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"Infecção pela *Leishmania chagasi*: papel dos receptores Toll-like 2 e 4 e alterações genotóxicas em camundongos BALB/c"

Dissertação apresentada ao Programa de Pós-Graduação em Doenças Tropicais da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP, para obtenção do título de mestre.

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Introdução

Introdução

As leishmanioses têm como agentes etiológicos, protozoários intracelulares obrigatórios pertencentes à ordem *Kinetoplastida,* família *Trypanosomatidae* e gênero *Leishmania.* Existem mais de 20 espécies deste protozoário, que podem ser transmitidas ao homem por, aproximadamente, 30 diferentes espécies de flebotomíneos[1]. A leishmaniose visceral é causada pela *Leishmania infantum (L. infantum)* e *Leishmania donovani (L.donovani)* no Velho Mundo; e pela *Leishmania chagasi (L.chagasi)* no Novo Mundo, mais especificamente na América do Sul[2,3].

De acordo com a Organização Mundial de Saúde (OMS), a leishmaniose visceral é uma antropozoonose endêmica, que apresenta ampla distribuição mundial em 88 países, com prevalência superior a 12 milhões de indivíduos, 1,5 a 2 milhões de casos novos por ano e 350 milhões de pessoas habitando áreas de risco. Com esses dados, a leishmaniose tornou-se, mundialmente, uma das 6 mais importantes doenças infectoparasitárias[4-12]. Cerca de 90% dos casos do mundo concentram-se em Bangladesh, nordeste da Índia, Nepal e Sudão (Velho Mundo) e nordeste do Brasil (Novo Mundo). No Brasil, ocorrem de 3000 a 5000 casos por ano, com cerca de 4000 novos casos nos últimos anos[13]. A maior incidência está no Nordeste, com 92% do total das notificações, seguida pelas regiões Sudeste (4%), Norte (3%) e Centro-Oeste (1%). O primeiro caso humano autóctone confirmado no estado de São Paulo ocorreu no município de Araçatuba em 1999[14]. Desde então, a transmissão da doença vem sendo descrita em vários municípios, de todas as regiões do Brasil, exceto na região Sul. A doença ocorre em zonas rurais, periurbanas e

urbanas de grandes centros como Rio de Janeiro (RJ), Belo Horizonte (MG), Araçatuba, Bauru, Marília, Presidente Prudente (SP), Corumbá (MS) entre outros. A leishmaniose visceral está registrada em 19 dos 27 estados do Brasil[15].

O ciclo de vida da leishmania ocorre nos hospedeiros vertebrados, como nos roedores, homens e canídeos, estes últimos são os principais reservatórios; e nos invertebrados, como dípteros hematófagos da família *Phlebotomidae*, a qual pertencem os gêneros *Phlebotomus* e *Lutzomyia*[16,17]. A espécie *Lutzomyia longipalpis*, encontrada no Novo Mundo, é o vetor da leishmaniose visceral americana (LVA), apresenta-se como pequeno mosquito de 2 a 3 mm, de hábitos peridomésticos e intradomeciliares[18].

A leishmania pode se apresentar sob duas morfologias: a promastigota flagelar, encontrada no intestino dos vetores e a amastigota, que se desenvolve intracelularmente no hospedeiro vertebrado. Somente as fêmeas dos flebotomíneos transmitem a doença por inoculação das formas promastigotas na pele, estas, por sua vez, são internalizadas por células dendríticas e macrófagos da derme e transformam-se em amastigotas pela perda dos flagelos. Elas multiplicam-se e sobrevivem nos fagolisossomos através de uma complexa interação parasita – hospedeiro. As amastigotas disseminam-se através dos sistemas vasculares e linfáticos e infectam outros monócitos e macrófagos do sistema retículo-endotelial, resultando em infiltração da medula óssea, hepatoesplenomegalia e, em algumas vezes, aumento dos l infonodos[19-21].

A imunidade protetora na leishmaniose está associada com eficiente resposta imune celular, e é caracterizada pela produção de citocinas, tais como, IFN-γ, TNF-α e IL-12[22,23]. Inicialmente, após a fagocitose da forma promastigota, os macrófagos e as células dendríticas liberam IL-12, que estimula as células Natural Killer (NK) a secretar, precocemente, o IFN-γ. Em combinação com esta citocina, a IL-12 induz a diferenciação e proliferação das células $TCD4^{+}$Th1, as quais produzirão mais IFN-γ. Macrófagos infectados secretam TNF-α, que age em sinergismo com IFN-γ para ativar e induzir a atividade antiparasitária desta célula[1,24,25]. Uma vez ativado, o macrófago é capaz de produzir moléculas efetoras tóxicas aos parasitas, levando-os à destruição.

A IL12 bioativa (IL-12 p70) é um heterodímero consistindo das subunidades p35 e p40. É a principal citocina envolvida na diferenciação das células T em Th1 e na proliferação das mesmas[26-29]. Experimentos realizados com camundongos "*knockout*" para as subunidades da IL-12 têm mostrado, que a presença desta citocina é crucial para montar uma resposta protetora (Th1) contra leishmania, pois induz a produção de IFN-γ, levando ao controle e cura da infecção[3]. Vários estudos demonstram que o IFN-γ é a principal citocina responsável pela indução da atividade leishmanicida de macrófagos[30-32].

O TNF-α é produzido por macrófagos ativados, possui efeitos pleiotróficos e age em conjunto com IFN-γ para exercer a atividade leishmanicida mediada pelo óxido nítrico (NO)[33-38]. Wilhelm *et al.*[33]

demonstraram que camundongos B6. $TNF^{-/-}$ infectados pela *Leishmania major (L.major)* não controlaram o crescimento dos parasitas no baço, sugerindo que o controle da replicação da leishmania é dependente da produção de TNF-α e que não poderia ser compensado por outros mecanismos[33].

Os dois principais mediadores responsáveis pela destruição de parasitas, dentro de macrófagos ativados, são os radicais de oxigênio e nitrogênio, formados pela oxidase (NADPH) do "*burst"* respiratório e pela óxido nítrico sintase induzida (iNOS), respectivamente. Os intermediários reativos do oxigênio envolvidos na destruição das leishmanias, incluem ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e radical hidroxila (OH^-). O principal intermediário reativo do nitrogênio envolvido no controle deste patógeno é o NO[39,40].

Inibidores da iNOS, como N^G- monometil- arginina ($_L$-NMMA), impedem a produção de NO, e consequentemente, aumenta a sobrevivência e replicação das amastigotas em macrófagos murinos[39,41]. Camundongos deficientes de iNOS são suscetíveis à leishmaniose, demonstrando, dessa forma, o envolvimento dos radicais do nitrogênio na destruição da leishmania *in vivo*[42].

A leishmaniose visceral humana é caracterizada por depressão da imunidade celular e exacerbação da resposta imune humoral. A presença de anticorpos é útil para o diagnóstico sorológico, entretanto não desempenha papel protetor e, de fato, estão associados com as formas de não cura da leishmaniose[43,44]. Indivíduos capazes de controlar a infecção apresentaram resposta Th1 específica com produção de IFN-γ e IL-12[45,46]. Por outro lado,

pacientes que desenvolveram leishmaniose visceral apresentaram resposta Th1 comprometida e produção de IL-10 aumentada[47,48].

O modelo de leishmaniose visceral mais estudado é com a linhagem de camundongo BALB/c infectados com *L. donovani* ou *L. chagasi*. Apesar de esta linhagem ser considerada suscetível, a infecção progride durante as duas primeiras até a quarta semana, e então é controlada pela resposta imune do hospedeiro[49].

A suscetibilidade à leishmaniose é, usualmente, relacionada à indução de resposta Th2 e consequente produção de IL-4, e de outros componentes que desativam macrófagos, como IL-10, TGF-β e prostanglandina E2. A inibição da produção de RNA mensageiro (RNAm) da iNOS em macrófagos tem sido associada com a produção de TGF-β e IL-10, citocinas relacionadas com exacerbação da doença[51,52].

Estudos realizados com pacientes apresentando leishmaniose visceral aguda demonstraram, que os mesmos apresentaram níveis plasmáticos, simultaneamente, elevados de IFN-γ e IL-10, e níveis inalterados de IL-12 [19,52,53]. A produção de IL-10 poderia influenciar o curso da infecção, por desativar macrófagos, inibindo a produção de TNF-α e de NO. Além disso, a IL-10 pode inibir parcialmente a produção de IFN-γ, acentuando a desativação macrofágica e a persistência do parasita nessas células [52,54,55].

A IL-10 também apresenta papel benéfico em algumas infecções causadas por protozoários, como observado no estudo de Gazzinelli *et al.*[56], os quais sugeriram, que esta citocina apresenta papel protetor contra os efeitos

prejudiciais da resposta imune celular exacerbada durante a infecção aguda pelo *Toxoplasma gondii (T.gondii),* já que camundongos IL-10$^{-/-}$ morreram após a produção de níveis elevados de citocinas inflamatórias, como IL-12, TNF-α e IFN-γ[56].

Alguns estudos demonstraram que o TGF-β estimula a progressão ou impede a cura da leishmaniose em modelos murinos[57-61]. Esta citocina altera o fenótipo inato do macrófago, por inibir a produção de TNF-α, iNOS e também, de IFN-γ pelas células NK, além de aumentar a expressão da arginase (enzima que cataliza a conversão de L-arginina em L-ornitina e uréia). Com a diminuição da iNOS e aumento da arginase há diminuição de NO e aumento das poliaminas, favorecendo o crescimento do parasita[57,62-64]. Barral-Neo *et al.*[59] relataram, em modelo de leishmaniose visceral murina, que o TGF-β pode representar um meio de suprimir a resposta Th1, de forma independente da expressão das citocinas Th2, ou seja, o TGF-β pode submodular as respostas Th1, causando suscetibilidade à doença

Estudo realizado com cultura de macrófagos humanos infectados pela *L.chagasi* demonstrou que a fagocitose estimulou o aumento de TGF-β biologicamente ativo, sem alterar a quantidade de RNAm sintetizado[57]. Alguns relatos utilizando camundongos infectados pela *L.chagasi* também observaram presença de níveis elevados de TGF-β [65,73]. Estes autores afirmam que o TGF-β tem capacidade de inibir a ativação de macrófagos e a proliferação e diferenciação das células T, além de contrabalancear os efeitos das citocinas proinflamatórias[57,65].

Belkaid *et al.*[66] observaram que durante a fase crônica da infecção pela *L.major*, aproximadamente, metade das células $TCD4^+$ presentes no sítio da infecção era Treg $CD25^+$ $Foxp3^+$ antígeno específicas, as quais inibiam a resposta das células T $CD25^-$ efetoras, através de mecanismos dependentes e independentes de IL-10. As células T regulatórias (Treg) produziram IL-10, citocina responsável pela manutenção da infecção crônica, enquanto que as células T $CD25^-$ produziram IFN-γ.

Atualmente, outra população de células estudada é a T helper 17 (Th17), independentemente regulada pelas células $TCD4^+$ e caracterizada como produtora de citocinas da família da IL-17, tais como IL-17, IL-21, IL-22, IL-23 e IL-26. Estas citocinas são altamente proinflamatórias, estimulam à produção de IL-6, TNF-α e quimiocinas pelas células endoteliais, epiteliais e monócitos, sendo também responsáveis por recrutar células para o sítio infeccioso [67-71].

Pitta *et al.*[67] mostraram que células mononucleares do sangue periférico (PBMCs) de indivíduos expostos à *L.donovani* induziram fortemente a expressão de IL-17 e IL-22. Essas citocinas poderiam atrair fortes efetores da imunidade inata e, posteriormente, recrutar células Th1 que, por sua vez, aumentam a atividade microbicida dos fagócitos. Portanto, de acordo com este autor, as células Th17 e Th1 poderiam desempenhar papéis complementares na proteção contra *L.donovani.*

O reconhecimento inicial de microrganismos é mediado por receptores celulares expressos em células da imunidade inata. Entre esses receptores destacam-se os semelhantes à toll (TLRs, toll-like receptors), através dos quais

as células são capazes de reconhecer microrganismos com conseqüente estimulação da fagocitose, ativação microbicida e produção de citocinas [72,73].

