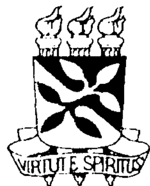

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IMUNOREGULAÇÃO E RESPOSTA IMUNE SISTÊMICA E TECIDUAL NA LEISHMANIOSE MUCOSA

LUCAS PEDREIRA DE CARVALHO

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LUCAS PEDREIRA DE CARVALHO

Orientador: **Prof[a]. Dra. AMÉLIA RIBEIRO DE JESUS**

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LUCAS PEDREIRA DE CARVALHO

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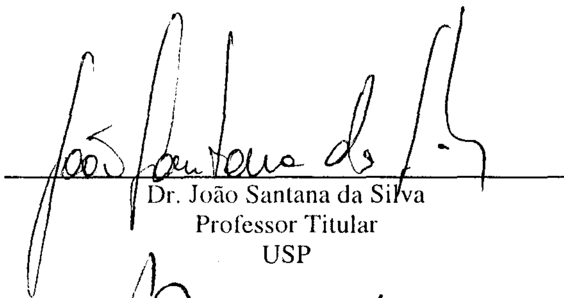

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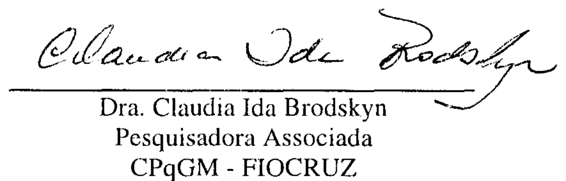

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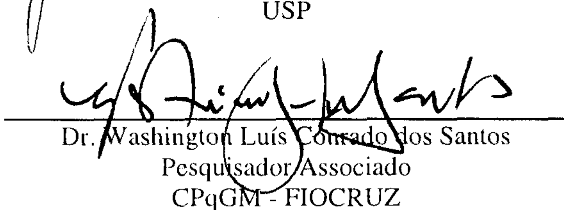

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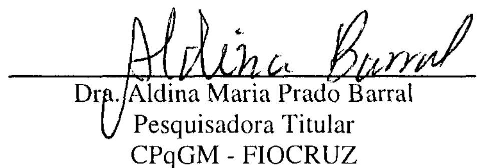

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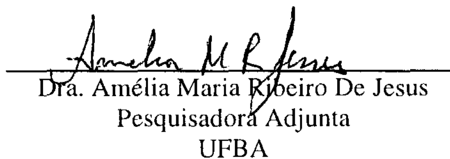

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SUMÁRIO

RESUMO

ABSTRACT

LISTA DE ABREVIATURAS

1 INTRODUÇÃO 01

1.1 Introdução Geral 01

1.2 O parasito e transmissão da doença 02

1.3 Aspectos epidemiológicos e formas clínicas 03

1.4 Aspectos imunológicos da leishmaniose 06

1.4.1 Modelos experimentais de leishmaniose 06

1.4.2 Imunopatoênese da leishmaniose tegumentar 08

1.4.3 Resposta imune a antígenos específicos de Leishmania 13

1.4.4 Resposta imune a nível tecidual 16

2 OBJETIVOS 18

3 ARTIGO 1 19

4 ARTIGO 2 20

5 ARTIGO 3 21

6 ARTIGO 4 22

7 DISCUSSÃO 23

8 CONCLUSÕES 35

9 PERSPECTIVAS FUTURAS 36

10 REFERÊNCIAS BIBLIOGRÁFICAS 37

11 ANEXO 55

LISTA DE ABREVIATURAS

CD	"Cluster of differentiation" (marcador de membrana de células)
CMSP	Células mononucleares de sangue periférico
HLA	Antígeno de histocompatibilidade leucocitário
HTLV-1	Vírus linfotrópico de células T humanas tipo 1
IFN-γ	Interferon-γ
IL	Interleucina
IL-10R	Receptor de interleucina-10
MHC	Complexo de histocompatibilidade principal
NK	Células matadoras naturais
NOD	Diabético não obeso
TGF-β	Fator de crecimento transformador 1-β
Th1	Linfócitos T auxiliares do tipo 1
Th2	Linfócitos T auxiliares do tipo 2
TNF-α	Fator de necrose tumoral-α
TT	Toxóide tetânico

RESUMO

IMUNOREGULAÇÃO E RESPOSTA IMUNE SISTÊMICA E TECIDUAL NA LEISHMANIOSE MUCOSA. **LUCAS PEDREIRA DE CARVALHO**. Na infecção humana por leishmania há uma complexa interação parasito-hospedeiro, levando a uma variedade de formas clínicas. Em áreas de transmissão de *L. braziliensis*, cerca de 3% dos pacientes com leishmaniose cutânea desenvolvem a forma mucosa da doença concomitantemente ou após cicatrização da ulcera. Células mononucleares do sangue periférico (CMSP) de pacientes com LM secretam maiores níveis de IFN-γ do que as de pacientes com LC quando estimuladas com antígeno solúvel de leishmania (SLA). A presente tese é composta de 4 trabalhos e teve como objetivo principal caracterizar a resposta imune de pacientes com LM e LC em células de sangue periférico e no tecido, e determinar fatores que possam estar envolvidos na resposta inflamatória exacerbada de pacientes com LM. Análise por citometria de fluxo demonstrou que a célula CD4+ é a principal célula produtora de IFN-γ em pacientes com LC ou LM. Na tentativa de modular a produção de IFN-γ de pacientes com LC ou LM anticorpos contra citocinas (anti-IL-2, anti-IL-12, e anti-IL-15) ou antígenos recombinantes (LACK e KMP11) indutores de IL-10 foram adicionados a culturas de CMSP estimuladas com SLA. A neutralização de IL-12 não teve efeito significativo na produção de IFN-γ em CMSP de pacientes com LC ou LM. A adição de anti-IL-2 suprimiu a produção de IFN-γ em 73% e 52% em pacientes com LC e LM, respectivamente. A adição de anti-IL-15 suprimiu 56% da produção de IFN-γ de pacientes com LC, mas não teve efeito significativo em pacientes com LM. A adição de LACK ou KMP11 a culturas de CMSP estimuladas com SLA suprimiu a produção de IFN-γ de pacientes com LC, mas não teve efeito significativo em pacientes com LM. Não houve diferença significante na expressão de moléculas co-estimulatórias entre CMSP de pacientes com LC e LM. A freqüência de células CD4+ expressando marcadores de ativação celular (CD28-, CD62L- e CD69+) foi maior em pacientes com LM quando comparados com pacientes com LC. A análise de biópsias de pacientes com LM e LC demonstrou que pacientes com LM apresentam maior freqüência de células CD4+ expressando IFN-γ, maior freqüência de células CD8+ positivas para Granzima A e menor freqüência de células expressando receptor de IL-10 quando comparados com pacientes com LC. O presente estudo mostrou que pacientes com LM apresentam uma maior freqüência de células CD4+ ativadas maior do que pacientes com LC. Essas células não são propriamente moduladas por antagonistas de citocinas envolvidas em diferenciação e proliferação linfocitária ou por antígenos que induzem IL-10, contribuindo também para este aspecto uma menor expressão de IL-10.

ABSTRACT

IMMUNE REGULATION AND SYSTEMIC AND TISSUE IMMUNE RESPONSE OF MUCOSAL LEISHMANIASIS. **LUCAS PEDREIRA DE CARVALHO.** Leishmania infection lead to a complex host-parasite relationship in humans, causing a large spectrum of clinical forms of disease. In areas o f *L braziliensis* transmission 3% of patients with cutaneous leishmanisis (CL) develops concomitantly or after healing of cutaneous ulcers mucosal leishmaniasis (ML). Peripheral blood mononuclear cells (PBMC) from ML patients secrete higher levels of IFN-γ than cells from CL patients upon stimulation with soluble leishmania antigen (SLA). The present thesis is composed of four manuscripts and had as the major aim, to characterize the systemic and tissue immune response of CL and ML patients and determine factors that can be involved in the exacerbated inflammatory response in ML patients. Flow cytometry analyses showed that $CD4^{+}$ T cells are the main source of IFN-γ in PBMC of CL and ML patients. In attempt to suppress IFN-γ in SLA-stimulated PBMC, blocking antibodies to cytokines (anti-IL-2, anti-IL-12, and anti-IL-15) and recombinant antigens (LACK and KMP11) that induce IL-10 were added to SLA-stimulated cultures. IL-12 neutralization had a week effect on IFN-γ suppression in CL and ML. Anti-IL-2 suppressed IFN-γ by 73% in CL and 52% in ML. Anti-IL-15 suppressed 56% of IFN-γ in CL but had no effect in ML. Addition of LACK or KMP11 to SLA-stimulated cultures was able to suppress SLA-stimulated IFN-γ production on CL patients, but had week effect on SLA-stimulated IFN-γ production from ML patients. There was no difference in the frequency of cells expressing co-stimulatory molecules or apoptosis between PBMC from CL and ML patients. The frequency of CD4+ cells expressing cell activation markers (CD28-, CD62L- and CD69+) were higher in ML patients when compared with CL patients. The biopsy analysis of ML and CL patients documented a higher expression of IFN-g in CD4+ cells, higher expression of Granzime A on CD8+ cells and lower expression of IL-10 receptor on ML patient when compared to CL. Overall, the data show that ML patients have more activated CD4+ T cells than CL patients. These cells are less modulated by antibodies against cytokines involved in differentiation and proliferation of lymphocytes or antigens with regulatory properties. ML cells were also poorly down-modulated by IL-10 induced by LACK and KMP11, a phenomenon that could be explained by the decrease in the expression of IL-10 receptor.

1.1 Introdução geral

As leishmanioses são causadas pelo protozoário intracelular obrigatório, Leishmania, e ocorre nos cinco continentes, sendo endêmicas em regiões tropicais e subtropicais. No Brasil são endêmicas tanto a leishmaniose visceral (LV), causada pela *L. chagasi*, quanto a leishmaniose tegumentar americana, causada principalmente pela *L. braziliensis, L. guianensis* e *L. amazonensis*. A leishmaniose tegumentar se apresenta com diferentes formas clínicas: leishmaniose cutânea (LC), leishmaniose mucosa (LM), leishmaniose disseminada (LD) e leishmaniose cutânea difusa (LCD). O principal mecanismo de defesa contra a Leishmania é a produção de IFN-γ, necessária para ativação de macrófagos e síntese de derivados do O_2 (ROI) e de nitrogênio (RNI). A leishmaniose se manifesta de forma polar, semelhante à hanseníase, como nos pacientes portadores de LV e LCD notando-se uma diminuição ou ausência de produção de IFN-γ, o que contribui para a multiplicação e disseminação do parasito; e num outro pólo da doença estão pacientes com LC e com LM. Células mononucleares (CMSP) de pacientes com LC e LM produzem altos níveis IFN-γ quando estimuladas com antígeno solúvel de Leishmania (SLA), o que controla a multiplicação de parasitos, ao tempo em que contribui para o dano tecidual observado nesses pacientes. **O principal objetivo desse estudo foi caracterizar a resposta imune de pacientes com leishmaniose cutânea e mucosa, e determinar fatores que possam explicar a exacerbação da resposta imune tipo 1 desses pacientes, bem como identificar formas de modular essa resposta imune.**

1.2. O parasito e transmissão da doença

A Leishmania é um protozoário intracelular obrigatório pertencente à família Trypanosomatidae que causa um amplo espectro clínico de doença denominada leishmaniose. O parasito normalmente infecta animais silvestres, constituindo-se numa zoonose, sendo acidentalmente transmitida para o homem por inseto do gênero phlebotominae, devido a uma mudança de habitat natural desses, por desmatamento ou construção de residências próximas às matas. Trinta espécies de flebotomíneos são distribuídas pelo mundo. As fêmeas desses hospedeiros intermediários sugam formas amastigotas do parasito ao se alimentarem de sangue de animais. Essas formas amastigotas transformam-se em promastigotas no intestino do flebótomo e migram para a probóscida como forma promastigota metacíclica. Essa forma evolutiva é injetada na pele do homen no ato do repasto sanguíneo. Ao penetrar na pele as formas promastigotas metacíclicas penetram em macrófagos do tecido conjuntivo, transformando-se em amastigotas, as quais possuem mecanismos evasivos aos produtos microbicidas dos macrófagos, proliferando nos fagolisossomos e disseminando-se para linfonodos regionais.

Vinte espécies de *Leishmania* são patogênicas para o homem (WHO, 2002), sendo que as comumente envolvidas na leishmaniose tegumentar são a *L. major, L. tropica* e *L aethiopica*, no Velho Mundo e *L. braziliensis, L. guianensis, L. amazonensis* e *L. mexicana*, no Novo Mundo. A LV é causada pela *L. donovani* e *L. infantum*, no velho mundo, e por *L chagasi* no Novo Mundo. A *L. braziliensis*, no Brasil, é o principal agente causal da leishmaniose tegumentar, seguida de *L amazonensis*. Existe uma associação entre algumas espécies e formas clínicas de leishmaniose. Assim, a *L. braziliensis* está associada à LC, LD e LM, a *L. amazonensis* com as formas LC, LD e LCD e mais raramente com LM e LV. Contudo, estudos recentes demonstraram presença de polimorfismos genéticos em

cepas de *L. braziliensis*, constituindo clados geneticamente distintos e associados a formas clínicas diferentes (Schriefer e cols., 2004).

1.3. Aspectos epidemiológicos e formas clínicas das leishmanioses

As leishmanioses ocorrem nos cinco continentes e são endêmicas em regiões tropicais e subtropicais de 88 países, onde cerca de 400 milhões de indivíduos residem em área de risco, com incidência anual estimada em 600.000 casos. Existem 12 milhões de casos estimados de leishmaniose em todo o mundo (WHO, 1998). A real prevalência da leishmaniose tegumentar no Brasil é difícil de ser estabelecida, devido à sub-notificações e ausência de diagnóstico. No entanto, o número de registros vem aumentando e no ano 2000 o coeficiente de detecção foi de 18,63/100.000 habitantes, segundo a Fundação Nacional de Saúde (FUNASA, 2000). Os indivíduos infectados por *L. chagasi* ou *L. donovani* podem permanecer assintomáticos, apresentar a forma sub-clínica da infecção ou evoluírem diretamente para a forma clássica da doença (Badaró e cols., 1986a). Na região nordeste brasileira foram registrados cerca de 90% dos casos de leishmaniose visceral no Brasil, tendo o estado da Bahia uma média de 500 - 600 casos por ano, principalmente nas regiões semi-áridas (Badaró e cols., 1986b). A leishmaniose tegumentar é encontrada em diversas regiões endêmicas distribuídas em praticamente todo o território nacional, predominando nas regiões de mata com desmatamento recente. Na Bahia, a área endêmica de Corte de Pedra, localizada no Sul do estado, numa região remanescente de Mata Atlântica, é a área com incidência mais elevada.

A infecção por Leishmania pode ser assintomática ou sub-clínica, demonstrada pela existência de cerca de 10% de indivíduos em áreas de leishmaniose tegumentar e cerca de 40% em áreas de LV, com uma reação intradérmica (Reação de Montenegro) de

hipersensibilidade tardia positiva a antígeno de Leishmania. Na área endêmica estudada por nosso grupo, a *L. braziliensis* tem sido a principal espécie diagnosticada nos últimos 25 anos. Pacientes com leishmaniose cutânea apresentam na maioria das vezes uma lesão ulcerada de fundo granuloso e bordas elevadas. Em alguns casos, duas ou mais lesões clinicamente ativas podem estar presentes. Lesões múltiplas são mais comuns em crianças e raras nos primeiros meses da doença.

Cerca de 5% dos pacientes com LC desenvolvem a forma mucosa da doença (LM), a qual pode aparecer concomitantemente ou mais comumente até 2 anos depois da LC. Alguns casos podem ocorrer 10-15 anos após a LC ou isoladamente, sem história de LC. A LM é uma das formas mais agressivas de leishmaniose tegumentar. A doença acomete principalmente a mucosa nasal, mas pode haver também envolvimento do palato, faringe e laringe. A importância da LM é devida não só ao intenso processo inflamatório que acompanha a doença, levando à destruição do septo nasal e aparecimento de lesões desfigurantes, mas também pelo alto índice de refratariedade ao tratamento com antimonial (cerca de 40%). Lessa desenvolveu uma escala de avaliação de gravidade das lesões mucosa, sendo o estágio I, nodulação sem ulceração; estágio II, ulceração superficial; estágio III, ulceração profunda; estágio IV, perfuração do septo; estágio V, deformidades na estrutura externa da pirâmide nasal (Machado e cols., Gazeta Médica da Bahia, *In press*).

A partir da década de 80 foi descrita uma forma clínica, a leishmniose disseminada (LD), caracterizada por lesões múltiplas emergente no Nordeste do Brasil, causada na maioria das vezes por *L. braziliensis*. A LD é definida como a presença de múltiplas lesões (> 10 lesões) em mais de uma parte do corpo, podendo ser acneiformes, nodulares ou ulceradas. Os pacientes habitualmente apresentam uma lesão, seguida de uma história de febre com aparecimento de múltiplas lesões distribuídas em todo o corpo, num quadro que

sugere disseminação hematogênica. O acometimento da mucosa nasal aparece com mais freqüência em indivíduos com LD (Turetz e cols., 2002).

A leishmaniose cutânea difusa (LCD) é uma forma clínica rara de leishmaniose, tendo como agente causal no Brasil a *L. amazonensis,* sendo mais encontrada na região Norte. Ausência de resposta ao tratamento ou recidiva após a terapêutica é um achado comum nesses pacientes. As lesões de pacientes com leishmaniose cutânea difusa, são na sua maioria, nodulares não ulceradas, em grande número e disseminadas ao longo do corpo. Macrófagos altamente parasitados são um achado comum em pacientes com LCD (Bryceson e cols., 1970; Bittencourt e cols., 1990).

Em áreas endêmicas de *L. braziliensis*, cerca de 10% dos moradores apresentam um teste cutâneo de hipersensibilidade tardia para antígeno de Leishmania positivo e não desenvolvem doença (Follador e cols., 2002). Esses indivíduos são classificados como portadores de uma infecção assintomática por *L. braziliensis.*

No Novo Mundo a leishmaniose visceral (LV), também denominada calazar, é causada pela *Leishmania chagasi.* No Mediterrâneo, o agente causal é a *Leishmania infantum* e na África e Ásia a doença é causada pela *Leishmania donovani* (WHO, 1990). A forma clássica da LV é grave, debilitante, tendo como achados clínicos principais: febre prolongada, perda de peso, anemia, hepatoesplenomegalia e hipergamaglobulinemia. Os pacientes com essa forma clínica evoluem para a morte se não tratados. Em áreas endêmicas de transmissão de *L. chagasi* a maioria dos indivíduos infectados não desenvolvem LV, podendo apresentar uma infecção oligossintomática com cura espontânea ou uma forma assintomática de infecção por *L. chagasi.*

1.4. Aspectos imunológicos da leishmaniose

1.4.1. Modelos experimentais de leishmaniose

Os modelos experimentais vêm contribuindo não só para o melhor conhecimento da imunologia básica como também para o entendimento da patogênese da leishmaniose. Mosmann e colaboradores, utilizando modelos experimentais de leishmaniose com resistência (C57BL/6) ou susceptibilidade (BALB/c) à infecção, demonstraram que a população de células T CD4+ podia ser subdividida em sub-populações baseadas na produção de citocinas após estimulação *in vitro* (Mosman e cols., 1986). As células CD4+ Th1 secretam IFN-γ e IL-2, enquanto que células CD4+ Th2 secretam IL-4, IL-5, IL-10 e IL-13 (Mosmann e cols., 1989). Esses conhecimentos trouxeram diversos avanços no entendimento das doenças infecciosas, alérgicas e autoimunes.

O importante papel da resposta imune na imunopatogênese da leishmaniose experimental vem sendo bem estudado a partir desses conhecimentos. No entanto, muitos aspectos permanecem obscuros, sendo alvo de estudos recentes. Vários estudos vêm sendo realizados avaliando diferentes comportamentos da doença: resposta imune de animais a cepas diferentes de Leishmania, o papel dos produtos da saliva do flebótomo na infecção, o efeito da infecção prévia na reinfecção, o papel da persistência do parasito no hospedeiro. Por ser um organismo intracelular obrigatório, o principal mecanismo de defesa contra a Leishmania é a produção de IFN-γ, necessária para ativação de macrófagos e síntese de derivados do oxigênio, (ROI) e do nitrogênio (RNI) (Sher e cols., 1983; Scott e cols., 1983; Scott e cols., 1988). Assim, foi demonstrado que a resposta Th1 ou tipo 1, está associada com produção de IL-2, IFN-γ e TNF-α, observadas em camundongos C57BL/6, com

resistência ao patógeno e cura espontânea da lesão, ao passo que a resposta tipo 2, caracterizada por produção de IL-4 e IL-5, observadas em camundongos BALB/c, com susceptibilidade à infecção está associada a proliferação e disseminação do parasito (Scott e cols., 1988; Scott e cols., 1989). Adicionalmente, tem sido proposto que no camundongo BALB/c, uma linhagem única de células T CD4+ possui um receptor específico (Vβ4Vα8) que reconhece o LACK (receptor homólogo de Leishmania para proteína kinase ativada). Esta linhagem de células é responsável pelo aumento da IL-4 no BALB/c, devido a baixa afinidade de seus receptores de células T por peptídeo-MHC (Kopf e cols., 1996; Launois e cols., 1997). Em camundongos BALB/c infectados por *L. major*, a citocina IL-4 pode inibir a proteção conferida pelo IFN-γ (Lehn e cols., 1989; Liew e cols., 1989). Logo, camundongos desta mesma linhagem infectados por *L. braziliensis* produzem níveis significativamente mais baixos de IL-4 do que os camundongos BALB/c infectado por *L. major*. Assim, os camundongos BALB/c infectados por *L. braziliensis* controlam a infecção somente com os níveis de IFN-γ que eles produzem (Titus e cols., 1994). Esses dados sugerem que a cepa de *L. braziliensis* difere da *L. major* biologicamente, sendo capaz de ativar a produção de IFN-γ mesmo em camundongos BALB/c. Scott e cols. demonstraram que a presença de altos níveis de IFN-γ no momento da infecção induzem a uma resposta Th1, enquanto que baixos níveis de IFN-γ induzem resposta imune Th2 (Scott, 1991). Outra citocina importante no que diz respeito a resistência à infecção por Leishmania é a IL-12. Camundongos resistentes deficientes de IL-12 tornam-se susceptíveis, mesmo na ausência de IL-4 (Park e cols., 2002). Belkaid e cols. demonstraram em camundongos C57BL/6, os quais são um bom modelo de resistência à infecção por *L. major*, desenvolvendo uma úlcera transitória que cura espontaneamente (Belkaid e cols., 1998),

uma população de células CD4+CD25+ (célula T regulatória) com características supressoras, dependente ou não de IL-10. Essas células são capazes de prevenir doenças autoimunes através da supressão de linfócitos auto-reativos (Shevach e cols., 2002). Ensaios em C57BL/6 demonstraram que células CD4+CD25+ estão presentes no sítio de infecção por Leishmania, junto com células CD4+CD25-, as quais são efetoras, impedindo a eliminação completa do parasito porém, levando à cura da lesão havendo reativação desta quando há um desequilíbrio entre sub-populações de células CD25+ e CD25- (Mendez e cols., 2004, Belkaid e cols., 2002).

