas geográficas, inclusive urbanas, tem constituído um grande problema de saúde pública (Brazil, 2006; de Castro, 2006).

A transmissão da leishmaniose ocorre durante o processo de repasto sangüíneo de insetos do gênero *Phlebotomus* (velho mundo) e *Lutzomya* (novo mundo), quando formas promastigotas infecciosas do parasito são inoculadas no hospedeiro vertebrado. Logo após a penetração, as formas promastigotas são fagocitadas por macrófagos, passando a viver dentro dos vacúolos parasitóforos. Nos vacúolos, o parasita assume a forma amastigota, passando por ciclos intracelulares de multiplicação que levam à população de parasitas a um crescimento exponencial (Weigle & Saraiva, 1996; De Almeida *et al*., 2003). O elevado parasitismo tecidual gera no hospedeiro um processo de reatividade celular diretamente relacionado aos mecanismos de resistência e também ao estabelecimento da patologia (Mosser, 1990; Revisto por Hepburn *et al*., 2003; Gollob *et al*., 2005).

As manifestações clínicas da leishmaniose são variáveis e dependem da associação das características da espécie do parasita e da resposta imunológica do hospedeiro. Entre os componentes do sistema imunológico do hospedeiro que podem interferir e determinar as manifestações clínicas da doença, a imunidade mediada por células parece ser um dos parâmetros determinantes (Gollob *et al.*, 2005).

No Brasil, a espécie *Leishmania viannia braziliensis* (*L. braziliensis*) é a mais freqüente e amplamente distribuída, ocorrendo em todo o país, exceto ao norte do rio Amazonas. A doença causada pela infecção com esta espécie, em humanos, é caracterizada por uma ou mais úlceras na pele (leishmaniose cutânea) ou, menos freqüentemente, por lesões mucosas metastáticas (leishmaniose mucosa) (Ampuero *et al*., 2006; Lessa *et al*., 2007). As lesões decorrentes da infecção por *L. braziliensis* iniciam-se pela picada do flebótominio fêmea infectado que, ao inserir sua probóscide, provoca a laceração dos vasos sanguíneos formando um pool (poço) hemorrágico onde ele se alimenta (Ribeiro 1987; Almeida *et al*., 2003).

Em humanos observa-se uma grande variabilidade no espectro clínico da doença, que varia desde formas brandas de regressão espontânea à forma visceral, que pode levar à morte (Liew & O'Donnell, 1993; Da-Cruz & Pirmez, 2005). Até o momento as formas clínicas bem caracterizadas da doença são: leishmaniose cutânea (LC), mucosa, disseminada e visceral. Independentemente da forma clínica, as lesões são a conseqüência patológica mais importante da leishmaniose uma vez que, em muitos casos, as lesões são mutilantes e debilitantes, comprometendo a vida econômica e social do indivíduo afetado.

A LC é a forma mais branda e comum da doença, caracterizada por lesões exclusivamente cutâneas, ulcerosas ou não, que cicatrizam após algumas semanas ou meses. Esta forma da doença parece estar associada a reações de hipersensibilidade tardia e a presença de baixos títulos de anticorpos circulantes (Jones *et al*., 1987). Já a forma mucosa é caracteriza pela lesão da mucosa sem o acometimento cutâneo concomitante (Jones *et al*., 1987; Singer *et al*., 1975). Os indivíduos acometidos por esta forma clínica apresentam lesões desfigurantes, geralmente localizadas no nariz e na boca, podendo também afetar a laringe e a traquéia. A forma cutânea difusa, geralmente observada em indivíduos anérgicos ou, tardiamente, em pacientes tratados de calazar, é caracterizada pelo aparecimento de lesões

crônicas e disseminadas, associadas à presença de inúmeros parasitas no interior de macrófagos e fortes reações de hipersensibilidade tardia (Castellano *et al*., 2009; Cunningham *et al*., 2002). Na leishmaniose visceral ou calazar, a forma mais grave da doença, desenvolvida por alguns indivíduos infectados com parasitas do complexo *L. donovani, L. infantum, L. chagasi,* os parasitas apresentam acentuado tropismo pelo baço, fígado, medula óssea e tecidos linfóides. Esta forma da doença está relacionada com a presença de elevados títulos de anticorpos circulantes, ativação policlonal de linfócitos B e uma extrema inibição das reações de hipersensibilidade tardia.

Mecanismos de formação de lesões: reatividade inflamatória

A infecção pelo parasita é o estímulo inicial para desencadear uma cascata de eventos que culminará no estabelecimento da lesão inflamatória. O recrutamento das células para o sítio inflamatório depende de uma reação complexa que ocorre em tecidos conectivos vascularizados e envolvem componentes da matriz extracelular e do tecido, vasos sanguíneos e células circulantes. As primeiras células a serem recrutadas são os neutrófilos. Os linfócitos T e B antígenos-específicos são recrutados e podem permanecer no local por vários dias. Este recrutamento celular acontece em resposta a mediadores solúveis, como as quimiocinas e citocinas, que são produzidos por células presentes no local onde se estabelece a inflamação e onde as células do sistema imune passam a realizar suas funções efetoras (Magalhães *et al*., 1986). A resposta inflamatória inicialmente estabelecida é essencial para a resolução da infecção. No entanto, ela também origina as lesões características da doença, que podem agravar-se caso a resposta imunológica não seja modulada.

As quimiocinas são proteínas secretadas que relacionam-se a várias funções biológicas, entre elas inflamação e recrutamento celular (Ward *et al*., 1998). O recrutamento seletivo de células por quimiocinas que são produzidas nos sítios inflamatórios pode ser

considerado um mecanismo que contribui para o balanço local entre os subtipos celulares e o estabelecimento da reação imune dentro do tecido inflamado (Siveke *et al.*, 1998).

Tipos diferentes de citocinas modulam a reação inflamatória, participando como mediadores da resposta imunológica, ativando-a e muitas vezes controlando-a. Além das citocinas desempenharem papel essencial em condições patológicas, de maneira geral, elas também desempenham funções importantes na manutenção da resposta celular. Apesar das várias classificações descritas recentemente, dois principais grupos de citocinas podem ser descritos de acordo com suas funções pró e anti-inflamatórias. Entre as citocinas pró-inflamatórias, destaca-se TNF-α, que é produzido por linfócitos T, células NK e macrófagos. A atividade de TNF-α é mais intensa na presença de IFN-γ, IFN-α e IFN-β (Aggarwal & Eessalu, 1987), e inibida por várias citocinas incluindo IL-4, IL-10, IL-13 e TGF-β. O TNF-α também é uma importante citocina envolvida nos mecanismos de apoptose e estímulo de células endoteliais e macrófagos a secretarem quimiocinas (Beutler, 1995; Bharat *et al.*, 2000).

Outra importante citocina pró-inflamatória é o IFN-γ. Ele é produzido principalmente por linfócitos T e pelas células NK e sua principal função é ativar os macrófagos, tanto nas respostas imunes inatas como nas respostas imunes adquiridas mediadas por células. Além disso, Gollob e colaboradores, em 1996, demonstraram que IFN-γ sozinho modula o desenvolvimento de células T $CD4^+$ virgens em direção ao perfil Th1 (Gollob *et al.*, 1996).

A IL-10 é uma importante citocina anti-inflamatória produzida principalmente por macrófagos ativados, células B1 e por células T. Esta citocina possui a capacidade de reduzir a expressão do MHC-II e de moléculas co-estimulatórias, como também de inibir a produção de outras citocinas, tais como IFN-γ e TNF-α (Fiorentino *et al.*, 1989; Bogdan & Nathan 1993). Desta forma, IL-10 possui papel anti-inflamatório evitando a exacerbação das reações imunológicas inatas e adquiridas. Além desta citocina controlar a função das populações

celulares Th1, ela também modula as respostas do tipo Th2 (Mocci & Coffman, 1995; Muraille *et al*., 1998).

As citocinas, além de participarem da comunicação intercelular no sítio inflamatório modulando a resposta, são importantes no controle da indução de expressão de leucointegrinas e seus ligantes, favorecendo a migração de linfócitos ativados para os sítios inflamatórios (Prober *et al*., 1987; Stolpen *et al*., 1988). Neste sentido, trabalhos que caracterizem esse infiltrado inflamatório são de extrema importância. Alguns estudos já realizaram uma caracterização parcial do infiltrado inflamatório decorrente da infecção por *L. braziliensis* (Magalhães *et al*., 1986; Barral *et al*., 1987; Conceição-Silva *et al*, 1990; Pirmez *et al*., 1990; Da-Cruz *et al*., 2005). No entanto ainda não foram realizados estudos sistemáticos comparativos entre lesões cutâneas e mucosas, causadas pela infecção com esse parasita, em relação à determinação da composição do infiltrado celular, da produção de citocinas e moléculas envolvidas com citotoxicidade e da identificação de suas fontes produtoras. Desta forma, nosso trabalho traz uma inovação para literatura no sentido de caracterizar o infiltrado celular produtor dessas importantes moléculas do sistema imune e sua comparação com as diferentes formas clínicas causadas por *L. braziliensis*.

Para melhor compreender os mecanismos imunorregulatórios envolvidos na leishmaniose, elaboramos a seguinte hipótese para a formação da lesão, representada na Figura 1 e descrita a seguir. A lesão decorrente da infecção por *L. braziliensis*, que pode levar ao desenvolvimento tanto da forma clínica cutânea quanto da mucosa, inicia-se com o processo de repasto sanguíneo pelo inseto vetor. Formas promastigotas da *Leishmania* são inoculadas e fagocitadas por células apresentadoras de antígeno que irão migrar para o linfonodo ativando outras células presentes neste órgão linfóide. As células ativadas caem na corrente sanguínea e migram para o sítio da lesão. Neste local, onde houve a inoculação do parasita, ocorre ativação celular e liberação de citocinas que vão atuar nas células endoteliais

induzindo a expressão de moléculas de adesão, favorecendo o recrutamento de células para o sítio inflamatório. Os iniciadores mais importantes da adesão são as três selectinas expressas em leucócitos (L-selectina), células endoteliais (P e E-selectina) e em plaquetas ativadas (P-selectina). Os leucócitos, ao entrarem em contato com as células endoteliais por essas moléculas de adesão, iniciam um processo de rolamento através do endotélio vascular. Outras moléculas de adesão presentes nos leucócitos, tais como LFA-1 e Mac-1 pertencentes à família das integrinas, reconhecem as moléculas ICAM-1 e ICAM-2 no endotélio vascular e são responsáveis pelo processo de adesão estável dos leucócitos ao endotélio. Uma vez estabelecida essa ligação, as células poderão responder aos estímulos quimiotáticos provenientes de mediadores solúveis, as quimiocinas, e realizar a migração tecidual, processo denominado diapedese. As citocinas também estimulam as células a secretarem quimiocinas que estão envolvidas com recrutamento celular e quimiotaxia. Por outro lado, células presentes neste infiltrado inflamatório também secretam moléculas citotóxicas como granzima A e oxido nítrico (NO), que estão envolvidas com reatividade inflamatória e outros mecanismos como destruição tecidual (Mackay & Rosen, 2000). Em conjunto, a infecção pelo parasita é o estímulo inicial para desencadear uma cascata de eventos que culminará em uma maior ativação celular, em um maior recrutamento celular e, conseqüentemente, em uma inflamação mais grave. Esta inflamação, enquanto necessária para ativar os mecanismos parasiticidas, precisa ser posteriormente controlada para que não cause destruição tecidual.

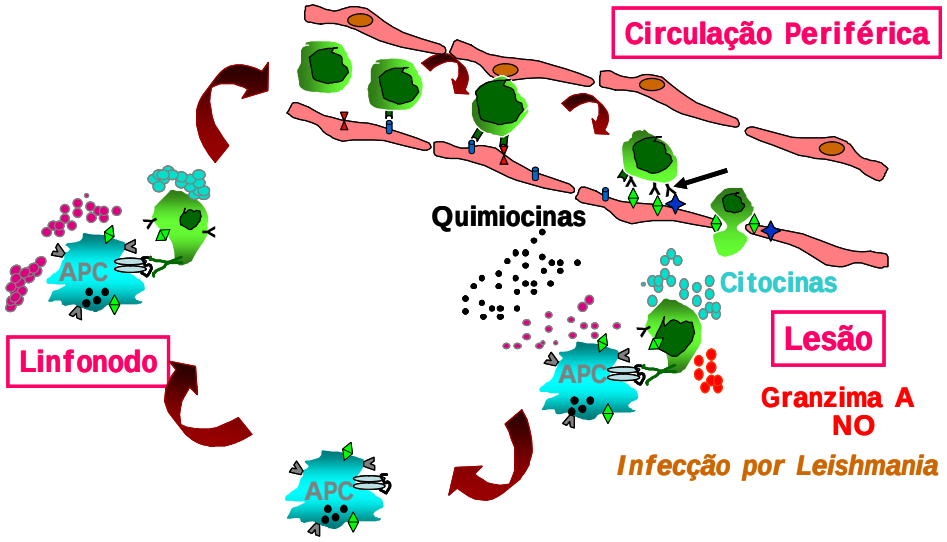

Figura 1- **Hipótese para a formação das lesões decorrentes da infecção por *Leishmania.*** A infecção inicia-se com o processo de repasto sanguíneo pelo inseto vetor. Formas promastigotas da *Leishmania* são inoculadas e fagocitadas por APCs que irão migrar para o linfonodo ativando outras células. Estas caem na corrente sanguínea e migram para o sítio da lesão, onde ocorre ativação celular e liberação de citocinas que vão atuar nas células endoteliais induzindo o recrutamento de células e a diapedese. As citocinas também estimulam as células a secretarem quimiocinas, envolvidas com recrutamento celular e quimiotaxia. Por outro lado, células presentes neste infiltrado inflamatório também secretam moléculas citotóxicas como granzima A e NO, envolvidas com reatividade inflamatória e outros mecanismos como destruição tecidual. APC = célula apresentadora de antígeno, NO= óxido nítrico.

Resposta imunológica de pacientes com leishmaniose cutânea e mucosa

A leishmaniose cutânea é caracterizada por uma variedade de aspectos imunológicos. Indivíduos que apresentam a forma cutânea localizada da doença e são classificados como respondedores, desenvolvem lesões bem definidas. As células desses indivíduos, quando reestimuladas *in vitro* com antígenos do parasita, são capazes de proliferar, sendo esta proliferação acompanhada pela alta produção de IFN-γ (Carvalho *et al*., 1985; Mendonça *et al.* 1986; Carvalho *et al*. 1994; Cabello *et al*., 1995; Bacellar *et al*., 2002). De forma interessante, Bottrel e colaboradores em 2001, demonstraram que células mononucleares do sangue periférico (CMSP) de pacientes com LC, estimuladas com o antígeno solúvel de

Leishmania (SLA), induzem um aumento na freqüência de linfócitos produtores de IFN-γ e TNF-α e que as sub-populações de células T $CD4^+$, seguidas de $CD4^-CD8^-$ e $CD8^+$ são as maiores produtoras de IFN-γ, nessa ordem (Bottrel *et al*., 2001). Um dado interessante deste estudo, foi que observou-se que os linfócitos T $CD4^-CD8^-$, uma população celular minoritária na circulação sanguínea humana (Shivakumar *et al*., 1989) são responsáveis pela produção de aproximadamente 45% do IFN-γ em pacientes com LC. Posteriormente, Antonelli e colaboradores (2006) demonstraram que a subdivisão destes linfócitos em TCR α/β^+ ou TCR γ/δ^+ define sub-populações funcionalmente distintas, produtoras de IFN-γ e IL-10, respectivamente. Uma vez que esta população linfocitária possui características de resposta eficiente em períodos iniciais da resposta imune (Gollob *et al.*, 2008), é possível que desempenhem papel fundamental nos mecanismos de controle do parasita e, subsequentemente, da reatividade inflamatória, em períodos iniciais da leishmaniose.

Trabalhos que utilizaram fragmentos de lesões de pele de pacientes infectados por *L. braziliensis*, e que avaliaram o perfil de citocinas, mostraram o predomínio de resposta do tipo Th1, caracterizada por altos níveis de IFN-γ, nas lesões cutâneas difusa antes e após tratamento com antimônio (Bomfim *et al.*, 1996). Tapia e colaboradores (1993) avaliaram, por RT-PCR, a presença de RNA mensageiro para citocinas em lesões de pacientes com LC localizada desencadeada por *L. braziliensis*. Esses autores verificaram níveis elevados de expressão de IFN-γ e níveis bastante reduzidos das citocinas IL-5 e IL-10 (Tapia *et al*., 1993). Outro estudo utilizando a mesma estratégia experimental foi realizado por Pirmez e colaboradores (1993) e mostrou a expressão aumentada de IL-2, IFN-γ, e linfotoxina em lesões cutâneas de pacientes infectados por *L. braziliensis* (Pirmez *et al*., 1993).

Antonelli e colaboradores (2004), analisando CMSP de pacientes com LC estimuladas com SLA, observaram uma correlação positiva entre as células produtoras das citocinas pró-inflamatórias IFN-γ e TNF-α quanto destas com a citocina anti-inflamatória IL-10. Já em

2005, o mesmo grupo demonstrou que pacientes LC infectados por *L. braziliensis* que apresentavam lesões maiores também possuíam maiores freqüências de células produzindo IFN-γ e TNF-α (Antonelli *et al*., 2005). Correlações positivas entre o tamanho da lesão e a produção *in situ* de IFN-γ também foram previamente observadas em pacientes infectados com *L. guyanensis*, enquanto que o perfil Th1 das lesões correlacionou-se negativamente com a carga parasitária das mesmas (Bourreau *et al.*, 2003). Além disso, estudos prévios mostraram uma produção preferencial de IL-10 no início do desenvolvimento da forma cutânea da infecção que, posteriormente, é sobrepujada pelo perfil inflamatório, com produção predominante de IFN-γ (Rocha *et al*., 1999). Já o predomínio do perfil Th2, evidenciado pela produção *in situ* de IL-4, foi observado no início da infecção por *L.guyanensis*, sendo sobreposto pelo perfil Th1 com a evolução da infecção (Bourreau *et al.*, 2003). De forma interessante, Gomes-Silva e Bittar demonstraram que o controle mútuo das citocinas IFN-γ e IL-10 é de importância crucial para a regulação das respostas imunes em pacientes infectados por *L. braziliensis* (Bittar *et al*., 2007; Gomes-Silva *et al*., 2007). Embora estes estudos tenham trazido importantes informações a respeito das citocinas produzidas no sítio das lesões, decorrentes da infecção humana com *L. braziliensis*, outras questões ainda ficaram por responder, como por exemplo, quais os tipos celulares responsáveis pela produção dessas citocinas.

A forma clínica mucosa da leishmaniose é caracterizada por lesões desfigurantes que são, geralmente, localizadas no nariz e na boca, podendo afetar também a laringe e a traquéia (Castes, 1984 e 1993). Diferentemente da forma cutânea, não é observada a cura espontânea da leishmaniose mucosa (LM) (Marsden, 1996). A LM tem sido considerada uma patologia associada à exacerbação da resposta imune inflamatória, causando uma extensa destruição do tecido (Carvalho *et al*., 1985; Brodskyn *et al*., 1997; Carvalho *et al*., 1993). Linfócitos circulantes de pacientes com leishmaniose mucosa produzem altos níveis de IFN-γ e TNF-α

em resposta ao estímulo por antígenos do parasita (Carvalho *et al*. 1985; Ribeiro-de-Jesus *et al.* 1998). Bacellar e colaboradores demonstraram que CMSP de pacientes com LM, analisadas por citometria de fluxo, expressam altos níveis de IFN-γ e TNF-α e possuem uma habilidade diminuída de modulação da resposta celular por IL-10 e TGF-β (Bacellar *et al*. 2002).

A expressão da molécula relacionada com ativação recente, como CD69 e a molécula co-estimuladora CD28, já foi avaliada em pacientes com leishmaniose (Kemp *et al*. 1999, Antonelli *et al*. 2004, Antonelli *et al*. 2005, Gaze *et al*., 2005). Recentemente, Carvalho e colaboradores demonstraram que pacientes com LM apresentam um aumento nas frequências de células $CD4^{+}CD69^{+}$, $CD4^{+}CD62^{Low}$ e $CD4^{+}CD28^{-}$ quando comparadas com pacientes LC (Carvalho *et al*., 2007). Este resultado também foi observado por Gaze e colaboradores em 2005 (Gaze *et al*., 2005). Trabalhos anteriores também já sugeriram uma hiper-ativação nos pacientes com LM, comparados com LC (Bacellar *et al*. 2002, Amato *et al*. 2003). Estes resultados sugerem que pacientes com LM apresentam uma maior ativação celular, o que possibilita uma maior freqüência de ativação de células T efetoras, contribuindo, desta forma, para a manutenção de uma resposta inflamatória persistente, bem como um aumento nos danos teciduais na LM.

Em relação à resposta imune *in situ* na LM, análises microscópicas de fragmentos de lesões revelaram uma reação inflamatória intensa, com predomínio de linfócitos e macrófagos, e pouco ou nenhum parasita (Bittencourt, 1991). Alguns autores mostraram que citocinas Th1 e Th2, como IFN-γ, TNF-α, IL-4 e IL-10, são produzidas no sítio das lesões em pacientes com leishmaniose mucosa (Caceres-Dittmar *et al.* 1993; Pirmez *et al.* 1993).

As manifestações clínicas da leishmaniose dependem das complexas interações entre a resposta imunológica do hospedeiro e as características de virulência do parasita. Neste contexto, a utilização de modelos experimentais tem sido uma excelente ferramenta para

estudo dos eventos imunorreguladores relacionados à infecção por *Leishmania*. Teixeira e colaboradores, em 2005, inocularam duas cepas de *L. braziliensis*, uma do Ceará (H3227) e uma da Bahia (BA788) em camundongos BALB/c com objetivo de averiguar a resposta imunológica frente às diferentes cepas do parasita. Estes autores observaram que os camundongos infectados com a cepa H3227 desenvolvem lesões mais graves e com intenso infiltrado inflamatório, composto principalmente por neutrófilos e macrófagos, quando comparado aos infectados pela cepa BA788. Além disso, a cepa H3227 induziu uma maior expressão das quimiocinas CCL2/MCP-1, CCL3/MIP-1a, XCL1/linfotactina-1, CXCL1/KC e CCL11/eotaxina que são importantes para o recrutamento de neutrófilos e macrófagos, o que justificaria o maior número destas células no sitio inflamatório. Estes resultados deixam claro que diferentes cepas de uma mesma espécie podem desencadear diferentes respostas imunológicas e, conseqüentemente, diferentes formas clínicas da leishmaniose.