Os TLRs, proteínas transmembrânicas tipo I, evolutivamente conservados entre insetos e mamíferos, também encontrado em plantas, foram primeiramente identificados como molécula essencial para polarização dorsoventral durante o desenvolvimento embrionário de *Drosophila* e, posteriormente, mostrou ser importante na resposta imune antifúngica de moscas adultas[74-77]. Esses receptores são expressos por macrófagos, células dendríticas imaturas, mastócitos, eosinófilos, neutrófilos, linfócitos T e B, além das células epiteliais, endoteliais, cardiomiócitos e adipócitos[74,78,79].

Os TLRs caracterizam-se por apresentar dois domínios, o extracelular, que contém repetições ricas em leucina (LRR) e o intracelular, que possui homologia com o receptor para interleucina 1, denominado Toll/IL-1R (TIR)[74,80,81].Todo TLR sinaliza através da proteína adaptadora MyD88 (fator de diferenciação mielóide), que também contém o domínio Toll/IL-1R[82]. Após ativação do MyD88 pode ocorrer translocação do fator NF-kβ para o núcleo, com consequente transcrição dos genes de citocinas e enzimas. Além disso, a via dependente de TRIF pode também ser ativada, a qual interage com TRAF 6 e RIF 1, mediando assim a ativação de NF-kβ[82]. O NF-Kβ, maior fator de transcrição e regulador pleiotrópico na expressão de muitos genes, tem a função de ligar-se à sequência alvo de DNA para iniciar o processo de transcrição[83].

Esses receptores reconhecem certos constituintes microbianos denominados padrões moleculares associados ao patógeno (PAMPs), que são moléculas produzidas e conservadas pelos microorganismos, e não pelas células do hospedeiro, sendo essenciais para o metabolismo e sobrevivência do patógeno[84]. O alto grau de conservação dos TLRs ocorre devido a não mutagenicidade dos PAMPs[85,86]. Os TLRs reconhecem, individualmente, um repertório distinto, mas limitado de PAMPs, dentre os exemplos de pares ligantes bem caracterizados citamos TLR-4 e LPS, TLR-5 e flagelina, TLRs-1/2/6 e lipoproteínas e TLRs – 3/7/8/9 e diferentes ácidos nucléicos [87,88].

A estimulação dos TLRs por produtos microbianos induz a síntese de substâncias antimicrobicidas e citocinas proinflamatórias, bem como, a ativação de células dendríticas, as quais regulam positivamente moléculas co-estimulatórias e aumenta a expressão de antígenos da molécula do Complexo Principal de Histocompatibilidade (MHC), que consequentemente, torna mais efetiva à apresentação de antígenos aos linfócitos T e B. Quando a resposta imune inata não for suficiente para eliminar a infecção, ocorre a indução da resposta imune adaptativa. As células apresentadoras de antígenos (APCs), quando ativadas pelos TLRs, liberam níveis elevados de citocinas proinflamatórias, como o TNF-α, IL-6, interleucina 8 (IL-8) e IL-12, quimiocinas e óxido nítrico. Ocorre também aumento da expressão de moléculas co-estimulatórias como, o CD40, CD80 e CD86. Estas alterações na função das APCs induzem resposta imune adaptativa e determinam a diferenciação das células T em Th_1, que promovem imunidade celular ou em Th_2, que direcionam resposta humoral[79].

A superfície celular da leishmania é composta por moléculas ligada as GPI (glicosilfosfatidilinositol), que são responsáveis pela ativação dos TLRs. Nas formas promastigotas, as principais moléculas que se ligam às GPIs são as lipofosfoglucanas (LPGs), as quais são consideradas fator de virulência multifuncional necessárias para o estabelecimento da infecção[89-93]. Outras moléculas presentes na superfície da leishmania como: glicoproteína gp63, proteofosfoglucanas e fosfatases ácidas estão diretamente ligadas à patogenicidade e sobrevivência do parasita. Contudo não se sabe se desempenham algum papel nas interações com o hospedeiro[93-97].

As LPGs de *L.major* estimulam células NK humanas e macrófagos de camundongos, através da ligação ao TLR-2[98,99]. Becker *et al.*[99] relataram que LPGs purificadas das formas promastigotas metacíclicas e procíclicas de *L.major* estimularam células NK humanas, levando a translocação nuclear do NF-KB, com aumento da produção de IFN-γ e TNF-α, bem como, da expressão de RNA mensageiro do TLR-2.

De Veer *et al.*[98] mostraram que camundongos deficientes da proteína MyD88 são mais suscetíveis a infecção pela *L. major*. Estes autores ainda demonstraram que a LPG deste parasita através da ligação ao TLR-2 estimulou macrófagos a secretar citocinas, como IL-12 e TNF-α, provavelmente via proteína MyD88. Esses achados sugerem o importante papel dos TLRs na resposta imune inata contra *L. major* e que a MyD88 é essencial para gerar resposta imune protetora.

Recentemente, Flandin *et al.*[100] demonstraram que macrófagos estimulados com IFN-γ, induziram expressão de TLR-2 e TLR-3, os quais atuaram no reconhecimento das formas promastigotas da *L. donovani*. Além disso, foi detectado o envolvimento destes receptores na indução da produção de NO e TNF-α.

Kropf *et al.*[101] observaram, em experimentos *in vivo* com camundongos, o envolvimento do TLR-4 no controle da infecção pela *L. major*, possivelmente, através da indução da expressão da iNOS. A ativação da iNOS leva a síntese de NO e destruição da leishmania. Camundongos TLR-4 competentes apresentaram correlação da elevada destruição do parasita com aumento da expressão da iNOS, já camundongos TLR-4 deficientes foram mais permissivos ao crescimento da leishmania. Neste sentido, esses autores confirmaram a importância do TLR-4 no desenvolvimento da resposta imune inata contra a infecção pela *L. major.*

A infecção pela leishmania pode estar associada a mutações no DNA, decorrentes de alterações na resposta imune, podendo ser responsável pelo aumento da suscetibilidade a transformações malignas[102-105].

Tem sido relatado que diversos agentes infecciosos são carcinogênicos[106-108]. Com base nesses estudos, estimou-se no ano de 2000 que 1,8 milhões de casos de câncer são decorrentes de agentes infecciosos, a maior parte relacionada a viroses, como a causada pelo Papillomavirus, vírus da hepatite C e, em menor proporção, às infecções bacterianas e parasitárias causadas pelo *Mycobacterium* sp*, Helicobacter pylori* (*H.pylori*) *e*

Trypanossoma cruzi (T.cruzi)[109-111]. Além disso, associação entre leishmaniose e câncer tem sido demonstrada através do envolvimento direto deste parasita na ocorrência de lesões malignas, especialmente de pele e membranas mucosas e também, como fator que predispõe ao câncer[104-105].

O NO é uma importante molécula efetora produzida pela iNOS, principalmente quando macrófagos são ativados por citocinas. O mecanismo de ação desta molécula para destruir a leishmania não está completamente elucidado, mas acredita-se que possa atuar, em conjunto com as espécies reativas do oxigênio para danificar o DNA mitocondrial, proteínas e lipídeos[112-115]. Este metabólito pode atuar covalentemente com o ferro intracelular, reagindo com grupos prostéticos Fe-S de enzimas sensíveis como a aconatase, complexo I e II de cadeia de transporte de elétron mitrocondrial, entre outras, resultando na formação do complexo ferro-nitrosil, inativação e degradação destas enzimas, com conseqüente parada da replicação do parasita. Outro efeito leishmanicida do NO é a fragmentação do DNA, característica do processo de morte celular programada ou apoptose[116]. Desta forma, estudos têm demonstrado que o controle da infecção pela leishmania é dependente da produção de NO[39,117]. No entanto, a elevada expressão da iNOs em resposta a estímulos inflamatórios, resulta na produção de elevadas quantidades de NO, sendo este processo envolvido na patogênese da leishmaniose[118].

Vários estudos têm verificado que o NO é produzido primariamente para atacar microrganismos ou outros corpos estranhos por nitração, oxidação e clorinação[5,119]. Entretanto, a expressão da iNOs e geração de níveis elevados

de NO durante a inflamação podem causar alterações no DNA da célula infectada, como também induzir danos em células adjacentes do hospedeiro. Acúmulo de danos no DNA, ao longo do tempo, pode causar modificações nas células, as quais podem tornar-se mutagênicas ou carcinogênicas[120]. Vários estudos têm relatado a capacidade de o NO promover mudanças no DNA através da oxidação do DNA e nitrosilação protéica [119,121-124].

Na leishmaniose cutânea, Kocyigit *et al.*[5] mostraram que os radicais do oxigênio produzidos pelo hospedeiro, durante a infecção, induziram danos no DNA em células infectadas ou adjacentes. Em modelo experimental de infecção aguda com *T.cruzi* foram observados níveis elevados de danos no DNA, detectados pelo ensaio do cometa em células do baço e coração de camundongos, indicando que provavelmente, os altos níveis de NO detectados estariam relacionados com tais danos[108].

Em conjunto, a análise dos trabalhos da literatura mostra, de forma consistente, que o perfil de citocinas presente na interação entre hospedeiro e leishmania pode ser eficiente para eliminação do parasita ou permitir sua multiplicação nos tecidos do hospedeiro, causando doença progressiva. Apesar dos mecanismos celulares e moleculares desse processo não estarem totalmente compreendidos, nos últimos anos, trabalhos têm mostrado o envolvimento dos TLRs na interação parasita-hospedeiro. No entanto, não existem na leishmaniose visceral, estudos com o objetivo de avaliar a expressão desses receptores, correlacionando com a produção de citocinas e NO durante a infecção. Além disso, não há relatos na literatura a respeito de alterações genotóxicas causadas pela *L.chagasi*.

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Capítulo I

Receptors and cytokines in leishmaniasis

Analysis of the expression of Toll-Like Receptors 2 and 4 and cytokine profile during *Leishmania chagasi* experimental infection

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Abstract

Toll-like receptors (TLRs) present in innate immune cells recognize pathogen molecules and influence on immunity to control the host-parasite interaction. Our objective was to evaluate the mRNA expression of TLR-2 and 4, expression and production of IL-12, IFN-γ, TNF-α, IL-17, IL-10 and TGF-β and NO production during infection with *Leishmania chagasi* and correlate TLR2 and 4 expressions with cytokines production and NO. Infection resulted in increased TLR2, TLR4, IL-17, TNF-α and TGF-β expression at the beginning of infection, with a decrease at the final phase in according the parasitic load; IFN-γ and IL-12 decreased at the peak of parasitemia and increased at the final phase; IL-10 increased during the whole period under analysis. With respect to cytokines and NO production, TGF-β, TNF-α and IL-17 showed high rates at the initial phase, and IFN-γ and IL-12 showed high rates at the final phase; IL-10 and NO showed increasing production during the infection period evaluated. There was a positive correlation of TLR2 and 4 with TNF-α, IL-17, NO, IL-10 and TGF-β at the beginning of infection, and with TNF-α, IL-17 and TGF-β at the end. Our data suggest that *L. chagasi* was in contact with host's cells via TLR2 and 4, which resulted in cytokine modulation. This interaction could be considered as pathogenic mechanism in visceral leishmaniasis.

Keywords: *Leishmania chagasi* - cytokines - TLR2 - TLR4

Introduction

Leishmaniasis still represents a serious public-health problem. It is endemic in 88 countries, affecting 12 million people worldwide. Its visceral form, caused by *L. donovani, L. infantum and L. chagasi* (species present in Brazil), is prevalent in tropical and subtropical regions, and 90% of cases occur in Bangladesh, Brazil, India, Nepal and Sudan (Desjeux 2001, Verma et al. 2010, Goto & Prianti 2009). The infection may be asymptomatic, oligosymptomatic or progressive, and the most severe cases result in splenomegalia, fever, pancytopenia, hypergammaglobulinemia and weight loss (Badaro et al. 1986). During active visceral leishmaniasis, the parasite multiplies within cells of the mononuclear phagocyte system in the spleen, liver and bone marrow, thus becoming fatal if untreated (Goto & Prianti 2009).