1.4.2. Imunopatogênese da Leishmaniose Tegumentar Humana

Assim como em camundongos, no homem também o controle da infecção contra a Leishmania é mediado por células Th1, através da ativação de macrófagos por IFN-γ. Contudo, a doença humana é ainda mais complexa, com um espectro clínico e imunológico amplo, podendo ser exemplificado por diversas variações do ambiente, do parasito ou do hospedeiro, incluindo: 1- variações nas populações ou no número de vetores transmissores. 2- tamanho do inóculo de parasitos. 3- efeito da saliva dos flebótomos na infecção inicial. 4- espécies, cepas e variações genéticas do parasito. 5- estado nutricional do hospedeiro. 6- co-infecções. 7- variações genéticas do hospedeiro que interfiram na resposta imune. Alguns desses aspectos vêm sendo estudados sendo demonstrado que uma deficiência da resposta imune Th1, protetora, está relacionada com multiplicação do parasito, estando associada às formas mais graves de leishmaniose, como a LV e a LCD. Nessas formas clínicas, a ausência de produção de IL-2 e IFN-γ foi documentada (Carvalho e cols., 1985; Barral e cols., 1995), havendo uma deficiência de ativação de macrófagos e disseminação

do parasito sistemicamente (na LV) ou na pele (na LCD). Nas formas LM e LC há uma forte resposta tipo 1, a qual está associada ao controle da multiplicação do parasito (Ribeiro de Jesus e cols., 1998; Bacellar e cols., 2002). Nessas formas de doenças há o desenvolvimento de lesões, apesar de poucos parasitos na lesão. A forma mucosa da doença é ainda mais agressiva, sendo a resposta Th1 nesses pacientes, ainda mais intensa que na LC (Bacellar e cols., 2002). Nesse contexto, à semelhança da hanseníase, a LTA apresenta formas polares da doença. Na LC e LM existe uma forte resposta tipo 1, o que controla a multiplicação do parasito, porém induz lesão tecidual (Ribeiro de Jesus e cols., 1998), enquanto que na LCD, a diminuição ou ausência de resposta tipo 1 favorece à multiplicação e disseminação de Leishmania (Bonfim e cols., 1996; Turetz e cols., 2002). Na leishmaniose disseminada (LD) observa-se uma produção de IFN-γ em níveis mais baixos do que nos pacientes de LC, podendo justificar a disseminação de parasitos e aparecimento de múltiplas lesões. Essa produção pode ser suficiente para conter a multiplicação de parasitos, sendo as lesões desses pacientes diferentes daquelas observadas nos pacientes com LCD, com a presença de linfócitos e poucos parasitos, ulcerando na sua evolução (Bittencourt e cols., 1990). Não está claro porque esses indivíduos desenvolvem lesões múltiplas, porém, fatores como quimiocinas e moléculas de adesão ou um retardo na produção de IFN-γ poderiam explicar esses achados.

A exemplo do que ocorre em outras doenças infecciosas e parasitárias, o estudo da resposta imune de indivíduos que apresentam uma resposta imune à infecção e não desenvolvem doença aparente, consiste numa boa forma de entender os mecanismos imunológicos de proteção contra a infecção. Follador e cols, comparando a resposta imune de indivíduos sem história de doença e com teste cutâneo de hipersensibilidade tardia positivo, a antígeno

de Leishmania (infecção sub-clínica) com a resposta de indivíduos com LC, demonstrou que esses indivíduos têm uma resposta tipo 1 em níveis bem menores do que aqueles com LC, sugerindo que a resposta tipo 1 modulada, com concentrações menores de IFN-γ pode ser suficiente para a proteção contra o parasito sem induzir inflamação tecidual (Follador e cols., 2002). Não são conhecidos, porém, os fatores que levam esses indivíduos a não manifestarem doença, pois a produção de IFN-γ em resposta a antígenos nesses indivíduos é mais baixa do que em indivíduos com LD. Fatores como variações na virulência ou no tamanho do inoculo, ou a rapidez com que a resposta imune se inicia devido a variações genéticas do hospedeiro poderiam explicar essas diferentes formas clínicas da doença. Conclui-se que, muitos aspectos da história natural e os mecanismos que influenciam na imunopatogênese da leishmaniose permanecem obscuros.

A resposta imune de pacientes com LC foi bem estudada. Pacientes com LC apresentam teste intradérmico de Montenegro positivo e alta resposta linfoproliferativa para antígeno solúvel de Leishmania (SLA). Células mononucleares do sangue periférico (CMSP) de pacientes com LC secretam altos níveis de IFÑ-γ e TNF-α e baixos níveis de IL-5 e IL-10 quando estimuladas com SLA (Bacellar e cols., 2002). E a célula CD4+ é o tipo celular que contribui com a maior parte da produção de IFN-γ,. tanto no sangue periférico quanto a nível tecidual, embora haja participação de células CD8+ na produção dessa citocina (Antonelli e cols., 2004). A análise histopatológica da lesão mostra infiltrado mononuclear e poucos parasitos, geralmente destruídos (Bittencourt e cols., 1991). Adicionalmente, é observada uma predominância de RNA mensageiro de citocinas tipo 1 como a IL-2, IFÑ-γ e TNF-α (Pirmez e cols., 1993). Da-Cruz e cols (2002) mostraram que a razão de células CD4/CD8 é maior em indivíduos com doença ativa e que o número dessas células tende a

se igualar após terapêutica com antimonial pentavalente, sendo sugerido um papel da célula CD8+ na cura da lesão (Da-Cruz e cols., 2002). Brodskyn e cols. também observaram a presença de células CD8+ com atividade citotóxica contra macrófagos de pacientes com LM infectados *in vitro* (Brodskyn e cols., 1997). Evidências de que a resposta inflamatória é responsável pelo aparecimento da úlcera na leishmaniose cutânea tem sido acumulada: 1) o tratamento de pacientes com lesão inicial não previne o aparecimento de úlcera (Machado e cols., 2002). 2) as concentrações de IFN-γ e TNF-α diminuem após tratamento (Ribeiro de Jesus e cols., 1998). 3) indivíduos com teste de Montenegro positivo que não desenvolvem lesão (forma sub-clínica da infecção por *L. braziliensis*) apresentam níveis mais baixos de IFN-γ e TNF-α do que pacientes com leishmaniose cutânea (Follador e cols., 2002). Machado e cols demonstraram não só que o tratamento precoce da leishmaniose cutânea (< 20 dias de lesão) não previne o crescimento da úlcera cutânea, como também nessa situação, cerca de 50% dos pacientes apresentam pior resposta ao tratamento. Adicionalmente, demonstraram a presença de vasculite e marcador de citotoxicidade (Tia-1) nessas lesões iniciais (Machado e cols., 2002a; 2002b). Esses dados sugerem que a resposta inflamatória é importante para o desenvolvimento da úlcera. A razão pela qual, a despeito de existir uma forte resposta tipo 1, e não existir uma completa destruição de parasitas, não é completamente entendida. A exemplo do que ocorre em modelos experimentais resistentes de doença, pode ser que células T regulatórias modulem a reação inflamatória a nível tecidual, e os mecanismos adaptativos do parasito mantenham essa complexa interação com o hospedeiro, favorecendo sua sobrevivência. Certamente, os mecanismos adaptativos tanto do parasito como do hospedeiro sucederam em preservar

ambas as espécies, podendo a persistência do parasito ser importante para a proteção do hospedeiro contra a reinfecção.

A resposta imune de pacientes com LM foi menos estudada devido à menor freqüência dessa forma clinica de doença. Bacellar e cols. demonstraram que CMSP de pacientes com LM secretam concentrações ainda mais elevadas de IFN-γ e TNF-α quando estimuladas com SLA do que às observadas nos pacientes com LC. A célula CD4+ é responsável pela maior parte da produção de IFN-γ no sangue periférico. Os linfócitos também representam cerca de 60 a 90% das células produtoras de TNF-α seguido por macrófagos (Bacellar e cols., 2002). Além disso, essa resposta inflamatória exacerbada não é devidamente modulada, desde que CMSP desses pacientes secretam concentrações mais baixas de IL-10, quando estimuladas com SLA, e quando comparadas às dos pacientes com LC. Adicionalmente, diferente dos pacientes com leishmaniose cutânea, a adição de IL-10 exógena em culturas de CMSP estimuladas com SLA, não é capaz de modular a produção de IFN-γ em pacientes com LM.

A nível tecidual a resposta imunológica observada em pacientes com leishmaniose mucosa difere daquela encontrada no sangue periférico. Na lesão de pacientes com leishmaniose mucosa observou-se RNA mensageiro tanto para citocinas do tipo 1, quanto do tipo 2 (Pirmez e cols., 1993). É possível que a produção tecidual de citocinas Th2 seja uma tentativa de modular uma intensa resposta Th1. A presença dessas citocinas pode estar contribuindo para dificultar a eliminação do parasito na lesão. Todavia, alguns estudos sugerem que a resposta imune exacerbada é responsável pelo dano tecidual, pois as lesões são ainda mais pobres em parasitos do que as de LC, havendo grande destruição tecidual e presença de TNF-α nas áreas de necrose. O papel do TNF-α na patogênese da LM é

também sugerido pelo fato de que estes pacientes apresentam grandes quantidades da forma solúvel de TNF-α, biologicamente ativa e deletéria (Da-Cruz e cols., 1996). Além dos estudos imunológicos, ensaios clínicos com ênfase na inibição de citocinas como o TNF-α têm contribuído para o entendimento da imunopatogênese na leishmaniose mucosa. Tratamento da leishmaniose com inibidor de TNF-α (pentoxifilina), associado ao antimonial pentavalente, diminui o tempo de cura de pacientes virgens de tratamento (Machado e cols, manuscrito enviado para publicação), e é eficaz no tratamento de indivíduos refratários ao antimonial (Lessa e cols., 2001) .

Motivo pelos quais pacientes com LC ou LM apresentam resposta imune exacerbada e não modulada não é bem elucidado. Múltiplos fatores podem contribuir para a hiperativação da resposta imunológica nesses pacientes, dentre eles: 1- um desequilíbrio de sinais co-estimulatórios positivos (B7-CD28), (CD40-CD40L) e negativos (B7-CTLA-4). 2- um aumento da produção de citocinas envolvidas na ativação e proliferação linfocitária (IL-2, IL-12 e IL-15). 3- a diminuição da apoptose e a alta freqüência de células T ativadas. **A primeira hipótese desse estudo é que pacientes com LC ou LM têm linfócitos com diminuição da capacidade de serem modulados.**

1.4.3. Resposta imune a antígenos específicos de Leishmania

Alguns componentes antigênicos do parasito são capazes de induzir resposta imune diferenciada, não só no tipo de resposta imunológica observada nesses pacientes, como também no tipo de célula ativada. Alguns antígenos recombinantes de Leishmania induzem resposta tipo 1, enquanto outros induzem resposta regulatória. A maioria dos estudos que visam isolar antígenos de Leishmania tem como objetivo identificar moléculas que

induzam uma resposta tipo 1 e conseqüentemente a possibilidade de serem utilizadas como vacina (Skeiky e cols., 1998), baseado no conhecimento de que essa resposta está envolvida na ativação de macrófagos a destruir Leishmania e, no controle da infecção. Esse pressuposto é verdadeiro para as formas clínicas de leishmaniose associadas a grande multiplicação de parasitos e onde a imunopatogênese está ligada a uma ausência de resposta tipo 1. Entretanto, pacientes com LC ou LM apresentam resposta inflamatória intensa e fracamente modulada o que inviabiliza o estudo com antígenos candidatos a vacina que induzam intensa resposta tipo 1 nesses pacientes, havendo inclusive o risco de provocar um aumento na freqüência da forma mucosa por vacinas que induzam esse tipo de resposta isoladamente. **A segunda hipótese desse estudo é que diferentes antígenos recombinantes de Leishmania induzem padrões de resposta imune diferenciada através da ativação de diferentes tipos celulares.**

Dentre os antígenos recombinantes indutores de resposta tipo 1, destacam-se o LeIF (Leishmania homologue of the eukaryotic initiation factor 4A) que induz produção de IL-12 (Skeyk e cols., 1995; 1998) que contribui para a diferenciação de células Th0 para células Th1. A Gp63 é uma glicoproteína expressa em abundância em várias espécies de Leishmania, capaz de induzir proliferação linfocitária e produção de IFN-γ em pacientes com LC e LM e em indivíduos curados de leishmaniose (Russo e cols., 1991), assim como em camundongos (Walker e cols., 1998).

Outros antígenos de Leishmania, no entanto, induzem resposta tipo 2, a exemplo do LACK e do KMP11. O LACK (Leishmania homologue of the mammalian receptor for activated C kinase-reactive) constitui cerca de 0,03% do total de proteínas da Leishmania e sua expressão é essencial para que ocorra infecção. Em modelo experimental, o LACK

induz predominantemente resposta imune tipo 2 com rápida secreção de IL-4 (Launois e cols., 1997). O KMP11 é uma proteína de membrana com peso molecular de 11 kDa que foi primeiramente descrita em *L. donovani* (Jardim e cols., 1995a; 1995b) . Em 1997, Berberich e colaboradores desenvolveram estudos para analisar aspectos moleculares e imunológicos da proteína KMP11 em *L. infantum* (Berberch e cols., 1997). Esta proteína foi previamente utilizada no diagnóstico de cães infectados naturalmente por *L. infantum* (Nieto e cols., 1999). A expressão de KMP11 diminui consideravelmente quando a Leishmania atinge a fase estacionária (Berberich e cols., 1998).

O rH2A e o rH3 são histonas, sendo o rH2A uma proteína recombinante purificada de promastigota de *Leishmania infantum* com peso molecular de 14 kDa (Nieto e cols., 1999; Soto e cols., 1992) e assim como o KMP11 foi utilizado para diagnóstico de cães infectados por *L. infantum* (Nieto e cols., 1999). Esta histona é mais abundante em promastigotas na fase logarítimica, do que nas mesmas células em culturas estacionárias (Berberich e cols., 1998; Soto e cols., 1992; 1995). Em um trabalho com cães infectados com *L. infantum* a presença de anticorpos contra a histona H2A foi um indicador de que anticorpos anti-histona podiam estar envolvidos no processo patológico direcionando para a progressão da doença (Nieto e cols., 1999).

Além do tipo do antígeno, é sabido que células apresentadoras de antígenos e o ambiente de citocinas onde se processa a resposta imune, interferem na diferenciação de uma resposta predominantemente tipo1 ou tipo 2 (Sabin e cols., 1995). Antígenos que induzem uma ativação de células CD4+ Th1 podem ser utilizados como vacinas devido à sua capacidade de gerar células T de memória, enquanto que antígenos que ativam as células da imunidade inata (macrófagos e neutrófilos) podem induzir citocinas regulatórias capazes de modular a resposta imune. **A Terceira hipótese do presente estudo é que**

antígenos recombinantes com propriedades regulatórias são capazes de modular a resposta inflamatória de pacientes com LM ou LC.

1.4.4. Resposta imune a nível tecidual

Ênfase tem sido dada nos últimos anos a resposta imunológica *in situ*. Estudos em humanos são habitualmente realizados com células de sangue periférico e tem sido aventado que nem sempre estes dados da resposta imune sistêmica refletem o que está ocorrendo a nível tecidual. Na LM, enquanto no sangue periférico há uma predominância de uma resposta tipo 1 a nível tecidual, a expressão de citocinas tanto do tipo 1 quanto do tipo 2, tem sido documentada (Pirmez e cols., 1993; 1999). A análise da resposta imune *in situ* nas leishmanioses tem sido prejudicada pela limitação de células obtidas em biópsias, o que impede a realização de estudos funcionais. Por isso, a maioria dos estudos realizados com material obtido de lesão tem se limitado a analisar a expressão de citocinas através da técnica de reação de cadeia polimerase, a qual oferece resultados qualitativos ou semi-quantitativos. Mais recentemente, a microscopia confocal tem sido utilizada permitindo um melhor estudo da resposta imune no tecido.

Discrepâncias entre a resposta imune no sangue periférico e no tecido, têm sido observadas em várias doenças. Enquanto os níveis séricos de IgE não são diferentes em pacientes com candidíase vaginal recorrente e candidíase vaginal esporádica, níveis elevados dessa imunoglobulina podem ser documentados no fluido vaginal (Witkin e cols., 1989). Também, enquanto no sangue periférico existe diminuição ou ausência de produção de IFN-γ em células estimuladas com antígeno de *Candida albicans* em pacientes com candidíase vaginal recorrente a nível vaginal, células T reativas têm sido observadas nessas

pacientes (Carvalho e cols. 2003; Piccinni e cols., 2002). A análise da resposta imune no líquor de pacientes com doença inflamatória do sistema nervoso tem contribuído bastante para o entendimento da patogênese da esclerose múltipla, da mielopoatia associada ao HTLV-1 (Hickey e cols., 1991; Narikaua e cols., 2005) e da Chorea de Sydenham (Korn-Lubetzki e cols., 2004).

No modelo experimental de leishmaniose, a análise da resposta é feita predominantemente em células obtidas de linfonodos para o qual migram as células da lesão ou com células obtidas do sítio da infecção. Na lesão, estudos recentes têm dado ênfase à importância de células T regulatórias na manutenção de parasitos na lesão de C57BL/6 (Belkaid e cols., 2003).

No caso da LC e principalmente da LM pode-se argumentar que enquanto a nível do sangue periférico há predomínio de resposta tipo 1, a nível tecidual há diminuição desse tipo de resposta o que permitiria a manutenção da infecção. Vários achados atestam contra essa possibilidade, como a existência de um predominante infiltrado inflamatório com escassez de parasitos (Bittencourt e cols., 1991) e o fato de que as lesões de pacientes com LC e LM são predominantemente localizadas sem tendência à disseminação. **A quarta hipótese desse estudo é que a resposta imune a nível tecidual na LC e na LM é semelhante à observada no sangue periférico.**

2. Objetivo Geral

Caracterizar a resposta imune de pacientes com LC e LM.

Objetivos específicos

1-Avaliar aspectos de modulação da resposta imune de pacientes com LC e LM.

1a. Caracterizar o fenótipo celular responsável por produção de IFN-γ em pacientes com leishmaniose cutânea e pacientes com leishmaniose mucosa.

1b. Avaliar a capacidade de anticorpos monoclonais anti-IL-2, anti-IL-12 e anti-IL-15 de modular a produção de IFN-γ de pacientes com LC e LM.

1c. Determinar a freqüência de células expressando moléculas co-estimulatórias em pacientes com LC e LM.

1d. Determinar a freqüência de células T ativadas em pacientes com LC e LM.

2- Avaliar a resposta imune de pacientes com leishmaniose cutânea e pacientes com leishmaniose mucosa a antígenos recombinantes de Leishmania, e o perfil de células produtoras de citocinas em resposta a esses antígenos.

3- Avaliar a capacidade de antígenos recombinantes de Leishmania de modular a produção de IFN-γ de pacientes com leishmaniose cutânea e pacientes com leishmaniose mucosa.

4- Comparar a resposta imune na lesão de pacientes com LC e LM, avaliando os tipos de células presentes, o perfil de citocinas e a expressão de receptor de IL-10 nas células das lesões de LC e LM.

A hipótese desse estudo é que pacientes com LC e LM tem diminuição da capacidade de modulação da produção de IFN-γ.

Resumo artigo 1:

Differential immune regulation of activated T cells between cutaneous and mucosal leishmaniasis as a model of pathogenesis.

Foi demonstrado anteriormente que pacientes com LM secretam mais IFN-γ e TNF-α do que células de LC, e que essa resposta inflamatória em LM é fracamente modulada desde quando células desses indivíduos secretam baixos níveis de IL-10 quando estimuladas com antígeno solúvel de Leishmania (SLA). No presente estudo foi documentado que as células CD4+ são as principais fontes de IFN-γ. Nenhuma diferença no perfil de moléculas co-estimulatórias (HLA-DR, CD-80, CD-86, CD-40 or CD-40L) foi observada entre pacientes com LC e LM. Anticorpos monoclonais anti-IL-2 e anti IL-15 diminuíram a produção de IFN-γ de PBMC de pacientes com LC e em menor grau a de pacientes com LM. Um aumento da freqüência de células CD4+ expressando marcadores de ativação (CD28-, CD69+ e CD62L-) celular foi observado em pacientes com LM. Esses dados indicam que pacientes com LM apresentam uma maior resposta inflamatória, com maior número de células T ativadas do que pacientes com LC. Além disso, esse estudo demonstrou que células de pacientes com LM têm menor capacidade de serem moduladas quando comparadas com LC.