Os diferentes padrões de progressão da doença estão relacionados a perfis distintos de produção de citocinas, estabelecidos em resposta à infecção (Bacellar *et al*., 2002). Alguns autores sugerem que o curso clínico da leishmaniose esteja associado a padrões locais de produção de citocinas. Porém, embora um número razoável de trabalhos relacionados ao envolvimento das citocinas nas diferentes fases da resposta imunológica humana contra *Leishmania* esteja disponível na literatura, poucos deles estabelecem relações entre a síntese dessas proteínas e as suas fontes produtoras nas lesões de pacientes infectados pela *Leishmania*. A identificação direta das fontes de citocinas, entre outros produtos celulares, é de importância inquestionável, pois fornece um conhecimento básico para qualquer possibilidade de manipulação da resposta imunológica.

Resposta imunológica de pacientes grávidas

As citocinas são abundantes no útero grávido. Qualquer distúrbio no delicado balanço imunológico que ocorre na interface materno-fetal pode resultar em perda do concepto ou

outras complicações gestacionais (Robertson *et al.*, 1994; Fried *et al.*, 1998; Rivera *et al.*, 1998).

Enquanto certas citocinas são benéficas para o sucesso da gravidez, foi proposto que a deficiência nessas citocinas poderia levar à placentação deficiente, crescimento subnormal e, possivelmente, à morte fetal (Clark e Chaouat, 1989). Citocinas pró-inflamatórias tais como IFN-γ, TNF-α e citocinas anti-inflamatórias, como a IL-10, foram investigadas como potenciais reguladoras desse equilíbrio que se estabelece entre mãe e embrião (Piccotti *et al.*, 1997; Fried *et al.*, 1998; Darmochwal-Kolarz, 1999; Brown *et al.*, 2000; Piccinni *et al.*, 2000). Durante a gestação, é desejável manter um equilíbrio entre os dois tipos de citocinas, ao passo que alterações desse equilíbrio podem favorecer ou prejudicar o desenvolvimento embrionário (Garcia-Lloret *et al.*,1994*)*.

Jenkins e colaboradores em 2000, utilizando o método de ELISA, analisaram o soro de mulheres grávidas e concluíram que o sucesso gestacional estava associado com níveis aumentados de IL-10, enquanto o aborto estava associado com níveis aumentados de IFN-γ. Isso sugere que o aumento da produção de IL-10 pode ser uma resposta natural a antígenos do trofoblasto, enquanto respostas que levam a uma maior produção de IFN-γ são aberrantes e podem, portanto, ter importante papel na falência reprodutiva. Condizente com o que foi relatado acima, Chaouat e colaboradores demonstraram que citocinas inflamatórias, tais como IL-2, TNF-α e IFN-γ, interrompem uma gravidez normal quando injetadas em fêmeas de camundongos grávidas (Chaouat *et al.*, 1990).

Uma revisão recente da literatura demonstrou que o número de mulheres gestantes com leishmanioses, principalmente a forma visceral, tornou-se mais freqüente (Figueiró-Filho *et al.*, 2004). Os estudos nessa área ainda são escassos e existem controvérsias sobre a segurança da administração do antimônio durante o período gestacional (Figueiró-Filho *et al.*, 2004). O antimonial pentavalente é a droga de escolha para o tratamento da leishmaniose visceral em

gestantes a partir do 2º trimestre (Utilli *et al*. 1995). No entanto, Caldas e colaboradores (2003), contra indicam sua utilização em gestantes, por ultrapassarem a barreira placentária podendo causar impregnação neural com desenvolvimento de retardo mental nos recém-nascidos.

Assim, estudos que visam gerar informações com relação à imunidade celular de mulheres grávidas na resposta à infecção com *Leishmania* são de extrema importância. Neste trabalho, analisamos 26 mulheres grávidas, infectadas por *L. braziliensis* com o objetivo de compreender os mecanismos imunorregulatórios envolvidos na progressão das lesões durante o período gestacional antes e após o tratamento com o antimonial pentavalente. Nossas análises certamente contribuirão para um entendimento dos mecanismos imunorregulatórios envolvidos com a patologia das leishmanioses cutânea e mucosa em mulheres grávidas.

Hipóteses de trabalho

A proposta básica deste projeto de pesquisa consiste na determinação da composição do infiltrado inflamatório decorrente da infecção por *Leishmania*, identificando as populações celulares que o compõe, determinando a expressão de citocinas, seus receptores, moléculas efetoras como granzimas e iNOS, envolvidas na resposta imune desencadeada pela infecção por *L. braziliensis.* O estudo foi conduzido na forma de análises comparativas, seja entre pacientes com formas clínicas distintas, em fases diferentes de uma mesma forma clínica ou entre gestantes e não gestantes. As hipóteses que nortearam o desenvolvimento desse trabalho são: (1) que lesões dos pacientes com a forma clínica mucosa da leishmaniose estão associadas a uma resposta inflamatória exacerbada; (2) que os eventos imunológicos iniciais são importantes para a evolução clínica da leishamaniose cutânea; (3) que pacientes grávidas possuem infiltrado inflamatório exacerbado e lesões mais exuberantes do que mulheres não-grávidas.

Acreditamos que a caracterização comparativa do infiltrado inflamatório nas lesões dos pacientes com LC e LM, e de pacientes com diferentes tempos de evolução da LC, através da avaliação da expressão das citocinas e determinação de suas fontes celulares tornam-se extremamente importantes, uma vez que diferentes padrões da progressão da doença estão correlacionados a perfis de citocinas distintos, estabelecidos em respostas à infecção e correlacionados à gravidade da doença. Acreditamos ainda que a infecção por *Leishmania* em grávidas possa desencadear alterações no sistema imune materno durante a gravidez. Assim, a caracterização comparativa do infiltrado inflamatório em lesões de pacientes grávidas com LC, e pacientes não grávidas pode auxiliar no entendimento dessa complexa interação.

Assim, esta tese tem como **objetivo geral**, definir características fenotípicas e funcionais dos infiltrados inflamatórios que compõem as lesões decorrentes da infecção por *Leishmania braziliensis*, em pacientes em diferentes períodos da forma cutânea, em pacientes com diferentes formas clínicas, e em pacientes grávidas ou não-grávidas portadoras de LC.

Para que o objetivo geral pudesse ser alcançado, delineamos os seguintes **objetivos específicos:**

(1) Analisar a expressão de IFN-γ, TNF-α, IL-10, granzima A, iNOS e do receptor para IL-10 em associação com os marcadores fenotípicos CD4, CD8 e CD68 em lesões de pacientes com LM e LC;

(2) Analisar a expressão de IFN-γ, IL-10 e granzima A em associação com os marcadores fenotípicos CD4, CD8 e CD68 em lesões de pacientes com LC com aproximadamente 15 (cutâneos inicias) ou 60 (cutâneos tardios) dias de infecção;

(3) Analisar comparativamente aspectos macroscópicos e histopatológicos em lesões de pacientes com LC, grávidas ou não.

Os resultados obtidos serão apresentados a seguir, no formato de quatro artigos. O primeiro artigo, já publicado, avaliou comparativamente o infiltrado inflamatório de lesões de pacientes com LC e LM, analisando a expressão de IFN-γ, IL-10, TNF-α, granzima A, iNOS e do receptor para IL-10, e identificando suas fontes celulares (objetivo específico 1). O segundo artigo, já publicado, avaliou comparativamente o infiltrado inflamatório de lesões de pacientes com LC com aproximadamente 15 (cutâneos iniciais) ou 60 (cutâneos tardios) dias de infecção, analisando o infiltrado inflamatório, a expressão de IFN-γ, IL-10 e granzima A em associação com os marcadores fenotípicos CD4, CD8 e CD68 (objetivo específico 2). O terceiro artigo, já publicado, descreve a análise comparativa dos aspectos macroscópicos e histopatológicos em lesões de pacientes com LC, grávidas ou não, submetidas ou não ao tratamento com antimônio (objetivo específico 3). O quarto artigo, já publicado, combina aspectos teóricos, discutindo mecanismos de imunoregulação na leishmaniose humana e integra aspectos estudados nos 3 objetivos específicos, focalizando no possível papel que células T $CD4^{-}CD8^{-}$ possam desempenhar na leishmaniose humana.

Em conjunto, este estudo apresenta finalidades múltiplas que visam gerar informações sobre o papel das diferentes populações celulares durante a infecção pela *Leishmania*. Nossas análises contribuirão para o entendimento dos mecanismos imunorregulatórios e de destruição tecidual envolvidos na patologia das leishmanioses cutânea e mucosa. Estudos como os aqui propostos serão essenciais para nortear qualquer intervenção alternativa de tratamento, beneficiando a população infectada.

CAPÍTULO II - ARTIGOS RESULTANTES

Artigo # 1: Daniela R. Faria,[1] Kenneth J. Gollob,[2] José Barbosa, Jr.,[3,4] Albert Schriefer,[5] Paulo R. L. Machado,[5] Hélio Lessa,5 Lucas P. Carvalho,[5] Marco Aurélio Romano-Silva,[4] Amélia R. de Jesus,[5] Edgar M. Carvalho,[5] and Walderez O. Dutra[1] - **"Decreased in situ expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis"**. Infection and immunity, 73: 7853 – 7859 (2005).

INFECTION AND IMMUNITY, Dec. 2005, p. 7853–7859 Vol. 73, No. 12
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.12.7853–7859.2005

Decreased In Situ Expression of Interleukin-10 Receptor Is Correlated with the Exacerbated Inflammatory and Cytotoxic Responses Observed in Mucosal Leishmaniasis

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Received 1 June 2005/Returned for modification 11 July 2005/Accepted 20 August 2005

Human infection with *Leishmania braziliensis* can lead to cutaneous leishmaniasis (CL) or mucosal leishmaniasis (ML). We hypothesize that the intense tissue destruction observed in ML is a consequence of an uncontrolled exacerbated inflammatory immune response, with cytotoxic activity. For the first time, this work identifies the cellular sources of inflammatory and antiinflammatory cytokines, the expression of effector molecules, and the expression of interleukin-10 (IL-10) receptor in ML and CL lesions by using confocal microscopy. ML lesions displayed a higher number of gamma interferon (IFN-γ)-producing cells than did CL lesions. In both ML and CL, $CD4^+$ cells represented the majority of IFN-γ-producing cells, followed by $CD8^+$ cells and $CD4^-$ $CD8^-$ cells. The numbers of tumor necrosis factor alpha-positive cells, as well as those of IL-10-producing cells, were similar in ML and CL lesions. The effector molecule granzyme A showed greater expression in ML than in CL lesions, while inducible nitric oxide synthase did not. Finally, the expression of IL-10 receptor was lower in ML than in CL lesions. Thus, our data identified distinct cytokine and cell population profiles for CL versus ML patients and provide a possible mechanism for the development of ML disease through the demonstration that low expression of IL-10 receptor is present in conjunction with a cytotoxic and inflammatory profile in ML.

The control of a given infection relies on the ability of our organism to mount an efficient immune response that leads to the control of the infectious agent. However, in addition to the need for an early response that will trigger killing mechanisms, further control of the response is critical for the establishment of pathology or cure. This coordinated action of the immune system involves mobilization of inflammatory mechanisms and antiinflammatory, modulatory responses. Thus, a failure to control an exacerbated inflammatory response can be a major cause of pathology and morbidity. Determination of the mechanisms involved with the development of pathology in human disease will open new possibilities of immunological intervention for prevention of pathology.

A great deal of information concerning the dynamics of immune responses to infectious agents, particularly the role of T helper 1 (Th1) and Th2 populations, have come from studies of cellular reactivity to *Leishmania* parasites (33). The insights obtained from these experimental studies provided critical knowledge toward the understanding of several other diseases. Thus, study of the immune response in human leishmaniasis, while clarifying the mechanisms involved in the establishment of protective and pathogenic responses in this important disease, will also aid in the comprehension of other diseases where the control of inflammatory responses is crucial.

While it is generally accepted that infection with different species of *Leishmania* leads to the establishment of different clinical forms, the same species of this parasite may also lead to different diseases, demonstrating that the host's immune response is essential for disease pathogenesis. In humans, infection with *Leishmania braziliensis* causes different forms of American cutaneous leishmaniasis, such as the localized and disseminated forms, as well as mucosal disease. Localized cutaneous leishmaniasis (CL) is characterized by the appearance of a single or a few ulcerated skin lesions and a relatively effective response to conventional antimonial treatment (19). Approximately 3% of patients previously affected by CL may develop the mucosal disease (29). Mucosal leishmaniasis (ML) is marked by the disfiguring nature of the associated lesions, usually involving nasal or oropharyngeal mucosal areas. Treatment of ML often requires more than one course of conventional antimonial therapy or even the use of more-toxic drugs such as amphotericin B or immunomodulatory approaches (20).

Previous studies have demonstrated that CL is associated with high in vitro proliferative responses to parasite-derived antigens (12, 13, 14). Moreover, increased production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) has been observed in both in vitro responses and in situ analysis of CL lesions (5, 31). Interestingly, interleukin-10 (IL-10) production was also detected in patients with CL by

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several different methodological approaches (4, 9). An increase in the frequency of IL-10- as well as IFN-γ-producing cells was recently seen (3). These findings suggest that the mild nature of CL may be related to the early establishment of efficient parasite-killing mechanisms, associated with the control of exacerbated inflammatory responses. Comparative analysis of the immune responses of CL and ML patients has shown that peripheral blood mononuclear cells (PBMC) from individuals that develop ML display a higher proliferative response to parasite antigens and higher levels of IFN-γ and TNF-α production, associated with lower levels of IL-10, than those from CL patients (5). Furthermore, addition of IL-10 to in vitro cultures led to modulation of antigen-induced IFN-γ production by PBMC from CL but not ML patients, suggesting that an uncontrolled inflammatory response is related to the severe tissue destruction observed in ML (5). However, the mechanisms responsible for this uncontrolled response have not yet been determined.

In this work, we investigated the question of whether the destructive ML lesions are associated with a high inflammatory response induced by the local production of inflammatory cytokines, cytotoxic molecules, and decreased modulatory responses. Thus, we evaluated the expression and cellular sources of the inflammatory cytokines IFN-γ and TNF-α, and of the antiinflammatory cytokine IL-10, in lesions from patients with CL and ML by using triple-staining confocal microscopy analysis. We also evaluated the expression of the effector molecules granzyme A and inducible nitric oxide synthase (iNOS) by cells from inflammatory lesions of CL and ML patients, correlating their expression with cytotoxic mechanisms involved in tissue damage in both clinical forms. Finally, we analyzed the expression of the IL-10 receptor as a possible mechanism for the exacerbated in situ response in ML. Our results clearly characterize ML as an example of how an uncontrolled response can lead to pathology, and they should be taken into consideration when vaccines and/or therapies based on the induction of inflammatory responses are proposed.

MATERIALS AND METHODS

Patients. The patients analyzed in this study were from Corte de Pedra, an area of endemicity for *Leishmania braziliensis* infection, located 280 km southwest of Salvador, the capital of Bahia state, Brazil. All patients were volunteers, and informed consent was obtained from all individuals prior to collection of lesion material. Medical care and patient evaluation and characterization were under the responsibility of Edgar Carvalho, with participation of a dermatologist and ear-nose-throat specialists to identify typical leishmaniasis skin or mucosal lesions, respectively. Diagnosis of leishmaniasis was performed based on clinical and laboratory criteria. Detection of suggestive skin or mucosal lesions was associated with a positive skin Montenegro test, parasite isolation, and/or histopathological analysis to confirm a diagnosis of CL or ML. For all CL and ML cases, parasite species were typed to confirm that the disease was due to *L. braziliensis* infection. CL patients enrolled in this study ($n = 14$) presented with a single ulcerated lesion and had not been previously diagnosed with or treated for leishmaniasis. ML patients ($n = 7$) presented with nasal lesions and, at the time of biopsy collection, did not display concomitant cutaneous disease. At the time of sample collection, the ages of the active lesions were estimated at 30 to 45 days for both CL and ML lesions. While the estimated times of CL and ML lesion development were comparable, ML patients had been exposed to leishmaniasis previously, since they had previously had cutaneous disease, which was healed at the time when mucosal disease was diagnosed. The interval between the cure of CL and the diagnosis of ML was variable. The estimated time of lesion development was based on questioning of the patients, together with the intervals of patient examinations in the area of endemicity. Treatment was offered to all patients as needed despite their enrollment in this project. However, CL and ML patients were not under treatment when samples were collected. Lesions were collected either at the Corte de Pedra health care facility or at the Serviço de Imunologia, Hospital Universitário Edgar Santos, UFBA, in Salvador. Skin biopsy specimens were taken from the borders of active lesions, using a 4-mm-diameter punch, after the application of a local anesthetic. Mucosal lesions were obtained by excision of a small fragment (approximately 3 mm, on average) using a scalpel, after local anesthetic application. Lesions were maintained in a 30% sucrose solution for approximately 30 min at 4°C; then they were transferred to OCT Tissue Tek freezing medium and immediately placed in dry ice. The material was stored at −70°C until analysis. The Ethical Committees of the Bahia and Minas Gerais Federal Universities approved all procedures involved in this study.

Histological and immunofluorescence staining. Individual 4- to 5-μm cryosections were placed in saline-precoated slides and fixed for 10 min with acetone. Slides were incubated with phosphate-buffered saline for 15 min and subjected either to hematoxylin-eosin staining or to immunofluorescence using specific monoclonal antibodies. Standard hematoxylin-eosin staining was performed to ensure tissue integrity as well as for evaluation of the intensity and location of the inflammatory infiltrate. Immunofluorescence reactions involved incubation with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies directed to surface receptors (CD4 clone S3.5, CD8 clone 3B5, CD68 clone Ki-M7, or IL-10 receptor clone B7-H1) or intracellular molecules (granzyme A clone CLB-GA28, iNOS or IFN-γ clone B27, IL-10 clone 9D7, or TNF-α clone 20A4), respectively. Sections were incubated with antibody mixtures overnight at 4°C. After staining, preparations were extensively washed with phosphate-buffered saline, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), and mounted using antifade mounting medium (Molecular Probes). Slides were kept at 4°C, protected from light, until acquisition in a laser scanning confocal microscope (Zeiss). Isotype controls were analyzed separately to confirm the lack of nonspecific staining. Monoclonal antibodies were purchased from Caltag (Burlingame, CA), except for the anti-IL-10 receptor monoclonal antibody, which was purchased from Becton Dickinson (San Jose, CA).

Confocal analysis. Imaging was performed with a Bio-Rad MRC 1024 laser scanning confocal system running LaserSharp 3.0 software coupled to a Zeiss microscope (Axiovert 100) with a water immersion objective (40×, 1.2 numerical aperture). A water-cooled argon UV laser (488 nm) or a krypton/argon laser was used to excite the preparation (through its 363-nm, 488-nm, or 568-nm line), and light emitted was selected with band-pass filters (522/35 for FITC and DAPI, 598/40 for PE). For each section, the inflammatory infiltrate present in the connective tissue adjacent to the epithelia was located and an area presenting with a uniform infiltrate was selected for analysis. Within this inflammatory area, a minimum of three images (fields) were collected. Image analysis and processing were performed with LaserSharp (Bio-Rad), Confocal Assistant, Adobe Photoshop, and Image Tool software. Analyses were performed by counting the total number of cells in the three fields acquired and calculating the average number of cells per section for each patient. This calculation was performed for each parameter analyzed, allowing for determination of the total number of inflammatory cells (total number of $DAPI^+$ cells within the inflammatory infiltrate), the number of FITC or PE single-positive cells, and the number of double-positive cells. The counts were performed blindly, the results were expressed as the average number of cells per field for each parameter for each patient, and then the values were averaged for each group. The results are representative of two experiments per patient. Intensities of IFN-γ and IL-10 receptor expression were determined using Pseudocolor software. This analysis allows for the conversion of pixels into numbers, providing a numerical analysis of intensity in relation to the number of pixels in the area analyzed. This number was then corrected for the number of cells present in the area analyzed.

Statistical analysis. Statistical analysis of the data was performed using JMP statistical software from SAS. The comparisons of means for a given parameter were performed using a nonparametric (one tailed, considering unequal variance of groups) *t* test. Results were considered statistically different when the analysis returned a P value of <0.05.

RESULTS

ML lesions display a more intense inflammatory infiltrate due to recruitment of $CD4^+$ and $CD8^+$ cells than CL lesions. The intensity of the inflammatory infiltrate in lesions from CL and ML patients was determined using conventional histological analysis, as well as by counting the number of DAPI-positive cells per field using confocal microscopy, as described

TABLE 1. Total numbers of inflammatory cells and numbers of $CD4^+$, $CD8^+$, and $CD68^+$ cells in CL and ML lesions

Clinical form	Total no. of cells[a]			
	$DAPI^+$	$CD4^+$	$CD8^+$	$CD68^+$
CL (n = 14)	4,580 ± 1,784	1,059 ± 757	1,069 ± 542	1,313 ± 580
ML (n = 7)	6,664 ± 1,821	1,671 ± 480	1,846 ± 801**	1,822 ± 908

[a] Counted based on expression of specific fluorescent markers as described in Materials and Methods. Double asterisks indicate that the difference between ML and CL lesions is statistically significant at a P value of 0.05.

in Materials and Methods. The average number of cells was significantly higher in ML lesions than in CL lesions, demonstrating the occurrence of a more intense inflammation in ML (Table 1). The inflammatory infiltrates were predominantly composed of mononuclear cells in both CL and ML lesions. Moreover, while the numbers of $CD68^+$ cells in CL and ML lesions were not statistically different, the numbers of $CD4^+$ and $CD8^+$ cells were higher in ML than in CL lesions (Table 1). Although increases in $CD4^+$ and $CD8^+$ cells were observed in ML lesions, the CD4/CD8 ratio was similar between groups. Lastly, the relative percentages of $CD4^+$, $CD8^+$, and $CD68^+$ cells did not differ between groups (data not shown).

Cellular sources of cytokines in ML and CL lesions. The expression of the inflammatory cytokines TNF-α and IFN-γ was evaluated in lesions from CL and ML patients. The analysis showed that the total numbers of cells expressing TNF-α were not statistically different between groups (Table 2). Evaluation of the cellular sources of TNF-α demonstrated that $CD68^+$ cells are the main cell population expressing this cytokine in both CL and ML lesions (Table 2) and that approximately 50% of the $CD68^+$ cells are committed to expression of TNF-α in CL and ML lesions (Table 2). While the total expression of TNF-α was not statistically different when ML and CL lesions were compared (Table 2), the total number of IFN-γ-expressing cells was higher in lesions from ML than from CL patients (Fig. 1A). Not only the number of IFN-γ^+ cells, but also the intensity of expression of this cytokine, was higher in lesions from patients with ML than in those from patients with CL (Fig. 1B). We also observed that ML patients displayed a statistically higher frequency of $CD4^+$ IFN-γ^+ cells than CL patients (Fig. 1A), and although the average numbers of $CD8^+$ IFN-γ^+ cells were higher in ML than in CL lesions, statistical significance was not achieved ($P < 0.06$) (Fig. 1A). Our analysis of the contributions of $CD4^+$, $CD8^+$, and $CD4^-$ $CD8^-$ cells toward the production of IFN-γ showed that $CD4^+$ cells are the main source of IFN-γ in ML and CL lesions (43% ± 21% and 51% ± 11%, respectively), followed by $CD8^+$ (38% ± 10% and 40% ± 15% for CL and ML, respectively) and $CD4^-$ $CD8^-$ (21% ± 15% and 10% ± 4% for CL and ML, respectively) cells.

