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ESTUDO DE ASPECTOS INICIAIS DA INFECÇÃO POR *Leishmania spp.*

MARCOS CÉLIO DE ALMEIDA

Salvador - Bahia - Brasil
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MARCOS CÉLIO DE ALMEIDA

Orientador: MANOEL BARRAL-NETTO

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MARCOS CÉLIO DE ALMEIDA

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RESUMO

ESTUDO DE ASPECTOS INICIAIS DA INFECÇÃO POR *Leishmania* spp . Co-estimulação, adesão e metástase. MARCOS CÉLIO DE ALMEIDA. Uma vez inoculadas na pele tanto cepas dermatotrópicas como viscerotrópicas de *Leishmania* spp podem disseminar-se pelo organismo. Acredita-se que haja uma disseminação inicial do parasita. Além disso, as etapas inicias da infecção por *Leishmania* spp são desconhecidas. Há poucas informações relacionadas à infecção de monócitos humanos, a célula hospedeira inicial que eventualmente encontra-se parasitada no sangue circulante, principalmente no que diz respeito a moléculas costimulatórias e de adesão. Sabe-se por outro lado que o padrão de expressão dessas moléculas poderá direcionar a resposta imune do hospedeiro, que em última análise determina o padrão da doença. Monócitos humanos e macrófagos isolados por adesão ou por gradientes de Percoll foram infectados e estimulados com LPS ou IFNγ. A expressão de moléculas co-estimulatórias e de adesão foi mensurada usando a citometria de fluxo. A detecção de citocinas foi feita por ELISA. Os ensaios de adesão foram feitos em placas de plástico previamente sensibilizadas com componentes da matriz extracelular. A infecção por *L.(L.) chagasi* não induziu, em monócitos e macrófagos, a expressão de CD54, IL-12, ou TNFα. A infecção inibiu a expressão de IL-12 (p40) , CD54 e HLA-DR em monócitos estimulados com LPS, assim como a expressão de HLA-DR e HLA-ABC em macrófagos estimulados com IFNγ. Houve uma correlação negativa na expressão de CD54 e CD86 tanto em monócitos como em macrófagos. Monócitos infectados se mostraram menos aderentes ao plástico e à fibronectina e apresentaram uma diminuição da expressão de CD11b. Evidenciamos aqui o caráter escapatório da infecção por *L.(L.) chagasi* não induzindo moléculas proinflamatórias e co-estimulatórias. A não indução de moléculas co-estimulatórias e proinflamatórias, a inibição da expressão das moléculas HLA de classe I e II e o baixo parasitismo podem deixar o sistema imune, pelo menos nas fases iniciais, em ignorância ou anergia. A perda de adesão observada, *in vitro,* em monócitos infectados, as células mais precocemente infectadas, pode ser relevante para a disseminação inicial do parasita, *in vivo.*
Palavras-Chave: monócitos, co-estimulação, adesão, metástase, citocinas, L*eishmania* .

SUMMARY

STUDIES OF INITIAL ASPECTS OF *Leishmania* spp. INFECTION. Costimulation, adhesion and metastasis. MARCOS CÉLIO DE ALMEIDA. Once inoculated in skin both dermatotropic and viscerotropic *Leishmania* spp can disseminate. It is believed that there is an initial spreading of the parasite. Furthermore, the initial steps of *Leishmania* spp infection in humans are largely unknown. There is limited information on the *Leishmania* infected human monocytes, the infected circulating cell and the first cells that the parasite lives in, particularly related to costimulatory and adhesion molecules. On the other hand both adhesion and costimulatory molecules may direct the immune response that ultimately determine the clinical spectrum of the disease. Human monocytes and macrophages, isolated by adherence or Percoll gradients, were infected and stimulated with LPS or IFNγ. Cell expression of adhesion and costimulatory molecules were measured using flow cytometry. Cytokine production was detecded by ELISA. Cell adhesion was measured in cell matrix coated plastic plates. *L.(L.) chagasi* infection does not induce CD54, IL-12 or TNF-α, potent proinflammatory cytokines and down modulates CD11b expression in monocytes. LPS stimulated IL-12 (p40) levels, CD54 and HLA-DR expression are diminished in infected monocytes as as well as IFN-γ stimulated HLA-DR and HLA-ABC expression in infected macrophages. There is a negative correlation between CD54 and CD86 expression in both monocytes and macrophages. *L.(L.) chagasi* infected monocytes were less adherent to plastic and fibronectin. We show here an evasive behavior of *L.(L.) chagasi* infection not inducing costimulatory and proinflammatory molecules on human monocytes. The depressed expression of class I and II molecules, absence of key proinflammatory cytokines , impaired expression of costimulatory molecules and the low parasite density could leave the immune system, at least in its initial phases, in anergy or ignorance. The *in vitro* loss of adherence of infected monocytes, which are probably the first infected cells, could be related to initial dissemination of parasite *in vivo*.
Key Words: monocytes, costimulation, adhesion, metastasis, CD54, cytokines, *Leishmania*

SUMÁRIO

.

1 INTRODUÇÃO

1.1 A fase inicial na leishmaniose humana

O gênero *Leishmania*, um grupo de protozoários flagelados pertencentes à família Tripanosomatidae e à ordem Cinetoplastida, possui em seu ciclo biológico duas fases: a de amastigota e a de promastigota. Os amastigotas, que vivem no interior dos vacúolos lisossomais presentes nos monócitos e macrófagos do hospedeiro vertebrado, são ingeridas pela fêmea hematófoga do flebotomíneo quando esta realiza um repasto sanguíneo (LAINSON 1987; CHANG et al. 1990; ALEXANDER et al. 1999). No tubo digestivo do inseto estas formas se transformam em promastigotas e vivem extracelularmente: livres, aderidas à cutícula ou às microvilosidades intestinais (KILLICK-KENDRICK 1990).

São vetores das diversas *Leishmania* spp somente insetos da subfamília *Phlebotominae*, com o gênero *Phlebotomus* abrigando apenas subgêneros e espécies do Velho Mundo, e *Lutzomyia*, abrigando subgêneros e espécies do Novo Mundo.

Nesses insetos hematófagos, o parasita desenvolve um ciclo complexo já notado pelos primeiros investigadores nesse campo (ADLER & THEODOR 1931; KILLICK-KENDRICK 1990). Assim, clones de *L. (L.) mexicana* obtidos de *Lu. longipalpis* não foram capazes de infectar hamsteres (*Mesocricetus auratus*), quando inoculados após três dias de crescimento no inseto, mas se tornavam progressivamente infectivos a partir do 4° dia de desenvolvimento no inseto, aumentando a capacidade infectiva até o 7° dia (SACKS & PERKINS 1985). Coincidentemente, o pico da capacidade infectiva ocorre no momento em que um novo repasto sanguíneo será feito. O inseto realimentando-se no hospedeiro vertebrado regurgita entre 1 e 1000 metacíclicos (ADLER & THEODOR 1935;

WARBURG & SCHLEIN 1986), fechando o ciclo biológico. Claramente, esta coordenação de eventos permite ao parasita uma chance ótima de infectar o hospedeiro vertebrado e se perpetuar.

O inseto hematófago, para atingir o sistema vascular do hospedeiro vertebrado, realiza uma solução de continuidade em sua pele, usando a probóscide, e gera um pequeno lago sanguíneo, onde se alimenta (RIBEIRO 1987). Esta é uma interação complexa. Tem-se mostrado que o local da inoculação (POULTER & PANDOLPH 1982; CONSTANT et al. 2000), inclusive se dérmica ou subcutânea influencia o desenvolvimento da lesão (NICOLAS et al. 2000). Neste particular, devido ao comprimento da probóscide o flebotomíneo atingirá apenas os vasos mais superficiais da derme, na região da junção dermo-epidérmica. Sem a ação vasodilatadora da sua saliva, devida principalmente à ação do maxadilan, o flebotomíneo teria inclusive dificuldades para atingir os capilares dérmicos (RIBEIRO et al. 1984). Um embate trava-se então entre a resistência do hospedeiro e a estratégia do inseto para a obtenção de sangue. O hospedeiro atua com seus mecanismos imunológicos, dentre eles os epidérmicos (MOLL 1993), que serão dos

essa agressão, através do sistema de cininas (vasoconstricção), coagulação (trombose) e fagocitário-neutrofílico, poderia anular ou diminuir a competência vetorial, dificultando ou impedindo a capacidade do flebotomíneo de obter sangue (GILLESPIE et al. 2000). No entanto, a saliva do flebotomíneo possui potentes elementos vasodilatadores, maxadilan (*Lutzomyia*) e adenosina (*Phlebotomus*), anti agregante plaquetário, apirase (*Lutzomyia* e *Phlebotomus*), e estimuladores da produção de prostraglandina E2 no hospedeiro, entre os mais conhecidos, que contrabalançam essa resposta. Em geral, a estratégia vetorial inibindo a inflamação e diminuindo a sensibilização do hospedeiro será fundamental para a transmissão do parasita (GILLESPIE et al. 2000). Demonstrou-se, por exemplo, que a sensibilização prévia de camundongos ao sonicado de glândula salivar de *Phlebotomus papatasii* elimina os efeitos exacerbadores da saliva na infecção por *L(L.) major,* notadamente a geraçâo precoce de IL-5 e IL-4 na epiderme (BELKAID et al. 1998). Assim, a picada prévia de flebotomíneos não infectados confere certo grau de proteção à infecção (KAMHAWI et al. 2000) e peptídeos protetores na saliva têm sido identificados (VALENZUELA et al. 2001). Revisões recentes sobre o papel da saliva do inseto encontram-se em GILLESPIE et al. 2000, KAMHAWI 2000, SACKS & KAMHAWI, 2001.

Mais tarde, o desenvolvimento de uma resposta TH1 ou TH2, será fundamental para a contenção (resistência) ou o desenvolvimento mais ou menos intenso do parasita (REINER & LOCKSLEY 1995). Basicamente, três teorias têm sido propostas para explicar a divergência TH1/Th2 na leishmaniose experimental (REINER & LOCKSLEY 1995): I-. Peptídeos diferentes estimulam diferentes grupos de clones TH1 ou TH2. II- Um padrão particular de citocinas e cofatores, produzido por células acessórias do sistema imune inato, seria o motivo da divergência. III- Células T provinientes de uma ou outra linhagem de

camundongos , sob estimulação, teriam propensão inata em desenvolver-se para um dos polos da resposta, assim clones de célula T do camundongo C57BL/6 direcionariam-se para TH1, e de BALB/c para TH2. Quanto à primeira hipótese, identificou-se um peptídeo, LACK (*L. major* antigen Leishmania homologue of mammalian RACK1), que reconhecido por células Vβ4Vα8 CD4+ do camundongo BALB/c induz a produção, por essas células, de IL-4, de 12 a 14 horas após a infecção, direcionando a resposta para TH2 (LAUNOIS et al. 1997; LAUNOIS et al. 1999). No entanto, esta resposta pode ser modulada para TH1, por fatores como baixas dose do antígeno (MENON & BRETSCHER 1998); produção precoce de IL-12 ou IFNγ (SCOTT 1991; SCHARTON-KERSTEN & SCOTT 1995; DOHERTY & COFFMAN 1996), administração de IL-4 até 12 horas do início da infecção (HIMMELRICH et al. 2000; BIEDERMANN et al. 2001). Conjecturou-se que uma produção diferencial de IL-12 por células dendríticas de camundongos da linhagem BALB/c e C57BL/6 ao parasita pudesse explicar essa divergência (VON STEUBT ET AL 1998). Contudo mostrou-se, posteriormente, que células dendríticas de ambas linhagens produzem IL-12 ao fagocitarem amastigotas (VON STEUBT et al 2000). Está claro, então, que os padrões da resposta inata na fase inicial terão papel fundamental no desenvolvimento da resposta TH1 ou TH2, e a resposta não é fixa para cada linhagem de camundongo (SCHARTON-KERSTEN & SCOTT 1995; DOHERTY & COFFMAN 1996). Nessa situação inicial, um dos aspectos fundamentais é a caracterização da célula apresentadora de antígeno infectada, porque sua interação com o linfócito iniciará e será decisiva para estruturar a resposta adquirida (DE ALMEIDA et al 2002. enviado para publicação).

Por razões que aduzimos a seguir, no lago sanguíneo inicial a célula fagocítica, onde o crescimento parasitário ocorrerá, será o monócito, embora não seja aí o fagócito mais abundante (ALMEIDA 2002). Os neutrófilos, células de vida curta (48hs), estão em maior número e parecem desempenhar um papel maior na eliminação do parasita do que na sua hospedagem (CHANG 1981). Essa célula, no entanto, é capaz de secretar citocinas IL-12, TGF-β (TACCHINI-COTTIER et al. 2000) e mesmo apresentar antígenos via MHC classe II sob ação do GM-CSF (GOSSELIN et al. 1993). O neutrófilo infectado apoptótico poderia também constituir uma importante fonte inicial de antígeno para as células dendríticas (DE ALMEIDA 2002). Os macrófagos não estão presentes no lago sanguíneo, e são escassos, ao redor do lago sangüineo recém formado, na pele humana normal, não inflamada (URMACHER 1997). Quanto à célula dendrítica epidérmica, é possível que mesmo estando os promastigotas acessíveis a ela, em caso de um inóculo excessivo (LIRA et al 2000), esta não seja capaz de fagocitá-los (VON STEBUT et al. 1998; VON STEBUT et al. 2000), ou tenha um um grau de fagocitose limitado (KONECNY et al. 1999; MAROVICH et al. 2000). Claramente isso reforça nosso modelo, ao sugerir um processo sequencial de infecção monócito/macrófago → célula dendrítica. Reforça também a importância dos neutrófilos infectados apoptóticos, que constituiriam a primeira fonte de antígenos de *Leishmania* spp para as células dendríticas (DE ALMEIDA 2002).

Tem-se aqui então um modelo, descrito acima, que se aplica à transmissão de todas as *Leishmania* spp. para seus hospedeiros vertebrados. Essa afirmação deve ser válida principalmente quanto aos aspectos qualitativos do modelo. Variações devem ocorrer no número de parasitas regurgitados por diferentes espécies de flebotomíneo, no volume do lago sanguíneo inicial e na composição da saliva, para citar aqueles aspectos em que há

poucos ou inexistentes estudos. A capacidade vetorial de uma mesma espécie de flebotomíneo, por exemplo, pode variar dependendo das condições ambientais, e é de se esperar também uma seleção nos parasitas em decorrência desse fato (SCHLEIN & JACOBSON 2001)

No nosso entender, esse modelo é o racional de uma série de trabalhos experimentais focalizando os aspectos mais iniciais da leishmaniose (DOMINGUEZ & TORANO 1999; BELKAID et al. 2000; LIRA et al. 2000). No entanto, o modelo murino mais frequentemente empregado da leishmaniose tem diferenças importantes com a leishmaniose humana, e as extrapolações são muitas vezes limitadas. Por exemplo, a infecção em camundongo não reproduz as diferentes formas da leishmaniose humana, notadamente as formas mucosas e viscerais (MELBY et al. 2001). Há claro envolvimento do NO na eliminação intracelular do parasita no camundongo e não no homem (STENGER et al. 1996). As resposta TH1 e TH2 são bem distintas na leishmaniose murina , mas não na humana (REINER & LOCKSLEY 1995). Há, por outro lado, necessidade da aplicação desse modelo ao estudo da leishmaniose humana. No entanto, os imperativos éticos restringem a análise de muitos desses aspectos da leishmaniose humana a estudos *in vitro.* É fundamental então que se desenvolvam técnicas e se acrescentem elementos ao modelo *in vitro,* que o tornem mais próximo da realidade.

2 PUBLICAÇÕES E OBJETIVOS

Tendo esses aspectos em vista, três publicações foram geradas relacionadas a esse tópico.

1. A primeira publicação descreve uma metodologia de obtenção de monócitos em suspensão a partir do sangue periférico. (DE ALMEIDA et al. 2000)

2. A segunda publicação estabelece um racional para a utilização de parâmetros usados em experimentos *in vitro,* notadamente o inóculo infectivo, e suas implicações para exprimentos *in vitro,* que procurem simular os aspectos mais iniciais da infecção por *Leishmania* spp.(DE ALMEIDA 2002).

3. Uma terceira publicação, sabendo-se que o padrão de expressão de moléculas co-estimulatórias, de adesão e citocinas está implicado na geração das resposta TH1 e TH2, estudou este padrão em monócitos e macrófagos humanos infectados com *L.(L.) chagasi.* (DE ALMEIDA et al 2002. *Leishmania (Leishmania) chagasi* infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage. Enviado para publicação

2.1 Publicação 1

DE ALMEIDA, M. C.; SILVA, A. C.; BARRAL, A.; BARRAL NETTO, M. "A simple method for human peripheral blood monocyte isolation." **Mem Inst Oswaldo Cruz, 95**: 221-223., 2000

SHORT COMMUNICATION

A Simple Method for Human Peripheral Blood Monocyte Isolation

Marcos C de Almeida/*, Alan C Silva, Aldina Barral, Manoel Barral Netto/+

Centro de Pesquisas Gonçalo Moniz-Fiocruz, Rua Valdemar Falcão 121, 40295-001 Salvador, BA, Brasil
*Departamento de Patologia, FCS, Universidade de Brasília, DF, Brasil

We describe a simple method using percoll gradient for isolation of highly enriched human monocytes. High numbers of fully functional cells are obtained from whole blood or buffy coat cells. The use of simple laboratory equipment and a relatively cheap reagent makes the described method a convenient approach to obtaining human monocytes.

Key words: monocytes - percoll - gradient-isolation

Macrophages and monocytes are largely used in immunological research especially for the study of intracellular parasites. The ideal method for monocyte isolation combining simplicity, cheapness, purity and high yield does not exist (Seljelid & Pertoft 1981, Bennett & Breit 1994). We propose here a simple two step procedure for obtaining highly purified human monocytes.

The most common procedure is monocyte isolation by adherence after Ficoll-Hypaque purification of peripheral blood mononuclear cells (PBMC) (Bennett & Breit 1994). Monocyte isolation by adherence, although simple, has several disadvantages: high lymphocyte contamination, low flexibility, high manipulation and monocyte transient activation (Haskill et al. 1988, Bennett & Breit 1994). Lymphocyte contamination in the first hour after adherence may be high, being as high as 40-50% after two washes and 30% even after five washings. It has been reported that in the first 24 h, after four vigorous washes, 25% of remaining cells are lymphocytes (Bennett et al. 1992). The degree of lymphocyte contamination when separating monocytes by the adherence method is probably related to percentage of lymphocytes in PBMC, the amount of PBMC laid for adherence, number of washes, strength of washing and time of adhesion. These aspects can make this method variable from donor to donor and from researcher to researcher. Alternative methods are immune-selection, centrifugal elutriation and density gradients. Immune selection is too expensive for daily routine and for large volumes of blood. Centrifugal elutriation, although the method of choice for larger volumes of blood, requires expensive equipment and a specialized technician. Several kinds of density gradients are available, both continuous and discontinuous. Pumps and ultracentrifuges, in general expensive equipment, are necessary for performing continuos gradients.

We report here on the results using a two step procedure with single gradients in each step. First using a Ficoll-Hypaque gradient (density = 1.070 g/ml) and afterwards a slight hyperosmolar Percoll gradient (density = 1.064 g/ml). Percoll solutions were done as follows: first an isosmotic Percoll was prepared as usually mixing one volume NaCl 1.5 M with nine volumes of Percoll (Pharmacia, density = 1.130 g/ml). The Percoll gradient was done mixing 1:1 (v/v) isosmotic Percoll with PBS/Citrate (NaH_2PO_4 1.49 mM; Na_2HPO_4 9.15 mM; NaCl 139.97 mM; $C_6H_5Na_3O_7 .2H_2O$ 13mM; pH 7.2). Both gradients were centrifuged at 25-35°C, 400 g for 35 min. Percentage of monocytes after the Percoll gradient was higher than 90% using morphology, histochemistry or FACS analysis (Table I, Figure). The cells were viable and functional and able to be cultivated in suspension or attached to plastic, plastic bound fibronectin, collagen or laminin (not shown). Further indications of functionality were secretion of large amounts of TNF-α after LPS stimulation, phagocytose of latex particles and *Leishmania* promastigotes both in suspension and after adherence (Table II). Eighty

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+Corresponding author. Fax: +55-21-356.2593. E-mail: mbarral@cpqgm.fiocruz.br
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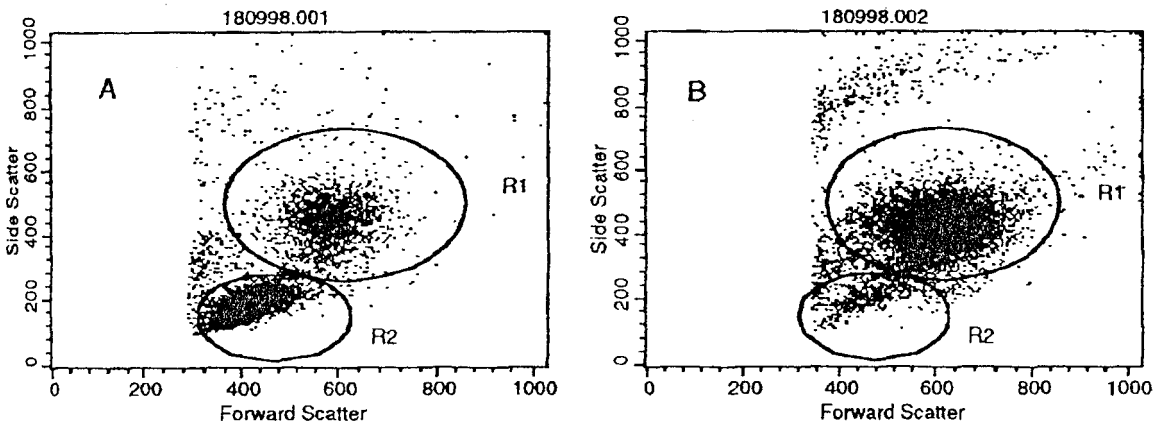
TABLE I

Monocyte yields and phenotypic analysis after Percoll gradient in relation to blood volumes, buffy-coats or number of peripheral blood mononuclear cells from different donors

Source of material	Monocytes recovered ($X10^6$)	Viabilility[a] (%)	Peroxidase activity (%)$^+$	Cytofluorimetry analysis
Blood (ml)				
20	4	85	92	ND
20	7.8	94.2	92.5	ND
20	10.2	96	ND	96.7% CD64+
40	12	90	95	ND
40	7.2	92	90	ND
53	22.8	95	98	96.6% CD14+
PBMC ($X10^6$)				
24.8	7.8	>90	ND	96.9% HG
76	25.1	>90	ND	95.4% HG
170	37.2	100	ND	94.8% CD14+CD3-
204	32	>90	ND	94% CD14+CD3-[b]
345	64	>90	ND	93.9% HG
372	134	>90	ND	98% CD14+CD3-
Buffy-coat				
1/2 blood bag	88	>90	ND	98% CD14+CD3-
1/2 blood bag	94.6	>90	ND	89.9% CD14+CD3-

a:measured by Trypan blue dye exclusion test.; *b*: 1.2% of CD19+CD3; HG: height and granularity; ND: note done; PBMC: peripheral blood mononuclear cells; +: presence of peroxidase activity was detected incubating at room temperature air dried cytospin preparations with 3,3"-diaminobenzidine plus H_2O_2.