Running title: Immune regulation in mucosal leishmaniasis

Differential Immune Regulation of Activated T Cells Between Cutaneous and Mucosal Leishmaniasis as a Model for Pathogenesis

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Abstract

In both cutaneous (CL) and mucosal leishmaniasis (ML) there is a predominant type 1 immune response and a strong inflammatory response at the lesion site with few parasites. Several immunological factors could explain the mechanisms behind the exacerbated type 1 response seen in ML as compared to CL patients, thus in the present study the frequency of cells expressing co-stimulatory molecules, apoptotic profiles, and T cell activation was determined in both ML and CL patients. Additionally, the ability of neutralizing antibodies to IL-2, IL-12 and IL-15 to down-modulate IFN-γ production in supernatants of Leishmania antigen (SLA), and PPD stimulated peripheral blood mononuclear cells (PBMC) was determined. No differences were found in the frequency of monocytes expressing HLA-DR or co-stimulatory molecules (CD80, CD86, CD40 or CD40-L) in CL versus ML, nor in the frequency of apoptotic cells. Interestingly, in CL anti-IL-2 and anti-IL-15 significantly suppressed antigen specific IFN-γ production from PBMC, while in ML only anti-IL-2 suppressed IFN-γ production. Moreover, anti-IL-2 suppressed IFN-γ production in CL to a greater extent than in ML ($P<0.05$). Finally, a higher frequency of CD4+ T cells expressing CD28-, CD69+ and $CD62L^{low}$ phenotype were observed in ML as compared to CL. These data indicate that an exacerbated type 1 response in ML is differentially regulated and associated with increased numbers of activated effector T cells. We hypothesize that these differentiated T cells are not appropriately down modulated and maintain a persistent inflammatory response leading to the tissue damage observed in ML.

Introduction

American cutaneous leishmaniasis (CL) is a disease characterized by a cutaneous ulcer predominantly on the lower limbs. In areas of *L. braziliensis* transmission, ~3% of patients with active or past CL develop mucosal leishmaniasis (ML), a disease that affects predominantly the nose, leading to tissue damage and occasionally disfiguring facial lesions (15). Visceral leishmaniasis (VL) and diffuse cutaneous leishmaniasis (DCL) are associated with impaired T cell response against parasite antigens (10, 12). In contrast, patients with CL and ML have a strong type 1 immune response to soluble leishmania antigen (SLA) (6). It is well known that Th1 mediated immunity is important for the control of leishmania infection and that oxidants produced by IFN-γ activated macrophages are the main final effector molecules killing leishmania (27, 28). However, evidence has accumulated that an exaggerated T cell response is a cause of pathologic lesions in CL and even more in ML. This evidence includes the following: 1) Lymphocytes from individuals with CL or ML produce high amounts of IFN-γ and TNF-α, two important pro-inflammatory cytokines, in peripheral blood and tissue (6, 24); 2) The lesions are characterized by infiltration of lymphocytes and plasma cells and parasites are rare or absent at the lesion sites (7); 3) During the initial stages of CL (lesions with less than 20 days old), granulomatus vasculitis precedes the appearance of the ulcer (19); 4) There is a correlation between the frequency of inflammatory cytokine producing T cells and lesion size (3); 5) Drugs that down-modulate the immune response associated with antimony therapy increase the cure rate and decrease the healing time of cutaneous and mucosal lesions (1, 17).

The immunopathogenesis of CL and ML is dependent on a complex interplay involving parasite and host factors. Whereas in peripheral blood there is a very strong

production of type 1 cytokines that decreases with the resolution of lesions (6), both type 1 and type 2 cytokines are observed in tissue (24). On the parasite side of pathogenesis, recent studies have shown that *L. braziliensis* from CL and ML are polymorphic and there is an association between genetically distinct parasite isolates and the different clinical forms of disease (24). We have previously shown that, in comparison with cells from patients with CL, SLA-stimulated peripheral blood mononuclear cells (PBMC) from individuals with ML secrete higher levels of IFN-γ and TNF-α and lower amounts of IL-10 (6). High levels of TNF-α are detected in the sera during active disease, and these decrease after therapy (13). Moreover, the exacerbated T cell response of PBMCs from individuals with ML is not appropriately modulated by IL-10 and TGF-β in vitro (6). However, the mechanisms responsible for the refractory inflammatory response in ML patients are still unknown. Factors that could be involved in inducing an exaggerated T cell response in such patients include high expression of co-stimulatory molecules, decreased expression of IL-10 receptor, decreased T cell apoptosis, increased number of memory / activated T cells, and/or lack of regulatory T cells. In the current study, PBMCs from CL and ML patients were compared regarding: 1) expression of co-stimulatory molecules, 2) frequency of apoptotic cells, 3) frequency of activated/effector T cells, and 4) ability of anti-IL-2, anti-IL-12 and anti-IL-15 to modulate the antigen specific IFN-γ response.

Materials and Methods

Patients

Twenty-two ML patients were selected and matched by age (± 10 if >20 years old and ± 5 if <20 years old) with 22 CL patients. Patients were recruited from the health post of Corte de Pedra, a region with high transmission of *L. braziliensis* in southeast Bahia,

Brazil. This health post is reference center for cases of leishmaniasis in 22 municipalities. Every year, approximately 20 ML cases and 800 CL cases are diagnosed in the health post. The patients entered in this study were not malnourished and were HIV negative. The diagnosis of CL and ML was based on characteristic clinical findings, a positive skin test reaction (>5 mm) to *Leishmania* antigen, and either isolation of the parasite or histopathological findings characteristic of these diseases. All patients were evaluated prior to therapy. Informed consent was obtained from all patients and controls or guardians of minors. The Ethics Committee of H ospital Professor Edgard Santos, and NIH-registered IRB committee approved this study. Healthy subjects (N = 4) with past history of tuberculosis were used as controls in some experiments.

Antigen

L. braziliensis soluble leishmania antigen (SLA) was obtained from an isolate of a ML patient as previously described (11). Briefly, parasites were harvested in stationary phase of growth, freeze and thawed five times, sonicated, and centrifuged to remove insoluble materials. The protein concentration was measured in the supernatant of this soluble leishmania antigen (SLA). In some cultures a tuberculin purified protein derivative (PPD – CT68) (Connaught laboratories, Ontario, Canada) was used.

Cell culture and cytokine assays

PBMCs were isolated from heparinized venous blood by ficoll-hypaque gradient centrifugation. After washing three times in 0.9% NaCl, the PBMCs were resuspended in RPMI 1640 culture medium (GIBCO BRL, Grand Island, NY) supplemented with 10% human AB serum, 100 IU/ml of penicillin and 100 mg/ml of streptomycin. Because enough cells were not obtained to perform all type of experiments in all patients, paired samples of

CL and ML cells were performed for each experiment. For cytokines assays, PBMCs from ML and CL patients were adjusted to 3×10^6 cells/ml, placed in 24-well plates, and stimulated with SLA (10 μg/ml). Monoclonal antibodies anti-IL-2, anti-IL-12 or anti-IL-15, or recombinant CTLA-4 (R&D Systems, Minneapolis, MN) were added to some SLA stimulated or unstimulated cultures. Dose-response curves were performed, and showed that optimal concentrations for monoclonal antibodies was 10 μg/ml, and the optimal concentration for recombinant CTLA-4 was 5 μg/ml. Cultures were incubated for 72 hours at 37°C with 5% CO2. Supernatants were collected and stored at –70 °C. The levels of IFN-γ were measured by the ELISA sandwich method using commercial reagents (R&D Systems, Minneapolis, MN). The results are expressed in pg/ml or in mean of IFN-γ suppression.

Evaluation of surface cell markers and apoptosis

The frequency of surface molecules on cells was detected by FACS (Becton Dickinson, Palo Alto, CA) in PBMCs from 8 CL and 8 ML patients. After washing three times, PBMCs were placed in 96-well U-bottom plates at a concentration of 2×10^5 cells/well of RPMI 1640 (GIBCO BRL, Grand Island, NY), supplemented with 10% human AB Rh+ serum (SIGMA Chemical Co. St. Louis, MO), 100 IU/ml penicillin and 100 μg/L streptomycin. Staining for surface and intracellular markers was performed as previously described (4). Briefly, PBMC were cultured for 20 hours at 37°C and 5 % CO_2. During the last four hours Brefeldin A (1 μg/ml) was added. After centrifugation, cells were incubated for 20 min at 4°C with a 1:20 dilution of PE-labeled surface antibodies anti-CD4, anti-CD8 and anti-CD14 (PE–labeled) (25). Cells were fixed with 2% formaldehyde in PBS, permeabilized with saponin, and stained for 30 min with 1:4 FITC-labeled anti-

CD80, anti-CD86, anti-CD40, anti-CD40L, anti-CTLA-4, anti-CD69, anti-CD62L, anti-CD28 or anti-HLA-DR in PBS as we previously described (Pharmingen, San Diego, CA) (8). Isotype control antibodies were IgG1 and IgG2a. FACS data were based upon two gated regions based on cell size and granularity: R1, which contained only lymphocytes and blasts; and R2, a macrophage region. Apoptosis was detected by binding of Annexin V to CD4+ and CD8+ cells by flow cytometry using a commercial kit (Becton Dickinson, Palo Alto, CA).

Statistical analysis

Considering that the values did not have a Gaussian distribution, the Mann-Whitney test was used to compare data between CL and ML patients. Paired analyses were performed by Wilcoxon matched-pairs test.

Results

Inflammatory profile of ML and CL patients

In order to confirm our previous findings concerning the exacerbated inflammatory nature of the cellular immune response in ML as compared to CL patients (6), we determined the levels of IFN-γ in supernatants of mononuclear cells from cultures of CL and ML patients (Figure 1). IFN-γ production by PBMCs from ML patients was significantly higher than PBMCs from patients with CL (12751±13871 pg/ml versus 3567±5973 pg/ml; p=0.001). Next using flow cytometry we also confirmed our previous observation (6, 8) that CD4 T cells were the major source of this IFN-γ from both CL and ML patients (data not shown).

Expression of co-stimulatory molecules and effect of addition of CTLA-4 on SLA-stimulated PBMC cultures

To evaluate whether the higher IFN-γ production observed in cultures from ML patients was due to an increase in co-stimulatory molecules, the frequency of cells expressing HLA-DR, CD80, CD86, CD40, CD40L and CTLA-4 was determined in PBMCs by FACS analysis (Figure 2). The frequency of cells expressing HLADR was equal in the two groups of patients. The frequency of CD14+ antigen presenting cells expressing CD80, CD86, CD40 and of CD4+ T cells expressing CD40L was higher in ML than in CL, but these differences did not reach statistical significance. There was also a tendency towards decreased frequency of $CD4^+$ T cells expressing CTLA-4 in ML compared to CL patients' PBMCs.

Ability of anti-IL-2, anti-IL-12, anti-IL-15 or CTLA-4 to suppress IFN-γ *production*

IL-12 is a cytokine produced by antigen presenting cells and is involved in Th1 cell differentiation. IL-2 is the main T cell growth factor and IL-15, in addition to inducing lymphocyte proliferation, protects cells from apoptosis. To determine the role of IL-2, IL-12 and IL-15 in SLA specific IFN-γ production, monoclonal antibodies were used to neutralize these cytokines in SLA stimulated cultures (Figure 3). Addition of anti-IL-2 to SLA-stimulated PBMC from CL patients (Figure 3A), decreased IFN-γ production by 70±17% (range 41% to 100%) ($p<0.0001$). In SLA-stimulated PBMC from ML patients, anti-IL-2 decreased IFN-γ production by 47±28% (Figure 3B, range 0% to 79%) ($p<0.0001$). Neutralization of IL-12 had a weak effect, decreasing SLA-induced IFN-γ production from PBMC of CL patients by 42±38% (Figure 3C, range 0% to 100%)

(p=0.078) and in ML patients by 30±24% (Figure 3D, range 0% to 57%) (p=0.147). A differential immune regulation of T cells between CL and ML was observed when IL-15 was neutralized by specific antibody. While addition of the monoclonal antibody anti-IL-15 to SLA-stimulated PBMC decreased the IFN-γ production from CL patients by 39±29% (Figure 3E, range 0% to 87%) (p=0.003) neutralization of IL-15 in ML patients only decreased by 11±18% the IFN-γ production (Figure 3F, range 0% to 53%) (p=0.098).

In spite of the ability of anti-IL-2 to modulate SLA-induced IFN-γ production in both CL and ML patients, IFN-γ suppression was more pronounced in cultured PBMCs from CL than from ML patients after neutralization of IL-2 (Figure 4, p=0.03). Moreover, whereas anti-IL-15 down modulated IFN-γ production in PBMC cultures from individuals with CL, there was no significant decrease of IFN-γ levels in PBMCs from ML patients after neutralization of IL-15 (Figure 4).

To determine whether the diminished ability of monoclonal antibody anti-IL-2 or anti-IL-15 to down regulates IFN-γ production in PBMCs from ML patients was antigen-specific, we neutralized IL-2 or IL-15 in PPD-stimulated PBMC cultures from PPD skin test positive CL (N = 6) and ML patients (N = 7). As shown in Figure 6 neutralization of IL-2 suppressed PPD-induced IFN-γ production in PBMC culture supernatants from CL patients 84±15% (range 62% to 100%) (p=0.031), and in cultured PBMCs from ML patients by 57±31% (range 4% to 91%) (p=0.031).

CTLA-4 is a down regulatory molecule, which competes with CD28 binding to the co-stimulatory molecules CD80 and CD86. Addition of CTLA-4 poorly down modulate IFN-γ production in SLA-stimulated PBMC of CL and ML patients (30% of reduction),

while it suppressed IFN-γ production from PPD-stimulated PBMC of health controls up to 98% (data not shown).

Apoptosis

The frequency of apoptotic cells was evaluated in PBMC preparations from five CL and five ML patients. Annexin V staining was assessed using flow cytometric analysis of gated cells in the blast and lymphocyte regions to analyze PBMCs cultured in presence or absence of SLA. There was no statistically significant difference between the frequencies of total apoptotic cells in CD4+ or CD8+ T cells population from CL or ML patients (Table 1).

Activation of lymphocyte populations from CL and ML patients

The frequency of CD28, CD69 and CD62L expressing T cells was evaluated *ex vivo* using PBMC from 5 CL and 5 ML patients by flow cytometry (Figure 6). As a measure of direct recent T cell activation, the marker CD69 was used which demonstrated a higher frequency of CD4+CD69+ cells in cultured PBMCs from ML (3.3±1.8%) than from CL patients (0.93±0.85%) (p=0.031). Moreover, a higher frequency of CD4+CD28- T cells was also seen in PBMC from ML (8±3%) as compared to CL patients (4±1%) (p=0.031). Finally, a higher frequency of CD4+CD62L^{low} cells was observed in PMBCs from ML (29±13%) patients when compared to CL patients (11±4%) (p=0.007).

Discussion

Immune responses against the *Leishmania* sp. protozoa are known to be influenced primarily by cell-mediated responses. An impairment in type 1 immune responses can lead to parasite multiplication and dissemination (10, 25). However, in CL and even more so in

ML patients, lymphocytes produce large amounts of IFN-γ and TNF-α, although few parasites can be found at the lesion site (7). We have previously shown that patients with ML have an exaggerated type 1 immune response in comparison to CL patients, and the ML response is not appropriated down-modulated by IL-10 (6). We hypothesize that a persistent and non-modulated inflammatory response might be the major factor responsible for the extensive tissue damage observed in ML.

The current study extends previous observations from our group and others showing that type 1 immune responses are elevated in both CL and ML. During both diseases, CD4+ T cells are the main source of IFN-γ (6, 8). The concept that an exacerbated type 1 T cell response participates in the pathogenesis of tegumentary leishmaniasis has not been well established, however recent findings have shown that a higher frequency of IFN-γ and TNF-α producing lymphocytes are associated with larger lesion sizes (3). In the classical model of experimental CL, susceptible BALB/c mice infected with *L. major* develop a type 2 response that suppresses the curative type 1 immune response. In contrast resistant C57BL/6 mice develop a type 1 immune response and control infection (27, 28). During human disease several cytokines including IFN-γ, TNF-α, IL-4 and IL-10 are expressed at the lesion site (18, 20, 21). Previously, we showed that during the initial phase of *L. braziliensis* infection there is a decrease in the type 1 immune response (25). Very quickly, however, the response changes and a strong antigen-specific production of IFN-γ and TNF-α is documented (25). More recently, experimental models in which a few parasites are transmitted by phlebotomine sand flies demonstrate that disease pathology is mediated by T cells (2). During human disease it has also been shown that unusual forms of tegumentary leishmaniasis such as diffuse cutaneous leishmaniasis (DCL) are associated with absence of

IFN-γ (24). Therefore, it has been established that IFN-γ is important in control of parasite multiplication and dissemination. However, the maintenance of this response without the appropriate control conferred by regulatory cytokines such as IL-10, might be responsible for the tissue damage observed in ML patients.

Evidence that the ML lesion is predominantly maintained by inflammatory response is the response to treatment with pentoxiphylline, an inhibitor of TNF-α (17). In the current study we showed that the enhanced T cell response of PBMC cultures from ML patients is not appropriately down modulated by anti-IL-12, anti-IL-15 or CTLA-4. We also showed that anti-IL-2 is less effective in down regulating IFN-γ production by PBMCs from ML than from CL patients. Moreover, ML patient PBMCs had a higher frequency of activated effector T cells than PBMCs from CL patients. These data suggest that peripheral blood T cells are more activated in ML than in CL patients *ex vivo,* possibly explaining why they are not easily modulated *in vitro.*

Several immunological parameters are consistent with the more exacerbated T cell response in ML than in CL PBMCs. Expression of co-stimulatory molecules is important for T cell activation, and a decrease in the rate of apoptosis can maintain activated cells after antigen stimulation. Although we did not detect a difference in the frequency of apoptotic cells between CL and ML patient lymphocytes, ML patients did express enhanced numbers of activated/memory T cells and there was a tendency toward increased in expression of co-stimulatory molecules, and decreased CTLA-4 expression in those patients. It is known that blocking CTLA-4 in experimental leishmaniasis enhances IL-12 and IFN-γ secretion (22) as well as antimony-associated leishmania killing (23). Here the addition of recombinant CTLA-4 to SLA-stimulated PBMC cultures did not decrease IFN-γ

production in CL or ML patients, while it suppresses IFN-γ production in PPD-stimulated PBMC from control patients.

Cytokines involved in T cell differentiation and proliferation (IL-2, IL-12 and IL-15) are important in the maintenance of T cell activation. IL-12 is important for T cell differentiation and proliferation. Neutralization of IL-12 suppressed *Staphylococcus epidermidis*-induced IFN-γ release (30) and exogenous addition of IL-12 restored in vitro T cells responses in VL (9). During the current study the ability of anti-IL-12 to modulate SLA-induced IFN-γ production was decreased in both ML and CL patients, likely reflecting the fact that these cells are already differentiated toward a Th1 type phenotype and no longer depend on IL-12. Neutralization of IL-2 modulated SLA-induced IFN-γ production to a greater extent in PBMC cultures from CL than from ML cases. This demonstrated that high levels of IFN-α, derived primarily from $CD4^{+}$ T cells in these patients, were dependent on IL-2. The greater effect of anti-IL-2 on CL patient T cells suggested a global lack of inhibitory effects that was not just limited to antigen-specific T cells. IL-15 induces lymphocyte proliferation and protects cells from apoptosis, thereby generating memory T cells (14), and anti-IL-15 can down-modulate lymphocyte proliferation (5). In our study, neutralization of IL-15 down-modulated SLA-induced IFN-γ secretion by T cells from CL patients but had weak effect on T cells from ML patients. The difference in SLA response between CL and ML patients could indicate many of these cells are recently activated, or that, similar to the PPD-responsive memory cells, these have become IL-15 independent (16, 31). Our data concerning the higher expression of CD69+ cells supports this hypothesis. Neutralization of IL-2 decreased PPD-induced IFN-γ production in CL and ML

neutralization of IL-15 did not have effect. A possible reason for this phenomenon could be due PPD-specific CD4+ memory T cells not been dependent on IL-15.

As a consequence of antigen stimulation, lymphocytes become activated and later differentiate to effector and memory T cell. Increasing number of cells expressing activation markers have been documented in other chronic inflammatory diseases (26, 29). We found the frequency of CD4+ T cells expressing activation markers was significantly higher in lymphocytes from ML than from CL patients. It is likely that these are effector T cells, which could explain not only the increased in IFN-γ production in ML, but also the inability of these cells to be modulated by neutralization of cytokines or exogenous addition of regulatory cytokines such as IL-10 and TGF-β (6).

The data from this study document the extent of the exaggerated type 1 immune response in lymphocytes from patients with ML in comparison to CL, both diseases caused by the same species of *Leishmania*, i.e. *L. braziliensis*.

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Table 1. Frequency of apoptotic lymphocytes from CL and ML patients. Apoptosis was evaluated by flow cytometry after SLA stimulation and Annexin staining. Results represent the mean ± SD from 4 patients.

% of cells (Annexin +)	CL	ML	p value[a]
Total PBMC	20 ± 7%	19 ± 3%	0.885
Within CD4+ population	25 ±15%	27 ±14%	>0.999
Within CD8+ population	28 ± 7%	28 ± 4%	>0.999

[a] Mann-Whitney

Figure Legends

Figure 1. IFN-γ production on supernatants of cultures of PBMC from CL and ML patients. Cultures of PBMC from 22 CL and 22 ML patients were incubated for 72 hours in presence of SLA (10 μg/ml). IFN-γ levels were measured by ELISA sandwich and results are expressed in pg/ml. Statistical comparisons were done by Mann-Whitney test.