Protective immunity is associated with efficient cellular response and the production of cytokines such as IL-12, IFN-γ and TNF-α (Dye 1996, Pinelli et al. 1995). Initially, after promastigote phagocytosis, dendritic cells and macrophages release IL-12, which stimulates NK cells to precociously secrete IFN-γ. The latter induces differentiation and proliferation of $TCD4^{+}$Th1 cells, which will produce more IFN-γ (Cunningham 2002). This cytokine is responsible for activating macrophages. They begin to produce TNF-α, which acts in synergism with IFN-γ, thus increasing phagocytosis and inducing leishmanicidal activity by these cells through the production of metabolites from nitrogen (NO) and oxygen (Cunningham 2002, Ray et al. 2000, Belosevic et al. 1989, Bodgan

et al. 1990, Liew et al. 1990, Theodos et al. 1991, Wilhelm et al. 2001). On the other hand, susceptibility in leishmaniasis is associated with the Th2 profile, characterized by the predominance of the humoral response and cytokine production, such as that of IL-4 and IL-10 (Agnello et al. 2003, Sharma & Singh 2009). Immunoregulatory mechanisms, which favor the pathogen's resistance, immunopathologic control and establishment of chronic infection, occur when another population of cells, referred to as Treg, prevails. Such cells produce macrophage-deactivating cytokines, such as IL-10 and TGF-β, and are responsible for subregulating the Th1 and Th2 responses (Sharma & Singh 2009, Nylén et al. 2007, Belkaid et al. 2002, Suffia et al. 2006, Gantt et al. 2003). At present, the Th1 and Th2 paradigm is being reconsidered following the discovery of a novel lineage of effector $CD4^+$ T helper lymphocytes, called Th17 cells, wich produce IL-17 A and F, IL-21, IL-22, IL-23, IL-26 and TNF-α (Guedes et al. 2010, Dong 2008). The Th17 has been linked to the pathogenesis of several diseases inflammatory and autoimmune diseases, such as psoriasis, rheumatoid arthritis, colitis and toxoplasmosis (Guedes et al. 2010, Tesmer 2008). Only a few studies have assessed the role of IL-17 in protozoan infection, and it is not known whether this cytokine participates as defense mechanisms or in pathogenesis of these infections (Bacellar et al. 2009)

The specific detection of microorganisms by innate immunity cells is mediated by pattern recognition receptors (PRRs), germ line-encoded receptors that recognize microbial structures referred to as pathogen-associated molecular pattern (PAMPs) (Medzhitov 2007). Toll-Like Receptors (TLR) are essential PRR that mediate recognition of microbicidal structures, such as those

of parasites, as well as to induce the subsequent inflammatory and adaptive responses (Tuon et al. 2008). Some studies have shown the recognition of leishmania molecules by some members of TLRs (de Veer et al. 2003, Becker et al. 2003, Flandin et al. 2006). For instance, LPG of *L. major* stimulated macrophages to secrete cytokines, such as IL-12 and TNF-α, by bonding to TLR2 (de Veer et al. 2003). Another study has also shown that IFN-γ-stimulated macrophages induced TLR-2 and 3 expression, which participated in the recognition of promastigote forms of *L. donovani*. Additionally, the involvement of such receptors in the induction of NO and TNF-α production has been shown (Flandin et al. 2006). Similarly, Kropf et al. (2004) observed, in *in-vivo* experiments with mice, the involvement of TLR-4 in the control of *L. major* infection through iNOS increase, thus inducing NO synthesis.

In view of such observations and in the absence of information during experimental visceral leishmaniasis infection, studies conducted to evaluate the association between TLRs and cytokines of the Th1, Th2 and Th17 profiles during *L. chagasi* infection may contribute to a better understanding of the parasite/host relationship in that disease. This study aimed at evaluating the expression of TLR2 and 4, the expression and production of IL-12, IFN-γ, TNF-α, IL-17, IL-10, TGF-β, the production of NO and at correlating the results obtained from the expression of TLR2 and 4 with cytokine and NO production during infection with *L. chagasi*.

Materials and Methods

Animals, parasites and experimental infection - Balb/c female mice aged 8-10 weeks old were obtained from the breeding colony of the Tropical Disease Department. All animals received sterile water and food *ad libitum* throughout the experiment. All the procedures involving animals and their care were conducted in conformity with National and International Guidelines and were approved by the Ethics Committee for Animal Experimentation of the Botucatu School of Medicine - Unesp. The mice were divided into two groups: infected with *L. chagasi* (G2) and non-infected (G1). The animals were infected intravenously with a concentration of $1x10^7$ amastigotes/ml of *L. chagasi*, strain M6445, in a final volume of 0.1 ml per animal. The moments of sacrifice of the animals were determined by the previously estimated parasitic load. Five animals from each group were euthanized at days 1 (24 hours after infection, beginning of the acute phase), 3, 7, 14, 21, 28 (parasitemia peak), 35 (moment of decrease, beginning of the chronic phase) and 42.

Cytokine quantification (ELISA) - Cytokine production was assayed in the spleen, and tissue fragments were added to vials containing PBS (50 mg/ml) with a protease inhibitor cocktail (Complete, Roche). The tissue fragments were macerated, centrifuged, and the supernatants collected for cytokine quantification. The ELISA sets were IL-17, IL-10, IFN-γ, TNF-α, IL-12 and TGF-β (R&D, Minneapolis, MN), and procedures were performed according to the manufacturers' instructions. Optical densities were measured at 450 nm.

Results are expressed as picograms per milliliter. The limits of sensitivity for the different assays were as follows: IL-17, IL-10, TGF-β and TNF-α: 15 pg/ml; IFN-γ: 50 pg/ml and IL-12: 5 pg/ml.

Quantification of cytokines by Real-Time PCR assay - Total RNA from the splenic tissue was isolated using the TRIZOL reagent (Invitrogen) according to the manufacturers' instructions. cDNA was synthesized using 1 μg of total RNA through a reverse transcription reaction (ImProm-II TM Reverse Transcriptase, Promega). Real-time PCR quantitative mRNA analyses were performed in an ABI Prism 7300 SDS (Applied Biosystems) using the Applied Biosystems Power Sybr® Green for quantification of amplicons. The standard PCR conditions were as follows: 95ºC (10 min); 40 cycles of 95ºC (15 s) and 60ºC (1 min); followed by a standard denaturation curve. Real-Time PCR quantitative mRNA analyses were performed with the standard curve method, using sequences of mice primers present in the GenBank database (Table 1). The mRNA expression of cytokines was normalized to the level of β-actin mRNA.

Table I - Primers used in RT-PCR

Gene	Primers	Sequence
IL-12p40	F1 (foward)	5´-CCC AAG CAG GCC ACA GAA TTG AAA-3´
	R2 (reverse)	5´-AGT CAA ATC CAG AAC ATG CCG CAG-3´
IL-10	F1 (foward)	5´-GCC AAG CCT TAT CGG AAA TG-3´
	R2 (reverse)	5´-CAC CCA GGG AAT TCA AAT GC-3´
INF-γ	F1 (foward)	5´-AGA GGA TGG TTT GCA TCT GGG TCA-3´
	R2 (reverse)	5´-ACA ACG CTA TGC AGC TTG TTC GTG-3´
TNF-α	F1 (foward)	5´-CAT CTT CTC AAA ATT CGA GTG ACA A-3´
	R2 (reverse)	5´-TGG GAG TAG ACA AGG TAC AAC CC-3´
TGF-β	F1 (foward)	5´-AAC AAT TCC TGG CGT TAC CTT-3´
	R2 (reverse)	5´-CTG CCG TAC AAC TCC AGT GA -3´
IL-17	F1 (foward)	5´-ACC GCA ATG AAG ACC CTG AT -3´
	R2 (reverse)	5´-TCC CTC CGC ATT GAC ACA-3´
TLR-2	F1 (foward)	5´-TGT CTC CAC AAG CGG GAC TT -3´
	R2 (reverse)	5´-TTC GAT GGA ATC GAT GAT GTT G -3´
TLR-4	F1 (foward)	5´-TGA CAG GAA ACC CTA TCC AGA GTT-3´
	R2 (reverse)	5´-TCT CCA CAG CCA CCA GAT TCT-3´
β-actina	F1 (foward)	5´-AGA GGG AAA TCG TGC GTG AC-3´
	R2 (reverse)	5´-CAA TAG TGA TGA CCT GGC CGT-3´

Parasitic load - Parasitic load was determined by the microtitration technique in culture so that the moment of animal sacrifice could be defined. Fragments of the spleen and liver were weighed and then homogenized with a tissue titrator in 4ml of Schneider's drosophila medium, supplemented with 20% fetal bovine serum, inactivated by heating to 56°C for 30 minutes, penicillin (100U/ml), and streptomycin (50mg/ml). Under sterile conditions, wells in 96-well microtitration plates with 225μl of culture medium were prepared with successive 4:1 ratio dilutions, varying from 1 to $0.25x10^{-6}$. These were

incubated for 7 to 15 days at 26ºC-28ºC in a 5% CO_2 atmosphere, and then examined by an inverted microscope at 100x or 200x magnification. Positive titrate was considered with at least one parasite in the last dilution, which was used for parasitic load quantification as per Buffet et al. (1995).

Determination of nitric oxide (NO) production - NO_2^- production was evaluated by the colorimetric method based on Griess Reaction (Green et al. 1982). The same volume (100 ul) of the Griess Reagent, which contains NEED 0.1% (n-(1-naphthyl)ethylenediamine – Sigma Co. USA) diluted in distilled water and sulphanilamide (Sigma Co. USA) diluted in 5% H_3PO_4, was added to the supernatants obtained from the microcultures (100 Ul) of macrophages. The two reagents were mixed in similar volumes at the moment of reaction, and the samples (triplicates) were read by an automatic ELISA reader with a wavelength of 540 nm against a blank constituted by Griess Reagent. The results were expressed in micromoles of NO_2^-/1 x 10^6 cells from the standard curve established in each assay with known molar concentrations of NO_2 varying from 200 to 0.39 uM.

Statistical analysis - Results were statistically analyzed by ANOVA using the Graphpad Software (Graf Pad Instat 3.05, San Diego, CA, USA) and compared by the Tukey-Kramer Test. Spearman analysis was performed for correlation analysis between TLR2 and TLR4, with IL-12, IFN-γ, TNF-α, TGF-β, IL-10, IL-17 and NO. Significance was set at $p<0.05$

Results

Infection pattern in Balb/c mice infected with L. chagasi - Infection progression in Balb/c mice infected intravenously was measured by the total parasitic load in the spleen. Results showed that the parasitic load presented slight increase until the 7th day, greater increase on 14th day, a parasitemia peak on the 28th day and decrease from the 35th day p.i. (Figure 1).