Analysis of the antiinflammatory cytokine IL-10 was also performed. We observed that the total number of IL-10-expressing cells and the number of $CD68^+$ IL-10^+ cells did not differ between groups (Table 2). $CD68^+$ cells account for approximately 62 and 53% of the total expression of IL-10 in CL and ML lesions, respectively (Table 2), suggesting that 40 to 50% of the IL-10 present in the lesion sites comes from other cellular sources. These percentages were statistically equivalent when the two groups were compared. Interestingly, while approximately 80% of the $CD68^+$ cells expressed IL-10 in CL lesions, more than 90% of the $CD68^+$ cells from ML lesions expressed IL-10, showing a higher commitment of the monocytic population to the production of IL-10 in ML than in CL lesions (Table 2).

Cells from ML lesions display a lower intensity of expression of the IL-10 receptor than cells from CL lesions. Previous studies performed by our group demonstrated that addition of IL-10 to in vitro cultures led to modulation of antigen-induced IFN-γ production by PBMC from CL but not ML patients, suggesting a deficient response to IL-10 in ML. This finding, together with the fact that we observed an intense inflammatory response in ML lesions, led to the hypothesis that IL-10 unresponsiveness was due to low IL-10 receptor expression, which would lead to an exacerbated inflammatory response. Thus, we evaluated the expression of the IL-10 receptor in ML and CL lesions, determining the numbers and percentages of cells expressing this molecule, as well as its intensity of expression per cell. We observed that while the numbers of cells expressing the IL-10 receptor were similar in ML and CL lesions (Fig. 1C), the percentage of cells expressing this molecule (Fig. 2A) and the intensity of expression of the IL-10 receptor on a cell-by-cell basis (Fig. 2B) were lower in ML than in CL lesions.

Expression of granzyme A and iNOS in ML and CL lesions. We determined the expression of two effector molecules, granzyme A and iNOS, in lesions from CL and ML patients. Although the total number of cells expressing iNOS was apparently higher in CL lesions, our analyses did not show statistical significance ($P = 0.2$) (Table 3). However, the percentage of expression of $iNOS^+$ cells was higher in CL than in ML (Table 3). The total number of cells expressing granzyme A and the

TABLE 2. Numbers of TNF-α^+ and IL-10^+ cells and relative contributions of the $CD68^+$ population to the production of these cytokines in lesions from patients with CL and ML

Clinical form	TNF-α production				IL-10 production			
	No. of TNF-α^+ cells[a]		% Contribution of $CD68^+$ cells[b]	% Commitment of $CD68^+$ cells[c]	No. of IL-10^+ cells[a]		% Contribution of $CD68^+$ cells[b]	% Commitment of $CD68^+$ cells[c]
	Total	$CD68^+$			Total	$CD68^+$		
CL (n = 14)	1,144 ± 691	909 ± 547	78 ± 12	65 ± 21	2,025 ± 785	1,267 ± 598	62 ± 13	79 ± 16
ML (n = 7)	1,354 ± 993	1,148 ± 937	80 ± 12	56 ± 23	2,468 ± 1,597	1,148 ± 1,158	53 ± 17	91 ± 4[d]

[a] Counted based on expression of specific fluorescent markers for TNF-α or IL-10 either alone or together with CD68, as described in Materials and Methods.
[b] Calculated as the percentage of $CD68^+$ cells expressing the cytokine in relation to the total expression of the cytokine.
[c] Calculated as the percentage of $CD68^+$ cells expressing the cytokine in relation to total $CD68^+$ cells.
[d] Statistically significantly different from the value for CL ($P < 0.05$).

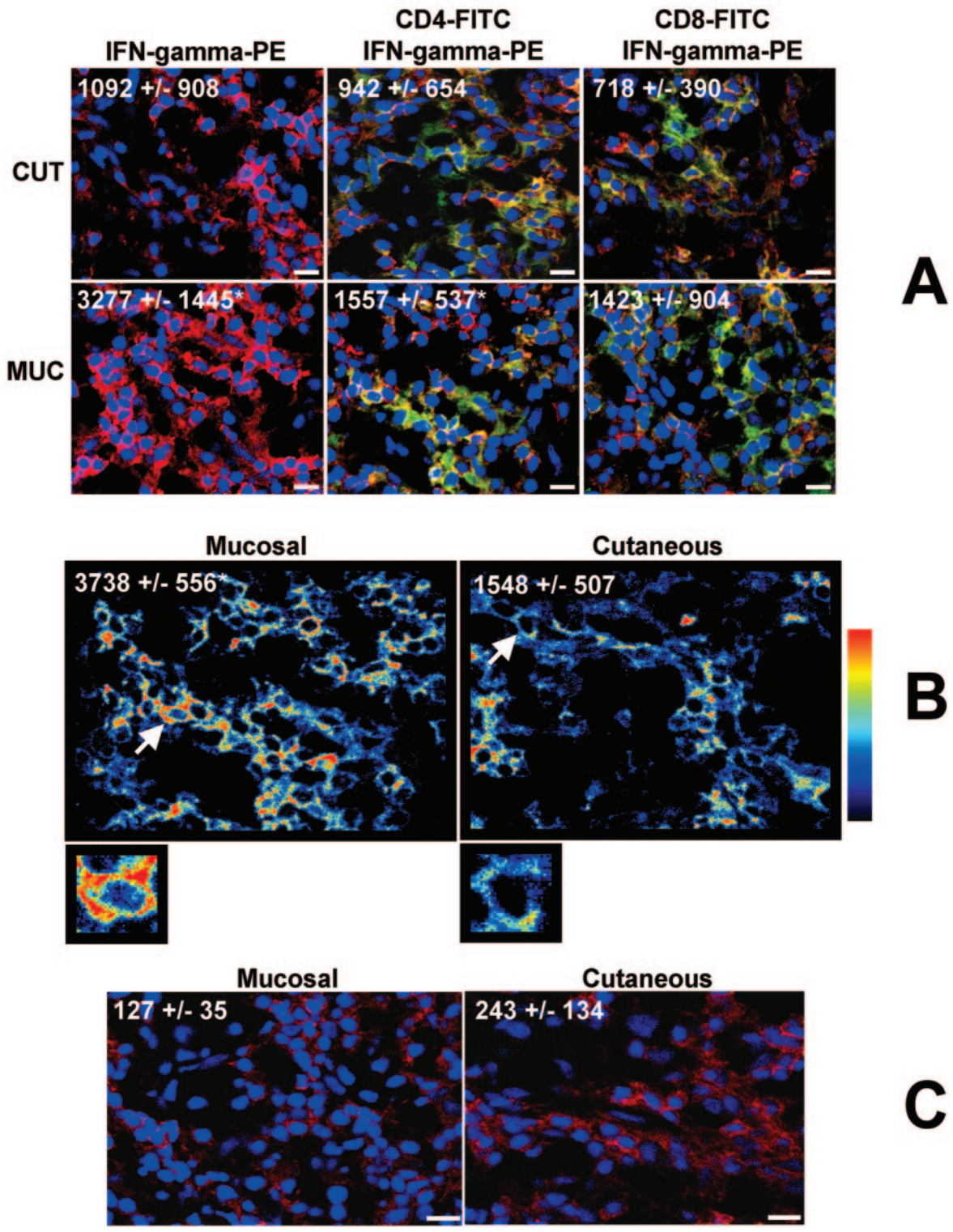

FIG. 1. Differential expression of IFN-γ and IL-10 receptor in CL and ML lesions. (A) Representative images from confocal microscopy analyses for determination of the numbers of total IFN-γ^+ cells, $CD4^+$ IFN-γ^+ cells, and $CD8^+$ IFN-γ^+ cells in CL (CUT) and ML (MUC) lesions. Tissue sections were stained with FITC-labeled anti-CD4 or anti-CD8 monoclonal antibodies and with PE-labeled anti-IFN-γ and were counterstained with DAPI as described in Materials and Methods. The three optical sections for each patient were obtained simultaneously with lines 363, 488, and 568 of the argon/krypton laser and the proper set of filters. Overlays for CD4 or CD8 (green), IFN-γ (red), and DAPI (blue) in CL and ML lesions are shown. Cells that are double positive for CD4 or CD8 and IFN-γ appear in yellow. These images are representative of each group. Values are the average ± standard deviation for each group following numerical determination of the number of positive cells for the indicated molecule(s). Asterisks indicate statistically significant differences (ML > CL) at a P value of <0.05. CL and ML lesions from 14 and 7 patients, respectively, were analyzed. Bar, 10 μm. (B) Representative analysis of the intensity of expression of IFN-γ in CL and ML lesions by using

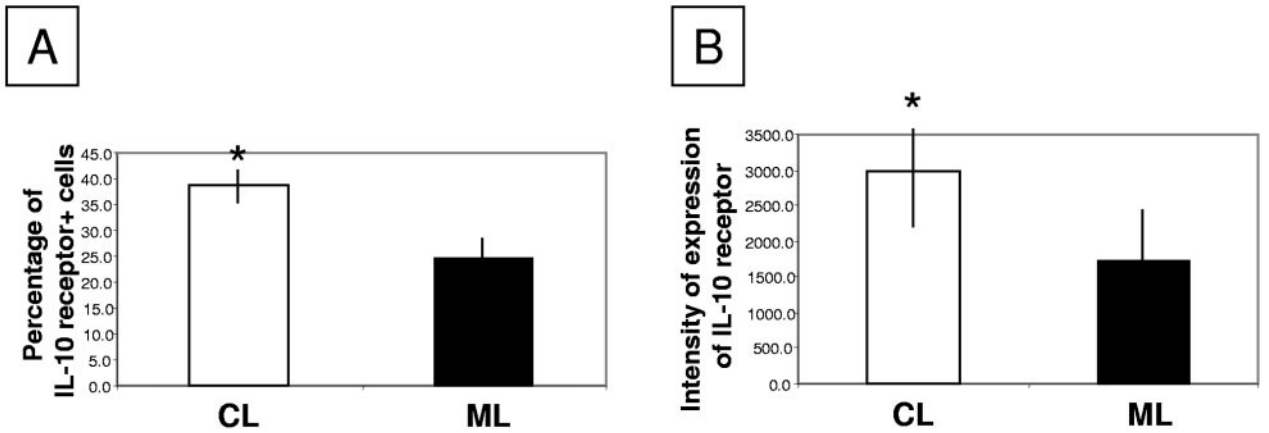

FIG. 2. Analysis of the expression of the IL-10 receptor in CL and ML lesions. (A) Percentage of cells expressing the IL-10 receptor in CL and ML lesions; (B) intensity of expression of the IL-10 receptor per cell. Tissue sections were stained with PE-labeled monoclonal antibodies against the IL-10 receptor and counterstained with DAPI, and the number and percentage of cells expressing the IL-10 receptor, as well as the intensity of its expression, were calculated as described in Materials and Methods. Asterisks indicate statistically significant differences between groups at a P value of <0.05. Nine CL and seven ML lesions were analyzed.

number of granzyme A^+ $CD8^+$ T cells were determined using double-staining confocal analysis. These studies demonstrated that ML lesions display a significantly higher number of granzyme A^+ cells than CL lesions (Table 3). Interestingly, the numbers of $CD8^+$ granzyme A^+ cells were similar in the two groups (Table 3). However, the commitment of the $CD8^+$ cell population to cytotoxic activity was higher in ML lesions, as shown by the higher percentage of $CD8^+$ cells expressing granzyme A (Table 3). Moreover, a statistically significant positive correlation was seen between total IFN-γ^+ and total granzyme A^+ cells in lesions from ML but not CL patients (data not shown). Further analysis also showed that while in CL lesions $CD8^+$ cells are responsible for 70% of the total granzyme A expression, in ML lesions this population contributes to approximately 50% of the total granzyme A expression. This shows that in CL the majority of the cells producing granzyme A are $CD8^+$, while in ML other cell populations are responsible for 50% of the total production.

DISCUSSION

Analyses of the immunological profile of PBMC from ML patients have suggested that the tissue pathology associated with this severe form of leishmaniasis is related to the establishment of an exacerbated inflammatory response, representing a polar hypersensitivity reaction to *Leishmania* infection (5, 12, 32). In this study, in which an extensive multiparameter confocal microscopy analysis of the inflammatory infiltrate in ML and CL lesions was performed, several points of evidence in favor of this hypothesis were revealed: (i) the inflammatory infiltrate in ML lesions is more intense than that observed in CL lesions; (ii) higher expression of the inflammatory cytokine IFN-γ and of the cytotoxic molecule granzyme A was observed in ML than in CL lesions; and (iii) the intensity of expression of the IL-10 receptor was lower in ML than in CL lesions. Thus, our work demonstrates the occurrence of in situ hyperactivation in ML lesions, likely due to down-regulation of the IL-10 receptor, and provides new insights toward the understanding of the complex mechanisms of immunopathology related to ML and CL.

Previous studies have demonstrated that the inflammatory infiltrate of CL and ML lesions is primarily composed of T cells, followed by macrophages and very few or no B cells or NK cells (9, 15, 32). These studies strengthen the argument that T cells play a critical role in leishmaniasis. A mixed cytokine profile, including expression of TNF-α, IFN-γ, IL-10, and IL-4, was detected in CL and ML lesions by using PCR and immunohistochemistry techniques (1, 11, 24, 25, 30, 31). The use of PCR, while allowing for a sensitive analysis, does not provide information on the intensity, composition, and architecture of the inflammatory infiltrate or on the cellular sources of the cytokines analyzed. By use of multiparameter confocal microscopy, we determined that $CD4^+$ T cells were the primary source of IFN-γ in both CL and ML, followed by $CD8^+$ T cells and $CD4^-$ $CD8^-$ cells. These results are similar to those we found previously when determining the sources of IFN-γ in PBMC from ML patients (5). However, $CD4^-$ $CD8^-$ cells appeared as the second major source of IFN-γ in PBMC from CL patients (9). This difference may reflect distinctive

Pseudocolor software as described in Materials and Methods. The predominance of red indicates a higher intensity of expression than the predominance of blue (see the color scale on the right). White arrows indicate the cells enlarged below. The asterisk indicates a statistically significant difference (ML > CL) at a P value of <0.05. Fourteen CL and seven ML lesions were analyzed. (C) Representative images from the confocal microscopy analysis for determination of the numbers of total IL-10 receptor-positive cells in CL and ML lesions. Tissue sections were stained with a PE-labeled monoclonal antibody against the IL-10 receptor as described in Materials and Methods. A total of nine CL and seven ML lesions were analyzed. Bars, 10 μm. The images are representative of one of two independent experiments for each individual lesion. Values are the average $\pm$ standard deviation for each group from the numerical analysis of the number of cells expressing the indicated molecule(s).

TABLE 3. Expression of iNOS and granzyme A in lesions from patients with CL and ML

Clinical form	iNOS expression		Granzyme A expression			
	No. of iNOS$^+$ cells[a]	% iNOS$^+$ cells	No. of granzyme A$^+$ cells[a]		% Contribution of CD8$^+$ cells[b]	% Commitment of CD8$^+$ cells[c]
			Total	CD8$^+$		
CL (n = 9)	1,274 ± 230	87 ± 6	924 ± 466	687 ± 391	70 ± 21	58 ± 13
ML (n = 7)	1,159 ± 231	64 ± 19[d]	1,562 ± 610[e]	950 ± 587	56 ± 14[d]	74 ± 17[e]

[a] Counted based on expression of specific fluorescence for either granzyme A or iNOS alone or granzyme A and CD8, as described in Materials and Methods.
[b] Calculated as the percentage of CD8$^+$ cells expressing granzyme A in relation to total granzyme A expression.
[c] Calculated as the percentage of CD8$^+$ cells expressing granzyme A in relation to total CD8$^+$ cells.
[d] Significantly lower than value for CL ($P < 0.05$).
[e] Significantly higher than value for CL ($P < 0.05$).

recruitment of T-cell subpopulations to lesion sites. We also determined that monocytes are the main source of TNF-α in CL and ML lesions, a finding that differs from those of our previous studies in that CD4$^+$ T cells were the main source of TNF-α among the PBMC from CL and ML (5, 9). It is possible that the local cytokine environment and the presence of parasites activate more TNF-α production by macrophages in the tissue. Our analysis showed that CD68$^+$ and CD68$^-$ cells contribute equally to the expression of IL-10 in CL and ML lesions, suggesting that T cells are also important in IL-10 production in the lesions.

IFN-γ, an inflammatory cytokine produced predominantly by T cells, is critical in eliciting cellular responses and is a potent mediator of *Leishmania* killing in synergy with other cytokines and effector mediators, such as nitric oxide (NO) (21, 22). On the other hand, it has been shown that NO can cause tissue damage (17, 28). Since similar frequencies of iNOS$^+$ cells were found in CL and ML lesions, it is possible that NO is not the only molecule involved in parasite killing or tissue damage in human leishmaniasis. In addition to NO induction, another important biological function of IFN-γ is the induction of cytotoxic activity, which is directly correlated with granzyme A and TIA-1 expression (2, 25, 36). We have previously shown that CL lesions display a high number of TIA-1$^+$ cells (23) and that cytotoxic activity is exacerbated in PBMC from ML patients (6). In this work, we observed that the number of granzyme A$^+$ cells was significantly higher in ML than in CL lesions. These data are consistent with the extensive tissue destruction observed in areas of mucosal commitment. Interestingly, while the main cells expressing granzyme A in CL lesions are CD8$^+$ T cells, a significant number of CD8$^-$ cells express granzyme A in ML lesions, pointing to the participation of other cell types in cytotoxic functions in ML. It is likely that CD4$^+$ T cells would account for the expression of granzyme A in ML lesions, since NK cells seem to be scarce in the ML inflammatory infiltrate (8). Moreover, it has been shown that the presence of inflammatory cytokines from Th1 cells can favor the development of CD4$^+$ cells that display cytotoxic functions (34). Also, granzyme A activity was previously assigned to *Leishmania*-reactive CD4$^+$ T cells in experimental models (16). These data suggest that cytotoxic CD4$^+$ T cells could play an important role in the pathogenesis of ML. Further studies need to be done to clarify this point.

While IFN-γ expression was increased in ML lesions over that in CL lesions, the expression of TNF-α, another important inflammatory cytokine, was not. Since the main sources of IFN-γ were CD4$^+$ and CD8$^+$ T cells, while the main sources of TNF-α were CD68$^+$ macrophages, it is possible that different mechanisms could be involved in the production of the two inflammatory cytokines. Recent studies have demonstrated that TNF-α is indeed present in ML lesions prior to and after specific treatment (1). The involvement of TNF-α in ML pathology was suggested previously, when pentoxifylline, a TNF-α inhibitor, was shown to be an efficient adjuvant treatment for ML patients refractory to conventional therapy (20). Previous studies have shown that inflammatory cytokines such as IFN-γ are able to induce expression of the TNF-α receptor (35). Since ML lesions display higher IFN-γ expression, it is possible that this cytokine is acting to increase the expression of the TNF-α receptor, which will be investigated in the future.

A potent antagonist of IFN-γ activities is the cytokine IL-10 (27). Our analysis of in situ IL-10 expression showed that CL and ML lesions have similar numbers of IL-10-expressing cells. Since the inflammatory infiltrate of ML lesions is significantly more intense than that of CL lesions, one would expect that the percentage of IL-10-expressing cells would be lower in ML than in CL lesions. Although statistical analysis of these data did not show significance, the IFN-γ/IL-10 ratio is higher in ML patients (0.4 and 0.7 for CL and ML, respectively), showing that the proportion of IFN-γ$^+$ cells is indeed higher in ML patients. Moreover, the finding that ML patients display low levels of IL-10 receptor adds supportive evidence to the hypothesis that ML patients have a poorly controlled inflammatory environment. Previous studies have shown that proinflammatory cytokines induce down-regulation of the IL-10 receptor (26), and we have shown that in vitro blocking of IL-10 is able to restore the production of IFN-γ in cultures of cells from CL but not ML patients stimulated with soluble leishmania antigen (5). Other antiinflammatory cytokines, such as IL-13 (10), IL-4 (31), and transforming growth factor β (18), may also be involved in the control of ML. Recent studies with the experimental model of *Leishmania major* infection have shown that CD4$^+$ CD25$^+$ regulatory T cells are accumulated in lesions and exert IL-10-dependent as well as IL-10-independent suppressive functions (7). The involvement of CD4$^+$ CD25$^+$ regulatory cells in human leishmaniasis has not been clarified to date and could present an important regulatory mechanism.

We show evidence that high expression of IFN-γ, increased cytotoxic activity, and low expression of the IL-10 receptor may be responsible for the lack of control of the inflammatory response in ML. By adding to our knowledge of immunoregulatory and pathogenic cellular mechanisms in American cuta-

neous leishmaniasis, these findings will be critical when interventions involving modulation of specific cell populations are considered.

ACKNOWLEDGMENTS

We are thankful to Roque Almeida for critical review of the manuscript.

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease (TDR), TMRC/NIH, the Howard Hughes Medical Institute, and PADCT/CNPq. W.O.D., E.M.C., M.A.R.-S., K.J.G., and A.R.D.J. are CNPq fellows, and D.R.F. is a CAPES fellow.

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Editor: W. A. Petri, Jr.

Artigo # 2: Faria, D.R.[1], Souza, P. E. A. [2], Durães F.V. [1], Carvalho, E.M.[3], Gollob, K.J.[4], Machado, P.R.[3], Dutra, W.O.[1] (2008) – **"Recruitment of CD8+ T cells expressing granzyme A is associated with lesion progression in human cutaneous leishmaniasis"** – Parasite Immunol. 2009, 31(8) : 432-439

Parasite Immunology, 2009, **31,** 432–439 DOI: 10.1111/j.1365-3024.2009.01125.x

Recruitment of $CD8^+$ T cells expressing granzyme A is associated with lesion progression in human cutaneous leishmaniasis

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SUMMARY

Human infection with Leishmania braziliensis *leads to the establishment of cutaneous leishmaniasis (CL), characterized by the appearance of skin lesions that progress from nonulcerated to ulcerated forms. Our goal was to characterize the immunological kinetics associated with this progression, comparing the cellular composition, cytokines and granzyme expression between lesions of patients with early (E-CL) and late stages (L-CL) of CL. Histopathological analysis showed that lesions from L-CL had more exuberant inflammatory infiltrate as compared to E-CL. Although E-CL and L-CL lesions were predominantly mononuclear, lesions from E-CL patients presented higher neutrophil and eosinophil counts than L-CL. While percentages of $CD4^+$ and of $CD68^+$ cells were slightly higher in L-CL, a fivefold increase of $CD8^+$ cells was observed in L-CL, as compared to E-CL. Moreover, $CD8^+$ T-cells from L-CL expressed significantly higher levels of granzyme A than E-CL. Interestingly, granzyme A expression was positively correlated with intensity of the inflammatory infiltrate in L-CL but not E-CL. Lastly, percentages of IFN-γ^+ and IL-10^+ cells were higher in L-CL as compared to E-CL, with $CD4^+$ T-cells and $CD68^+$ monocytes as the main sources of these cytokines, respectively. These results suggest that recruitment of $CD8^+$ granzyme A^+ T cells is involved in lesion progression in human CL.*

Keywords *$CD8^+$ T cells, granzyme, leishmaniasis, lesion, progression*

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Received: 24 November 2008
Accepted for publication: 19 March 2009

INTRODUCTION

Leishmaniasis, caused by infection with parasites of the *Leishmania* genus, affects millions of individuals worldwide causing serious morbidity and mortality. Individuals infected with *Leishmania* present with different clinical forms, depending on the species/strain of the parasite, as well as characteristics of the host's immune response (1). In Brazil, *L. braziliensis* is the most prevalent species, and can cause at least two major clinical forms: cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML). CL is the most frequent form and is clinically characterized by the presence of a single skin ulcer with elevated borders that can progress to spontaneous healing (2). While the self-healing of CL lesions appears to be associated with natural resistance, the immunological mechanisms of resistance have not been clearly defined. Nonself-healing lesions usually respond well to antimonial administration, although this treatment is toxic to patients (3). We have previously shown that early treatment fails to prevent ulcer formation in CL (4). Thus, understanding the immunological kinetics of lesion progression in CL is critical for better understanding the establishment of pathology and how it can be prevented.