Figure 2. Frequency of CD4+ or CD14+ expressing co-stimulatory molecules. PBMC from CL or ML patients were incubated for 72h in presence of SLA (10 μg/ml). Results represent means and standard deviation from a total of 8 patients from each group.

Figure 3. Ability of anti-IL-2 (20 μg/ml), anti-IL-12 (20 μg/ml) or anti-IL-15 (20 μg/ml) in suppress SLA (10 μg/ml)-induced IFN-γ production in 10 CL and 10 ML patients. IFN-γ levels were determined by ELISA on supernatants of PBMC cultures. A, C and E show mean ± SD of results using cultured PBMCs from CL patients; B, D, and F show results using PBMCs from ML patients. All conditions were tested in triplicate in each experiment. Statistical comparisons were done using the Wilcoxon matched-pairs test.

Figure 4. Down modulation of SLA-induced IFN-γ production by of anti-IL-2 (20 μg/ml) and anti-IL-15 is more evident in CL than in ML patients. A comparative analyses of the percentage of suppression modulated by anti-IL2 or anti-IL-15 is shown as the mean ± SD suppression in PBMC cultures from CL versus ML patients, analyzed by Mann-Whitney test. Data are compiled from the raw ELISA data shown in Figure 3.

Figure 5. Ability of anti-IL-2 (20 μg/ml) or anti-IL-15 (20 μg/ml) to suppress PPD (10 μg/ml)-induced IFN-γ production from PBMC cultures from 6 CL and 7 ML patients. Shown are the mean ± SD suppression. IFN-γ levels were performed by ELISA on

supernatants of PBMC cultures. Statistical differences between the degree of suppression in CL versus ML supernatants was performed using Mann-Whitney test.

Figure 6. Frequency of CD4+ cells expressing cell activation markers. PBMCs were incubated for 72h in presence of SLA (10 μg/ml). Results represent means and standard deviation from a total of 5 patients from each group. Conditions were tested in triplicate. Statistical comparisons were done using the Mann-Whitney test.

Figure 1

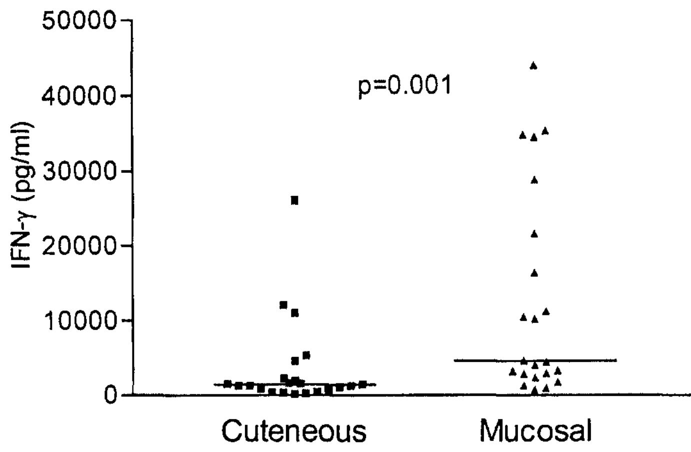


Figure 2

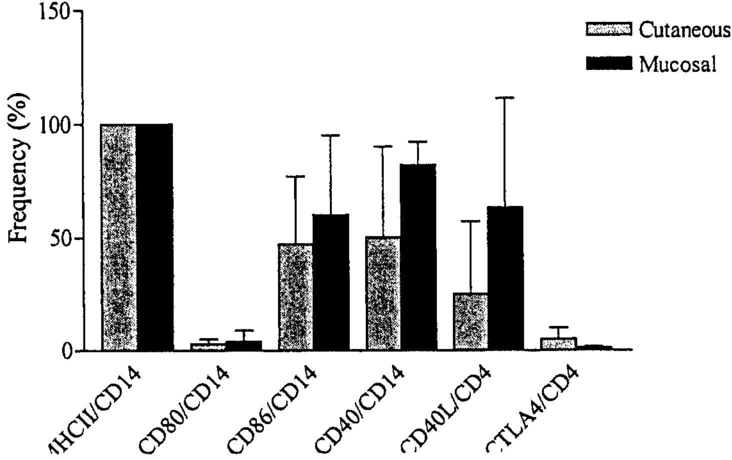
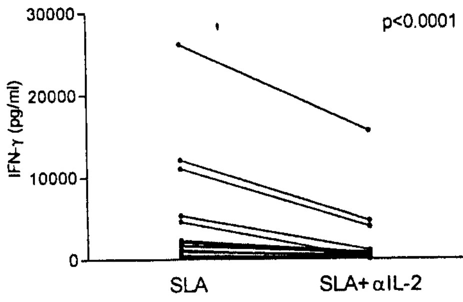


Figure 3

A



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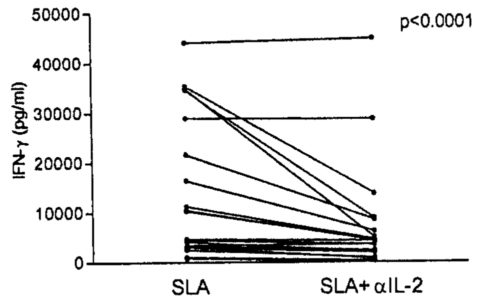

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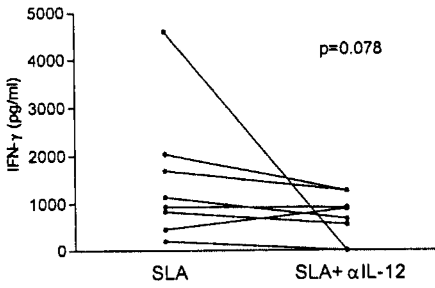

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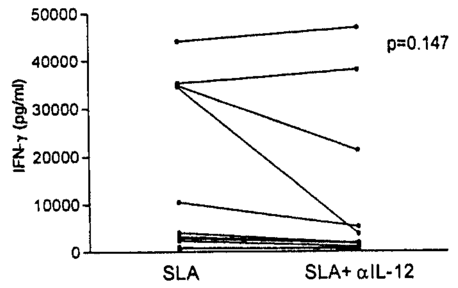

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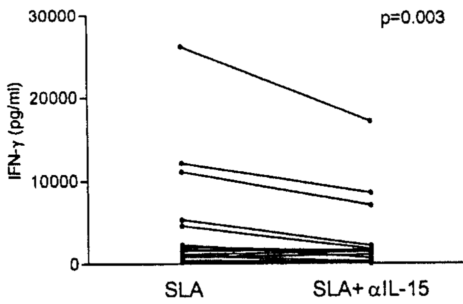

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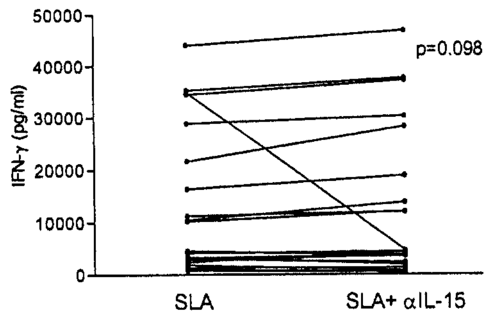


Figure 4

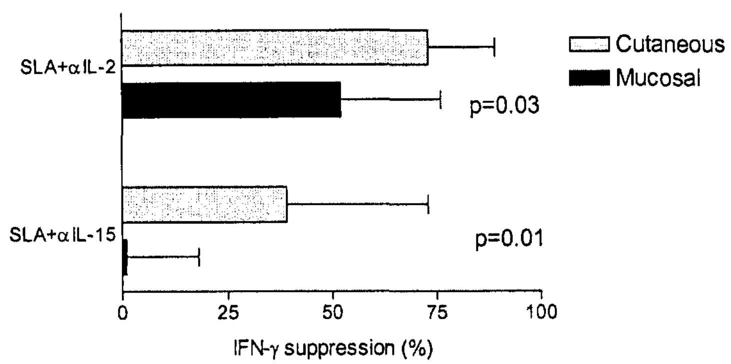


Figure 5

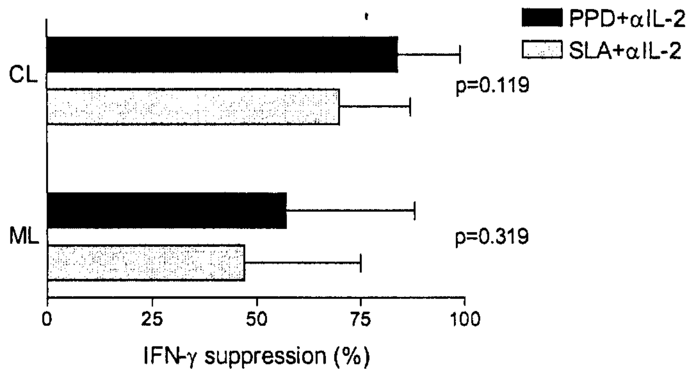
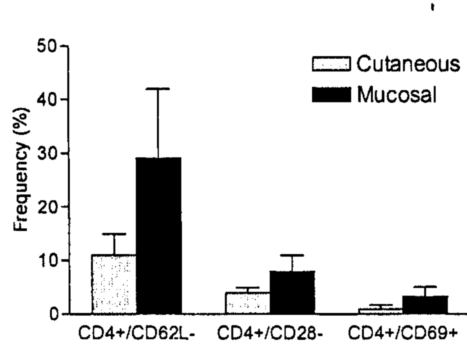


Figure 6



A hipótese desse estudo é que diferentes antígenos recombinantes de Leishmania induzem padrões de resposta imune diferenciada através da ativação de diferentes tipos celulares.

Resumo artigo 2:

Characterization of immune response to *Leishmania infantum* recombinant antigens.

Leishmanioses têm alta prevalência em países tropicais. A fim de aperfeiçoar os sistemas diagnósticos existentes baseados em proteínas totais de Leishmania, e identificar antígenos candidatos para desenvolvimento de vacinas, uma intensa busca para identificação de antígenos vem sendo realizada, utilizando técnicas de biologia molecular. Neste estudo, a resposta imune a três antígenos recombinantes de *L. infantum* foi avaliada. Células mononucleares (PBMC) de pacientes com leishmaniose cutânea produziram altos níveis de IL-10 após estimulação com KMP11, enquanto uma predominante produção de IFN-γ pode ser observada em culturas estimuladas com H2A e antígeno solúvel de Leishmania. Todos os antígenos recombinantes induziram baixos níveis de IL-5. A adição de KMP11 modulou a produção de IFN-γ (48%) nas culturas de células mononucleares de sangue periférico de pacientes com leishmaniose cutânea, estimulados com antígeno solúvel de Leishmania. Além disso, anticorpos (IgG) anti-KMP11 foram detectados no soro de todos os pacientes com LV e na maioria dos soros de pacientes com LC ou indivíduos com infecção assintomática por *L. chagasi*. KMP11 é reconhecido pelas células e soro de pacientes com diferentes formas clínicas da leishmaniose, induz produção de IL-10 e é capaz de modular a resposta imune tipo 1.
Experimento de depleção de linfócitos demostrou monócitos / macrófagos são as principais células produtoras de IL-10 após estímulo com KMP11.

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Original article

Characterization of the immune response to *Leishmania infantum* recombinant antigens

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Abstract

Leishmaniases have a high prevalence in tropical countries. In order to improve existing diagnostic systems based on total *Leishmania* proteins, and to identify antigen candidates for vaccine development, an intensive search for the identification of antigens was performed using molecular biology techniques. In this study, the immune response to three *L. infantum* recombinant antigens was evaluated. Upon stimulation with KMP11, mononuclear cells from leishmaniasis patients produced high levels of IL-10, while a predominant IFN-γ production could be observed in cultures stimulated with H2A and soluble *Leishmania* antigen. All the recombinant antigens induced very little IL-5. KMP11 decreased IFN-γ production by 48% in cultures of peripheral blood mononuclear cells from cutaneous leishmaniasis patients who had been stimulated with soluble *Leishmania* antigen. Furthermore, antibodies to KMP11 were detected in the sera from all patients with visceral leishmaniasis and in the majority of the sera from patients with cutaneous leishmaniasis or individuals with asymptomatic *L. chagasi* infection. Thus, KMP11 is recognized by cells and sera of patients with different clinical forms of leishmaniasis, and KMP11, through IL-10 production, proved to be a potent antigen in modulating type 1 immune response.

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Keywords: Leishmaniasis; Immuneregulation; IFN-γ; KMP11

1. Introduction

Leishmaniases are endemic in developing tropical countries, affecting ~600,000 people every year [1]. Due to the high cost of treatment, evidence of resistance and the toxicity generated by antimony therapy [2], the development of a vaccine must be considered an anticipated solution. Most studies aimed at identifying antigens from *Leishmania* sp. have searched for molecules with the ability to stimulate IL-2, IFN-γ and IL-12 [3–5], once the Th1 type immune response was known to be the major defense mechanism against *Leishmania* infection [6]. In fact, IFN-γ is the main cytokine implicated in the activation of macrophages to the killing of *Leishmania* [7]. The absence of a type 1 immune response to *Leishmania* antigen is documented in patients with visceral leishmaniasis and diffuse cutaneous leishmaniasis diseases characterized by parasite multiplication and dissemination [8,9]. However, in patients infected with *L. braziliensis*, the presence of strong IFN-γ and TNF-α production does not prevent development of the cutaneous or mucosal disease [10,11]. In fact, an exacerbated inflammatory response may be correlated with the tissue damage observed in cutaneous and mucosal leishmaniasis [11,12].

IL-10 is the main downregulatory cytokine of type 1 immune response inhibiting IL-12, IL-2, TNF-α and IFN-γ synthesis and decreasing macrophage activity [13–16]. Although high levels of IL-10 may be dangerous in patients with infections caused by intracellular pathogens, the modulation of an inflammatory response mediated by this cytokine

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is important for the regulation of a chronic inflammatory reaction [17,18]. In the present study, the immune response to three recombinant *L. infantum* antigens (H3, H2A and KMP11) was characterized in patients with cutaneous leishmaniasis and visceral leishmaniasis and in individuals with subclinical *L. chagasi* infection. The finding that one of these antigens (KMP11) induced high levels of IL-10 led us to determine the ability of KMP11 to modulate type 1 immune response observed in cutaneous leishmaniasis.

2. Material and methods

2.1. Patients

Cutaneous leishmaniasis patients (n = 13) were recruited from the health post of Corte de Pedra (southeast of Bahia state, Brazil), an area of *L. braziliensis* transmission, where clinical and immunological studies have been carried out for more than 20 years. All patients had active disease and were evaluated prior to therapy. Subclinical *L. chagasi*-infected individuals (n = 10) were recruited from the peri-metropolitan area of Natal, where a cohort study of *L. chagasi*-infected individuals has been followed for 5 years [19]. All persons with subclinical *L. chagasi* infection had antibodies to *Leishmania* antigen and had a positive delayed type hypersensitivity for more than 1 year without evidence of clinical symptoms related to kala-azar. Visceral leishmaniasis patients (n = 10) were admitted to the Hospital Universitário Prof. Edgard Santos, and diagnosis was confirmed by parasite isolation. Sera from visceral leishmaniasis patients were obtained prior to and after therapy. Healthy individuals (n = 20) were used as controls. The diagnosis of cutaneous leishmaniasis was performed by parasite isolation or by histopathological findings characteristic of cutaneous leishmaniasis and a positive skin test reaction with *Leishmania* antigen. Healthy employees (n = 20) living outside endemic areas of leishmania were used as controls. This study was approved by the ethical committee of the hospital, and informed consent was obtained from all patients.

2.2. Leishmania *antigens and antibody detection*

Two histones (H3 and H2A) and the 11-kDa surface protein (KMP11) were purified as previously described [20,21]. The leishmania species used in the present study were characterized by a World Health Organization center (MHOM). Soluble *Leishmania* antigen (SLA) was prepared from a strain of *L. amazonensis* (MHOM BR-86 BA-125) or from a strain of *L. chagasi* (MHOM BA-62) as previously described [22]. After a dose curve response had been performed, these recombinant antigens were used at a concentration of 10 μg/ml of culture.

An ELISA test was performed to detect antibodies against KMP11 using a 1:50 dilution of the sera as previously described for soluble *Leishmania* antigen [23]. The KMP11 antigen was used at a concentration of 100 ng/well. A serologic test for KMP11 antigen was considered positive when the OD was > 0.030, which represents the mean + three standard deviations of the OD observed when the sera of healthy subjects not exposed to *Leishmania* were used.

2.3. Cell culture and cytokine assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Hypaque gradient centrifugation. After being washed three times in 0.9% NaCl, the PBMCs were resuspended in RPMI-1640 culture medium (GIBCO BRL, Grand Island, NY) supplemented with 10% human AB serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Cells were adjusted to 3×10^6 cells/ml, placed in 24-well plates and stimulated with SLA (1 μg/ml), H3 (10 μg/ml), H2A (10 μg/ml), KMP11 (10 μg/ml) or *L. chagasi* antigen (1 μg/ml). Some of the cells were stimulated with soluble *Leishmania* antigen plus KMP11. To evaluate the origin of IL-10, we determined the amount of this cytokine in cultures containing adherent and non-adherent cells and by determination of the ability of KMP11 to downregulate IFN-γ production in non-adherent cells. For these experiments, macrophages were depleted by adherence to a plastic surface as previously described [24]. Monoclonal antibody anti-IL-10 (200 μg/ml) (Coffman, DNAX Institute, Palo Alto, CA) was added to some SLA-stimulated cultures. Cultures of the whole population of cells, adherent and non-adherent, were incubated for 72 h at 37 °C and 5% CO_2. Supernatants were collected and stored at −70 °C. The levels of IL-5, IL-10 and IFN-γ were measured by the ELISA sandwich method using reagents (R&D Systems, Minneapolis, MN). The results are expressed in pg/ml.

2.4. Statistical analysis

The statistical analysis was performed using the Mann Whitney test.

3. Results

The levels of IFN-γ, IL-5 and IL-10 in supernatants of PBMC cultures from cutaneous leishmaniasis patients in response to SLA and the recombinant antigens H3, H2A and KMP11 are shown in Fig. 1 . IFN-γ levels in cultures stimulated with H3 and H2A antigen were 234 ± 576 and 252 ± 429 pg/ml, respectively. These levels were lower than those observed in cultures stimulated with SLA (1003 ± 564 pg/ml). Low levels of IFN-γ (104 ± 120 pg/ml) were detected in 11 of the 13 (84%) supernatants of PBMC cultures stimulated with KMP11. IL-5 levels were significantly higher in supernatants of SLA-stimulated cultures than in those stimulated with recombinant antigens. In contrast with what was observed in the supernatants of PBMC of H2A- or SLA-stimulated cultures from cutaneous leishmaniasis pa-

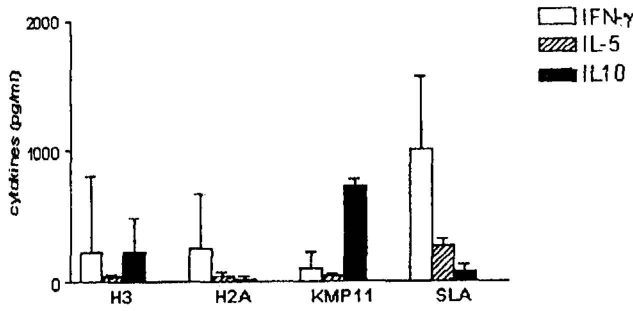

Fig. 1. IFN-γ, IL-5 and IL-10 levels in PBMCs from cutaneous leishmaniasis patients ($n = 13$) in response to H3 (10 μg/ml), H2A (10 μg/ml), KMP11 (10 μg/ml) and *L. amazonensis* (SLA) (1 μg/ml).

tients, in which production of IL-10 was absent or low, high levels of IL-10 were observed in KMP1-stimulated cultures (725 ± 54 pg/ml) and in the majority of the supernatants of H3-stimulated cultures (231 ± 157 pg/ml).

IFN-γ, IL-5 and IL-10 levels in the supernatants of PBMC cultures from six individuals with subclinical *L. chagasi* infection are shown in Fig. 2 . IFN-γ production predominated over that of IL-5 and IL-10 in these supernatants after stimulation of cultures with H3 or H2A. Although KMP11 induced higher ($P < 0.05$) levels of IL-10 (139 ± 121 pg/ml) than those observed after stimulation with SLA (24 ± 51 pg/ml), the amount of IL-10 was much lower ($P < 0.01$) than that observed in the PBMC cultures from cutaneous leishmaniasis patients. Only KMP11 was able to induce high levels of IL-10 on PBMC cultures of healthy subjects (669 ± 195 pg/ml).

Since a high production of IL-10 was observed after KMP11 stimulation, the ability of this antigen to modulate the Th1 type immune response in cutaneous leishmaniasis patients was tested in cell cultures after SLA stimulation (Fig. 3). The addition of KMP11 to SLA-stimulated cultures decreased the IFN-γ production from 1140 ± 761 to 441 ± 496 pg/ml ($P < 0.01$).

To confirm that the downregulation of IFN-γ was dependent on the high levels of IL-10 induced by KMP11, the neutralization of this cytokine was performed in cultures stimulated with KMP11 plus SLA; in four cutaneous leishmaniasis patients, addition of anti-IL-10 enhanced the IFN-γ production from 207 ± 212 to 728 ± 518 pg/ml (Fig. 4).