Representative side (granularity) and forward (height) light scatters (A) following Fycoll-Hypaque and (B) following Percoll gradient. The R1 gate corresponds to monocyte and R2 to lymphocyte populations. The R1+R2 (T) were considered to represent 100% of cell population (96.6% of acquisition events in (A) and 93.4% in (B): (A) R1/T x 100=21 and in (B) R1/T x 100=93.9.

to 90% from the monocytes laid on the Percoll gradient were recovered afterwards (Table III). The procedure yields similar results with small and large amounts of blood. When working with small amounts of blood (< 50 ml) for saving time we have used leukocyte rich plasma after dextran sedimentation.(Meerschaert & Furie 1994). When working with very large amounts of blood (> 200 ml) was better to take the buffy coat. It has been previously proposed a two step Percoll gradient for monocyte isolation with 90% of purity but with variable yield (Seljelid & Pertoft 1981). The most important pitfall of their method was that they advocate the use of defribrinated blood. This could lead to serious cell loss and activation. As they had observed, monocytes bind strongly to small microscopic blood clots. Platelets could also bind to monocytes forming clumps (Weyrich et al. 1996). The adequate blood anticoagulation is then critical. The use of sodium citrate in all the solutions

until the Percoll gradient avoids the use of defibrinatrion and platelet binding to monocytes (Roos & de Boer 1986), possibly the use of EDTA could have the same effect although we have not tested it. Platelet elimination can be easily done with low speed centrifugation (100 g) before or after the Percoll gradient although it implies in cell loss. Temperature is also a critical point. We prefer working during all the procedure at room temperature (25-35°C) as it has been shown that monocyte tends spontaneously to aggregate at lower temperatures (Mentzer et al. 1986) and platelets to be activated (White & Krivit 1967, Oliver et al. 1999). Finally as it has been shown (Fluks 1981, Boyum 1983) the monocyte purity can be improved by hyperosmotic density gradients. In conclusion the procedure devised here can be done with usual reagents and equipment of average laboratory, it is easily handled and provides a 90% pure population of monocytes.

TABLE II

Monocyte functional assays after Percoll gradient

TNF-α production 48 h after LPS stimulation (10ng/ml)	1,019 pg/ml[a]
Adherence to plastic	Yes
Phagocytose of latex beads	91%[b]
Phagocytose of *Leishmania (L) chagasi* promastigotes	94%[c]
Increased CD 54 expression 48 h after IFN-γ (100U/ml) stimulation	50%[d]

a: mean of five experiments (SD=778.7); 10^6monocytes/ml were cultivated in RPMI medium plus 2mM L-glutamine with 10% human blood serum; *b*: % of cells exhibiting latex beads after 12 h incubation with 10 beads/monocyte; one representative experiment; *c*: % of cells exhibiting amastigotes after 12 h incubation with ten promastigotes/monocyte; one representative experiment; *d*: mean percent increase of four experiments (SD=17.74).

TABLE III

Rate of monocyte recovery after Percoll gradient

PBMC ($x10^6$)	Monocytes[a] (%)	Monocytes ater percoll gradient ($x10^6$)	Yield (%)	Purity (% HG)
24.8	31	7.8	82.4	96.9
76	40.3	25.1	82.0	95.4
345	20.4	63.9	91	95.2

HG: height and granularity; PBMC: peripheral blood mononuclear cells, obtained from Ficoll-Hypaque gradient; *a*: the monocyte percentage from PBMC was determined as shown in Fig. 1.

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REFERENCES

Bennett S, Breit SN 1994. Variables in the isolation and culture of human monocytes that are of particular relevance to studies of HIV. *J Leukoc Biol 56*: 236-240.

Bennett S, Por SB, Stanley ER, Breit SN 1992. Monocyte proliferation in a cytokine-free, serum-free system. *J Immunol Methods 153*: 201-212.

Boyum A 1983. Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. *Scand J Immunol 17*: 429-436.

Fluks AJ 1981. Three-step isolation of human blood monocytes using discontinuous density gradients of Percoll. *J Immunol Methods 41*: 225-233.

Haskill S, Johnson C, Eierman D, Becker S, Warren K 1988. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J Immunol 140*: 1690-1694.

Meerschaert J, Furie MB 1994. Monocytes use either CD11/CD18 or VLA-4 to migrate across human endothelium *in vitro*. *J Immunol 152*: 1915-1926.

Mentzer SJ, Guyre PM, Burakoff SJ, Faller DV 1986. Spontaneous aggregation as a mechanism for human monocyte purification. *Cell Immunol 101*: 312-319.

Oliver AE, Tablin F, Walker NJ, Crowe JH 1999. The internal calcium concentration of human platelets increases during chilling. *Biochim Biophys Acta 1416*: 349-360.

Roos D, de Boer M 1986. Purification and cryopreservation of phagocytes from human blood. *Methods Enzymol 132*: 225-243.

Seljelid R, Pertoft H 1981. In DO Adams, PJ Edelson & HS Koren (eds), *Methods for Studying Mononuclear Phagocytes*, Academic Press, p. 201-205.

Weyrich AS, Elstad MR, McEver RP, McIntyre TM, Moore KL, Morrissey JH, Prescott SM, Zimmerman GA 1996. Activated platelets signal chemokine synthesis by human monocytes. *J Clin Invest 97*: 1525-1534.

White JG, Krivit W 1967. An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood 30*: 625-635.

2.2 Publicação 2

DE ALMEIDA, M. C. "Infective inoculum for *Leishmania*." **Trends Parasitol., 18**: 154-155, 2002.

Neil McFerran
The Centre for Peptide and Protein Engineering, Medical Biology Centre, The Queen's University of Belfast, 97 Lisburn Road, Belfast, UK BT9 7BL.

References
1 Prichard, R.K. (2001) Genetic variability following selection of *Haemonchus contortus* with anthelmintics. *Trends Parasitol.* 17, 445–453
2 Nogales, E. *et al.* (1998) Structure of the αβ tubulin dimer by electron crystallography. *Nature* 391, 199–203

Benzimidazole binding to *Haemonchus contortus* tubulin: a question of structure

Response from R.K. Prichard

M. Robinson *et al.*, present an interesting structural analysis. The model that was presented in my review was proposed on the evidence for the role of Phe200 and Phe167 in allowing high-affinity benzimidazole (BZM) binding [1]. The evidence that the amino acids Ser166, Phe167, Phe200 and Cys201 cluster together, the importance of having a planar BZM structure in order to obtain high-affinity binding, which could allow a staking interaction of the BZM phenyl ring with the two Phe residues at positions 167 and 200, the location of the approximately parallel B5 and B6 strands containing these residues [2] and the loss of high-affinity BZM binding when Phe at either position 200 or 167 becomes substituted with a hydroxy substituent (Tyr). However, the model that was proposed does require experimental analysis; therefore, I welcome their discussion. Robinson and colleagues make valuable comments based on measured separation distances of the Phe–Phe inter-rings and the orientation of the side chain of Cys201 in the crystalline structure obtained following taxol stabilization [2] and, in the absence of taxol, these restrictions might not apply. I would welcome further work so that we do understand the BZM binding site better, and hope that the model that I have proposed will stimulate further analysis.

Roger K. Prichard
Institute of Parasitology, McGill University, Macdonald College, 21 111 Lakeshore Road, St Anne-de-Bellevue, Quebec, Canada H9X 1C0.
e-mail: roger.prichard@staff.mcgill.ca

References
1 Prichard, R.K. (2001) Genetic variability following selection of *Haemonchus contortus* with anthelmintics. *Trends Parasitol.* 17, 445–453
2 Nogales, E. *et al.* (1998) Structure of the αβ tubulin dimer by electron crystallography. *Nature* 391, 199–203

Infective inoculum for *Leishmania*

The importance of a low-dose inoculum (LDI) for understanding the initial steps of *Leishmania* infection has recently been stressed [1,2]. When the sandfly bites the skin, a small blood-lake is formed [3] into which 1–1000 infective metacyclic promastigotes are delivered [4]. In one experiment, 33 egested promastigotes were obtained from most (75.6%) sandflies [4]. In LDI models, there is a long incubation period (6–8 weeks, similar to that in natural infections) [1,2,5] when parasite growth is not affected by the host immune system [1,2]. Human leishmaniasis also has a long incubation period of months or even years (e.g. visceral leishmaniasis takes 3–8 months) [6]. However, the initial steps of *Leishmania* infection in humans are not known, and ethical constraints restrict experiments to *in vitro* studies. It is proposed here, for the first time, a rationale for an *in vitro* LDI that would mimic an *in vivo* situation. Using human and mice phagocytes, most experimental designs (irrespective of *Leishmania* spp.) have used 5–10 infective *Leishmania* promastigotes per phagocytic cell, typically a macrophage. As we are aware, there is no clear reason for using this widely accepted number of promastigotes per phagocytic cell. We also have to consider that the mean number of parasites inside phagocytic cells in established diseases is related to the *Leishmania* spp. studied [e.g. macrophages are heavy infected in *Leishmania (L.) amazonensis* disease and parasites are rarely found in cells from *Leishmania (V.) braziliensis*-infected individuals].

The number of parasites found in established disease would not necessarily reflect the numbers present at the initial steps of infection. Following the inoculation of 100 metacyclics into the mouse footpad, amastigotes were not detectable in the first three weeks of infection. However, six–seven weeks after inoculation, the concentration of parasites per mg^{-1} of tissue (10^5) was 1000 times more than that found in chronic disease [1]. Considering that the infective bloodmeal volume is ~0.1 μl [7,8], then a range of 300–1000 phagocytes (normal values for adult blood) would have to be matched with a range of 1–1000 infective promastigotes. In the dermal bloodmeal lake, the first human cells to phagocytize the promastigotes are monocytes, neutrophils and eosinophils [9], and the residual macrophages of the human skin. Neutrophils and eosinophils must be considered when designing complex *in vitro* systems that simulate the initial steps of *Leishmania* infection (e.g. *in vitro* priming systems) because these cells can produce cytokines [10], and apoptotic infected neutrophils could represent an early source of *Leishmania* antigens for macrophages or dendritic cells (DC). However, neutrophils do not sustain parasite growth. Hence, the first cells that the parasite lives in are mainly the blood monocytes because human skin has only few macrophages. The number of parasites per phagocytic cell would then range 0.001–3. Considering the mean number of promastigotes egested by most of the sandflies when biting (~33), the most probable mean number of parasites per phagocyte would be between 0.03 and 0.1. As some metacyclics could be lysed by host complement, these numbers could be even lower. Of course some phagocytes, including antigen-presenting cells (APCs), will not become infected when the mean number of parasites is <1. This is in contrast with large-dose inoculum models, in which excessively released antigens and parasites would reach dermal and epidermal DC rapidly, thus inducing early activation of the immune system [1]. These considerations would be particularly relevant to experimental designs where antigen concentration, antigen distribution, number and the type of APC are important, as in lymphocyte activation systems leading to T helper (Th) 1 or Th2 cell differentiation [11,12].

http://parasites.trends.com 1471-4922/02/$ – see front matter

So taking these considerations into account, the infective inoculum that has been used in *in vitro* systems is too high and far from that expected for an *in vivo* situation. The predicted minimum number of parasites per infected APC and the low number of infected APC would have the best chance of generating few activated T cells [11], or inducing minimal changes in these APC and consequently fail to stimulate the adaptive branch of immune response. It could explain the long, silent phase of infection and absence of adaptive immune response, allowing undisturbed parasite growth inside permissive cells such as monocytes and macrophages [1,2] found in initial phases of natural models of *Leishmania* infection.

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Marcos Celio de Almeida
Centro de Pesquisas Gonçalo Moniz-Fiocruz, Laboratório-LIMI Rua Waldemar Falcão 121, Brotas, 40295-001, Salvador, BA, Brazil.
e-mail: marcoscelio@ig.com.br

References

1 Lira, R. *et al.* (2000) Evolution of lesion formation, parasitic load, immune response, and reservoir potential in C57BL/6 mice following high- and low-dose challenge with *Leishmania major*. *Infect. Immun.* 68, 5176–5182
2 Belkaid, Y. *et al.* (2000) A natural model of *Leishmania major* infection reveals a prolonged 'silent' phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J. Immunol.* 165, 969–977
3 Ribeiro, J.M.C. (1987) Role of saliva in blood-feeding by arthropods. *Annu. Rev. Entomol.* 32, 463–478
4 Warburg, A. and Schlein, Y. (1986) The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. *Am. J. Trop. Med. Hyg.* 35, 926–930
5 Almeida, M.C. *et al.* (1993) Metacyclogenesis of *Leishmania (Viannia) braziliensis in vitro*: evidence that lentil lectin is a marker of complement resistance and enhanced infectivity. *Trans. R. Soc. Trop. Med. Hyg.* 87, 325–329
6 Pearson, R.D. and Sousa, A.Q. (1996) Clinical spectrum of leishmaniasis. *Clin. Infect. Dis.* 22, 1–13
7 Guevara, P. *et al.* (2001) Green fluorescent protein-tagged *Leishmania* in phlebotomine sand flies. *J. Med. Entomol.* 38, 39–43
8 Nieves, E. and Pimenta, P.F.P. (2000) Development of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* in the sand fly *Lutzomyia migonei* (Diptera: Psychodidae). *J. Med. Entomol.* 37, 134–140
9 Dominguez, M. and Torano, A (1999) Immune adherence-mediated opsonophagocytosis: the mechanism of *Leishmania* infection. *J. Exp. Med.* 189, 25–35
10 Tacchini-Cottier, F. *et al.* (2000) An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. *J. Immunol.* 165, 2628–2636
11 Hosken, N.A. *et al.* (1995) The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J. Exp. Med.* 182, 1579–1584
12 Shankar, A.H. and Titus, R.G. (1997) The influence of antigen-presenting cell type and interferon-γ on priming and cytokine secretion of *Leishmania major*-specific T cells. *J. Infect. Dis.* 175, 151–157

Hydrogen production in *Giardia intestinalis*, a eukaryote with no hydrogenosomes

Giardia intestinalis, the commonest protozoal cause of upper intestinal infections (2.8×10^8 estimated cases per year [1]), is a waterborne parasite with a worldwide distribution. Although usually self-limiting, infections can be life threatening in nutritionally deprived or immunocompromised individuals, or in the very young or elderly. Almost always designated as an ancient and primitive anaerobic flagellate, it has many bacterial characteristics (pyrophosphate-biased energy metabolism, arginine dihydrolase and other anaerobic fermentation pathways, and no mitochondrial pathways of energy production) [2]. *Giardia intestinalis* (syn. *lamblia, duodenalis)* is referred to in textbooks as an early-branching eukaryote, but this parasite has almost certainly been misplaced [3] on the lowest branch of the eukaryotic tree [4]. Evidence accumulated from molecular studies indicates that this amitochondriate protist has nuclear genes for a mitochondrial heat shock protein, related to hsp60 chaperonin [5,6]. The lack of mitochondria and peroxisomes, and possession of only a rudimentary Golgi body [7] might not hint at a primitive status but rather represent the secondary loss of characteristics during evolution as a parasite [8].

We now report that, although the organism has no identifiable hydrogenosomes, it nevertheless does produce H_2 under anaerobic conditions [9]. We have used fluorescent-labeled monoclonal antibodies to typical hydrogenosomal enzymes (e.g. malate enzyme and succinyl-CoA synthetase α and β subunits and the large granule fraction all purified from *Trichomonas vaginalis* [10]) to locate hydrogenosomes by confocal laser scanning microscopy. No discrete localization of typical hydrogenosomal epitopes was evident.

Mass spectrometric monitoring through a Mylar (polyethylene terephthalate) membrane, permeable only to H_2 and CO_2 but not to ethanol, indicated H_2 evolution (2 nmol min^{-1} per 10^7 organisms) only under strictly anaerobic conditions [9]. Other fermentation products include L-alanine, ethanol, acetate and CO_2 [11,12]. The introduction of 10 μM O_2 inhibited H_2 production completely. The capability for H_2 generation might be necessary for cellular redox balance. Control mechanisms employed in an environment where O_2 supply is limited, and spatial and time-dependent fluctuations for apportionment of reducing equivalents between products remain to be investigated, but the redox ratio of nicotinamide nucleotide coenzymes NAD(P)$^+$ to NAD(P)H has been shown to be involved [12]. Hydrogenase activity linked to methyl viologen has now been demonstrated in anaerobic incubations using cell-free extracts [9].

Extensive mechanistic research on hydrogenases indicates that evolutionary relationships can be elucidated [13]. This first report of a hydrogenase in *G. intestinalis* paves the way for molecular studies [14] which could help phylogenetic studies on *G. intestinalis* as has been the case for *Entamoeba histolytica* [15]. The complete sequence of an iron-only hydrogenase has also been identified in the *Giardia* genome recently (see http://www.ncbi.nlm.nih.gov:80).

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David Lloyd*
James R. Ralphs
Janine C. Harris
Microbiology (BIOSI 1, Main Building), Cardiff University, PO Box 915, Cardiff, UK CF10 3TL.
*e-mail: Lloydd@cardiff.ac.uk

2.3 Publicação 3

DE ALMEIDA, M. C. ; CARDOSO, S. A.; BARRAL-NETTO, M *Leishmania (Leishmania) chagasi* infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage. 2002 (Enviado para publicação).

Leishmania (Leishmania) chagasi INFECTION ALTERS THE EXPRESSION OF CELL ADHESION AND COSTIMULATORY MOLECULES ON HUMAN MONOCYTE AND MACROPHAGE

M. C. De Almeida [1,2] S. A.Cardoso[1] and M. Barral-Netto[1].

1. Faculdade de Medicina – Universidade Federal da Bahia and Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, BA, Brazil;
2. Departamento de Patologia, UnB, DF, Brazil

Short title: *Leishmania (Leishmania) chagasi* INFECTION ALTERS ...

The work was done at:.Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, BA, Brazil;

Address for correspondence:

Marcos Celio De Almeida

Universidade de Brasília(UnB)
Campus Universitário Darcy Ribeiro
Faculdade de Medicina
Departamento de Patologia(PTL)
ASA NORTE. CEP:70910-900 . FAX NUMBER: 00 55 61 2723355
BRASÍLIA.DF.
BRASIL
E-mail: marcalmo@unb.br

SUMMARY

The initial steps of *Leishmania* infection in humans are largely unknown. There is limited information on the *Leishmania* infected human monocytes, the first cells that the parasite lives in, particularly related to costimulatory molecules. We show here that *L.(L.) chagasi* infection avoids inducing proinflammatory molecules and has strikingly down modulating effects on human monocytes or macrophages. It does not induce CD54, IL-12 or TNF-α, potent proinflammatory cytokines and down modulates CD11b expression in monocytes. LPS stimulated IL-12 (p40) levels, CD54 and HLA-DR expression are diminished in infected monocytes as well as IFN-γ stimulated HLA-DR and HLA-ABC expression in infected macrophages. There is a negative correlation between CD54 and CD86 expression in both monocytes and macrophages. The depressed expression of class I and II molecules, absence of key proinflammatory cytokines and impaired expression of costimulatory molecules induced by *L.(L.) chagasi* could leave the immune system, at least in its initial phases, in anergy or ignorance.

Key words: costimulation, CD54, CD11b, CD86, cytokines, *Leishmania.*

INTRODUCTION

The early steps of *Leishmania* infection are of utmost importance in determining whether a Th1 predominance, leading to resistance, or a Th2 advantage, related to susceptibility, occurs.(Reiner & Locksley 1995).

Recently arrived monocytes are probably the first permissive human cells to phagocyte *Leishmania* in the blood pool formed by the sand fly bites (Ribeiro 1987) followed by the scarce residual macrophages present in the normal human skin (Milon et al. 1995; Urmacher 1997). Human neutrophils are short-lived cells involved in clearing the parasite (Chang 1981) but not in sustaining the parasite growth. Monocytes and macrophages could play distinct roles in *Leishmania* infection (Cervia et al. 1993; Milon et al. 1995).

Inside the macrophage the parasite has several strategies for evading the host immunological system (Reiner 1994; Buates & Matlashewski 2001). One of these strategies is the down modulation or not induction of molecules involved directly or indirectly in antigen presentation. For example *L.(L.) donovani* infection has been shown to down modulate the expression of MHC I and II molecules (Reiner et al. 1987).

Studies in mice are controversial, concerning the ability of *Leishmania* infection to induce changes in the expression pattern of costimulatory molecules of monocyte/macrophage. No changes on costimulatory molecules expression were found on macrophages from the resistant C57BL/6 mice upon infection with *L.(L.) donovani* (Saha et al. 1995) or with

L.(L.) major (von Stebut et al. 1998). *L.(L.) donovani* infection of macrophages from susceptible BALB/c mouse marginally increases CD54 (Saha et al. 1995) but fails to trigger CD80 (Kaye et al. 1994). *L.(L.)major* was also not able to change the low level of CD80 expression and the basal level of CD86 expression in human macrophages (Brodskyn et al. 2001). The pattern of expression of costimulatory molecules has been implicated in driving the immune system to a Th1 or a Th 2 response leading to secretion of cytokines that could activate the macrophage and arrest the infection or burst it (Kaye 1995; Hunter & Reiner 2000). These initial steps of *Leishmania* infection in humans are largely unknown, and there is limited information on the response of the *Leishmania* infected human monocytes, particularly related to costimulatory molecules.

Herein we report on the expression of costimulatory molecules, cytokines as well as HLA, on human blood monocytes and on "in vitro" differentiated macrophages upon infection with *L.(L.) chagasi*.

MATERIALS AND METHODS

The parasite

L.(L.) chagasi (MHOM/BR/BA 305) was isolated from a patient with visceral leishmaniasis. The isolate was inoculated in the spleen of a Syrian hamster (*Mesocricetus auratus*), seeded in Schneider's insect culture medium (Sigma, Saint Louis, MO, USA) plus 10% of fetal calf blood serum, at 25^0 C. In its first subculture several samples of the parasite were collected and frozen in liquid nitrogen. Stocks were thawed and cultivated in

same conditions until the 10^{th} "in vitro" subcultures. The parasites were collected at stationary growth phase for in vitro infection of human monocytes and macrophages.