Different patterns of cytokine expression by T cells are related with severity of *L. braziliensis* infections in humans. It has been demonstrated that circulating $CD4^+$ T cells of CL patients, stimulated *in vitro* with soluble *Leishmania* antigen, express IFN-γ, but not IL-4 and IL-5, indicating the presence of specific Th1 cells in this form of the illness (5). Moreover, analysis of intralesional cytokine gene expression showed that the Th1 cytokine mRNAs (IL-2, IFN-γ and lymphotoxin) were present in CL (6,7). Coutinho *et al.* (8) have demonstrated a predominant production of IFN-γ by T cells from cured CL patients, suggesting a protective role for this cytokine in CL (9). However, ML, a more aggressive clinical form that can also arise from *L. braziliensis* infection, is associated with exuberant

production of IFN-γ and TNF-α (10) and a deficient control of inflammation due to low expression of IL-10 receptor (11). Moreover, we have shown that expression of granzyme A is evident in lesions from CL and is even more abundant in the severe ML form, suggesting a role for this molecule in tissue destruction (11). It is clear that a balance between immunoregulatory mechanisms that lead to parasite control vs. tissue destruction is critical for determining pathology or protection in human leishmaniasis.

With regard to the clinical evolution of CL, previous studies have shown that circulating T cells from patients with early *L. braziliensis* infection, characterized by approximately 15 days of illness and the presence of nonulcerated lesion, displayed a down-modulated Th1-type response as compared to cells from patients with late-stage CL (L-CL), characterized by approximately 60 days of illness and the presence of ulcerated lesion (12). Understanding the kinetics of establishment of the cutaneous lesion, through the determination of which cells are recruited to the lesion site, what is the level of expression of immunomodulatory cytokines and cytotoxic molecules will provide new insight towards the understanding of the mechanism involved with the pathology associated with human CL. Thus, the aim of this study was to compare the cellular composition, cytokine and granzyme expression in lesions of patients with early CL (E-CL) and L-CL, to better understand the immunological mechanisms involved in the clinical evolution of CL. Our data show that E-CL and L-CL are predominantly composed of mononuclear cells, although E-CL has a higher frequency of polymorphonuclear (PMN) cells. Moreover, we demonstrated that the more exuberant inflammatory infiltrate observed in L-CL is consistent with lesion ulceration and is associated to higher IFN-γ expression and the recruitment of $CD8^+$ T cells expressing granzyme A. Importantly, an ongoing immunoregulation is present, characterized by the increased frequency of IL-10 in L-CL, consistent with further lesion resolution usually observed in CL patients.

MATERIALS AND METHODS

Patients

The patients analysed in this study were from Corte de Pedra, an endemic area for *L. braziliensis*, located 280 km south-west of Salvador, in the state of Bahia, Brazil. All patients were volunteers, and informed consent was obtained from all individuals prior to collection of lesion material. Diagnosis of leishmaniasis was performed based on clinical and laboratory criteria. Detection of suggestive popular or ulcerated cutaneous lesions was associated with a positive skin Montenegro test, parasite isolation and/or histopathological analysis to confirm a diagnosis of CL. Patients were classified as E-CL (approximately 15 days of illness, nonulcerated lesion) or L-CL (approximately 60 days of illness, ulcerated lesion), as previously established by us (4). For all cases, parasite species were typed to confirm that the disease was due to *L. braziliensis* infection. E-CL patients enrolled in this study ($n = 6$) or L-CL patients ($n = 9$) presented with a single nonulcerated or ulcerated lesion, respectively, and had not been previously diagnosed with or treated for leishmaniasis. At the time of sample collection, the time of the active lesions were estimated among 15 (E-CL) or 30–60 days (L-CL), as reported by the patients themselves. Treatment was offered to all patients as needed despite their enrolment in this project and was administered after sample collection. E-CL and L-CL patients were not under treatment when samples were collected. Lesions were collected at the Corte de Pedra healthcare facility. The Ethical Committees of Universidade Federal de Minas Gerais and Universidade Federal da Bahia approved all procedures involved in this study.

Sample obtention

Skin biopsy specimens were taken from the borders of active lesions using a 4-mm-diameter punch, as routinely done by us and others, as the central area of the lesion comprises necrotic and haemorrhagic areas. In the case of early lesions, biopsies were collected from the papule of the nonulcerated lesions, after the application of a local anaesthetic. Lesions were maintained in a 30% sucrose solution for 30 min at 4°C and then transferred to OCT Tissue Tek freezing medium and immediately placed in dry ice. The material was stored at −70°C until analysis.

Histological and immunofluorescence staining

Individual 4–5 μm cryosections were placed in silane-precoated slides and fixed for 10 min with acetone. Slides were incubated with phosphate-buffered saline for 30 min and subjected either to haematoxylin–eosin (HE) staining or to immunofluorescence staining using specific monoclonal antibodies. Standard HE staining was performed to ensure tissue integrity as well as for the evaluation of the intensity and composition of the inflammatory infiltrate. Immunofluorescence reactions involved incubation with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labelled monoclonal antibodies directed to surface receptors (CD4 clone S3·5, CD8 clone 3B5 or CD68 clone Ki-M7) and intracellular molecules (granzyme A clone CLB-GA28, IFN-γ clone or B27, IL-10 clone 9D7) respectively. Sections were incubated with antibodies mixture

overnight at 4°C. After staining, preparations were extensively washed with phosphate-buffered saline, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), and mounted using Antifade mounting medium (Molecular Probes, Carlsbad, CA, USA). Slides were kept at 4°C, protected from light, until acquisition in a laser scanning confocal microscope (Zeiss, Thornwood, WV, USA). Isotype controls were analysed separately to confirm the lack of nonspecific staining. Monoclonal antibodies were purchased from Caltag (Burlingame, CA, USA).

Light microscopy and confocal analysis

Haematoxylin–eosin stained sections were analysed using a light microscopy (Axiovert, Zeiss). We acquired the data using a power magnification of 400×, and the frequencies of neutrophils, eosinophils and mononuclear cells were expressed as absolute numbers or percentage of the total cell count. A total of 16 fields/sample were acquired for the histological analysis.

Confocal analysis were performed using a Meta-510 Zeiss laser scanning confocal system running LSMix software coupled to a Zeiss microscope (Axiovert 100) with an oil immersion Plan-Apochromat objective (63×, 1·2 numerical aperture) and Bio-Rad (Hercules, CA, USA) MRC 1024 laser scanning confocal system running LaserSharp 3.0 software coupled to a Zeiss microscope (Axiovert 100) with a water immersion objective (40×, 1·2 numerical aperture). A water-cooled argon UV laser (488 nm) or a krypton/argon laser was used to excite the preparation (through its 363, 488 or 568 nm line), and light emitted was selected with band-pass filters (522/35 for FITC or 598/40 for PE). For DAPI visualization a mercury lamp were used to excite the preparation (through its 20/80 nm line), and light emitted was selected with band-pass filters (363/90 for DAPI). For each section, the inflammatory infiltrate present in the connective tissue adjacent to the epithelia was located and an area presenting with an uniform infiltrate was selected for analysis. Within this inflammatory area, a minimum of six images (fields) were collected. Image analysis and processing were performed with LSMix (Zeiss) or LaserSharp (Bio-Rad), Confocal Assistant, Adobe Photoshop and Image Tool software. Analyses were performed by counting the total number of cells in six to nine fields acquired and calculating the average of cells number per field for each patient. This procedure was performed for each parameter analysed, allowing determination of the total number of inflammatory cells (total number of DAPI+ cells within the inflammatory infiltrate), the number of FITC or PE single-positive cells, and the number of double-positive cells. The counts were performed blindly, the results were expressed as the average of cells number per field for each parameter for each patient, and then the values were averaged for each group. The results are representative of two experiments per patient.

Statistical analysis

Statistical analysis of the data was performed using JMP statistical software from SAS (Cary, NC, USA) and BioEstat 3.0 statistical software. The comparisons of percentage for a given parameter between the groups were performed using the nonparametric *t*-test or Mann–Whitney test. Spearmann correlation analysis test was also performed. Results were considered statistically different when the analysis returned a $P < 0{\cdot}05$.

RESULTS

Distinct inflammatory profiles in E-CL and L-CL lesions

The composition of the inflammatory infiltrate in lesions from E-CL and L-CL patients was determined using conventional histological analysis as described in 'Materials and methods'. Sections stained with HE from E-CL patients were compared to L-CL patients. Histopathological analysis of lesions from patients with both stages of CL showed a keratinized stratified squamous epithelium, presenting hyperkeratosis, parakeratosis, acanthosis and a large number of cells with hydropic degeneration in the prickly layer (not shown). Diffuse chronic inflammation with predominantly mononuclear cell infiltration was observed in the dense connective tissue (Table 1) classified as productive and exudative type. Quantitative analysis showed that lesions from patients with E-CL presented higher percentage of PMN cells ($P = 0{\cdot}02$), neutrophils ($P = 0{\cdot}04$) and eosinophils ($P = 0{\cdot}01$) when compared to lesions from L-CL (Table 1). However, the frequency of PMN cells in both groups was very low in relation to the total inflammatory infiltrate (<2%). It was observed that the intensity of the inflammatory infiltrate (number of inflammatory cells per field) was higher in lesions from patients with L-CL than E-CL ($P = 0{\cdot}004$) (Table 1).

Lesions from L-CL display a higher proportion of $CD8^+$ cells than E-CL lesions

Analysis of the frequencies of $CD68^+$, $CD4^+$ and $CD8^+$ cells was performed using confocal microscopy. The absolute numbers of these cell populations increased in L-CL, as compared to E-CL, compatible with the more exuberant inflammatory infiltrate observed in the former group. Thus, we determined the percentages of these populations within

Table 1 Total number of inflammatory cells per area and frequencies of mononuclear and polymorphonuclear (neutrophil and eosinophil) cells in lesions from patients with early cutaneous leishmaniasis and late cutaneous leishmaniasis

	E-CL (n = 6)				L-CL (n = 12)				
Parameter	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum	P-value
Number of total cells/field	200·96	188·87	118·44	302·34	388·74	395·63	233·75	551·09	0·0037
% Mononuclear cells	97·92	97·97	97·13	98·45	98·66	98·95	96·83	99·45	0·0492
% Polymorphonuclear cells	2·73	2·83	2·17	3·25	1·61	1·17	0·66	3·80	0·0192
% Neutrophils	2·08	2·03	1·55	2·88	1·35	1·05	0·55	3·17	0·0492
% Eosinophils	0·65	0·58	0·34	1·22	0·26	0·18	0·07	0·62	0·0131

Haematoxilin–eosin stained sections were analysed using light microscopy. All connective tissue from each lesion was evaluated in a power magnification of 400×. The comparisons of percentage for a given parameter between the groups were performed using the nonparametric Mann–Whitney test.
E-CL, early cutaneous leishmaniasis; L-CL, late cutaneous leishmaniasis.

the infiltrates of both forms, to access the contribution of each population to the overall inflammatory infiltrate, comparing between E-CL and L-CL. Our analysis showed that the percentages of $CD4^+$ cells and $CD68^+$ cells were higher in L-CL as compared to E-CL (Figure 1). Strikingly, a five-fold increase in the percentage of $CD8^+$ cells was observed in L-CL when compared to E-CL group (Figure 1).

Recruitment of $CD8^+$ T cells expressing a cytolytic molecule, granzyme A, is associated with lesion ulceration in CL

To evaluate whether the establishment of CL lesions was associated to cytotoxic responses, we determined the frequency of cells expressing granzyme A, a cytolytic molecule, in E-CL and L-CL lesions. Our results showed that the expression of granzyme A by cells within the inflammatory infiltrate was over five times higher in L-CL that E-CL (Figures 2 and 3b). Moreover, while approximately 20% of the $CD8^+$ cells from E-CL expressed granzyme A, approximately 50% of the $CD8^+$ cells from L-CL expressed this cytolytic molecule. Interestingly, a positive correlation was observed between the intensity of the inflammatory infiltrate and the number of $CD8^+$ granzyme A+ cells in lesions from L-CL but not E-CL (Figure 2). These results suggest that the expression of granzyme A by $CD8^+$ T cells is involved in tissue destruction during the progression of CL to the ulcerated stage.

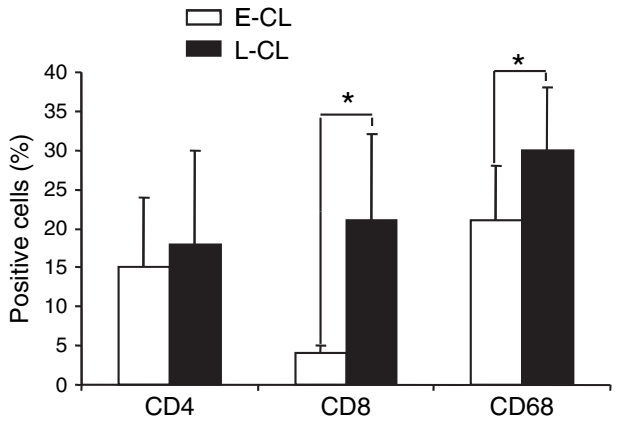

Figure 1 Percentages of T $CD4^+$, T $CD8^+$ and $CD68^+$ cells in the inflammatory infiltrate from early cutaneous leishmaniasis (E-CL) (n = 6) and late cutaneous leishmaniasis (L-CL) (n = 9) lesions. Frozen tissue sections were stained with FITC-labelled anti-CD4, anti-CD8 or anti-CD68 monoclonal antibodies and were counterstained with DAPI as described in 'Materials and methods'. Results are expressed as bars of the mean percentages for each group. Standard deviation indicated by above line bars. Asterisks indicate statistically significant differences between groups at $P < 0{\cdot}05$.

Ongoing immunoregulatory mechanism is observed during the evolution of CL lesions

The frequencies of cells expressing the pro-inflammatory cytokine IFN-γ or the anti-inflammatory cytokine IL-10 were determined using confocal analysis. The results showed that the percentages of cells expressing IFN-γ and IL-10 were higher in L-CL as compared to E-CL (Figure 3a and b). However, no differences were observed as for the sources of these cytokines between the groups, with $CD4^+$ and $CD68^+$ cells as the main sources of IFN-γ and IL-10 respectively.

DISCUSSION

The most common clinical form of the disease caused by *L. braziliensis* is localized CL, which is characterized by single or multiple ulcerated dermal lesions that usually heal spontaneously. Patients with a short period of illness (<2 weeks) have acneiform lesions or small bleeding ulcers that clearly differ from the classical CL ulcer observed in patients with L-CL. We have previously shown that early

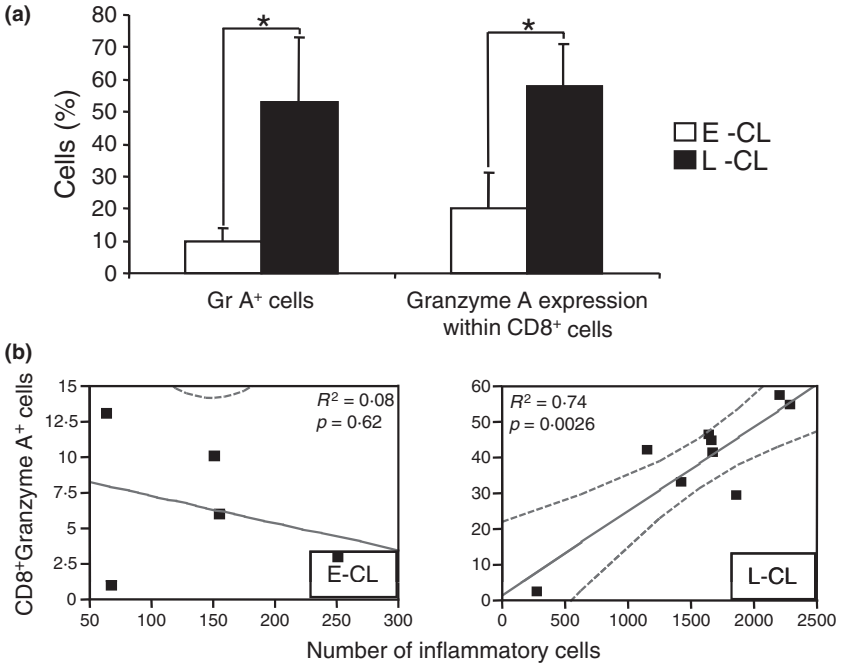

Figure 2 (a) Percentages of cells expressing granzyme A (Gr A+) and % granzyme A expression within $CD8^+$ T cells in early cutaneous leishmaniasis (E-CL) (n = 6) and late cutaneous leishmaniasis (n = 9) lesions. Asterisks indicate statistically significant differences between groups at $P < 0.05$. (b) Correlation analysis between the frequency of $CD8^+$ granzyme A+ cells and intensity of inflammatory infiltrate in lesions from E-CL and L-CL.

treatment of CL fails to prevent ulcer development (4). In the present study, we compared the cellular composition, cytokine and granzyme A expression in lesions between individuals with E-CL and L-CL to better understand the progression of this disease towards lesion formation.

The composition of the inflammatory infiltrate in lesions from E-CL and L-CL patients was determined using conventional histological analysis, and it was verified that both clinical forms showed diffuse chronic inflammation, with the presence of dense fibrous connective tissue classified as productive and exudative type with a predominantly mononuclear cellular infiltrate. Moreover, lesions from patients with E-CL presented higher frequencies of PMN neutrophils and eosinophils when compared to lesions from L-CL. Previous studies had documented that ulcerated lesions from CL patients are mainly composed of mononuclear cells (11,13). Although the frequency of PMN cells is low in relation to mononuclear cells (<2%), the presence of PMN cells in E-CL suggests a role for these cells during early infection. A recent paper using the murine model of *Leishmania* infection has demonstrated a role for PMN cells, specifically neutrophils, in hosting the parasite during early infection, favouring parasite survival (14). Although we did not evaluate the frequency of *Leishmania*-infected PMN cells in E-CL lesions due to material limitation and preservation, it is possible that these cells also host the parasite in early stages of human infection. Further analysis to evaluate parasite burden in E-CL and L-CL will be performed. Rocha *et al.* (12) showed that a down regulation of the Th1-type response occurs during the early phases of *L. braziliensis* infection. Moreover, they suggested that this phenomenon would allow the parasite survival and growth, leading to the development of disease.

We observed that the intensity of inflammation was higher in lesions from patients with L-CL than E-CL, compatible with lesion progression. Furthermore, this increased inflammatory infiltrate was associated with a striking recruitment of $CD8^+$ T cells to L-CL lesions. We hypothesized that this increased inflammatory infiltrate was associated with the expression of the inflammatory cytokine IFN-γ. IFN-γ has been considered a critical cytokine involved in the pathogenesis of CL, due to its parasiticidal ability and also due to its inflammatory activity (11,15). Our data showed an increase in IFN-γ expression in L-CL as compared to E-CL lesions, which is consistent with our hypothesis. We have previously shown that $CD4^+$ Th1 cells are responsible for the majority of IFN-γ production in peripheral blood mononuclear cells (PBMC) from CL patients (5). Recently, our group demonstrated that $CD4^+$ cells represented the majority of IFN-γ-producing cells, followed by $CD8^+$ cells and $CD4^-CD8^-$ cells in PBMC and lesions from CL patients (11,16). The analyses performed here showed that $CD4^+$ T cells are the main source of IFN-γ in L-CL and also E-CL, accounting for over 50% of the IFN-γ expression in E-CL and 75% in L-CL. Thus, with progression of the disease, a higher frequency of $CD4^+$ T cells become engaged with expression of this cytokine, although statistical analysis of these data did not show significance (P = 0·06). Our suggestion that IFN-γ is associated with disease progression is in accor-

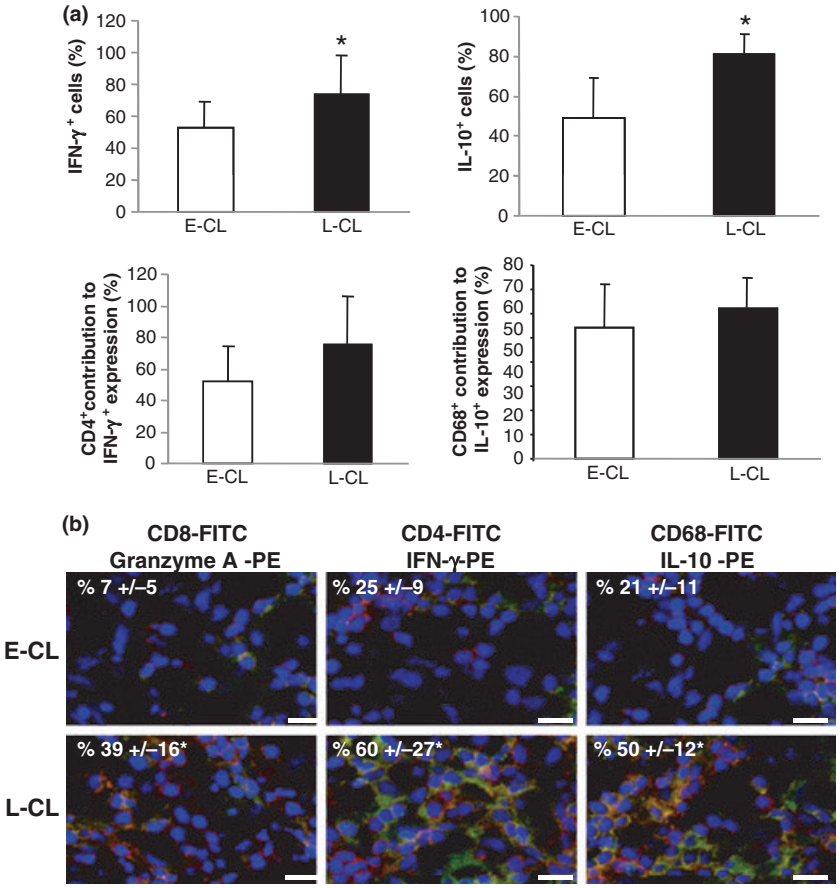

Figure 3 (a) Percentages, in early cutaneous leishmaniasis (E-CL) (n = 6) and late cutaneous leishmaniasis (n = 9) lesions, of cells expressing IFN-γ and IL-10 and the percentage contribution of $CD4^+$ and $CD68^+$ cells for the expression of IFN-γ and IL-10 respectively. Frozen tissue sections were stained with FITC-labelled anti-CD4 or anti-CD68 monoclonal antibodies and with PE-labelled anti-IFN-γ or anti-IL-10 antibody, and were counterstained with DAPI as described in 'Materials and methods'. Standard deviation indicated by above line bars. Asterisks indicate statistically significant differences between groups at $P < 0{\cdot}05$ (t-test). (b) Representative images from confocal microscopy analyses for determination of the frequencies of $CD8^+$ granzyme A, $CD4^+$ IFN-γ+ and $CD68^+$ $IL\text{-}10^+$ cells in E-CL and L-CL lesions. Frozen tissue sections were co-stained with FITC-labelled anti-CD8 or anti-CD4 or anti-CD68 monoclonal antibody and PE-labelled anti-granzyme A or anti-IFN-γ or anti-IL-10, and were counterstained with DAPI as described in 'Materials and methods'. The three optical sections for each patient were obtained simultaneously with 363, 488 and 568 nm line of the argon/krypton laser and the proper set of filters. The overlay for CD4 or CD8 or CD68 (green), granzyme A or IFN-γ or IL-10 (red) and DAPI (blue) in E-CL and L-CL lesions is shown. The cells that are double positive for each pair of staining ($CD8^+$ granzyme A^+ or $CD4^+$ IFN-γ+ or $CD8^+$ IL-10+) appear in yellow. These images are representative of each group. Values represent the average ± standard deviation of each group following numeric determination of the percentage of positive cells for the indicated molecules. *Indicates statistically significant differences at $P < 0{\cdot}05$. The bar = 10 μm.

dance with previous studies showing that the higher the frequencies of IFN-γ or TNF-α producing circulating T lymphocytes, the larger the ulcerated lesion of CL patients (17).