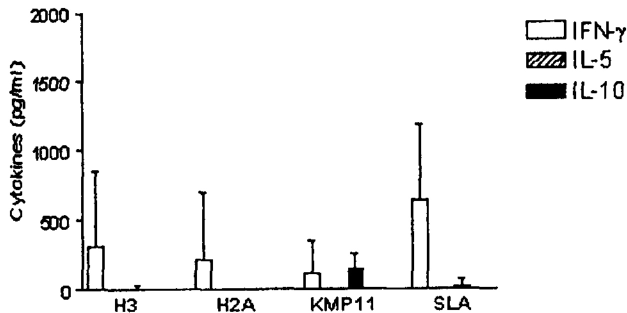

Fig. 2. IFN-γ, IL-5 and IL-10 levels in PBMCs from asymptomatic *L. chagasi*-infected patients ($n = 6$) in response to H3, H2A, KMP11 and *L. chagasi* (SLA) antigen.

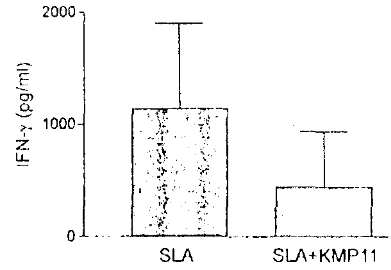

Fig. 3. Ability of KMP11 to suppress IFN-γ production in SLA-stimulated lymphocyte cultures from cutaneous leishmaniasis patients.

Two analyses were performed to determine the source of IL-10. First, we evaluated whether the suppression of IFN-γ production mediated by KMP11 in cultures stimulated with *Leishmania* antigen could be observed in the population of non-adherent cells. We also determined the production of IL-10 in cultures of adherent as well as non-adherent cells and in cultures of adherent cells stimulated with KMP11. Depletion of adherent cells abrogates the ability of KMP11 to suppress IFN-γ production (Fig. 5). While addition of KMP11 to PBMC cultures stimulated with *Leishmania* antigen suppressed IFN-γ production by 74%, no suppression of

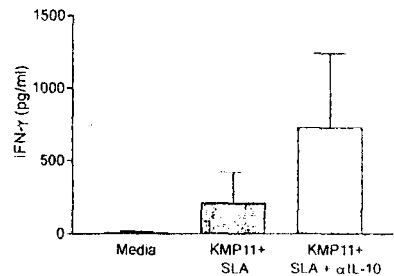

Fig. 4. Anti-IL-10 monoclonal antibody (50 μg/ml) enhanced IFN-γ production in SLA (1 μg/ml) plus KMP11 (10 μg/ml)-stimulated cultures from four cutaneous leishmaniasis patients.

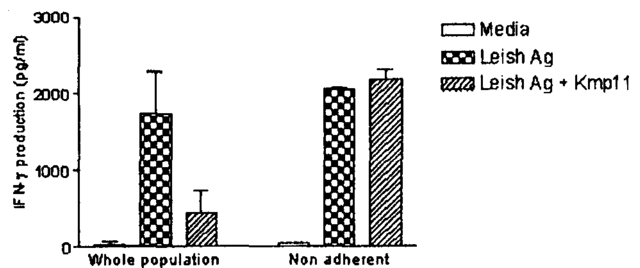

Fig. 5. Evaluation of the ability of KMP11 (10 μg/ml) to downregulate IFN-γ production in SLA-stimulated PBMCs (whole population) and in cells depleted of macrophages (non-adherent cells) of cutaneous leishmaniasis patients.

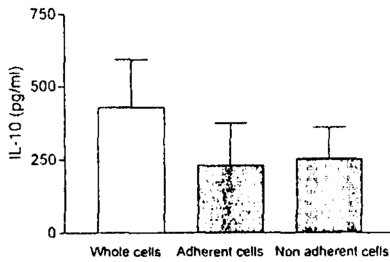

Fig. 6. IL-10 levels in KMP11-stimulated cultures of different cell populations from cutaneous leishmaniasis patients (n = 4).

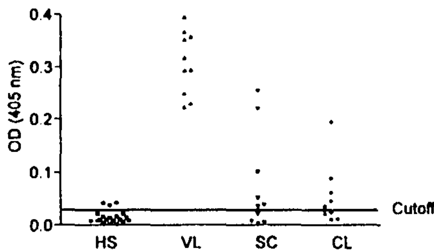

Fig. 7. Anti-KMP11 IgG titers of healthy subjects (HS), visceral leishmaniasis patients (VL), subclinical *L. chagasi*-infected individuals (SC) and cutaneous leishmaniasis patients (CL).

IFN-γ production was observed when KMP11 was added to cultures of non-adherent cells stimulated with *Leishmania*antigen (Fig. 5). It should be noted that while the percentage of macrophages in PBMC cultures as measured by esterase staining was 24 ± 5% in the mononuclear cells, it fell to 2 ± 1.8% in the non-adherent cell population. When IL-10 was measured in supernatants of adherent and non-adherent KMP11-stimulated cells, no significant difference was detected (Fig. 6).

Antibody levels to KMP11 were also determined in the serum samples from visceral leishmaniasis, subclinical *L. chagasi* infection, cutaneous leishmaniasis and healthy subjects (Fig. 7). Anti-KMP11 IgG antibodies were observed in serum samples of all visceral leishmaniasis patients and in sera of some individuals with subclinical *L. chagasi* infection and cutaneous leishmaniasis patients. Antibodies to KMP11 were not detected in the majority of sera from healthy subjects not exposed to leishmania infection.

4. Discussion

The search for parasite antigens able to induce an immune response has been predominantly associated with the identification of proteins that may be used for serodiagnosis or vaccine development [3–5]. In this study, we characterized the immune response to three *L. infantum* recombinant antigens that were recognized by sera of visceral leishmaniasis and cutaneous leishmaniasis patients. We found that the KMP 11-kDa antigen induced high levels of IL-10 in PBMC cultures of patients with cutaneous leishmaniasis, in individuals with asymptomatic *L. chagasi* infection and in healthy subjects. Characterization of the cellular immune response was performed in patients with cutaneous leishmaniasis and in individuals with subclinical *L. chagasi* infection, since it is known that a T-cell response develops when cells from these individuals are stimulated with SLA [25,26]. Although the three antigens were to some extent able to stimulate PBMCs from these individuals, the response was quite variable. The three antigens induced lower amounts of IFN-γ and IL-5 than did SLA. Additionally, the number of individuals who produced these cytokines upon stimulation with the recombinant antigens was quite variable. In contrast, there was a high production of IL-10 in KMP11-stimulated cultures in 18 of 19 individuals with leishmaniasis. This antigen induced a higher level of IL-10 in PBMCs from patients with cutaneous leishmaniasis than in PBMCs from individuals with an *L. chagasi* infection. The documentation that KMP11 induces IL-10 in PBMCs from healthy subjects suggests that this cytokine is produced by macrophages or that KMP11-like epitopes may be found in other antigens, thus making the majority of the subjects respond to it. Our data suggest that the source of IL-10 can be both macrophages and lymphocytes, since macrophage depletion abrogated the ability of KMP11 to suppress IFN-γ production and because of the finding that IL-10 was produced by both the adherent and non-adherent cells. Additionally, the explanation for finding different levels of IL-10 in patients with cutaneous leishmaniasis and those who had subclinical *L. chagasi* infection would be the participation of lymphocytes in IL-10 secretion. It cannot be ruled out that macrophages from different individuals produce different amounts of IL-10 after stimulation with KMP11. The observation of antibodies against KMP11 only in patients infected with *Leishmania* is evidence that this antigen induces an acquired immune response.

By modulating macrophages and T-cell functions, IL-10 is the major regulatory cytokine of the inflammatory and type 1 immune response [14]. In diseases caused by intracellular parasites, high production of IL-10 may be dangerous to the host due to its ability to decrease type 1 immune response and macrophage activation. This would allow multiplication of the parasites, as we observed in patients with visceral leishmaniasis, and diffusion of cutaneous leishmaniasis [27,28]. However, IL-10 may be crucial in modulating the inflammatory response associated with tissue damage. In fact, in patients with cutaneous and mucosal leishmaniasis due to *L. braziliensis*, the high type 1 immune response, when not appropriately modulated, may cause tissue damage [11,12]. Modulation of the immune response by IL-10 has also been suggested to protect animals from tissue destruction in autoimmune diseases such as diabetes and experimental acute immune encephalitis [29,30]. We showed that KMP11 was able to downregulate IFN-γ production in patients with cuta-

neous leishmaniasis and that this effect is dependent on the modulation of IL-10, since neutralization of IL-10 by monoclonal antibody abrogates the regulatory effect of this antigen. Since KMP11 is predominantly expressed in promastigotes and only expressed at very low levels in amastigotes [31], it is unlikely that this antigen is detrimental to the host by significantly decreasing type 1 immune response needed for parasite control. The observation that exogenous KMP11 downregulates IFN-γ production indicates that this antigen may play a role in modulating the exaggerated type 1 immune response observed in chronic inflammatory diseases.

Acknowledgements

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A hipótese do presente estudo é que antígenos recombinantes com propriedades regulatórias negativas são capazes de modular a resposta inflamatória de pacientes com LM ou LC.

Resumo artigo 3:

Effect of LACK and KMP11 on IFN-γ production by peripheral blood mononuclear cells from cutaneous and mucosal leishmaniasis.

As propriedades imuno modulatórias dos antígenos recombinantes Kinetoplasmídeo proteína de membrana – 11 (KMP11) e receptor homólogo para proteína C kinase ativada da *Leishmania* (LACK) em pacientes com leishmaniose cutânea (LC) e em pacientes com leishmaniose mucosa (LM) foram avaliadas. A média das concentrações de interferon-γ (IFN-γ) em sobrenadantes de CMSP estimuladas com antígeno solúvel de Leishmania (SLA) de pacientes com LM e LC foram 5625 ± 2333 pg/ml e 4422 ± 3665 pg/m, respectivamente. IFN-γ não foi detectado em sobrenadantes de CMSP estimuladas com KMP11 ou LACK. A concentração de interleucina-10 (IL-10) em sobrenadantes de CMSP estimuladas com SLA, KMP11 e LACK em pacientes com LM foi 13 ± 12 pg/ml, 285 ± 388 pg/ml e 802 ± 483 pg/ml, respectivamente. A adição de KMP11 ou LACK às CMSP, estimuladas com SLA, de pacientes com LC e LM aumentou a produção de IL-10 ($P<0.05$). A adição de KMP11 diminuiu as concentrações de IFN-γ em 52% em pacientes com LC e em 19% de pacientes com LM. A adição de LACK às culturas estimuladas com SLA diminuiu as concentrações de IFN-γ em 58% nos pacientes com LC e em 30% nos pacientes com LM. A neutralização de IL-10 inibiu o efeito modulador do LACK e do KMP11sobre a produção de IFN-γ. A capacidade moduladora do LACK e do KMP11 pode servir para atenuar as doenças inflamatórias crônicas. Entretanto, em algumas condições clínicas, como demonstrado na LM, estas moléculas não são capazes para suprimir a resposta de IFN-γ, mesmo induzindo a produção de IL-10.

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Effect of LACK and KMP11 on IFN-γ Production by Peripheral Blood Mononuclear Cells from Cutaneous and Mucosal Leishmaniasis Patients

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Abstract

The immune modulatory properties of recombinant antigens Kinetoplasmid membrane protein-11 (KMP11) and *Leishmania* homologue of receptors for activated C kinase (LACK) in cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) patients were evaluated. The mean levels of interferon-γ (IFN-γ) in soluble leishmania antigen (SLA) stimulated peripheral blood mononuclear cells (PBMC) of ML and CL patients were 5625 ± 2333 pg/ml and 4422 ± 3665 pg/ml, respectively. IFN-γ was not detected in cultures stimulated with KMP11 or LACK. Interleukin-10 (IL-10) concentration in SLA, KMP11 and LACK-stimulated PBMC of ML patients was 13 ± 12 pg/ml, 285 ± 388 pg/ml and 802 ± 483 pg/ml, respectively. Addition of KMP11 or LACK to SLA-stimulated PBMC of CL and ML patients enhanced IL-10 production ($P < 0.05$). Addition of KMP11 decreased IFN-γ levels by 52% in CL patients and by 19% in ML patients. Addition of LACK to SLA-stimulated cultures decreased IFN-γ levels by 58% in CL patients and by 30% in ML patients. Neutralization of IL-10 abrogated the downregulatory effect of LACK and KMP11. The modulatory properties of LACK and KMP11 are due to induction of IL-10 production and may be helpful for attenuating chronic inflammatory diseases. However, in some clinical conditions, as demonstrated for ML, these molecules are not able to suppress the IFN-γ response, even inducing IL-10 production.

Introduction

Leishmaniasis is a disease with a global distribution, affecting 12 million people worldwide with 600,000 new cases per year, caused by various strains of *Leishmania* species [1]. Cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) caused by *Leishmania braziliensis* are the main clinical forms of leishmaniasis found in Northeast Brazil [2]. A well-delineated ulcerated lesion with elevated borders characterizes the cutaneous lesions. Around 3% of CL patients develop mucosal disease [2] characterized by an intense inflammatory reaction with the absence of or few parasites at the lesion site [3]. Because the T-helper type 1 immune response characterized by interferon (IFN)-γ, interleukin (IL)-2 and tumour necrosis factor (TNF)-α production is known to be protective against *Leishmania* infection [4, 5], many studies attempted to identify molecules that stimulate these responses. However, peripheral blood mononuclear cells (PBMC) from both CL and ML patients produce high amounts of IFN-γ and TNF-α when stimulated with soluble leishmania antigen (SLA) [6], and evidence has been accumulating that tissue damage in these diseases may be due to the host immune response [6–8]. For instance, (1) treatment with antimony of early cutaneous lesions does not prevent the development of ulcers [9]; (2) SLA-stimulated PBMC from ML patients produce higher levels of IFN-γ and TNF-α and lower levels of IL-10 than PBMC from CL patients [6]; (3) IFN-γ and TNF-a levels fall after successful antimony therapy [10, 11] and (4) drugs that down modulate the immune response combined with antimony increase the cure rate and decrease the healing time of mucosal lesions [8].

Multiple host and parasite factors are likely to influence if a lesion develops during infection with leishmania. To date, little attention has been given to the role of specific

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parasite antigens in leishmaniasis. The tissue damage in CL and ML is probably in part due to a dominant type I inflammatory response trigged by leishmania antigens. However, it is likely that other specific parasite antigens are able to modulate inflammatory response in these patients. Identification of these molecules may provide perspectives for immunotherapy.

The *Leishmania* homologue of receptors for activated kinase C (LACK) is known to induce a high frequency of $CD4^+$ T cells that secrete IL-4 in BALB/*c* mice, which are known to be associated with exacerbation of *Leishmania major* infection [12]. Moreover, the LACK-specific response has recently been correlated with a downregulation of monocyte activity in human CL [13]. Kinetoplasmid membrane protein-11 (KMP11) is present in a wide range of trypanosomatids [14] and has been used in serological tests in canine visceral leishmaniasis. This antigen is known to induce more IL-10 than IFN-γ in CL patients [15]. Thus, the aim of the present study was to evaluate the effect of KMP11 and LACK in downregulate the high type 1 immune responses observed in ML and CL patients.

Materials and methods

Patients. CL ($n=11$) and ML patients ($n=11$), matched by sex and age (±10 years), were selected from the *L. braziliensis* transmission endemic area of Corte de Pedra in the Southeast of Bahia state, Brazil. Diagnosis of leishmaniasis was made by the presence of a typical skin or mucosal lesion and parasite isolation or by one of the two following criteria: histopathology or delayed-type hypersensitivity skin test to *Leishmania* antigen. All subjects had active disease, positive *Leishmania* skin test (>5 mm) and were evaluated prior to therapy. Exclusion criteria were age <5 or >60 years, pregnancy or diseases (diabetes and HIV) that would interfere in the immune response or having started treatment for leishmaniasis. This study was approved by the ethical committee of the Hospital Universitário Prof Edgard Santos, and an informed consent was obtained from all participants or their parents or guardians if patients were less than 18 years old.

Antigen preparation and cytokine production in PBMC-stimulated cultures. SLA was prepared from a strain isolated from a ML patient as previously described [16]. The recombinant antigens LACK and KMP11 were prepared as previously described [14, 17]. In both LACK and KMP11 preparations, lipopolysaccharide (LPS) was determined by Limulus amoebocyte lysate assay. No LPS was detected in the LACK or KMP11 antigens. Treatment of the KMP11 preparation with polymixin B did not modify the ability of this antigen to induce IL-10 and to downmodulate IFN-γ production. A dose – response curve was performed, and the antigens were used at a concentration of 10 μg/ml (SLA and LACK) and 5 μg/ml (KMP11).

PBMC were isolated from heparinized venous blood by Ficoll – Hypaque (Organon Teknika, Durham, NC, USA) gradient centrifugation. After washing three times in 0.9% NaCl, the PBMC were resuspended in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% human AB serum (SIGMA Chemical Co, St. Louis, MO, USA), 100 IU/ml penicillin and 100 mg/l streptomycin. Cells were adjusted to 3×10^6 cells/ml, placed in 24-well plates and stimulated with SLA (10 μg/ml), KMP11 (5 μg/ml) or LACK (10 μg/ml) or with SLA plus KMP11 or LACK. Neutralization of IL-10 by monoclonal antibody (R & D Systems, Minneapolis, MN, USA) (20 μg/ml) was performed in four experiments. The PBMC were incubated for 72 hours at 37 °C and 5% CO_2. Supernatants were collected and stored at −70 °C. The levels of IFN-γ, IL-5 and IL-10 were measured by the enzyme-linked immunosorbent assay sandwich method using commercial reagents (R & D Systems). Results are expressed in pg/ml based on a standard curve with recombinant cytokines.

Determination of cytokine producing cells. Evaluation of the frequency of cytokine-producing cells by fluorescence-activated cell sorter (Becton Dickinson, Palo Alto, CA, USA) was performed in the last four CL and ML patients enrolled in the study. After washing three times, PBMC were counted and placed in 96-well U-bottom plates at a concentration of 2×10^5 cells/200 μl of RPMI-1640 (Gibco BRL) supplemented with 10% human AB Rh^+ serum (SIGMA Chemical Co), 100 IU/ml penicillin and 100 mg/l streptomycin. Staining for surface and intracellular markers was performed as previously described. Briefly, PBMC were cultured for 20 hours at 37 °C and 5% CO_2, and during the last 4 hours, Brefeldin A (1 μg/ml) was added. After centrifugation, cells were incubated for 20 min at 4 °C with surface antibodies with a 1:20 dilution of anti-CD4, anti-CD8 and anti-CD14 (Pharmingen, San Diego, CA, USA) [18]. Cells were fixed with 2% formaldehyde in PBS, permeabilized with a solution of saponin and stained for 30 minutes, using a 1:4 dilution of anti-IL-10-PE (Pharmingen). The antibodies concentrations were previously established in our laboratory according to previous publication from our group and others [19]. Isotype-control antibodies (IgG1 and IgG2a) were used. To analyze the frequency of IL-10-producing cells, two regions were created based on cell size and granularity: R1, which contained only lymphocytes and blasts; and R2, a macrophage region.

Statistical analysis. The comparison between the immunological responses of CL and ML patients to SLA with their response to SLA plus KMP11 or SLA plus LACK was performed by Wilcoxon matched-pairs analysis. A nonparametric test was chosen considering that the samples did not follow a Gaussian distribution. An α of 5% ($P=0.05$) on a two-sided table was considered for significance.

Results

Cytokine profile from ML and CL patients

A total of 11 CL patients and 11 ML patients were included in this study. There were no significant differences between the mean ages of CL and ML. The disease duration ranged from 30 to 90 days in both groups, and all patients were evaluated prior to therapy. The levels of IFN-γ, IL-5 and IL-10 in PBMC supernatants stimulated by SLA, LACK and KMP11 of ML and CL patients are represented in Fig. 1. The mean level of IFN-γ in supernatants of SLA-stimulated PBMC of ML and CL patients were 5625 ± 2333 pg/ml and 4422 ± 3665 pg/ml, respectively (Fig. 1A,B). IFN-γ was not detected in PBMC supernatants from ML or CL patients stimulated with KMP11 or LACK. Absence or low levels of IL-5 were detected in PBMC supernatants of both the groups of patients stimulated with SLA, KMP11 or LACK. The levels of IL-10 (mean ± SD) in SLA-stimulated PBMC supernatants from ML patients was 13 ± 12 pg/ml, while in KMP11- and LACK-stimulated PBMC supernatants were 285 ± 388 pg/ml and 802 ± 483 pg/ml, respectively (Fig. 1A). The level of IL-10 in SLA-stimulated PBMC supernatants from CL patients was 20 ± 25 pg/ml, while in KMP11- and LACK-stimulated PBMC supernatants were 277 ± 132 pg/ml and 1072 ± 414 pg/ml, respectively (Fig. 1B).

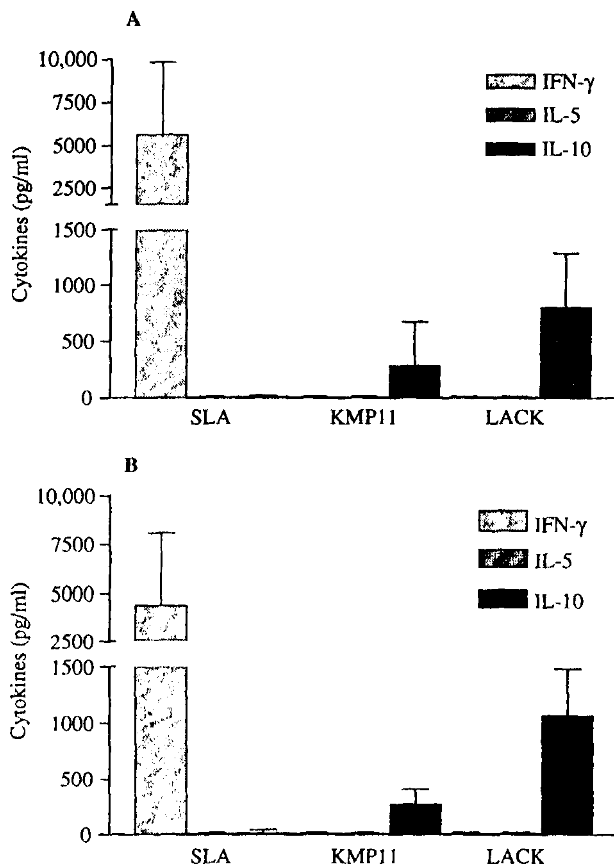


Figure 1 Cytokines profile in peripheral blood mononuclear cell supernatants from mucosal leishmaniasis patients ($n = 5$) (A) and cutaneous leishmaniasis patients ($n = 5$) (B) stimulated with soluble leishmania antigen (10 µg/ml), Kinetoplasmid membrane protein-11 (5 µg/ml) or *Leishmania* homologue of receptors for activated C kinase (10 µg/ml).