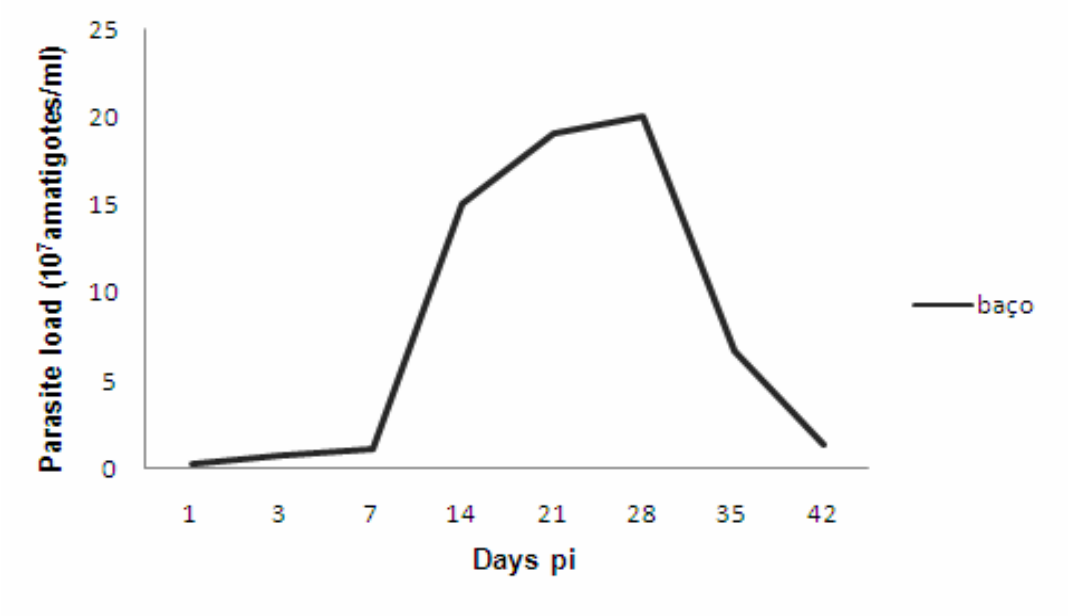

Fig. 1: Total parasitic load in the spleen of Balb/c mice during the course of visceral infection with *L. chagasi*. The data means are representative of two independent experiments (Five mice per group).

L. chagasi induced mRNA expression of TLR2 and 4 - In order to determine whether the infection with *L. chagasi* had an effect on the expression of TLR2 and 4, mRNA levels were evaluated. Infection with *L. chagasi* induced greater mRNA expression of TLR4 at all studied moments when compared to G1. mRNA expression of this receptor in G2 was significantly higher on days 1, 3, 7, 14 and 21 and 28 p.i. in relation to days, 35 and 42 (Fig. 2A). The animals in G2 also showed higher mRNA expression of TLR2 in relation to G1 at all studied moments. In G2, TLR2 mRNA expression was significantly higher on days 14, 21 and 28 p.i. It was also observed that, at the beginning of infection (days 1, 3 and 7), the expression of such receptor was higher than that on days 35 and 42 p.i. (Fig. 2B).

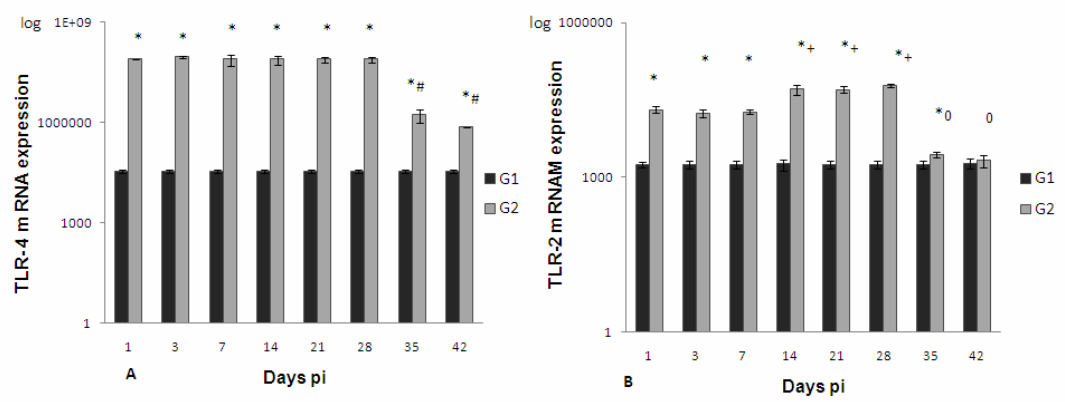

Fig. 2: mRNA expression of Toll-Like Receptors 2 and 4 during *L. chagasi* infection. TLR 2 and 4 were quantified in the splenocytes of infected (G2) and non-infected (G1) Balb/c mice by Real-Time (PCR) on days 1, 3, 7, 14, 21, 28, 35 and 42 p.i. The data (mean ± SEM) are representative of two independent experiments (five mice per group). Statistically significance between groups is indicated.*p<0,05 *vs* G1; # p<0,05 *vs* G2 1-28;0 p<0,05 *vs* G2 1-28; + p<0,05 *vs* G2 1-7, 35 and 42.

mRNA expression and the production of proinflammatory cytokines and Th17 during infection with L. chagasi - Given that IFN-γ and IL-12 have been considered to be the major protective cytokines in visceral leishmaniasis, in addition to the observation that TNF-α and IL-17 show an inflammatory role in several parasitic infections, we investigated the expression of such cytokines during the period of infection evaluated. In G2, high IFN-γ mRNA expression was detected in relation to G1 from the 3rd to the 14th days and to the end of infection (35th and 42nd days), with higher mRNA expression on days 3, 7 and 14, followed by days 35 and 42 p.i. Production in G2 showed to be similar at the beginning, lower from the 14th to the 28th days and higher at the end of infection (35th and 42nd days) when compared to that in G1. The highest IFN-γ production occurred on the 42nd day p.i. With respect to IL-12, mRNA expression was significantly higher in G2 if compared to G1 during the period of infection evaluated. In G2, mRNA expression increased from the 1st to the 21th days p.i. and decreased on the 28th day p.i. Such expression was significantly higher on days 14 and 21 in G2 when compared to that on the other days. Higher expression of such mRNA was also detected on days 28, 35 and 42 if compared to that at the beginning of infection (days 1 and 3). IL-12 production in G2 was significantly lower on days 7, 14, 21 and 28 p.i. when compared to that in G1; and higher on days 35 and 42 p.i. In G2, increased IL-12 production at the end of infection (days 35 and 42 p.i.) was statistically significant when compared to that on days 7, 14, 21 and 28 ($p<0.001$) (Figures 3 A and B).

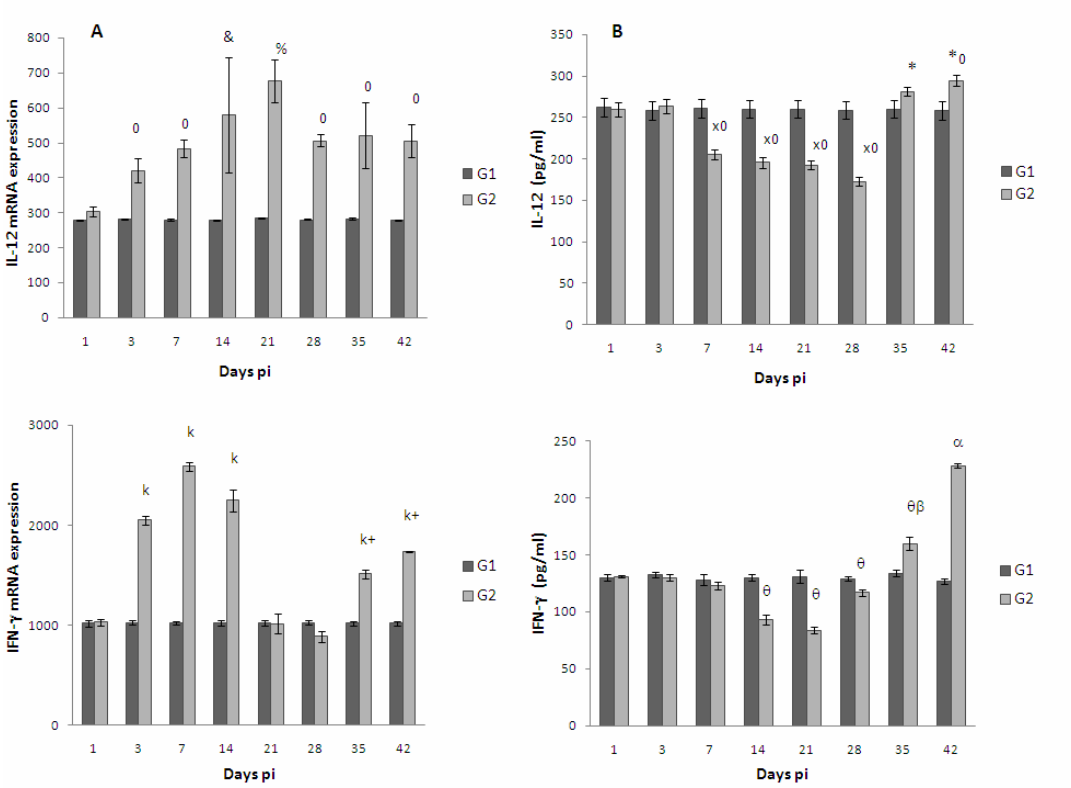

Fig. 3: IFN-γ and IL-12 mRNA expression and production during *Leishmania chagasi* infection. IFN-γ and IL-12 were quantified in the splenocytes of infected (G2) and non-infected (G1) Balb/c mice by Real-Time PCR (A) and ELISA (B) on days 1, 3, 7, 14, 21, 28, 35 and 42 p.i. The data (mean ± SEM) are representative of two independent experiments (five mice per group). Statistically significance between groups is indicated. k p<0,001 *vs* G1 and G2 1,21,28; + p <0,001 *vs* G2 3,7,14; θ p<0,01 *vs* G1; β p<0,05 *vs* G2 14-28; α p<0,001 *vs* G1 and G2 1-35; 0 p<0,05 *vs* G1 and G2; & p<0,01*vs* G2 1; % p<0,001 *vs* G2 1; x p <0,001 *vs* G2 1, 3; * p<0,001 *vs* G2 7-28.

High TNF-α and IL-17 expression and production were detected in the whole period of infection from the 3rd day in relation to G1. In G2, increasingly higher TNF-α mRNA production was observed until the 14th day, which continued until the 28th day. Steady increase was observed in G2 for IL-17 expression and production until the 28th day, with a decrease on days 35 and 42 (end of infection) (Figures 4A e B).

There was inverse correlation between parasitic load and IFN-γ and IL-12 ($p<0.05$) production, and a direct correlation between parasitic load and IL-17 and TNF-α expression and production ($p<0.001$) during the infection.