Another important biological function of IFN-γ is the induction of cytotoxic activity, which is directly correlated with granzyme A and TIA-1 expression (18–20). Previous studies using murine infection with *Leishmania major* have shown that the frequency of T cells expressing granzyme A was significantly higher in susceptible BALB/c than in resistant C57BL/6 mice (20). Also, we have previously demonstrated a higher frequency of granzyme A expression in lesions from ML patients, as compared to CL (11) and that CL lesions display a high number of $TIA\text{-}1^+$ cells (21). In this work, we observed that the percentages of granzyme A^+ cells and $CD8^+$ granzyme A^+ cells were significantly higher in L-CL than in E-CL lesions. These data suggest that the higher frequency of cells expressing granzyme A is consistent with the extensive tissue destruction observed in L-CL and the main cell subpopulation involved in this process is the $CD8^+$, which were preferentially recruited to L-CL. Thus, execution of cytolytic func-

tion by $CD8^+$ T cells may be an important mechanism of tissue destruction in CL. Moreover, this cytolytic function could also be important for parasite killing. While eliminating the parasite, cell death would occur due to cytotoxicity and thus, tissue destruction and parasite elimination would be concomitant mechanisms. Our previous study showed that ML lesions have higher inflammation and tissue destruction that CL lesion (11) and it has been shown that ML lesions have lower parasite burden (2) that CL, strengthening the idea of a connection between parasite killing and tissue destruction.

In humans, Th1 and Th2 lymphocytes, as well as macrophages, can produce IL-10, a key macrophage deactivating cytokine (22). IL-10 also promotes decreasing of IFN-γ production and has been associated with parasite dissemination, as seen in human visceral leishmaniasis and diffuse CL (23–25). Experimental models of CL have shown that IL-10 produced by naturally occurring T regulatory cells is crucial for persistent *L. major* infection and generation of protective memory (26). On the other hand, we have shown that a lack of response to IL-10 is associated to the hyperactive immune response seen in ML patients (11). Our analysis of *in situ* IL-10 expression showed an increase in the expression of this cytokine in L-CL as compared to E-CL. Previous studies performed by us have shown a positive correlation between IFN-γ and IL-10 expression by circulating leukocytes from CL (27). Here we also observed an increase in IFN-γ and IL-10 expression in L-CL. Moreover, we observed that $CD68^+$ cells are the main source of IL-10 during both stages of the disease. The increase in IL-10 expression at later stages of CL may be important for the control of the inflammatory response and further healing of the lesion. This hypothesis is supported by the fact that ML patients do not respond to IL-10, have more intense inflammation than CL patients, and importantly, macrophages from ML patients do not display co-production of TNF-α and IL-10 as do CL patients (11,28).

Taken together, our data indicate that the progression of CL lesions from nonulcerated to ulcerated states is associated with the presence of an intense inflammatory infiltrate, possibly amplified by the presence of IFN-γ, and the recruitment of potentially cytolytic $CD8^+$ granzyme A^+ cells. These analyses bring new insights towards the understanding of the progression of human CL lesions.

ACKNOWLEDGEMENTS

The authors are grateful to the financing agencies CNPq, FAPEMIG, CAPES and NIH/TMRC. We also would like to thank Drs Alda Cruz, Cristina Guatimosim, Fátima Noronha e Annamaria Vago for critical review of the manuscript and valuable suggestions and Dr José Barbosa Júnior for valuable help with the confocal figures.

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Artigo # 3: Morgan, D.J.[1], Guimaraes, L.H.[2], Machado, P.R.[2], D'Oliveira A.Jr.[2], Almeida, R.P.[2], Lago, E.L.[2], Faria, D.R.[3], Tafuri, W.L.[3], Dutra, W.L.[3], and Carvalho, E.M.[2] – **"Cutaneous leishmaniasis during pregnancy: exuberant lesions and potential fetal complications - Clinical Infectious Diseases"**. Clinical Infect Dis. 2007, 45: 478-482.

Cutaneous Leishmaniasis during Pregnancy: Exuberant Lesions and Potential Fetal Complications

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Cutaneous leishmaniasis affects millions of people worldwide. After observations of atypical lesions in pregnant women at the health centers of Corte de Pedra, Brazil, 9 years of records were reviewed, and 26 pregnant patients were identified. A retrospective case-control study revealed that lesions in pregnant women were much larger than those in nonpregnant patients in an age- and sex-matched group (mean area, 6.08 cm^2 vs. 1.46 cm^2; $P = .008$), and many lesions had an exophytic nature. Despite foregoing treatment until after delivery, response to pentavalent antimony therapy was favorable (rate of cure with 1 course of treatment, 85%). High rates of preterm births (10.5%) and stillbirths (10.5%) were reported. Cutaneous leishmaniasis during pregnancy produces distinct lesions and may have adverse fetal effects.

Worldwide, leishmaniasis affects >12 million people in 88 countries, with a yearly incidence of 2 million cases [1]. The majority of cases are cutaneous leishmaniasis (CL), which is most common in adolescents and young adults from rural areas of extreme poverty [2]—a population with a high fertility rate. Pregnancy is associated with improvement of most inflammatory diseases [3] and an increased susceptibility to many infectious agents, including *Malaria* species [4] and *Listeria monocytogenes* [5]. Moreover, during pregnancy, many infections are associated with adverse fetal outcomes [6]. In the case of leishmaniasis, infection with the viscerotropic form has been described during pregnancy, resulting in vertical transmission and fetal loss when treatment failure occurs [7]. After occasional observations of atypical CL during pregnancy, we retrospectively reviewed all cases of CL and mucocutaneous leishmaniasis (ML) seen at a reference center, identifying gravid patients (a standard screening question). We report clinical aspects of these cases, including lesion size and impact on pregnancy outcome. In addition, a retrospective case-control study comparing lesion size and response to therapy was performed.

Methods. The study was performed at the Corte de Pedra Reference Center for Tegumentary Leishmaniasis in Bahia, Brazil, which has been in operation for >20 years [2]. Yearly, >600 patients are treated for CL and ML at this center.

We manually reviewed charts for all patients with CL or ML who were seen at the referral center during the period 1997–2005, selecting patients who were pregnant and had signs of leishmaniasis. Cases were defined by inclusion criteria of a definite diagnosis of CL or ML as the combination of a compatible lesion and (1) biopsy results showing amastagotes or compatible histopathologic findings, (2) positive culture results from a lesion aspirate specimen, or (3) positive Leishmanin test results. Exclusion criteria were incomplete documentation of pregnancy or of postpartum follow-up. Control subjects were age-matched (within 5 years of age) and sex-matched; the 2 consecutive patients with definite leishmaniasis who were evaluated after each case patient were chosen as control subjects. Probable CL or ML was defined as a compatible lesion with lack of definitive test results.

At the initial visit, patient weight, lesion size and location, and the number of lesions were recorded, and past medical history was evaluated in a standard manner by 1 nurse. All women of childbearing age were evaluated for pregnancy. Leishmanin testing was performed at the initial visit. The initial lesion size was the size of the lesion recorded at the initial visit. The maximum lesion size was the size of the largest documented lesion. All patients found to be pregnant were followed up clinically without treatment for definitive leishmaniasis (i.e., pentavalent antimony compounds) until after delivery.

This study was approved by the Committee of Ethics of The Federal University of Bahia (Salvador, Brazil) and the institutional review board of Weill Medical College of Cornell University (New York, NY). Laboratory studies were performed in the university laboratory using standard commercial techniques. Histopathologic examination was performed in the pa-

Received 12 January 2007; accepted 18 April 2007; electronically published 5 July 2007.

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Clinical Infectious Diseases 2007; 45:478–82

1058-4838/2007/4504-0013$15.00
DOI: 10.1086/520017

thology department. Slides were reexamined by 2 readers (D.R.F. and W.L.T.), who examined each slide for dermal and epidermal changes, the nature of the inflammatory infiltrate, and the presence of amastigote forms. The isolates were characterized at the *Leishmania* Collection of the Oswaldo Cruz Institute (Rio de Janeiro, Brazil) by multilocus enzyme electrophoresis, as described elsewhere [8].

Data were entered into Excel (Microsoft). Lesion areas were calculated as ellipses. The Mann-Whitney *U* test (Wilcoxon rank sum test) and Pearson rank test were performed using Stata, version 7.0 (Stata). $P < .05$ was considered to be statistically significant.

Results. We identified 27 pregnant patients among ~4200 people with suspicion of leishmaniasis. Of the 27 pregnant patients, there were 8 patients with probable leishmaniasis and 18 with definite leishmaniasis. One patient was excluded because of lack of postpartum follow-up information.

The characteristics of 26 patients with leishmaniasis during pregnancy are presented in table 1. Lesions appeared at a mean of 18-weeks gestation (95% CI, 13–21 weeks). Descriptions of vegetative, exophytic, or atypical lesions were found in 11 (42%) of 26 patient charts (figure 1). No manifestations of CL developed prior to pregnancy in any of the patients. Exophytic lesions were nonsignificantly correlated with trimester of pregnancy ($P = .338$, by Pearson rank test; $R^2 = 0.046$).

Lesions showed documented postpartum improvement in 3 patients prior to treatment; nonetheless, these patients subsequently received standard treatment (figure 2). Two patients (7.7%) initiated pentavalent antimony treatment during the first trimester but stopped treatment when pregnancy was discovered (after 7 days of treatment in 1 patient and after 13 days of treatment in the other patient). Both patients continued to have active lesions throughout their pregnancy, and neither woman had an adverse fetal outcome.

Nineteen patients provided information regarding pregnancy complications: 2 (10.5%) of 19 patients delivered preterm, 2 (10.5%) experienced a stillbirth, and 15 (79%) reported normal deliveries (table 1). Cutaneous lesions in patients who expe-

Table 1. Clinical and laboratory findings for 26 pregnant patients with probable and definite leishmaniasis, compared with findings for 36 nonpregnant control subjects with definite cutaneous leishmaniasis.

	Patients with cutaneous leishmaniasis	
Variable	Pregnant patients ($n = 26$)	Nonpregnant control subjects ($n = 36$)
Clinical finding		
Disseminated lesions[a]	3/26 (11.5)	0
Mucosal disease	2/26 (7.7)	0
Recurrent disease	2/26 (7.7)	0
Exophytic lesions documented	11/26 (42.3)	0
Week of pregnancy at lesion appearance (range)	18 (13–21)	NA
Treatment		
Glucantime		
One 20-day course	22/26 (84.6)	29/36 (80.5)
Two 20-day courses	2/26 (7.7)	7/36 (19.5)
Topical paramomycin	2/26 (7.7)[b]	0
No treatment	1/26 (3.4)	0
Azithromycin	2/26 (7.7)[c]	0
Fetal effects		
Preterm birth	2/19 (10.5)	NA
Stillbirth	2/19 (10.5)	NA
Reported normal birth	15/19 (79.0)	NA
Laboratory finding		
Positive culture result	7/11 (63.6)	3/7 (42.8)
Compatible biopsy result	11/11 (100)	4/4 (100)
Amastigotes	2/11 (18)	0

NOTE. Data are no. (%) of patients, unless otherwise indicated. NA, not applicable.

[a] Defined as >10 lesions.

[b] One patient subsequently received 1 course of glucantime therapy.

[c] Both patients received 1 course of glucantime therapy.

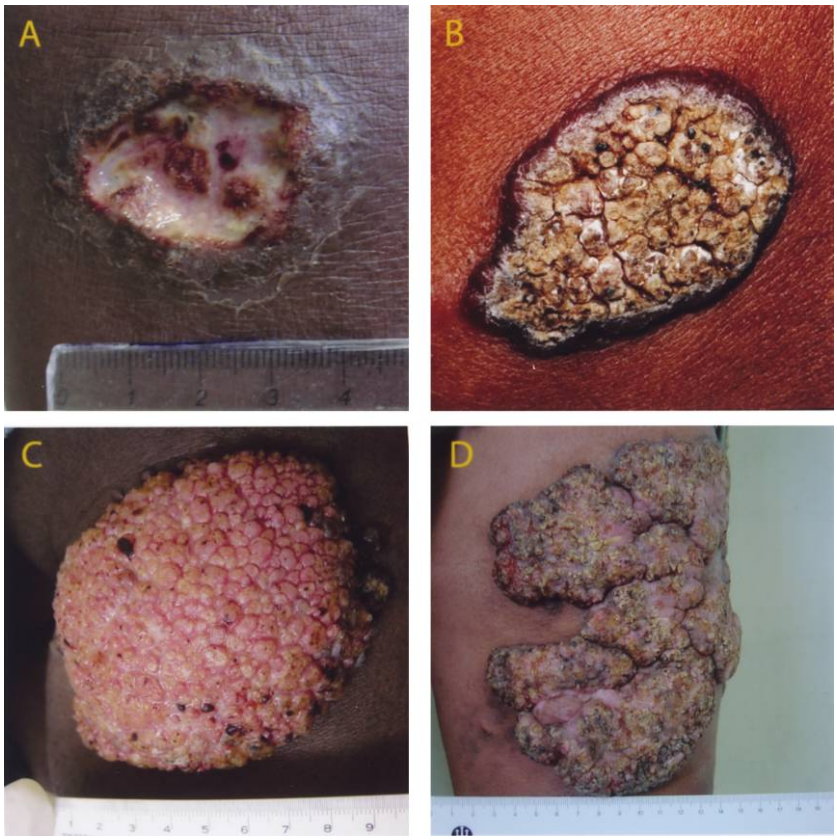

Figure 1. Appearance of cutaneous leishmaniasis during pregnancy. *A,* Typical, well-demarcated ulcer with raised borders on a patient's leg. *B,* Mildly raised, verrucous lesion on a patient's back. Massive, vegetative lesions on a patient's buttock *(C)* and thigh *(D).* Rulers represent centimeters.

rienced a preterm birth or stillbirth did not differ from those in patients who experienced normal deliveries with respect to clinical characteristics or trimester of onset of infection.

Biopsy specimens from pregnant individuals had an inflammatory exudate that was more intense than that typically found in CL, with a predominance of neutrophils, which is not typically observed. Culture results were positive for *Leishmania* species in 7 of 11 patients examined. Five specimens were no longer viable. Two specimens were typed as *Leishmania braziliensis* by multilocus enzyme electrophoresis.

Eighteen patients with definite leishmaniasis were compared with 36 age- and sex-matched control subjects. No difference was found between pregnant patients and nonpregnant control subjects with regard to the median size of the leishmanin delayed-type hypersensitivity test result (induration, 1.77 cm^2 [interquartile range (IQR), 1.13–2.53 cm^2] vs. 1.77 cm^2 [IQR, 0.86–2.98 cm^2]), median duration of lesions prior to the first visit (1.25 months [IQR, 1.0–2.0 months] vs. 1.0 month [IQR, 1.0–2.0 months]), median number of lesions (1.0 lesions [IQR, 1.0–2.0 lesions] vs. 1.0 lesion [IQR, 1.0–2.0 lesions]), and median number of treatment courses (1.0 course [IQR, 1.0–2.0 courses] vs. 1.0 course [IQR, 1.0–2.0 courses]). Both median initial lesion area (6.08 cm^2 [IQR, 1.88–12.01 cm^2] vs. 1.46 cm^2 [IQR, 0.79–3.78 cm^2]; $P = .008$, by Mann-Whitney *U* test) and median maximal lesion area (14.46 cm^2 [IQR, 5.50–54.95 cm^2] vs. 1.46 cm^2 [IQR, 0.79–3.78 cm^2]; $P < .001$, by Mann-Whitney *U* test) were significantly larger among pregnant women than among control subjects.

Discussion. This study demonstrates the influence of pregnancy on the clinical manifestations of CL in a region with *L. braziliensis* transmission. Patients who presented with CL while pregnant had much larger lesions than did nonpregnant women (median initial lesion area, 6.08 cm^2 vs. 1.46 cm^2), despite showing no difference in disease duration. Lesion size was also larger among our patients than among patients seen in a historical cohort from the same region who did not receive treatment (median lesion area, 4 cm^2; IQR, 3–5 cm^2) [9]. In contrast to the typical presentation of a well-demarcated ulcer with raised borders, lesions were frequently of a cauliflower appearance, which raised concern for other diseases, such as chromomycosis, yaws, or neoplasms. Although not previously reported, more-exuberant CL involving other species, including *Leishmania major,* has been observed during pregnancy in Northern Africa (H. Louzir, personal communication).

In a C57BL/6 mouse *L. major* model, larger CL lesions occurred during pregnancy, which correlated with decreased Th1

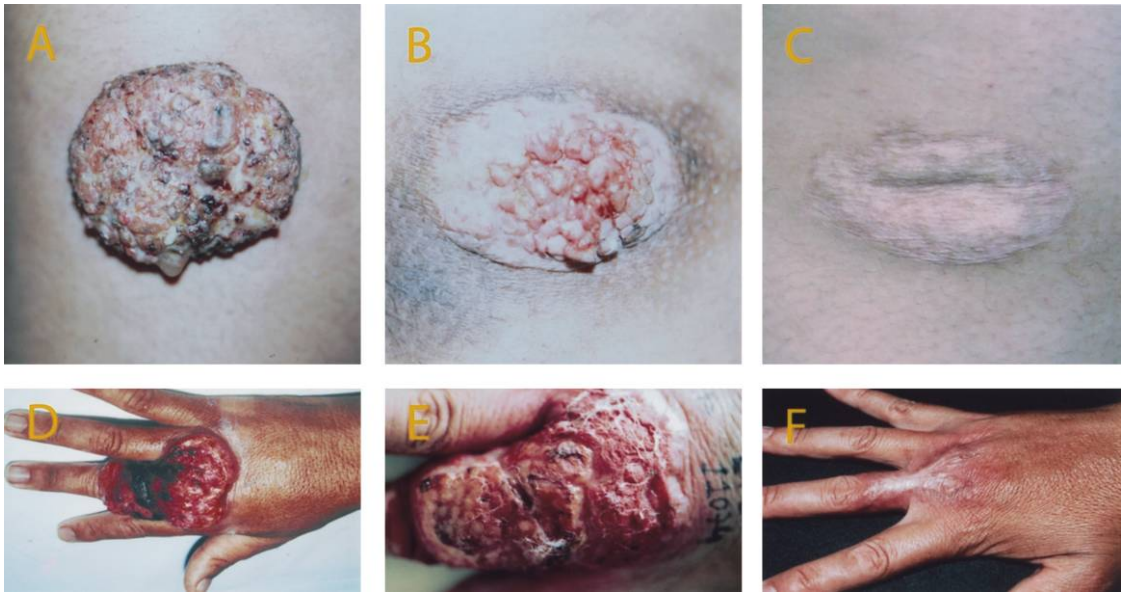

Figure 2. Spontaneous improvement of cutaneous leishmaniasis postpartum. Raised, atypical lesions seen during pregnancy *(A* and *D),* 1–2 months postpartum prior to treatment *(B* and *E),* and after 1 course of pentavalent antimony treatment *(C* and *F).*

cytokine production [10]. The human cell-mediated immune response is altered during pregnancy [11], with an overcompensation immediately after delivery. Because the main histopathological difference in lesions in pregnant women with typical lesions was increased, neutrophilic infiltration and fibrinoid necrosis, differential neutrophil signaling, or activation may play a specific role in development of atypical lesions.

Standard treatment of CL caused by *L. braziliensis* is 20 days of intravenous pentavalent antimony compound, which is potentially abortogenic. Because of this concern, only 2 patients received antimony during pregnancy (the 2 patients stopped treatment after they realized they were pregnant). Of note, these patients experienced full-term deliveries of healthy infants, although their lesions were not cured until after delivery. Because spontaneous cure has been reported to occur after delivery [9], the merit of different treatments cannot be evaluated. No patients in this study were cured while pregnant. No patients developed mucosal disease, although the small sample size limits its generalizations.

An unexpected finding was the high rate of preterm births and stillbirths. Various maternal infections, including malaria [4], listeriosis [5], and visceral leishmaniasis [7], are associated with fetal complications. In a murine model of CL, cutaneous infections increased the rate of implantation failure and fetal reabsorption [12]. In northeastern Brazil as a whole, infant mortality is high (~38 of 1000 infants die per year) [13]. The rates observed in this study are 3-fold higher than the normal rates for the region; however, the small size of this study limits conclusions regarding adverse fetal outcome.

This study is limited, because we did not measure the host immune response, including HIV seropositivity, which could modify disease presentation. In addition, our study was retrospective and, therefore, had no formalized protocol for treatment or data collection.

CL during pregnancy is characterized by larger lesions with a highly atypical, exophytic appearance. No therapy is known to cure disease during pregnancy, although postpartum cure has been found to be complete. CL during pregnancy has a notably different clinical presentation and may increase the risk of fetal complications. It is important for physicians who are caring for patients in regions where disease is endemic to recognize this presentation.