LACK and KMP11 upregulate IL-10 production in SLA-stimulated PBMC of ML and CL patients

KMP11 and LACK induced predominantly IL-10; therefore, the ability of these recombinant antigens to enhance IL-10 production in SLA-stimulated PBMC of CL and ML patients was evaluated (Fig. 2). The levels of IL-10 in SLA-stimulated PBMC of CL and ML patients were 20 ± 25 pg/ml and 13 ± 12 pg/ml, respectively. Addition of KMP11 to SLA-stimulated PBMC of CL and ML patients enhanced IL-10 production to 124 ± 101 pg/ml and 215 ± 229 pg/ml, respectively ($P < 0.05$), Mann – Whitney test. Addition of LACK to SLA-stimulated PBMC of CL and ML patients enhanced IL-10 production to 774 ± 340 pg/ml and 652 ± 403 pg/ml, respectively ($P < 0.05$), Mann – Whitney test. IL-10 levels in KMP11-stimulated PBMC of CL and ML were 277 ± 132 pg/ml and 285 ± 388 pg/ml, respectively, and LACK-stimulated PBMC of CL and ML were 1072 ± 414 pg/ml and 802 ± 483 pg/ml, respectively (Fig. 2).

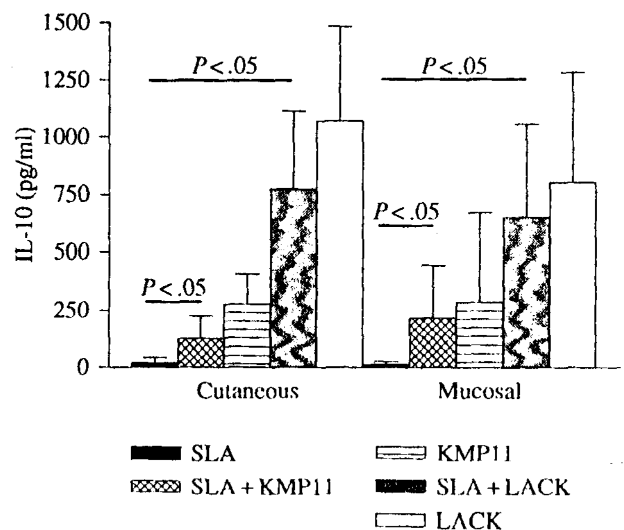


Figure 2 Enhancement of interleukin-10 production by the addition of Kinetoplasmid membrane protein-11 (5 µg/ml) or *Leishmania* homologue of receptors for activated C kinase (10 µg/ml) on soluble leishmania antigen-stimulated (10 µg/ml) peripheral blood mononuclear cell cultures of cutaneous ($n = 5$) and mucosal leishmaniasis ($n = 5$) patients. Wilcoxon matched pairs.

LACK and KMP11 downmodulate IFN-γ production by SLA-induced PBMC of CL patients but has a lower effect in ML patients

To test the ability of KMP11 and LACK to downmodulate IFN-γ production induced by SLA, these recombinant antigens were added into SLA-stimulated PBMC from CL and ML patients. The level of IFN-γ in SLA-stimulated PBMC cultures from CL patients was 4756 ± 5054 pg/ml and from ML patients was $14{,}542 \pm 21{,}792$ pg/ml. Addition of KMP11 to SLA-stimulated cultures decreased by 52% (2743 ± 3600 pg/ml) ($P < 0.05$) the IFN-γ production of CL patients, while a weak modulation (19%) was observed in ML patients ($11{,}367 \pm 19{,}904$ pg/ml). Addition of LACK to SLA-stimulated cultures decreased by 58% (698 ± 875 pg/ml) ($P < 0.05$) the IFN-γ production in CL patients and 30% ($10{,}962 \pm 16{,}735$ pg/ml) in ML patients (Fig. 3). To determine whether the neutralization of IL-10 was able to block the suppressive effect mediated by KMP11, anti-IL-10 monoclonal antibody was added to PBMC containing SLA and KMP11. Neutralization of IL-10 of SLA plus KMP11-stimulated cultures from four patients enhanced IFN-γ from 207 ± 212 to 728 ± 518 pg/ml (data not shown).

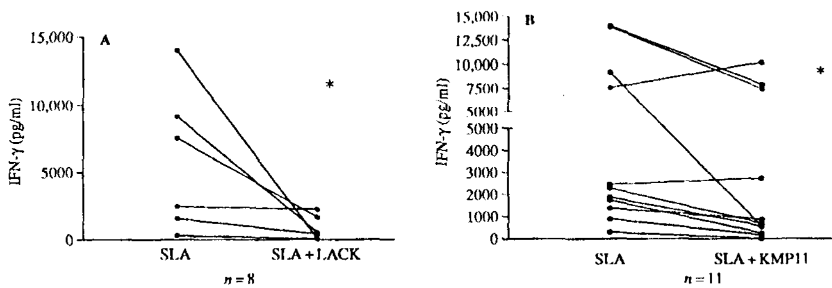
Phenotype of IL-10-secreting cells

Evaluation of the source of IL-10 production in PBMC after stimulation by KMP11 or LACK was performed by staining these cells for CD4, CD8 and CD14 and determination of the frequency of cells producing IL-10. (Fig. 4). In ML patients, $46 \pm 11\%$ of $CD14^+$, $9 \pm 9.9\%$ of $CD8^+$ and $4.8 \pm 5.2\%$ of $CD4^+$ cells were positive for IL-10 after LACK stimulation. Stimulation with KMP11 led to a lower percentage of cells expressing IL-10 than LACK did: $CD14^+$ ($5.4 \pm 7.4\%$), $CD8^+$ ($1.9 \pm 1.8\%$) and $CD4^+$ ($1.1 \pm 0.7\%$) (Fig. 4A). In CL patients, $5.3 \pm 1.3\%$ of $CD14^+$, $0.7 \pm 0.3\%$ of $CD8^+$ and $1.3 \pm 0.6\%$ of $CD4^+$ cells were positive for IL-10 after LACK stimulation (Fig. 4B). After KMP11 stimulation, $4 \pm 0.4\%$ of $CD14^+$ cells, $1.6 \pm 0.2\%$ of $CD8^+$ and $1.3 \pm 1.1\%$ of $CD4^+$ were positive for IL-10 (Fig. 4B).

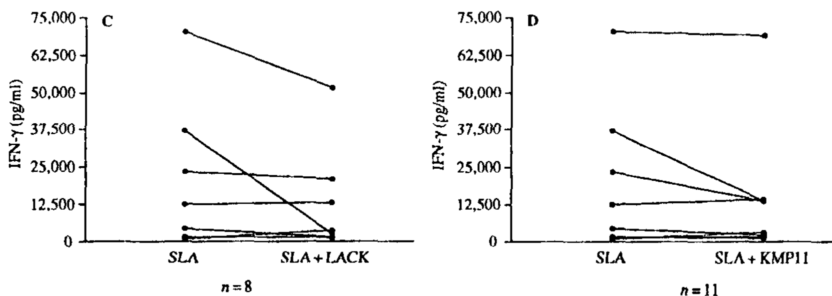
Discussion

Macrophage activation by IFN-γ has been recognized as the major mechanism of *Leishmania* killing. The absence of T-cell responses to *Leishmania* antigens is associated with parasite dissemination in experimental animals [4] and development of multiple nodular cutaneous lesions or visceral disease in humans [20, 21]. Although the type 1 immune response is protective against *Leishmania* infection, evidence has accumulated regarding the role of pro-inflammatory cytokines in the pathogenesis of CL and ML. In these diseases, high levels of IFN-γ and TNF-α are produced [6], macrophages are activated [22], and the subsequent nonmodulated immune response is known to be associated with tissue damage. Moreover, drugs that downmodulate the immune response are effective as adjuvant therapy for cutaneous and mucosal leishmaniasis [8, 23]. Here, we show that two recombinant *Leishmania* antigens (LACK and KMP11) induce production of IL-10 and are able to downregulate IFN-γ production.

Cutaneous Leishmaniasis Patients



Mucosal Leishmaniasis Patients



*P<.05

Figure 3 Suppression of interferon-γ production in soluble leishmania antigen stimulated peripheral blood mononuclear cells of cutaneous leishmaniasis patients (A and B) and mucosal leishmaniasis patients (C and D) after addition of *Leishmania homologue of receptors for activated C* kinase (10 μg/ml) or Kinetoplasmid membrane protein-11 (5 μg/ml). Wilcoxon matched pairs.

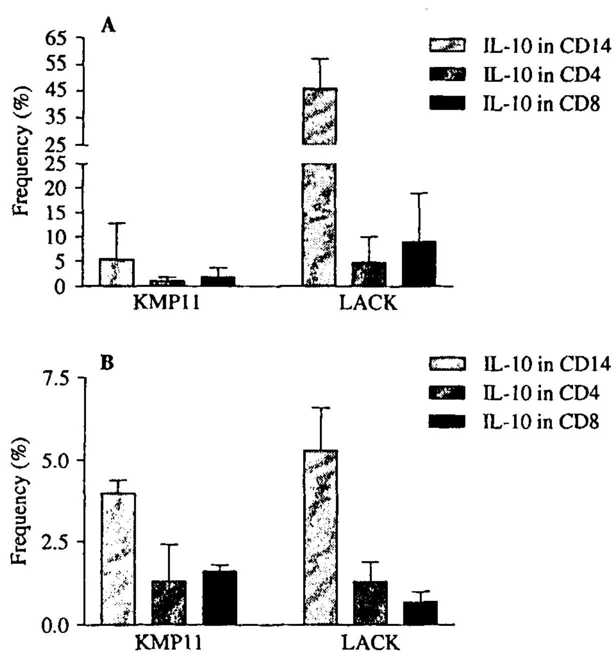


Figure 4 Frequency of interleukin-10 (IL-10)-producing cells from mucosal ($n = 4$) (A) and cutaneous leishmaniasis patients ($n = 4$) (B) expressing IL-10 after Kinetoplasmid membrane protein-11 (5 μg/ml) or *Leishmania* homologue of receptors for activated C kinase (10 μg/ml) stimulation. Results represents the frequency of IL-10-producing cells within the population of $CD14^+$, $CD4^+$or $CD8^+$ cells.

However, ML patients present a even higher IFN-γ production in response to *Leishmania* antigen, and the recombinant antigens have a weaker downregulatory effect in PBMC of these patients.

The immunological responses to several *Leishmania* recombinant antigens have been evaluated, and emphasis has been given to the identification of antigens associated with type 1 immune responses. Antigens such as GP-63, GP-42, hsp-70 and hsp-83 have been described as T-cell antigens that induce mainly IFN-γ production [24–26]. The *Leishmania* elongation initiation factor has been described as a potent inducer of macrophage-derived IL-12, providing differentiation of Th0 to Th1 cells [27]. The present study describes two recombinant *Leishmania* antigens, LACK and KMP11, that downregulate IFN-γ production. Bourreau *et al.* have shown that LACK induces IFN-γ production in PBMC of healthy subjects, even before exposure to leishmania, and these responses significantly increased after exposure to the infection [18]. However, these results were obtained with individuals without disease who have, as previously shown, a different type of immune response from patients with CL or ML [28].

The immune response in CL and ML is characterized by high type 1 immune responses (IFN-γ and TNF-α) and low or absent IL-10 production [6]. Here, it was shown in PBMC from ML and CL patients that both KMP11 and LACK are able to induce IL-10, alone and in the presence of SLA. It is known that an exaggerated type 1 immune response may downregulate differentiation and expansion of Th2 cells thereby decreasing synthesis of type 2 cytokines [29]. Although both CD4 and CD8 T cells were triggered by LACK and KMP11, macrophages were the main sources of IL-10. Because LACK and KMP11 act predominantly on macrophages, the production of IL-10 may occur independent of Th2 cells and could induce IL-10 production even during active disease. It is known that LACK antigen presents a single epitope (amino acid 156–173) responsible for the induction of IL-10 synthesis [30]. KMP11 also induces IL-10 production through an unknown mechanism.

The reason why SLA did not induce IL-10 may be due to relatively low levels of LACK and/or KMP11 in our antigenic preparations. These antigens can be expressed in low amounts in *L. braziliensis* due to competing effects of other antigens in the complex mixture of SLA.

Although LACK and KMP11 had an overall downregulatory effect on IFN-γ in cultures stimulated with SLA, this was not seen in all patients. In one CL patient, LACK did not suppress IFN-γ production in response to SLA. In two CL patients, KMP11 did not suppress the IFN-γ production in response to SLA. Future studies are needed to evaluate if the lack of suppression is associated with different clinical outcomes. The downregulatory effect of these antigens was dependent on IL-10, Because neutralization of this cytokine abrogated the inhibition of IFN-γ synthesis. It has also been shown that neutralization of the LACK-induced IL-10 leads to a higher frequency of TNF-α-producing monocytes. This demonstrates that LACK-induced IL-10 regulates not only lymphocyte produced IFN-γ but also monocyte activity [13].

The modulatory actions of LACK and KMP11 were more pronounced in PBMC of CL patients. In the PBMC of ML patients, IL-10 is induced by these recombinant antigens but with little suppressive effect on IFN-γ production. A previous study by our group has shown a decreased ability of exogenous IL-10 in downregulate IFN-γ production [6]. The decreased modulatory effect of IL-10 in ML patients may be due to a decreased expression of IL-10 receptors in cells from these patients or due to the fact that the activated T cells from ML patients are not properly modulated by IL-10. Both the hypotheses are being tested.

The failure of antimony therapy in preventing the development of *Leishmania* ulcers in CL patients in early stage of the infection [9] and the high rate of antimony therapy failure in ML further support the theory that the inflammatory response plays an important role in the pathogenesis of CL and ML. Modulation of the immune response may play an important role in therapy of both CL and ML diseases. Lastly, the identification of *Leishmania* antigens with the ability to downmodulate inflammatory type 1 response holds promise not only for the new treatments

of human leishmaniasis, but also for the identification of possible approaches for the treatment of other inflammatory or autoimmune diseases.

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A hipótese desse estudo é que pacientes com LM apresentam maior resposta inflamatória a nível tecidual do que pacientes com LC.

Resumo artigo 4:

Decreased in situ expression of IL-10 receptor is correlated to the exacerbated inflammatory and cytotoxic response observed in lesions from ML.

A despeito das evidências de que CMSP de pacientes com LM secretam mais IFN-γ e TNF-α do que os de LC e que uma resposta imune tipo 1 exacerbada está relacionada com a patologia da LC e principalmente da LM, estudos realizados através da técnica de PCR e expressão de citocinas em células da lesão têm mostrado que a nível tecidual tanto citocinas tipo 1 como tipo 2 são expressas nessas doenças. No presente trabalho, a caracterização da resposta imune de pacientes com LC e LM foi realizada através da microscopia confocal com a utilização de anticorpos contra marcadores de superfície de células e contra citocinas. Foi documentado que a freqüência de células expressando TNF-α foi semelhante entre pacientes com LC e LM, enquanto que maior número de células expressando IFN-γ foi encontrado em pacientes com LM. Tanto na LC como na LM a principal célula produtora de IFN-γ foi a célula CD4+. Houve também uma maior freqüência de células CD8+ expressando Granzima A em pacientes com LM, do que em pacientes com LC. Adicionalmente, a expressão do receptor de IL-10 foi menor em pacientes com LM do que em pacientes com LC. Esses dados sugerem que a maior freqüência de células expressando IFN-γ e Granzima A e a baixa expressão do receptor de IL-10 observada em pacientes com LM, levam a uma resposta inflamatória não controlada que contribui com a patologia da doença.

DECREASED IN SITU EXPRESSION OF IL10 RECEPTOR IS CORRELATED WITH THE EXACERBATED INFLAMMATORY AND CYTOTOXIC RESPONSES OBSERVED IN MUCOSAL LEISHMANIASIS.

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Running title: Mechanism of inflammation in human leishmaniasis

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Abstract

Human infection with *Leishmania braziliensis* can lead to cutaneous (CL) or mucosal (ML) diseases. 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 anti-inflammatory cytokines, the expression of effector molecules, and the expression of IL-10 receptor in lesions from ML and CL lesions using confocal microscopy. ML lesions displayed a higher number of IFN-gamma producing cells than did CL lesions. In both ML and CL, CD4+ cells represented the majority of IFN-gamma producing cells, followed by CD8+ cells and CD4-CD8- cells. The numbers of TNF-alpha+ cells, as well as IL-10 producing cells were similar in ML and CL lesions. The effector molecule, granzyme A, showed greater expression in ML than CL lesions, while iNOS did not. Finally, the expression of IL-10 receptor was lower in ML than CL lesions. Thus, our data identified distinct cytokine and cell population profiles between CL and ML patients, and provides a possible mechanism for the development of ML disease through the demonstration that a low expression of IL-10 receptor is present in conjunction with a cytotoxic and inflammatory profile in ML.

Key words: lesion, leishmaniasis, cytotoxicity, cytokines, IL-10 receptor

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 few ulcerated skin lesions and a relatively effective response to conventional antimonial treatment (19). Approximately 3% of the 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 oro-faringeal 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 IFN-gamma and TNF-alpha has been observed in both *in vitro* responses as well as *in situ* analysis of CL lesions (5, 31). Interestingly, IL-10 production was also detected in patients with CL, using several different methodological approaches (4, 9). An increase in the frequency of IL-10 as well as IFN-gamma 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 response of CL and ML patients have shown that peripheral blood mononuclear cells (PBMC) from individuals that develop ML display a higher proliferative response to parasite antigens, higher levels of IFN-gamma and TNF-alpha production, associated with lower levels of IL-10, as compared to CL patients (5). Furthermore, addition of IL-10 to *in vitro* cultures led to modulation of antigen-induced IFN-gamma 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 hypothesis 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-gamma and TNF-alpha, and of the anti-inflammatory cytokine IL-10 in lesions from patients with CL and ML, using triple-staining confocal microscopy analysis. We also evaluated the expression of the effector molecules, granzyme A and iNOS, by cells from the inflammatory lesions from CL and ML patients, correlating their expression with cytotoxic mechanisms involved in tissue damage in both clinical forms. Finally, analysis of the expression of IL-10 receptor as a possible mechanism for the exacerbated *in situ* response in ML was investigated. Our results clearly characterize ML as an example of how an uncontrolled response can lead to pathology, and should be taken under consideration upon proposition of vaccines and/or therapies based on the induction of inflammatory responses.

2. Patients, materials and methods

2.1 Patients

The patients analyzed in this study were from Corte de Pedra, an endemic area for *Leishmania braziliensis* infection, located 280 km southwest from 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 Dr. Edgar Carvalho, with participation of a dermatologist and ear-nose-throat specialists to identify typical leishmaniasis skin or mucosal lesions, respectively. Diagnosis for leishmaniasis was performed based on clinical and laboratorial criteria. Detection of suggestive skin or mucosal lesions was associated to positive skin Montenegro test, parasite isolation and/or histopathological analysis to confirm diagnosis of CL or ML. For all CL and ML cases parasite species were typed to confirm that disease was due to *L. braziliensis* infection. Cutaneous patients enrolled in this study (total n=14) presented with a single ulcerated lesion and had not been previously diagnosed or treated for leishmaniasis. Mucosal patients (total n=7) presented with nasal lesions and, at the time of biopsy collection, and did not display concomitant cutaneous disease. At the time of sample collection, the age of the active lesions were estimated between 30 and 45 days for both CL and ML lesions. While the estimated time of CL and ML lesion development was comparable, ML patients have had previous exposure to leishmaniasis, since they had previous cutaneous disease, which was healed at the time when mucosal disease was diagnosed in these patients. The time interval between the cure of CL and the diagnosis of ML was variable. The estimated time of lesion development was based on questioning the patients, together with the intervals of patient examinations in the endemic area. 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 Corte de Pedra health care facility or at the Serviço de Imunologia, Hospital Universitário Professor Edgar Santos, UFBA, in Salvador. Skin biopsies were taken from

the borders of active lesions, using a 4mm diameter punch, after the application of local anesthetic. Mucosal lesions were obtained by excision of a small fragment (approximately 3mm, in average) using a scalpel, after local anesthetics application. Lesions were maintained in a 30% sucrose solution for approximately 30 minutes at 4^0C and then transferred to OCT Tissue Tek freezing media and immediately placed in dry ice. The material was stored at -70^oC until analysis. The Ethical Committees of Bahia and Minas Gerais Federal Universities approved all procedures involved in this study.