Isolation of monocytes and parasite infection

Monocyte isolation was performed as previously described (de Almeida et al. 2000) with slight modifications. Briefly, a two step procedure with single gradients in each step was used. PBMC were isolated from buffy coats (obtained from normal blood donors) by centrifugation over a Ficoll-Hypaque (400g, 35 min, 25-35^{0}C). The PBMC were washed three times with PBS/Citrate (1.49 mM $Na_2H_2PO_4$; 9.15 mM Na_2HPO_4 ; 139.97 mM NaCl ,13mM $C_6H_5Na_3O_7.2H_2O$; pH 7.2) (100g,15 min, 25-35^{0}C) to avoid platelet contamination. Around 10^8 PBMC were then incubated (15-30 min, 37^{0}C) in 15ml of a solution formed by nine parts of RPMI 1640 medium (Sigma) supplemented by 2mM L-glutamine,15mMHEPES, 10% human serum and 50μg/ml gentamcin and one part of trisodium citrate ($C_6H_5Na_3O_7.2H_2O$) 3,8%(w/v) in 50 ml conical polypropylene tubes with loosed lids in a CO_2 incubator. This suspension was then layered on top of a 15ml Percoll gradient [1:1 (v:v) isosmotic Percoll plus PBS/Citrate] and centrifuged (400g, 35 min, 25-35^{0}C). The percentage of monocytes recovered on top of the gradient was higher than 90% with more than 90% viable cells (de Almeida et al. 2000). Cells were washed two times in PBS before cultivation on cell culture medium. Some experiments were done using monocytes purified by adherence using a previous established protocol (Wahl & Smith 1994), in this case the percentage of contaminant lymphocytes was 20%-30%. Monocyte percentage was determined using morphology and peroxidase activity detection in cytospin preparations, height and granularity characteristics in light scattering, and in some samples

double staining with fluorescein isothiocyanate (FITC) and r-phycoerythrin (PE)–conjugated mouse anti-human monoclonal antibodies (mABs): CD14 (PE) and CD3 (FITC) . Monocytes were cultivated at 1 X 10^6 ml in 1ml RPMI 1640 medium (Sigma, Saint Louis, MO, USA) with 10% human AB serum, 2mM L-glutamine, 50μg/ml gentamicin and 15mM HEPES in 24 well plates in a 5% CO_2 incubator at 37°C. The monocytes were immediately infected (10:1 parasite/human cell ratio) or left to differentiate to macrophages for seven days, in this case, half of the cell culture medium was replenished with new complete medium each 48 hours. Macrophages were also infected at a 10:1 parasite/human cell ratio. Two hours after the infection the wells were gently washed with RPMI 1640 to remove non infective parasites. New medium was then replenished. At this time cells were also stimulated with LPS (10 ng/ml)(Sigma, Saint Louis, MO, USA) or recombinant human IFN-γ (Roussel-Uclaf, Romainville,France) (10-100 U/ml). After 48 hours in a 5% CO_2 incubator at 37°C cell free supernatant was collected and cells scrapped for flow cytometry analysis. On our hands 90% of the monocytes or macrophages were infected after 48 hours.

Flow cytometry analysis.

Surface markers were analyzed by direct immunofluorescence staining using the following FITC or PE–conjugated mouse anti-human monoclonal antibodies (mABs): CD11b (PE), CD54 (PE), CD80 (FITC), HLA-ABC (FITC), HLA-DR (PE),CD14 (PE),CD3 (FITC) purchased from PharMingen (San Diego,CA, USA).The biotin conjugated mouse anti-

human mABs CD49e, CD86 purchased from PharMingen (San Diego, CA, USA) were detected with indirect immunofluorescence using the biotin-streptavidin system. The mouse isotype controls $IgG_{1,K}$ (PE,FITC), $IgG_{2a,k}$ (PE) were also purchased from PharMingen (San Diego, CA, USA). Briefly, the scrapped cells were washed in cold PBS/BSA1%/N_3Na 0,1%. The cell pellet was suspended in PBS/Human serum (v: v) (200 µl; 5 X 10^6cells/ml) for 15 min at 4^0C. To 20 µl of this suspension were mixed a previous titrated amount of mABs and left 30 min at 4^0 C in the dark. The cell suspension was then washed in cold PBS. The cells for direct immunofluorescence were suspended in 400µl of cold PBS and ready to be analyzed. The pellets of cells labeled with biotin conjugated antibodies were suspended in 20µl of PBS and added 3µl of streptavidin-PE (Becton Dickinson, San Jose, CA.USA) for 15 min at 4^0C. These cell were then washed and suspended in 400µl of cold PBS. The fluorescence was analyzed by FACScan (Becton Dickinson, San Jose, CA.USA). Dead cells and residual lymphocytes were excluded according to their forward and side scatter characteristics. The mean fluorescence intensity of each experimental condition (MFI) was measured excluding the fluorescence of the respective isotype controls, and the streptavidin non-specific binding for biotin labeled antibodies. Five thousand events were analyzed for each sample using the Cell Quest software (Becton Dickinson, San Jose, CA.USA). Relative fluorescence (RF) was defined as the mean fluorescence intensity of each experimental condition (MFI) relative to the mean fluorescence intensity of its control (CT) considered as 100%, RF= MFI/CT X 100.

Cytokine detection.

TNF-α, IL-10 and IL-12 (p40) determination was done by commercial sandwich immunoassay (DuoSET ELISA; Genzime diagnostics- Cambridge, MA, USA) as manufacturer's instructions.

Statistical analysis.

Data were analyzed using de GraphPad Prism version 3.00 for Windows. Comparisons of multiple groups with control group were performed with one-way analysis of variance (ANOVA) using the Dunnet's post test and the comparison between previously selected pairs of groups using the Bonferroni's method. (GraphPad software, San Diego California USA). The level of significance adopted was $p< 0.05$.

RESULTS

Pattern of expression of surface molecules on infected cells.

With the exception of very low CD80 cell positivity (measured only on macrophages) the frequency of cell positivity to markers here measured was higher than ninety per cent in human monocytes and between sixty and ninety per cent in human macrophages(data not shown).. When human monocytes were infected by *L.(L.) chagasi* CD11b expression was diminished (mean R.F=63.35 $\pm$ 19.09 SD; $p<0.01$) (Figure 1,A and B). There was also a trend towards reduced expression of CD54 (eight out of nine blood donors) and increased expression of CD86 (four out of six blood donors) but the differences were not statistically significant. Infection of human macrophages by *L.(L.) chagasi* lead to a decreased expression HLA-DR (mean RF = 74.46 $\pm$ 25.08 SD; $p<0.05$) and an increased CD86

expression (mean RF = 120.2 ± 19.96 SD; $p<0.05$) (Figure 1, C and D). No changes were observed in the expression of CD54 (mean RF = 92.75), CD49e (mean RF = 101.9), HLA-ABC (mean RF = 97.03) or CD11b (mean R.F.=107.6) as shown in Figure 1C. Interestingly, upon infection there was a negative correlation between CD54 and CD 86 expression both on monocytes (Figure 2A, $r = -0.9222$, $p< 0.01$) and on macrophages (Figure 2B, $r = - 0.7478$, $p<0.05$).

Effect of infection on LPS-induced monocyte/macrophage stimulation.

Infection had a powerful depressant effect on LPS-induced CD54 expression of human monocytes ($p<0.001$) (Figure 3, A and B) and macrophages ($p<0.001$) Figure 3C. As shown in Figure 3 (C and D), *L.(L) chagasi* infection diminishes HLA-ABC and HLA-DR expression of LPS stimulated macrophages ($p<0.001$ for each pair). Infection did not change CD86 and CD49e expression of LPS stimulated macrophages or monocytes. Effect of LPS-stimulated HLA-ABC and HLA-DR expression was not evaluated in infected monocytes.

Effect of infection on IFNγ induced monocyte/macrophage stimulation.

As shown in Figure 4, addition of IFNγ to infected cells had a synergistic effect on CD86 expression on both monocytes (A and B) and macrophages (C and D) ($p<0.05$ and $p<0.001$ respectively). *L.(L.) chagasi* infection did not change the CD49e expression on IFNγ stimulated human macrophages or monocytes. Infection reduced the stimulatory action of IFNγ on CD54 expression of monocytes ($p<0.01$), (Figure 4A) but not on macrophages. Inhibitory effect of infection on IFNγ was also found on expression of HLA-ABC

($p<0.001$) and HLA-DR ($p<0.01$) on human macrophages (Figure 4C). Effect of infection on IFNγ stimulation was not measured for HLA-ABC and HLA-DR in human monocytes.

Cytokine production.

L (L) chagasi infection *per si* did not induce IL-10, IL-12 (p40) (data no shown for macrophages) or TNF-α production in either monocytes or macrophages, as shown in Figure 5A and B for TNF-α in monocytes and macrophages and in Figure 6A for IL-10 and Figure 6B for IL-12 (p40) in monocytes. Both cell populations were able to produce, TNF-α (Figure 5A and B), IL-10 or IL-12(p40) (Figure 6A and B) upon LPS stimulation (10ng/ml). Additionally *L.(L.) chagasi* infection did not diminish TNF-α production by LPS-stimulated human monocytes and macrophages (Figure 5A and B), or IL-10 in monocytes (not done on macrophages), but diminished IL-12 (p40) production by LPS-stimulated human monocytes (mean of 391.7 pg/ml ± 527.6 SD; n=5) (LPS vs LPS-infection ,$p<0.05$) (Figure 6B)(not done on macrophages).

DISCUSSION

A direct relationship between costimulatory molecules, particularly CD80 and CD86, and a Th1 or Th2 response in established leishmaniasis models is not clear (Murphy et al. 1997; Hunter & Reiner 2000). Blockage of CD86 reduced parasitism in BALB/c mice (with a predominant Th2 response), implicating CD86 in production of Th2 cytokines and disease induction (Brown et al. 1996; Tsuyuki et al. 1997). In contrast blocking CD86 in cultures of *L.(L.) major*-infected human macrophages plus peripheral blood lymphocytes

lead to a significant inhibition of IFN-γ production (Brodskyn et al. 2001), a classical Th1 cytokine. An apparent contrary result was also obtained in *L.(L.) donovani* model of visceral leishmaniasis where CD86 blocking increased IFN-γ production and reduced parasite burden (Murphy et al. 1997). A possible explanation for these results is that a more complex picture could be involved in driving lymphocytes to Th1 or Th2 response (Kim et al. 1999). Whereas CD80 or CD86 expression leads to high IL-4 and IL-10 production by naive CD4 T cells, co-expression of CD54 plus CD80 or CD 86 resulted in decreased IL-4 and IL-10 production (Luksch et al. 1999). Additionally, co-stimulation by CD80 and CD54, but not by CD80 or CD54 alone, leads to rapid TNF-α cytotoxicity, tumor rejection and generation of memory T cells (Nishio & Podack 1996). Moreover, both B7 and CD54 may co-regulate activation-driven maturation of T cells (Damle et al. 1992). We are reporting here for the first time that a parasite infection leads to increased CD86 expression simultaneously to decreased CD54 expression in both monocytes and macrophages. In human lepromatous leprosy, characterized by marked immunosupression, high CD54 with low B7 expression has been shown (Agrewala et al. 1998). It is possible that avoidance of simultaneous expression of these two molecules, and consequently optimal costimulatory activity (Damle et al. 1992; Kim et al. 1999; Camacho et al. 2001), is an escape mechanism exploited by *L.(L.) chagasi* and *Mycobacterium leprae*. On the other hand, *Trypanosoma cruzi* infected mouse macrophages, having upregulated CD86 expression levels and maintained high basal levels of CD54 expression, showed strong costimulatory activity towards the Th1 side of the immune response (Frosch et al. 1997). Reinforcing this point , a leishmania antigen, capable of up regulating both CD54

and B7 in human macrophages and monocyte derived dendritic cells, induced IL-12 production and Th1-type T cell response in PBMC (Probst et al. 1997). Noteworthy, IFN-γ treated infected macrophages were able to up-regulate CD54 and CD86 expression. Finally, previous clinical trials have shown successful IFN-γ treatment of human visceral leishmaniasis (Badaro & Johnson 1993).

CD54 (ICAM-1) is a multifunctional molecule with important functions in homotypic and heterotypic cell to cell adhesion, binding LFA-1 on lymphocytes or monocyte/macrophages and CD11b on monocytes and macrophages (Diamond et al. 1990; Dustin & Springer 1991). Indeed CD54 acts in T-cell costimulation (Kim et al. 1999) and granuloma formation (Ritter & McKerrow 1996). CD54 expression induces IFN-γ by co-stimulated T cells (Kim et al. 1999), conversely, as we show here, IFN-γ was able to increase CD54 expression on both monocytes and macrophages. *L.(L.) chagasi* infection had a tendency to decrease CD54 expression in monocytes besides down modulating CD54 expression in LPS or IFN-γ stimulated monocytes. Additionally, we report for the first time that CD11b has its expression diminished by *Leishmania* infected human monocytes but not macrophages, as recently shown for mice LFA-1 and MAC-1 α chains gene expression in *L.(L.) donovani* infected mouse macrophages (Buates & Matlashewski 2001). Thus the probably *L.(L.) chagasi* lowering of the strength of monocyte adhesion to lymphocyte, could impair IFN-γ production, increase the antigen dose required for T cell costimulation, and influence the Th1/ Th2 balance (Luksch et al. 1999). The parasite inhibition of CD11b expression could be a previously unreported escape mechanism, as CD11b has recently been shown to play a significant role in LPS induced IL-12 secretion, and CD11b

deficient macrophages had diminished nuclear translocation of NF-κB (nuclear factor κB) and reduced MAPK (mitogen-activated protein kinase) phosphorylation (Perera et al. 2001). In addition, CD11b has been shown to be involved in platelet-activating factor synthesis (PAF) by human monocytes (Elstad et al. 1994). Thus, diminished CD11b expression might interfere with production of essential host protecting agents, PAF (Lonardoni et al. 2000) and IL-12 (Reiner et al. 1994). Such a situation would be particularly relevant at the initial phases of *Leishmania* infection when monocytes are the first and most numerous parasited antigen presenting cells.

We as others have found that *Leishmania* infection does not induce TNF-α or IL-12 production in human monocytes (Reiner et al. 1990; Ghalib et al. 1995; Sartori et al. 1997) or macrophages but inhibits IL-12 production by LPS stimulated monocytes. Also, in agreement with previous studies using healthy human PBMC (Ghalib et al. 1993; Sartori et al. 1997) instead of visceral leishmaniasis patients PBMC (Ghalib et al. 1993), *Leishmania* infection did not induce IL-10 release in human monocytes or macrophages. The binding of the parasite to its receptor (CD11b), and the low CD11b expression on human monocytes, could explain most of these findings as it has been shown that binding to human CD11b, or CD11b deficiency, inhibits LPS-induced IL-12 production with unchanged levels of TNF-α (Marth & Kelsall 1997; Perera et al. 2001) or IL-10 (Marth & Kelsall 1997). This may seem surprising as in human disease there is high IL-10 (Gasim et al. 1998) and TNF-α (Barral-Netto et al. 1991) serum levels, and both TNF-α and IL-10 explain most of the aspects of active disease as immune suppression (Carvalho

et al. 1994) cachexia and fever (Barral-Netto et al. 1991). On the other hand, infected monocytes or macrophages, under LPS stimulation, are able to produce similar amounts of TNF-α or IL-10 to their uninfected controls. Thus in the evolution to active disease other factors as expansion of IL-10 producing T cell clones, ingestion of IgG-opsonized amastigotes (Kane & Mosser 2001), the frequent bacterial infections patients have (Andrade et al. 1990) or immune complex mediated cell activation (Carvalho et al. 1983; Crawford et al. 1985) could explain TNF-α and IL-10 production. Is also possible that IL-10 act as a counter regulatory mechanism induced by TNF-α production (Wanidworanun & Strober 1993). Both absence of IL-12 and high IL-10 are main features linked to visceral leishmaniasis, and its probably not by chance that the parasite, even before being phagocitozed, starts inhibiting IL-12 secretion. IL-12 is a main driving Th1 cytokine at initial phases of leishmania infection and able to restore *in vitro* patients visceral leishmaniasis PBMC Th1 responses to leishmania antigens, notwithstanding the IL-10 presence (Ghalib et al. 1995).

Human visceral leishmaniasis has an incubation period of months or even years (Pearson & Sousa 1996). In models of leishmaniasis simulating natural infection there is also a long silent phase of the infection lasting for 6 to 8 weeks (Almeida et al. 1993; Belkaid et al. 2000; Lira et al. 2000) when parasite growth is undisturbed by the host immune system without inducing pathology (Belkaid et al. 2000). Indeed in both cutaneous and visceral human infections subclinical infections have been described (Pearson & Sousa 1996). The data presented here is in agreement with these observations as *L.(L.) chagasi*

fails to induce TNF-α production and impairs IL-12 production besides decreasing CD54 expression, all important proinflammatory molecules (Camacho et al. 2001).

Additionally, such an infection has down modulatory effects on LPS or IFN-γ stimulation. The evasive behavior of *L.(L.) chagasi* infection in human phagocytic cells also includes lack of CD11b and CD80 induction in human macrophages (data not shown) and down modulation of CD11b on monocytes. Noteworthy, CD18 deficient mice macrophages produce less NO and have impaired leishmanicidal activity (Schonlau et al. 2000). It is tempting to speculate that depressed expression of class I and II molecules, absence of key proinflammatory cytokines and not fully expression of costimulatory molecules in monocytes or macrophages conceal *Leishmania* leaving the immune system, at least in initial phases, in anergy (Kaye 1995) or ignorance (de Almeida 2002). This ignorance may probably be broken when, due to parasite multiplication, the parasite or its antigens reach other cell, as dendritic cells, since in such cells these down regulatory events are not operative (Gorak et al. 1998; von Stebut et al. 1998; Marovich et al. 2000).

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FIGURE LEGENDS

Figure 1. Mean fluorescence intensity (MFI) of cell surface molecules relative to uninfected control (CT), R.F.=(MFI/CT) x100. Human monocytes (A) and macrophages (C) were infected with *L.(L) chagasi*. The broken line shows the control level. (A) *L.(L.) chagasi infection* decrease CD11b expression on human monocytes, $p<0.01$. (C) CD86 expression is increased on infected macrophages, ($p<0.05$) and HLA-DR expression decreased, ($p<0.05$). Representative histograms are shown for monocytes, CD11b (B), and macrophages, CD86 (D): infected cells (INFEC), uninfected cells (CT), isotype control (ISO) and streptavidin-PE (STREP). Error bar means one standard deviation.

Figure 2. Correlation between Mean fluorescence intensity (MFI) of CD54 and CD86 relative to uninfected control (CT), R.F.=(MFI/CT) x100. Human monocytes (A) and macrophages (B) were infected with *L(L) chagasi* as described in the text. r = correlation coefficient, $p<0.01$.

Figure 3. Mean fluorescence intensity (MFI) of cell surface molecules relative to uninfected control (CT), R.F.=(MFI/CT) x100. R.F. of infected monocytes (A) or macrophages (C) (hatched bars) stimulated with LPS 10 ng/ml for 48 hours were compared to LPS alone (black bars). See details in text. The broken line shows the control level (cells without stimulation). (A) Infection counteracts the stimulatory effect of LPS on CD54 expression of human monocytes, $p<0.001$. Each bar represents the mean of four

(CD86), five (CD49e) and six experiments (CD54). (C) Infection counteracts the stimulatory effect of LPS on CD54 ($p<0.001$), HLA-ABC ($p<0.001$) and HLA-DR ($p<0.001$) expression of human macrophages. Each bar represents the mean of three (CD49e), six (CD 54,HLA-ABC and HLA-DR) and eight experiments (CD86).). Representative histograms are shown for monocytes, CD54 (B), and macrophges, HLA-DR (D): infected cells (INFEC), LPS stimulation alone (LPS), LPS plus infection (L+I), and isotype control (ISO).). Error bar means one standard deviation.

Figure 4. Mean fluorescence intensity (MFI) of cell surface molecules relative to uninfected control (CT), R.F.=(MFI/CT) x100. R.F. of infected monocytes (A) or macrophages (C) (hatched bars) stimulated with 100U/ml of IFNγ for 48 hours were compared with IFNγ stimulation alone (black bars). See details in text. The broken line shows the control level (cells without stimulation). (A) Infection counteracts the stimulatory effect of IFNγ on CD54 expression of human monocytes, $p<0.01$, and primes human monocytes for IFNγ stimulation of CD86, $p<0.05$. Each bar represents the mean of four (CD86), five (CD49e) and six experiments (CD54). (C) Infection counteracts the stimulatory effect of IFNγ on HLA-ABC and HLA-DR expression of human macrophages, $p<0.001$and $p<0.01$ respectively. Primes human macrophages for IFNγ stimulation of CD 86, $p<0.001$. Each bar represents the mean of three (CD49e), six (CD 54,HLA-ABC and HLA-DR) and eight experiments (CD86).. Representative histograms are shown for monocytes, CD86 (B), and macrophages, CD86 (D): IFNγ stimulation alone (IFN), IFNγ stimulation plus infection (I+I), and streptavidin-PE (STREP). Error bar means one standard deviation.

Figure 5. TNFα levels in culture supernatants of uninfected and infected *L.(L.) chagasi* human monocytes (A) and macrophages (B) under several conditions. CT: uninfected cells. INFEC: infected cells. LPS: LPS stimulation alone (10ng/ml). L+I: LPS (10ng/ml) stimulation after 2 hours of infection. IFN: IFNγ (10-100 U) stimulation alone. I+I: IFNγ (10-100 U) stimulation after 2 hours of infection.

Figure 6. IL-10 (A) and IL-12 (B) levels in culture supernatants of uninfected and infected *L.(L.) chagasi* human monocytes (A) and (B) under several conditions. CT: uninfected cells. INFEC: infected cells. LPS: LPS stimulation alone (10ng/ml). L+I: LPS (10ng/ml) stimulation after 2 hours of infection. IFN: IFNγ (10-100 U) stimulation alone. I+I: IFNγ (10-100 U) stimulation after 2 hours of infection.(B) Infection diminished IL-12 (p40) production by LPS-stimulated human monocytes (LPS vs LPS-infection ,$p<0.05$).