Acknowledgments

We thank E. Cupolillo and the Leishmania Collection of the Oswaldo Cruz Institute (Rio de Janeiro, Brazil), for performing multilocus enzyme electrophoresis identification of *Leishmania braziliensis,* M. Glesby, for statistical and editorial suggestions, and W. Johnson, for research guidance.

Financial support. National Institutes of Health (T32 AI07613 to D.J.M.). E.M.C. is supported by the Brazilian Foundation for Support of Research.

Potential conflicts of interest. All authors: no conflicts.

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Artigo # 4: Gollob, K.J.[1], Antonelli, L.R.[2], Faria, D.R[3], Keesen T.S.L.[1], Dutra, W.O[3] – **"Immunoregulatory mechanisms and CD4-CD8- (double negative) T cell subpopulations in human cutaneous leishmaniasis: a balancing act between protection and pathology"**. Int Immunopharmacology. 2008, 8(10) : 1338-1343.

International Immunopharmacology (2008) **8**, 1338–1343

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Immunoregulatory mechanisms and CD4–CD8– (double negative) T cell subpopulations in human cutaneous leishmaniasis: A balancing act between protection and pathology

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Received 22 October 2007; received in revised form 14 March 2008; accepted 17 March 2008

KEYWORDS
Cutaneous leishmaniasis;
Cytokines;
T lymphocytes;
Th1;
NK T cells;
Double negative T cells

Abstract

Cellular immune responses directed against protozoan parasites are key for controlling pathogen replication and disease resolution. However, an uncontrolled, or improperly controlled, response can be deleterious to the host in terms of both allowing for the establishment of pathology, as well as less effective establishment of memory responses. Human cutaneous leishmaniasis is a disease caused by the infection with *Leishmania* spp. following a bite from the sandfly, the natural vector of this disease. Tens of millions worldwide are currently infected with *Leishmania* and no effective vaccines have been developed to date. In the face of the complexity presented by the interaction between a host (humans) with the parasite, *Leishmania*, and the fact that this parasite is inoculated by another complex, biologically active, vector, the sandfly, it is clearly important to study the immunoregulatory mechanisms that are induced in humans naturally infected by this parasite if we hope to develop effective vaccines and immunotherapeutic treatments in the future. Our laboratory has focused over the years on the study of the local and systemic T cell response during the first episode of cutaneous leishmaniasis suffered by individuals before they undergo antimony treatment. The goal of this review is to briefly outline our findings with hopes of putting our most recent studies concerning the dichotomy between alpha/beta TCR and gamma/delta TCR expressing, CD4–CD8– (double negative–DN) T cells in the context of a balanced immune response against *Leishmania* and to discuss the implications of these findings toward our understanding of human leishmaniasis.

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doi:10.1016/j.intimp.2008.03.016

1. Human leishmaniasis — a complex disease resulting from the host–parasite–vector interaction

Human leishmaniasis is caused by the infection with the obligate intracellular protozoan parasite, *Leishmania*, and is expressed as one or more of several possible clinical forms including principally the visceral and tegumentary diseases (localized cutaneous, mucosal or disseminated) [1]. Several factors influence the progression of an infected individual into these clinical forms of disease including, but not limited to, host genetics (protective and susceptibility factors), host environment and previous immunological experience, parasite genetics (unique species and sub-strain characteristics leading to tropism differences, different virulence factors and immunogenicity differences), and the influence of the vector, phlebotomine sandflies. Obviously, the elucidation of the role that all of these factors have on the progression of disease is a daunting task requiring the work of specialists in many fields of research, and here we will focus our discussion on the immune response of individuals infected with *Leishmania braziliensis* and presenting with cutaneous disease. The majority of the results we discuss were obtained by studying peripheral blood mononuclear cells (PBMC) or lesions from individuals during their first manifestations of cutaneous disease as identified by the presence of an ulcerated lesion. The choice to study this clinical form is several fold, based on both practical considerations and on scientific grounds. Practically, it is the most prevalent clinical form in Brazil and scientifically, it is the clinical form that, amongst the clinical forms caused by *L. braziliensis* infection, is the most benign and leads to a fairly high rate of cure and subsequent immunologic protection amongst the population [2–4]. Thus, by studying individuals with this clinical form, we can have an idea as to the nature of the immune response that leads to the formation of a lesion, followed by control of the infection, and long lived immunity in a high percentage of individuals.

2. Protection vs. pathology vs. memory...a three-way balancing act

The study of the cellular immune response and determination of cytokine profiles in both murine models and in humans has been determined over the years through the application of a number of quantitative and semi-quantitative methodologies. These methods have ranged from single-cell cytoplasmic staining of cytokines within specific cell populations to ELISA from culture supernatants or body fluids, and quantitative PCR. In tissues investigators have used in situ detection of cytokines by confocal or fluorescent microscopy and cytokine specific probes. For quite some time it has been known that a biased Th1 response in animal models of *Leishmania major* infection lead to control of parasite replication and cure of the animal, while a biased Th2 response leads to an exacerbated infection [5–7]. This picture has grown in complexity over the years and we now appreciate nuances of the model including the important role of IL-10 in regulating the response [8,9]. Over the years, dozens of studies in animal models have helped to elucidate the cellular and molecular mechanisms involved during the immune response against *Leishmania* spp, and have pointed to the importance of many factors that influence the progression of disease following infection with *Leishmania* [10–14]. These factors vary from the strain of the infecting parasite, to the method of inoculation, and host genetics, to name a few. Thus, these models continue to provide insights towards understanding basic mechanisms of immunity and memory, as well as clarify factors involved in complex interactions between host and pathogen. However, interpretation and extrapolations of results obtained from animal models to the human realm should be performed carefully due to complex differences between the systems.

It has recently been demonstrated in a number of animal models that cellular immune responses resulting in a sterile cure of *L. major* infection, lead to poor long term protection or memory responses [11,15,16]. Moreover, in human cutaneous disease caused by *L. braziliensis* infection, we have found that an exacerbated, Th1 response (determined using flow cytometry, ELISA, and confocal microscopy) is associated with severe pathology in mucosal disease [17–19], and even in relatively controlled responses in cutaneous disease, higher frequencies of IFN-gamma producing CD4+ T cells, as determined using flow cytometry, are associated with larger lesions [20]. Thus, it seems likely that a balance between responses that induce leishmaniacidal activities, those that could induce pathology, and those that maintain persistence, may be the most desirable response in the natural human response directed against *L. braziliensis* infection (Fig. 1).

Using single-cell cytokine staining to identify directly subpopulations of lymphocytes producing immunoregulatory cytokines in human cutaneous disease caused by *L. braziliensis*, we demonstrated that indeed, CD4+ Th1 cells are the major source of IFN-gamma with very little to undetectable CD4+ T cells producing IL-4 or IL-5. In this same study we also identified CD4+ T cells and monocytes as important sources of IL-10 [21] (Fig. 1). Interestingly, however, upon further study we found that IL-10 production by lymphocytes was not associated with lower frequencies of TNF-alpha or IFN-gamma producing T cells, but rather, the two were positively correlated, indicating a coordinate regulation in the frequency of CD4+ T cells producing cytokines capable of activating leishmaniacidal activities (IFN-gamma and TNF-alpha) and those producing the down regulatory cytokine, IL-10 [22]. This finding points to an active immunoregulation of cells with protective and potentially pathogenic potentials, together with IL-10 producing T cells. The immediate biological activity of *Leishmania* antigen specific T cells to produce IL-10 with down-modulatory activities on host monocytes was also demonstrated, and thus, while there is not a direct correlation between higher frequencies of IL-10 producing lymphocytes and lower frequencies of IFN-gamma producing T cells, there is a greater decrease in monocyte activation (as measured by TNF-alpha production) in individuals with higher frequencies of antigen specific, IL-10 producing lymphocytes [22]. Interestingly, it was recently suggested that a lower IFN-gamma/IL-10 ratio could be associated with a more favorable prognosis amongst individuals infected with *L. braziliensis* [23]. Thus, co-production of IL-10 could be critical for both controlling pathology, as well as regulation of an "over-active" Th1 response. As mentioned above, the failure to regulate the Th1 response could lead to the poor formation of long lived, effective memory. Whether in the human disease these cells producing

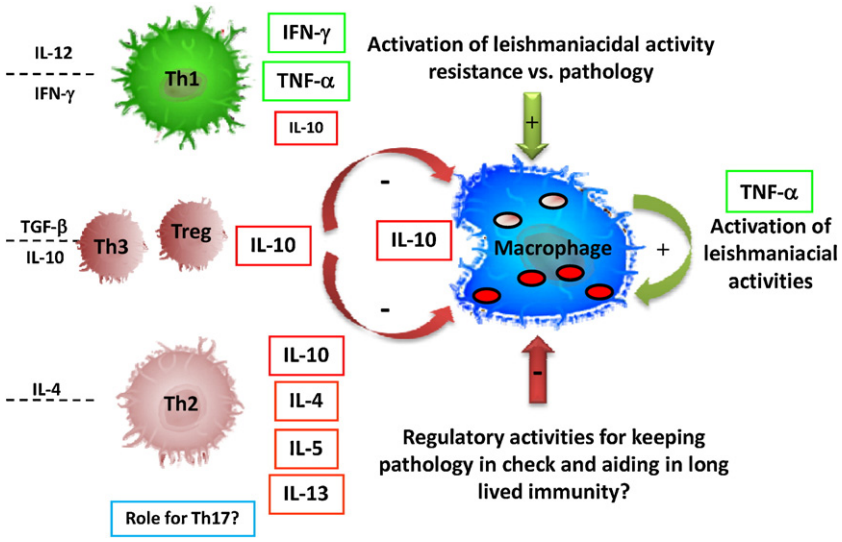

Figure 1 CD4+ T lymphocyte balance and activation of macrophages for control of *Leishmania*. Classically, Th1 and Th2 CD4+ T cell subsets act to control the leishmaniacidal activity of host monocytes and macrophages. While this model still explains many aspects of the cellular immune response in human and mouse models of leishmaniasis, several other subpopulations are also involved in the regulation of effective leishmaniacidal immune responses. The cytokines on the far left are important for differentiation of the given cell populations and the cytokines to the right of each T cell subset are key immunoregulatory cytokines that can be produced by the given subset. Importantly, IL-10, a down-modulatory cytokine, can be produced by several T cell subpopulations (Th2, Th1, Treg and Th3), as well as by infected or activated macrophages. The same is true for TNF-alpha which can be produced by both T cells and activated macrophages. In addition, it is clear that these responses must be controlled for the limitation of pathology and likely to allow persistence and thus, maintenance of an effective memory response. The possible role of Th17 cells in the induction of pathology or protection in human leishmaniasis has not been determined, but represents another possibly important population in the dynamics of the response following *Leishmania* infection. Several cellular sources of important immunoregulatory cytokines exist, including not only subpopulations of CD4+ T cell, but also the macrophage itself and other leukocytes and granulocyte populations not highlighted in this figure.

IFN-gamma and IL-10 are the same cell population, or different cells, has not yet been determined. As pointed out in our study, it has been recognized for many years that human Th1 cells can also produce IL-10 as described by Sornasse et al. [24], and it now appears that mouse T cells can do the same as recently shown following *L. major* infection [25,26]. Thus, it seems clear that active human cutaneous leishmaniasis is associated with a multifaceted regulated Th1 type response with components that will aid in the activation of host macrophages for activation of leishmaniacidal activities, as well as cytokines that aid in the control of this response (Fig. 1). Many other cell types and cytokines not discussed in this review could clearly play an important role in the immunoregulation of human cutaneous disease including Th17 cells, Treg and Th3 populations, some of which have been investigated in human and animal models of disease [10,27]. The future elucidation of the role that these populations play in protection and pathology is clearly important to understanding the overall regulatory mechanisms of human leishmaniasis.

While CD4+ T cells are clearly an important source of cytokines for activation of leishmaniacidal activities, it is equally clear that several other cell types play an important role in establishment of the overall cytokine microenvironments important for both initiation and differentiation of effective T cell immune responses, as well as for local immune responses at the site of infection [18]. On this note, when studying the cellular sources of immunoregulatory cytokines from cutaneous leishmaniasis patients, we found that the second most prevalent cell type responsible for IFN-gamma production was lymphocytes negative for both CD4 and CD8 [21]. Based on this finding, further studies were carried out to determine the possible role of these cells in immunity and pathology [28].

3. CD4−CD8− (double negative—DN) T cells in human cutaneous leishmaniasis: alpha/beta DN T cells likely have a role in protection and pathology, while gamma/delta DN T cells could play a more important role in negative regulation

In addition to classic CD4+ and CD8+ T cells, there is a minority subpopulation of T cells that express neither CD4 nor CD8, thus termed double negative (DN) T cells. Within this DN T cell population several subpopulations can be found. In the broad sense, they contain T cells expressing either the gamma/delta or alpha/beta TCR, and within each of these populations, several subpopulations can be defined. Amongst the alpha/beta TCR DN T cells in humans, studies have identified cells of both negative-regulatory nature [29,30], as well as associated with several autoimmune disorders [31–33]. Of further interest are the alpha/beta TCR+ DN T cells that are restricted to CD1 presented antigens, of which some express a restricted TCR and often recognize lipid antigens presented by one of the CD1 family

of molecules. These T cells often express a restricted, or invariant, TCR, and have been classified by many as invariant NK T cells. These are of particular interest due to their potent cytokine producing activity and highly activated profile as reviewed by others [34–37]. In particular, CD1 restricted NK T cells were recently implicated in the host immune response to *Leishmania* [38] and a MHC class II restricted subset of DN T cells in protection to mycobacteria infection in mice [39].

In our work by Bottrel et al., we determined that DN lymphocytes were the second most prevalent cell type producing IFN-gamma in human cutaneous leishmaniasis, and that this IFN-gamma production was seen after short term cultures with media alone, as well as after stimulation with soluble *Leishmania* antigen (SLA) [21]. Given the fact that we identified these cells solely on their positioning within the lymphocyte gate and their lack of expression of CD4 and CD8, further studies were required to determine if they were in fact T cells, and if so, determine what TCR they expressed, either alpha/beta or gamma/delta. In non-infected individuals, earlier studies had demonstrated that over 80% of peripheral blood DN T cells expressed the gamma/delta TCR. Moreover, several studies in human leishmaniasis had been performed that demonstrated a possible role for gamma/delta T cells [40–42]. Strikingly, our findings published by Antonelli et al. [28] first identified that the great majority of DN T cells in the circulation from cutaneous leishmaniasis patients express the alpha/beta TCR. In fact, this population makes up approximately 75% of the DN T cells in cutaneous leishmaniasis patients, with the remainder expressing the gamma/delta TCR. In contrast, as had been shown earlier, we also observed that non-infected controls, have an inverted ratio with alpha/beta TCR expressing DN T cells making up at most 20% of the DN T cells in the circulation, and the great majority express the gamma/delta TCR. Upon studying the activation state of the DN T cells, it became clear that they express a hyper-activated *ex vivo* profile based on the expression of CD69 and the expression of IFN-gamma and

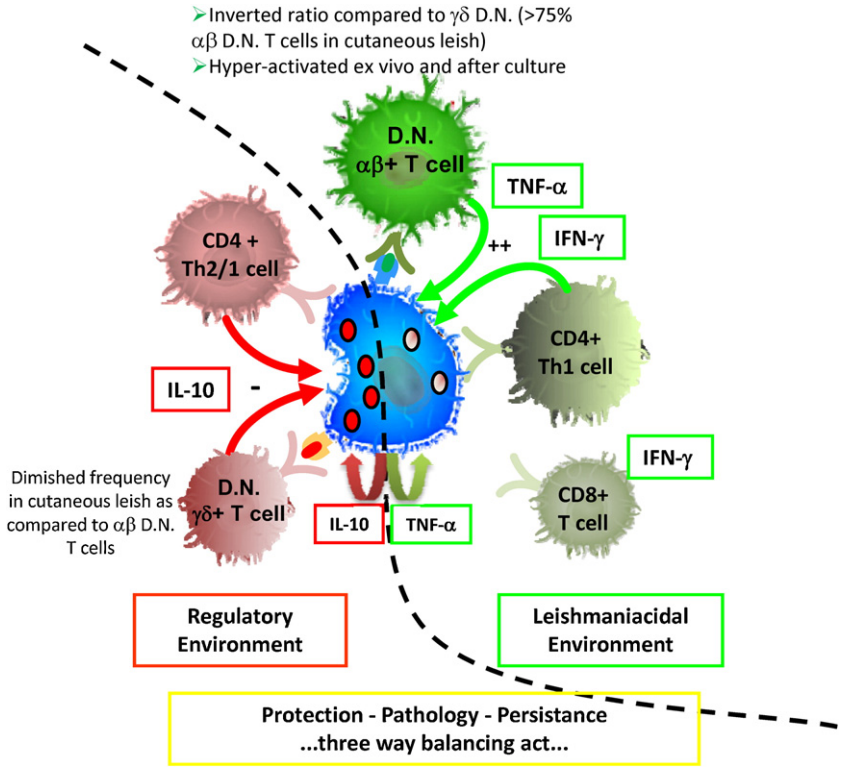

Figure 2 αβ+ DN T cells contribute to a leishmaniacidal immune environment while, γδ + DN T cells appear to contribute more towards a down regulatory environment in human cutaneous leishmaniasis caused by *L. braziliensis*. Several cellular sources contribute to the overall immune environment in human cutaneous leishmaniasis. Depending on the dynamics of the interaction between these cell populations, their relative frequency, and the timing of their appearance and activation, each cell type could not only influence the effector cellular immune response, but also shape the cytokine microenvironment necessary for subsequent T cell differentiation. IFN-gamma and TNF-alpha are both key for optimal macrophage activation and for creating a microenvironment beneficial for driving Th1 cell development. Both CD4+ Th1 cells and αβ+ DN T cells are important sources of these cytokines in cutaneous disease. IL-10 is an important cytokine for down regulation of inflammatory responses and controlling macrophage activation. It comes from several sources in human cutaneous disease including CD4+ T cells, DN T cells (the balance in γδ+ DN T cells is skewed toward IL-10 as compared to the αβ+ DN T cells) and monocytes/macrophages. For several years it has been known that IL-10 is a poor indicator of Th1 and Th2 subsets in humans, and in cutaneous disease we see a co-regulation of IL-10 production along with IFN-gamma or TNF-alpha. The overall balance of these cell types and the cytokines they produce will determine if an immune response is effective at controlling the parasite and limiting pathology, while allowing for some degree of persistence which may be important for long lived and effective memory. The diagram is based on work by our group and represents the balance between subpopulations taken from PBMC of infected cutaneous leishmaniasis patients.

TNF-alpha after 20 h culture with media alone. This profile of hyper-activation *ex vivo*, coupled with cytokine production in the absence of exogenously added antigen, adds to the concept that these cells could be highly involved in an active response following infection by *Leishmania*, and may play an important role in the innate response as well. Moreover, this profile is different from what is seen for both CD4 or CD8 cells, where we see antigen induced activation and cytokine production, but very little production *ex vivo* or after media alone cultures [21]. Thus, the DN T cell subpopulation, and in particular, the alpha/beta DN T cell subpopulation seems to fit into a highly activated T cell subpopulation producing biologically relevant cytokines for the activation of monocytes and macrophages.

As a means of determining their physiologic role we further characterized the cytokine profiles of the alpha/beta and the gamma/delta DN T cells and demonstrated that the alpha/beta DN T cell subpopulation expresses high IFN-gamma or TNF-alpha to IL-10 ratios (approximately 20 and 10, respectively) after stimulation with SLA. Interestingly, these ratios are approximately twelve to fifty times higher than that seen for the gamma/delta DN T cell subpopulation for IFN-gamma/IL-10 and TNF-alpha/IL-10, respectively. Thus, the role of the alpha/beta DN T cells is likely skewed toward activation of leishmaniacidal activity and possibly in pathology (if not regulated), while the gamma/delta DN T cells would be more active as negative regulators of this activity (Fig. 2). Finally, it is noteworthy that in media alone, the alpha/beta DN T cells display a more balanced cytokine profile with a diminished IFN-gamma/IL-10 ratio. Thus, it is possible that different subpopulations of alpha/beta DN T cells are being activated in the conditions of *ex vivo* and media alone as compared to after stimulation with SLA. It is clear that DN NKT cells will be contained within the alpha/beta DN T cell population and further studies are being carried out to determine their role in the overall profile we have identified.

Clearly, the identification of what antigen presenting molecule(s) and antigen(s) are responsible for activation of the DN T cells in human leishmaniasis is an important step for understanding the role these cells play in induction of protective or pathogenic immune responses, as well as their possible role in the maintenance and generation of memory responses.

Acknowledgements

We would like to thank the collaboration over the past ten years of Dr. Edgar Carvalho and his group at Hospital Edgard Santos, UFBA, in Salvador, Bahia, Brazil, without which the studies described in this overview of our work would not have been possible. We would also like to thank the following funding agencies for support of our work over the years: WHO/TDR, CNPq, FINEP, FAPEMIG, and NIH-NIAID.

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CAPÍTULO III - DISCUSSÃO

A participação de diferentes populações celulares na resposta imune durante a LC, assim como os mecanismos que levam à cura ou patologia têm sido extensivamente explorados. No entanto, uma análise comparativa da contribuição relativa de cada subpopulação *in situ*, comprometida com a produção de diferentes mediadores em duas formas clínicas distintas da leishmaniose, cutânea e mucosa, começou a ser estudada com o desenvolvimento desta tese com a utilização de microscopia confocal. Para um melhor conhecimento dos fatores envolvidos com o direcionamento e balanço da resposta imune do hospedeiro, neste trabalho foram realizadas avaliações que podem ser divididas em três etapas principais: (1) análise da expressão de IFN-γ, TNF-α, IL-10, granzima A, iNOS e o receptor para IL-10 em associação com os marcadores fenotípicos CD4, CD8 e CD68 em lesões de pacientes com LC e LM, (2) análise da expressão de IFN-γ, IL-10 e granzima A em associação com os marcadores fenotípicos CD4, CD8 e CD68 em lesões de pacientes com LC com 15 (cutâneos iniciais) ou 60 (cutâneos tardios) dias de infecção (3) análises comparativa de aspectos clínicos e histopatológicos de pacientes grávidas infectadas por *L. braziliensis.* Discutimos, ainda, o papel de células T CD4-CD8- nos mecanismos imunoregulatórios na LC.

No Brasil, a espécie *Leishmania braziliensis* é a principal responsável pela geração de duas formas clínicas diferentes da leishmaniose em humanos: a forma cutânea e a forma mucosa. A cura espontânea da leishmaniose é observada em inúmeros pacientes com LC, os quais podem, inclusive, desenvolver imunidade protetora contra o parasita. Acredita-se que a resposta imune celular caracterizada pela produção predominante de citocinas do tipo Th1, como IFN-γ, esteja envolvida com a cura (Tapia *et al*., 1993; Bomfim *et al*., 1996). Entretanto, aproximadamente 3% dos pacientes previamente acometidos pela forma clínica cutânea desenvolvem a forma clínica mucosa, com ou sem o envolvimento concomitante de lesões cutâneas (Castes *et al*., 1984).