2.2 Histological and immunofluorescence staining

Individual 4-5um cryosections were placed in saline-precoated slides and fixed for 10 minutes with acetone. Slides were incubated with phosphate buffered saline (PBS) for 15 minutes and submitted to either hematoxilin-eosin (HE) staining or to immunofluorescence using specific monoclonal antibodies. Standard HE staining was performed to assure tissue integrity, as well as evaluation of the intensity and location of the inflammatory infiltrate. Immunofluorescence reactions involved incubation with FITC or PE-labeled monoclonal antibodies directed to surface receptors (CD4-cloneS3.5, CD8-clone 3B5, CD68-clone Ki-M7 or IL-10 receptor-clone B7-H1) or intracellular molecules (granzymeA-cloneCLB-GA28, iNOS or IFN-gamma-cloneB27, IL-10-clone 9D7 or TNF-alpha-clone 20A4), respectively. Sections were incubated with antibody mixtures overnight at 4^0C. After staining, preparations were extensively washed with PBS, counter-stained with DAPI and mounted using anti-fade mounting media (Molecular Probes, USA). Slides were kept at 4^0C, protected from light, until acquisition in a laser scanning confocal microscope (Zeiss). Isotype controls were added to the immunofluorescence reactions, to confirm the lack of non-specific staining. Monoclonal

antibodies were purchased from Caltag (Burlingame, CA), except for anti-IL-10 receptor monoclonal antibody, purchased from Becton-Dickinson (San Jose, CA).

2.3 Confocal analysis

Imaging was performed with a Bio-Rad MRC 1024 laser scanning confocal system running the software LASERSHARP 3.0 coupled to a Zeiss microscope (Axiovert 100) with a water immersion objective (40x, 1.2 NA). A water-cooled argon UV laser (488nm) or a krypton/argon laser was used to excite the preparation (through its 363nm line, 488nm line or 568nm 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 3 images (fields) were collected. Image analysis and processing were performed with the software LASERSHARP (Bio-Rad), Confocal Assistant, ADOBE PHOTOSHOP and Image Tool. Analyses were performed by counting the total number of cells in the 3 fields acquired and calculating the average of cells/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, as well as the number of double positive cells. The counts were performed blindly and the results were expressed as the average of cells/field for each parameter for each patient, and then the values were averaged for each group. The results are representative of two experiments/patient. Determination of intensity of IFN-gamma and IL-10 receptor expression was performed using the Pseudocolor software. This analysis allows for the

conversion of pixels in numbers, providing a numerical analysis of intensity in relation to the number of pixels in the analyzed area. This number was then corrected for the number of cells present in the analyzed area.

2.4 Statistical analysis

Statistical analysis of the data was performed using the JMP statistical software from SAS. The comparisons of means for a given parameter were done using non-parametric (one tailed, considering unequal variance of groups) t-Test. Results were considered statistically different when the analysis returned a $p < 0.05$.

3. Results

3.1 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 in Material and Methods. The average number of cells was significantly higher in lesions from ML, as compared to CL, 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 number of CD68+ cells was not statistically different between CL and ML lesions, the numbers of CD4+ and CD8+ cells were higher in ML, as compared to CL lesions (Table 1). Although an increase in CD4+ and CD8+ cells was observed in ML lesions, the ratio

CD4/CD8 was similar between groups. Lastly, the relative percentage of CD4+, CD8+ and CD68+ cells did not differ between groups (data not shown).

3.2 Cellular sources of cytokines in ML and CL lesions.

The expression of the inflammatory cytokines TNF-alpha and IFN-gamma was evaluated in lesions from CL and ML patients. The analysis showed that the total numbers of cells expressing TNF-alpha were not statistically different between groups (Table 2). Evaluation of the cellular sources of TNF-alpha 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-alpha in CL and ML lesions (Table 2). While the total expression of TNF-alpha was not statistically different when comparing ML and CL lesions (Table 2), the total number of IFN-gamma-expressing cells was higher in lesions from ML, as compared to CL patients (Figure 1A). Not only the number of IFN-gamma+ cells was higher in ML lesions, but also the intensity of expression of this cytokine was higher in lesions from patients with mucosal as compared to cutaneous disease (Figure 1B). We also observed that ML patients display a statistically higher frequency of CD4+IFN-gamma+ cells than do CL (Figure 1A) and although the average numbers of CD8+IFNgamma+ cells were higher in ML as compared to CL, statistical significance was not achieved ($p<0.06$; Figure 1A). Our analysis of the contribution of CD4+, CD8+ and CD4-CD8- cells toward the production of IFN-gamma showed that CD4+ cells are the main source of IFN-gamma in ML and CL (43+/-21 and 51+/-11, respectively), followed by CD8+ (38+/-10 and 40+/-15 for CL and ML, respectively) and CD4-CD8- cells (21+/-15 and 10+/-4 for CL and ML, respectively).

Analysis of the anti-inflammatory 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 between 40 and 50% of the IL-10 present in the lesion sites comes from other cellular sources. These percentages were statistically equivalent comparing the two groups. Interestingly, while approximately 80% of the CD68+ cells expressed IL-10 in CL lesions, over 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).

3.3 Cells from ML lesions display lower intensity of expression of IL-10 receptor, as compared to cells from CL lesions.

Previous studies performed by our group demonstrated that addition of IL-10 to *in vitro* cultures lead to modulation of antigen-induced IFN-gamma 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 IL-10 receptor in ML and CL lesions, determining the number and percentage of cells expressing this molecule, as well as its intensity of expression per cell. We observed that while the number of cells expressing the IL-10 receptor was similar in ML and CL lesions (Figure 1C), the percentage of cells expressing this molecule (Figure 2A), as well as the intensity of expression of the IL-10

receptor in a cell per cell basis (Figure 2B) was also lower in ML, as compared to CL lesions.

3.4 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. Our results showed that although the total number of cells expressing iNOS was apparently higher in CL lesions, these analyses did not show statistical significant (p=0.2) (Table 3). However the percentage of expression of iNOS+ cells was higher in CL as compared to ML (Table 3). The total number of cells expressing granzyme A, as well as the number of granzyme A+ CD8+ T cells, was 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 number of CD8+GranzymeA+ cells was similar between groups (Table 3). However, the commitment of the CD8+ cell population to cytotoxic activity is higher in ML lesions, as shown by the higher percentage of CD8+ cells expressing granzymeA (Table 3). Moreover, a statistically significant positive correlation was seen between total IFN-gamma+ 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.

4. 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 (12,5,32). In this study, performing an extensive multiparameter confocal microscopy analysis of the inflammatory infiltrate in ML and CL lesions, several points of evidence in favor of this hypothesis were reveled: (1) the inflammatory infiltrate in ML lesions is more intense than the one observed in CL lesions; (2) higher expression of the inflammatory cytokine IFN-gamma and of the cytotoxic molecule, granzyme A, were observed in ML as compared to CL lesions; and (3) the intensity of expression of IL-10 receptor was lower in ML than CL lesions. Thus, our work demonstrates the occurrence of an *in situ* hyper-activation in ML lesions, likely due to a down regulation of IL-10 receptor, and provides new insights towards 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 are 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-alpha, IFN-gamma, IL-10 and IL-4, was detected in CL and ML lesions 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 as to the intensity, composition and

architecture of the inflammatory infiltrate, nor to the cellular sources of the analyzed cytokines. Through the use of multiparameter confocal microscopy, we determined that CD4+ T cells were the primary source of IFN-gamma in both CL and ML, followed by CD8+ T cells and CD4-CD8- cells. These results are similar to the ones previously found by us when determining the sources of IFN-gamma in PBMC from ML patients (5). However, CD4-CD8- cells appeared as the second major source of IFN-gamma in PBMC from CL patients (9). This difference may reflect distinctive recruitment of T cell subpopulations to lesion sites. We also determined that monocytes are the main source of TNF-alpha in CL and ML lesions, which differ from our previous studies in that CD4+ T cells were the main source of TNF-alpha 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-alpha 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-gamma, 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-gamma 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, as compared to CL lesions. This data is 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- express granzyme A in ML lesions, pointing to the participation of other cell types in cytotoxic functions in ML leishmaniasis. 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 the expression of IFN-gamma was increased in ML as compared to CL lesions, the expression of TNF-alpha, another important inflammatory cytokine was not. Since the main sources of IFN-gamma were CD4+ and CD8+ T cells, while the main sources of TNF-alpha 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-alpha is indeed present in ML lesions prior to and after specific treatment (1). The involvement of TNF-alpha in ML pathology was previously suggested, when pentoxifylline, a TNF-alpha 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-gamma are able to induce the expression of the TNF-alpha receptor (35). Since ML lesions display higher expression of IFN-gamma, it is possible that this cytokine is acting towards the increase of expression of TNF-alpha receptor, which will be investigated in the future.

A potent antagonist of IFN-gamma 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 the cutaneous, one would expect that the percentage of IL-10 expressing cells would be lower in ML, as compared to CL lesions. Although statistical analysis of this data did not show significance, the ratio IFN-gamma/IL10 is higher in ML patients (0.4 and 0.7 for CL and ML respectively), showing that the proportion of IFN-gamma+ to IL-10+ 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 pro-inflammatory cytokines induce a 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-gamma in cultures of cells from CL, but not ML patients stimulated with SLA (5). Other anti-inflammatory cytokines may also be involved in the control of ML such as IL-13 (10), IL-4 (31) and TGF-beta (18). Recent studies in the experimental model of *L. 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-gamma, increased cytotoxic cytotoxic activity and the low expression of IL-10 receptor may be responsible for the lack of control of the inflammatory response in ML. By adding to our knowledge in immunoregulatory and pathogenic cellular mechanisms in American cutaneous leishmaniasis, these findings are critical when considering interventions that will involve modulation of specific cell populations.

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Table 1: Total number of inflammatory cells and numbers of CD4+, CD8+ and CD68+ cells in CL and ML lesions.

Clinical form	Total number of*			
	DAPI+ cells	CD4+ cells	CD8+ cells	CD68+ cells
CL (n=14)	4580 +/- 1784	1059 +/- 757	1069 +/- 542	1313 +/- 580
ML (n=7)	**6664 +/- 1821***	**1671 +/- 480***	**1846 +/- 801****	1822 +/- 908

* Numbers of cells were counted based on expression of specific fluorescent marker, as indicated and described in Materials and Methods.

** Statistical significance: ML>CL; $p<0.05$

Table 2: Number of TNF-alpha+ and IL-10+ cells and relative contribution of CD68+ population to the production of these cytokines in lesions from patients with CL and ML

Clinical form	**Total TNF-α+ cells***	**TNF-α+ CD68+ cells***	**% contribution of CD68+ cells****	**% commitment of CD68+ cells*****	**Total IL-10+ cells***	**IL-10+ CD68% cells***	**% contribution of CD68+ cells****	**% commitment of CD68+ cells*****
CL (n=14)	1144 +/- 691	909 +/- 547	78 +/- 12	65 +/- 21	2025 +/- 785	1267 +/- 598	62 +/- 13	79 +/- 16
ML (n=7)	1354 +/- 993	1148 +/- 937	80 +/- 12	56 +/- 23	2468 +/- 1597	1148 +/- 1158	53 +/- 17	**91 +/- 4********

* Numbers of cells were counted based on expression of specific fluorescent markers for either TNF-alpha or IL-10 alone or TNF-alpha or IL-10 and CD68, as indicated and described in Materials and Methods.

** % contribution of CD68+ cells to TNF-alpha expression was calculated as the percentage of CD68+ cells expressing TNF-alpha in relation to the total TNF-alpha expression.

*** % commitment of CD68+ cells to TNF-alpha expression was calculated as the percentage of CD68+ cells expressing TNF-alpha in relation to the total CD68+ cells.

**** Statistically significant difference; $p<0.05$

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

Clinical form	iNOS expression*		Granzyme A+ cells*			
	Number of iNOS+ cells	% iNOS+ cells	Total	CD8+ granzymeA+	% Contribution of CD8+ cells****	Commitment of CD8+ cells to granzyme A expression*****
CL (n=9)	1274 +/- 230	87 +/- 6	924 +/- 466	687 +/- 391	70 +/- 21	58 +/- 13
ML (n=7)	1159 +/- 231	**64 +/- 19****	**1562 +/- 610*****	950 +/- 587	**56 +/- 14****	**74 +/- 17*****

* Numbers of cells were counted based on expression of specific fluorescence for either granzyme A or iNOS alone or granzyme and CD8, as indicated and described in Materials and Methods.

** Statistical analysis showed ML < CL; $p<0.05$

*** Statistical analysis showed ML > CL; $p<0.05$

**** % Contribution of CD8+ cells to granzyme A expression was calculated as the percentage of CD8+ cells expressing granzyme A in relation to the total granzyme A expression.

***** % Commitment of CD8+ cells to granzyme A expression was calculated as the percentage of CD8+ cells expressing granzyme A in relation to the total CD8+ cells.

Figure legends:

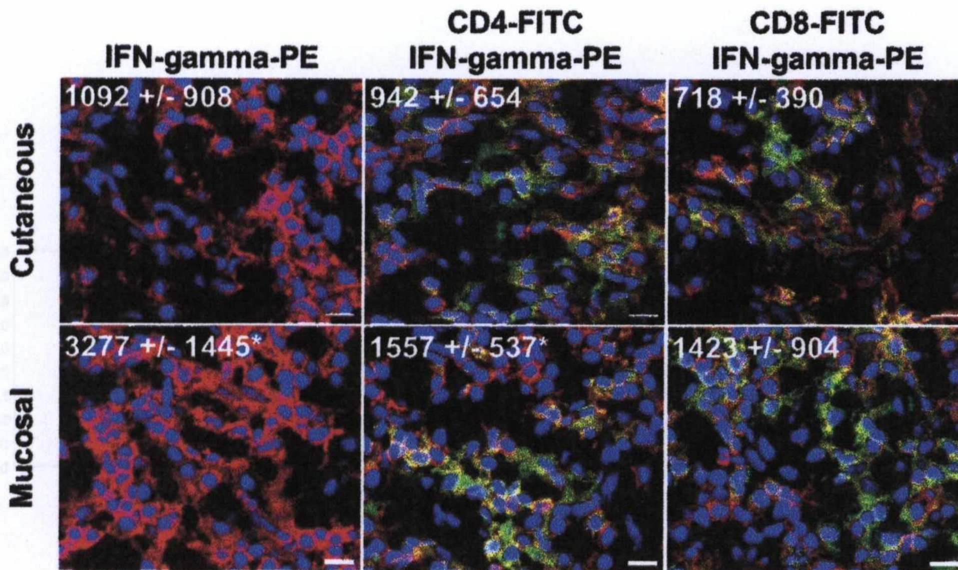
Figure 1: *Differential expression of IFN-gamma and IL-10 receptor in CL and ML lesions.* (A) Representative picture of the confocal microscopy analysis for determination of the number of total IFN-gamma+ cells, number of CD4+IFN-gamma+ cells and number of CD8+IFN-gamma+ cells in CL and ML lesions. Tissue sections were stained with FITC labeled monoclonal antibodies anti-CD4 or CD8, PE-labeled anti-IFN-gamma, and counter stained with DAPI, as described in Material and Methods. The three optical sections for each patient were obtained simultaneously with 363, 488 and 568 line of the argon/krypton laser and the proper set of filters. The overlay for CD4 or CD8 (green), IFN-γ (red) and DAPI (blue) in CL and ML lesions is showed. The cells that are double positive for CD4 or CD8 and IFN-γ appear in yellow. These images are representative of each group. Values represent the average +/- standard deviation of each group following numeric determination of the number of positive cells for the indicated molecule(s). * indicates statistically significant differences (ML>CL) using $p<0.05$. (n=14 CL and 7 ML). The bar = 10μm.

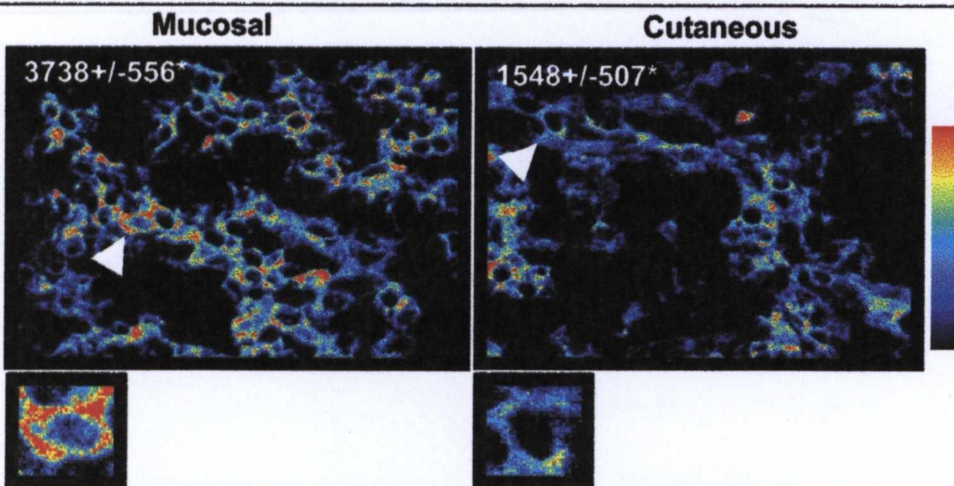
(B) Representative analysis of the intensity of expression of IFN-gamma in CL and ML lesions using the Pseudocolor analysis, as described in Materials and Methods. The predominance of red indicates higher intensity of expression as opposed to the predominance of blue (see scale in the right side of figure). White arrows indicate the cells shown in the larger image. * indicates statistically significant differences (ML>CL) using $p<0.05$. (n=14 CL and 7 ML).

(C) Representative picture of the confocal microscopy analysis for determination of the number of total IL-10 receptor (IL-10R)+ cells in CL and ML lesions. Tissue sections were stained with PE labeled monoclonal antibody anti-IL-10R, as described in Material and Methods. (n=9 CL and 7 ML). For all analysis the Bar = 10μm, and the images are representative of one of two independent experiments for each individual lesion. Values represent the average +/- standard deviation of the numerical analysis of the number of cells expressing the indicated molecule(s) for each group.

Figure 2. *Analysis of the expression of IL-10 receptor in CL and ML lesions*. (A) percentage of cells expressing IL-10 receptor in CL and ML lesions, and (B) intensity of expression of IL-10 receptor per cell. Tissue sections were stained with PE labeled monoclonal antibodies anti-IL-10 receptor and counter stained with DAPI, and the number, percentage and intensity of expression of IL-10 receptor was calculated as described in Material and Methods. * indicates statistically significant differences between groups, using $p<0.05$. (n=9 CL and 7 ML).

Figure 1, Faria et al.





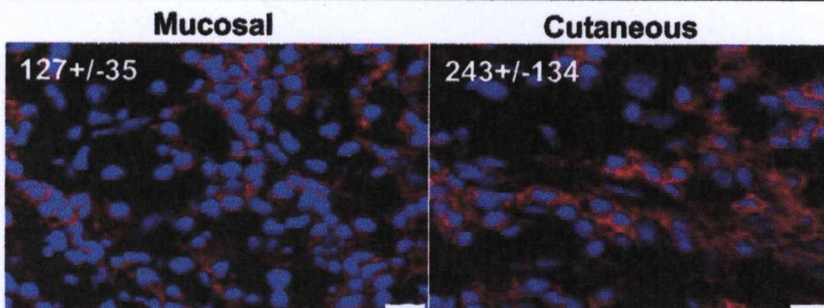
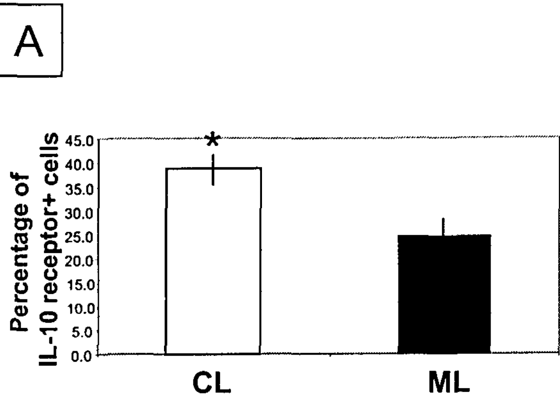
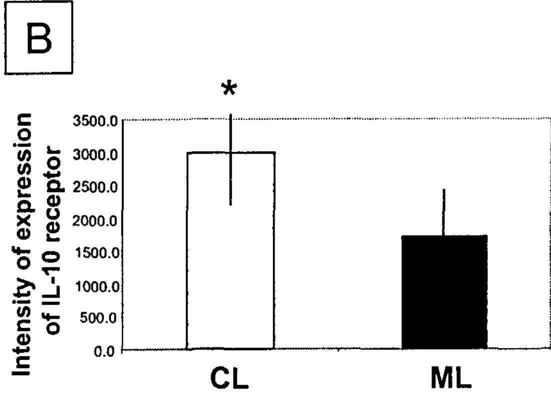


Figure 2, Faria et al.





7. Discussão

A presente tese é composta por 4 manuscritos que representam resultados de uma linha de pesquisa que visa caracterizar a resposta imune na LC e LM, avaliar o papel da resposta imune na patogênese dessas doenças e avaliar a nível tecidual a resposta imunológica desses pacientes. Foi documentado que tanto a nível de sangue periférico quanto a nível tecidual, a principal célula envolvida na produção de IFN-γ foi a célula CD4+, embora *in situ* houvesse um menor número de células CD8+ expressando IFN-γ. Uma contribuição importante foi a demonstração de que existe um número maior de células T expressando marcadores de ativação celular em pacientes com LM, o que pode explicar o fato de existir uma resposta imune mais exacerbada na LM, como também o fato dessas células serem menos dependentes de IL-2 e IL-15. Foi também observado que determinados antígenos recombinantes de Leishmania têm a propriedade de induzir IL-10, fenômeno não só observado em pacientes com leishmaniose, como em controles sadios e, que esses antígenos podem modular a resposta imune através da produção de IL-10 de pacientes com LC, embora essa modulação não seja observada na LM. Finalmente, a hipótese de que a resposta imune exacerbada participa da patogênese da doença foi reforçada com as evidências citotoxicidade a nível tecidual, grande freqüência de células expressando citocinas pró-inflamatórias e a existência de uma menor expressão de receptor de IL-10 na LM.