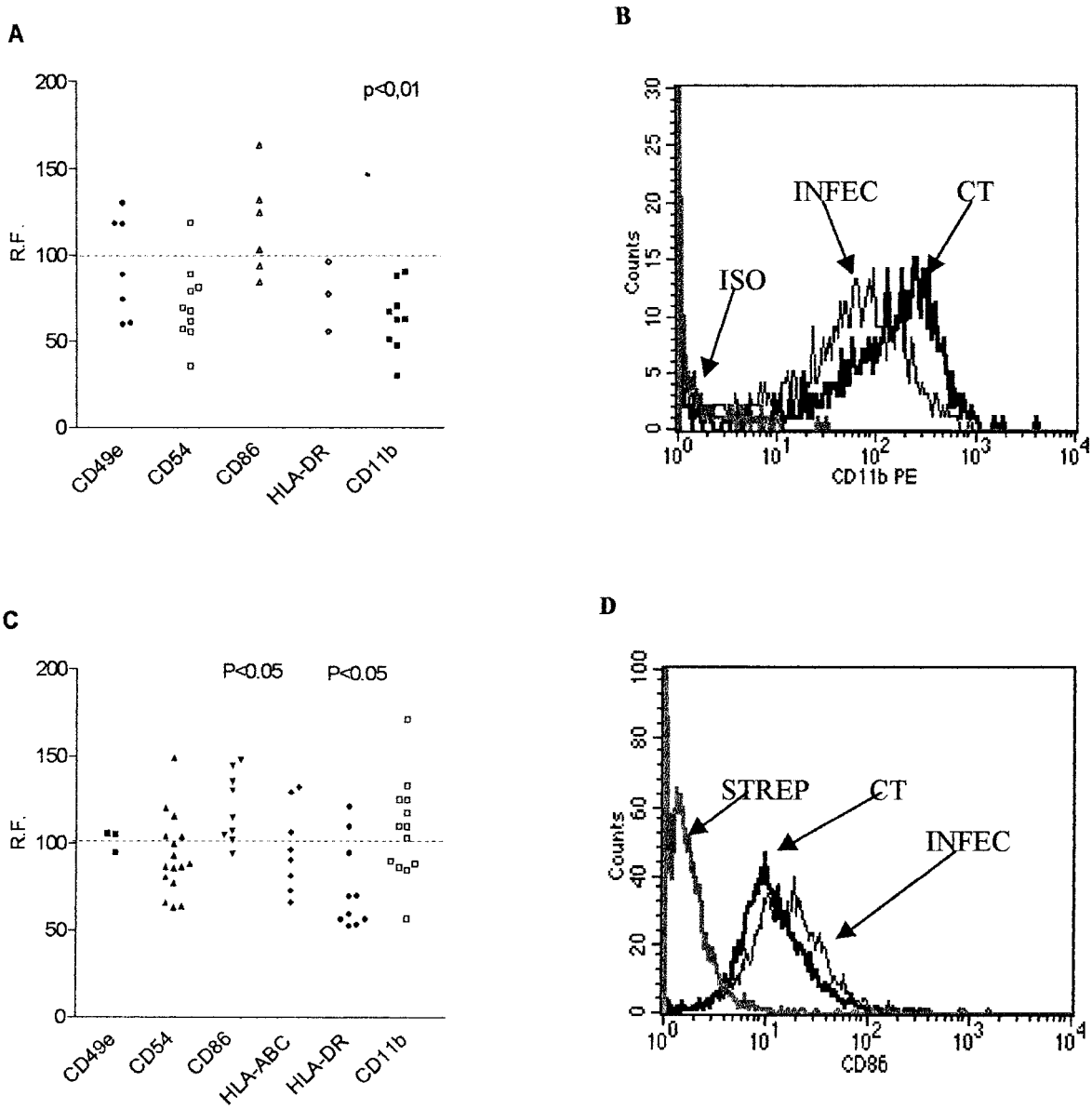

Figure 1.
Marcos Célio de Almeida and Manoel Barral Netto

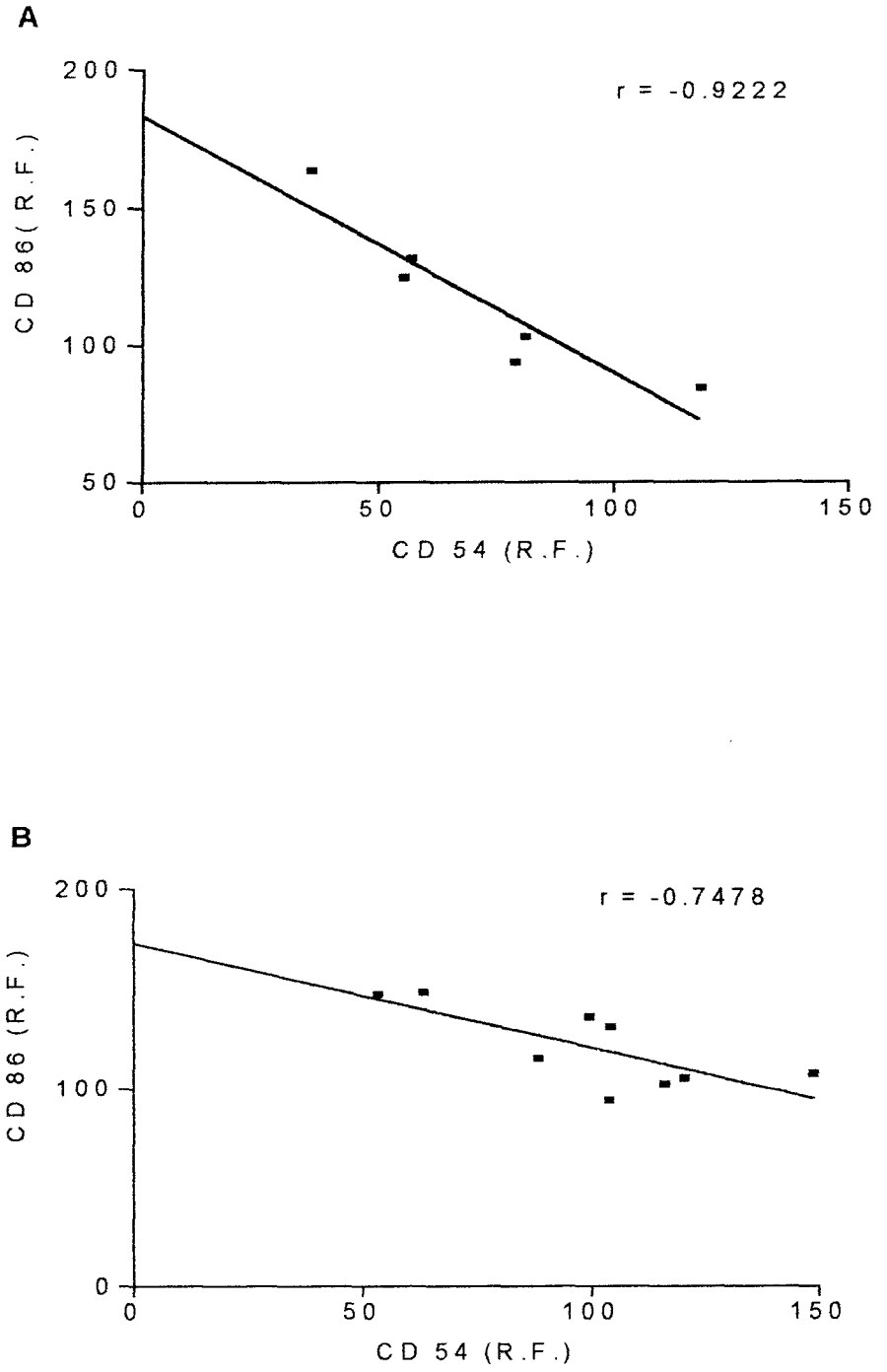

Figure 2.

Marcos Célio de Almeida and Manoel Barral Netto

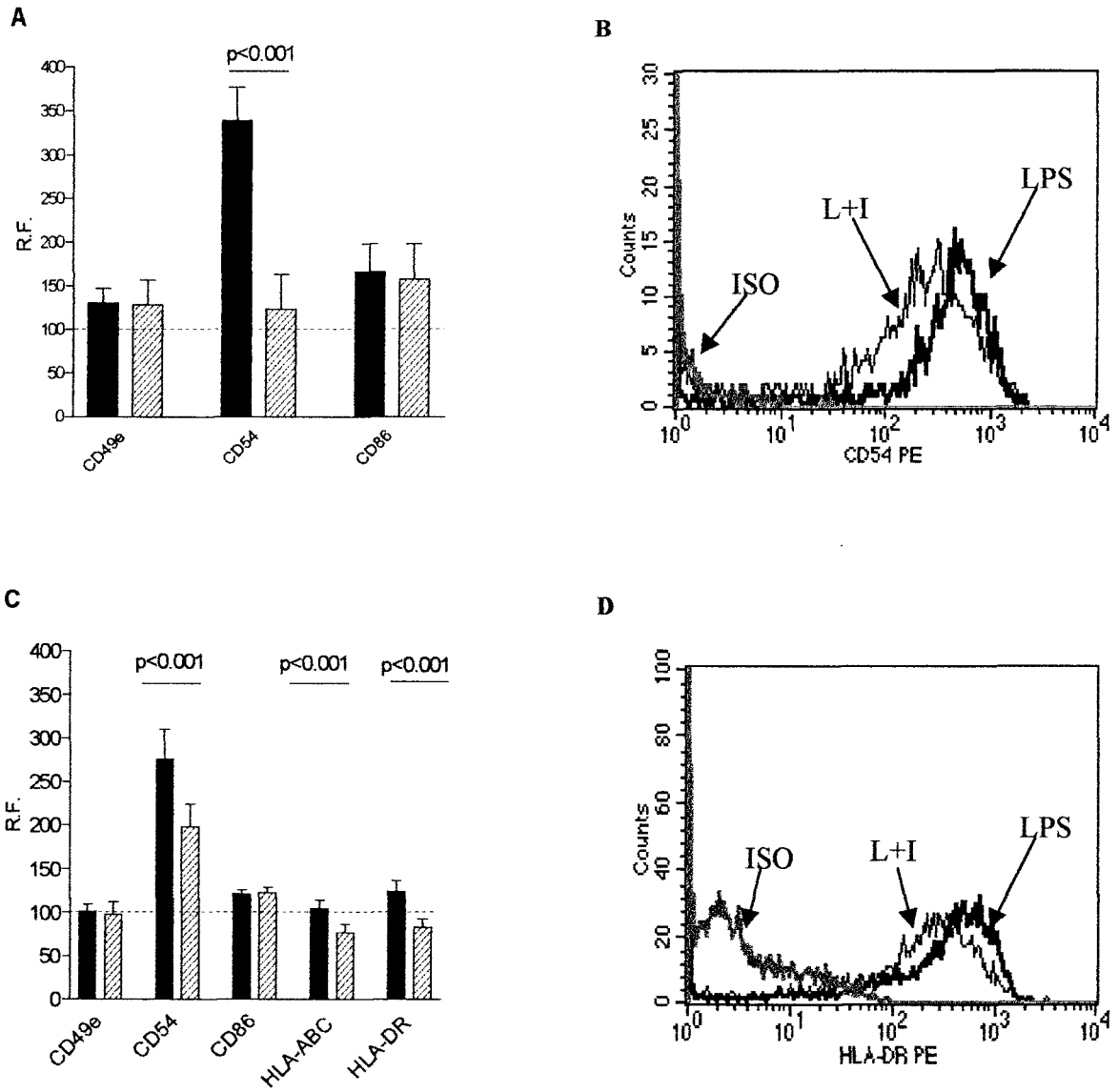

Figure 3.

Marcos Célio de Almeida and Manoel Barral Netto

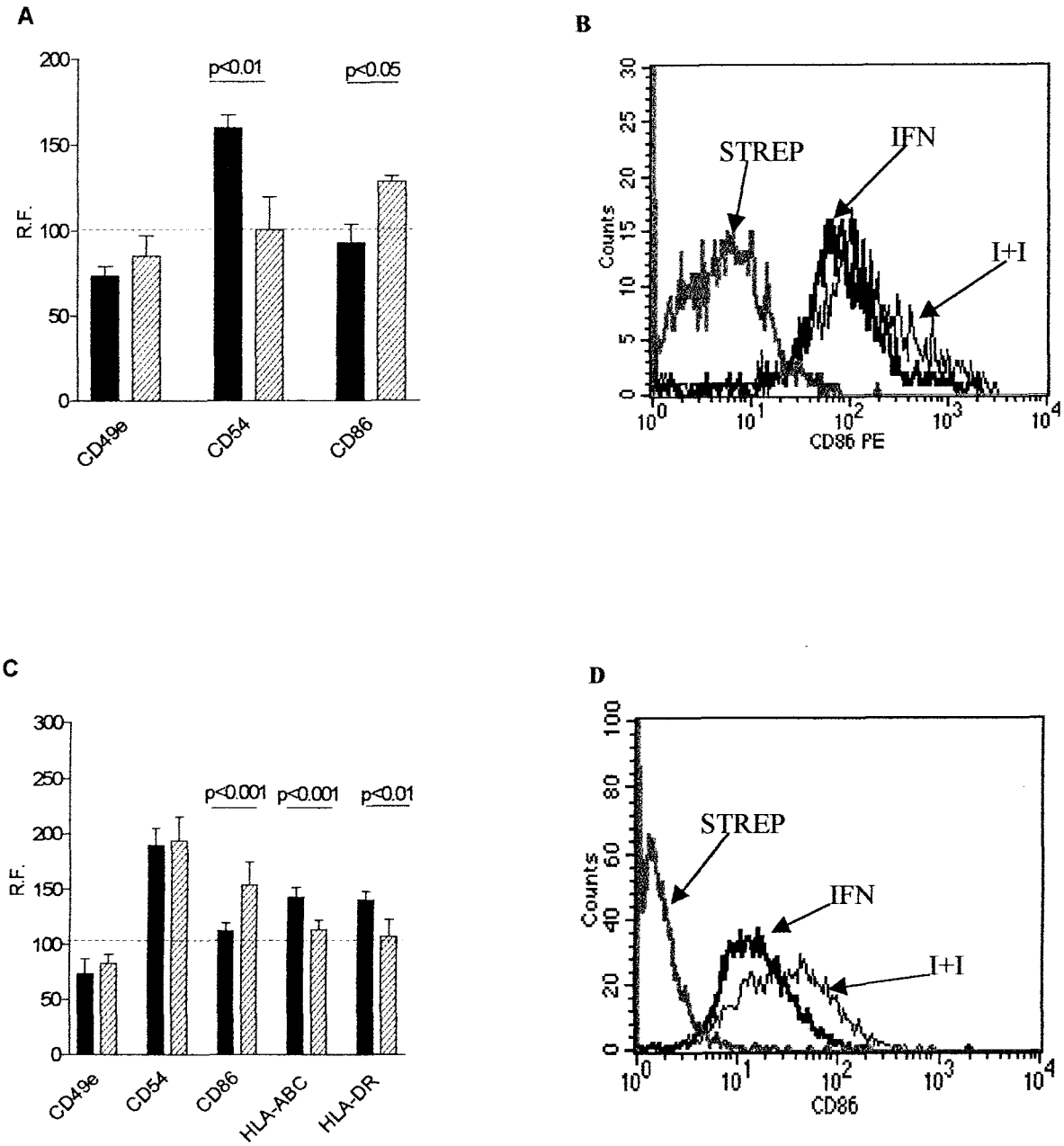

Figure 4.

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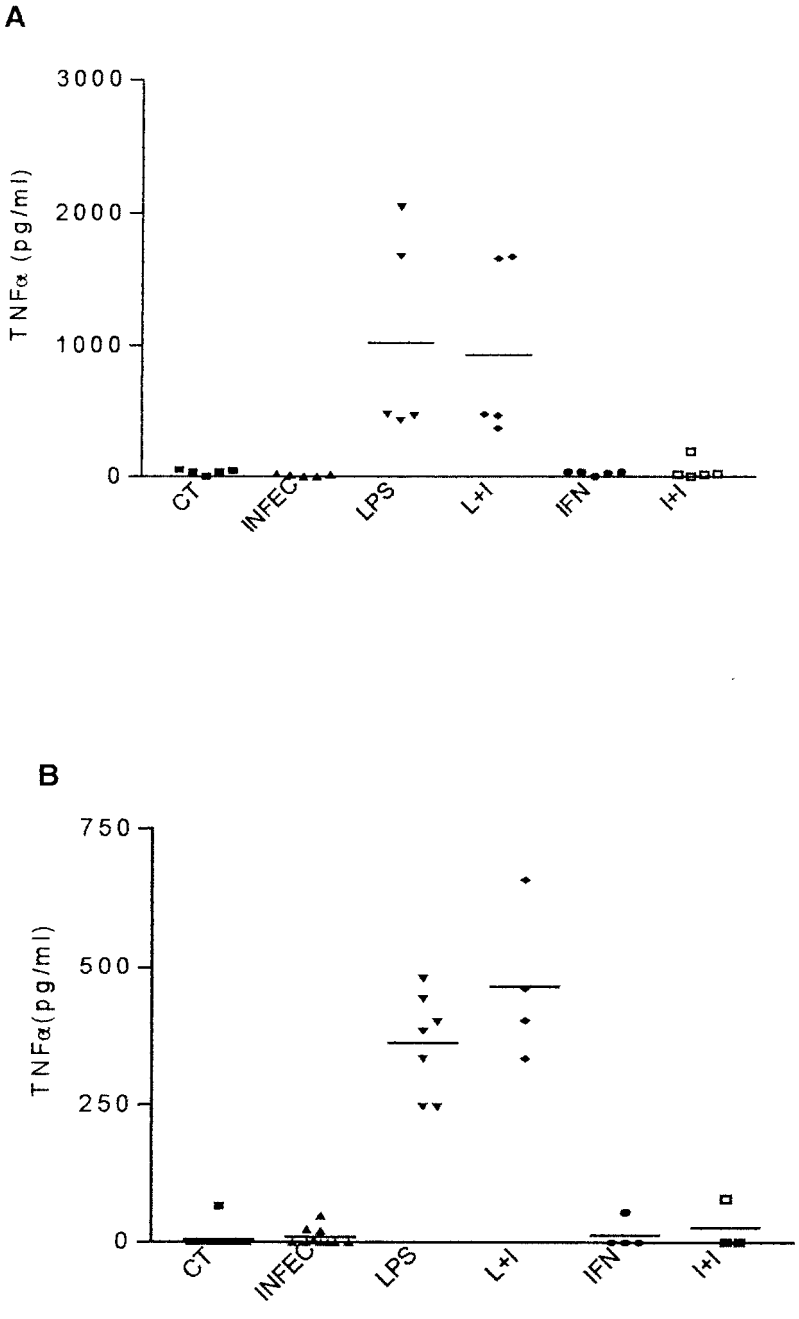


Figure 5.

Marcos Célio de Almeida and Manoel Barral Netto

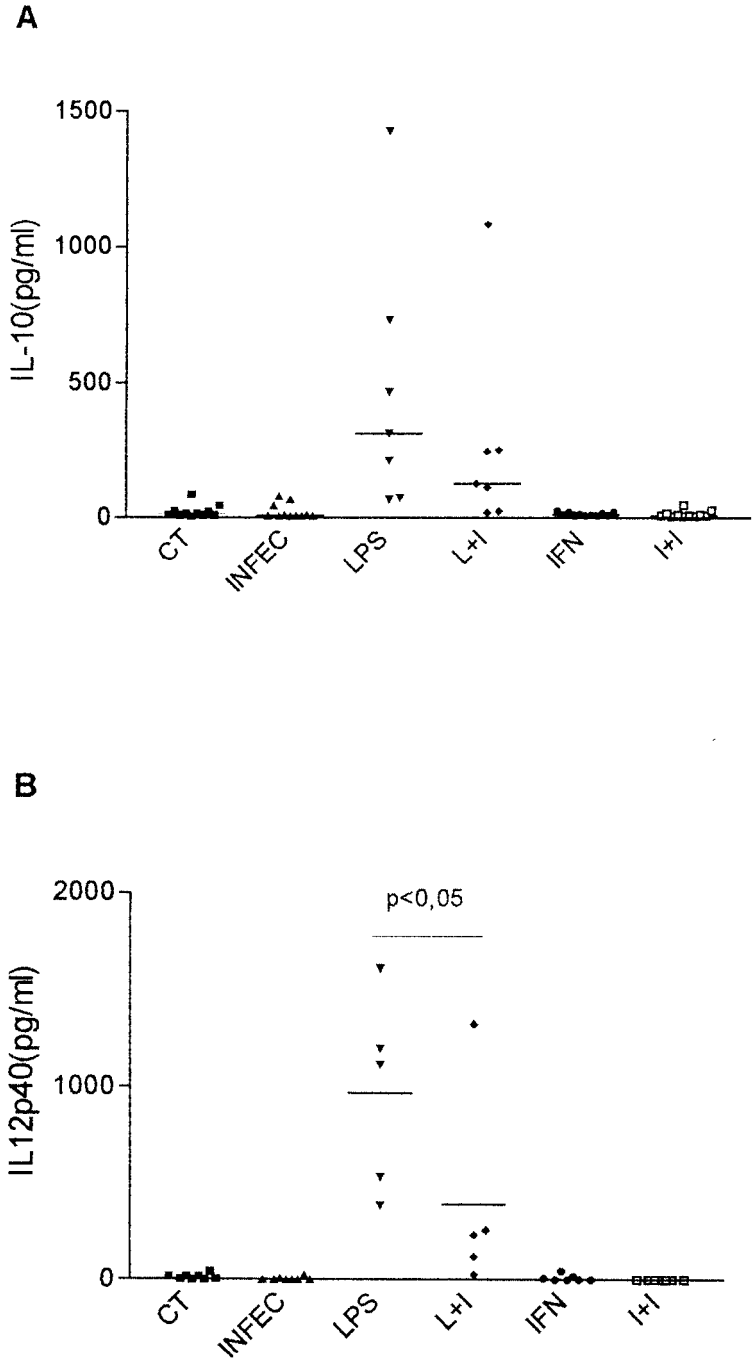


Figure 6.

Marcos Célio de Almeida and Manoel Barral Netto

1 REFERENCES

2

3 Agrewala, J. N.; Kumar, B. and Vohra, H., 1998. Potential role of B7-1 and CD28
4 molecules in immunosuppression in leprosy. Clin. Exp. Immunol., 111: 56-63.

5

6 Almeida, M. C.; Cuba, C. A.; de Sa, C. M.; Pharoah, M. M.; Howard, K. M. and Miles, M.
7 A., 1993. Metacyclogenesis of Leishmania (Viannia) braziliensis in vitro: evidence that
8 lentil lectin is a marker of complement resistance and enhanced infectivity. Trans. R. Soc.
9 Trop. Med. Hyg., 87: 325-9.