A patogênese da LM tem sido associada à hiperativação de células T e a conseqüente exacerbação da resposta inflamatória, envolvendo extensa destruição tecidual (Carvalho *et al*., 1985). Embora o exato mecanismo patogênico da LM não tenha sido totalmente esclarecido, as análises de caracterísiticas imunológicas de CMSP de pacientes acometidos por esta forma clínica sugerem que uma hiper-reatividade celular causada pela falta de controle da resposta imune leve ao estabelecimento da doença mucosa (Bacellar *et al*., 2002).

Com o objetivo de melhor entender os fatores que controlam o desenvolvimento e a manutenção de respostas imunes protetoras e patogênicas durante a leishmaniose humana, realizou-se um estudo detalhado, determinando o fenótipo celular, a produção de citocinas e outros mediadores e suas fontes produtoras, comparando fragmentos de lesões de pacientes infectados por *L. braziliensis*, apresentando as formas clínicas cutânea e mucosa.

Uma importante citocina na resposta imune à *Leishmania* é o IFN-γ, produzida, principalmente, por células T $CD4^+$, $CD4^-CD8^-$(DN), $CD8^+$ e por células NK estimuladas por IL-12. A citocina IFN-γ é a principal ativadora de macrófagos, que produzirão TNF-α, citocina indutora da atividade leishmanicida que agirá nessas células infectadas (Murray *et al*., 1983; Trinchieri, 1994). Alguns trabalhos, analisando a reatividade de células mononucleares do sangue periférico (CMSP) de pacientes com LC e LM através de técnicas de citometria de fluxo, RT-PCR e Elisa, detectaram altos níveis de IFN-γ (Bottrel *et al*., 2001; Bacelar *et al*., 2002; Ribeiro-de-Jesus *et al*., 1998; Carvalho *et al*., 1985). A produção de IFN-γ também foi detectada em biopsias de lesões de pacientes com LC e LM, sugerindo a importância dessa citocina nestas manifestações clínicas da leishmaniose tegumentar (Tapia *et al*., 1993; Caceres - Dittmar *et al*., 1993; Pirmez *et al*., 1993).

Nossos resultados demonstraram que as lesões de pacientes com a forma clínica mucosa possuem maior número de células produtoras de IFN-γ quando comparadas com as lesões de pacientes com a forma clínica cutânea. Existe uma hipótese, baseada no alto nível de

IFN-γ encontrado em lesões de pacientes com forma clínica mucosa, de que o baixo parasitismo tecidual observado em lesões de pacientes com a forma clínica mucosa pode ser explicado pela ativação celular mediada por IFN-γ, já que esta citocina é uma importante indutora da atividade citotóxica da célula. Embora as citocinas inflamatórias IFN-γ e TNF-α sejam capazes de inibir o crescimento do parasita no início da infecção (Trinchieri *et al*., 1989), está claro que respostas exageradas podem ser deletérias para o hospedeiro. Assim, uma falta de controle dos níveis de IFN-γ e TNF-α dirigindo funções efetoras podem aumentar a doença mucosa, ao invés de levar à cura das lesões.

A fim de determinarmos as fontes produtoras de IFN-γ, em lesões de pacientes com ambas as formas clínicas da leishmaniose, foram realizadas análises comparativas da freqüência de células T $CD4^+IFN\text{-}\gamma^+$, $CD8^+IFN\text{-}\gamma^+$ e análise de correlação entre a freqüência de células T $CD4^+IFN\text{-}\gamma^+$, $CD8^+IFN\text{-}\gamma^+$ *versus* o número total de células expressando IFN-γ.

Nas análises comparativas nós observamos um maior número de células T $CD4^+IFN\text{-}\gamma^+$ em lesões mucosas do que em lesões cutâneas. Além disso, observamos em ambas as formas clínicas uma correlação positiva entre as células T $CD4^+IFN\text{-}\gamma^+$ e o número total de células expressando IFN-γ. Este dado nos indica que estas células T $CD4^+$ são a principal fonte produtora de IFN-γ em lesões decorrentes da infecção por *L. braziliensis*. Embora as células T $CD4^+$ contribuam com a maior parte da produção total de IFN-γ, outras fontes celulares produtoras de IFN-γ foram detectadas nas lesões, entre elas os linfócitos T $CD8^+$ e os linfócitos T DN. Em lesões cutâneas, linfócitos T $CD8^+$ contribuem com aproximadamente 38% da produção total de IFN-γ, enquanto que os linfócitos T DN contribuem, em média, com 22% da produção total dessa citocina. Já na forma mucosa, observamos que células T $CD8^+$ contribuem com 43%, enquanto que linfócitos T DN contribuem com 9%. Assim, nós demonstramos que a produção de IFN-γ em lesões de pacientes com LC e LM conta com a

participação de mais de uma fonte celular, sendo a maior contribuição de células T $CD4^+$, seguida por células T $CD8^+$ e, finalmente, por linfócitos T DN.

Bottrel e colaboradores (2001), avaliando as fontes produtoras de IFN-γ em CMSP de pacientes com LC através da citometria de fluxo, observaram que a principal fonte produtora de IFN-γ são as células T $CD4^+$, seguida de linfócitos T DN e, finalmente, por linfócitos T $CD8^+$. Numa análise comparativa aos nossos dados, houve uma inversão da expressão de IFN-γ por células T DN e $CD8^+$ na forma cutânea. Diferente da constante freqüência das células T DN no sangue periférico de indivíduos sadios, as freqüências observadas nos pacientes com leishmaniose variam amplamente. Considerando que estas células apresentam intenso perfil de ativação, a inversão das células T DN com as células $CD8^+$ poderia ser explicada pela morte celular induzida por ativação. De fato, estudo em camundongos e humanos infectados com *Leishmania* mostram aumento de Fas solúvel, entretanto o aumento da expressão de Fas *in situ* foi detectado somente em humanos (Kowrany *et al.*, 2001). Outra possibilidade seria um recrutamento mais expressivo de células T $CD8^+$ para o sítio da lesão quando comparado ao recrutamento de células T DN. De fato, esta última possibilidade nos parece mais verídica, uma vez que o número de células T DN está aumentado no sangue (Bottrel *et al*., 2001; Antonelli *et al.*, 2007) e reduzido na lesão (Faria *et al.*, 2005). Avaliações semelhantes ainda não foram concluídas em CMSP de pacientes com LM, mas estão sendo realizadas por nosso grupo.

Outra importante citocina inflamatória é o TNF-α, produzido por linfócitos T, células NK e macrófagos (Van Deuren *et al*., 1992). O papel desta citocina tem sido relacionado à ativação de células que albergam a *Leishmania* causando a diminuição da carga parasitária. Além disso, estudos anteriores mostraram que TNF-α está presente em lesões de indivíduos com as formas cutânea e mucosa da leishmaniose (Melby, *et al.*, 1994; Lessa *et al*., 2001). No entanto, a contribuição das células $CD68^+$ para sua produção *in situ* não tinha sido

demonstrada, antes deste trabalho. Observou-se que o número de células $CD68^+TNF\text{-}\alpha^+$ e o número de células expressando TNF-α foram similares em ambas as formas clínicas da doença. Além disso, nossos resultados mostram que aproximadamente 78% das células $CD68^+$ estão comprometidas com a expressão de TNF-α em lesões de pacientes com LC e 80% das células $CD68^+$ estão comprometidas com a expressão de TNF-α em lesões de pacientes com LM, demonstrando o comprometimento dessa população celular com a produção dessa importante citocina inflamatória.

Da-Cruz e colaboradores em 1996 (Da-Cruz *et al.*, 1996) detectaram altos níveis de TNF-α em pacientes com LM. Após o tratamento e a cura, esses níveis foram reduzidos, sugerindo que a produção excessiva dessa citocina seja um dos fatores associados à progressão da doença. O envolvimento de TNF-α na exacerbação da resposta inflamatória resultando no agravamento das lesões dos pacientes com LM foi também sugerido por Lessa e colaboradores em 2001 (Lessa *et al.*, 2001). Estes autores utilizaram a pentoxifilina, um inibidor de TNF-α, como adjuvante no tratamento com antimônio obtendo sucesso na cura de pacientes com a forma clínica mucosa resistente ao tratamento com antimônio isolado. Apesar de não ter-se observado aumento na produção de TNF-α nas lesões de pacientes com LM quando comparado com LC, observou-se alta expressão de IFN-γ nas lesões dos primeiros pacientes. Ainda assim, TNF-α pode estar envolvido no agravamento da doença uma vez que tem sido descrito na literatura que IFN-γ induz a expressão do receptor de TNF-α, favorecendo a ação desta citocina (Tannenbaum *et al.*, 1993).

Como mencionado acima, na leishmaniose humana as citocinas inflamatórias IFN-γ e TNF-α tem papel determinante no controle e resolução da infecção inicial. A ativação de macrófagos por estas citocinas inflamatórias leva à produção de óxido nítrico (NO) resultando na morte do parasita (Liew *et al.*, 1990). Apesar desta essencial função do NO durante a leishmaniose, a falta de controle de sua produção pode causar dano a tecidos (Grisham *et al.*,

2002; Napoli, 2002). Um de nossos interesses neste trabalho foi verificar se existia diferença na expressão de NO nos pacientes com LM e LC. Como a medida de NO *in situ* é inviabilizada, escolhemos uma medida indireta da presença da molécula, que é a medida de expressão da iNOS. iNOS é a forma indutível da enzima óxido nítrico sintase, cuja expressão é correlacionada aos níveis de NO (Aktan, 2004). Nossos resultados mostram uma maior freqüência de células expressando iNOs em lesões de pacientes com LC quando comparadas com lesões de pacientes com LM. Desta forma, esses resultados sugerem que, nas lesões de pacientes com a forma clínica cutânea, a destruição dos parasitas pode estar relacionada com a participação dessa molécula. Já o baixo parasitismo tecidual, observado em lesões de pacientes com a forma clínica mucosa, poderia ser explicado pela ativação celular, por citocinas inflamatórias, relacionada à indução da produção de reativos do oxigênio.

Outro importante mecanismo responsável pelo controle do parasita no sítio da lesão é a citotoxicidade mediada por células T. A citotoxicidade mediada por células T $CD8^+$ ocorre através da liberação de grânulos citotóxicos, incluindo a granzima A. Nosso trabalho mostra que lesões de pacientes com LM apresentam maior freqüência de células expressando granzima A quando comparadas com as lesões de pacientes LC. De forma interessante, dados da literatura mostram que respostas "exageradas" podem ser deletérias para o hospedeiro. Assim, a falta de controle desta resposta pode levar ao agravamento da doença, mediado por uma citotoxicidade celular exacerbada, como é observado em lesões de pacientes com LM.

Trabalhos da literatura demonstram experimentalmente a participação das células T $CD8^+$ na imunidade anti-*Leishmania* (Muller, 1991; JO *et al*.,1989). Na leishmaniose humana, porém, o papel dessas células ainda permanece obscuro. A citocina inflamatória IFN-γ também é uma importante indutora da atividade citotóxica de linfócitos T $CD8^+$, que está diretamente correlacionada à granzima A e TIA-1 (Anderson *et al.*, 1990; Barral-Netto *et al*., 1995; Machado *et al*., 2002). Já foi descrito que, em pacientes com a forma clínica cutânea, as

lesões possuem um grande número de células TIA-1^+ e que esta população é heterogênea, composta principalmente de linfócitos T $CD8^+$ e células NK (Anderson *et al*., 1990; Barral-Netto *et al*., 1995; Machado *et al*., 2002). De maneira interessante, os nossos dados mostram que as células T $CD8^+$ são as principais fontes de granzima A em lesões de pacientes com a forma clínica cutânea. No entanto, em pacientes com LM, a maior produção de granzima A é atribuída às células $CD8^-$, indicando que outras populações celulares estejam comprometidas com esta produção. Apesar das células NK produzirem granzima A, estas encontram-se escassas em lesões de pacientes com LM, indicando que as células T $CD4^+$ sejam as principais fontes deste mediador citotóxico nas lesões de pacientes com a forma mucosa analisadas nesse trabalho (Esterre *et al*., 1994; *apud* Ribeiro-de-Jesus *et al*., 1993). De fato, as citocinas inflamatórias IFN-γ e TNF-α, presentes abundantemente em lesões de pacientes com LM, podem favorecer o desenvolvimento de células T $CD4^+$ que possuem funções citotóxicas (Mosmann *et al*, 1991).

Como demonstrado por nós, o número de células expressando IL-10 foi similar em ambas as formas clínicas analisadas, embora o infiltrado inflamatório seja mais intenso em LM do que LC. Além disto, Bacellar e colaboradores, 2002, demonstraram que o bloqueio *in vitro* de IL-10 com anticorpos monoclonais foi capaz de restaurar a produção de IFN-γ em culturas de células de pacientes com LC, estimuladas com SLA, mas não de pacientes com LM. Esses dados nos levaram a postular que uma deficiência na expressão do receptor dessa citocina reguladora estaria relacionada à deficiência na resposta a essa citocina por células de pacientes com a forma mucosa da doença. Avaliamos então a expressão do receptor de IL-10, observando uma freqüência reduzida de células expressando esse receptor em lesões de pacientes com LM. A intensidade de expressão do receptor de IL-10 também foi menor em lesões de pacientes com LM do que em lesões de paciente com LC. Como mencionado anteriormente, as células presentes nas lesões dos pacientes com LM são altamente

comprometidas com a produção de citocinas inflamatórias. De fato, Michel e colaboradores, 1997, mostraram que citocinas pró-inflamatórias induzem uma regulação negativa da expressão do receptor de IL-10. Nossos resultados sugerem que IFN-γ produzido por células presentes na lesão pode estar reduzindo a expressão do receptor de IL-10 e, dessa forma, comprometendo a regulação da resposta em pacientes com a forma clínica mucosa da leishmaniose. No entanto, pacientes com a forma clínica cutânea também apresentam um grande número de células expressando IFN-γ, mas apresentam uma maior freqüência de células expressando o receptor de IL-10 quando comparado com as lesões de pacientes com a forma clínica mucosa. Trabalhos da literatura já demonstraram que as manifestações clínicas da leishmaniose dependem das complexas interações entre a resposta imunológica do hospedeiro, que está ligada a fatores genéticos, e as características de virulência do parasita. Salhi e colaboradores recentemente, avaliando lesões de pacientes com LC, observaram que monócitos do sangue e células T $CD4^+$ reguladoras são as principais fontes produtoras de IL-10 na lesão. Estes mesmos autores encontraram que indivíduos com polimorfismo de IL-10-819C/T possuem maiores lesões e conseqüentemente são maiores produtores dessa citocina (Salhi *et al*., 2008). Desta forma, acreditamos que pacientes com a forma clínica mucosa também possam apresentar polimorfismo para expressão tanto da citocina IL-10 quanto para o seu receptor. No futuro, o estudo deste polimorfismo poderá trazer novas informações que contribuirão para o melhor entendimento dos mecanismos envolvidos na patologia da leishmaniose.

Portanto acreditamos que, em ambas as formas clínicas, a migração de células envolvidas na reposta anti-parasita para os sítios das lesões estão comprometidas com a expressão de IFN-γ e TNF-α, o que pode estar exercendo funções de ativação de macrófagos e culminando com a morte dos parasitas. Já em lesões de pacientes com LM, os achados de que essas citocinas são sintetizadas em excesso e de que há uma expressão reduzida do

receptor para IL-10 sugerem que esses eventos possam estar envolvidos na geração de respostas patogênicas ao hospedeiro (Figura 2).

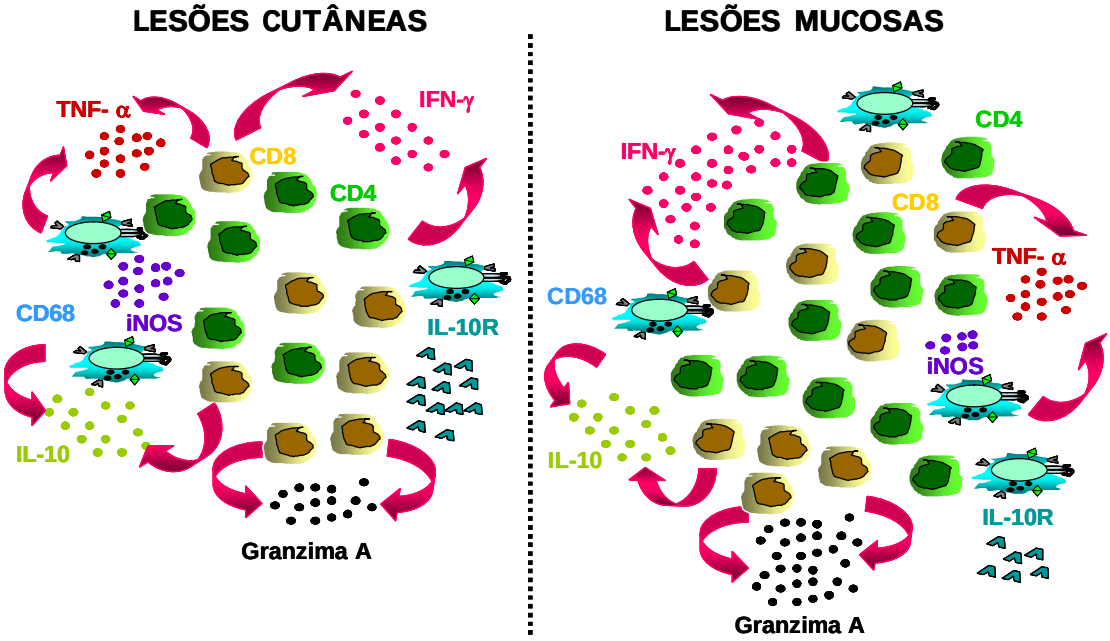

Figura 2- **Interpretação dos resultados observados em lesões de pacientes com LC e LM decorrentes da infecção por *L. braziliensis*.** A figura mostra o processo desencadeado pela infecção, após a ativação celular. Observou-se um infiltrado inflamatório mais intenso e um maior número de células expressando IFN-γ e granzima A em lesões de pacientes com LM, comparando-se com LC. Além disso, observou-se uma baixa expressão do receptor de IL-10 (IL-10R) em lesões de pacientes com LM.

Portanto, nossas análises certamente contribuirão para um entendimento dos mecanismos imunorregulatórios envolvidos com a patologia das leishmanioses cutânea e mucosa em humanos e serão fundamentais para um posterior desenvolvimento de intervenções relacionado à modulação de populações celulares específicas.

Como comentado anteriormente, a leishmaniose tegumentar americana pode levar ao desenvolvimento de manifestações clínicas diferentes que podem ser classificadas como: LC, mucocutânea e cutânea difusa. Dentre estas, a forma clínica mais freqüente é a LC localizada,

tendo como característica o desenvolvimento de lesões únicas ou múltiplas na derme, que usualmente respondem à terapia antimonial (Carvalho *et al*., 1985; Carvalho *et al*., 1994; Cabello *et al*., 1995). Pode haver comprometimento de linfonodos regionais (Barral *et al*., 1995; Souza *et al.*, 1995; Weigle *et al*., 1996), levando a um quadro de linfoadenopatia granulomatosa, que geralmente antecede a lesão cutânea. O período de incubação da *Leishmania* pode levar poucos dias ou meses. Uma pequena pápula vermelha aparece no sítio da picada, mostrando uma infiltração com presença de linfócitos, macrófagos e granulócitos (Weigle *et al*., 1996; Murray *et al*., 2005). A cura espontânea pode ocorrer entre dois meses e um ano, contudo lesões secundárias podem surgir (Dowlati *et al*., 1996; Cunningham *et al*., 2002).

Neste trabalho, nós realizamos análise comparativa do infiltrado inflamatório em lesões de pacientes com LC com aproximadamente 15 dias de infecção (cutânea inicial) e com aproximadamente 60 dias de infecção (cutânea tardia), com a finalidade de compreender os mecanismos envolvidos na progressão ou cura desta patologia.

Alguns autores já demonstraram que o infiltrado inflamatório de lesões de pacientes com as formas clínicas cutânea é composto por células T, com uma baixa ocorrência de macrófagos (Castes *et al*., 1983; Esterre *et al*., 1994; Faria *et al*., 2005). De forma interessante, os cortes de lesões de pacientes com a forma clínica cutânea inicial apresentaram um maior número de neutrófilos e granulócitos, quando comparados com lesões tardias. Esses dados sugerem maior participação de células da fase aguda da inflamação nas lesões iniciais quando comparadas às lesões tardias. Entretanto, a freqüência de células polimorfonucleares em ambos os grupos é baixa em relação ao infiltrado inflamatório total (menos de 2%). Dessa forma, em ambos os grupos a inflamação é caracterizada por predomínio absoluto de células mononucleares. É possível que a interação entre o parasita e as células teciduais na fase inicial da doença estimule a produção de citocinas e quimiocinas que ainda estimulem o

recrutamento de células polimorfonucleares mesmo 15 dias após o surgimento das lesões. Recentemente, Zandbergen e colaboradores (2004) demonstraram que os leucócitos polimorfonucleares são as primeiras células fagocíticas a encontrarem as formas promastigotas de *Leishmania* na pele. Neutrófilos infectados secretam altos níveis da quimiocina MIP-1β que atraem macrófagos. Macrófagos humanos fagocitam prontamente polimorfonucleares infectados apoptóticos *in vitro*, facilitando o estabelecimento da infecção nestas células (Zandbergen *et al*., 2004). A baixa freqüência de leucócitos polimorfonucleares encontrada nas lesões cutâneas sugere predomínio de recrutamento de células mononucleares para formação do infiltrado inflamatório em resposta à infecção pela LC. A significativa redução das freqüências de células polimorfonucleares nas fases tardias das lesões poderia ser explicada pela alteração nos padrões de produção de citocinas e quimiocinas, alterando a expressão de moléculas de adesão e consequentemente favorecendo ainda mais o recrutamento de células mononucleares do sangue em detrimento das células polimorfonucleares. Pelo fato das células polimorfonucleares apresentarem curto tempo de vida, cerca de 24 a 48 horas, mesmo em ambientes inflamatórios, a redução do recrutamento dessas células a partir do sangue levaria ao rápido decréscimo de suas freqüências nos tecidos.

Análises comparativas em CMSP de pacientes com LC, apresentam uma alta resposta proliferativa quando estimuladas com antígenos de *Leishmania*, associada a altos níveis de produção de IFN-γ e TNF-α (Bottrel *et al*., 2001; Bacellar *et al*., 200; Bittar et *al*., 2007). Nossos resultados mostram que a intensidade do infiltrado inflamatório é maior em lesões de pacientes com a forma clínica cutânea tardia quando comparados com lesões iniciais, sugerindo um maior recrutamento celular no decorrer da infecção. Estes resultados em conjunto, nos levam a pensar sobre a hipótese de que a injúria provocada pelo parasita aumenta a freqüência de células expressando IFN-γ e TNF-α e que a alta freqüência de células no sítio inflamatório pode ser devido a ações diretas ou indiretas de IFN-γ e TNF-α;

uma vez que estas citocinas são capazes de induzir a expressão de moléculas de adesão, levando a um maior recrutamento celular para esse sítio de infecção (Gemmell *et al*., 1997).