A destruição de agentes infecciosos e consequentemente o controle das infecções é dependente de uma resposta imune eficaz. A ausência ou diminuição da resposta imunológica é habitualmente acompanhada de disseminação do agente agressor. Os microorganismos intracelulares como a Leishmania causam infecções crônicas e o papel da

diminuição da resposta imune celular na patogênese de doenças é bem documentado. É amplamente conhecida a associação entre a síndrome da imunodeficiência adquirida (AIDS) com a tuberculose, candidíase, toxoplasmose e leishmaniose visceral (Desjeux e cols., 2004; Floyd e cols., 1997; Sebitloane e cols., 2005; Rodier e cols., 2003). O mecanismo de defesa contra agentes intracelulares é feito através de células CD8+ citotóxicas (Stenger e cols., 2001) e da ativação de macrófagos por IFN-γ e conseqüente destruição dos microorganismos (Murray e cols., 1983). Nesses casos, a resposta imune protetora é associada com uma resposta tipo 1, sendo facilmente compreendido o desenvolvimento de doenças como a LV e a LCD, nas quais existe uma diminuição dessa resposta e conseqüente multiplicação e disseminação do parasito (Carvalho e cols. 1985, Sacks e cols., 1987; Barral e cols., 1995). A importância da resposta imune na patogênese das doenças infecciosas tem sido evidenciada em várias doenças, entre as quais: hanseníase, malária, tuberculose e doença de Chagas (Arruda e cols., 1994; Cunha-Neto e cols., 1996; Artavanis-Tsakonas e cols., 2003; Kaplan e cols. 1991). Embora em todas as condições tenha sido bem documentada que a deficiência da resposta Th1 é associada com disseminação do processo infeccioso (Cardillo e cols., 1996), vários estudos têm comprovado que na fase crônica dessas enfermidades a patologia está relacionada não só com o microorganismo mas, com a resposta imune exacerbada e não controlada. Em algumas dessas condições o mecanismo envolvido na destruição tecidual tem sido melhor estudado. A malária cerebral está relacionada com uma forte produção de TNF-γ e IFN-γ (Artavanis-Tsakonas e cols., 2003) e na doença de Chagas, o aumento de quimiocinas e citocinas inflamatórias, assim como a presença de células T auto-reativas, tem sido associada com a miocardite crônica chagásica (Cunha-Neto e cols., 1995). Na leishmaniose

tegumentar estudos prévios têm destacado a possibilidade da lesão tecidual estar relacionada a uma resposta imune tipo 1 exacerbada e não controlada. Essas evidências incluem: 1) desproporção entre o número de parasitos e lesão tecidual; 2) presença de células CD4+ e CD8+ nas lesões de LC e LM; 3) redução dos níveis de IFN-γ e TNF-α após a cura dessas doenças; 4) presença de TNF-α nas lesões de LM; 5) maior produção dessas citocinas em células de pacientes com LM do que com LC, com maior destruição tecidual na LM; 6) incapacidade do tratamento com antimonial de LC na fase pré-ulcerativa de prevenir o aparecimento da úlcera (Machado e cols., 2002); 7) capacidade da pentoxifilina, droga inibidora da produção de TNF-α, quando associadas ao antimonial, e GM-CSF, citocina que modula a resposta imune, de reduzir o tempo de cura de pacientes com LM e pacientes com LC e de curar pacientes com LM e LC refratários ao tratamento com antimonial (Almeida e cols., 1999; Báfica e cols., 2003; Lessa e cols., 2001). Consequentemente, dá suporte ao papel de uma resposta imune exacerbada na patogênese dessa doença, a observação de que indivíduos infectados por *L. braziliensis* mas, que não desenvolvem doença apresentam uma resposta imune modulada. As análises comparativas da resposta imune desses indivíduos, que são considerados como possuidores de uma infecção sub-clínica, com pacientes com LC demonstram que os indivíduos sub-clínicos apresentam menor produção de IFN-γ e maior produção de IL-5 do que pacientes com LC (Follador e cols., 2002).

Embora a LM ocorra na quase totalidade das vezes em pacientes que têm ou tenham tido LC e em virtude de em ambas condições existir um aumento da produção de citocinas pró-inflamatórias, a exacerbação da resposta imune e a gravidade da lesão tecidual é mais evidente na LM do que na LC. Dessa forma, no presente estudo, a resposta imune foi

estudada nessas duas doenças, tendo como principais objetivos estudar os mecanismos envolvidos na exacerbação da resposta imune, avaliar a resposta imune a antígenos recombinantes e estudar a resposta imune *in situ*. Vários fatores podem ser responsáveis por uma resposta imune exacerbada como a documentada na LC e na LM. É bem conhecido que aspectos relacionados com a apresentação antigênica têm um papel importante na magnitude da resposta imune. No camundongo tem sido mostrado que células dendríticas da pele (células de Langherans) migram do sítio da infecção com *L. major* para o linfonodo, local onde são estimuladas as células específicas para *L. major*. A capacidade dessas células em se constituírem células apresentadoras de antígeno com grande potência é em grande parte mediada pela expressão de moléculas co-estimulatórias (Moll e cols., 2000). Nesse contexto tem sido documentado que a interação entre CD40-CD40L é fundamental para ocorrência da resistência da *L. major*, desde que animais deficientes de CD40 ou CD40L são altamente susceptíveis à infecção por *L. major*. No presente estudo moléculas co-estimulatórias foram estudadas, no sentido de se avaliar se uma maior freqüência de células expressando essas moléculas, poderia estar relacionada com a resposta imune exacerbada do tipo 1 documentado em pacientes com LM. Neste estudo, não foram encontradas diferenças na expressão dessas moléculas entre pacientes com LC e LM. Não foi também observado a existência de um maior número de células em apoptose em pacientes com LC. A apoptose é uma das formas de modulação da resposta imune, através da morte programada de células ativadas. Mas, a magnitude da resposta imune na LM em relação à LC não pode ser relacionada com um aumento na expressão de moléculas co-estimulatórias, nem com uma diminuição de células em apoptose.

Várias citocinas participam da diferenciação, proliferação e ativação de células T. A IL-12 é a principal citocina responsável pela diferenciação de células Th0, para células

Th1 e a capacidade de anti-IL-12 de suprimir produção de IFN-γ, já foi demonstrada na infecção por *Staphylococcus epidermidis* (Stuyt e cols., 2003). Entretanto, a neutralização de IL-12 não teve efeito significativo nos níveis de IFN-γ dos pacientes com LC ou LM após estimulação com SLA. A IL-2 e a IL-15 interagem com o mesmo receptor e tem grande importância na proliferação linfocitária. A leucemia de células T do adulto é uma doença associada à infecção do vírus linfotrópico de células T humana (HTLV) e se caracteriza por uma proliferação desregulada das células T infectadas (Azimi e cols., 1999). Nessa doença, em comparação com indivíduos somente portadores do vírus HTLV-1 a neutralização da IL-2 e da IL-15 tem uma menor capacidade de suprimir a proliferação linfocitária (Azimi e cols., 1999). No presente estudo, neutralização dessas citocinas foi realizada no sentido de determinar se a produção dessas moléculas era necessária para manutenção da resposta imune exacerbada. Algumas diferenças foram encontradas quando a capacidade da anti-IL-2 e da anti-IL-15, em modular a resposta imune na leishmaniose cutânea e na leishmaniose mucosa. Enquanto, que a neutralização de IL-15 diminuiu a produção de IFN-γ de pacientes com LC, a neutralização dessa citocina não teve efeito significativo na produção de IFN-γ de pacientes com LM. Embora a neutralização de IL-2 tenha modulado a produção de IFN-γ de pacientes com LC e LM, a supressão dessa citocina foi significativamente maior nos pacientes com LC. Esses dados indicam que as células T de pacientes com LM têm uma menor dependência de IL-2 e de IL-15, sugerindo tratar-se de células T em fase final de ativação celular. Apóia esta hipótese, a observação da existência de uma maior freqüência de células CD4+ expressando marcadores de ativação celular. Adicionalmente, a neutralização de IL-2 foi mais eficaz em suprimir a produção de IFN-γ em pacientes com LC, do que em pacientes com LM. Após a estimulação com SLA a

neutralização dessa citocina reduziu de modo significante a produção de IFN-γ em células estimuladas com PPD indicando que a capacidade da célula de ser modulada, tem em certa extensão relação com o antígeno.

A neutralização de IL-15 foi capaz de modular a produção de IFN-γ de pacientes com LC, porém não teve efeito significativo na produção de IFN-γ de pacientes com LM. A diminuição da capacidade de anti-IL-15 de modular a produção de IFN-γ dos pacientes com LM pode ser explicada pelo fato de que pacientes com LM apresentam maior número de células CD4+ ativadas/memória (CD28-, CD62L- e CD69), do que pacientes com LC. Outros prováveis fatores para explicar esses achados são: 1) os anticorpos anti-IL-15 não são capazes de neutralizar totalmente essa citocina na LM, por uma captação rápida da célula. 2) as células T de pacientes com LM já estariam ativadas e imortalizadas por IL-15 que foi produzida *in vivo*, não tendo portanto, modulação *in vitro* após a adição de anticorpo monoclonal anti-IL-15.

O interesse pelo estudo da resposta imune a antígenos específicos de Leishmania teve início na década de 80, quando vários antígenos foram isolados baseados nas diferenças entre os seus pesos moleculares e outros, após determinar o sequenciamento de seus genes que foram produzidos em larga escala, a maioria em vetores *E. coli.* Nessa ocasião, o principal interesse era a identificação de antígenos de Leishmania que fossem indutores de forte resposta Th1, os quais pudessem ser utilizados como vacinas, com base nos conhecimentos observados em modelos de leishmaniose, onde as lesões são associadas a ausência de resposta Th1 e multiplicação de parasitos (Scott e cols., 1989). O conhecimento que a imunopatogênese da LC e da LM é mediada por uma resposta imune exacerbada e não controlada, tem aberto perspectiva para o estudo de moléculas que

tenham a capacidade de regular a resposta imune. O interesse na identificação de moléculas que possam modular a resposta imunológica tem também, aumentado pela documentação de que algumas infecções como a causada pelo *S. mansoni* tem não só a propriedade de modular tanto a resposta tipo 1 como tipo 2, mas também de atenuar doenças inflamatórias e doenças autoimunes. Por exemplo, tem sido observado que camundongos NOD infectados com *S. mansoni* ou os quais ovos de *S. mansoni* foram inoculados desenvolvem menos diabetes do que animais não infectados (Cooke e cols., 1999) e que a infecção por esse parasito atenua as manifestações clinicas da encefalite autoimune experimental, modelo animal da esclerose múltipla (La flamme e cols., 2003). Mais recentemente, tem sido observado que a infecção pelo *S. mansoni* diminui a resposta tipo 2 a aeroalérgenos (Araújo e cols., 2004) e atenua as manifestações clínicas da asma (Medeiros e cols., 2004). Essa propriedade que tem o *S. mansoni* de modular tanto a resposta tipo1 quanto a resposta tipo 2 tem sido em grande parte relacionada com a produção de IL-10. No presente estudo, a resposta imune a 4 antígenos recombinantes de Leishmania foi inicialmente caracterizada e posteriormente analisada a capacidade dos antígenos que induziam IL-10 de modular a resposta imune em pacientes com LC e LM. A capacidade de antígenos de Leishmania de induzirem resposta imune tipo 1, a exemplo do H3 e do H2A, é sem dúvida, de grande importância para o desenvolvimento de vacinas para regiões onde a LV ou LCD são endêmicas, onde células de memória H3 ou H2A-específicas poderiam contribuir para produção de IFN-γ e ativação de macrófagos. Entretanto, em pacientes com LC ou LM, nos quais a resposta inflamatória tem importante papel na patogênese da doença, a imunização com moléculas indutoras de resposta imune tipo 1 pode exacerbar o processo inflamatório observado nesses pacientes. A documentação de que KMP11 e LACK induzem secreção

de IL-10 por macrófagos, e que esses antígenos são capazes de modular a produção de IFN-γ em pacientes com LC é um achado importante e que pode ter repercussão na modulação de resposta imune em doenças inflamatórias. Como a principal fonte de IL-10 após estimulação com LACK e KMP11 é o macrófago, e esses antígenos são capazes de induzir produção de IL-10 em indivíduos sadios, trabalhos devem ser realizados no sentido de avaliar a capacidade desses antígenos de modular a resposta imune de pacientes com doença inflamatória crônica e doenças autoimunes. É importante enfatizar que, apesar da existência de moléculas com propriedades regulatórias como o LACK e o KMP11 na Leishmania, o SLA induz pouca secreção de IL-10 em indivíduos com LC ou LM. Talvez, a baixa expressão dessas moléculas no parasito na fase de preparação do antígeno, explique esse fenômeno. O LACK, por exemplo, representa apenas 0,03% do total de proteínas da Leishmania e a expressão do KMP11 é consideravelmente diminuída durante a fase estacionária das promastigotas. Diferente dos achados de que LACK e KMP11 são capazes de modular a produção de IFN-γ em pacientes com LC, nos experimentos *in vitro* com pacientes com LM, mesmo tendo estes antígenos a capacidade de induzir altos níveis de IL-10, a adição de KMP11 ou LACK à culturas de CMSP estimuladas com SLA não foram capazes de modular a produção de IFN-γ. A diminuição da expressão de receptor de IL-10 em linfócitos de pacientes com LM como observado na publicação n. 4 indica ser esta uma das razões pela qual a adição de IL-10 exógena (Bacellar e cols., 2002) ou de antígenos com propriedades regulatórias através da secreção de IL-10, não suprimirem a produção de IFN-γ de pacientes com LM. O achado de que pacientes com LM apresentam mais células T CD4+ ativadas do que pacientes com LC também explica, em parte, a incapacidade de modulação da produção de IFN-γ nesses pacientes.

Um outro aspecto importante é a observação de que indivíduos residentes em área de transmissão de *L. braziliensis* que apresentam sorologia e teste de Montenegro positivo mas não desenvolvem doença têm um tipo de resposta imune não polarizada (tipo 1 e tipo 2). Esses indivíduos com infecção sub-clínica por *L. braziliensis*, têm uma resposta imune modulada, a qual é capaz de controlar a multiplicação parasitária e não causar danos ao hospedeiro (Follador e cols., 2002). Não estão ainda esclarecidos fatores do hospedeiro que influenciem na resposta imune e que possam estar contribuindo para o espectro clínico diverso das leishmanioses. Recentemente, tem sido demonstrada a influência de polimorfismos genéticos em genes que regulam a resposta imune na magnitude da resposta imune. É possível que estes polimorfismos expliquem variações nas respostas dos indivíduos ao parasito, resultando numa resposta imune diferenciada e conseqüente diversidade de formas clínicas. Cabrera observou uma associação de genes que induzem alta expressão de TNF-α com a LM (Cabrera e cols., 1995). Por outro lado, o parasito deve também exercer um papel importante na resposta imune e contribuir para a exacerbação da doença, como vem sendo demonstrado em alguns estudos que mostram associação entre formas diversas de leishmaniose e espécies, ou mesmo entre parasitos da mesma espécie com diferenças genéticas (Schriefer e cols., 2004; Indiani de Oliveira e cols., 2004).

A análise da resposta imune tecidual contribuiu não só para comparar a resposta imune *in situ* de pacientes com LC e LM, mas também para avaliar se os achados em CMSP são também documentados a nível do tecido. No presente estudo, tanto semelhanças como diferenças com o que foi documentado sistemicamente foram observados pela análise da resposta imune *in situ*. No contexto geral, os achados teciduais dão suporte à hipótese de que a patologia na LC e principalmente da LM é mediada pela resposta imune. Foi

confirmado o dado observado em CMSP (trabalho 1) que pacientes com LC e principalmente pacientes com LM apresentam grande quantidade de células expressando IFN-γ e que a célula CD4+ é a principal célula produtora desta citocina. Evidência de citotoxicidade tem sido observada na leishmaniose em modelos experimental e em humanos (Brodskyn e cols., 1997; Barral-Netto e cols., 1995; Smith e cols., 1991). Em pacientes com leishmaniose tegumentar a resposta citotóxica contra macrófagos infectados por Leishmania foi observada (Brodskyn e cols., 1997) e essa resposta citotóxica foi maior em pacientes com LM do que com LC. Previamente tinha sido documentado a expressão de Tia-1, marcador de resposta citotóxica, em pacientes com LC (Machado e cols., 2002) e aqui nós documentamos a nível tecidual de células expressando de Granzima A como também foi observado que na LM existia uma freqüência maior de células expressando este marcador de citotoxicidade.

O TNF-α e a IL-10 têm sido considerados citocinas chaves na patogênese da LM. Em CMSP os níveis de TNF-α são significativamente mais elevados em pacientes com LM do que em pacientes com LC (Bacellar e cols., 2002) e a cura da LM é associada com redução dos níveis de TNF-α no soro e no sobrenadante de CMSP (Da-cruz e cols., 2002; Ribeiro de Jesus e cols., 1998). Adicionalmente, pacientes com LM refratários ao tratamento com antimonial foram curados quando pentoxifilina, droga inibidora de TNF-α foi adicionada ao antimonial (Lessa e cols., 2001). Enquanto, em CMSP de pacientes com LC e LM a principal fonte de TNF-α pode ser células CD4+, CD8+ e macrófagos (Bacellar e cols., 2002; Antonelli e cols., 2004), no presente trabalho a análise da resposta imune *in situ*, não mostrou diferença entre a freqüência de células expressando TNF-α entre pacientes com LC e LM e a principal fonte dessa citocina foi o macrófago. Enquanto no

sangue o nível de TNF-α em sobrenadantes de culturas é bem mais alto do que os níveis de IL-10 (Bacellar e cols., 2002), no tecido a freqüência de células expressando TNF-α e IL-10 foi similar e bastante elevada. Embora as diferenças entre os dados do sangue periférico e os achados teciduais possam estar relacionadas com o fato que *in situ*, nas leishmanioses, a expressão dessas citocinas é diferente do observado em CMSP, deve-se levar em conta dois aspectos: primeiro, enquanto no sangue periférico a avaliação da resposta imune é feita após estimulação com SLA, o estudo a nível tecidual avalia, além da estimulação mediada por antígenos parasitários, outros estímulos, principalmente representados por antígenos de bactérias. É bem conhecido que tanto as úlceras cutâneas quanto as úlceras mucosas são contaminadas por bactérias. Nesse caso não pode ser afastada que a semelhança entre a freqüência de células expressando TNF-α na lesão entre pacientes com LC e LM esteja refletindo a produção dessa citocina à produtos bacterianos ou à própria bactéria. Nesse raciocínio o significado de um grande número de células expressando IL-10 para a patologia da LC e LM fica também comprometida, desde que na resposta imune inata, a produção de TNF-α é seguida de uma produção de IL-10 possivelmente na tentativa de modular os efeitos deletérios do TNF-α.

É interessante o fato de que mesmo havendo um grande número de células expressando IL-10 tanto na LC como na LM, a percentagem de células expressando receptor de IL-10 e a intensidade da expressão desses receptores foi menor na LM do que na LC. Previamente tinha sido documentado que CMSP de pacientes com LM não eram apropriadamente moduladas por IL-10 (Bacellar e cols., 2002). No trabalho n°. 3 esses dados foram reforçados pela documentação que embora LACK e KMP11 tivessem capacidade de induzir a produção de IL-10 em pacientes com LM, enquanto esses antígenos

modulam a produção de IFN-γ de pacientes com LC, não conseguem suprimir a produção de IFN-γ de pacientes com LM. Um dos fatores que pode explicar esse fenômeno em adição ao aumento da freqüência de células T expressando marcadores de ativação celular (trabalho 1), é a diminuição de receptores de IL-10, fato confirmado pelos estudos a nível tecidual em pacientes com LM.

Os dados do presente estudo confirmam e estendem as observações prévias de que pacientes com LC e LM apresentam uma intensa resposta Th1. A natureza da resposta imune a nível de sangue periférico e a nível do tecido mostrou que essas duas formas clínicas são parecidas. Também, embora ambas as doenças apresentarem uma resposta imune associada à destruição de parasitos, patologia é também representada por lesões ulceradas e destrutivas. Esses estudos também demonstraram que em comparação com LC, CMSP de pacientes com LM não são apropriadamente moduladas em relação a produção de IFN-γ. Essa dificuldade na modulação está relacionada a uma diminuição de expressão de receptor de IL-10 em células de pacientes com LM e aumento de células T ativadas expressando marcadores como CD28-, CD62L- e CD69+.

8. Conclusões

1- A maior frequência de células T CD4+ ativadas na leishmaniose mucosa impedem a apropriada modulação da resposta imune e tem participação na manutenção da resposta inflamatória e dano tecidual.

2- KMP11 e LACK são antígenos indutores de IL-10 com capacidade de modular a produção de IFN-γ em pacientes com leishmaniose cutânea mas sem ação moduladora na leishmaniose mucosa.

3- A nível tecidual a resposta imune na leishmaniose mucosa é mais exacerbada do que na leishmaniose cutânea e a diminuição da intensidade da expressão e do número de células expressando receptor de IL-10 podem contribuir para a manutenção da exacerbação da resposta imune.

9. Perspectivas futuras

1. Avaliar o papel de células dendríticas em induzir um maior número de células efetoras na LM.

2. Avaliar o papel de outras citocinas nesse descontrole da resposta Th1, sendo a IL-27 uma das possibilidades.

3- Avaliar a resposta imune de indivíduos com a forma sub-clínica da doença com a finalidade de entender os mecanismos envolvidos na manutenção de uma resposta imune capaz de controlar a multiplicação de parasitos mas não causar doenças.

4- Avaliar o papel de células T regulatória na modulação da resposta Th1 exacerbada de pacientes com LC e LM.

10. Referências

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