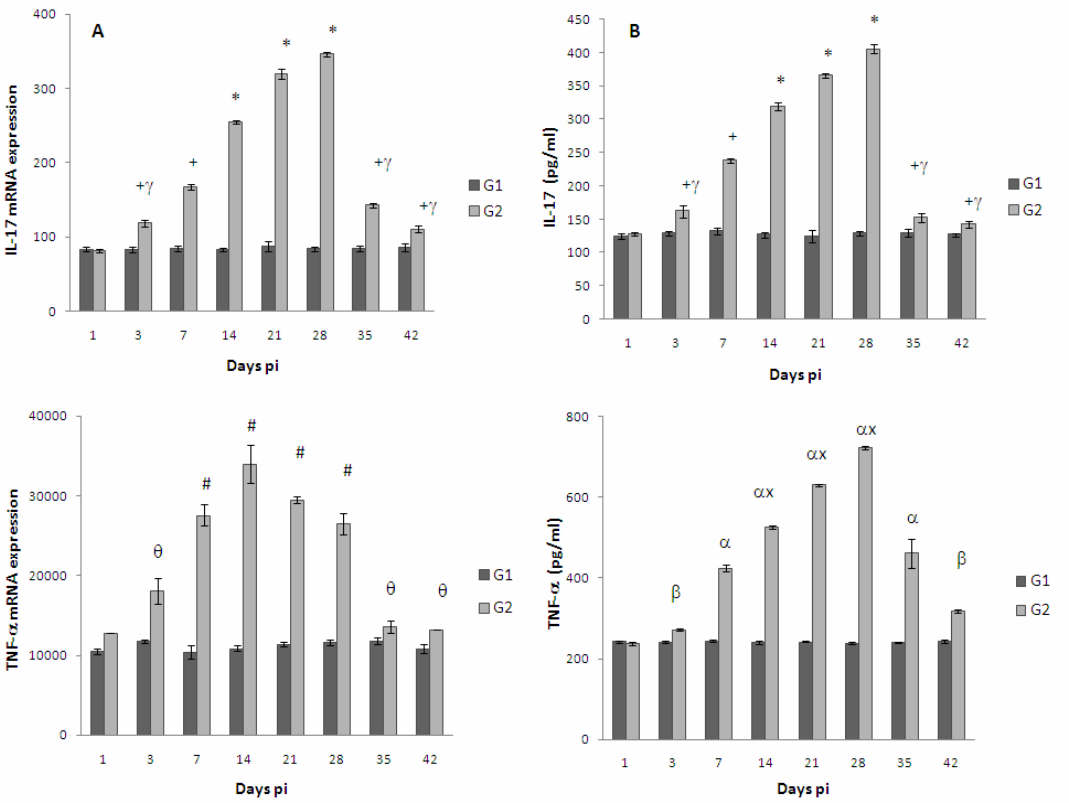

Fig. 4: TNF-α and IL-17 mRNA expression and production during *Leishmania chagasi* infection. TNF-α and IL-17 were quantified in the splenocytes of infected (G2) and non-infected (G1) Balb/c mice by Real-Time PCR (A) and ELISA (B) on days 1, 3, 7, 14, 21, 28, 35 and 42 p.i. The data (mean ± SEM) are representative of two independent experiments (five mice per group). Statistically significance between groups is indicated. * p<0,001 *vs* G1 and G2 1,3,7,35,42; + p < 0,05 *vs* G1 and G2 1; γ p < 0,05 *vs* G2 7-28; θ p< 0,01 *vs* G1 and G2 7-28; # p< 0,01 *vs* G1 and G2 1,3,35,42; β p<0,05 *vs* G1 and G2 7, 35; α p <0,001 *vs* G1 and G2 1,3,42; x p <0,01 *vs* G2 7,35.

mRNA expression and production of TGF-β and IL-10 during infection with L. chagasi - Since the participation of TGF-β and IL-10 in the control of inflammatory processes and in the persistence of parasites has been demonstrated, we evaluated the expression and production of such cytokines during *L. chagasi* infection. The animals in G2 showed higher mRNA expression and TGF-β production than that detected for G1 during the whole evaluated period from the 3rd day over the course of infection, which was followed by a decrease on days 35 and 42 p.i. Similarly to what occurred to TGF-β, mRNA expression and IL-10 production in G2 were higher from the 3rd day and during the whole period of infection; nevertheless, such increase was steady during the period of infection evaluated. In G2, the highest IL-10 production occurred on days 35 and 42 p.i (Figures 5A and B).

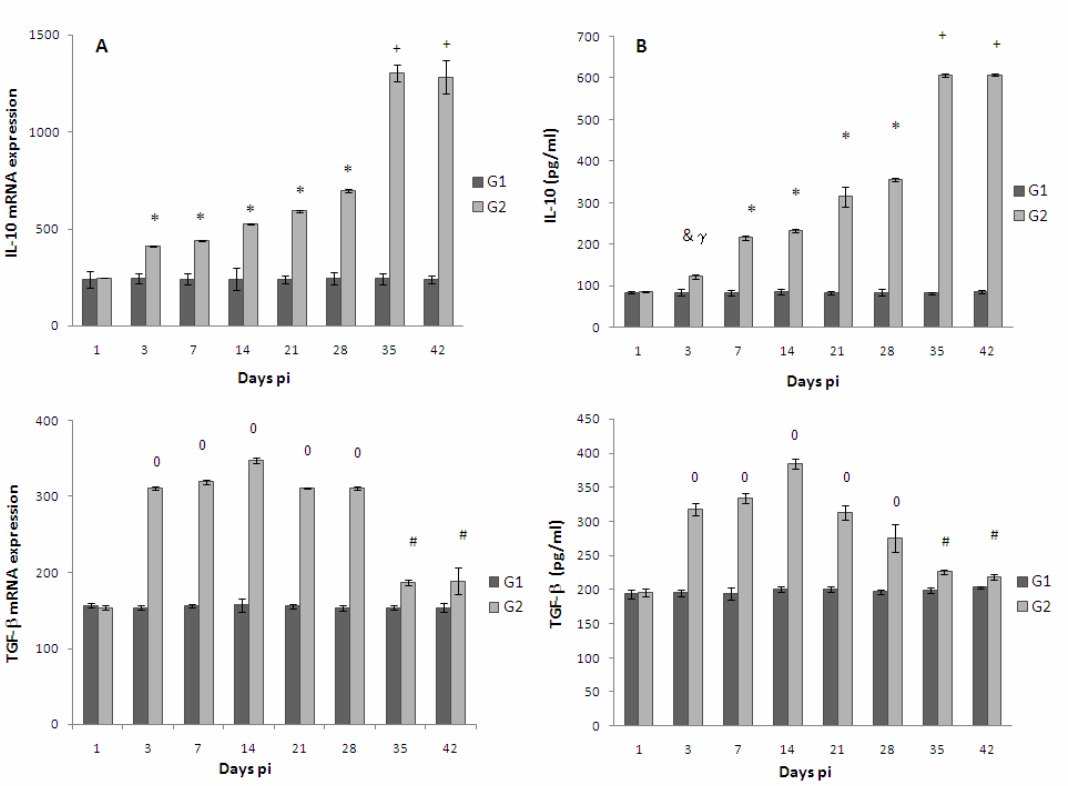

Fig. 5: mRNA expression and production of TGF-β and IL-10 during *Leishmania chagasi* infection. TGF-β and IL-10 were quantified in the splenocytes of infected (G2) and non-infected (G1) Balb/c mice by Real-Time PCR (A) and ELISA (B) on days 1, 3, 7, 14, 21, 28, 35 and 42 pi. The data (mean ± SEM) are representative of two independent experiments (five mice per group). Statistically significance between groups is indicated. **p*<0,001 *vs* G1 and G2 1, 35, 42; + *p*<0,001 *vs* G1 and G2 1-28; & *p*<0,05 *vs* G1 and G2 1; γ *p*<0,01 *vs* G2 7-28; 0 *p*<0,001 *vs* G1 and G2 35,42; # *p*<0,05 *vs* G1 and G2 1.

NO production during L. chagasi infection - NO production by splenic cells during *L. chagasi* infection are shown in Figure 5. NO production by splenic cells in G2 was significantly higher than that by animals in G1 from the 14th day p.i. In G2, the production of such metabolite was maintained in steady levels until the 7th day p.i. From the 14th day, such levels increased until the end of the experiment.

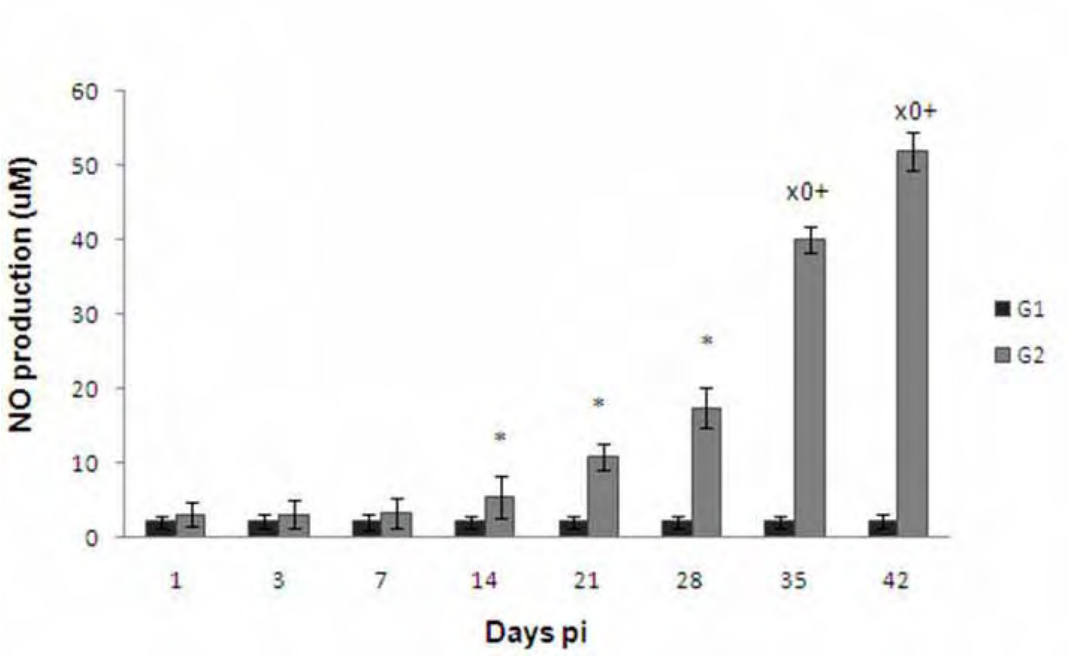

Fig. 6: Nitric oxide (NO) production during *Leishmania chagasi* infection. NO was quantified in the splenic macrophages of infected (G2) and non-infected (G1) Balb/c mice on days 1, 3, 7, 14, 21, 28, 35 and 42 p.i. The data (mean ± SEM) are representative of two independent experiments (five mice per group). Statistically significance between groups is indicated. **p*<0,05 *vs* G1; x *p*<0,001 *vs* G1; 0 *p*<0,001 *vs* G2 1-14; + *p*<0,05 *vs* G2 21,28.

Correlation between the expression of TLR2 and 4 and production of cytokines and NO during L. chagasi infection - The results of the correlation analysis between the levels of expression of TLR2 and 4 and the production of cytokines and NO are shown in Tables 2 and 3. A positive correlation was observed between TLR2 and TNF-α, IL-17, NO, IL-10 and TGF-β at the beginning of infection and between that receptor and TNF-α, IL-17, TGF-β at the end of infection. Also, an inverse correlation was found between TLR2 and IL-12 and IFN-γ at the beginning of infection and between that receptor and IL-12, IFN-γ, NO, and IL-10 at the end. (Table II). Similar results were obtained for TLR4 (Table III).

TABLE II – Correlation between the expression of TLR2 and production of cytokines and NO during *L.chagasi* infection

		TRL2							
		1	3	7	14	21	28	35	42
IL-12	r	-0,37	-0,43	-0,89	-0,87	-0,83	-0,53	-0,43	-0,6
	p	0,042	0,039	0,018	0,029	0,035	0,027	0,017	0,009
IFN-γ	r	-0,52	-0,21	-0,58	-0,84	-0,27	-0,96	-0,32	-0,22
	p	0,028	0,067	0,022	0,032	0,059	0,002	0,0053	0,0076
TNF-α	r	-0,67	-0,39	0,78 *	0,81*	0,96*	0,89*	0,63*	0,85*
	p	0,139	0,043	0,047	0,046	0,001	0,016	0,047	0,049
IL-17	r	-0,26	0,82*	0,98*	0,63*	0,79*	0,88*	0,25*	0,1*
	p	0,66	0,044	0,001	0,046	0,048	0,019	0,048	0,045
IL-10	r	-0,49	0,4	0,933*	0,8*	0,07*	0,81*	-0,59	-0,54
	p	0,318	0,342	0,006	0,045	0,005	0,049	0,21	0,26
TGF-β	r	-0,02	0,97*	0,26*	0,89*	-0,32	-0,95	0,67*	0,85*
	p	0,955	0,009	0,049	0,017	0,05	0,002	0,013	0,016
NO	r	0,69	0,44	0,78*	0,13*	0,93*	0,98*	-0,04	-0,83
	p	0,127	0,97	0,001	0,007	0,007	0,005	0,009	0,037

* Statistically significant differences among expression of TLR2 and production of cytokine and NO are shown as $p<0,05$

TABLE III – Correlation between the expression of TLR4 and production of cytokines and NO during *L.chagasi* infection