Durante uma infecção parasitária, apesar do sistema imune controlar o número de parasitas e algumas vezes conferir resistência à reinfecção, este também pode induzir patologia associada ao parasitismo (Brener &Gazzinelli, 1997; Urban *et al*., 2005). As células T desempenham um papel muito importante nesses eventos tanto diretamente, por mediarem respostas celulares, quanto indiretamente, na regulação da produção de anticorpos. Analisando, de forma comparativa, a freqüência de células T $CD4^+$ e $CD8^+$ entre os diferentes tempos de infecção observamos que a freqüência de células $CD8^+$ é mais elevada em lesões de pacientes com a forma clínica cutânea tardia em relação à forma clínica cutânea inicial. O mesmo não foi observado para as células $CD4^+$. Da Cruz e colaboradores em 1994, avaliaram a freqüência de células T $CD4^+$ e $CD8^+$ após cinco dias de cultura na presença de antígenos de *L. braziliensis*. A comparação dos resultados obtidos antes e após o tratamento dos pacientes revelou um aumento na porcentagem de células T $CD8^+$ e uma diminuição da freqüência de células T $CD4^+$ após o tratamento. Dessa forma, estes resultados sugerem que as células T $CD8^+$ podem estar envolvidas no mecanismo de ativação ou morte celular, já que estas células têm um papel tanto na produção de citocinas inflamatórias quanto na produção de moléculas citotóxicas na LC.

Nossos resultados mostraram que a freqüência de células expressando IFN-γ é maior em lesões de pacientes com a forma clínica cutânea tardia quando comparado à forma clínica cutânea inicial. Assim, IFN-γ é uma importante citocina para o recrutamento e conseqüente progressão da doença.

As células T $CD4^+$ são essenciais para a resolução da infecção por *Leishmania*, em função de sua capacidade de produzir tanto citocinas inflamatórias quando modulatórias. A principal contribuição dessas células, sobretudo no início da infecção, está relacionada com a

produção de IFN-γ que, juntamente com TNF-α sob a influência de IL-12 e IL-18, age na ativação de macrófagos infectados pelos parasitas (Wei *et al*., 1999). Nossos dados indicam que as células $CD4^+$ são a principal fonte produtora de IFN-γ nos diferentes tempos de infecção, aumentando a expressão desta citocina durante a progressão da doença. Estes resultados estão de acordo com dados publicados anteriormente que avaliaram as fontes produtoras de IFN-γ *in situ* e *in vitro* de pacientes com LC e LM, observaram que a principal fonte produtora de IFN-γ são as células T $CD4^+$ (Bottrel *et al*., 2001; Faria *et al*., 2005). No entanto, duas outras populações de células T contribuem de maneira importante na produção de IFN-γ na leishmaniose – células T $CD8^+$ e células T $CD4^-CD8^-$ (DN) (Bottrel *et al*., 2001; Faria *et al*., 2005).

A população de células T DN expressa em sua superfície o receptor de células T (TCR-αβ ou γδ), mas não expressa os co-receptores CD4 e CD8. Estas células já foram identificadas no timo e órgãos periféricos de camundongos e de humanos (Crispe *et al*., 1987; Toribio *et al*., 1988; Groh *et al*., 1989; Londei *et al*., 1989). Em humanos, elas possuem características de policlonalidade (Brooks *et al*., 1993). Embora represente uma freqüência muito baixa na maioria dos indivíduos saudáveis, as células T DN apresentam-se em números aumentados na ocorrência de diversas patologias, sobretudo em algumas doenças auto-imunes e imunodeficiências (Shivakumar *et al*., 1989; Sieling *et al*., 2000; Ohga *et al*., 2002).

Recentemente, foi demonstrado que células T αβ DN de pacientes com LC, estimuladas com SLA, apresentaram freqüências mais altas de ativação e produção de IFN-γ e TNF-α. Já as células T γδ DN apresentaram uma maior expressão de IL-10, o que poderia indicar o potencial imunorregulador dessas células (Antonelli *et al*., 2006; revisto por Gollob *et al*., 2008). Dessa forma, fica claro que apesar da baixa freqüência no sangue periférico das células T DN de pacientes com leishmaniose, as células T αβ DN expressam um perfil inflamatório mais intenso que as células T DN dos indivíduos sadios. O intenso perfil de ativação

apresentado por células T DN sugere que estas desempenham importante papel nas respostas imunes durante a infecção por *Leishmania* (Antonelli *et al.*, 2004; Gollob *et al.*, 2008).

Neste trabalho, nós demonstramos que embora as células T $CD4^+$ contribuam com a maioria da produção total de IFN-γ, outras fontes celulares produtoras de IFN-γ foram detectadas nas lesões de pacientes com LC e LM, entre elas os linfócitos $CD4^-CD8^-$. Nós detectamos uma contribuição significativa, uma média de 21% em cutâneos e 10% em mucosos da produção total de IFN-γ, por parte dos linfócitos $CD4^-/CD8^-$. Recentemente, Antonelli e colaboradores (2006) demonstraram que as células T DN de pacientes com leishmaniose cutânea expressam um perfil de prévia estimulação antigênica, ativação recente e forte potencial inflamatório quando comparadas às células T DN de indivíduos não apresentando a doença. Além disso, as células T DN dos pacientes apresentaram freqüências mais altas de células T ativadas e células produzindo IFN-γ e TNF-α. Entretanto, células destes mesmos pacientes expressaram freqüências mais baixas de células produzindo IL-4 e IL-10 que as células T $CD4^+$ e $CD8^+$ dos mesmos pacientes. Em conjunto, esses dados sugerem que as células T DN podem estar envolvidas no desenvolvimento de respostas imune na leishmaniose humana, auxiliando inicialmente no desenvolvimento de um ambiente propício para o desenvolvimento de uma resposta Th1, necessária para a diminuição da carga parasitária. No entanto, vale ressaltar que um melhor entendimento das subpopulações de células T DN se torna fundamental para uma melhor compreensão destas células na proteção e patologia da leishmaniose humana, uma vez que os tipos de citocinas por ela produzidos, além de contribuir para a resolução da infecção, estão associados à patologia nas formas mais graves da doença.

Os linfócitos T $CD8^+$ medeiam diferentes mecanismos efetores no combate às infecções, como a secreção de quimiocinas e/ou a indução de atividade citolítica (linfócitos T citotóxicos) (Andersen *et al*., 2006). Neste trabalho, observamos que lesões de pacientes com

LC tardia apresentam uma maior freqüência de células granzima A^+ e células $CD8^+$granzima A^+ quando comparadas com as lesões de pacientes com a forma clínica cutânea inicial. Portanto a crescente destruição tecidual observada ao longo da infecção pode estar relacionada com a alta expressão de granzima A. De forma interessante, os nossos dados também mostram que as células T $CD8^+$ são as principais células responsáveis pela expressão de granzima A em lesões de pacientes com a forma clínica cutânea tardia. Estes dados sugerem que células T $CD8^+$ citotóxicas podem ter uma importante função na destruição de tecidos em lesões de pacientes com a forma clínica cutânea tardia, uma vez que a expressão exacerbada pode ser deletéria para o paciente.

Neste trabalho, observamos que o número de células expressando IL-10 é maior em lesões de pacientes com a forma clínica cutânea tardia em relação à forma clínica cutânea inicial. Devemos mencionar que, em fases iniciais da LC localizada, Rocha e colaboradores (1999) observaram uma diminuição da produção de IFN-γ e uma elevada síntese de IL-10 por CMSP de pacientes com leishmaniose cutânea inicial, indicando que altos níveis de IL-10 poderiam inibir a produção de citocinas inflamatórias, desativando atividade macrofágica ou afetando a apresentação de antígenos pela diminuição de expressão de moléculas co-estimulatórias e MHC-II (Ding *et al.*, 1993; de Waal *et al.*, 1991). Além disso, a presença concomitante de altas freqüências de células produzindo IFN-γ e TNF-α indica um bom prognóstico para a doença, e a presença de IL-10 sugere a possível modulação dos efeitos patológicos ocasionados pelas primeiras (Bittar *et al*., 2007; Gomes-Silva *et al.*, 2007). Recentemente, Anderson e colaboradores em 2005 demonstraram que a transferência da cepa *L. major* de lesões de pacientes com a forma persistente da doença para camundongos resistentes levou à falta de controle da doença, relacionando esse fenômeno com ausência de modulação da resposta Th1 ao invés de falta de resposta do tipo Th2 (Anderson *et al*., 2005). Estes dados sugerem que a IL-10 é importante no controle da resposta Th1, como também na

imunopatologia observada na leishmaniose humana, estando envolvida na modulação e progressão nas fases iniciais e, principalmente tardias em pacientes com LC.

Quanto à fonte produtora de IL-10 no sítio da lesão, observamos que as lesões de pacientes com a forma clínica cutânea inicial e tardia apresentam freqüências semelhantes de células $CD68^{+}IL\text{-}10^{+}$. Estes dados sugerem que as células $CD68^{+}$ são cruciais no controle das manifestações clínicas na LC e que estão envolvidas não só nas fases iniciais, mas em todo o decorrer da infecção.

A análise de múltiplos parâmetros nas lesões de pacientes com LC com 15 ou 60 dias de infecção permitiu a determinação das fontes celulares de citocinas, indicando que células T $CD4^{+}$ são as principais produtoras de IFN-γ, as células $CD8^{+}$ são as principais produtoras de granzima A e macrófagos são importantes fontes de IL-10. Estas informações são críticas quando consideramos intervenções que envolverão modulações específicas nas populações celulares.

Em resumo, os nossos dados mostram que tanto com 15 dias quanto com 60 dias de infecção a inflamação é do tipo crônica com predomínio absoluto de células mononucleares e que o perfil de produção de citocinas foi diferentes nos tempos de infecção. Entretanto, a interação entre o parasita e as células teciduais na fase inicial da doença estimula a produção de citocinas e quimiocinas, alterando a expressão de moléculas de adesão e conseqüentemente favorecendo um maior recrutamento de células mononucleares do sangue em detrimento das células polimorfonucleares nas fases mais tardias da infecção. Assim, a intensidade de inflamação (infiltrado inflamatório) é maior em lesões de pacientes com a forma clínica cutânea tardia quando comparados com lesões iniciais. Além disso, a elevada frequência de células CD8+ expressando granzima A é correlacionada com a destrução tecidual observada na fase tardia da doença (Figura 3).

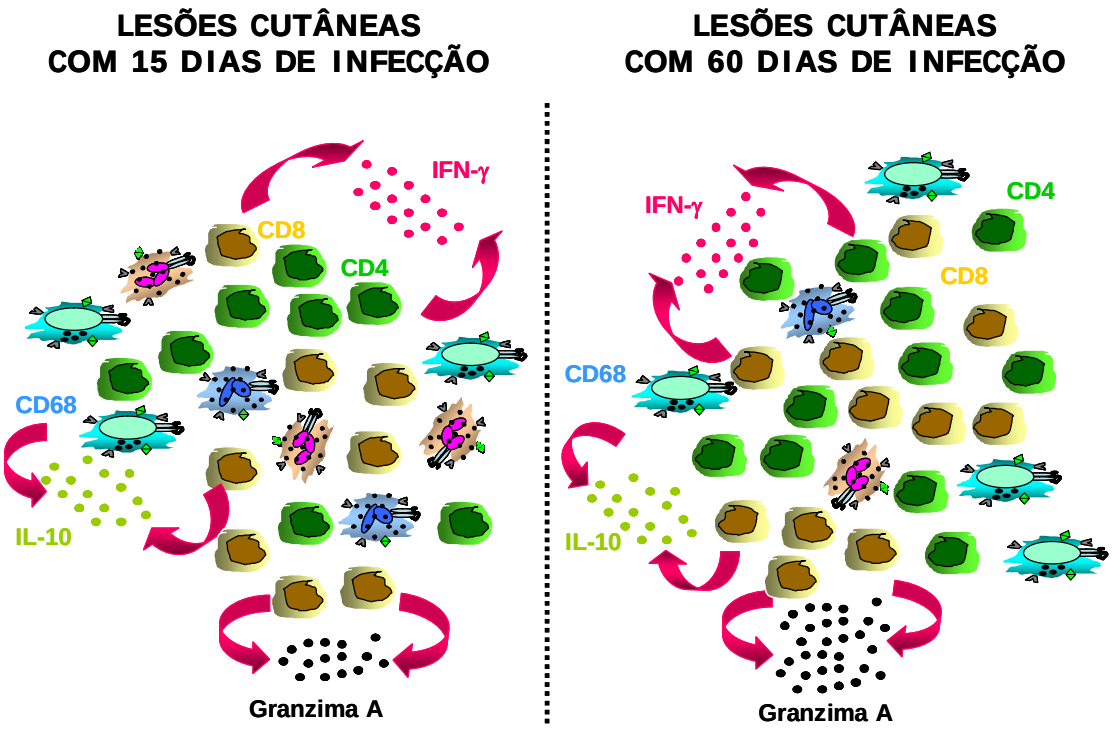

Figura 3- **Interpretação dos resultados observados em lesões de pacientes com LC com 15 (cutâneos iniciais (LC-I)) ou 60 (cutâneos tardios – (LC-T)) dias de infecção por *L. braziliensis*.** A figura mostra o processo desencadeado pela infecção, após a ativação celular. Observou-se um elevado número de células polimorfonucleares nas lesões de pacientes com LC-I e o recrutamento preferencial de células T $CD8^+$ em LC-T. Além disso, observou-se uma maior expressão de granzima A em lesões de LC-T.

Como mencionado anteriormente, um bom prognóstico para LC está relacionado ao predomínio da resposta Th1, que é caracterizada pela produção de IFN-γ e TNF-α e pela ativação de macrófagos (Liew *et al*., 1999). Pacientes infectados por *L. braziliensis* que apresentam este perfil podem evoluir para cura espontânea.

A concentração de citocinas do tipo Th1 juntamente com as citocinas Th2 também pode ser crítica em certas fases da gravidez. As citocinas são altamente pleiotrópicas e multipotentes, pois uma mesma citocina pode mediar efeitos diferentes em diferentes concentrações e em diferentes períodos da gestação. Algumas citocinas tipo Th1, como o TNF-α, podem ter papel importante no período peri-implantação e no estabelecimento da

gravidez, mediando a comunicação entre células do embrião e células uterinas maternas (Tartakovsky & Ben-yair, 1991; Haimovici & Anderson, 1993; Chard, 1995). Citocinas do tipo Th2, como IL-4, induzem a produção de HCG pelo trofoblasto e o HCG estimula a produção de progesterona pelo corpo lúteo na gravidez. A progesterona estimula a secreção de citocinas Th2 e inibe a secreção de citocinas Th1(Piccinni *et al*., 1995; Saito, 2000).

Assim, as citocinas do tipo Th2 provavelmente contribuem para a manutenção da gravidez, controlando os sistemas imune e endócrino (Saito, 2000). Como os níveis de citocinas são controlados geneticamente, tal controle pode ter um papel importante no curso de uma resposta imune durante o processo reprodutivo (Pociot *et al.*, 1993).

Krishnan e colaboradores (1996) mostraram que camundongos fêmeas infectados durante o período de gravidez apresentam uma redução na resposta do tipo Th1 e, com isso, a resolução da lesão causada pela *Leishmania* parece ficar comprometida (Krishnan *et al*., 1996). Em um trabalho recente em que fui co-autora (Morgan *et al*., 2007), foi observado que pacientes grávidas que desenvolveram a doença apresentaram lesões maiores quando comparadas com lesões de pacientes com LC não grávidas (média 6,08 cm^2 *versus* 1,46 cm^2; p=0,008). Também foi observado que as grávidas com LC apresentam um percentual de 10,5 de nascidos a pré-termo e potenciais complicações fetais. Estes resultados sugerem que a redução na resposta do tipo Th1 em grávidas infectadas por *Leishmania* pode estar relacionada ao desenvolvimento de lesões mais graves além de complicações na gravidez.

Realizamos análises histopatológica em lesões de pacientes grávidas apresentando a forma clínica cutânea da leishmaniose. Em geral, os cortes apresentaram uma intensa resposta inflamatória. Esta resposta é caracterizada pela presença de tecido conjuntivo denso do tipo exsudativo com infiltrado inflamatório difuso com linfócitos, plasmócitos, macrófagos e rico em neutrófilos. Estes dados sugerem que o agravamento das lesões em grávidas pode ser decorrente de uma ativação ou sinalização diferencial, principalmente em neutrófilos.

Sabe-se que na LC há um predomínio da resposta do tipo Th1, o que contrasta com a resposta encontrada em mulheres no período gestacional, caracterizada por predominância de células T do tipo Th2. Em pacientes grávidas infectadas por *L. braziliensis* encontramos características clínicas indicativas de ambas as respostas, como o tamanho aumentado das lesões, condizendo com a resposta Th2 e nascimentos prematuros, característicos de uma resposta Th1. Portanto, acreditamos que a infecção por *Leishmania* em pacientes grávidas possa ocasionar um desequilíbrio entre as respostas do tipo Th1 e Th2, levando ao agravamento das lesões cutâneas nestas pacientes (Figura 4). Estes estudos continuam em nosso laboratório, pretendendo esclarecer a presença das citocinas no sítio da inflamação em grávidas e não-grávidas com leishmaniose.

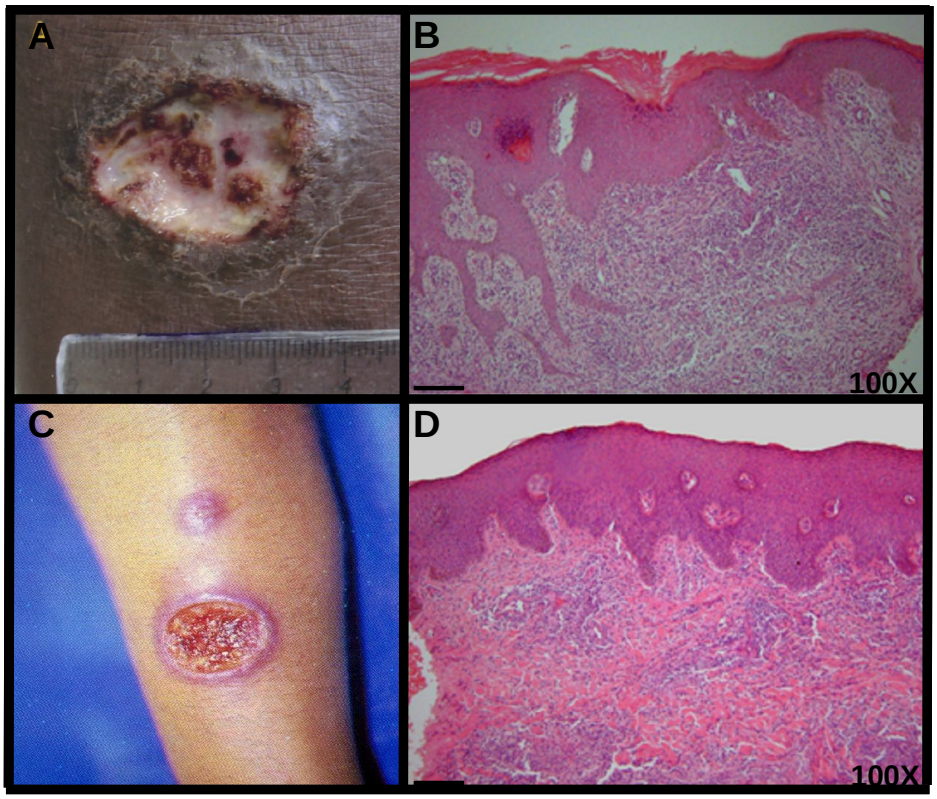

Figura 4- Análise macroscópica (A e C) e microscópica (B e D) de lesões de pacientes com leishmaniose cutânea grávidas (A e B) ou não-grávidas (C e D) decorrentes da infecção por *L. braziliensis*. Cortes corados em H&E. Barras 30µm.

Embora esteja claro que a produção de mediadores imunológicos seja fundamentalmente responsável pelo estabelecimento e cura da leishmaniose, os mecanismos envolvidos na progressão e/ou resolução da doença não foram completamente estabelecidos. Especificamente, a geração de células parasito específicas, a cinética de produção de citocinas pró e anti-inflamatórias e suas fontes produtoras, bem como potencial patológico e imunorregulatório, evidenciado também por outras moléculas, podem contribuir amplamente para o entendimento da resposta imunológica do hospedeiro na proteção contra *Leishmania*. A utilização de técnicas de imunofluorescência para a detecção de citocinas e suas fontes produtoras poderão auxiliar na elucidação do intricado mecanismo de estabelecimento da leishmaniose.

Em conjunto, este trabalho demonstra uma complexa rede composta por diferentes tipos celulares e seus produtos durante a leishmaniose humana. A necessidade das interações celulares para a manutenção de uma resposta imune efetiva e ao mesmo tempo benigna ao hospedeiro pode ser demonstrada claramente na avaliação das lesões de pacientes com a forma mais branda da doença. Por outro lado, a falta de modulação da resposta inflamatória, exemplificada pela baixa expressão do receptor de IL-10, leva ao desenvolvimento de lesões agressivas observadas em pacientes com a forma clínica mucosa. Além disso, a caracterização das alterações causadas nas lesões de pacientes com leishmaniose em resposta a diferentes tratamentos foi descrita neste estudo. Assim, estes dados chamam a atenção para uma nova abordagem no conhecimento da imunopatogênese da leishmaniose humana, podendo futuramente levar a novas abordagens terapêuticas.

CAPÍTULO IV - CONCLUSÕES

Como conclusões nossos resultados indicaram que:

A baixa expressão do receptor de IL-10 in situ pode estar relacionada a uma resposta imune exacerbada encontrada em pacientes com LM, indicando que esses pacientes apresentam uma habilidade reduzida de modular a reatividade inflamatória.

A maior freqüência de células expressando granzima A correlaciona-se com a maior destruição tecidual observada em lesões de pacientes com LC tardia.

A LC durante a gravidez está correlacionada com lesões maiores, compostas por um infiltrado inflamatório exudativo mais intenso e com um maior número de neutrófilos, como também complicações no decorrer da gestação, gerados por conseqüência do desequilíbrio entre as respostas do tipo Th1 e Th2.

As células T $\gamma\delta$ ou $\alpha\beta$ DN podem estar envolvidas tanto na proteção como patologia da leishmaniose humana, já que os tipos de citocinas (IFN-γ e IL-10) por elas produzidas, além de contribuir para a resolução da infecção, estão associados à patologia nas formas mais graves da doença

CAPÍTULO VI - REFERÊNCIAS BIBLIOGRÁFICAS

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