10

11 Andrade, T. M.; Carvalho, E. M. and Rocha, H., 1990. Bacterial infections in patients with
12 visceral leishmaniasis. J. Infect. Dis., 162: 1354-9.

13

14 Badaro, R. and Johnson, W. D., Jr., 1993. The role of interferon-gamma in the treatment of
15 visceral and diffuse cutaneous leishmaniasis. J. Infect. Dis., 167 Suppl. 1: S13-7.

16

17 Barral-Netto, M.; Badaro, R.; Barral, A.; Almeida, R. P.; Santos, S. B.; Badaro, F.; Pedral-
18 Sampaio, D.; Carvalho, E. M.; Falcoff, E. and Falcoff, R., 1991. Tumor necrosis factor
19 (cachectin) in human visceral leishmaniasis. J. Infect. Dis., 163: 853-7.

20

21 Belkaid, Y.; Mendez, S.; Lira, R.; Kadambi, N.; Milon, G. and Sacks, D., 2000. A natural
22 model of Leishmania major infection reveals a prolonged "silent" phase of
23 parasite amplification in the skin before the onset of lesion formation and immunity. J.
24 Immunol., 165: 969-77.

Brodskyn, C. I.; DeKrey, G. K. and Titus, R. G., 2001. Influence of costimulatory molecules on immune response to Leishmania major by human cells in vitro. Infect. Immun., 69: 665-72.

Brown, J. A.; Titus, R. G.; Nabavi, N. and Glimcher, L. H., 1996. Blockade of CD86 ameliorates Leishmania major infection by down-regulating the Th2 response. J. Infect. Dis., 174: 1303-8.

Buates, S. and Matlashewski, G., 2001. General suppression of macrophage gene expression during Leishmania donovani infection. J. Immunol., 166: 3416-22.

Camacho, S. A.; Heath, W. R.; Carbone, F. R.; Sarvetnick, N.; LeBon, A.; Karlsson, L.; Peterson, P. A. and Webb, S. R., 2001. A key role for ICAM-1 in generating effector cells mediating inflammatory responses. Nat. Immunol., 2: 523-9.

Carvalho, E. M.; Andrews, B. S.; Martinelli, R.; Dutra, M. and Rocha, H., 1983. Circulating immune complexes and rheumatoid factor in schistosomiasis and visceral leishmaniasis. Am. J. Trop. Med. Hyg., 32: 61-8.

Carvalho, E. M.; Bacellar, O.; Brownell, C.; Regis, T.; Coffman, R. L. and Reed, S. G., 1994. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. J. Immunol., 152: 5949-56.

Cervia, J. S.; Rosen, H. and Murray, H. W., 1993. Effector role of blood monocytes in experimental visceral leishmaniasis. Infect. Immun., 61: 1330-3.

Chang, K. P., 1981. Leishmanicidal mechanisms of human polymorphonuclear phagocytes. Am. J. Trop. Med. Hyg., 30: 322-33.

Crawford, G. D.; Wyler, D. J. and Dinarello, C. A., 1985. Parasite-monocyte interactions in human leishmaniasis: production of interleukin-1 in vitro. J. Infect. Dis., 152: 315-22.

Damle, N. K.; Klussman, K.; Linsley, P. S.; Aruffo, A. and Ledbetter, J. A., 1992. Differential regulatory effects of intercellular adhesion molecule-1 on costimulation by the CD28 counter-receptor B7. J. Immunol., 149: 2541-8.

de Almeida, M. C., 2002. Infective inoculum for *Leishmania*. Trends Parasitol., 18: 154-155.

de Almeida, M. C.; Silva, A. C.; Barral, A. and Barral Netto, M., 2000. A simple method for human peripheral blood monocyte isolation. Mem. Inst. Oswaldo Cruz, 95: 221-3.

Diamond, M. S.; Staunton, D. E.; de Fougerolles, A. R.; Stacker, S. A.; Garcia-Aguilar, J.; Hibbs, M. L. and Springer, T. A., 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). J. Cell Biol., 111: 3129-39.

Dustin, M. L. and Springer, T. A., 1991. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Annu. Rev. Immunol., 9: 27-66.

Elstad, M. R.; Parker, C. J.; Cowley, F. S.; Wilcox, L. A.; McIntyre, T. M.; Prescott, S. M. and Zimmerman, G. A., 1994. CD11b/CD18 integrin and a beta-glucan receptor act in concert to induce the synthesis of platelet-activating factor by monocytes. J. Immunol., 152: 220-30.

Frosch, S.; Kuntzlin, D. and Fleischer, B., 1997. Infection with Trypanosoma cruzi selectively upregulates B7-2 molecules on macrophages and enhances their costimulatory activity. Infect. Immun., 65: 971-7.

Gasim, S.; Elhassan, A. M.; Khalil, E. A.; Ismail, A.; Kadaru, A. M.; Kharazmi, A. and Theander, T. G., 1998. High levels of plasma IL-10 and expression of IL-10 by keratinocytes during visceral leishmaniasis predict subsequent development of post-kala-azar dermal leishmaniasis. Clin. Exp. Immunol., 111: 64-9.

Ghalib, H. W.; Piuvezam, M. R.; Skeiky, Y. A.; Siddig, M.; Hashim, F. A.; el-Hassan, A. M.; Russo, D. M. and Reed, S. G., 1993. Interleukin 10 production correlates with pathology in human Leishmania donovani infections. J. Clin. Invest., 92: 324-9.

Ghalib, H. W.; Whittle, J. A.; Kubin, M.; Hashim, F. A.; el-Hassan, A. M.; Grabstein, K. H.; Trinchieri, G. and Reed, S. G., 1995. IL-12 enhances Th1-type responses in human Leishmania donovani infections. J. Immunol., 154: 4623-9.

Gorak, P. M.; Engwerda, C. R. and Kaye, P. M., 1998. Dendritic cells, but not macrophages, produce IL-12 immediately following Leishmania donovani infection. Eur. J. Immunol., 28: 687-95.

Hunter, C. A. and Reiner, S. L., 2000. Cytokines and T cells in host defense. Curr. Opin. Immunol., 12: 413-8.

Kane, M. M. and Mosser, D. M., 2001. The Role of IL-10 in Promoting Disease Progression in Leishmaniasis. J. Immunol., 166: 1141-1147.

Kaye, P. M., 1995. Costimulation and the regulation of antimicrobial immunity. Immunol. Today, 16: 423-7.

Kaye, P. M.; Rogers, N. J.; Curry, A. J. and Scott, J. C., 1994. Deficient expression of co-stimulatory molecules on Leishmania-infected macrophages. Eur. J. Immunol., 24: 2850-4.

Kim, J. J.; Tsai, A.; Nottingham, L. K.; Morrison, L.; Cunning, D. M.; Oh, J.; Lee, D. J.; Dang, K.; Dentchev, T.; Chalian, A. A.; Agadjanyan, M. G. and Weiner, D. B., 1999. Intracellular adhesion molecule-1 modulates beta-chemokines and directly costimulates T cells in vivo. J. Clin. Invest., 103: 869-77.

Lira, R.; Doherty, M.; Modi, G. and Sacks, D., 2000. Evolution of lesion formation, parasitic load, immune response, and reservoir potential in C57BL/6 mice following high- and low-dose challenge with Leishmania major. Infect. Immun., 68: 5176-82.

Lonardoni, M. V.; Russo, M. and Jancar, S., 2000. Essential role of platelet-activating factor in control of Leishmania (Leishmania) amazonensis infection. Infect. Immun., 68: 6355-61.

Luksch, C. R.; Winqvist, O.; Ozaki, M. E.; Karlsson, L.; Jackson, M. R.; Peterson, P. A. and Webb, S. R., 1999. Intercellular adhesion molecule-1 inhibits interleukin 4 production by naive T cells. Proc. Natl. Acad. Sci. U S A, 96: 3023-8.

Marovich, M. A.; McDowell, M. A.; Thomas, E. K. and Nutman, T. B., 2000. IL-12p70 production by Leishmania major-harboring human dendritic cells is a CD40/CD40 ligand-dependent process. J. Immunol., 164: 5858-65.

Marth, T. and Kelsall, B. L., 1997. Regulation of interleukin-12 by complement receptor 3 signaling. J. Exp. Med., 185: 1987-95.

Milon, G.; Del Giudice, G. and Louis, J. A., 1995. Immunobiology of Experimental Cutaneous Leishmaniasis. Parasitol. Today, 11: 244-247.

Murphy, M. L.; Engwerda, C. R.; Gorak, P. M. and Kaye, P. M., 1997. B7-2 blockade enhances T cell responses to Leishmania donovani. J. Immunol., 159: 4460-6.

Nishio, M. and Podack, E. R., 1996. Rapid induction of tumor necrosis factor cytotoxicity in naive splenic T cells by simultaneous CD80 (B7.1) and CD54 (ICAM-1) co-stimulation. Eur. J. Immunol., 26: 2160-4.

Pearson, R. D. and Sousa, A. Q., 1996. Clinical spectrum of Leishmaniasis. Clin. Infect. Dis., 22: 1-13.

Perera, P. Y.; Mayadas, T. N.; Takeuchi, O.; Akira, S.; Zaks-Zilberman, M.; Goyert, S. M. and Vogel, S. N., 2001. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. J. Immunol., 166: 574-81.

Probst, P.; Skeiky, Y. A.; Steeves, M.; Gervassi, A.; Grabstein, K. H. and Reed, S. G., 1997. A Leishmania protein that modulates interleukin (IL)-12, IL-10 and tumor necrosis factor-alpha production and expression of B7-1 in human monocyte-derived antigen-presenting cells. Eur. J. Immunol., 27: 2634-42.

Reiner, N. E., 1994. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. Immunol. Today, 15: 374-81.

Reiner, N. E.; Ng, W. and McMaster, W. R., 1987. Parasite-accessory cell interactions in murine leishmaniasis. II. Leishmania donovani suppresses macrophage expression of class I and class II major histocompatibility complex gene products. J. Immunol., 138: 1926-32.

Reiner, N. E.; Ng, W.; Wilson, C. B.; McMaster, W. R. and Burchett, S. K., 1990. Modulation of in vitro monocyte cytokine responses to Leishmania donovani. Interferon-gamma prevents parasite-induced inhibition of interleukin 1 production and primes

monocytes to respond to Leishmania by producing both tumor necrosis factor-alpha and interleukin 1. J. Clin. Invest., 85: 1914-24.

Reiner, S. L. and Locksley, R. M., 1995. The regulation of immunity to Leishmania major. Annu. Ver. Immunol., 13: 151-77.

Reiner, S. L.; Zheng, S.; Wang, Z. E.; Stowring, L. and Locksley, R. M., 1994. Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. J. Exp. Med., 179: 447-56.

Ribeiro, J. M. C., 1987. Role of saliva in blood-feeding by arthropods. Annu. Ver. Entomol., 32: 463-478.

Ritter, D. M. and McKerrow, J. H., 1996. Intercellular adhesion molecule 1 is the major adhesion molecule expressed during schistosome granuloma formation. Infect. Immun., 64: 4706-13.

Saha, B.; Das, G.; Vohra, H.; Ganguly, N. K. and Mishra, G. C., 1995. Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on Leishmania-infected macrophages and its implication in the suppression of cell-mediated immunity. Eur. J. Immunol., 25: 2492-8.

Sartori, A.; Oliveira, M. A.; Scott, P. and Trinchieri, G., 1997. Metacyclogenesis modulates the ability of Leishmania promastigotes to induce IL-12 production in human mononuclear cells. J. Immunol., 159: 2849-57.

Schonlau, F.; Scharffetter-Kochanek, K.; Grabbe, S.; Pietz, B.; Sorg, C. and Sunderkotter, C., 2000. In experimental leishmaniasis deficiency of CD18 results in parasite dissemination associated with altered macrophage functions and incomplete Th1 cell response. Eur. J. Immunol., 30: 2729-40.

Tsuyuki, S.; Tsuyuki, J.; Einsle, K.; Kopf, M. and Coyle, A. J., 1997. Costimulation through B7-2 (CD86) is required for the induction of a lung mucosal T helper cell 2 (TH2) immune response and altered airway responsiveness. J. Exp. Med., 185: 1671-9.

Urmacher, C. D. , 1997. Normal skin. In:Stenberg, S. S. (Ed.), Histology for pathologists. Lippincott-Raven, Philadelphia ,pp. 25-45.

von Stebut, E.; Belkaid, Y.; Jakob, T.; Sacks, D. L. and Udey, M. C., 1998. Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity. J. Exp. Med., 188: 1547-52.

Wahl, L. M. and Smith, P. D. , 1994. Isolation of monocyte/macrophage populations. In:Coligan, J. E., Kruibeek, A. M., Margulies, D. H., Shevach, E. and Strober, W. (Ed.), Current Protocols in Immunology. John Wiley &Sons,Inc, New York ,pp. 7.6.1-7.6.8.

Wanidworanun, C. and Strober, W., 1993. Predominant role of tumor necrosis factor-alpha in human monocyte IL-10 synthesis. J. Immunol., 151: 6853-61.

3 DISCUSSÃO GERAL

3.1 Estratégia geral do parasita na fase inicial da infecção

É uma lei geral dentro da microbiologia: quanto maior a dose do agente infeccioso maior a chance de se estabelecer a infecção, já que um pequeno número de parasitas pode ser eliminado antes que estabeleça a infecção. Paradoxalmente, a infecção natural pelas *Leishmania* spp parece não utilizar essa lei - o flebotomíneo inocula cerca de 100 formas metacíclicas (WARBURG & SCHLEIN 1986). Uma série de fatores no entanto permite ao parasita penetrar na célula hospedeira e estabelecer a infecção. Em primeiro lugar, metacíclicos têm maior resistência à lise pelo sistema complemento (SACKS 1989). Metacíclicos de *L(L.). major* não somente são mais resistentes à lise pelo sistema complemento como também interagem com esse sistema de maneira diferente em relação às formas procíclicas (PUENTES et al. 1990). As formas procíclicas ativam a via alternativa do sistema complemento enquanto as formas metacíclicas ativam a via clássica, mesmo na ausência de soro imune, sendo as metacíclicas fracas ativadoras da via alternativa. Na superfície celular de metacíclicos de *L.(L.) major*, surpreendentemente, o componente C3 é depositado na sua forma ativa, hemolítica, C3b. Uma lipofosfoglicana (LPG) do parasita se liga a essa fração do sistema complemento (PUENTES et al. 1988). Nesses parasitas, essa molécula é modificada na sua composição de açúcares, simultaneamente com um aumento do número de suas subunidades (PIMENTA et al. 1992). Esse aumento de subunidades corresponde estruturalmente a uma maior espessura da membrana celular das formas metacíclicas, que impede o complexo de ataque à

membrana C5-9 atingir o citoplasma, e formar o poro molecular que levaria à lise (PIMENTA et al. 1991). O parasita será assim mais facilmente fagocitado, sem ser lisado. Recentemente, mostrou-se ainda que mecanismos de imunoaderência mediados por anticorpos naturais do tipo IgM permitiriam, devido à sua cinética extremamente rápida, a fagocitose antes que o complemento provocasse a lise de todos parasitas (DOMINGUEZ & TORANO 1999). Também mecanismos de conversão de C3b - C3bi estariam operando no CR1, receptor tipo I do complemento, em eritrócitos, ou mesmo através da gp63, metaloproteinase abundante na superfície do parasita (BRITTINGHAM et al. 1995; DOMINGUEZ & TORANO 1999). Dessa maneira, mesmo em número mínimo, o parasita poderá se estabelecer no interior da célula hospedeira. Inoculado, o parasita, devido às condições iniciais, se encontrará em uma densidade parasitária extremamente baixa, 0,03-0,1 parasita por célula fagocítica (DE ALMEIDA. 2002). Uma vez estabelecida a infecção, longe de constituir uma desvantagem a baixa densidade parasitária poderá permitir `as *Leishmania* spp passarem desapercebidas ao sistema imune, e criar um ambiente menos hostil à sua sobrevivência

Nós mostramos ainda que no interior do monócito/macrófago *L.(L.) chagasi* provoca vários efeitos modulatórios negativos e não induz uma série de moléculas proinflamatórias. A infecção não induziu CD54, IL-12 ou TNF-α, potentes moléculas proinflamatórias (SUTTERWALA & MOSSER 1999; CAMACHO et al. 2001). Modulou negativamente CD11b em monócitos, modulou negativamente a ação estimulatória do LPS na expressão de CD54, IL-12 (p40) e HLA-DR assim como a a ação estimulatória do IFN-γ sobre a expressão do HLA-DR e do HLA-ABC em macrófagos humanos. Não trata-se aqui de uma depressão generalizada das funções celulares. Na verdade, a infecção induziu um

aumento da expressão do CD86 nos monócitos e macrófagos. Observou-se ainda uma correlação negativa na expressão de CD54 e CD86 tanto em monócitos como em macrófagos. Além disso, a expressão de CD49e e a produção tanto do TNF-α como da IL-10 induzidas por LPS ficaram intactas.

É importante notar que os efeitos modulatórios negativos foram mais freqüentes em monócitos que macrófagos. Isto pode ser relevante, principalmente na fase inicial da infecção, quando o monócito é a célula inicialmente infectada. Trabalho recente em macrófagos de camundongos mostrou que cerca de 40 % dos genes estudados são regulados negativamente durante a infecção por *L(L.) donovani* (BUATES & MATLASHEWSKI 2001), incluindo genes para fatores de transcrição da família NFκβ/Rel que regulam genes ligados à resposta imune e inflamação. A maioria dos genes que exibiram uma regulação negativa a tiveram em baixos níveis (~2), com poucos genes apresentando sua expressão aumentada (BUATES & MATLASHEWSKI 2001). Tal aspecto se correlaciona com nossos dados. A infecção *per se,* nos nossos experimentos, de nove moléculas analisadas diminuiu a expressão de duas moléculas (CD11b, HLA-DR) e aumentou a expressão apenas de uma (CD86). Outros autores (MOROVICH *et al* 2000; VON STEUBT et al 1998) estudando a interação metacíclico-macrófago não identificaram alteraçãoes do estado basal de secreção das citocinas, notadamente IL-12, moléculas co-estimulatórias ou de MHC classe II. A não identificação dessas alterações, por estes autores, pode, em parte, dever-se a que as alterações, principalmente em macrófagos, são em poucas moléculas, e em geral o efeito não é pronunciado, conforme nossos dados e os de outros mostram (BUATES & MATLASHEWSKI 2001). Além disso, muitos desses dados de citometria de fluxo têm sido apresentados apenas qualitativamente, o que também

dificulta a detecção de pequenas alterações. Alguns autores (SAHA et al 1995), por outro lado, têm relatado alterações em uma ou outra molécula, dependendo da linhagem do camundogo estudado. Então, camundongos BALB/c infectados com *L(L) donovani* apresentam pequeno aumento da expressão de CD54, mas não são observadas alterações em camundongos C57BL/6 (SAHA et al 1995). Não deixa , no entanto, de ser surpreendente que o parasita, ao infectar o macrófago, altere pouco ou não altere o estado basal de expressão de moléculas co-estimulatórias e citocinas. Isso pode fazer com que a infecção passe despercebida nas suas fases mais iniciais, ou seja, haveria ignorância do sistema imune (DE ALMEIDA 2002). Ignorância pode ser definida como um estado em que clones de células T funcionais, e potencialmente reativos a um determinado antígeno, não reagem a a este antígeno (ABBAS et al 2000). Na anergia, por outro lado, a célula T se torna incapaz de reagir ao antígeno. Dentre os fatores que favoreceriam o estado de ignorância estariam: Presença do antígeno em quantidades muito pequenas, incapazes de levar à ativação (VOEHRINGER et al. 2000), sítios imunoprivilegiados (KURTS et al. 1999), baixo numero de células reativas, não apresentação por células apresentadoras profissionais, não ativação da célula apresentadora de antígeno (SHEVACH 1999). Até onde sabemos não há qualquer aplicação, anterior a DE ALMEIDA 2002, desse termo a uma doença parasitária. Quais seriam as vantagens e desvantagens desse mecanismo de escape para o parasita. Uma óbvia desvantagem seria que, ao infectar em baixo número, o parasita poderia ser prematuramente eliminado pelo sistema imune natural. No entanto, como discutimos acima, algumas características próprias do parasita permitem que ele tenha relativo sucesso aqui. Também, contribuirá em muito para esse sucesso, que a célula que o parasita infectará será permissiva ao seu crescimento. Outra vantagem, quando a ignorância for quebrada, devido ao próprio crescimento parasitário, o parasita poderá ter

modulado o microambiente do hospedeiro, de modo a não tê-lo tão hostil a si. Outro possível aspecto benéfico ao parasita, tentativas frustadas de infectar poderiam não gerar imunidade. Uma vez estabelecida a imunidade, por outro lado, o parasita poderia ter muitas dificuladdes em reeinfectar o mesmo hospedeiro. Haveriam razões, inerentes ao crescimento ou biologia do parasita no seu vetor, que expliquem por que o parasita infecta em tão baixo número? Parece que sim. O crescimento excessivo do parasita no seu vetor poderia interferir desfavoravelmente com a fisiologia do seu vetor (KILLICK-KENDRIC 1987). O crescimento excessivo do parasita necessitaria de abundantes nutrientes, no entanto, o seu ciclo de diferenciação inicia-se após o respasto sanguíneo (KILLICK-KENDRIC 1987), e soluções de açúcares, que são ingeridas pela fêmea do flebotomíneo posteriormente, levam a uma queda no número de parasitas *in vitro* (SCHLEIN et al. 1987). Finalmente a probóscide do vetor, onde os metacíclicos se localizam, é um dos locais mais pobres em nutrientes do tubo digestivo do inseto (KILLICK-KENDRIC 1987).
O aumento da expressão de CD 86 constitui uma exceção, no quadro geral da regulação das moléculas testadas. Contudo, por vir isoladamente, sem um aumento concomitante de outras moléculas co-estimulatórias, CD 54, CD 80 e dos HLA de classe I e II pode induzir anergia ou morte de clones de células T (KAYE et al. 1994; DE ALMEIDA et al 2002 .Enviado para publicação). Sob estimulação, no entanto, os efeitos regulatórios negativos se estenderam e se acentuaram (CD54, HLA-DR, HLA-ABC e IL-12). Então, efeitos inibitórios que não eram aparentes durante a infecção (por exemplo para CD 54 e IL-12), tornaram-se evidentes, quando a célula hospedeira foi submetida a um estímulo inflamatório. Isso reforça a idéia que o parasita evita a resposta inflamatória. Dessa maneira, é possível ainda que inúmeras outras ações do parasita sobre sua célula hospedeira venham a se manifestar, na dependência das diferentes condições ambientais. Então,

inicialmente tanto a saliva do vetor como o próprio parasita convergem para uma ação antinflamatória.