		TLR4							
		1	3	7	14	21	28	35	42
IL-12	r	-0,15	-0,73	-0,42	-0,6	-0,73	-0,25	-0,95	-0,99
	p	0,77	0,035	0,04	0,013	0,049	0,016	0,002	0,001
IFN-γ	r	-0,016	-0,87	-0,96	-0,97	-0,86	-0,96	-0,99	-0,52
	p	0,098	0,024	0,022	0,001	0,025	0,001	0,028	0,047
TNF-α	r	0,2	0,76	1*	0,95*	0,11*	0,71*	0,66*	0,12*
	p	0,695	0,05	0,001	0,002	0,049	0,046	0,014	0,049
IL-17	r	-0,26	0,78*	0,65*	0,84*	0,78*	0,98*	0,84*	0,77*
	p	0,61	0,047	0,034	0,035	0,048	0,004	0,009	0,006
IL-10	r	-0,85	0,97*	0,78*	0,58*	0,98*	0,95*	-0,88	-0,97
	p	0,029	0,009	0,006	0,022	0,003	0,003	0,019	0,007
TGF-β	r	-0,48	0,49*	0,8*	0,98*	-0,32	-0,95	0,67*	0,32*
	p	0,32	0,035	0,047	0,02	0,037	0,001	0,013	0,048
NO	r	-0,96	-0,72	0,1*	0,43*	0,58*	0,88*	-0,98	-0,2
	p	0,001	0,102	0,001	0,038	0,022	0,02	0,006	0,0069

* Statistically significant differences among expression of TLR4 and production of cytokine and NO are shown as $p<0,05$

Discussion

Although studies have suggested an involvement of TLRs in the initial interation of Leishmania with the immune system, the role played by TLRs in visceral leismaniasis is still unclear. In this context, we aimed at evaluating the expression of TLR2 and 4, expression and production of IFN-γ, TNF-α, IL-17, IL-10, TGF-β and production of NO during the acute and chronic phases of *L. chagasi* infection in Balb/c mice. Additionally, we correlated the expression of receptors with the production of cytokines and of NO. It was found that *L. chagasi* induced mRNA expression of TLR2 and 4 during the period of infection evaluated, thus indicating that they are likely to be involved in parasitic recognition. These results are in agreement with others showing that the parasite binds to both TLR2 and 4 to enter the host's cell (Kropf et al. 2004, Flandin et al. 2006) We also found that, at the peak of parasitemia, there was higher expression of TLR4 and particularly of TLR2. A possible explanation for this result is that the parasite and/or cytokines can modulate the expression of these receptors. Hence, at the very beginning of infection, parasites interact with cells, thus activating them through TLR2 and 4 and, consequently, this activation process increases the expression of such receptors. This idea is supported by studies showing that the LPG from *L. major* is recognized by TLR2 on NK cells, leading their activation with consequent increase in TLR2 levels on cells surface (Becker et al 2003). Additionally, macrophages activated by IFN-γ showed higher TLR2 expression on their surface (Flo et al. 2001). In an experimental model of neurocysticercosis, increased TLRs 11-13 expression was observed in the acute phase as compared to the chronic phase. Acute

infection was associated with the Th1 response. However, a Th1/Th2 response pattern was detected in the chronic phase and associated with decrease in expression of these receptors, thus contributing to reduce tissue damage induced by excessive inflammation (Bibhuti et al. 2008).

We also found that *L. chagasi* infection resulted in cytokine modulation. The mechanism responsible for cytokine production probably involves TLRs. Parasite components lead to the activation of TLR2 and 4, with the resulting production of cytokines (Tuon et al. 2008). With respect to IL-12 and IFN-γ, it was found that, although the expression of such cytokines was high in the infected animals, production by cells during the initial phase of infection remained low and smaller in relation to non-infected animals. These findings can be explained by the fact that the process of translation of those cytokines was prevented, and the mRNA transcribed from the specific DNA sequence of IL-12 and IFN-γ was not decoded to specify the synthesis of these cytokines (proteins) (Maier et al. 2009). Our results show that low IL-12 and IFN-γ production was followed by high parasitic load; hence, the high parasitic load observed at the beginning and in the intermediate phase of infection may be due to the low release of these cytokines by cells. On the other hand, the decreased parasitic load in the chronic phase would be related to the increased production of such cytokines. These results are in agreement with others showing that protective role played by IL-12 and IFN-γ during leishmania infection by promoting the Th1 response in addition to controlling parasitic replication and resolving the infection (Cummings et al. 2010, Watford et al. 2004, Hernandez-Pando et al. 1997). Some studies have shown low or absent IFN-γ or IL-12 production in the supernatant of PBMC culture stimulated with

leishmania antigen, according to our results (Cillari et al. 1995, Ghalib et al. 1995, Kumar et al. 2001) At the beginning of infection, high IL-12 and IFN-γ mRNA expression was detected, and it was not translated into production. This may have occurred due to the action of suppressive mechanisms, such as soluble factors, different cell types and interactions among cytokines. The presence of such mechanisms was shown in studies using peripheral blood mononuclear cells (PBMC) (Barral et al. 1986, Carvalho et al. 1989, Bacellar et al. 1996)**.**

In this study, it was also shown that *L. chagasi* infection resulted in mRNA expression and IL-17 production, which followed the profile of the parasitic load, showing a response characteristic that was opposite to that of IFN-γ and of IL-12. Additionally, in a previous study (unpublished data) using the same infection model a direct correlation between IL-17 and TNF-α and IL-6 was observed. They were related to an intense inflammatory process and tissue damage. Hence, our results suggest that IL-17 is not involved in infection control, but in the pathogenesis mechanisms leading to disease development. These results are in agreement with those from other studies showing that, in experimental infection of susceptible Balb/c animals with *Leishmania major*, there was high IL-17 production as compared to resistant C57BL/6 animals. They also showed that the development of lesions and susceptibility to infection would be related to IL-17 production, resulting in a larger recruitment of neutrophils to asymptomatic sites, since Balb/c mice had failed in lesion to exacerbation of the lesions, but also showed similar IFN-γ and IL-10 production levels (Anderson et al. 2009, Kostka et al. 2009). In relation to TNF-α levels, they also followed the parasitic load profile. Such cytokine is essential for

normal response associated with parasite elimination; however, its excessive or inappropriate production may cause damage to the host (Bradley 2008, Robak et al. 1998). With this regard, in a study on visceral leishmaniasis, high TNF-α concentrations were related to disease progression (Kaye et al. 2004). Additionally, TNF-α inhibition healed ulcers in patients with American cutaneous leishmaniasis (Ribeiro de Jesus et al. 2008). According to these studies, our results also suggest that TNF-α is not involved to protection, but to tissue damage and pathogenesis. NO production increased during infection, and, similarly, the presence of a large amount of NO was reported in the supernatant of murine macrophages infected with *L. chagasi* and stimulated with IFN-γ, which reflects the importance of that effector molecule in leishmanicidal activity (Gantt et al. 2001). Although the levels of such metabolite were high at the beginning o infection, such increase did not result in parasite destruction in the initial phase. Such destruction occurred only at the beginning of the chronic phase, probably when the levels of that molecule sufficiently increased to eliminate the parasites.

In leishmaniasis, as in other infections, IL-10 and TGF-β have been identified as important cytokines involved in the homeostatic mechanism, limiting the tissue damage caused by extensive inflammation. Nevertheless, they also favor pathogenic persistence (Belkaid et al. 2001). In visceral leishmaniasis, IL-10 has been detected in lymph nodes, serum and the spleen (Cillari et al. 1995, Ghalib et al. 1995, Ansari et al. 2006, Kenney et al. 1998). These findings are in agreement with our results, which show the presence of high IL-10 levels in the spleen, particularly at the end of infection. However, at the beginning of infection, lower IL-10 levels, but still higher than those in non-

infected animals, were detected, in addition to low IFN-γ and IL-12 levels. This suggests the involvement of IL-10 in the suppression of these cytokines, a process that probably did not occur, since IFN-γ and IL-12 production at the end of infection were high, despite the high IL-10 levels. With respect to TGF-β, results show that, besides its involvement in IL-17 induction together with IL-6, it may also be related to suppression of protective mechanisms, acting directly or indirectly on IFN-γ and IL-12 production, since high TGF-β levels were detected at the beginning of infection, followed by low IFN-γ and IL-12 production, and the opposite occurred at the end. High mRNA expression and TGF-β production were also detected in the spleen, and according to our results, it was associated with suppressive mechanisms (Nylén et al. 2007, Li et al. 1999).

In summary, our findings suggest that TLR2 and 4 play an important role in cytokine modulation, as shown by the high production of TNF-α, IL-17, NO, IL-10 and TGF-β, in agreement with the high expression of TLR2 and 4 at the beginning of infection (acute phase) and low production of TNF-α, IL-17 and TGF-β at the end (chronic phase), in agreement with the lower expression of these receptors. Our study points to the idea that *L. chagasi* interaction with cells via TLR2 and 4 could be considered as a pathogenicity mechanism of this parasite, which would use the host receptors of innate immunity to infect cells and to guarantee its own multiplication.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Capítulo II

DNA damage and nitric oxide production in experimental infection with *L. chagasi*

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Trabalho a ser submetido em Mutation Research – Genetic Toxicology and Environmental Mutagenesis

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Abstract

Leishmania chagasi, which causes visceral leishmaniasis in South America, is an obligate intracellular protozoan. Nitric oxide (NO), produced by macrophages during inflammatory response, is one of the main microbicide mechanisms involved in the process of destroying this parasite. However, there is no information on DNA damage caused by *L. chagasi* and NO's involvement in this process. Thus, the purpose of this work was to evaluate whether *L. chagasi* infection can cause DNA damage in peripheral blood and spleen cells of Balb/c mice, and whether this damage is related to NO production. Balb/c mice were distributed into two groups: Control (non-infected) and infected with *L. chagasi.* Single-cell gel electrophoresis (also known as comet assay) was used to measure DNA damage in peripheral blood and spleen cells, and Griess reaction to determine NO production in the spleen. The results obtained showed that *L. chagasi* was able to induce DNA damage in peripheral blood and spleen cells of infected mice. In relation to NO, macrophages from control group challenged with *L. chagasi* showed a significant production ($p<0,05$) when compared to that obtained with non-challenged cells with parasite. Treatment of spleen cells with NG-monomethyl-L-arginine (LNMMA) induced significant reduction of NO and DNA damage in these cells ($p<0,05$). Taken together, our results indicate that *L. chagasi* induces high levels of DNA damage in peripheral blood and spleen cells and NO not only cause the killing of the parasite, but also induce high levels of DNA damage in adjacent cells.

Keywords: *Leishmania chagasi*, DNA damage, NO production

1. Introduction

Visceral leishmaniasis is generally caused by *Leishmania donovani* and *Leishmania infantum,* in the Old World, and *Leishmania chagasi,* in the New World, including Brazil. It is a widely distributed endemic zoonosis, found in 88 countries around the world, with a prevalence exceeding 12 million individuals [1]. Infection leads to a variety of outcomes ranging from asymptomatic infection to active disease, which is characterized by fevers, cachexia, hepatosplenomegaly and suppressed immune response [2].

It has been reported that various infectious agents are carcinogenic to humans. Based on these studies, it is estimated that almost 8-17% of global cancers burden are caused by infectious agents, the majority related to viruses such as Papillomavirus, hepatitis C, and in lesser proportions by infectious bacteria and parasites caused by *Mycobacterium, Helicobacter pylori*, and *Trypanossoma cruzi* [3-6]. Association between leishmaniasis and cancer has been demonstrated through its direct involvement in the occurrence of malignant lesions, especially of the skin and mucous membranes, and also as a predisposing factor for cancer [7, 8, 6].