3.2 Fase inicial e evolução da infecção: estados sub-clínicos, doença, reativação e metástase

Uma vez instalada, a infecção leishmaniótica poderá se desenvolver como doença ou permanecer em estado subclínico (PEARSON & SOUSA 1996; NICOLAS et al. 2000; BELKAID et al. 2001; UZONNA & BRETSCHER 2001). Em geral, este estado subclínico permitirá o surgimento da doença cutâneo-mucosa (MARSDEN 1986) ou da leishmaniose dérmica pós-kalazar (ZIJLSTRA & EL-HASSAN 2001) (ambas de provável natureza metastática), ou estados de reativação que ocorrem após episódios de imunodepressão tanto nas formas viscerais como cutâneas. Aqui o padrão da doença pode mudar, por exemplo, na SIDA indivíduos com doença originalmente cutânea podem desenvolver doença visceral (HERNANDEZ et al. 1993). Então, não somente a espécie de *Leishmania* é importante na definição do padrão de doença mas também o hospedeiro e, particularmente, seu sistema imune (BELLI et al. 1999). Sabe-se, por exemplo, que espécies de *Leishmania* que são patogênicas para o homem, se desenvolvem, em seus reservatórios naturais, em orgãos que não os preferencias da doença humana, apararentemente sem provocar lesões (LAINSON et al. 1981).

Embora a doença humana tenha uma divisão clínica de origem anatômica isso não significa que a infecção esteja restrita aos sítios em que a lesão é mais aparente. Assim, parasitas classicamente de localização cutânea tem sido encontrados em sítios viscerais (BARRAL et al. 1986), e casos de doença cutânea por *L(L.) chagasi* (BELLI et al. 1999) e *L.(L.)*

infantum (DEL GIUDICE et al. 1998) têm sido descritos. Da mesma forma, é freqüente a localização de leishmânias na pele do cão no Kalazar canino (MARZOCHI et al. 1985). A explicação mais provável para as diferentes manifestações clínicas e, em particular, da lesão mucosa, é uma disseminação hematogênica (PESSOA & BARRETO 1948). Corroboram essa hipótese, os achados da forma disseminada (COSTA et al. 1986), a presença de parasitas em células sanguíneas circulantes (MARTINEZ et al. 1992; GUEVARA et al. 1994; MAGILL et al. 1994), e o acometimento dos linfonodos com úlcera cutânea primária e mesmo na ausência desta (PESSOA & BARRETO 1948; LLANOS CUENTAS et al. 1984; AL-GINDAN et al. 1989; AZADEH et al. 1994; BARRAL et al. 1995).

A disseminação hematogênica poderia então ser inicial ou tardia. Uma disseminação linfática inicial, porém, parece provável já que a retirada prévia dos linfondos de drenagem contribui para a disseminação do parasita (POULTER & PANDOLPH 1982; REED et al. 1986; LASKAY et al. 1995). Qualquer que seja a via, o parasita é transportado nos vacúolos lisossomais dos monócitos do hospedeiro, onde vive . Torna-se então necessário, para o entendimento do processo metastático, o esclarecimentno de como se dá o trânsito de monócitos infectados no organismo parasitado.

Um primeiro grupo de moléculas de adesão, as selectinas ou lectinas de adesão, compõe-se de três conjuntos de moléculas: as selectinas leucocitárias (L), as selectinas endoteliais (E) e as selectinas plaquetárias (P) que assim se denominam por serem expressas respectivamente em leucócitos, células endoteliais e plaquetas. As selectinas são glicoproteínas transmembrana de cadeia única com a porção aminoterminal expressa extracelularmente. Seus ligantes são também sialomucinas (CARLOS & HARLAN 1994).

O processo de ligação lectina-ligante nas três moléculas L ,E e P é em geral de baixa afinidade e inicialmente gera a aderência leucocitária ao endotélio, que sob pressão do fluxo sanguíneo, ocasiona o rolamento de leucócitos na superfície endotelial (SPRINGER 1995). Um outro grupo, o das integrinas, são proteínas leucocitárias heterodiméricas que se ligam a vários membros da superfamília das imunoglobulinas no endotélio. As integrinas ligam-se ainda a várias proteínas da matriz extracelular como o colágeno, laminina e fibronectina (CARLOS & HARLAN 1994). Na matriz extracelular se ligam às proteínas com sequências de aminoácidos Arg-Gly-Asp (RGD) - fibronectina e vitronectina - Asp-Gly-Glu-Ala (DGEA) - colágeno tipo I e Glu-Ile-Leu-Asp-Val (EILDV) - fibronectina. Três grupos básicos são descritos, simplificadamente, com base nas três unidades beta inicialmente descritas: integrinas contendo cadeias beta 1 (moléculas VLA), que participam principalmente na adesão celular à matriz extracelular; integrinas contendo cadeias beta 2 (moléculas LFA 1,MAC 1), que participam na adesão leucocitária ao endotélio e na fagocitose como receptores do complemento (MAC 1 ,CR4); as integrins contendo cadeias beta 3 , que incluem o receptor para vitronectina, participando na adesão à matriz extrcelular. Pares de integrina -ligante incluem VLA-1 (CD49aCD29)-colágeno; LFA-1 (CD11aCD18)-ICAM-1; MAC-1 (CD11bCD18)-iC3b ou ICAM-1; CD11cCD18-iC3b (CARLOS & HARLAN 1994).

No processo de adesão leucocitária ao endotélio, três etapas básicas são conhecidas: uma etapa inicial, a da ligação das selectinas com seus ligantes, que é uma etapa de baixa afinidade e de regulação limitada; uma etapa intermidiária em que a ativação celular por citocinas e quimiocinas regulam a terceira etapa por aumento na expressão e/ou afinidade das integrinas (BUTCHER 1991). Assim, esta modulação das integrinas é mediada por outros receptores celulares como TCR, receptores para interleucinas e quimiocinas. Por

exemplo, a afinidade de LFA-1 (célula T) por ICAM-1 aumenta quando há ligação do TCR ao MHC CLASSE I da mesma maneira que a afinidade do LFA-1 neutrofílico aumenta por ICAM-1 em resposta a IL-8 (DUSTIN & SPRINGER 1989; SPRINGER 1995).

De qualquer maneira, para que o processo metastático se dê, a célula infectada deverá se deslocar do tecido em direção ao sangue, ou seja, em sentido contrário ao processo atualmente mais conhecido. É fundamental então que ela se torne menos aderente, em especial às proteínas da matriz extracelular. Entre as proteínas da matriz: colágeno, laminina e fibronectina, a adesão se dá fundamentalmente à fibronectina (Figuras 1, A e B. **Resultados Anexos**). Em ensaios de adesão mostramos que a infecção de monócitos mas não de macrófagos diminui a adesão ao plástico e à fibronectina (Figuras 1 e 2. **Resultados Anexos**). Essa diminuição da adesão à fibronectina não se deve a uma diminuição na expressão de seu principal receptor, CD49e, como mostram nossos dados para monócitos (DE ALMEIDA et al. 2002. Enviado para publicação). No entanto, não podemos descartar uma redução em sua afinidade pela fibronectina (DUSTIN & SPRINGER 1989). Outras moléculas por nós analisadas, CD49d e CD49f, apresentaram expressão muito baixa (dados não mostrados). Um receptor que pode estar envolvido no processo de adesão é CD11b. Já foi demonstrado que CD11b media a ligação de monócitos humanos ao plástico (MCNALLY & ANDERSON 1994). CD11b pode se ligar também, entre outros, ao fibrinogênio, à fibrina e a componentes da matriz extracelular como laminina (JIANG et al. 1994). A infecção diminuiu a expressão de CD11b em monócitos mas não em macrófagos e, monócitos infectados tiveram menor adererência tanto ao plástico como à fibronectina. É particularmente interessante que essa redução tenha se dado nos monócitos, que são justamente as células encontradas parasitadas no sangue circulante. De acordo, camundongos deficientes em CD 18(-/-), infectados por *L(L.)major,* apresentam maior

disseminação do parasita do que o tipo selvagem, embora esse estudo tenha focalizado mais os efeitos imunológicos dessa deficiência (SCHONLAU et al. 2000).

Em macrófagos humanos, mesmo utilizando proporções muito elevadas de promastigota por macrófago (até 30:1) não evidenciamos alteração na adesão ao plástico. No entanto, com concetrações maiores (até 60:1) houve perda de adesão ao plástico (dados mostrados na fig 2), estando as células não aderentes viáveis pelo método do azul tripan. O que significaria, em termos da infecção natural, essas concentrações tão elevadas de parasitas? Seguindo-se a inoculação de 100 metacíclicos, houve um crescimento parasitário de ~1000 vezes até imediatamente antes do surgimento da resposta adaptativa , 6-7 semanas após a inoculação (BELKAID et al 2000; LIRA et al 2000). Partindo de nossas estimativas (DE ALMEIDA 2002) iniciaríamos então com uma densidade parasitária de 0,03-0,1 por fagócito, e atingiríamos de 30-100 parasitas por fagócito em 6-7 semanas após a infecção, desconsiderando o afluxo monocitário. Embora esse parasitismo possa parecer exagerado, não é incomum encontrá-lo em casos em que há baixa ou ausência da resposta imune adquirida, como parece ser o caso, nessa fase mais inicial da infecção por *Leishmania* spp (LIRA et al 2000; BELKAID et al 2000; DE ALMEIDA 2002). Dados experimentais têm mostrado que o macrófago suporta, sem qualquer alteração de sua viabilidade, cargas parasitárias em torno de 100 parasitos por macrófago (CHANG & DWYER 1978). Então, pelos nossos dados de adesão, a disseminação do parasita dar-se-ia, mais facilmente, quando o parasitismo fosse mais intenso, ou seja, imediatamente antes do surgimento da imunidade adqurida, na fase inicial da infecção, ou quando há depressão dessa imunidade na fase crônica da infecção. Resumindo, no início da infecção, o parasita, devido à ignorância do sistema imune, se multiplicaria exponencialmente no interior de monócitos e macrófagos, levando à perda de adesão à matriz extracelular, e atingiria sítios distantes.

Nesse momento, o surgimento da imunidade adqurida confinaria o crescimento parasitário aos locais aonde o parasita esivesse mais adaptado para proliferar (ver discussão a seguir). Essa hipótese é compatível com alguns dados clínicos e experimentais. Por exemplo, inoculando-se *L.(L.) donovani* na pele de hamsters houve um crescimento intenso do parasita nesse local até 4 semanas após a infecção. Nesse momento, com o surgimento de uma resposta granulomatosa, o parasita desapareceu da pele, mas disseminou-se para sítios viscerais, onde produziu o quadro típico da leishmaniose visceral (WILSON et al 1987). Observamos também que *L.(V.)braziliensis,* inoculada em sítios viscerais do hamster, não produz sintomatologia clínica, mas metastatisa tardiamente para pele, onde produz lesões exuberantes (ALMEIDA et al 1996). Na fase crônica, a doença pode apresentar focos de reativação/disseminação, dependendo se há alterações localizadas na imunidade, como devido ao aumento de IL-10 na pele de pacientes com leishmaniose cutânea pós –kalazar, ou traumatismos cutâneos e transferência de células CD4+, pré sensibilizadas ao antígeno do parasita, em infecções de camundongos da linhagem BALB/c por *L(L)amazonensis* (BERTHO et al. 1994). Permanecem esses fatores, infelizmente, ainda desconhecidos, no caso da doença mucosa. Na SIDA, por outro lado, a reativação/disseminação será sistêmica.

Uma vez que o parasita tenha se metastatisado, poderá inicialmente ficar quiescente nesses locais devido às condições não ideais à sua multiplicação, tais como temperatura (BERMAN & NEVA 1981; SACKS et al. 1983; SCOTT 1985; CALLAHAN et al. 1996) (cepas dermatrotópicas vs viscerotrópicas) imunidade orgão específica (ALMEIDA et al. 1996; WILSON et al. 1998; ENGWERDA & KAYE 2000) e fatores do microambiente não esclarecidos (hipótese do solo e da semente). Neste particular, pudemos demonstrar (ALMEIDA et al. 1996), através de modelo experimental, que sítios viscerais (fígado, baço

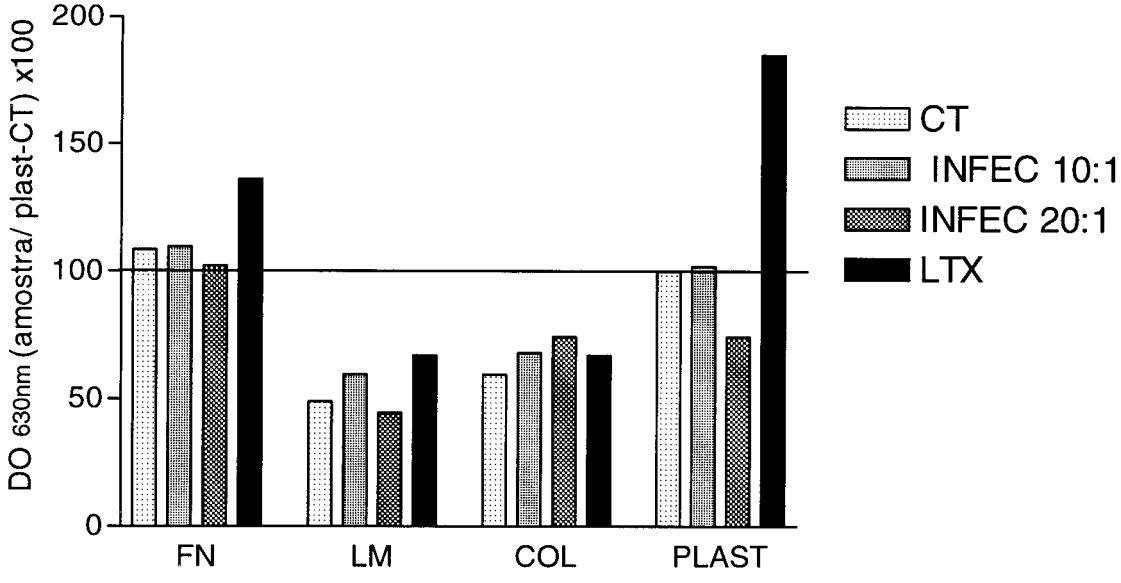
e linfonodos) principalmente no caso de *L(L.) braziliensis* podem constituir um sítio de dormência relativa e fonte de parasitas para disseminação hematogênica.). Acometimento de orgãos internos foi, também, recente observada com *L(L) major* infectando camundongos BALB/c (NICOLAS et al. 2000). Tem-se mostrado que a presença de parasitas em áreas cicatriciais (SCHUBACH et al. 1998), em linfonodos (LLANOS CUENTAS et al. 1984; BARRAL et al. 1992; MOLL et al. 1993; AZADEH et al. 1994; ZIJLSTRA & EL-HASSAN 2001), no interior de fibroblastos (CHANG 1978; BOGDAN et al. 2000) e células dendríticas (MOLL et al. 1993; MOLL et al. 1995; BELKAID et al. 2001) podem constituir sítios de abrigo do parasita em estados subclínicos da infecção. Estudos recentes (DOHERTY & COFFMAN 1996; BELKAID et al. 2001; UZONNA et al. 2001) mostram que um elemento importante para o estabelecimento da doença subclínica é o inóculo em baixas doses, tanto em camundongos tidos como sensíveis (BALB/c) como naqueles tidos como resistentes à infecção (C57BL/6). Os estudos concordam também num aspecto, que a manutenção do estado de equilíbrio se dá basicamente pelo equilíbrio das respostas TH1 e TH2. É até certo ponto surpreendente que inóculos maiores ($\sim 10^6$) polarizem a resposta para TH1 em camundongos resistentes e para TH2 em camundongos susceptíveis, enquanto inóculos similares àqueles da infecção natural ($\sim 10^2$) induzem uma resposta mista em ambas as linhagens e, principalmente, no camundongo de linhagem C57BL/6. O parasita, ao contrário do que se pensava, poderá ser eliminado mesmo no hospedeiro susceptível pela polarização da resposta para TH1 (UZONNA & BRETSCHER 2001; UZONNA et al. 2001). É também fundamental a resposta TH2 (IL-10) na manutenção do estado subclínico em camundongos C57BL/6. Quando do inóculo em baixas doses, estes camundongos, na fase crônica da infecção, desenvolvem uma resposta com produção de IL-10 e IFN-γ, inclusive com alta frequência de células dérmicas T

CD4+ produzindo ambas as citocinas. Bloqueada a resposta TH2, usando anticorpos bloqueadores do receptor da IL-10, o hospedeiro igualmente se cura (BELKAID et al. 2001). Inesperadamente, nos estados subclínicos o parasita tem maiores chances de ser retransmitido ao seu vetor natural (LIRA et al. 2000).

Os mecanismos evolutivos selecionaram, então, a condição ótima que permite a infecção e a retransmissão a partir de um hospedeiro que não se sabe *a priori* se resistente ou susceptível, perpetuando o ciclo parasitário. Uma vez rompido o equílibrio, o parasita poderá disseminar-se, levando o hospedeiro à morte , ou ser eliminado pelo sistema imune do hospedeiro. Em ambas as situações termina o ciclo parasitário .

4 RESULTADOS ANEXOS

A


DO 630nm (amostra/ plast-CT) x100
200
150
100
50
0
FN
LM
COL
PLAST
CT
INFEC 10:1
INFEC 20:1
LTX

B

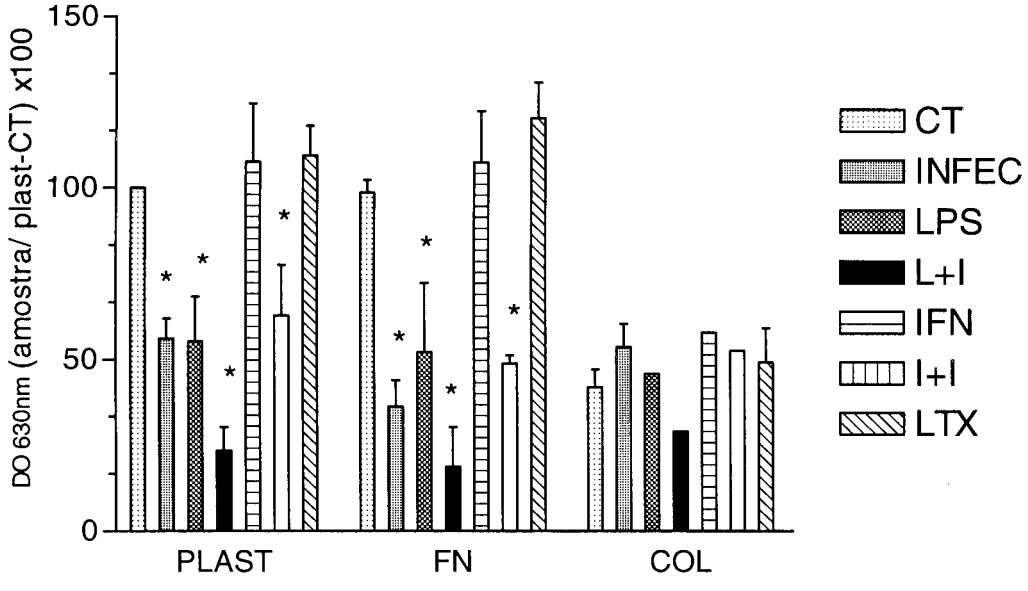

DO 630nm (amostra/ plast-CT) x100
150
100
50
0
PLAST
FN
COL
CT
INFEC
LPS
L+I
IFN
I+I
LTX

Fig1. Adesão de macrófagos (A) e monócitos (B) humanos ao plástico (PLAST), fibronectina (FN), colágeno (COL) e laminina (LM) em diferentes condições experimentais após 48 horas da infecção com *L.(L.)chagasi.* (A) Adesão de macrófagos humanos diferencidos *in vitro e* infectados na proporção de 10 e 20 promastigotas de *L.(L.)chagasi* para cada macrófago (INFEC10:1 e 20:1 respectivamente) e macrófagos não infectados (CT) após 48horas. (B) A adesão de monócitos humanos ao plástico e à fibronectina após 48hs em diferentes situações experimentais. CT: células não infectadas. INFEC: células infectadas 10:1. LPS: estimulação com LPS (10ng/ml). L+I: estimulação com LPS (10ng/ml) duas horas após a infecção. IFN: estimulação com IFNγ (10-100 U). I+I: estimulação com IFNγ (10-100 U) duas horas após a infecção. Placas plásticas para realização de Elisa, previamente sensibilizadas com componentes da matriz (10 μg/ml-overnight), com bloqueio de PBS/BSA a 3% (p/v) foram utilizadas nos ensaios. Após 48 horas os poços foram lavados com RPMI a jatos de pipeta nos pontos imaginários 3, 6, 9 e 12 horas de cada poço, a seguir foram fixados em metanol e corados com cristal violeta aquosa 1% (p/v); lavados em água e deixados secar. Adaptado de (BURKE et al. 1995). A absorbância foi lida a 630nm em um espectrofotômetro de placa. Os valores estão em percentuais da absorbância da adesão dos monócitos/macrófagos em relação ao plástico controle. Com monócitos foram realizados 7-4 experimentos para as condições de adesão em plástico, 3 experimentos em fibronectina e 2 experimentos em colágeno. Cada barra representa um desvio padrão da média. *, $p<0,05$ em relação aos respectivos controles.