Control and protection against *Leishmania* infection are mediated by Th1 type response, and IFN-γ secreted by Th1 cells is the most potent macrophage activating cytokine leading to host resistance to infection with *Leishmania* parasites [9]. The generation of nitric oxide (NO) through the up regulation of inducible NO synthase by IFN-γ and TNF-α, is the critical macrophage effector mechanism involved in the control of parasite replication [5, 6, 10]. NO is synthesized when L-arginine is converted to citrulline by inducible nitric oxide synthase (iNOs) enzyme activity. N^G-monomethl-L-arginine (L-NMMA) inhibits

this conversion increasing amastigote survival and replication in murine macrophages [11], as well as increasing lesion size and parasite load, and also induce the immediate reactivation of infection in resistant animals. The mechanism by which NO action destroys this parasite is not fully established [12, 13], but it is believed to act with reactive oxygen intermediates to damage mitochondrial DNA, proteins, and lipids [14]. NO can act covalently with intracellular iron, reacting with prosthetic Fe-S groups of sensitive enzymes, such as aconatase, mitochondrial electron transport chain complex I and II, among others, resulting in the formation of ferrous-nitrosyl, deactivation and degradation of these enzymes, with a consequent stop in replication. In this way studies have shown that control of *Leishmania* infection is dependent on NO production [9]. However, elevated iNOs expression in response to an inflammatory stimulus, results in the production of high NO levels, the process involved in leishmaniasis pathogenesis [15]. It has also been suggested that iNOs expression and elevated NO generation during inflammation can cause host cell damage and also induce alterations in host DNA. Accumulation of DNA damage over time can cause changes in cells, making them mutagenic or carcinogenic [16]. Studies have reported NO's involvement to promote changes in DNA through DNA oxidation and protein nitrosilation [17- 19].

Considering the strong evidence linking NO production, DNA damage, and carcinogenesis [20], and although there are various studies demonstrating the role of NO in visceral leishmaniasis pathogenesis, there is no report in the literature on DNA damage mainly caused by *L. chagasi*. Thus, the purpose of present study was to evaluate whether infection by *L .chagasi* can cause DNA damage in peripheral blood and spleen cells from Balb/c mice and if this damage is related to NO production.

2. Materials and methods

2.1 Experimental groups

Twenty 8-12 week old isogenic female Balb/c mice were used; they were kept at the Tropical Diseases Research Laboratory Animal Centre, Department of Tropical Diseases, Botucatu School of Medicine. They weighed approximately 30g and were kept in an environment at 25ºC-27ºC, with a 12h day 12h night cycle with food and water *ad libitum.* Animals were distributed into two groups: G1 (control): non-infected animals; and G2: animals infected with *Leishmania chagasi*. The Ethical Committee for Animal Research, Botucatu Medical School (UNESP), approved the experimental protocols.

2.2. Determining parasite load to define moment of sacrifice

Parasite load was determined by microtitration technique in culture so that animal sacrifice moment could be defined. Fragments of the spleen were weighed and then homogenized with a tissue titrator in 4ml of Schneider's drosophila medium, supplemented with 20% fetal bovine serum, inactivated by heating to 56^{o}C for 30 minutes, penicillin (100U/ml), and streptomycin (50mg/ml). Under sterile conditions, wells in 96 well microtitration plates with 225μl of culture medium were prepared with successive 4:1 ratio dilutions, varying from 1 to $0.25x10^{-6}$. These were incubated for 7 to 15 days at 26ºC-28ºC in a 5% CO_2 atmosphere, and then examined by inveted microscope at 100x or 200x magnification. Positive titrate was considered with at least one parasite in the last dilution, which was used for parasite load quantification as per Buffet *et al* [21].

2.3. Infection of animals

Amastigotes forms of *L. chagasi* (cepa M6445) were supplied by São Paulo Institute of Tropical Medicine Protozoology Laboratory – USP School of Medicine, maintained in hamster at the Tropical Diseases Research Laboratory Animal Centre, Department of Tropical Diseases, Botucatu School of Medicine – UNESP, and obtained from infected hamster spleen. The mice were infected by intravenous route with a concentration of $1x10^7$ amastigotes/ml in a final volume of 0.1ml per animal [22].

After 28 days infection (peak parasitemia), 10 animals from each group were sacrificed by intraperitonal administration of 0.4% sodium pentobarbital (1ml/Kg). The thoracic cavity was exposed, and cardiac puncture performed to collect blood (1ml). Then the spleen was aseptically removed for the experiments of DNA damage.

2.4. Isolation and culture of spleen cells

After spleen removal from each animal, splenocytes were isolated as per Fenech *et al.* [23]. Briefly, spleen cells were disrupted in endotoxin-free RPMI 1640 (Sigma, ST Louis, MO, USA), supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), penicillin (100U/ml), and streptomycin (100µl/ml). Cell suspensions were collected, and 10 ul of this suspension was used for experiments on DNA damage in G1 and G2. Later, the splenocytes from group G1 were isolated using the Ficoll-Hypaque gradient method. Splenocytes were then washed twice in RPMI containing heat-inactivated fetal

calf serum, added to 96-well flat-bottom tissue culture plates (Nunc, Life Tech. Inc., Maruland, MA, USA) at $1x10^6$ cells/mL, and cultured for 24h in the presence or absence of N(G)-monomethyl-L-arginine (LNMMA), 500μM, the nitric oxide inhibitor (Sigma, ST Louis, MO, USA) and in the presence or absence of $1x10^4$ epimastigotes of *L. chagasi*, at 37°C in a humidified 5% CO_2 atmosphere. These experiments were conducted to evaluate the role of NO in DNA damage

2.5. DNA damage

The protocol used for both peripheral blood and spleen cells was as per Sasaki *et al.* [24] with some modifications. Namely, 10μl of peripheral blood and spleen cells were added to in 120μl 0.5% low melting point agarose at 37ºC and added to slides previously covered with 1.5% regular agarose, and covered with a coverslip. After solidifying agarose in a refrigerator, coverslips were removed and slides immersed in a lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris-HCl buffer, pH10, 1% sodium sarcosinate with 1% Triton X-100, and 10% DMSO) for one hour. Prior to electrophoresis, slides were washed in PBS and placed in alkaline solution (pH>13) for 20 minutes, then submitted to electrophoresis for 20 minutes at 25V, 300mA. After electrophoresis, slides were neutralized with 0.4M Tris-HCl buffer (pH7.5), fixed in absolute ethanol and stored awaiting analysis. Independently, positive controls (blood and spleen cells) were treated with 100μM H_2O_2 for 5 minutes, in triplicate, to ensure assay sensitivity and reproducibility. All steps were performed under low light conditions. Slides were stained with ethidium bromide (100μl per slide at

20µl/mL). A total of 50 nucleoids per slide were examined at 400x magnification using an immunofluorescence microscope connected to an image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, Suffolk, UK). DNA damage was measured by analysing “tail intensity” (% of migrated DNA) and tail moment [the product of tail length (DNA migration) and fraction of DNA in the comet tail, i.e. % DNA in the tail] [25]. As the groups showed statistical differences between these parameters, we chose “tail moment” to present our results.

2.6. Macrophage NO production

NO production was quantified by the accumulation of nitrite in supernatants using the standard Griess assay [26]. Briefly, 100µl of the collected supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide) (Sigma, ST Louis, MO, USA) diluted in 5% H_3PO_4, and 0.1% Naphthylethylenediamine (Sigma, ST Louis, MO, USA) in 96-well flat-bottom tissue culture plates (Nunc, Life Tech. Inc., Maruland, MA, USA). Absorbance at 540nm was determined by ELISA reader. Conversion of absorbance to micromolar concentrations of NO was obtained using a standard curve of known $NaNO_2$ concentration diluted in distilled H_2O. All measurements were performed in triplicate and expressed as micromolar concentrations of NO.

2.7. Statistical analysis

Comet assay results were analysed as per Wiklund & Agurell [27]. Tail moment data were first transformed into square root values; infected animals were compared with controls by ANOVA, followed by post hoc analysis (Tukey test) if a significant effect was detected. Nitric oxide production was analysed by ANOVA, followed by the Tukey test. Significance level was 5%.

3. Results

Infected animals and controls were killed on the 28th day after infection, previously established as peak parasitemia (Fig. 1). All animals presented positive for *L. chagasi* infection, by spleen *imprints* analysis, under optical immersion microscope. No animal died during the experiment. Fig. 2 shows DNA damage levels (tail moment) in peripheral blood and spleen cells by comet assay (single cell gel assay). Peripheral blood and spleen cells from infected animals (G2) presented significant induced DNA damage compared to controls (G1) ($p<0.05$). However significant differences were not detected between blood and spleen cells.

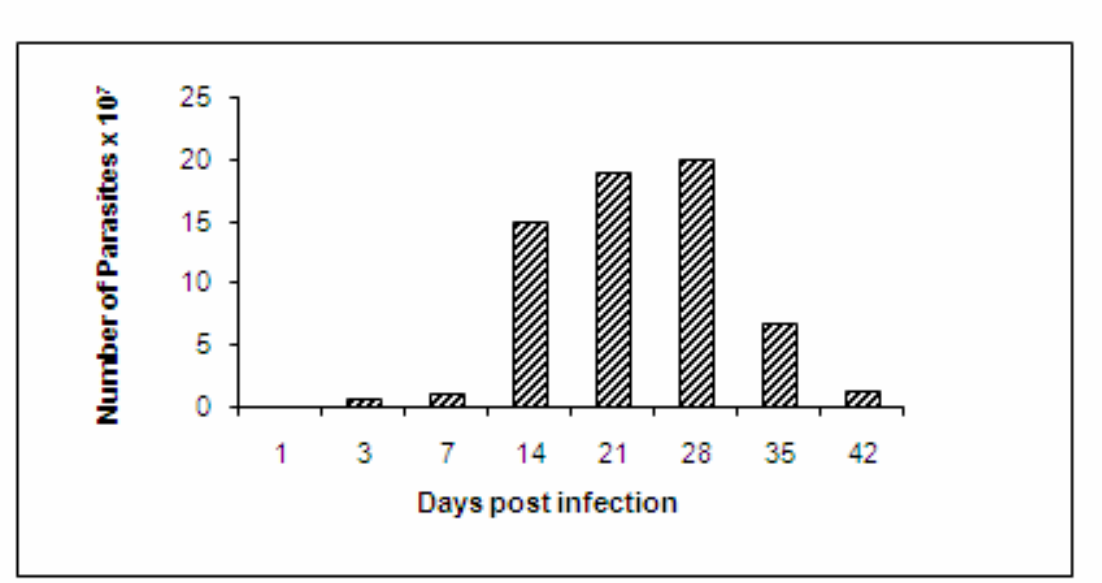

Fig. 1: Parasite load in Balb/c mice spleen during i.v. infection with 1 x 10^7 *L. chagasi* amastigotes. The data (mean) are representative of two independent experiments (five mice per group).

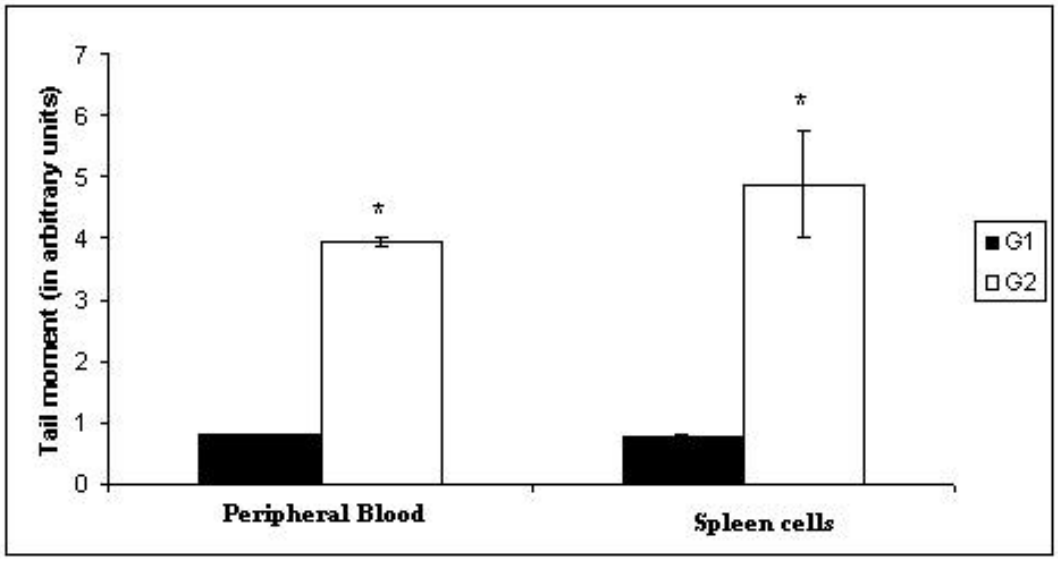

Fig. 2: DNA damage in peripheral blood and spleen cells of non-infected (G1) and *L. chagasi* infected (G2) Balb/c mice. Results are expressed as mean and standard deviation of tail moment and are representative of two independent experiments (ten mice per group); *p<0.05, compared to G1.