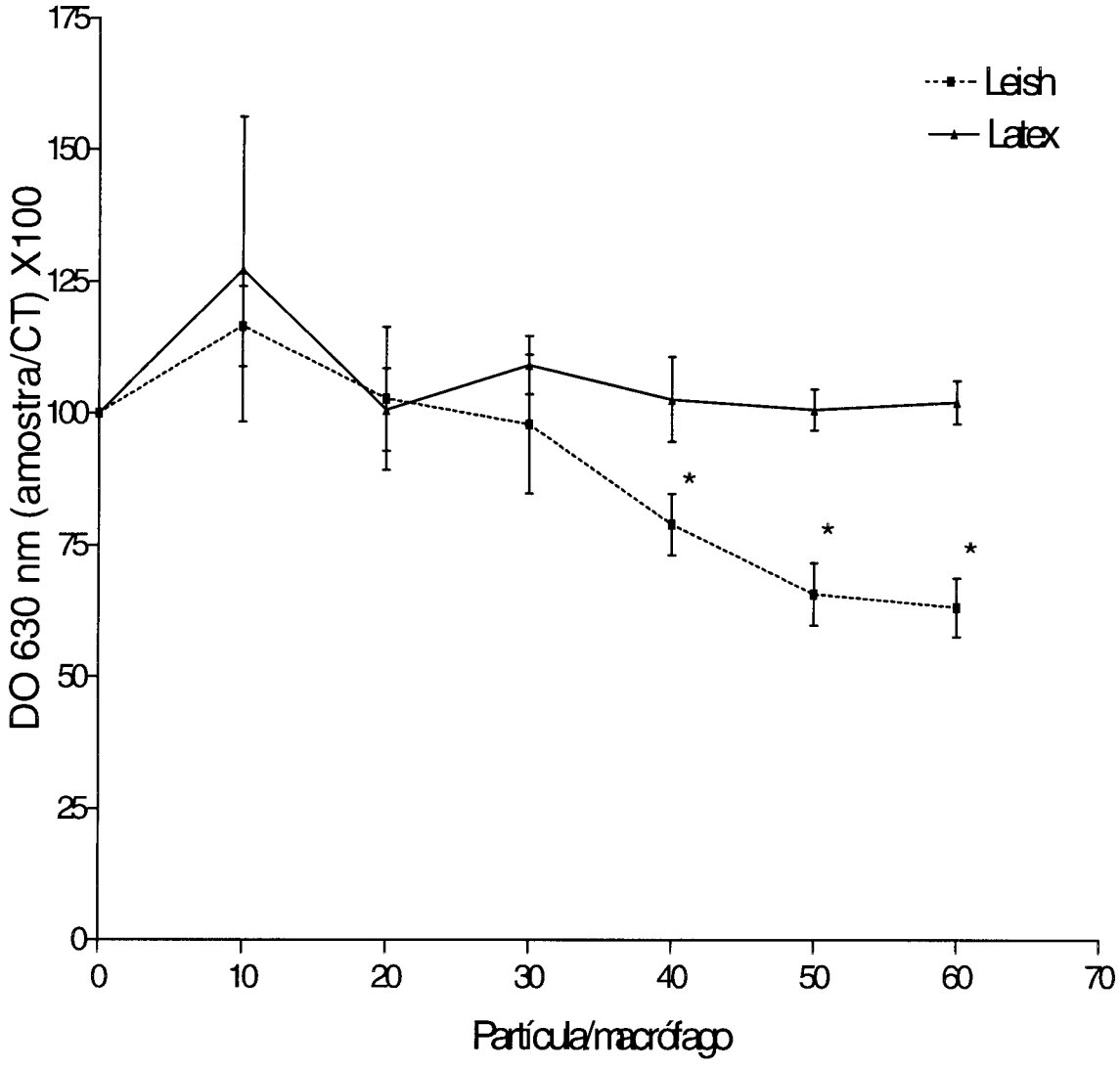


Fig 2. Adesão de macrófagos humanos ao plástico, após 48 horas, com diferentes inóculos de promastigotas e partículas de Látex. Leish: Promastigotas de *L.(L.) chagasi.* . A absorbância foi lida a 630nm em um espectrofotômetro de placa. Os valores estão em percentuais da absorbância da adesão dos monócitos/macrófagos, nas diferentes situações experimentais, em relação ao plástico controle. Foram realizados quatro exprimentos para a condição experimental com *Leishmania* e dois com Látex. Cada barra representa um desvio padrão da média. *,$p<0,05$ em relação ao Látex.

5 CONCLUSÕES

1. A infecção por *L.(L.) chagasi*, ao modular negativamente ou não induzir em monócitos e macrófagos humanos várias moléculas de caráter proinflamatório, notadamente CD54, IL-12 , TNFα, assim como os HLA de classe I e II, pode deixar o sistema imune, nas fases mais iniciais da infecção, em ignorância ou anergia.

2. A infecção por *L.(L.) chagasi* tem efeitos inibitórios, mais acentuados em monócitos que macrófagos humanos. Em monócitos infectados há diminuição na expressão de CD11b e da adesão ao plástico e à fibronectina. Sendo o monócito a célula mais precocemente infectada, esses efeitos podem ser relevantes para a disseminação inicial do parasita.

3. Na fase inicial da infecção, um número minímo de parasitas por célula apresentadora de antígeno, associado a alterações mínimas nessas células, e a um baixo número de células infectadas, dificultaria a geração de clones de célula T reativos a antígenos do parasita. Isto ajudaria a explicar, nas fases mais iniciais da infecção, a ausência da resposta imune adaptativa, e o crescimento irrestrito do parasita no interior de monócitos e macrófagos.

4. As informações existentes em relação à infecção natural pedem um ajuste de algumas variáveis dos sistemas *in vitro,* principalmente no que se refere ao número de parasitas do inóculo, e aos componentes celulares do sistema. Isto é particularmente relevante para sistemas *in vitro* que procuram simular a geração da resposta imune adaptativa. O sistema mais próximo da infecção natural seria o inóculo infectivo em sangue total, procurando-se atingir uma densidade parasitária inicial em torno de 0,03-0,1 parasita por célula apresentadora de antígeno.

6 REFERÊNCIAS BIBLIOGRÁFICAS

ABBAS, A. K.; LICHTMAN, A. H.; POBER, J. S. Immunologic Tolerance**. Cellular and molecular immunology**. Philadelphia: W.B. Saunders company, 2000. p: 208-231.

ADLER, S. & THEODOR, O. "Investigations on Mediterranean Kala azar I-V." **Proc. R. Soc.,London, Ser. B, 108**: 447-502, 1931

ADLER, S. & THEODOR, O. "Investigations on Mediterranean Kala azar VII-X." **Proc. R. Soc. ,London, Ser. B, 116**: 494-544, 1935

ALEXANDER, J.; SATOSKAR, A. R.; RUSSELL, D. G. "Leishmania species: models of intracellular parasitism." **J Cell Sci, 112 Pt 18**: 2993-3002., 1999

AL-GINDAN, Y.; KUBBA, R.; EL-HASSAN, A. M.; OMER, A. H.; KUTTY, M. K.; SAEED, M. B. "Dissemination in cutaneous leishmaniasis. 3. Lymph node involvement." **Int J Dermatol, 28**: 248-54., 1989

ALMEIDA, M. C.; CUBA-CUBA, C. A.; MORAES, M. A.; MILES, M. A. "Dissemination of Leishmania (Viannia) braziliensis." **J Comp Pathol, 115**: 311-6., 1996

AZADEH, B.; SELLS, P. G.; EJECKAM, G. C. ; RAMPLING, D. "Localized Leishmania lymphadenitis. Immunohistochemical studies." **Am J Clin Pathol, 102**: 11-5., 1994

BARRAL, A.; BADARO, R.; BARRAL-NETTO, M.; GRIMALDI, G., JR.; MOMEM, H.; CARVALHO, E. M. "Isolation of Leishmania mexicana amazonensis from the bone marrow in a case of American visceral leishmaniasis." **Am J Trop Med Hyg, 35**: 732-4., 1986

BARRAL, A.; BARRAL-NETTO, M.; ALMEIDA, R.; DE JESUS, A. R.; GRIMALDI JUNIOR, G.; NETTO, E. M.; SANTOS, I.; BACELLAR, O.; CARVALHO, E. M. "Lymphadenopathy associated with Leishmania braziliensis cutaneous infection." **Am J Trop Med Hyg, 47**: 587-92., 1992

BARRAL, A.; GUERREIRO, J.; BOMFIM, G.; CORREIA, D.; BARRAL-NETTO, M.; CARVALHO, E. M. "Lymphadenopathy as the first sign of human cutaneous infection by Leishmania braziliensis." **Am J Trop Med Hyg, 53**: 256-9., 1995

BELKAID, Y.; HOFFMANN, K. F.; MENDEZ, S.; KAMHAWI, S.; UDEY, M. C.; WYNN, T. A.; SACKS, D. L. "The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure." **J Exp Med, 194**: 1497-506., 2001

BELKAID, Y.; KAMHAWI, S.; MODI, G.; VALENZUELA, J.; NOBEN-TRAUTH, N.; ROWTON, E.; RIBEIRO, J.; SACKS, D. L. "Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis." **J Exp Med, 188**: 1941-53., 1998

BELKAID, Y.; MENDEZ, S.; LIRA, R.; KADAMBI, N.; MILON, G.; SACKS, D. "A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity." **J Immunol, 165**: 969-77., 2000

BELLI, A.; GARCIA, D.; PALACIOS, X.; RODRIGUEZ, B.; VALLE, S.; VIDEA, E.; TINOCO, E.; MARIN, F.; HARRIS, E. "Widespread atypical cutaneous Leishmaniasis caused by Leishmania (L.) Chagasi in Nicaragua." **Am J Trop Med Hyg, 61**: 380-5., 1999

BERMAN, J. D.& NEVA, F. A. "Effect of temperature on multiplication of Leishmania amastigotes within human monocyte-derived macrophages in vitro." **Am J Trop Med Hyg, 30**: 318-21., 1981

BERTHO, A. L.; SANTIAGO, M. A.; COUTINHO, S. G. "An experimental model of the production of metastases in murine cutaneous leishmaniasis." J Parasitol, 80: 93-9., 1994.

BIEDERMANN, T.; ZIMMERMANN, S.; HIMMELRICH, H.; GUMY, A.; EGETER, O.; SAKRAUSKI, A. K.; SEEGMULLER, I.; VOIGT, H.; LAUNOIS, P.; LEVINE, A. D.; WAGNER, H.; HEEG, K.; LOUIS, J. A.; ROCKEN, M. "IL-4 instructs TH1 responses and resistance to Leishmania major in susceptible BALB/c mice." **Nat Immunol, 2**: 1054-60., 2001

BOGDAN, C.; DONHAUSER, N.; DORING, R.; ROLLINGHOFF, M.; DIEFENBACH, A.; RITTIG, M. G. "Fibroblasts as host cells in latent leishmaniosis." **J Exp Med, 191**: 2121-30., 2000

BRITTINGHAM, A.; MORRISON, C. J.; MCMASTER, W. R.; MCGWIRE, B. S.; CHANG, K. P.; MOSSER, D. M. "Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis." **J Immunol, 155**: 3102-11., 1995

BUATES, S. & MATLASHEWSKI, G. "General suppression of macrophage gene expression during Leishmania donovani infection." **J Immunol, 166**: 3416-22., 2001

BURKE, F.; ROZENGURT, E.; BALKWILL, F. R. Measurament of proliferative, cytolytic, and cytostatic activity of cytokines. In:F. R. BALKWILL. **Cytokines.** Oxford: Oxford University Press, 1995.p. 279-296.

BUTCHER, E. C. "Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity." **Cell, 67**: 1033-6., 1991

CALLAHAN, H. L.; PORTAL, I. F.; BENSINGER, S. J.; GROGL, M. "Leishmania spp: temperature sensitivity of promastigotes in vitro as a model for tropism in vivo." **Exp Parasitol, 84**: 400-9., 1996

CAMACHO, S. A.; HEATH, W. R.; CARBONE, F. R.; SARVETNICK, N.; LEBON, A.; KARLSSON, L.; PETERSON, P. A.; WEBB, S. R. "A key role for ICAM-1 in generating effector cells mediating inflammatory responses." **Nat Immunol, 2**: 523-9., 2001

CARLOS, T. M. & HARLAN, J. M. "Leukocyte-endothelial adhesion molecules." **Blood, 84**: 2068-101., 1994

CHANG, K. P. "Leishmania infection of human skin fibroblasts in vitro: absence of phagolysosomal fusion after induced phagocytosis of promastigotes, and their intracellular transformation." **Am J Trop Med Hyg, 27**: 1084-96., 1978

CHANG, K. P. "Leishmanicidal mechanisms of human polymorphonuclear phagocytes." **Am J Trop Med Hyg, 30**: 322-33., 1981

CHANG, K. P.; CHAUDHURI, G.; FONG, D. "Molecular determinants of Leishmania virulence." **Annu Rev Microbiol, 44**: 499-529, 1990

CHANG, K. P. & DWYER, D. M. "Leishmania donovani. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes." **J Exp Med, 147**: 515-30., 1978.

CONSTANT, S. L.; LEE, K. S.; BOTTOMLY, K. "Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential Th2-type differentiation." **Eur J Immunol, 30**: 840-7., 2000

COSTA, J. M.; MARSDEN, P. D.; LLANOS-CUENTAS, E. A.; NETTO, E. M.; CARVALHO, E. M.; BARRAL, A.; ROSA, A. C.; CUBA, C. C.; MAGALHAES, A. V.; BARRETO, A. C. "Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases." **J Trop Med Hyg, 89**: 319-23., 1986

DE ALMEIDA, M. C.; SILVA, A. C.; BARRAL, A.; BARRAL NETTO, M. "A simple method for human peripheral blood monocyte isolation." **Mem Inst Oswaldo Cruz, 95**: 221-3., 2000

DE ALMEIDA, M. C. "Infective inoculum for *Leishmania*." **Trends Parasitol., 18**: 154-155, 2002.

DEL GIUDICE, P.; MARTY, P.; LACOUR, J. P.; PERRIN, C.; PRATLONG, F.; HAAS, H.; DELLAMONICA, P.; LE FICHOUX, Y. "Cutaneous leishmaniasis due to Leishmania infantum. Case reports and literature review." **Arch Dermatol, 134**: 193-8., 1998

DOHERTY, T. M. & COFFMAN, R. L. "Leishmania major: effect of infectious dose on T cell subset development in BALB/c mice." **Exp Parasitol, 84**: 124-35., 1996

DOMINGUEZ, M. & TORANO, A. "Immune adherence-mediated opsonophagocytosis: the mechanism of Leishmania infection." **J Exp Med, 189**: 25-35., 1999

DUSTIN, M. L. & SPRINGER, T. A. "T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1." **Nature, 341**: 619-24., 1989

ENGWERDA, C. R. & KAYE, P. M. "Organ-specific immune responses associated with infectious disease." **Immunol Today, 21**: 73-8. 00001549 00001549, 2000

GILLESPIE, R. D.; MBOW, M. L.; TITUS, R. G. "The immunomodulatory factors of bloodfeeding arthropod saliva." **Parasite Immunol, 22**: 319-31., 2000

GOSSELIN, E. J.; WARDWELL, K.; RIGBY, W. F.; GUYRE, P. M. "Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3." **J Immunol, 151**: 1482-90., 1993

GUEVARA, P.; ROJAS, E.; GONZALEZ, N.; SCORZA, J. V.; ANEZ, N.; VALERA, M.; RAMIREZ, J. L. "Presence of Leishmania braziliensis in blood samples from cured patients or at different stages of immunotherapy." **Clin Diagn Lab Immunol, 1**: 385-9., 1994

HERNANDEZ, D.; RODRIGUEZ, N.; MARTINEZ, C.; GARCIA, L.; CONVIT, J. "Leishmania braziliensis causing visceral leishmaniasis in a patient with human immunodeficiency virus infection, identified with the aid of the polymerase chain reaction." **Trans R Soc Trop Med Hyg, 87**: 627-8., 1993

HIMMELRICH, H.; LAUNOIS, P.; MAILLARD, I.; BIEDERMANN, T.; TACCHINI-COTTIER, F.; LOCKSLEY, R. M.; ROCKEN, M.; LOUIS, J. A. "In BALB/c mice, IL-4 production during the initial phase of infection with Leishmania major is necessary and sufficient to instruct Th2 cell development resulting in progressive disease." **J Immunol, 164**: 4819-25., 2000

JIANG, Y.; ZHU, J. F.; LUSCINSKAS, F. W.; GRAVES, D. T. "MCP-1-stimulated monocyte attachment to laminin is mediated by beta 2- integrins." **Am J Physiol, 267**: C1112-8., 1994

KAMHAWI, S. "The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of Leishmania infections." **Microbes Infect, 2**: 1765-73., 2000.

KAMHAWI, S.; BELKAID, Y.; MODI, G.; ROWTON, E.; SACKS, D. "Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies." **Science, 290**: 1351-4., 2000

KAYE, P. M.; ROGERS, N. J.; CURRY, A. J. ; SCOTT, J. C. "Deficient expression of co-stimulatory molecules on Leishmania-infected macrophages." **Eur J Immunol, 24**: 2850-4., 1994.

KILLICK-KENDRIC, R. Biology of *Leishmania* in Phlebotomine sandflies. In:W. K.ILLICK-KENDRIC & PETERS, R. eds. The Leishmaniases in Biology and Medicine. London: Academic Press, 1987.p. 392-460.

KILLICK-KENDRICK, R. "The life-cycle of Leishmania in the sandfly with special reference to the form infective to the vertebrate host." **Ann Parasitol Hum Comp, 65**: 37-42., 1990

KONECNY, P.; STAGG, A. J.; JEBBARI, H.; ENGLISH, N.; DAVIDSON, R. N.; KNIGHT, S. C. "Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro." **Eur J Immunol, 29**: 1803-11., 1999.

KURTS, C.; SUTHERLAND, R. M.; DAVEY, G.; LI, M.; LEW, A. M.; BLANAS, E.; CARBONE, F. R.; MILLER, J. F.; HEATH, W. R. "CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose." **Proc Natl Acad Sci U S A, 96**: 12703-7., 1999.

LAINSON, R.; SHAW, J. J.; POVOA, M. "The importance of edentates (sloths and anteaters) as primary reservoirs of Leishmania braziliensis guyanensis, causative agent of "pianbois" in north Brazil." **Trans R Soc Trop Med Hyg, 75**: 611-2, 1981

LAINSON, R. S., J. J. Evolution, classification and geographical distribution. In:W. K.-K. PETERS, R. **The Leishmaniases in Biology and Medicine**. London: Academic Press, 1987.p. 1-120.

LASKAY, T.; DIEFENBACH, A.; ROLLINGHOFF, M.; SOLBACH, W. "Early parasite containment is decisive for resistance to Leishmania major infection." **Eur J Immunol, 25**: 2220-7., 1995

LAUNOIS, P.; SWIHART, K. G.; MILON, G.; LOUIS, J. A. "Early production of IL-4 in susceptible mice infected with Leishmania major rapidly induces IL-12 unresponsiveness." **J Immunol, 158**: 3317-24., 1997.

LAUNOIS, P.; HIMMELRICH, H.; TACCHINI-COTTIER, F.; MILON, G.; LOUIS, J. A. "New insight into the mechanisms underlying Th2 cell development and susceptibility to Leishmania major in BALB/c mice." **Microbes Infect, 1**: 59-64., 1999.

LIRA, R.; DOHERTY, M.; MODI, G.; SACKS, D. "Evolution of lesion formation, parasitic load, immune response, and reservoir potential in C57BL/6 mice following high- and low-dose challenge with Leishmania major." **Infect Immun, 68**: 5176-82., 2000

LLANOS CUENTAS, E. A.; CUBA, C. C.; BARRETO, A. C.; MARSDEN, P. D. "Clinical characteristics of human Leishmania braziliensis braziliensis infections." **Trans R Soc Trop Med Hyg, 78**: 845-6, 1984

MAGILL, A. J.; GROGL, M.; JOHNSON, S. C.; GASSER, R. A., JR. "Visceral infection due to Leishmania tropica in a veteran of Operation Desert Storm who presented 2 years after leaving Saudi Arabia." **Clin Infect Dis, 19**: 805-6., 1994

MAROVICH, M. A.; MCDOWELL, M. A.; THOMAS, E. K.; NUTMAN, T. B. "IL-12p70 production by Leishmania major-harboring human dendritic cells is a CD40/CD40 ligand-dependent process." **J Immunol, 164**: 5858-65., 2000.

MARSDEN, P. D. "Mucosal leishmaniasis ("espundia" Escomel, 1911)." **Trans R Soc Trop Med Hyg, 80**: 859-76, 1986

MARTINEZ, J. E.; ALBA; ARIAS, L.; ESCOBAR, M. A.; SARAVIA, N. G. "Haemoculture of Leishmania (Viannia) braziliensis from two cases of mucosal leishmaniasis: re-examination of haematogenous dissemination." **Trans R Soc Trop Med Hyg, 86**: 392-4., 1992

MARZOCHI, M. C.; COUTINHO, S. G.; DE SOUZA, W. J.; DE TOLEDO, L. M.; GRIMALDI JUNIOR, G.; MOMEN, H.; PACHECO RDA, S.; SABROZA, P. C.; DE SOUZA, M. A.; RANGEL JUNIOR, F. B. et al. "Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, parasitological, therapeutical and epidemiological findings (1977-1983)." **Mem Inst Oswaldo Cruz, 80**: 349-57., 1985

MCNALLY, A. K. & ANDERSON, J. M. "Complement C3 participation in monocyte adhesion to different surfaces." **Proc Natl Acad Sci U S A, 91**: 10119-23., 1994

MELBY, P. C.; CHANDRASEKAR, B.; ZHAO, W.; COE, J. E. "The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response." **J Immunol, 166**: 1912-20., 2001

MENON, J. N. & BRETSCHER, P. A. "Parasite dose determines the Th1/Th2 nature of the response to Leishmania major independently of infection route and strain of host or parasite." **Eur J Immunol, 28**: 4020-8., 1998

MOLL, H. "Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis." **Immunol Today, 14**: 383-7., 1993

MOLL, H.; FLOHE, S.; BLANK, C. "Dendritic cells seclude Leishmania parasites that persist in cured mice- -a role in the maintenance of T-cell memory?" **Adv Exp Med Biol, 378**: 507-9, 1995

MOLL, H.; FUCHS, H.; BLANK, C.; ROLLINGHOFF, M. "Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells." **Eur J Immunol, 23**: 1595-601., 1993

NICOLAS, L.; SIDJANSKI, S.; COLLE, J. H.; MILON, G. "Leishmania major reaches distant cutaneous sites where it persists transiently while persisting durably in the primary dermal site and its draining lymph node: a study with laboratory mice." **Infect Immun, 68**: 6561-6., 2000

PEARSON, R. D. & SOUSA, A. Q. "Clinical spectrum of Leishmaniasis." **Clin Infect Dis, 22**: 1-13., 1996

PESSOA, S. B. & BARRETO, M. P. **Leishmaniose tegumentar americana.**Rio de Janeiro: Ministério da Educação e Saúde., 1948

PIMENTA, P. F.; SARAIVA, E. M.; SACKS, D. L. "The comparative fine structure and surface glycoconjugate expression of three life stages of Leishmania major." **Exp Parasitol, 72**: 191-204., 1991

PIMENTA, P. F.; TURCO, S. J.; MCCONVILLE, M. J.; LAWYER, P. G.; PERKINS, P. V.; SACKS, D. L. "Stage-specific adhesion of Leishmania promastigotes to the sandfly midgut." **Science, 256**: 1812-5., 1992

POULTER, L. W. & PANDOLPH, C. R. "Mechanisms of immunity to leishmaniasis. IV. Significance of lymphatic drainage from the site of infection." **Clin Exp Immunol, 48**: 396-402., 1982

PUENTES, S. M.; DA SILVA, R. P.; SACKS, D. L.; HAMMER, C. H.; JOINER, K. A. "Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9." **J Immunol, 145**: 4311-6., 1990

PUENTES, S. M.; SACKS, D. L.; DA SILVA, R. P.; JOINER, K. A. "Complement binding by two developmental stages of Leishmania major promastigotes varying in expression of a surface lipophosphoglycan." **J Exp Med, 167**: 887-902., 1988

REED, S. G.; ANDRADE, Z. A.; ROTERS, S. B.; INVERSO, J. A.; SADIGURSKY, M. "Leishmania mexicana amazonensis infections in 'resistant' inbred mice following removal of the draining lymph node." **Clin Exp Immunol, 64**: 8-13., 1986

REINER, S. L. & LOCKSLEY, R. M. "The regulation of immunity to Leishmania major." **Annu Rev Immunol, 13**: 151-77, 1995

RIBEIRO, J. M.; ROSSIGNOL, P. A.; SPIELMAN, A. "Role of mosquito saliva in blood vessel location." **J Exp Biol, 108**: 1-7., 1984

RIBEIRO, J. M. C. "Role of saliva in blood-feeding by arthropods." **Annu Rev Entomol, 32**: 463-478, 1987

SACKS, D. & KAMHAWI, S. "Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis." **Annu Rev Microbiol, 55**: 453-83, 2001

SACKS, D. L. "Metacyclogenesis in Leishmania promastigotes." **Exp Parasitol, 69**: 100-3., 1989

SACKS, D. L.; BARRAL, A.; NEVA, F. A. "Thermosensitivity patterns of Old vs. New World cutaneous strains of Leishmania growing within mouse peritoneal macrophages in vitro." **Am J Trop Med Hyg, 32**: 300-4., 1983

SACKS, D. L. & PERKINS, P. V. "Development of infective stage Leishmania promastigotes within phlebotomine sand flies." **Am J Trop Med Hyg, 34**: 456-9., 1985

SAHA, B.; DAS, G.; VOHRA, H.; GANGULY, N. K.; MISHRA, G. C. "Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on Leishmania-infected macrophages and its implication in the suppression of cell-mediated immunity." **Eur J Immunol, 25**: 2492-8., 1995

SCHARTON-KERSTEN, T. & SCOTT, P. "The role of the innate immune response in Th1 cell development following Leishmania major infection." **J Leukoc Biol, 57**: 515-22., 1995

SCHLEIN, Y.; BORUT, S.; GREENBLATT, C. L. "Development of sandfly forms of Leishmania major in sucrose solutions." **J Parasitol, 73**: 797-805., 1987.

SCHLEIN, Y. & JACOBSON, R. L.. Hunger tolerance and Leishmania in sandflies. **Nature, 414**: 168, 2001

SCHONLAU, F.; SCHARFFETTER-KOCHANEK, K.; GRABBE, S.; PIETZ, B.; SORG, C.; SUNDERKOTTER, C. "In experimental leishmaniasis deficiency of CD18 results in parasite dissemination associated with altered macrophage functions and incomplete Th1 cell response." **Eur J Immunol, 30**: 2729-40., 2000.

SCHUBACH, A.; MARZOCHI, M. C.; CUZZI-MAYA, T.; OLIVEIRA, A. V.; ARAUJO, M. L.; OLIVEIRA, A. L.; PACHECO, R. S.; MOMEN, H.; CONCEICAO-SILVA, F.; COUTINHO, S. G.; MARZOCHI, K. B. "Cutaneous scars in American tegumentary leishmaniasis patients: a site of Leishmania (Viannia) braziliensis persistence and viability eleven years after antimonial therapy and clinical cure." **Am J Trop Med Hyg, 58**: 824-7., 1998

SCOTT, P. "Impaired macrophage leishmanicidal activity at cutaneous temperature." **Parasite Immunol, 7**: 277-88., 1985

SCOTT, P. "IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis." **J Immunol, 147**: 3149-55., 1991

SHEVACH, E. M. Organ-specific autoimmunity. In: W. E. Paul. **Fundamental immunology.** New York: Lippincott -Raven, 1999. Chapter 34.

SPRINGER, T. A. "Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration." **Annu Rev Physiol, 57**: 827-72, 1995

STENGER, S.; DONHAUSER, N.; THURING, H.; ROLLINGHOFF, M.; BOGDAN, C. "Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase." **J Exp Med, 183**: 1501-14., 1996

SUTTERWALA, F. S. & MOSSER, D. M. "The taming of IL-12: suppressing the production of proinflammatory cytokines." **J Leukoc Biol, 65**: 543-51., 1999

TACCHINI-COTTIER, F.; ZWEIFEL, C.; BELKAID, Y.; MUKANKUNDIYE, C.; VASEI, M.; LAUNOIS, P.; MILON, G.; LOUIS, J. A. "An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major." **J Immunol, 165**: 2628-36., 2000

URMACHER, C. D. Normal skin. In:S. S. STENBERG. **Histology for pathologists**. Philadelphia: Lippincott-Raven, 1997.p. 25-45.

UZONNA, J. E. & BRETSCHER, P. A. "Anti-IL-4 antibody therapy causes regression of chronic lesions caused by medium-dose Leishmania major infection in BALB/c mice." **Eur J Immunol, 31**: 3175-84., 2001

UZONNA, J. E.; WEI, G.; YURKOWSKI, D.; BRETSCHER, P. "Immune elimination of Leishmania major in mice: implications for immune memory, vaccination, and reactivation disease." **J Immunol, 167**: 6967-74., 2001

VALENZUELA, J. G.; BELKAID, Y.; GARFIELD, M. K.; MENDEZ, S.; KAMHAWI, S.; ROWTON, E. D.; SACKS, D. L.; RIBEIRO, J. M. "Toward a defined anti-Leishmania vaccine targeting vector antigens: characterization of a protective salivary protein." **J Exp Med, 194**: 331-42., 2001

VOEHRINGER, D.; BLASER, C.; GRAWITZ, A. B.; CHISARI, F. V.; BUERKI, K.; PIRCHER, H. "Break of T Cell Ignorance to a Viral Antigen in the Liver Induces Hepatitis." **J Immunol, 165**: 2415-2422, 2000.

VON STEBUT, E.; BELKAID, Y.; JAKOB, T.; SACKS, D. L.; UDEY, M. C. "Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity." **J Exp Med, 188**: 1547-52., 1998.

VON STEBUT, E.; BELKAID, Y.; NGUYEN, B. V.; CUSHING, M.; SACKS, D. L.; UDEY, M. C. "Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis." **Eur J Immunol, 30**: 3498-506., 2000.

WARBURG, A. & SCHLEIN, Y. "The effect of post-bloodmeal nutrition of Phlebotomus papatasi on the transmission of Leishmania major." **Am J Trop Med Hyg, 35**: 926-30., 1986

WILSON, M. E.; YOUNG, B. M.; DAVIDSON, B. L.; MENTE, K. A.; MCGOWAN, S. E. "The importance of TGF-beta in murine visceral leishmaniasis." **J Immunol, 161**: 6148-55., 1998

ZIJLSTRA, E. E. & EL-HASSAN, A. M. "Leishmaniasis in Sudan. Post kala-azar dermal leishmaniasis." **Trans R Soc Trop Med Hyg, 95 Suppl 1**: S59-76., 2001

7 TRABALHO ANEXO

ALMEIDA, M. C.; CUBA-CUBA, C. A.; MORAES, M. A.; MILES, M. A. "Dissemination of Leishmania (Viannia) braziliensis." **J Comp Pathol, 115**: 311-316., 1996

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SHORT PAPER

Dissemination of *Leishmania (Viannia) braziliensis*

M. C. Almeida, C. A. Cuba-Cuba, M. A. P. Moraes and M. A. Miles*

*Laboratorios de Parasitologica e Patologia, Universidade de Brasilia, 70910-900 Brasilia, DF, Brazil and * Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK*

Summary

Destructive human mucocutaneous leishmaniasis may appear many years after the primary cutaneous infection with *Leishmania (Viannia) braziliensis*. Hamsters (*Mesocricetus auratus*) were infected with metacyclic *L. braziliensis* promastigotes. It was found that secondary metastatic visceral lesions could arise from a primary cutaneous lesion, or secondary cutaneous lesions from a primary visceral lesion. Parasites in the viscera were shown to be viable, multiplying and capable of metastasis to either secondary visceral or cutaneous sites. The finding of an early metastasis in the wall of a small cutaneous vessel indicates that dissemination can occur by the haematogenous route. Slow growing organisms in viscera may thus be a source for late metastasis to mucocutaneous sites or for systemic relapse after immunosuppression.

Leishmania (Viannia) braziliensis, the agent of cutaneous leishmaniasis, causes destructive mucosal disease in man, usually years after the primary cutaneous lesions. It has been suggested that early haematogenous dissemination of parasites to sites where they lie dormant for years might explain the origin of metastases to the mucosae (Costa *et al.*, 1986). In support of this hypothesis there have been reports of disseminated cutaneous disease in man (Costa *et al.*, 1986) and parasites have been demonstrated in healthy mucosal sites, in lymph nodes (Moraes *et al.*, 1993), in deep visceral sites (Souza *et al.*, 1989) and in circulating blood cells (Martinez *et al.*, 1992). Some of these observations were, however, sporadic and could not be confirmed by others (Silveira *et al.*, 1989).

The experimental disease due to *L. braziliensis* in hamsters (*Mesocricetus auratus*) has controversial aspects that parallel those found in the human disease. Thus, metastatic spread has been reported occasionally in hamsters (Kahl *et al.*, 1991); spread from primary cutaneous lesions to the viscera has been described by some authors (Peterson *et al.*, 1988) but not by others (Rey *et al.*, 1990); while some authors succeeded in producing disseminated disease after immunosuppression (Wilson and Lollini, 1980), with the demonstration of parasitized circulating blood cells. The inconsistent results in the hamster model may in part be due to the fact that only recently has a reliable system become available for culturing the infective (metacyclic) promastigote form of *L. braziliensis in vitro* (Almeida *et al.*, 1993).

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It is not clear whether parasites found in visceral sites in hamsters are shed continuously from cutaneous lesions, and are perhaps non-viable; or whether they are sequestered and multiplying *in situ*, with the capacity for further metastatic spread to form new visceral or cutaneous lesions. This report assesses the capacity of *L. braziliensis* to spread and describes some histopathological aspects of secondary lesions in hamsters. The WHO reference strain *L. braziliensis* MHOM/BR/83/LTB300 was used throughout.

In a first experiment, metacyclic promastigotes (500), obtained as described by Almeida *et al.* (1993), were injected into the left hind paw of 20 adult male hamsters. Three months later the animals were killed and aspirates from the liver were cultured on Difco USAMRU blood agar (Walton *et al.*, 1977). Smears from both liver and spleen were stained by Giemsa's method; tissue samples from the draining lymph nodes, liver and spleen were fixed in neutral 10% formalin and sections were stained with haematoxylin and eosin (HE). For the liver, 75% (15/20) of cultures, 25% (5/20) of Giemsa-stained smears, and 20% (4/20) of histopathological sections gave positive results. For the spleen, amastigotes were seen in 65% (13/20) of the smears and in 60% (12/20) of the sections. Of the draining lymph-node sections, 89% (16/18) contained demonstrable amastigotes.

In a second experiment, four adult male hamsters and one female were anaesthetized and inoculated in the spleen with 10^4 metacyclic promastigotes. The animals were examined weekly for cutaneous lesions. Aspirates from metastatic lesions were cultured on blood-agar. After 3 months, three hamsters were killed and aspirates from liver and spleen were cultured; in two of these three animals the number of parasites in the spleen was quantified by the agar-plating technique (Hill, 1983). The two remaining animals died before the third month of infection, but tissue samples were examined histopathologically. Three of the five hamsters inoculated in the spleen developed metastatic cutaneous lesions in the paws and cultures from these secondary sites gave positive results. All cultures of spleen and liver aspirates gave positive results. Of the two animals examined quantitatively, one (with no cutaneous lesions) had $2{\cdot}4 \times 10^7$ amastigotes in the spleen, and the other (with cutaneous metastasis) $5{\cdot}6 \times 10^7$. The three animals that developed metastatic cutaneous lesions also had lesions in the liver and lymph nodes. The two remaining animals had lesions in the liver and spleen and one also had lymph node lesions.

The microscopical findings in spleen, liver and lymph nodes were similar in both experiments. Histiocytic aggregates surrounded by plasma cells, a moderate number of reactive lymphocytes and a few eosinophils were found in the spleen and lymph nodes. In the lymph nodes the aggregates were found in the cortex and medulla, without accompanying follicular hyperplasia. In the spleen and lymph nodes, the histiocytic aggregates were less cohesive than in the liver and they were found chiefly in the red pulp. The randomly distributed hepatic lesions were slight, with small and compact histiocytic aggregates surrounded by plasma cells and rare eosinophils (Fig. 1A). The cutaneous metastic lesions (Fig. 1B) showed numerous polymorphonuclear cells, many with fragmentation, and numerous dispersed and richly parasitized macrophages. In one animal an early metastatic cutaneous lesion was found

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The availability of our in-vitro system for producing metacyclic promastigotes with the LBT300 strain (Almeida *et al.*, 1993) made possible this simple re-evaluation of the dissemination of *L. braziliensis*. The study confirmed that *L. braziliensis* could produce a secondary metastatic visceral lesion from a primary cutaneous lesion, and a secondary cutaneous lesion from a primary visceral lesion. The parasites in the viscera were viable, multiplying and capable of metastasis to secondary visceral and cutaneous sites. Metastatic visceral lesions were found irrespective of whether the primary site of inoculation was visceral or cutaneous (Schnur, 1976). Hill (1988) reported that *Leishmania amazonensis* amastigotes accumulated in the viscera as a result of being shed from primary cutaneous lesions; in our study, increase of *L. braziliensis* amastigotes in the viscera was explicable only by multiplication *in situ*. As far as we are aware, agents of New World cutaneous leishmaniasis have been isolated from deep visceral sites on only two occasions, *L. braziliensis* by Souza *et al.* (1989) and *L. amazonensis* by Barral *et al.* (1986), but lymphadenopathy due to *L. braziliensis* (and *Leishmania tropica*) has been reported in the absence of cutaneous lesions (Moraes *et al.*, 1993). The histopathological features of lymphadenitis in man, irrespective of the infecting *Leishmania* species, resembles that in hamsters, and is reminiscent of lymphadenitis due to *Toxoplasma gondii*, except that central necrosis of histiocytic aggregates is seen only in man; this might explain, at least in part, the more efficient elimination of organisms from man than from the hamster (Moraes *et al.* 1993). Growth of *L. braziliensis* is more abundant at cutaneous sites than at visceral sites and this may reflect the differential growth rates of viscerotropic and dermotropic *Leishmania* at temperatures above 34°C. However, tissue-specific aspects of host defence, such as the production of more granulomata in the liver than in the skin and the stronger tuberculoid reaction in draining lymph nodes than in skin (Ridley and Ridley, 1984), may play a role; such tissue-specific reactions are apparent in schistosomiasis (Hirata *et al.*, 1993). The scarcity of reports of "visceralization" of *L. braziliensis* may be due to the few attempts that have been made to isolate organisms from viscera and the difficulties associated with culture.

Dominant or quiescent forms are known in a variety of bacterial, fungal and protozoal infections, associated with a poor response to chemotherapy and a tendency to produce relapse. There is evidence that residual *Leishmania* infections can occur in healthy human nasal mucosa, in lymph nodes in the absence of an associated cutaneous lesion, in scar tissue, and in the mouse model (Aebischer *et al.*, 1993; Moraes *et al.*, 1993). By the polymerase chain reaction, *L. braziliensis* has even been detected in the blood of patients considered to have been cured, or in the blood of persons resident in endemic areas but never known to have suffered from leishmaniasis (Guevara *et al.*, 1994). In mice, hamsters and man, infections with *Leishmania* spp. (including *L. braziliensis*), considered to be clinically cured, may relapse if the host becomes immunosuppressed (Wilson and Lollini, 1980; Rossell *et al.*, 1992). Disseminated disease due to *L. braziliensis* has been reported in AIDS patients (Machado *et al.*, 1992), and visceral leishmaniasis due to *Leishmania infantum* is considered an opportunistic disease in Europe (Gorgolas and Miles, 1994).

Our findings support the hypothesis that quiescent or slow-growing organisms in deep visceral sites are a source for late metastasis to mucocutaneous sites and for systemic relapse after immunosuppression. The discrete early cutaneous metastasis in the wall of a small cutaneous vessel (Fig. 1C) clearly supports the view that metastasis occurs by the haematogenous route (Martinez *et al.*, 1992; Bertho *et al.*, 1994).

Acknowledgments

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References

Aebischer, T., Moody, S. F. and Handman, A. (1993). Persistence of virulent *Leishmania major* in murine cutaneous leishmaniasis: a possible hazard for the host. *Infection and Immunity*, **61**, 220–226.

Almeida, M. C., Cuba-Cuba, C. A., Sá, C. M., Pharoah, M. M., Howard, L. M. and Miles, M. A. (1993). Metacyclogenesis of *Leishmania (Viannia) braziliensis*. *In vitro* evidence that lentil lectin is a marker of complement resistence and enhanced infectivity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 325–329.

Barral, A., Badaro, R., Barral-Netto, M., Grimaldi, G., Momen, H. and Carvalho, E. M. (1986). Isolation of *Leishmania mexicana amazonensis* from bone marrow in a case of American visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene*, **35**, 732–734.

Bertho, A. L., Santiago, M. A. and Coutinho, S. G. (1994). An experimental model of the production of metastases in murine cutaneous leishmaniasis. *Journal of Parasitology*, **80**, 93–99.

Costa, J. M. L., Marsden, P. D., Llanos-Cuentas, E. A., Netto, E. M., Carvalho, E. M., Barral, A., Rosa, A. C., Cuba, C. C., Magalhaes, A. V. and Barreto, A. C. (1986). Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. *Journal of Tropical Medicine and Hygiene*, **89**, 313–323.

Gorgolas, M. de and Miles, M. A. (1994). Visceral leishmaniasis and AIDS. *Nature*, **372**, 734.

Guevara, P., Rojas, E., Gonzalez, N., Scorza, J. V., Anez, N., Valera, M. and Ramirez, J. L. (1994). Presence of *Leishmania braziliensis* in blood samples from cured patients or at different stages of immunotherapy. *Clinical and Diagnostic Laboratory Immunology*, **1**, 385–389.

Hill, J. O. (1983). Quantitation of *Leishmania tropica major* by its ability to form distinct colonies on agar-based media. *Journal of Parasitology*, **69**, 1068–1071.

Hill, J. O. (1988). Pathophysiology of experimental leishmaniasis. The role of parasite physiology in the development of metastatic disease. *American Journal of Tropical Medicine and Hygiene*, **39**, 256–260.

Hirata, M., Takushima, M., Kage, M. and Fukuma, T. (1993). Comparative analysis of hepatic pulmonary and intestinal granuloma formation around freshly laid *Schistosoma japonicum* eggs in mice. *Parasitology Research*, **79**, 316–321.

Kahl, L. P., Byram, J. E., David, J. R., Comerford, S. A. and Von Lichtenberg, F. (1991). *Leishmania (Viannia) braziliensis*: comparative pathology of golden hamsters infected with isolates from cutaneous and mucosal lesions of patients residing in Tres Bracos, Bahia, Brazil. *American Journal of Tropical Medicine and Hygiene*, **44**, 218–232.

Machado, E. S., Braga, M. P., Cruz, M. A., Coutinho, S. G., Vieira, A. R. V., Rutowitsch, M. S., Cuzzi-Maya, T., Grimaldi, G. Jr and Menezes, J. A. (1992).

Disseminated American mucocutaneous leishmaniasis caused by *Leishmania braziliensis braziliensis* in a patient with AIDS: a case report. *Memórias do Instituto Oswaldo Cruz*, **87**, 487–492.

Martinez, J. E., Arias, A. L., Escobar, M. A. and Saravia, N. G. (1992). Haemoculture of *Leishmania (Viannia) braziliensis* from two cases of mucosal leishmaniasis: re-examination of haematogenous dissemination. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 392–394.

Moraes, M. A. P., Corrieia Filho, D. and Santos, J. B. (1993). Linfadenopatias na leishmaniose tegumentar americana. Consideraçoes sobre dois casos. *Revista da Sociedade Brasileira de Medicina Tropical*, **26**, 181–185.

Peterson, N. E., Costa, M. A. F., Vexenat, J. A., Netto, E. M., Marsden, P. D., Magalhaes, A. V. and Barreto, A. C. (1988). Recovery of *Leishmania (Viannia) braziliensis* from inoculated hamsters. *Journal of Parasitology*, **74**, 512–514.

Rey, J. A., Travi, B. L., Valencia, A. Z. and Saravia, N. G. (1990). Infectivity of the subspecies of the *Leishmania braziliensis* complex *in vivo* and *in vitro*. *American Journal of Tropical Medicine and Hygiene*, **43**, 423–631.

Ridley, D. S. and Ridley, M. J. (1984). Late cutaneous leishmaniasis: immunopathology of tuberculoid lesions in skin and lymph nodes. *British Journal of Experimental Pathology*, **65**, 337–346.

de Rossell, R. A., de Duran, R. J., Rossell, O. and Rodriguez, A. M. (1992). Is leishmaniasis ever cured? *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 251–253.

Schnur, L. F. (1976). The dissemination of American slow and fast leishmanias in Syrian hamsters. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **74**, 277–278.

Silveira, F. T., Lainson, R., Shaw, J. J., Ishikawa, E. A., Souza, A. A. A. and Braga, R. R. (1989). Sobre a sensibilidade da cultura de leucocitos circulantes na deteçao de *Leishmania* no sangue periférico de pacientes com leishmaniose tegumentar. *Revista da Sociedade Brasileira Medicina Tropical*, **22**, 143–146.

Souza, A. Q., Façanho, M. C., Pompeu, M. L., Vasconcelos, I. A. B., Borges Filho, G. M., Homem, H. and Vasconcelos, A. W. (1989). Visceral leishmaniasis in Ceará caused by *Leishmania braziliensis braziliensis*. *Memórias do Instituto Oswaldo Cruz*, **84**, Suppl. II, 35.

Walton, B. C., Shaw, J. J. and Lainson, R. (1977). Observations on the *in vitro* cultivation of *Leishmania braziliensis*. *Journal of Parasitology*, **63**, 1118–1119.

Wilson, H. R. and Lollini, L. O. (1980). *Leishmania braziliensis braziliensis*: metastatic infection in golden hamsters. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **74**, 833.

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