To establish whether this DNA damage is related to NO production, experiments were performed to detect NO levels in macrophages (MØ) from non-infected mice (G1) challenged with *L. chagasi*. Fig. 3 shows that MØ not challenged with *L. chagasi* liberated basal NO levels, while levels in MØ challenged with *L. chagasi* were significantly higher ($p<0.05$). Subsequently, NO involvement in inducing DNA damage was evaluated by adding L-NMMA, a competitive inhibitor for L-arginine. Control experiments were conducted to determine the best L-NMMA dose (data not shown). Results from Fig. 3 show that NO production by MØ challenged with *L. chagasi* was significantly inhibited in the presence of this inhibitor. Fig. 4 shows DNA damage levels (tail moment) in culture of spleen cells by comet assay (single cell gel assay). Spleen cells challenged with *L. chagasi* presented significant induced DNA damage when compared to spleen cells not challenged ($p<0.05$). Addition of L-NMMA in culture of spleen cells reduced significantly the DNA damage, proving NO's involvement in this process.

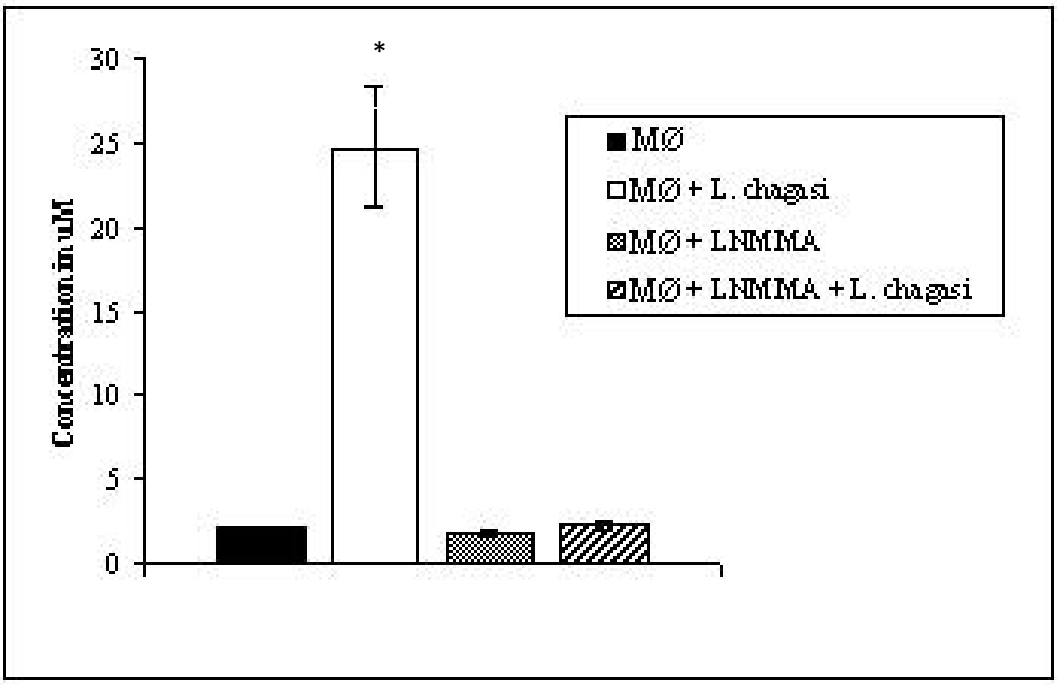

Fig. 3: Nitric oxide (NO) production by macrophages of Balb/c mice (G1) treated or no with LNMMA, challenged or no with promastigotes of *L. chagasi*. The data (mean ± sem) an representative of two independent experiments (ten mice); *p<0.05 compared to MØ, MØ treated with LNMMA and MØ treated with LNMMA and challenged with *L. chagasi*.

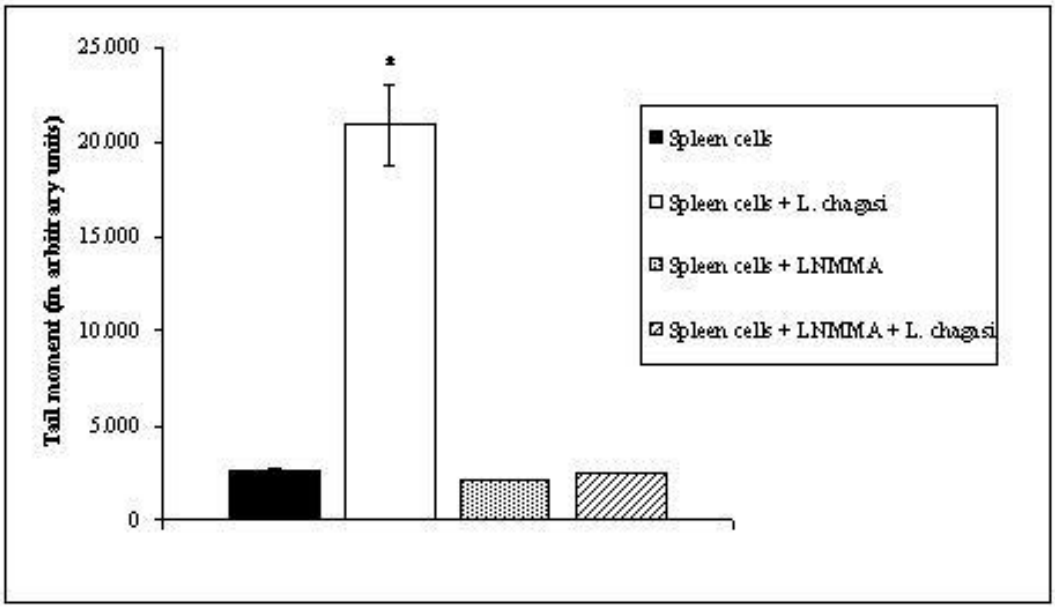

Fig. 4: DNA damage in culture of spleen cells of Balb/c mice (G1) treated or no with LNMMA, challenged or no with promastigotes of *L. chagasi*. The data (mean ± sem) an representative of two independent experiments (ten mice); *p<0.05 compared to spleen cells, spleen cells treated with LNMMA and spleen cells treated with LNMMA and challenged with *L. chagasi*.

4. Discussion

In this study we investigated whether infection by *L. chagasi* could cause DNA damage in peripheral blood and spleen cells from Balb/c mice. Our results showed that cells from infected animals presented DNA damage similar to other studies that also verified that the parasites can induce DNA damage [6, 28, 29]. However, this is the first report in relation to genotoxicity caused by *L. chagasi.*

Leishmaniasis has a broad clinical spectrum, varying form a localized cutaneous lesion which is naturally cured to fatal visceral leishmaniasis, where the parasites spread from the infection point and invade mononuclear phagocytic system organs and tissues [30]. In this sense, peripheral blood and the spleen, compartments used in this study, are affected by parasite replication during infection, in contrast to other organs like the liver, maintaining infection throughout the course of visceral leishmaniasis [31]. Also during infection, these compartments maintain an intense interaction between the immune system and the parasite, because all participants of this system are present in large quantities [32, 33].

Peripheral blood and spleen cells have been used to monitor genetic damage caused by environmental factors using different methods, of which single-cell gel electrophoresis (comet assay), is a new, fast, and sensitive method of determining DNA damage in different situations, such as treatment with toxic agents, oxidative stress [34-36, 6]. Based on the above information,

we chose to use this method and these compartments to evaluate DNA damage in infected animals.

Resistance to infection from *L. chagasi* is correlated to Th1 cell activation and IFN-γ production, while susceptibility is associated with Th2 cell development and production of IL-4 and IL-10 [37]. Activation of cellular response mechanisms leads to an intense inflammatory process [15], which is important for destroying *Leishmania*, but which can also expose important organs to certain endogenous genotoxins [38, 6]. It seems that leishmaniasis is a predisposing factor for cancer [39, 40]. However, the carcinogenic mechanisms associated with infection and inflammation are not totally clear. Three main mechanisms have been proposed to explain the infection/carcinogenesis association: direct action by the infectious agent on host cells and tissues, immunosuppression and production of reactive nitrogen and oxygen intermediates. These mechanisms can directly cooperate through integration of viral DNA with the host genome and indirectly activating inflammatory cells and through the involvement of genetic and other host factors, to induce cancer in some cases of infection and inflammation association [41, 42].

In this study, the DNA damage found could have been induced by the presence of high NO concentrations in culture of spleen cells after challenge by *L. chagasi.* These results agree with various other works [43, 44]. The iNOS enzyme is induced and activated by cells during inflammation, and is regulated by pro-inflammatory cytokines such as TNF-α, IL1-β, IL-6, IFN-γ. Elevated

production of these cytokines can induce or activate iNOS, which produce high concentrations of NO [45]. In a study using the same experimental model, we observed that at infection peak, the period also evaluated in this study, high levels of TNF-α and IFN-γ were detected (data not shown), which could explain the elevated NO levels found in this study.

When we added L-NMMA to our study, the reduction in NO levels was detected at the same time as a reduced DNA damage in culture of spleen cells. Together these results can explain the genotoxicity found in spleen cells after infection by *L. chagasi*, as NO is associated with different types of breaks in DNA through reactive species generation, considered a tumour causing agent [46], due to extensive DNA damage [47]. Alteration in protein structure and function induced by reactive nitrogen intermediates react with proteins and modify aminoacid residues by oxidation, nitrosylation, and carbonylation. Also, despite various studies demonstrating the involvement of NO in inhibiting infection by *L. chagasi* through high concentration cytokine action, it can also induce oxidative stress [15]. These results agree with other authors who also verified that damage in host cellular DNA is associated with free radicals, liberated by lymphoid cells during their attempt to control infection. In cutaneous leishmaniasis, Kocyigit *et al*. [48], showed that the oxygen radicals produced by the host could induce DNA damage in infected cells. An earlier study by our group also demonstrated that acute infection in animals infected with *T. cruzi* induced DNA damage in spleen cells, and that this damage was probably related to high NO production [6].

In conclusion, our results indicate that *L .chagasi* induced high levels of DNA damage in peripheral blood and spleen cells. Probably NO not only played an important role in parasite destruction, but also by inducing high levels of DNA damage in adjacent cells. Future studies will be performed mainly to improve the evaluation of these mechanisms with inducing cancer.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Anexo

Anexo 1 – Parecer do Comite de Ética em Pesquisa.

unesp
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Comissão de Ética em Experimentação Animal

CERTIFICADO

*CERTIFICAMOS que o Protocolo n.º **608** sobre o Projeto de Pesquisa "**Infecção por Leishmania chagasi: papel dos receptores Toll-like 2 e 4 e alterações genotóxicas em camundongos BALB/c**" sob a responsabilidade de Lidiane Ritsue Nagashi, orientada pela Profª. Drª. Sueli Aparecida Calvi, com a colaboração de Marcondes- Machado J, Lima CRG, Cocato RA, Cezário GAP, Oliveira LRC e Bursaca VS, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva que os "**ratos**" são provenientes de Biotério convencional, sem condições de certificar a sanidade dos mesmos.*

Projeto de Pesquisa Aprovado em 31 de maio de 2.007

Profª Drª Regina Helena G. Martins
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA