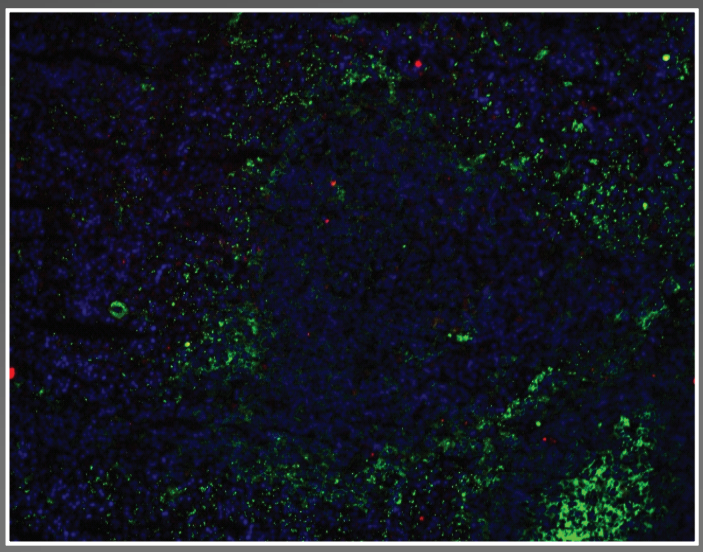
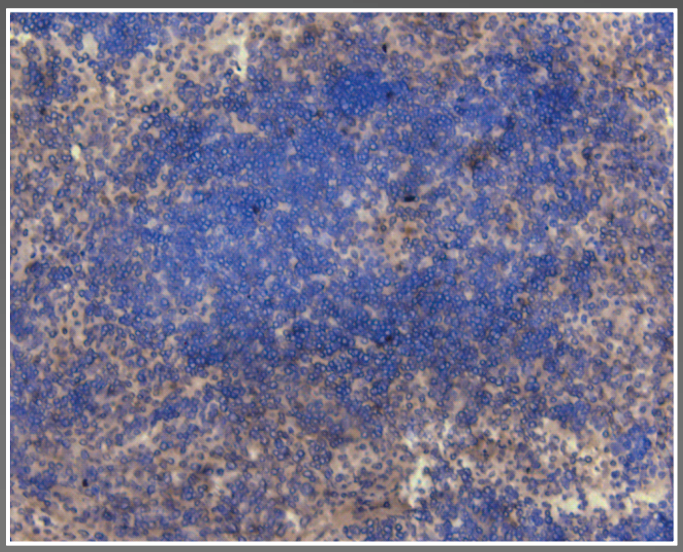
Papel do co-receptor PD-1 ("programmed cell death receptor 1") na infecção experimental por Trypanosoma cruzi

Fredy Roberto Salazar Gutierrez





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Universidade de São Paulo

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Departamento de Bioquímica e Imunologia

Pós-graduação em Imunologia Básica e Aplicada

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Ribeirão Preto

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Tese apresentada ao curso de Pós-Graduação em Imunologia Básica e Aplicada da Faculdade de Medicina de Ribeirão Preto – Universidade de São Paulo, para obtenção do grau de Doutor em Ciências – Área de concentração: Imunologia Básica e Aplicada

Orientador: Prof. Dr. João Santana da Silva

Ribeirão preto

2009

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Gutierrez, Fredy Roberto Salazar

Papel do co-receptor PD-1 ("*Programmed Cell Death Receptor 1*") na infecção experimental por *Trypanosoma cruzi*.

Tese de Doutorado, apresentada à Faculdade de Medicina de Ribeirão Preto/USP.

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Epígrafe

"(…) I gang my own gait and have never belonged to my country, my home, my friends, or even my immediate family, with my whole heart; in the face of all these ties I have never lost an obstinate sense of detachment, of the need for solitude--a feeling which increases with the years."

Albert Einstein, em "*The world as I see it*"

Dedicatória

A mi madre, por el amor, por los consejos, los inmensos e innúmeros sacrificios, Por el ejemplo, por la sabiduría. Por la dedicación y entrega y por el esfuerzo para poner a mi disposición las mejores opciones que encontró a su alcance. Por despertar en mi, desde muy pequeño, el gusto por la lectura y la curiosidad de aprender cosas nuevas cada dia. Por el tiempo sin dormir, pensando en su hijo ausente.

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(...) Minha mãe é um poema de branca cabeleira,
que tem à flor dos lábios um gesto de perdão.
Quando trás longa ausência regresso, ela me espera.
Me abraça como a criança e me beija com paixão

Minha mãe é pequena, igual uma violeta,
o doce está na sua alma, o pranto no adeus.

Minha mãe é como um cromo de mágica paleta
canção, dor, ternura... de tudo há na sua voz

Tradução livre de
"Carta a mi madre", de Julio Jaramillo

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Resumo

Durante a apresentação antigênica, a ativação eficiente dos linfócitos T depende de dois sinais: o primeiro é mediado pelo reconhecimento do antígeno através da interação TCR/MHC/peptídeo e o segundo pela interação dos co-receptores com os seus respectivos ligantes. CD28 e ICOS são co-receptores estimuladores, enquanto CTLA-4 e PD-1 inibem a ativação dos linfócitos. A sinalização através de CTLA-4 e PD-1 está envolvida na manutenção da tolerância periférica e pode ser indiretamente favorecedora de mecanismos de escape da resposta imune a parasitas. De fato, *T. cruzi* induz CTLA-4 e é eliminado quando esta é inibida. Por outro lado, o envolvimento de PD-1 como um mecanismo de controle da resposta imune a *T. cruzi* não é conhecido. No entanto, existem evidências que mostram o desenvolvimento de cardiopatia e o surgimento de anticorpos contra componentes dos cardiomiócitos na deficiência de PD-1, eventos que também acontecem na infecção pelo *T. cruzi.*

No presente trabalho foi avaliada a hipótese de que PD-1 e seus ligantes participam do sistema de regulação da resposta imune durante a infecção por *T. cruzi.* Primeiramente, foi mostrado que este parasito induz a expressão de PD-1 e de seus ligantes PD-L1 e PD-L2 em células apresentadoras de antígeno, em células do baço e em linfócitos circulantes e teciduais de camundongos infectados. Experimentos envolvendo o bloqueio desta via de sinalização com anticorpos, bem como o uso de camundongos geneticamente deficientes para PD-1 mostrou que esses camundongos apresentam uma resistência aumentada frente à proliferação do parasito, tanto *in vitro* quanto *in vivo*. Confirmou-se ainda, a exacerbação da resposta inflamatória no tecido cardíaco após a inibição de PD-1 e PD-L1. Esta resistência envolve mecanismos dependentes da imunidade inata e adaptativa. Especificamente, os macrófagos deficientes de PD-1 apresentam uma maior produção de óxido nítrico e maior capacidade tripanocida quando comparados às células provenientes de animais selvagens. Ainda, foi mostrado que a deficiência de PD-1 leva à diminuição da população de células com fenótipo regulador, bem como diminuição da taxa de apoptose em linfócitos T $CD4^+$e $CD8^+$.

Esses dados permitem afirmar que o co-receptor PD-1 participa no controle do processo inflamatório induzido após a infecção *T. cruzi* no coração. Os possíveis alcances do novo conhecimento gerado neste trabalho sobre os conceitos fisiopatológicos da infecção são discutidos.

Abstract

During an infection, the quality and intensity of the adaptive immune response relays on the efficient T cell activation which requires two signals: the first one is mediated by the recognition of the complex MHC/peptide (in the surface of professional antigen presenting cells, APC) by the TCR (in the surface of T cells). The second signal is provided by the interaction of co-receptors expressed in T cells surface, with their ligands on APC. CD28 and ICOS are co-stimulator receptors, while CTLA-4 and PD-1 inhibit T cell activation. The signaling through CTLA-4 and PD-1 is involved in the maintenance of peripheral tolerance and can indirectly support the immune evasion by parasites. In fact, CTLA-4 is induced after infection by *T. cruzi* and its inhibition leads to a better control of parasite growth. However, the involvement of PD-1 as a possible immune regulatory mechanism during this infection remains unknown.

Some evidences indicate that PD-1 is particularly important to the generation and maintenance of immunological tolerance to cardiac antigens. In accordance, PD-1 deficient mice develop spontaneous myocarditis which is mediated mainly by anti-cardiac troponin I. As *T. cruzi* infection is able to trigger a strong inflammatory reaction at myocardium of infected hosts, even in the absence of substantial parasitism, we hypothesized that PD-1 could be involved in the regulation of immune response during *T cruzi* infection.

First, we showed that this parasite induces the expression of PD-1 and its ligands PD-L1 and PD-L2 in spleen as well as circulating cells in infected hosts. Experiments performed *in vivo* and *in vitro* with blocking antibodies and knockout mice showed improved control of parasite proliferation. We observed increased inflammatory reaction in mice with deficient PD-1 signaling. This increased inflammation involves innate and adaptive immune mechanisms, characterized by increased ability of phagocytes to kill the parasite, and reduced populations of cells with regulatory phenotype (CD4CD25FoxP3), along with reduced rate of apoptosis in $CD4^+$and $CD8^+$T cells.

These data lead us to conclude that PD-1 co-receptor do participate in the control of immune response induced by *T. cruzi*. We discuss the potential relevance of these novel evidences on the understanding of immunopathogenesis of the immune response triggered by *T. cruzi*.

Introdução

Trypanosoma cruzi é o agente etiológico da doença de Chagas (CHAGAS, C., 1909). Este protozoário intracelular é capaz de infectar o ser humano, assim como várias espécies de mamíferos domésticos e selvagens que habitam as áreas tropicais das Américas. *T. cruzi* está distribuído geograficamente entre os 42° de latitude norte (norte da Califórnia) e 43° de latitude sul (norte da Argentina e Chile). Na atualidade, a infecção tem uma prevalência de aproximadamente 13 milhões de pessoas. Cerca de 75 milhões de pessoas estão em risco de infecção nestas regiões. A incidência anual da infecção é de 300 mil casos (WHO, 2005).

Devido a isso, a doença é considerada endêmica em várias regiões da América Latina e persiste como a principal forma de doença cardíaca infecciosa no mundo (WHO, 2002; KIRCHHOFF, WEISS *et al.*, 2004). A transmissão pode ocorrer através das fezes contaminadas do vetor (80-90%), transplantes ou transfusão sanguínea (5-20%) ou de forma congênita (0.5-8%) (DIAS, 2000). A grande maioria das infecções acontece durante a infância, sendo que as formas cardíacas ou digestivas da enfermidade se manifestam em aproximadamente 30% dos indivíduos, geralmente depois de décadas de infecção assintomática (TAFURI, 1987).

Múltiplas condições sócio-econômicas favorecem a interação entre humanos e vetores. Por exemplo, a constante invasão do homem aos ambientes inabitados, onde o inseto transmissor é endêmico, o desmatamento e até fenômenos naturais podem contribuir para transmissão vetorial (WALTER, 2003; GUZMAN-TAPIA, RAMIREZ-SIERRA *et al.*, 2005). Tais situações prevalecem nos países mais pobres da América Latina.

A iniciativa dos países do cone sul, usando medidas que visam à erradicação do parasito do peridomicílio, mostrou-se extremamente efetiva para reduzir a transmissão vetorial, chegando a ser considerada erradicada em muitas regiões (DIAS e SCHOFIELD, 1998; YAMAGATA e NAKAGAWA, 2006).

Contudo, devido a deficiências na vigilância epidemiológica e à persistência de alguns focos endêmicos, surtos esporádicos, transmissão transfusional e oral, ausência de marcadores diagnósticos e prognósticos efetivos, bem como à ineficácia da terapêutica disponível, a doença de Chagas persiste como um motivo de séria preocupação para a saúde pública em vários países (TARLETON,

REITHINGER *et al.*, 2007; DIAS, PRATA *et al.*, 2008). Ademais, devido à falta de interesse em investimentos por parte da indústria farmacêutica para o desenvolvimento de novos fármacos e por ser uma doença típica de países pobres, foi catalogada como "doença negligenciada" pela Organização Mundial da Saúde (WHO, 2002; URBINA e DOCAMPO, 2003; GUHL, RESTREPO *et al.*, 2005).

As características gerais da tripanossomíase americana foram descritas há um século por Carlos Chagas. Ele descreveu o agente etiológico, a principal forma de transmissão da infecção e caracterizou suas manifestações clínicas. Esse nível de entendimento de uma patologia, alcançado por um só pesquisador constitui um fato excepcional em toda a história da medicina (CHAGAS, C., 1909).

Em 1907, enquanto participava de um programa governamental voltado para o controle da prevalência da malária e da febre amarela no estado de Minas Gerais, na localidade de Lassance, um engenheiro lhe relatou a existência local de um inseto hematófago que descia pelo interior das tendas e andava sobre a face dos trabalhadores à noite enquanto eles dormiam. Os insetos eram chamados "barbeiros" ou "chupança". Carlos Chagas começou a especular sobre a possibilidade de esses insetos serem hospedeiros naturais para algum tipo de microorganismo e mais tarde identificou nessas pessoas um parasito flagelado. Logo depois ele demonstrou que esse parasito, previamente desconhecido (denominado por ele *Schizotripanum cruzi* e mais tarde como *Trypanosoma cruzi)*, era capaz de infectar mamíferos, o que foi confirmado por diversos autores posteriormente (CHAGAS, C, 1909; BRENER, 1973). Ainda, Carlos Chagas buscou evidências de infecção causada por este parasito em animais e o isolou na corrente sanguínea de um gato doméstico. Pouco tempo depois ele foi procurado para avaliar uma criança de dois anos de idade chamada Berenice, que apresentava febre e era a proprietária do gato infectado. Ele demonstrou então que a febre era causada pelo mesmo organismo isolado do gato e ainda observou a ausência do parasito no sangue quando a paciente melhorou da febre. Nos anos subsequentes ele descreveu as características mais importantes da enfermidade crônica, incluindo as manifestações cardíacas, gastrointestinais e neurológicas, que eram endêmicas na região, além de descrever a transmissão congênita da infecção. Por esses trabalhos científicos ele recebeu varias menções honrosas e prêmios (CHAGAS, C., 1909).

Depois de quase duas décadas de relativo esquecimento, algumas descobertas notáveis foram realizadas na epidemiologia da doença de Chagas. O pesquisador argentino Salvador Mazza sugeriu

em 1936 que essa doença poderia ser adquirida através de transfusões sanguíneas (MAZZA, 1936) e depois Dias e Pellegrino descreveram que a incidência da doença podia ser controlada através da erradicação do vetor com inseticidas (DIAS e PELLEGRINO, 1948). No entanto, foi somente no inicio da década dos anos 90 quando a magnitude real da doença foi percebida, levando ao estabelecimento de políticas de saúde pública com o objetivo de evitar que pessoas infectadas fossem doadores de sangue e também de erradicar o vetor das casas. Essas estratégias têm sido extremamente bem sucedidas no controle dessa ameaça à saúde pública (MOREL, 1999).

As opções terapêuticas disponíveis para o tratamento da doença de Chagas são limitadas. O tratamento consiste basicamente de medidas de suporte, como o tratamento da insuficiência cardíaca. A terapia tripanocida (TT), constituída principalmente por nitrofuranos e imidazolicos, estabelecida durante a fase aguda da infecção, pode evitar a evolução para a fase crônica entre 50-70 % dos casos (FERREIRA, 1961). Embora alguns estudos experimentais ou com pacientes sugiram seu uso na cardiomiopatia chagásica crônica (CCC) ou durante a fase indeterminada, não existe evidencia clínica sistemática da efetividade da TT nessas fases da doença (VILLAR, MARIN-NETO *et al.*, 2002; GARCIA, RAMOS *et al.*, 2005; REYES e VALLEJO, 2005). Atualmente existem vários estudos visando testar o beneficio da TT em pacientes com CCC ou com a forma indeterminada da doença (MARIN-NETO, RASSI *et al.*, 2008).

Os agentes farmacológicos usados na TT foram desenvolvidos de maneira totalmente empírica e estão vigentes na terapia clínica há mais de duas décadas, apesar dos consideráveis efeitos adversos por eles produzidos (CASTRO, DE MECCA *et al.*, 2006) e de eficácia dependente do genótipo do parasito (FILARDI e BRENER, 1987). Por tanto, há necessidade de desenvolver novas terapias para o tratamento da doença de Chagas, que sejam mais eficazes e seguras que a atual. Abordagens terapêuticas inovadoras poderão exercer um papel essencial para o sucesso da terapia clínica desta doença parasitária (URBINA, 1999). Entre essas estratégias a imunoterapia visando modular a reação inflamatória tem atraído a atenção desde que a resposta imune do hospedeiro, além de contribuir para o controle eficaz do parasitismo, parece ser responsável pela indução de boa parte da patologia (DOSREIS, FREIRE-DE-LIMA *et al.*, 2005). Contudo, os mecanismos que desencadeiam a resposta inflamatória no coração ainda são motivos de controvérsia. Estudos anteriores demonstraram que as

respostas mediadas por células $CD3^+CD8^+$e em menor grau $CD3^+CD4^+$são cruciais no desenvolvimento da lesão inflamatória cardíaca e também no controle do parasitismo (TARLETON, SUN *et al.*, 1994; TARLETON, 1995).

A hipótese de que a doença de Chagas tem um componente autoimune teve suas primeiras fundamentações na descrição de respostas do tipo celular e humoral contra diversos autoantígenos no curso da infecção (ENGMAN e LEON, 2002; LEON e ENGMAN, 2003). A presença de autoanticorpos circulantes que se ligavam a antígenos cardíacos em pacientes e camundongos cronicamente infectados com *T. cruzi* (CUNHA-NETO, DURANTI *et al.*, 1995; GUILHERME, CUNHA-NETO *et al.*, 1995) chamou a atenção para um fenômeno de natureza alérgica/autoimune. Além disso, linfócitos T autorreativos contra componentes dos tecidos cardíaco e nervoso foram encontrados nestes indivíduos, o que levou à extensão do conceito, incluindo a imunidade mediada por células (TARLETON, ZHANG *et al.*, 1997).

Assim, a participação de fenômenos autoimunes de natureza humoral e celular tem sido descrita durante a infecção. No entanto, não existe acordo se essas reatividades imunológicas são desencadeadas por epitopos do parasito e acabam por reconhecer os tecidos do hospedeiro devido a "mimetismo molecular", ou se são induzidas por antígenos próprios, expostos durante destruição tecidual decorrente do processo inflamatório. Ainda, a relevância dessa autorreatividade na patogênese da doença de Chagas é motivo de discussão. Teorias mais recentes descrevem outros mecanismos como sendo os responsáveis pela patogenia (ARAUJO JORGE, BARBOSA *et al.*, 1986; HIGUCHI, DE BRITO *et al.*, 1993; JONES, COLLEY *et al.*, 1993; HIGUCHI, 1995; ROWLAND, LUO *et al.*, 1995; BELLOTTI, BOCCHI *et al.*, 1996; VAGO, MACEDO *et al.*, 1996; ANEZ, CARRASCO *et al.*, 1999; LAGES-SILVA, CREMA *et al.*, 2001; GIRONES e FRESNO, 2003; GUTIERREZ, GUEDES *et al.*, 2009).

Entre essas teorias, a desregulação da resposta induzida pelo parasito tem atraído o interesse das pesquisas. A resposta imune não controlada pode levar a dano tecidual exagerado, podendo em casos extremos conduzir a um estado de resposta inflamatória sistêmica. Embora a infecção seja capaz de induzir uma robusta e aparentemente descontrolada resposta imune celular do tipo Th1 no miocárdio, o parasito não é completamente eliminado dos tecidos (SCHIJMAN, VIGLIANO *et al.*, 2004).

Entre os fatores que caracterizam a resposta imune observada no coração durante a infecção experimental por *T. cruzi,* destaca-se a excessiva produção local de quimiocinas, bem como o aumento da expressão dos seus receptores nas células que migram para os focos inflamatórios no músculo cardíaco e outros tecidos (MACHADO, MARTINS *et al.*, 2000; ALIBERTI, SOUTO *et al.*, 2001; TEIXEIRA, GAZZINELLI *et al.*, 2002; LANNES-VIEIRA, 2003; GOMES, BAHIA-OLIVEIRA *et al.*, 2005; MACHADO, KOYAMA *et al.*, 2005).

O recrutamento de leucócitos para o coração envolve a participação de citocinas pró-inflamatórias, moléculas de adesão (LAUCELLA, SALCEDO *et al.*, 1996) e de componentes da matriz extracelular, coordenada por quimiocinas (TEIXEIRA, GAZZINELLI *et al.*, 2002; LANNES-VIEIRA, 2003). Esses leucócitos produzem grandes quantidades de NO (SILVA, MACHADO *et al.*, 2003) e de citocinas IFN-γ, IL-12 e TNF-α (SILVA, TWARDZIK *et al.*, 1991; GAZZINELLI, OSWALD *et al.*, 1992; SILVA, MORRISSEY *et al.*, 1992), bem como metaloproteinases de matriz extracelular (MMP) (GUTIERREZ, LALU *et al.*, 2008) e apresentam baixa ou inexistente produção de citocinas anti-inflamatórias como IL-4 ou IL-10 (GAZZINELLI, WYSOCKA *et al.*, 1996; GOMES, BAHIA-OLIVEIRA *et al.*, 2005).

Assim, a migração inicial de células para o tecido cardíaco deve ser controlada no sentido de minimizar a gênese de lesões teciduais, mas também sem permitir a persistência do agente infeccioso (MACHADO, MARTINS *et al.*, 2000). É possível que na ausência de um controle adequado da resposta imune haja maior formação de lesões cardíacas, como poderia ocorrer com a produção aumentada de IFN-γ observada em pacientes com cardiopatia chagásica (GOMES, BAHIA-OLIVEIRA *et al.*, 2003). Todavia, estratégias abordando os mecanismos naturais de regulação da resposta imune do hospedeiro têm sido pouco utilizadas na pesquisa de agentes quimioterápicos contra *T. cruzi* (CHAMOND, COATNOAN *et al.*, 2002).

O controle natural da resposta imune em animais infectados tem sido alvo de intenso estudo. Como resultado, sabe-se que IL-10, TGF-β, CTLA-4 e células T reguladoras $CD4^+CD25^+$ $FoxP3^+$ (Treg) constituem os principais mecanismos que participam no controle natural da resposta imune inflamatória.

A presença de células Treg no sítio inflamatório é frequentemente vista como um fator determinante na persistência de parasitos nos tecidos comprometidos, pois mantêm uma resposta imune efetora atenuada frente a agentes infecciosos (BELKAID, 2007). Recentemente foi sugerido que as células Treg não participam ativamente no controle da resposta inflamatória no miocárdio, porque o bloqueio do receptor de IL-2 de alta afinidade (CD25), uma das moléculas altamente expressas em células Treg, mas também expressa em células T ativadas, não exerceu efeitos significativos na resposta inflamatória miocárdica ou sistêmica (KOTNER e TARLETON, 2007). No entanto, esses dados contrastam com os achados em pacientes, em que uma maior frequência de células Treg foi detectada no sangue periférico de indivíduos com a forma indeterminada da doença (ARAUJO, GOMES *et al.*, 2007)(Guedes, Gutierrez *et al*, manuscrito em preparação). Além disso, nós mostramos que essas células migram para o tecido cardíaco durante a fase aguda da infecção e que o tratamento com o anticorpo anti-GITR (expresso quase que exclusivamente em células Treg) induziu aumento da miocardite, caracterizada pela intensa presença de células inflamatórias e produção de citocinas no tecido cardíaco, sugerindo que as células Treg são necessárias ao controle da resposta inflamatória miocárdica (MARIANO, GUTIERREZ *et al.*, 2008).

A falha nesses mecanismos reguladores parece ser responsável pela deterioração progressiva da função cardíaca durante a fase crônica da enfermidade (DUTRA, ROCHA *et al.*, 2005; VITELLI-AVELAR, SATHLER-AVELAR *et al.*, 2005; ARAUJO, GOMES *et al.*, 2007). De fato, as alterações estruturais observadas nas fases avanzadas da doença (ROSSI, 2001) são consequências do processo inflamatório local intenso e persistente durante o parasitismo tecidual, que pode ser inexaurível (TEIXEIRA, GAZZINELLI *et al.*, 2002; DUTRA, ROCHA *et al.*, 2005; GUTIERREZ, GUEDES *et al.*, 2009). A matriz extracelular cardíaca (MEC) também participa ativamente do processo de migração das células inflamatórias uma vez que estas células necessitam quebrar momentaneamente os componentes da MEC para estabelecer o processo inflamatório no tecido (SPINALE, 2002; TSURUDA, COSTELLO-BOERRIGTER *et al.*, 2004). As MMP são essenciais em processos de migração celular durante inflamação e regeneração tecidual. Além disso, as MMP são também capazes de modificar a estrutura molecular de componentes solúveis da resposta inflamatória, como por exemplo, citocinas e quimiocinas, atuando assim como moduladoras de sua atividade. As MMP são

particularmente ativas na MEC em resposta a diversos estímulos, desde a isquemia até a inflamação. Durante a progressão da falência cardíaca foi relatado aumento da atividade das MMP, principalmente de MMP-9. MMP-2 também participam do dano cardíaco causado por isquemia-reperfusão (CHEUNG, SAWICKI *et al.*, 2000). De fato, esta enzima é ativada na presença de peroxinitrito (WANG, SAWICKI *et al.*, 2002) e pode clivar a troponina I (RORK, HADZIMICHALIS *et al.*, 2006), numa maneira similar ao mecanismo exercido por outras MMP em outras doenças autoimunes cujas patogenias envolvem epitopos remanescentes da degradação de proteínas do hospedeiro (OPDENAKKER e VAN DAMME, 1994). A participação destas enzimas na geração de possível autoimunidade frente a esse antígeno cardíaco, bem como dos mecanismos imunológicos que garantem a manutenção da tolerância imunológica frente à troponina I cardíaca nas condições inflamatórias desencadeadas por *T. cruzi* permanece totalmente desconhecida. Contudo, o potencial terapêutico da inibição das MMP está apenas começando a ser analisado em outras doenças cardíacas inflamatórias (SCHULZ, 2007) e promete se constituir em um adjuvante na terapia de pacientes chagásicos.

Outro mecanismo de regulação da resposta imune é através da expressão das moléculas coestimuladoras, tanto positivas quanto negativas que pertencem à superfamília das imunoglobulinas e controlam a ativação de linfócitos (SHARPE e FREEMAN, 2002). Essas moléculas interagem com seus ligantes, presentes em células apresentadoras de antígeno (APC), proporcionando um sinal adicional no processo de ativação das células T durante a apresentação antigênica. Esse sinal pode ser ativador no caso de CD28 e ICOS ou inibidor no caso de CTLA-4 e PD-1.

As moléculas coestimuladoras mais amplamente estudadas são CD80, CD86 e CD40, que apresentam expressão diferencial em camundongos resistentes ou suscetíveis a *T. cruzi* (PLANELLES, THOMAS *et al.*, 2003). Além disso, recentemente foi descrito que *T. cruzi* é capaz de modular negativamente a expressão destas moléculas na superfície de células dendríticas, tornando-as incapazes de ativar eficientemente as células T (PONCINI, ALBA SOTO *et al.*, 2008). Sugere-se que essas células APC com pouca capacidade estimuladora de linfócitos seriam capazes de induzir células Treg, ou regular diretamente a resposta (PULENDRAN, BANCHEREAU *et al.*, 2001; ZHANG,

TANG *et al.*, 2004), indicando que as moléculas coestimuladoras apresentam um papel determinante na indução da resposta frente a esse patógeno.

CD28 é um dos principais co-receptores que promovem a ativação linfocitária, reduzindo o tempo do estímulo antigênico necessário para ativar tanto as células T *naive* quanto aquelas já primadas (SPERLING e BLUESTONE, 1996; SHARPE e FREEMAN, 2002). Consistentemente a ausência ou o bloqueio de CD28 em animais infectados por *T. cruzi* resulta em aumento da parasitemia e da mortalidade (MARTINS, CAMPANELLI *et al.*, 2004).

CTLA-4 ("*cytotoxic T lymphocyte antigen* 4") é uma glicoproteína da superfamília das imunoglobulinas expressa de maneira estritamente regulada, apenas em linfócitos T e que apresenta alta homologia com CD28. Com efeito, seus ligantes são os mesmos que os de CD28: CD80 e CD86. Contudo, CTLA-4 liga-se às moléculas B7 com afinidade 100 vezes maior que CD28 (WALUNAS, BAKKER *et al.*, 1996; THOMPSON e ALLISON, 1997; SHARPE e FREEMAN, 2002). O bloqueio tanto *in vivo* quanto *in vitro* de CTLA-4 leva a um aumento na produção de NO, assim como de IFN-γ em resposta a antígenos de *T. cruzi*. Esses achados se correlacionam com o aumento da resistência à infecção, sugerindo que esses co-receptores estão envolvidos na modulação da resposta contra esse parasito, através da produção de IFN-γ e NO (MARTINS, TADOKORO *et al.*, 2004). A expressão de CTLA-4 previne o desenvolvimento de patologias autorreativas ao restringir a ativação de linfócitos T. Além disso, CTLA-4 constitui um mecanismo crucial de controle da resposta imune por células Treg (KINGSLEY, KARIM *et al.*, 2002; MANZOTTI, TIPPING *et al.*, 2002).

PD-1 ("programmed cell death receptor1") é uma proteína transmembrana de 55 kDa pertencente à superfamília das imunoglobulinas que apresenta em torno de 20% de identidade com CD28, CTLA-4 e ICOS. PD-1 é um co-receptor inibitório cuja expressão é induzida em linfócitos T e B, após os processos de ativação celular. Possui dois resíduos de tirosina na cadeia citoplasmática, um dos quais está localizado na porção N-terminal, associado a um ITIM ("*Immunoreceptor Tyrosine-based Inhibitory Motiff*")(ISHIDA, AGATA *et al.*, 1992; WANG, BAJORATH *et al.*, 2003). Diferente dos outros membros da família de moléculas coestimuladoras, PD-1 é expresso tanto em timócitos quanto em linfócitos T e B ativados e, em geral, em todas as células ativadas de linhagem linfóide (ISHIDA,

AGATA *et al.*, 1992; AGATA, KAWASAKI *et al.*, 1996; NISHIMURA, AGATA *et al.*, 1996; SHARPE e FREEMAN, 2002).

A ligação de PD-1 com os seus ligantes leva a ativação da tirosina fosfatase, SHP-2 ("*Src-Homology*-2-*domain-containing protein tyrosine phosphatase* 2"), que é recrutada pelo resíduo de tirosina fosforilado no ITIMSM ("ITIM-*Switch Motiff*") do extremo intracitoplasmático do PD-1 e defosforila moléculas envolvidas na transdução de sinais pelos receptores de linfócitos T ("*T cell Receptor*", TCR) ou B ("*B cell Receptor*", BCR), nas cascatas intracelulares de quinases (TAMIR, DAL PORTO *et al.*, 2000; OKAZAKI, MAEDA *et al.*, 2001).

A importância da sinalização via PD-1 foi demonstrada em estudos de transplante, nos quais o tratamento com anticorpos bloqueadores das moléculas coestimuladoras junto á estimulação via PD-1 leva a permanência de enxertos por períodos prolongados (GAO, DEMIRCI e LI, 2003; GAO, DEMIRCI, STROM *et al.*, 2003; LEE, WANG *et al.*, 2003). O tratamento com anticorpos neutralizantes anti-PD-L1 aumenta a expressão de IL-2 e IFN-γ por células T que desenvolveram aloativação *in vivo*, implicando essa sinalização na inibição da síntese dessas citocinas (MAZANET e HUGHES, 2002; BENNETT, LUXENBERG *et al.*, 2003). Evidências indicam que PD-1 contribui para a manutenção de um estado de hiporresponsividade frente a antígenos próprios nos linfonodos periféricos, assim como nos sítios inflamatórios (BENNETT, LUXENBERG *et al.*, 2003; BLANK, BROWN *et al.*, 2003).

Camundongos deficientes em PD-1 apresentam resposta linfoproliferativa marginal e doenças autoimunes (NISHIMURA, NOSE *et al.*, 1999; NISHIMURA, OKAZAKI *et al.*, 2001), embora não tão precoces e graves como em animais deficientes de CTLA-4 (WATERHOUSE, PENNINGER *et al.*, 1995), sugerindo a participação de PD-1 na supressão da ativação e/ou proliferação de células T periféricas autorreativas.

A deficiência de PD-1 em camundongos C57BL/6 leva a hipergamaglobulinemia com posterior desenvolvimento de glomerulonefrite e artrite, enquanto que em animais BALB/c a deficiência de PD-1 leva ao aparecimento de cardiomiopatia dilatada autoimune mediada por anticorpos anti-troponina I cardíaca (cTnI, uma proteína do complexo troponina/tropomiosina específica do sistema contráctil dos cardiomiócitos), que altera o influxo de cálcio, levando à disfunção e dilatação cardíaca nesses animais

(NISHIMURA, MINATO *et al.*, 1998; NISHIMURA, NOSE *et al.*, 1999; NISHIMURA, OKAZAKI *et al.*, 2001; BLANK, BROWN *et al.*, 2003).

Estudos com animais duplamente deficientes (RAG-2$^{-/-}$ e PD-1$^{-/-}$) proporcionaram evidências de que a ausência de PD-1 mantém o desenvolvimento tímico de células T duplo negativas e aumenta a seleção negativa de células T duplo positivas. Isso é consistente com seu papel de controlar o limiar de sinalização durante o processo de seleção, permitindo nesses animais a saída de células T maduras que normalmente não sobreviveriam à seleção tímica (NISHIMURA, AGATA *et al.*, 1996). Essas alterações não foram observadas em animais duplamente deficientes para CTLA-4 e PD-1(CTLA-4$^{-/-}$ PD-1$^{-/-}$), mostrando que alterações no desenvolvimento tímico contribuem para o fenótipo autoimune de animais PD-1$^{-/-}$ (BLANK, BROWN *et al.*, 2003). Esses dados sugerem que CTLA-4 e PD-1 controlam a resposta imune em fases diferentes.

O receptor PD-1 contém na sua fração intracelular um "motiff" de "switch" de tirosina inibitória, que leva à inativação da cascata de proteínas quinases desencadeada pela interação do TCR e as moléculas coestimuladoras com o MHC, particularmente da ERK (KEIR, LATCHMAN *et al.*, 2005).

PD-L1 e PD-L2 são os ligantes para PD-1 e compartilham 38% de identidade entre si e 20 a 27% com os outros membros da família: CD80, CD86 e ICOSL (DONG, ZHU *et al.*, 1999; NISHIMURA, NOSE *et al.*, 1999; FREEMAN, LONG *et al.*, 2000; LATCHMAN, WOOD *et al.*, 2001; WANG, BAJORATH *et al.*, 2003). PD-L2 é praticamente restrito a células dendríticas, mas pode ser induzido em macrófagos via IL-4. Além disso, não foi detectada a sua expressão em linfócitos (ISHIDA, IWAI *et al.*, 2002). Já o PD-L1 é principalmente expresso em células apresentadoras de antígeno (monócitos e células dendríticas) de forma constitutiva (YAMAZAKI, AKIBA *et al.*, 2002). Após estimulação policlonal, praticamente todos os linfócitos expressam PD-L1.

A expressão desses ligantes pode ser detectada em tecidos linfóides e não linfóides e pode ser induzida em monócitos e outras células apresentadoras de antígeno (APC) em resposta a IFN-γ e LPS (YAMAZAKI, AKIBA *et al.*, 2002). Em macrófagos inflamatórios, a expressão dos ligantes é regulada diferencialmente na presença de células Th1 e Th2, sendo que, PD-L1 é induzido em vários tipos de células fagocíticas na presença de células Th1 e PD-L2 é expresso (somente em macrófagos inflamatórios) na presença de células Th2 (LIANG, LATCHMAN *et al.*, 2003). PD-L1, é

constitutivamente expresso em células dendríticas (DC) e pode ser expresso em outros tipos celulares em tecidos não linfóides, como por exemplo, as células cardíacas (LATCHMAN, WOOD *et al.*, 2001; LIANG, LATCHMAN *et al.*, 2003).

Apesar de ter homologia com CD28, PD-L1 carece do motivo MYPPPY, sequência encontrada nas moléculas CTLA-4 e CD28 que permite a ligação a CD80 e CD86. Por tanto, PD-L1 não se liga em CD28, CTLA-4 nem ICOS.

Sob estímulos inflamatórios ocorre um aumento da expressão de PD-L1 em APC e várias células de tecidos não linfóides como coração, pulmão, fígado, rim e células endoteliais que podem passar a expressar esse ligante (LIANG, LATCHMAN *et al.*, 2003). Especula-se que a expressão de PD-L1 nesses tecidos funcionaria como proteção contra células T efetoras potencialmente autorreativas (SHARPE e FREEMAN, 2002; GREENWALD, FREEMAN *et al.*, 2005). Igualmente, tem-se encontrado em células tumorais (*in vivo* e *in vitro*) um aumento da expressão dessa molécula, a qual é induzida por IFN-γ. A expressão de PD-L1 e PD-L2 nessas células tem-se associado à capacidade de escape do ataque direito de linfócitos T citotóxicos específicos (CTL) (IWAI, ISHIDA *et al.*, 2002). Foi demonstrado que o ligante PD-L1 expresso em algumas linhagens celulares de tumores, induz anergia ou apoptose de células T ativadas, através da produção de IL-10 ou da expressão de FasL, respectivamente (DONG, ZHU *et al.*, 1999; DONG, STROME *et al.*, 2002). Porém, os mecanismos que regulam a expressão desses ligantes permanecem desconhecidos.

A via de sinalização PD-1/PD-L1 está sendo objeto de intensa pesquisa na atualidade, já que um maior entendimento e uma adequada manipulação dessas novas vias coestimuladoras levariam à criação de estratégias para modular a resposta imune. Por exemplo, estudos em modelos experimentais de tumores demonstraram que o tratamento com anticorpos anti-PD-L1 incrementam a eficiência terapêutica da transferência adotiva de CTLs (STROME, DONG *et al.*, 2003). É de se esperar que tais abordagens terapêuticas possam ser de utilidade para imunoterapia de algumas doenças caracterizadas por autoimunidade ou perda da tolerância periférica (GAO, DEMIRCI e LI, 2003), como poderia ser o caso da doença de Chagas.

As células Treg expandem em detrimento do número de células $CD4^+$ efetoras e isso está associado a um aumento da expressão de PD-L1 em células Treg quando o hospedeiro é submetido à

imunoterapia com anticorpo agonista de CD40, adicionado da citocina IL-2 recombinante em um modelo experimental de tumor. Surpreendentemente, somente as células efetoras apresentam aumento na expressão de PD-1 em resposta a esse tratamento. Essas evidências apontam para PD-1 como um importante mecanismo de controle para a expansão de Treg. (ALDERSON, ZHOU *et al.*, 2008). Como salientado, o estudo da função de PD-1 na modulação da resposta imune em animais infectados com *T. cruzi* é extremamente interessante. É provável que exista uma associação entre o fenótipo observado na deficiência de PD-1 (que pode levar a cardiopatia) e o que ocorre em indivíduos infectados por *T. cruzi*, onde aproximadamente 30% desenvolvem cardiopatia.

A cardiopatia verificada em animais PD-$1^{-/-}$ é causada por anticorpos contra troponina I cardíaca. É possível que isto tenha uma relação com anticorpos reativos contra o endocárdio, estruturas vasculares e interstício (anti-EVI), descritos antigamente no soro de pacientes infectados (COSSIO, DIEZ *et al.*, 1974). Uma série de estudos mostrou a presença, tanto no soro de pacientes como de animais experimentalmente infectados com *T. cruzi*, de anticorpos que reconhecem componentes do miocárdio (BRENER, RAMIREZ *et al.*, 1983) (PONTES-DE-CARVALHO, SANTANA *et al.*, 2002; CUNHA-NETO, IWAI *et al.*, 2004). Concluiu-se na época, que esses anticorpos seriam formados em resposta a antígenos liberados de fibras miocárdicas parasitadas gerando reações de autoagressão que, atuando no coração, determinariam a cronificação da cardiopatia chagásica. Por outro lado, a presença de anticorpos anti-EVI no soro de pacientes chagásicos foi interpretada como consequência da presença de determinantes antigênicos comuns a *T. cruzi* em estruturas do miocárdio. Tal interpretação baseou-se no fato de que os anticorpos anti-EVI foram adsorvidos por antígenos de *T. cruzi*, enquanto que os anticorpos contra o parasito não foram adsorvidos por homogeneizado de miocárdio (SZARFMAN, COSSIO *et al.*, 1977).

A demonstração do envolvimento da via de sinalização por PD-1 na miocardite chagásica poderia sugerir uma alteração da tolerância periférica na gênese deste processo, ajudando no entendimento dos mecanismos de patogenicidade. Assim sendo, o objetivo deste trabalho foi estudar a participação e o papel de PD-1 na resposta imune durante a infecção experimental por *T. cruzi*.

Objetivos

O objetivo central deste estudo foi estudar o papel da via de sinalização de PD-1 na fisiopatogenia da infecção por *T. cruzi*.

Mais especificamente,

1. Verificar e analisar o perfil da expressão de PD-1 e de seus ligantes em células da resposta imune durante a fase aguda da infecção experimental por *T. cruzi*.
2. Verificar a presença de anticorpos anti-troponina cardíaca no soro de camundongos infectados por *T. cruzi*.
3. Estudar o efeito do bloqueio da via de sinalização de PD-1 na imunopatologia durante a fase aguda da infecção experimental por *T. cruzi*.
4. Verificar o perfil da resposta imune em camundongos geneticamente deficientes de PD-1.

Material e métodos

Animais e infecção experimental

Os experimentos foram realizados com camundongos C57BL/6 fêmeas, com idade entre seis e oito semanas, criada e mantida no Biotério dos Departamentos de Bioquímica e Imunologia e Biologia Celular, Molecular e Bioagentes Patogênicos. Em todos os experimentos realizados *in vivo*, foram utilizadas formas tripomastigotas da cepa Y de *T. cruzi* (SILVA e NUSSENZWEIG, 1953), mantidas por passagens seriadas em camundongos *Swiss*. Nos experimentos *in vitro*, o parasita foi mantido em cultura de fibroblastos derivados de rim de macaco (LC-MK2)(HULL, CHERRY *et al.*, 1956) mantida em meio RPMI suplementado com 10% de soro fetal bovino (SFB, Gibco Invitrogen, Carlsbad, CA EUA). Os camundongos foram inoculados com 1000 formas tripomastigotas de *T. cruzi* por via intraperitoneal (i.p.), diluídas em 0,2 mL de tampão salina fosfato ("*phosphate buffered saline*", PBS). Os experimentos envolvendo camundongos PD-$1^{-/-}$ foram realizados nos Estados Unidos. Estes camundongos possuem "*background"* genético de C57BL/6 e foram mantidos no biotério livre de patógenos específicos ("*specific pathogen free*," SPF) no *Cincinnati Children's Medical Center*. Como grupo controle, nesses experimentos foram usados camundongos C57BL/6 adquiridos no Jackson Laboratories (Bar Harbor, Maine EUA*).*

Anticorpos e tratamento

Os anticorpos bloqueadores anti-PD-1 (J43), anti-PD-L1 (MIH5) e anti-PD-L2 (TY25), foram cedidos pelos doutores Tasuku Honjo (*Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto Japan*) e Miyuki Azuma (*Department of Molecular Immunology, Tokyo Medical and Dental University, Tokyo Japan*). Nos experimentos de bloqueio *in vivo* com esses anticorpos, os camundongos foram distribuídos em 4 grupos (n=10) e tratados com: IgG de rato, anti-PD-1, anti-PD-L1 ou anti-PD-L2 e posteriormente infectados. Os animais foram inoculados por via intra-peritoneal (i.p.) com 250 µg de cada anticorpo, diluído em um volume de 0,2 mL de PBS.

Dois esquemas de tratamento foram utilizados: no primeiro, o tratamento foi iniciado 24 horas antes da infecção, sendo administradas doses iguais e sucessivas a cada 72 horas por duas semanas e o

grupo controle recebeu 250 µg de IgG de rato seguindo o mesmo esquema. Alternativamente, apenas uma dose de 250 µg de cada anticorpo foi administrada i.p., 24 horas antes da infecção. Os resultados obtidos com o segundo tratamento foram similares aos do primeiro.

Parasitemia

O número de parasitas no sangue periférico foi determinado em diferentes dias após a infecção, conforme descrito previamente (BRENER, 1962). Resumidamente, 0,005 mL de sangue da cauda foram colhidos e colocados sobre uma lâmina, sendo então coberta por uma lamínula 22 x 22 mm, de modo a se obter uma fina camada ocupando homogeneamente toda a superfície da lamínula. Essa preparação foi levada ao microscópio óptico, determinando-se o número de parasitas em um total de 100 campos, com um aumento final de 400x. Conhecendo-se a área de cada campo microscópico, foi possível inferir o número aproximado de parasitas por mL do sangue.

Extração de RNA e síntese de cDNA

A extração de RNA total foi realizada a partir de amostras de coração de animais com diferentes dias de infecção ou sadios. Foi utilizado o reagente Trizol (Invitrogen Carlsbad, CA EUA) de acordo com as recomendações do fabricante. De forma resumida, em cada amostra foi adicionado 1 mL de Trizol® e 200 µL de clorofórmio absoluto (Sigma, St Louis, MO, EUA) e as amostras centrifugadas a 12.000xg por 15 minutos a 4ºC. A fase aquosa foi transferida para um novo tubo e adicionados 500 µL de isopropanol absoluto. As amostras foram então incubadas por 15 minutos a -20ºC para a precipitação do RNA da fase aquosa. A seguir os tubos foram centrifugados a 12.000 g por 15 minutos a 4ºC. O *pellet* foi lavado em etanol 75%. Finalmente, as amostras de RNA foram resuspendidas em 30 µL de água tratada com Dietilpirocarbonato (DPC). 2 µg de RNA total foram utilizados para confecção do cDNA. Este foi sintetizado utilizando-se a enzima transcriptase reversa (Superscript II , Gibco) de acordo com as instruções do fabricante.

Extração de DNA genômico total

A extração de DNA total foi realizada a partir de amostras de tecido cardíaco de animais infectados com *T. cruzi*. Brevemente, 1 mL de tampão de digestão (Tris-HCl 50 mmol/L; EDTA 1

mmol/L; Tween20 0,5% em água ultra filtrada) foi adicionado em cada amostra, homogeneizando as amostras com seringa de 3 mL e agulha 38G11/2. Uma solução de proteinase K (20 mg/mL, Gibco) foi adicionada, em uma diluição final de 0,2 mg/mL, incubando a 56ºC, *overnight*. As amostras foram aquecidas a 95ºC por 8 minutos e tratadas com 6 µL de RNAse A (10 mg/mL) por 1 *horas* a 37ºC e 1 volume de fenol:clorofórmio:álcool isoamílico (Invitrogen) adicionado, invertendo as amostras 50 vezes por 15 minutos. Em seguida, as amostras foram centrifugadas a 3500xg por 5 minutos a 25ºC e a fase aquosa coletada num novo tubo, adicionando clorofórmio, homogeneizando e centrifugando a 3500xg por 5 minutos a 25ºC. Após isso, a fase aquosa foi coletada em um tubo novo, adicionando isopropanol, homogeneizando novamente e centrifugando a 3500xg por 5 minutos a 25ºC. Posteriormente, os sobrenadantes foram descartados, lavando o DNA com 3 volumes de etanol (70%) e centrifugando a 3500xg por 5 minutos a 25ºC, para em seguida descartar os sobrenadantes e deixar secar. O DNA foi ressuspendido em 150 µL de solução 10 mmol/L Tris-HCl pH8,5 e a concentração de cada amostras determinada no espectrofotômetro (Molecular Devices) a λ 260/280 ηm. As amostras foram então guardadas a 4ºC até o momento do uso na reação em cadeia da polimerase (PCR) em tempo real, com os iniciadores específicos para o gene do cinetoplasto do parasito.

Detecção de PD-1 e seus ligantes por PCR em tempo real

A quantificação da expressão do RNA mensageiro (mRNA) para PD-1, PD-L1 e PD-L2, bem como para β-actina foi analisada por PCR em tempo real, conforme descrito anteriormente (RAMOS-PAYAN, AGUILAR-MEDINA *et al.*, 2003). As sequências dos iniciadores utilizados encontram-se listados na tabela 1.

Nome	Sense	*Anti sense*
β-actina	5'-AGC TGC GTT TTA CAC CCT TT-3'	5'-AAG CCA TGC CAA TGT TGT CT-3
PD-1	5'-TTC AGG TTT ACC ACA AGC TGG-3'	5'-TGA CAA TAG GAA ACC GGG AA-3'
PD-L1	5'-GCT GAA AGT CAA TGC CCC ATA-3'	5'-TCC ACG GAA ATT CTC TGG TTG-3'
PD-L2	5'-TTG TCG GTG TGA TTG GCT TC-3'	5'-AAA AGG CAG CAC ACA GTT GC-3'
TcDNA	5'-GCT CTT GCC CAC AMG GGT GC-3'	5'-CCA AGC AGC GGA TAG TTC AGG-3'

Tabela 1 Sequências dos iniciadores usados para PCR em tempo real

PCR com transcrição reversa (RT-PCR)

Após a extração do RNA total e obtenção do cDNA, 2µg de cDNA foram usados para reação de PCR usando os reagentes do kit TaqMan (Promega, Madison Wi, EUA), de acordo com as indicações

do fabricante, usando os iniciadores listados na tabela 2 e o termociclador (PTC-100, MJ-Research, Walthman MA EUA). O resultado da PCR foi usado para eletroforese em gel de agarose 2% contendo 0,5 µg/mL de brometo de etídio (Sigma) para permitir visualizar as bandas sob luz ultravioleta. Os géis foram fotografados e a análise densitomêtrica das imagens foi realizada com o software QuantityOne v4.4.1. (BioRad). Os resultados da densitometria foram apresentados em unidades arbitrárias.

Cultura de células dendríticas (CD) derivadas da medula óssea

As células precursoras de CD foram obtidas da medula óssea de fêmures e tíbias de camundongos. Para diferenciação em CD maduras essas células progenitoras mielóides foram cultivadas em micro placas de 24 poços na densidade de 1 x 10^6 células por poço, em RPMI suplementado com GM-CSF (50 ηg/mL) e IL-4 (10 ηg/mL) conforme descrito previamente (LUTZ, KUKUTSCH *et al.*, 1999).

Nome	*Sense*	*Anti sense*
β-actina	5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'	5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'
NOS2	5'-CCC TTC CGA AGT TTC TGG CAG C-3'	5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'
Arginase 1	5'-ATG GAA GAG ACC TTC AGC TAC-3'	5'-GCT GTC TTC CCA AGA GTT GGG-3'
IL-6	5'-CTTCATGTACTCCAGGTAG-3'	5'-TTCCATCCAGTTGCTTCTTG-3'
IL-10	5'-CGGGAAGACAATAACTG-3'	5'-CATTTCCGATAAGGCTTGG-3'
IL-12 p35	5'-TTGCCCTCCTAAACCACCTCAGTT-3'	5'-ATTCTGAAGTGCTGCGTTGATGGC-3'
IL-12 p40	5'-ACCTGTGACACGCCTGAAGAAGAT-3'	5'-ATTCCCGCCTTTGCATTGGACTTC-3'
IL-17A	5'-ATCATCCCTCAAAGCTCAGCGTGT-3'	5'-ACCAGCATCTTCTCGACCCTGAAA-3'
IFN-γ	5'-AACGCTACACACTGCATCTTGG-3'	5'-GACTTCAAAGAGTAACAGG-3'
SOCS1	5-CAGGTGGCAGCCGACAATGCGATC-3	5-CGTAGTGCTCCAGCAGCTCGAAAA-3
SOCS2	5-GGAAGTATGACTGTTAATGAAGCC-3	5-CCCAGATCGTACCGGTACATT-3
SOCS3	5-CCGGCTAGCATGGTCACCCACAGCAAG-3	5-TTTGGATCCTTAAAGTGGAGCATCATA-3
Stat-5b	5'-TGTTCAACATCAGCAGCAACCACC-3'	5'-TGTTGTAGTGAGGTTGAGGGCACA-3'
CCR5	5'-GAAACAAATTGCGGCTCAGCTCCT-3'	5'-AGCCCTGTGCCTCTTCTTCTCATT-3'
TNF-α	5'-GATCTCAAAGACAACCAACTAGTG-3'	5'-CTCCAGCTGGAAGACTCCTCCCAG-3'
T-bet	5'-ATCATCACTAAGCAAGGACGGCGA-3'	5'-ACAGCTCGGAACTCCGCTTCATAA-3'
Atg5	5'-CACCTCGGTTTGGCTTTGGTTGAA-3'	5'-TCGTTGTCCTGACTCAAGGTGGTT-3'
Beclin-1	5'-TGATGAAGGCATCCTGGCAGTGTA-3'	5'-TGTGTTTCAGCGTTCTGCAACTGG-3'

Tabela 2 Sequências dos iniciadores usados para RT-PCR.

Resumidamente, cinco camundongos foram sacrificados por experimento e seus fêmures e tíbias cuidadosamente removidos, as epífises seccionadas em ambiente estéril e as medulas lavadas com PBS utilizando uma seringa com agulha 13 x 4,5 mm. As células recém colhidas foram lavadas e centrifugadas a 350xg por 10 minutos, eliminando-se o sobrenadante e incubando o sedimento com tampão de lise por 4 minutos à temperatura ambiente (TA), sendo submetidos a um novo ciclo de centrifugação por 10 minutos. Em seguida, as células foram diluídas em RPMI-1640 suplementado com 10% de SFB (Cultilab) e distribuídas em placas de 24 poços (Corning) na concentração de 2,5 x 10^6 células/ml na presença das citocinas descritas inicialmente. O meio de cultura suplementado com

10% de SBF adicionado de citocinas foi substituído a cada três dias. Entre o sétimo e o nono dia de cultura as células foram fenotipadas por citometria de fluxo, para determinar a co-expressão de CD11b e CD11c, sendo a infecção da cultura realizada quando a porcentagem de células CD11c Cd11b foi maior ou igual a 85% (em média, no nono dia de diferenciação). Nesse período, as células foram cultivadas com lipopolissacárideo (LPS) de *Escherichia coli* (1mg/mL, Sigma) ou tripomastigotas na razão parasita:célula de 5:1, por 48 horas em câmara úmida a 37°C contendo 5% de CO_2.

Cultura de células do baço

Os baços foram coletados com técnica estéril, de camundongos *naive* ou infectados com *T. cruzi*. Os órgãos foram divulsionados com a ajuda do embolo de uma seringa em uma placa de Petri contendo 5 ml de PBS. As suspensões assim obtidas foram passada através de microfiltros de 40 µm estéreis de nylon (Falcon Cell strainers, BD Biosciences) e centrifugadas a 350xg para precipitar as células, o sobrenadante foi descartado as células resuspendidas em 2 a 4 mL de tampão de lise de hemácias. Após incubação de 4 minutos a 4ºC, as células foram novamente centrifugadas, prévia adição de 10 mL de RPMI suplementado com 10 % de SFB. Uma alíquota dessa suspensão celular foi utilizada para realizar a contagem de células viáveis na câmara de Neubahuer. As células foram então distribuídas em micro placas de 96 poços (Nunc) na densidade de 5 x 10^5 células viáveis por poço em um volume total de 200 µL.

Para avaliar a indução da expressão de PD-1, PD-L1 e PD-L2 em células T, as células do baço foram incubadas na presença de concanavalina A (ConA, 2µg/ml) e/ou *T. cruzi* (multiplicidade da infecção, 5:1). Após incubação por 18, 24, 48, 72 ou 96 horas em câmara úmida a 37°C contendo 5% de CO_2, as células foram retiradas da placa e a expressão de PD-1 em linfócitos $CD4^+$ou $CD8^+$foi avaliada por citometria de fluxo.

Para avaliar o efeito do bloqueio de PD-1 e seus ligantes sobre a atividade proliferativa dos linfócitos, as células do baço foram previamente marcadas com *Carboxyfluorescein diacetate succinimidyl Ester* (CFSE), usando os reagentes *CellTrace CFSE Cell Proliferation Kit* (Molecular Probes) de acordo com as recomendações do fabricante. Resumidamente, a suspensão de células foi ajustada pra 1 x 10^6 células por mL e estas incubadas por 8 minutos a 37ºC em uma solução contendo

10 µmol/L de CFSE em PBS estéril suplementado com 0,1 g/dL de albumina sérica bovina ("*bovine serum albumine*" BSA). As células foram então lavadas duas vezes com a adição de 5 mL vezes de RPMI gelado (4º C) suplementado com 10% de SFB, centrifugando a 350xg. Entre uma e outra lavagem por centrifugação, as células foram incubadas por 30 minutos em câmara úmida a 37°C contendo 5% de CO_2. As células foram então distribuídas em placas de fundo em "U" (Nunc) e incubadas durante 72 a 96 horas na presença ou não de anti-CD3 (500A2, eBiosciences) na concentração de 200 ng/mL (CHAPOVAL, NELSON *et al.*, 1995). Ainda, a essas culturas foram adicionandos cada um dos seguintes anticorpos: anti-PD-1 (J43), anti-PD-L1 (MIH5) ou anti-PD-L2 (TY25) (todos da eBiosciences), todos na concentração de 100 µg/mL. As células foram então retiradas da placa e o perfil de expressão de CFSE foi avaliado por citometria.

Cultura de macrófagos peritoneais

Para obtenção dos macrófagos peritoneais, foi usado o meio tioglicolato Brewer (Difco), diluído em água destilada e autoclavado. O meio foi estocado a TA protegido da luz por pelo menos um mês para permitir a formação de produtos derivados da glicosilação avançada (LI, BAVIELLO *et al.*, 1997). Os camundongos foram inoculados com 1 mL de Tioglicolato. Quatro a cinco dias após a inoculação, os animais foram sacrificados através de asfixia com CO_2 e a pele abdominal removida. A lavagem peritoneal foi então realizada com três a cinco mL de PBS gelado (4º C) para obtenção dos macrófagos peritoneais, tomando cuidado de não induzir hemorragia na cavidade peritoneal.

A suspensão assim obtida foi centrifugada a 350xg e re-suspendida em RPMI suplementado com 10% de SFB. As células foram contadas em câmara de Neubauer e distribuídas em placas de 96 poços (Nunc) ou em placas de 8 poços acopladas a lâminas (*Labtek,* Nunc) na densidade de 5 x10^5 células por poço, no volume de 200 µL. Após incubação com concentrações crescentes de IFN-γ durante 6 horas, as células foram infectadas com tripomastigotas de *T. cruzi* (na razão de 5 parasitos por célula) e incubadas por más 6 horas, para posteriormente trocar o meio de cultura e lavar duas vezes cada poço com adição de 200 uL de PBS estéril. Finalmente, as células foram re-suspendidas em 200 uL de RPMI suplementado com 10% de SFB. Após isso, as células foram mantidas por 48h em câmara

úmida a 37ºC contendo 5% de CO_2. As células foram então retiradas da placa para fenotipagem por citometria de fluxo.

Quando as células foram cultivadas em lâminas *Labtek*, as mesmas foram lavadas com PBS para retirar o meio de cultura e os poços retirados para realizar a coloração por Giemsa (Sigma) e montagem com Permount (Sigma). As lâminas foram examinadas ao microscópio e fotografadas (Olympus). As imagens obtidas foram utilizadas para contagem do número de parasitos por célula, contando 200-250 células por poço, usando o programa imageJ.

Isolamento de células inflamatórias do coração

Para isolar as células inflamatórias a partir do tecido cardíaco de animais infectados, os corações removidos de 5-10 animais infectados foram cortados em pequenos fragmentos de aproximadamente 1 mm^3 e incubados a 37°C por 1 hora em 5 mL de solução contendo 0,005g/mL de liberase CI (Roche, Basel, Suíça), preparada em RPMI incompleto (GIBCO). Posteriormente, o tecido foi triturado com ajuda do instrumento "Medimachine" (BD Biosciences) por 4 minutos. A seguir, acrescentou-se 2 mL de RPMI suplementado com 10% de SFB. Depois de duas lavagens com 3 mL de RPMI suplementado com 10% de SFB por centrifugação a 350xg durante 10 minutos a 4°C, as células foram suspendidas em 2 mL de PBS estéril e centrifugadas 350xg em gradiente de Ficoll-Hypaque (Amersham Biosciences) na proporção V/V de 1/3, por 30 minutos a 25 ^{o}C. Após coletar a camada rica em leucócitos e re-suspendê-la em 2 mL de PBS filtrado, 2 centrifugações adicionais a 350xg por 10 minutos a 4°C foram realizadas e as suspensões celulares homogeneamente distribuídas em tubos de citometria aproximadamente a razão de 1 x 10^6 células/mL para a fenotipagem com anticorpos.

Obtenção de células mononucleares a partir de células do sangue periférico de camundongos

O sangue foi coletado do plexo ocular de camundongos *naive* ou em diferentes momentos após a infecção com *T. cruzi* (3d, 7d, 14d, 20d, 218d). Aproximadamente um mL de sangue foi coletado de cada camundongo num microtubo contendo 0,1 mL de citrato de sódio 4g/dL em PBS como anticoagulante. A seguir, o sangue foi centrifugado a 350xg para separação do plasma e este último armazenado a -70°C para posteriores análises. As células do psedimento foram então ressuspensas em 2 mL de tampão de lise

de hemácias (cloreto de amônio 0.16 mol/L em tampão tris) e incubadas durante 4 minutos a 4°C. A seguir, foi acrescentado meio de cultura RPMI suplementado com 10% de SFB, centrifugando duas vezes a 350xg durante dez minutos a 4°C para retirar o excesso de tampão de lise. A viabilidade celular foi determinada com azul de trypan e as suspensões celulares homogeneizadas com PBS para $1x10^6$ células por mL e distribuídas em tubos de citometria.

Citometria de fluxo

A técnica de citometria de fluxo foi utilizada para as análises da expressão de PD-1 e seus ligantes na superfície dos diferentes tipos celulares analisados. As suspensões celulares foram obtidas diretamente das culturas ou preparadas a partir da digestão de tecido cardíaco ou da divulsão cuidadosa do tecido esplênico. Estas suspensões celulares foram filtradas com microfiltro de nylon de 40 μm (Falcon Cell strainers, BD Biosciences), contadas as células e ressuspensas à razão de 1×10^6 células/mL em 0,1 mL de sobrenadante da cultura de células produtoras de anti-CD16/CD32 (2.4G2) por 30 minutos a 4°C, em seguida incubadas nas mesmas condições com anticorpos monoclonais marcados com os diferentes fluorocromos (CD11c-PE, PD-L1-PE, PD-L2-PE, PD-1-PE, CD-3-PerCP, CD-4-FITC, CD-8-FITC, F480-FITC, IL-10-FITC, IL-17A-FITC, IFN-γ-FITC) ou conjugados não relacionados de mesmo isotipo (todos obtidos da Ebiosciences).

Após incubação com os anticorpos, as amostras foram centrifugadas a 350xg por 10 minutos a 4°C, desprezando o sobrenadante e re-suspendendo o sedimento em 1 mL de PBS filtrado adicionado com 0,1g/dL de BSA e 2mmol/L de EDTA. Após um novo ciclo de centrifugação, a suspensão celular foi fixada utilizando-se com 0,1 mL de PBS contendo formol a 1% antes de ser adquirida no citômetro de fluxo.

Para citometria de fluxo de miócitos as células foram extraídas pela digestão com Liberase (Roche) do tecido cardíaco de animais sadios ou infectados, como descrito para o isolamento de células inflamatórias do coração, com algumas modificações. Brevemente, as células extraídas após a digestão foram incubadas em meio RPMI suplementado com 10% de SFB durante 30 minutos em placas de Petri para retirada das células aderentes (fibroblastos e monócitos). A seguir, foi feita a fenotipagem para verificar a expressão de PD-1 e ligantes.

As amostras foram adquiridas no citômetro FACsorting (Becton and Dickson, San Jose, CA, USA) e as análises foram feitas usando o *Software* "Cell Quest" (BD) ou FlowJo (Tristar, Ashland, OR, EUA). Os experimentos realizados nos EUA foram adquiridos no citômetro FACsCantoII (BD). As porcentagens de células CD3, CD3CD4, CD3CD8, CD11b, CD11c, PD-1, PD-L1 ou PD-L2 foram calculadas dentro da "*gate*" de leucócitos, junto com a intensidade de expressão destas moléculas.

Marcação intracelular de citocinas em células isoladas dos tecidos de animais infectados com T. cruzi

Após obtenção da suspensão celular dos diferentes tecidos de camundongos infectados ou não com *T. cruzi*, a marcação de moléculas de superfície foi realizada como descrito no protocolo de fenotipagem celular, Posteriormente, as células foram fixadas com PBS contendo formaldeído 2% (200 uL por cada tubo contendo $1x10^6$ células). A seguir, as células foram precipitadas por centrifugação a 350 x g, previa adição de 1mL de uma solução contendo EDTA 2 mmoles/L e BSA 0,1g/dL em água destilada. À suspensão celular foram acrescentados 100 µL de saponina 0,01 g/dL (Merck, Darmstadt, Alemanha), contendo os anticorpos anti-citocinas conjugados a fluorocromos, na diluição de 1:200, incubada por 18 horas a 4°C protegidas da luz. As células foram centrifugadas a 350xg, previa adição de 1mL de PBS adicionado de BSA 0,1g/dL e EDTA 2 mmol/L. Após desprezar o sobrenadante, 100 µL de formaldeído 1% foi então adicionado a cada tubo e as células foram adquiridas no citômetro de fluxo.

Detecção de apoptose de linfócitos do baço

A detecção de apoptose foi realizada em células extraídas de baço de camundongos C57BL/6 normais ou PD-$1^{-/-}$ infectados com *T. cruzi* pela técnica de Anexina V(VERMES, HAANEN *et al.*, 1995). As células obtidas foram incubadas por 15 minutos com Anexina V e 7-Amino actinomicina (7-AAD) usando os reagentes *PE Annexin V Apoptosis Detection Kit I* (BD Pharmingen), de acordo com as indicações do fabricante e analisadas em até 45 minutos no citômetro.

Histologia

Amostras de coração, baço, fígado e músculo esquelético foram coletadas e fixadas em PBS contendo 10% de formol (PBS-F) durante 18-24 horas e posteriormente incluídas em blocos de parafina. E realizado, com auxílio de um micrótomo, cortes de 5 µm em lâminas e incubados a 60° C para fixação. Em seguida, foram lavados em xilol para retirar o excesso de parafina e re-hidratados com passagens em soluções contendo concentrações decrescentes de álcool (do absoluto a 80%). Os cortes re-hidratados foram corados com hematoxilina e eosina (H&E) e desidratados novamente, pela passagem em soluções com concentrações crescentes de álcool (80% a absoluto), lavados com xilol e cobertos com lamínulas.

Imunohistoquímica e imunofluorescência

Animais C57BL/6 infectados ou não com *T. cruzi* foram sacrificados, os corações e baços extraídos e congelados em meio de crio-preservação (OCT, Tissue Teck). Com ajuda de um criostato (Leica), foram feitos cortes de 5 µm de espessura a partir destes órgãos.

Brevemente, as lâminas contendo os cortes foram fixadas em acetona por 1 minuto e após 10 minutos a TA, foram congeladas a -80º C até o momento do uso. Anteriormente à realização da reação de imunofluorescência, as lâminas foram retiradas do freezer e mantidas a TA por 30 minutos. A ligação inespecífica do anticorpo foi bloqueada com anti-CD16/CD32 (2.4G2; *FcBlock,* BD Biosciences) na concentração de 5ug/mL, incubando por 30 minutos à TA. Em seguida, os anticorpos primários, conjugados ou não, foram acrescentados aos cortes na diluição correspondente para cada caso, no volume de 50uL, diluídos com PBS suplementado com 0,1g/dL de BSA.

Os cortes de tecido foram então utilizados para análise da presença do PD-1 e seus ligantes por imunofluorescência, usando os seguintes anticorpos: FITC-anti-PD-1 (J43, na diluição 1:50), PE-anti B7-H1 (MIH5, na diluição 1:100) e PE-anti-PD-L2 (TY25, na diluição 1:100), todos da BD Biosciences. Nos animais PD-1$^{-/-}$ infectados com *T. cruzi* (e nos controle C57BL/6) foi realizada a imunofluorescência para detecção de células CD8 e de fosfo-stat5 (pstat5) no tecido cardíaco utilizando o mesmo protocolo e os seguintes anticorpos: PE-antiCD8 (H35-17.2, eBioscience, diluição

1:100) e anti-pstat5 (cabra, policlonal, Santa Cruz, diluição 1:100). No caso da pstat5, foi necessária a incubação adicional com anticorpo secundário FITC-anti-IgG de cabra (Santa Cruz) na diluição 1:400

Nos tecidos de animais submetidos aos diferentes tratamentos, a detecção de iNOS foi analisada por imunohistoquímica, usando a técnica da peroxidase. Nesse caso, as lâminas contendo os cortes foram fixadas em acetona e tratadas com água oxigenada (H_2O_2), a 3% por 30 minutos para o bloqueio da peroxidase endógena. Em seguida, foram incubadas em leite desnatado a 3g/dL em PBS por 30 minutos, para bloquear a ligação inespecífica. As lâminas foram então incubadas por 18 horas em câmara úmida a 4ºC com anti-NOS2 (coelho, policlonal, Santa Cruz), na diluição 1:500 em PBS contendo 3g/dL de soro de leite. Após três lavagens, o anti-IgG de coelho (C101-167, BD Bioscienes) foi adicionado aos cortes, na diluição 1:1000 em PBS contendo 3g/dL de soro de leite. Após incubação durante 4 horas a 4ºC, a incubação seguinte foi realizada com o complexo avidina-biotina (ABC). Finalmente, os sítios imunorreativos foram revelados com diamino-benzidina (DAB). Os cortes de tecido foram corados com hematoxilina e as lâminas montadas em bálsamo para exame em microscopia óptica. Todos os passos (exceto o bloqueio dos sítios inespecíficos) foram seguidos de lavagem abundante em PBS.

Análise morfométrica e quantificação de imunohistoquímica

Após a reação de imunohistoquímica, as lâminas contendo os cortes de tecido cardíaco corado com diaminobenzidina (DAB) foram fotografadas usando um microscópio acoplado a uma câmera digital (Olympus). Posteriormente, foi utilizado o software livre ImageJ v. 1.39u (NIH) para análise da marcação em cada fotografia. As imagens foram sistematicamente editadas usando uma mascara de cor pré-estabelecida, de modo a selecionar unicamente as tonalidades de vermelho (correspondentes à reação) e posteriormente transformada em imagens binárias (branco e preto puro). Então, o aplicativo de análise de partículas foi executado, permitindo estabelecer a fração da área da imagem representada em preto em cada fotografia (em μm^2). Vinte imagens com aumento final de 200x foram analisadas por cada corte, de um total de 3 cortes não seriado por animal, cada grupo experimental continha 3 animais.

Quantificação da inflamação cardíaca

O processo inflamatório cardíaco induzido por *T. cruzi* é difuso, o que torna difícil a sua quantificação pelos métodos de processamento de imagens convencionais. Entretanto, esse processo é homogeneamente distribuído, dessa forma a proporção de células inflamatórias na superfície do corte é considerada representativa da proporção do volume. Sendo assim, foi usada a proporção volumétrica como medida da inflamação, por meio da contagem de células mononucleares presentes no corte, com o auxílio de uma ocular milimetrada com 100 pontos (Zeiss, Öberkohen, Germany), utilizando uma amplificação final de 400x. Foram analisados cinquenta campos por corte, em três cortes não seriados por cada animal, sendo analisados três corações de cada grupo. A inflamação foi expressa como porcentagem de tecido inflamado, (índice inflamatório) conforme descrito previamente (ROFFE, SOUZA *et al.*, 2007).

Dosagem de citocinas por ELISA

As concentrações das citocinas (IFN-γ, TNF-α, IL-10, IL-12p40) e quimiocinas (MCP-1, MIP1α, RANTES) no tecido cardíaco ou no soro foram mensuradas através de ensaio imuno-enzimático (ELISA) do tipo "*sandwich*". Brevemente, microplacas de alta afinidade (Corning Costar Europe Badhoevedorp, The Netherlands) foram sensibilizadas com anticorpo monoclonal específico para a citocina ou quimiocina alvo (PharMingen), diluído em tampão carbonato-bicarbonato 0,06M, pH 9,5 e incubadas por 12-16h a 4 ^{o}C. Após esse procedimento as placas foram lavadas três vezes em PBS contendo 0,05% de Tween 20 (PBS-T; Sigma Chemicals) em lavador automático de placas (ImmunoWash 1575, BioRad Laboratories, Hercules, CA), em seguida, incubadas a TA em PBS acrescido de leite em pó desnatado (5g/dL; Nestlé, Araçatuba, SP)(PBS-L), por 2h. Após esse período, a solução de leite foi descartada e aos poços foram adicionadas por mais um período de 12-14h, em duplicata, a curva-padrão das citocinas e as amostras diluídas em PBS-L. Em seguida, as placas foram lavadas 6 vezes com PBS-T e foi adicionado o anticorpo secundário específico conjugado com biotina (PharMingen). Após 1 hora a TA, as placas foram novamente lavadas e o conjugado avidina-peroxidase adicionado conforme as instruções do fabricante (Vector Laboratories). A incubação procedeu por mais 30 minutos e após 6 lavagens sucessivas com PBS-T, a revelação foi realizada com

o substrato orto-fenildiamina-2HCl (OPD) (Abbot Laboratories, Abbot Park, IL) diluído em tampão apropriado. A reação colorimétrica foi bloqueada após 10 minutos com ácido sulfúrico (Merck) 1N e a leitura realizada a 492 nm em leitor de microplacas (EMAX, Molecular Devices Corporation, Sunnyvale, CA). A concentração das diferentes citocinas nos sobrenadantes das culturas de células foi determinada com referência às leituras obtidas nos poços contendo diferentes diluições de concentração conhecida da citocina ou quimiocina recombinante (curva padrão).

Dosagem de Óxido Nítrico (NO)

O NO, derivado do nitrogênio guanidino terminal da L-arginina, é muito instável e decompõe-se espontaneamente em nitritos e nitratos. Por tanto, a produção de NO foi avaliada indiretamente pela mensuração da produção de nitrito (NO-2) mais nitrato (NO-3) no plasma dos animais. Após a coleta, o soro obtido foi submetido à redução de nitritos em nitratos pela incubação com a enzima nitrato redutase (Sigma) e a concentração de nitritos e nitratos mensurada pela reação colorimétrica de Griess (GRIESS, 1879; GREEN, TANNENBAUM *et al.*, 1981). Resumidamente, 50 µl de plasma foram adicionados a 50 µL de reagente de Griess e incubados por 10 minutos a 26°C. Em seguida, a absorbância foi determinada em filtro de 540 ηm em leitor de placas. Os resultados foram determinados pela comparação com a curva-padrão realizada com nitrito de sódio (Vetec) em concentrações de 100 a 0,9 µM.

Determinação da atividade de arginase

A atividade da arginase circulante foi determinada em amostras de soro dos animais pertencentes a cada grupo, com base na conversão de L-arginina em L-ornitina e uréia de acordo com a técnica previamente descrita (MELLERUP, 1967; CORRALIZA, CAMPO *et al.*, 1994), usando os reagentes *Quantichrom Arginase Kit* (BioAssay Systems, Hayward, CA, EUA). Resumidamente, 20 µL de soro foram incubados com 5µL de arginina durante 1 hora a 37º C em microplacas de ELISA de alta afinidade (Corning). Após esse período, foi adicionado uréia em todos os poços, para parar a atividade da arginase. Após 15 minutos de incubação a TA, a densidade ótica foi mensurada no espectrofotômetro (Molecular devices), a λ 430 ηm. O resultado foi utilizado para calcular as unidades

da enzima de acordo com as recomendações do fabricante, usando uma concentração padrão de uréia de 1 mmol/L. O limite mínimo de detecção destes reagentes é de 1U/L. Uma unidade da enzima é definida como a quantidade de enzima necessária para catalizar a formação de 1mmol de uréia por hora.

ELISA indireto para detecção de anticorpos anti-troponina I cardíaca

A proteína recombinante murina troponina I cardíaca foi doada pelos doutores Ziya Kaya e Stefan Göser do Department of Cardiology, University of Heidelberg, Alemanha. Microplacas de ELISA de alta afinidade (Corning) foram sensibilizadas com 1µg/mL de troponina recombinante, diluída em tampão carbonato de sódio 0,1 mol/L pH 9.5. Após incubação de 12-18 horas a 4°C, as placas foram lavadas 3 vezes com uma solução contendo Tween-20 0.05% em PBS, realizando posteriormente o bloqueio da reação inespecífica com soro de leite (Molico) 5% em PBS durante 1h a 37 ^{o}C. A seguir, as placas foram novamente lavadas e amostras de soro provenientes de camundongos não infectados ou em vários períodos após a infecção foram diluídas 1:50 em PBS e adicionadas, incubando por 2 horas a 37°C. Após novas lavagens, foi adicionado anti-IgG de camundongo conjugado a peroxidase na diluição de 1:5000 em PBS (Vector), incubando novamente por 2h a 37°C e lavando novamente as placas. A reação foi então revelada com TMB e a densidade ótica lida no espectrofotômetro (Molecular Devices) usando o filtro com comprimento de onda de 450 ηm.

Immunobloting para detecção de autoanticorpos anti troponina I

Para verificar a detecção de autoanticorpos no soro de camundongos *naive* ou infectados com *T. cruzi*, três camundongos C57BL/c não infectados foram sacrificados e seus corações extraídos e extratos de tecido cardíaco desses animais foram preparados usando uma solução contendo inibidor de proteases (Complete, Roche). Após dosagem de proteína nesse extrato pelo método do ácido bicinconínico (Sigma), quantidades iguais de proteína (10µg/línea) foram usadas para preparar uma eletroforese em gel de poliacrilamida (SDS- PAGE 12%). Após a corrida eletroforética, as proteínas contidas no gel foram transferidas para uma membrana de nitro celulose, com ajuda do instrumento ECL *semi-dry transfer unit* (Amersham). Para confirmar a qualidade da transferência, a membrana de nitro celulose foi corada com Coomasie Blue, o que permitiu visualizar as bandas de proteínas. Após a

transferência, cada línea da membrana foi incubada com soro proveniente de cada um dos diferentes animais (3 de cada grupo: naive, infectados com 14 dias ou infectados com 21dias). Para verificar que a reatividade do soro era frente à troponina I cardíaca, uma línea da eletroforese foi realizada com 10µg de proteína recombinante e a mesma línea foi incubada com o "pool" dos soros de animais com 21 dias de infecção, por serem os que possuem os maiores títulos de autoanticorpos, como demonstrado no ensaio de ELISA. Os segmentos da membrana foram incubados por 18 horas com anticorpo anti-IgG total de camundongo conjugado a peroxidase (Santa cruz) na diluição 1:2500 sob agitação. Após esta incubação, a membrana foi lavada e foi acrescentado o substrato enzimático e o revelador da reação (DAB Peróxido de hidrogênio). Após 10 minutos, foi possível verificar as bandas de reconhecimento específico.

Analise estatística

Para avaliar as diferenças entre os grupos experimentais e controles nos diversos parâmetros mesurados, foi usado o teste t-*student* ou ANOVA de uma via seguida, quando necessário, do teste de Newman-Keuls ou teste de comparação múltipla de Dunnett. No caso das curvas de mortalidade, foi realizada a análise de *qui* quadrado. O valor de p foi fixado em $\leq 0,05$. Todas as análises foram realizadas no software Prisma (GraphPad, San Diego, CA, EUA).

Resultados

Expressão de PD-1 e seus ligantes em camundongos infectados com T. cruzi

Inicialmente, testamos se há modulação da expressão de PD-1 durante a fase aguda da infecção por *T. cruzi*. Para tal, o nível de transcritos de mRNA para PD-1 e ligantes foi avaliada no tecido cardíaco de animais infectados durante os dias 14º e 20 da infecção e comparada com a expressão em tecido cardíaco de camundongos não infectados. Os resultados mostraram que há aumento dos transcritos de PD-1 no 14º e 20º dia após a infecção, quando comparados aos encontrados em animais não infectados (Figura 1 A). No caso de PD-L1, somente houve aumento significativo no 14º dia da infecção e não no 20º dia (Figura 1 B). Já no caso de PD-L2, não houve modificações significativas nos períodos analisados (Figura 1). Esses resultados permitem afirmar que existe modulação dos níveis de transcritos para PD-1 e seu ligante PD-L1 no tecido cardíaco durante a infecção por *T. cruzi.* Cabe ressaltar que as expressões de PD-1 e PD-L1 foram cinco vezes maiores em tecido cardíaco infectado do que no não infectado.

Para confirmar a expressão aumentada destas moléculas, foi realizada imunofluorescência nos tecidos cardíacos de camundongos normais ou no 20º dia após a infecção. Em concordância com os dados de PCR, os resultados mostraram ausência de expressão de qualquer das três moléculas em tecido cardíaco de animais não infectados (Figura 1 D, E F, G). Além disso, após a infecção, não foi possível detectar aumento da expressão de PD-1 (Figura 1 G) e sim um aumento da expressão de PD-L1 (Figura 1 H) e em menor grau de PD-L2 (Figura 1 I).

Por se tratar de uma infecção sistêmica, há participação do baço na geração da resposta imune frente a *T. cruzi*. A seguir, foi avaliada a expressão de PD-1 e de seus ligantes por imunofluorescência no tecido esplênico de camundongos infectados ou não com *T. cruzi*. A imunohistoquímica do tecido esplênico de animais não infectados mostrou uma expressão de PD-1 localizada em acúmulos de células na periferia dos centros germinativos (Figura 1 J). Já PD-L1 (Figura 1 K) e PD-L2 (Figura 1 L) apresentam expressões maiores que a de PD-1, contornando de maneira mais definida o centro germinativo. Foi possível estabelecer um grau moderado de possível co-localização da expressão de cada ligante com o receptor.

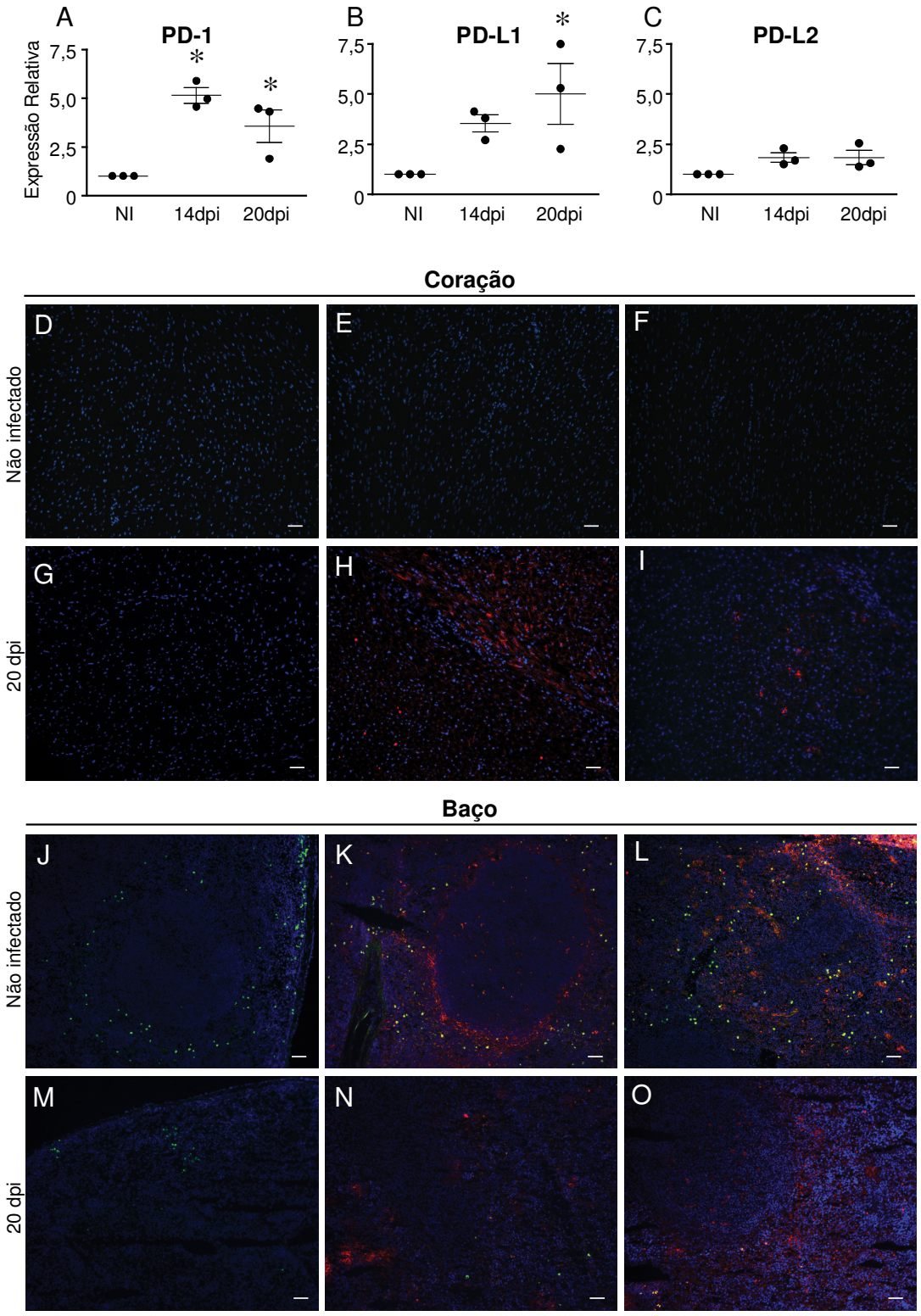

Figura 1. Expressão de PD-1 e de seus ligantes em tecido cardíaco e esplênico. Os níveis de transcritos de mRNA para PD-1, PD-L1 e PD-L2 em tecido cardíaco foi determinada por PCR em tempo real e a expressão relativa de cada mRNA (comparada ao não infectado), corrigida para β-actina é mostrada em cada caso. (**A-C**). O asterisco mostra significância estatística ($p<0,05$) na ANOVA de uma via seguida de teste de Newman-Keuls. **D-I**: microfotografias representativas da imunofluorescência realizada no tecido cardíaco proveniente de animais não infectados (**D-F**) ou no 20º dia após a infecção (**G-I**). **J-O**: microfotografias representativas da imunofluorescência realizada no tecido esplênico proveniente de animais não infectados (**J-L**), ou no 20º após a infecção (**M-O**). Verde: PD-1; Vermelho: PD-L1 (**E,H,K,N**) ou PD-L2 (**F,I,L,O**); azul: DAPI. A barra representa 20 µm.

No tecido de animais infectados foi notada a diminuição da expressão de PD-1 (Figura 1 M) e em menor grau a de PD-L1 (Figura 1 N), o que não aconteceu com PD-L2 (Figura 1 O). Além disso, os ligantes de PD-1 são expressos no centro germinativo. De maneira importante, a infecção por *T. cruzi* ocasiona uma hiperplasia do centro germinativo, levando a uma alteração da arquitetura normal do tecido esplênico (Figura 1 M-O). Consequentemente, as expressões de PD-1 (Figura 1 M), PD-L1(Figura 1 N) e PD-L2 (Figura 1 O) sofrem re-organização, com uma tendência à diminuição da expressão.

Estes resultados indicam modulação da expressão de moléculas da via de sinalização PD-1 durante a fase aguda da infecção experimental por *T. cruzi* em órgãos que participam na geração da resposta imune frente ao parasito.

Analise da expressão de PD-1 e ligantes em linfócitos T, NK e em APC ao longo da fase aguda da infecção

Para testar a hipótese de que *T. cruzi* poderia induzir expressão aumentada de PD-1 em linfócitos, tais células foram extraídas do sangue periférico, baço e coração de camundongos em vários períodos ao longo da fase aguda da infecção. A frequência da expressão de cada uma das moléculas: PD-1, PD-L1 e PD-L2 foi determinada nas subpopulações de linfócitos.

A análise das células isoladas do baço de camundongos não infectados ou em vários períodos ao longo da infecção, mostrou que durante a infecção por *T. cruzi*, há uma modulação da expressão de PD-1 em células $CD3^+CD4^+$ e em menor grau em $CD3^+CD8^+$ (Figura 2). Em células $CD3^+CD4^+$, é possível observar um aumento gradativo da expressão de PD-1 até o 15º dia, que diminui no 20º dia após a infecção (Figura 2 A). No caso das células $CD3^+CD8^+$, houve uma redução significativa da expressão de PD-1 a partir do 20º dia da infecção (Figura 2 D). É importante mencionar que a frequência de expressão de PD-1 nas duas populações oscila entre 0 e 15%, sendo maior na subpopulação de células T CD4.

A expressão de PD-L1 nas subpopulações de células T do baço ao longo da infecção foi levemente reduzida da frequência de células $CD3^+CD4^+$ e $CD3^+CD8^+$ expressando este marcador, quando comparada à frequência observada em animais não infectados (Figura 2 B, E).

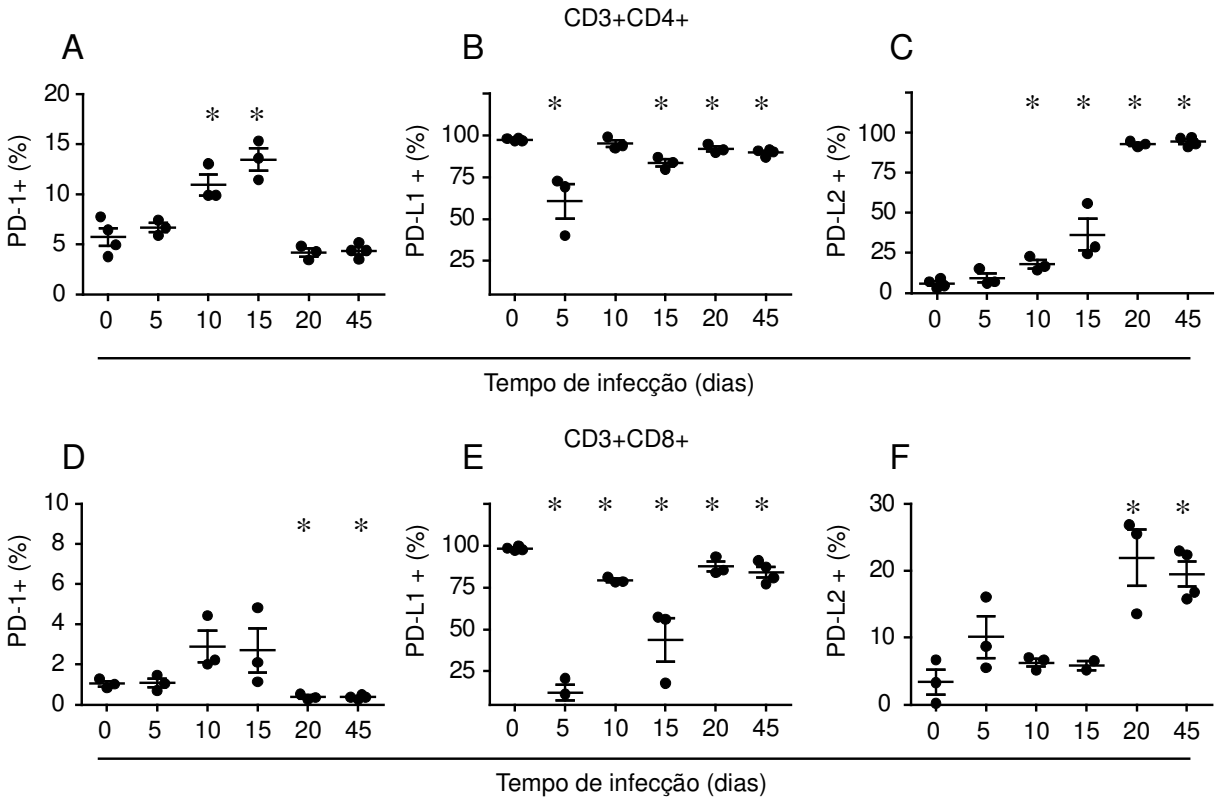

Figura 2. Expressão de PD-1 e ligantes em subpopulações de linfócitos do baço ao longo da infecção por *T. cruzi*. Linfócitos foram separados a partir do baço de camundongos não infectados (0) ou após a infecção por *T. cruzi* e a análise de citometria de fluxo realizada para estudar a expressão de PD-1 (**A,D**), PD-L1 (**B,E**) e PD-L2 (**C,F**) em células $CD3CD4^+$(**A, B, C**) E $CD3CD8^+$(**D, E, F**). Dados representativos de 3 experimentos. Os asteriscos representam a significância estatística ($p<0.05$) no teste de ANOVA de 1 via, seguido de Newman-Keuls.

Essa redução é mais acentuada no 5º dia após a infecção e ocorre tanto para as células $CD3^+CD4^+$ (Figura 2 B) quanto para as $CD3^+CD8^+$ (Figura 2 E). De maneira interessante, foi observado um aumento gradativo na expressão de PD-L2, ao longo da infecção, em todas as populações celulares analisadas (Figura 2 C,F). Este fato constitui uma novidade, pois não é descrita na literatura a expressão de PD-L2 em células $CD4^+$.

No caso das células extraídas do sangue, os resultados mostraram que durante a infecção por *T. cruzi* ocorre, no sangue periférico, uma diminuição inicial da frequência de células $CD3^+CD4^+$ e $CD3^+CD8^+$ expressando PD-1, PD-L1 ou PD-L2, com um aumento posterior das mesmas (Figura 3).

Estudos recentes referem expressão de PD-1 em células da resposta inata, especificamente em fagócitos e células APC. Embora os mecanismos reguladores para esta molécula nestes tipos celulares não tenham sido plenamente descritos, sabe-se que o PD-1 é capaz de controlar eficientemente a geração da resposta imune na fase inicial da imunidade inata (CHO, LEE *et al.*, 2008; HUANG, VENET *et al.*, 2009).

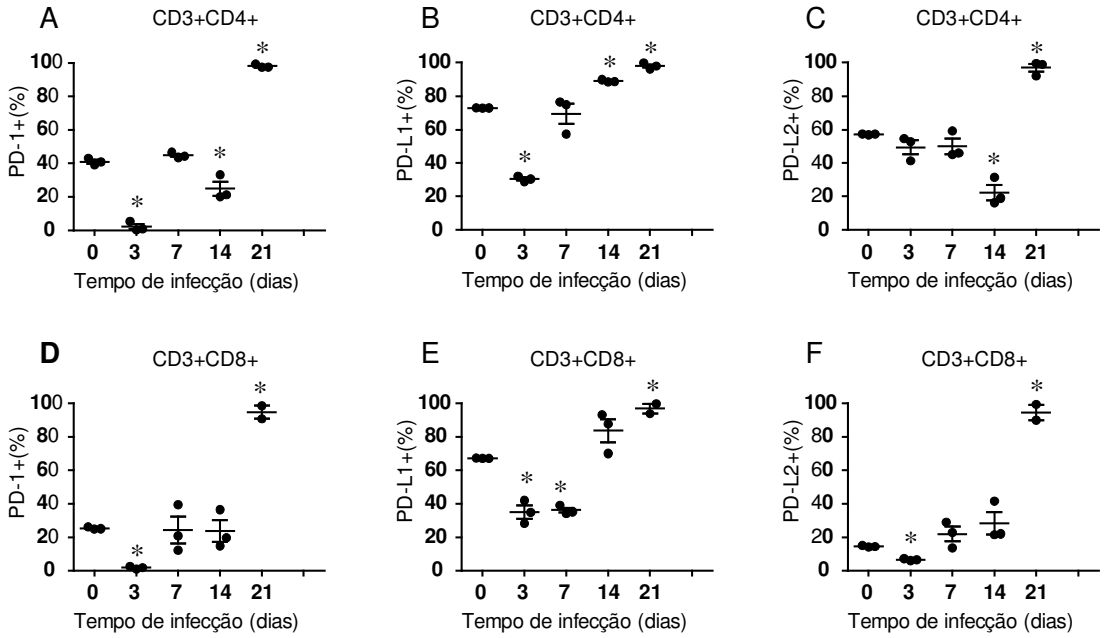

Figura 3. Expressão de PD-1 e dos seus ligantes em leucócitos isolados do sangue de camundongos ao longo da infecção com *T. cruzi*. A expressão de PD-1, PD-L1 e PD-L2 foram determinadas por citometria de fluxo em linfócitos do sangue periférico de camundongos normais ou infectados, analisando as g*ates* de células $CD3^+CD4^+$(**A-C**) ou $CD3^+CD8^+$(**D-F**) dentro da *gate* de linfócitos. Os gráficos representam média das frequências de células positivas para cada marcador (PD-1, PD-L1, PD-L2) e o erro padrão da média em cada caso (n=3). Os dados são representativos de 2 experimentos independentes. Os asteriscos representam a significância estatística ($p<0.05$) no teste de ANOVA de uma via, seguido de Newman-Keuls.

Para investigar se havia modulação da expressão de PD-1 em células NK e APC, tais células foram isoladas do baço em vários períodos após a infecção e a expressão de PD-1 foi avaliada por citometria de fluxo (Figura 4). Os resultados mostram, em geral, uma menor modulação da expressão de PD-1 em células $CD3^+NK^+$ ou $CD3^-NK^+$ após a infecção. Contudo, foi evidenciada uma diminuição significativa da porcentagem de células $CD3^+NK^+PD\text{-}1^+$ nos dias 15º e 45º após a infecção (Figura 4 A). Já no caso das células CD3-NK^+, somente houve redução significativa no 20º dia após a infecção (Figura 4 B).

No caso das células APC (Figura 4 C, D), foi avaliada a expressão de PD-1 em células $CD11b^+CD11c^+$ (Figura 4 C) e em células $CD11b^+$ (Figura 4 D), contidas na *gate* de monócitos. Os resultados mostraram aumento gradativo da expressão de PD-1 em ambos os tipos de APC no decorrer da infecção. Cabe ressaltar que nas células $CD11b^+Cd11c^+$, foi observada uma diminuição da expressão de PD-1 no dia 45º após a infecção (Figura 4 C), o que não aconteceu no caso das células $CD11b^+$ (Figura 4 D). Estes resultados demonstram que ocorre modulação da expressão das moléculas da via de sinalização por PD-1 durante a infecção aguda por *T. cruzi* em linfócitos do sangue periférico e em células apresentadoras de antígeno presentes no baço.

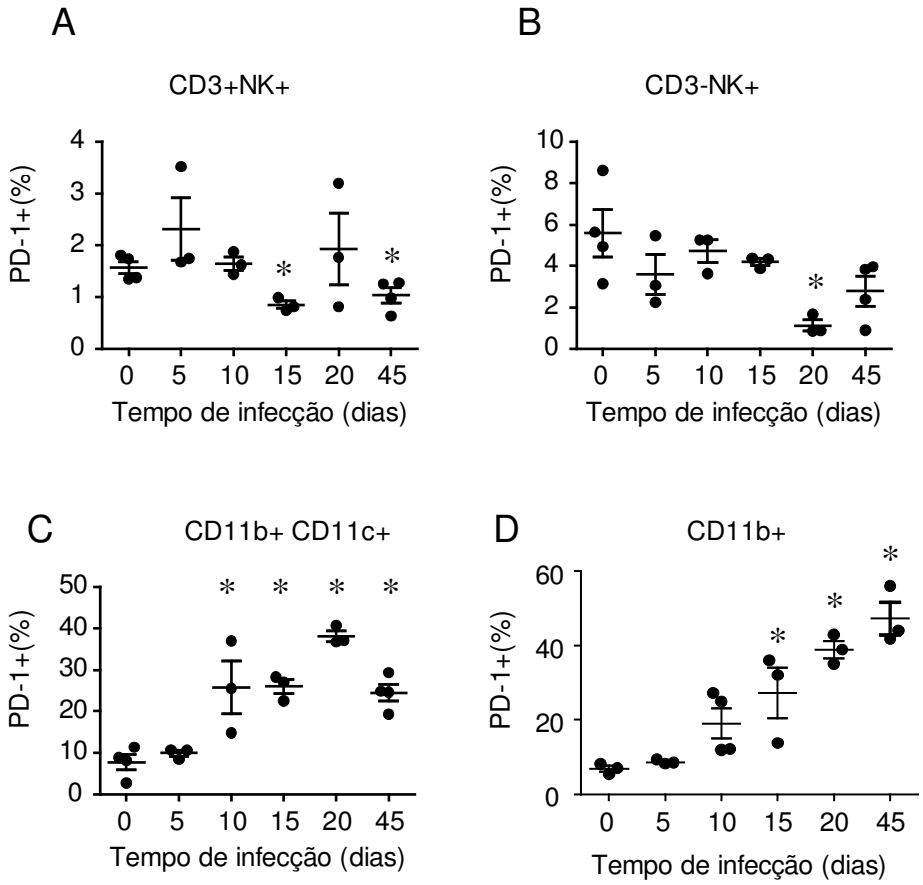

Figura 4. Expressão de PD-1 em células NK e APC isoladas do baço de camundongos ao longo da infecção com *T. cruzi.* A expressão de PD-1 foi determinada por citometria de fluxo em células do baço de camundongos normais ou infectados, analisando as g*ates* de linfócitos CD3NK (**A**) ou CD3-NK (**B**), ou monócitos CD11bCD11c (**C**) ou CD11b(**D**). Os gráficos representam média das frequências de células positivas para PD-1 dentro de cada *gate* e o erro padrão da média em cada caso (n=3). Os dados são representativos de 2 experimentos independentes. Os asteriscos representam a significância estatística ($p<0.05$) no teste de ANOVA de 1 via, seguido de Newman-Keuls.

Expressão de ligantes de PD-1 em CD e macrófagos induzida por T. cruzi in vitro

Dados da literatura referem que CD em repouso carecem da capacidade de induzir uma resposta imune linfocitária eficiente durante a apresentação antigênica, principalmente devido à sua capacidade coestimuladora deficiente. Além disso, é sabido que em condições onde há aumento da expressão dos ligantes de PD-1 durante a ativação das mesmas (HOCHWELLER e ANDERTON, 2005), a capacidade estimuladora de CD sobre linfócitos T também é comprometida. Estes ligantes desempenham um papel crucial na geração de anergia e na regulação da resposta imune.

Para avaliar a hipótese de que *T. cruzi* poderia estar induzindo expressão aumentada desses ligantes em CD *in vitro*, experimentos foram conduzidos, usando cultura de CD diferenciadas a partir de monócitos da medula óssea. Estas células foram cultivadas durante 48h na presença de *T. cruzi* ou

LPS. Após esse período, a expressão dos ligantes de PD-1 foi avaliada por citometria de fluxo e imunofluorescência. Os resultados mostram que a infecção por *T. cruzi* é capaz de induzir a expressão de PD-L1 e PD-L2 em CD *in vitro* (Figura 5). A capacidade de induzir expressão de PD-L1 foi tão eficiente quanto a do LPS (Figura 5 A,C). No caso da expressão de PD-L2, esse aumento da expressão foi menor e no caso de LPS houve uma redução da expressão (Figura 5 B, D). Cabe ressaltar que a expressão de PD-L2 na população de células avaliadas é mais heterogênea e um aumento na quantidade de células com maior expressão desta molécula foi encontrado após a infecção *in vitro* com *T. cruzi* (Figura 5 D).

Resultados similares foram verificados em macrófagos (dados não apresentados). Contudo, além da expressão de ligantes de PD-1, foi possível verificar o aumento da expressão de PD-1 em macrófagos, de maneira dose-dependente. A incubação de macrófagos peritoneais com quantidades crescentes de tripomastigotas vivos levou ao aumento gradativo da expressão de PD-1 na superfície destas células (Figura 6).

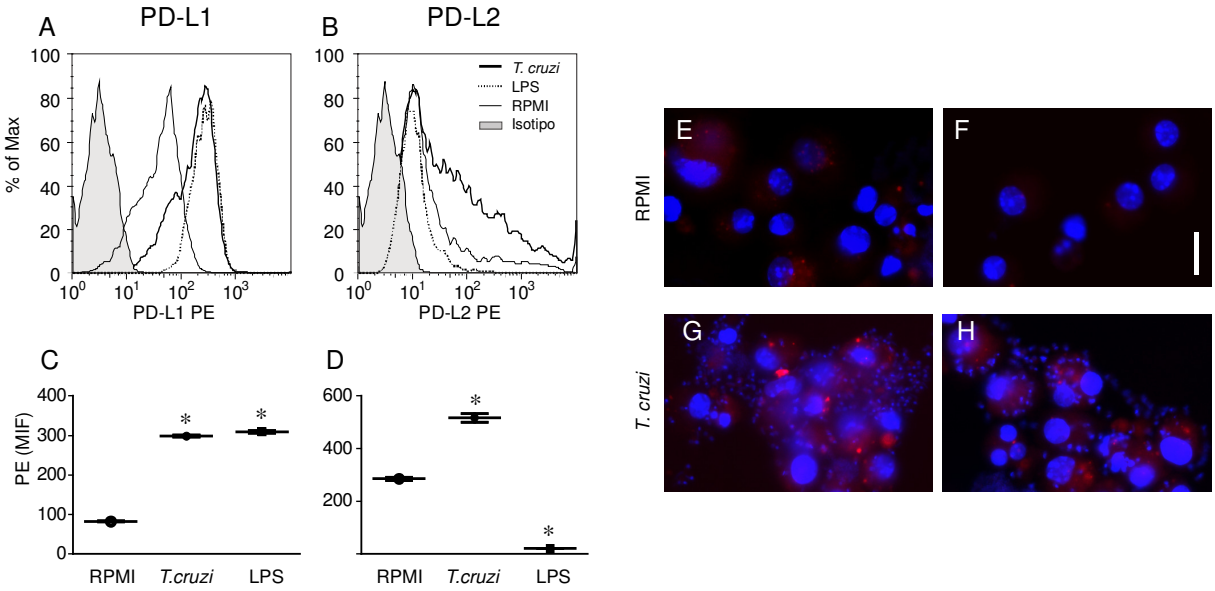

Figura **5. Expressão de ligantes de PD-1 em CD após a infecção com *T. cruzi*.** CD diferenciadas da medula óssea foram incubadas por 48 horas com tripomastigotas da cepa Y de *T. cruzi*, na relação de 5 parasitos:1célula [MOI=5]) ou com LPS (2µg/mL). A intensidade de expressão de PD-L1 (**A, C, E, G**) ou PD-L2 (**B, D, F, H**) na superfície celular foi avaliada por citometria de fluxo (**A-D**) ou microscopia de fluorescência (**E-H**). A barra vertical do lado inferior direito da microfotografia em F representa 10µm. Os asteriscos representam a significância estatística ($p<0.05$) no teste de ANOVA de 1 via, seguido de Newman-Keuls.

Expressão de PD-1e PD-L1 induzida por T. cruzi *em linfócitos* in vitro

Para verificar se *T. cruzi* possui a capacidade de induzir diretamente a expressão de PD-1 ou ligantes em linfócitos, foram realizados experimentos de cultura de células do baço, incubadas com *T.*

cruzi, ConA ou sem estímulo. Após diferentes períodos de incubação, a expressão de PD-1 e de seus ligantes foi determinada por citometria de fluxo (Figura 7). Os resultados mostraram que a incubação com *T. cruzi* modulou a expressão de PD-1, PD-L1 e PD-L2 predominantemente em células $CD3^+$(Figura 7 A, D, G) ou $CD8^+$ (Figura 7 C, F, I) e em menor grau em células $CD4^+$(Figura 7 B, E, H).

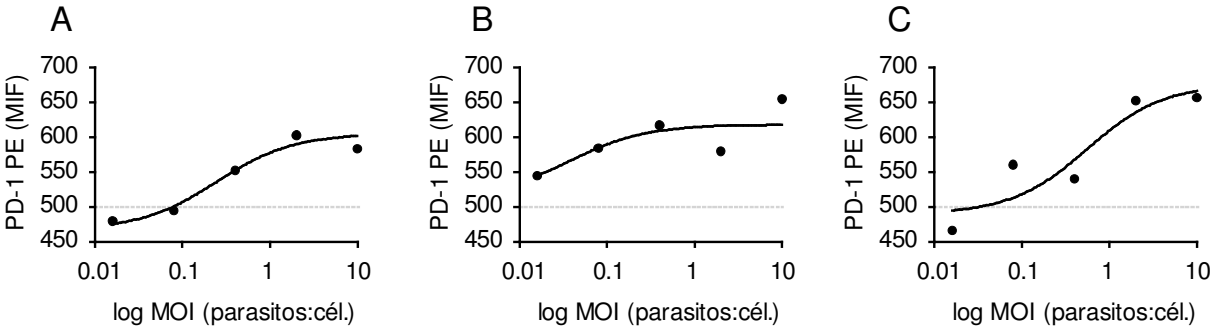

Figura 6. Expressão de PD-1 em macrófagos peritoneais após infecção com *T. cruzi*. Macrófagos peritoneais foram incubados com diferentes quantidades de parasito (MOI: *multiplicity of infection*) durante 48h. A figura mostra a média da intensidade de fluorescência (MIF) para PD-1, em células F-480 analisadas dentro da gate de monócitos. **A**, **B** e **C** correspondem a células provenientes de cada um de três camundongos C57BL/6 diferentes. A linha preta corresponde à curva ideal após análise de regressão linear no modelo *log* (agonista) *vs* resposta. A linha cinza tracejada corresponde à MIF do isotipo controle.

O nível de expressão de PD-1 em células $CD3^+$ totais e $CD3^+CD8^+$, foi maior nas primeiras 24 horas de incubação com *T. cruzi* ou ConA, diminuindo gradativamente até o período de 96 horas, onde não foi encontrada diferença na expressão de PD-1 por células cultivadas com *T. cruzi* quando comparadas com o meio de cultura Diferentemente, as células cultivadas na presença de ConA, mantiveram níveis aumentados de PD-1 em todos os períodos avaliados (Figura 7 A). Um perfil similar foi observado na expressão de PD-1 por células $CD3^+CD8^+$ (Figura 7 C). Porém, no caso da expressão de PD-1 por células $CD3^+CD4^+$, o nível de expressão foi diminuído 24 horas após a incubação com o parasito, apesar de aumentar nos períodos posteriores, com exceção do ponto de 96 horas, onde estava diminuída (Figura 7B). A expressão de PD-L1 apresentou um perfil similar ao observado por PD-1 em todas as subpopulações de células (Figura 7 D-F).

No caso da expressão de PD-L2, houve uma melhor modulação da expressão quando as células foram cultivadas na presença de ConA, apesar de que o parasito induziu diferenças estatisticamente significativas nos níveis de expressão (Figura 7 G-I). Estes resultados demonstram a modulação direta da expressão de PD-1 por linfócitos T na presença do parasito. Essa modulação é exercida principalmente em célula $CD3^+CD8^+$ e em menor grau em células $CD3^+CD4^+$.

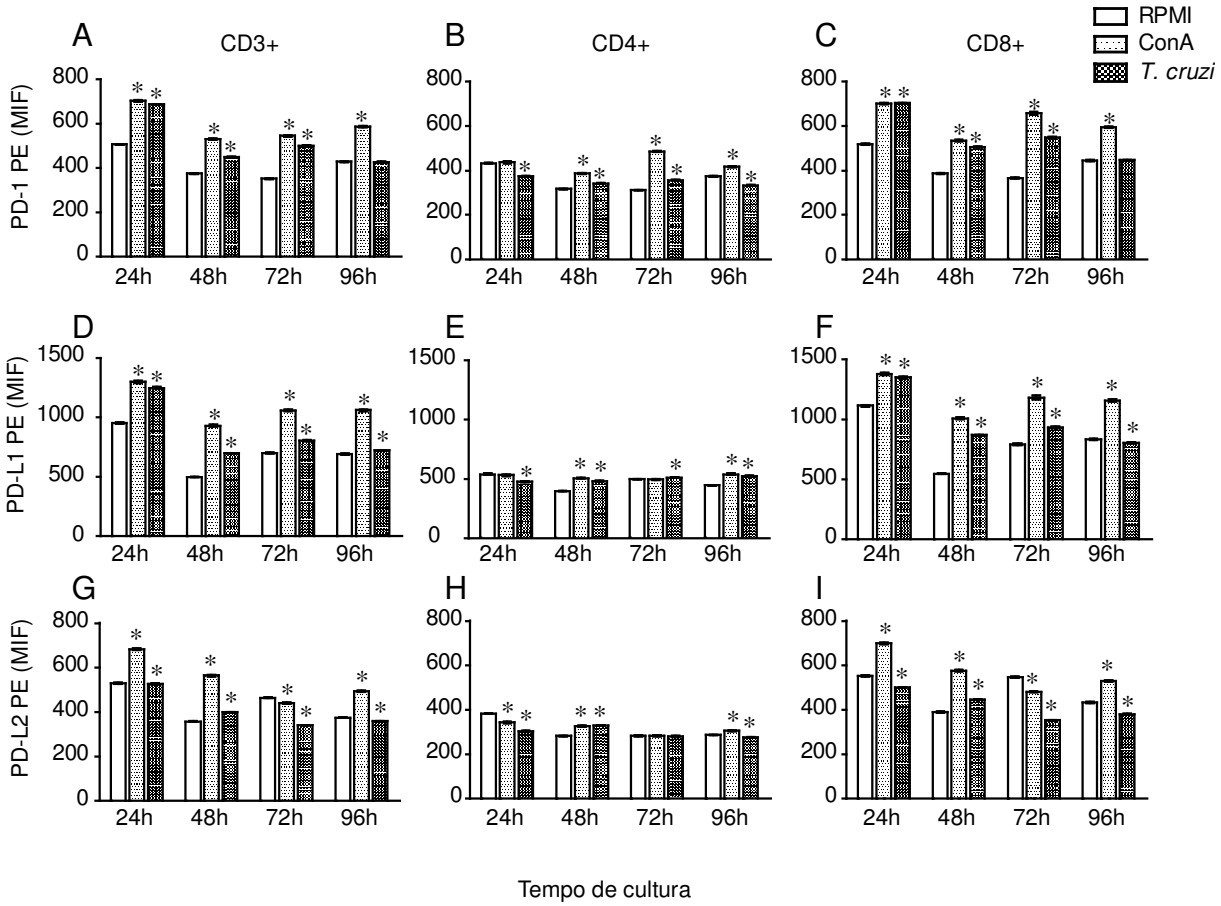

Figura 7. Expressão de PD-1 e ligantes em cultura de células do baço em diferentes tempos após estimulação com *T. cruzi* ou ConA. As células obtidas do baço foram cultivadas com ConA (2ug/mL) ou *T. cruzi* (5 parasitos por célula) e após 24, 48, 72 ou 96 horas a expressão de PD-1 (**A-C**), PD-L1 (**D-F**) ou PD-L2 (**G-I**) foi determinada nas populações de $CD3^+$(**A,D,G**), $CD3^+D4^+$ (**B,E,H**) ou $CD3^+CD8^+$(**C,F,I**). A figura mostra a média da intensidade de fluorescência para o canal PE e o erro padrão da média em cada *gate*. Os dados são representativos de dois experimentos independentes conduzidos com células de 3 animais. Os asteriscos indicam significância estatística ($p<0,05$) na ANOVA de uma via seguida de Dunnet.

Expressão de PD-1 e ligantes em leucócitos isolados do coração de camundongos infectados

Continuando a analisar a expressão de PD-1 e seus ligantes em células da resposta imune durante a infecção por *T. cruzi*, foi realizado o isolamento de células inflamatórias a partir de corações de camundongos infectados. Essa técnica permite caracterizar o fenótipo das células inflamatórias que migram para o coração durante a fase aguda da infecção experimental por *T. cruzi*. Devido à necessidade da obtenção de uma quantidade grande de células, esta técnica permite avaliar somente o período de maior intensidade da inflamação cardíaca (14 a 20 dias após a infecção, Figura 17). É possível obter leucócitos do tecido cardíaco de animais infectados no dia 14 após a infecção e a

expressão de PD-1 e seus ligantes não diferem dos observados no dia 20. Em torno da quarta semana de infecção, o tecido cardíaco apresenta redução na resposta inflamatória e com isso, a recuperação de leucócitos diminui novamente.

A Figura 8 mostra o fenótipo das células $CD3^+CD4^+$ (A-E) e $CD3^+CD8^+$ (F-J), em relação à expressão de PD-1 (Figura 8C e H), PD-L1 (Figura 8D e I) e PD-L2 (Figura 8E e J). Tanto as células $CD4^+$quanto as $CD8^+$ apresentaram uma frequência de positividade para PD-1 e PD-L1, superior a 85 %.

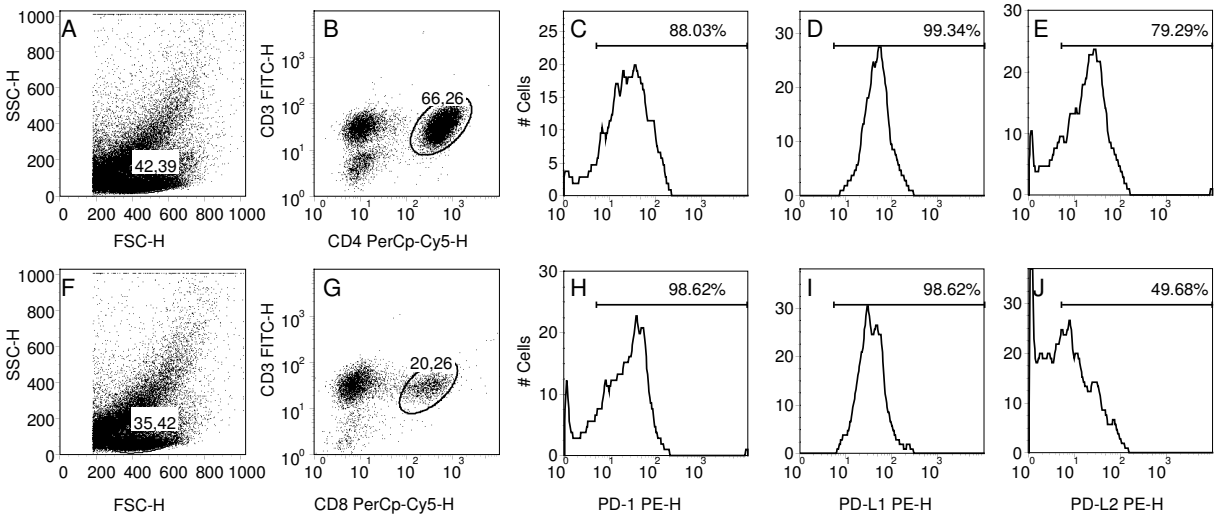

Figura 8. Expressão de PD-1 e de seus ligantes PD-L1 e PD-L2 em linfócitos T $CD4^+$(A-E) e $CD8^+$(F-J) extraídos do coração de camundongos infectados com *T. cruzi*. A Figura representa os histogramas da expressão de PD-1 (**C e H**), PD-L1 (**D e I**) e PD-L2 (**E e J**) em células $CD3^+CD4^+$(**B**) ou $CD3^+CD8^+$(**G**), analisadas dentro da *gate* de linfócitos (**A e F**). O valor em cada histograma representa a frequência relativa (%) e o histograma cinza representa o isotipo de controle. Os gráficos **A**, **B**, **F** e **G** são representativos da maneira como foram realizadas as *gates* para a análise para cada histograma. Os dados são representativos de 4 experimentos independentes.

A principal diferença encontrada na expressão destas moléculas em células $CD4^+$e $CD8^+$é em relação à PD-L2 (79.29% e 49.68 % das células $CD4^+$e $CD8^+$expressam PD-L2, respectivamente). Com isso, é possível afirmar que as células inflamatórias presentes no tecido cardíaco no período da maior intensidade da miocardite, apresentam alta expressão de PD-1, uma molécula envolvida na manutenção da tolerância periférica.

Efeito da expressão de PD-1 sobre a produção de citocinas por linfócitos $CD4^+$ou $CD8^+$

Para estudar o efeito da expressão de PD-1 na produção de citocinas por linfócitos durante a infecção por *T. cruzi,* tais células foram isoladas do sangue periférico e do tecido cardíaco e a presença

intracelular das citocinas IFN-γ, IL-10 ou IL-17 foi avaliada por citometria de fluxo. A figura 9 mostra a expressão destas três citocinas em linfócitos $CD4^+$ ou $CD8^+$do sangue.

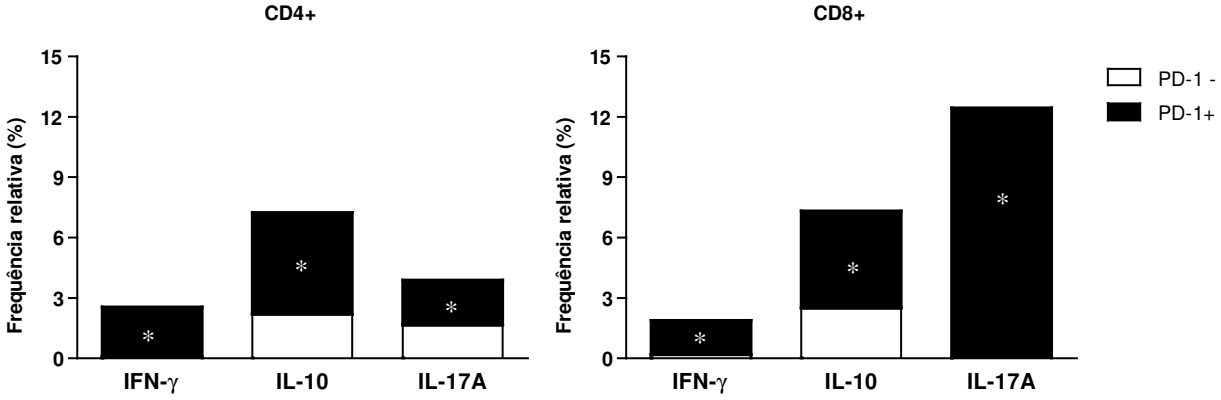

Figura 9. Expressão de PD-1 e sua relação com a produção de citocinas em linfócitos circulantes de animais infectados com *T. cruzi*. Camundongos infectados com *T. cruzi* foram sacrificados no 14º dia após a infecção e a expressão de citocinas intracelulares foi analisada nas *gates* de CD3CD4 e CD3CD8 e *sub-gates* foram feitas para ver a expressão de PD-1. A figura mostra a produção total de cada citocina em cada subtipo de linfócitos. As barras estão compostas por células $PD\text{-}1^+$ (preto) ou $PD\text{-}1^-$ (branco). Figura representativa de dois experimentos independentes com 3 animais. O asterisco indica $p<0,05$ no teste t-*student* comparando as populações de células PD-1- expressando a respectiva citocina.

Foi verificada uma quantidade significativamente maior de células PD-1 expressando cada uma das citocinas, nos dois subtipos celulares estudados. Cabe ressaltar que a maior expressão de IFN-γ foi detectado em células $CD4^+$ (2,56% *vs* 1,89% em CD8). IL-10 foi produzida de maneira similar por células $CD4^+$e $CD8^+$ (7,24% e 7,32% respectivamente). No caso da IL-17A, houve um predomínio das células $CD8^+$(12,47%, contra 3,9% das CD4; Figura 9).

Ainda, foi possível verificar uma relação entre a expressão de PD-1 e a produção das citocinas IFN-γ por células $CD4^+$e $CD8^+$, já que as células $CD4^+PD\text{-}1^+$ e $CD8^+PD\text{-}1^+$ constituíam proporções predominantes dentro das células produtoras dessa citocinas. Já no caso da IL-17, tal predomínio existe somente na população de células $CD8^+$ e não nas $CD4^+$.

Resultados similares foram encontrados analisando a produção de citocinas por células inflamatórias presentes no coração de animais infectados. A figura 10 mostra que a frequência de células produtoras de IFN-γ é maior em células $PD\text{-}1^+CD4^+$e $PD\text{-}1^+CD8^+$do que nas subpopulações $PD\text{-}1^-CD4^+$ e $PD\text{-}1^-CD8^+$.

Contudo, o mesmo não é observado em células $CD4^+$produtoras de IL-10, em que há produção é similar entre células $PD\text{-}1^-CD4^+$e $PD\text{-}1^+CD4^+$(10,8% e 9,15% respectivamente). No caso das células $CD8^+$ produtoras de IL-10, novamente foi verificado que aquelas positivas para PD-1 apresentam uma

frequência maior de produção desta citocina se comparadas às células PD-1$^-$CD8$^+$(16,75% contra 6,01).

Similar aos resultados observados no sangue, o efeito da expressão de PD-1 na produção de citocinas foi muito mais importante no caso das células CD8$^+$, onde 65,47% das células IL-10$^+$CD8$^+$(16,75% e 6,01%) também expressavam PD-1 e 73,6% no caso das células IL-17A$^+$CD8$^+$ (12,13% e 6,4%). Estes resultados estão de acordo com a idéia de que PD-1 desempenha um papel importante na resposta mediada por IL-17 durante a infecção por *T. cruzi*, como visto em alguns resultados recentes do nosso laboratório (manuscrito submetido), bem como reportes recentes da literatura (BABU, BHAT *et al.*, 2009; WANG, DEHGHANI *et al.*, 2009).

Modulação da expressão de PD-L1 em células do miocárdio de camundongos infectados

PD-L1 possui uma ampla expressão em células mielóides e não mielóides e em células não diretamente envolvidas na resposta imunológica, como células endoteliais do fígado e das ilhotas pancreáticas, entre outras. No caso do tecido cardíaco é provável que uma alteração da expressão de PD-L1 (expresso constitutivamente em cardiomiócitos) participe da miocardite intensa resultante da presença do parasito.

Para estudar o envolvimento de PD-L1 como modulador deste processo, as células miocárdicas foram separadas a partir de corações de animais saudáveis ou de animais infectados com *T. cruzi*, no pico da inflamação (Figura 11). Os resultados indicam uma diminuição quantitativa da expressão de PD-1, PD-L1 e PD-L2 nestas células durante o processo inflamatório (Figura11 C, D, G). Resultados similares foram obtidos pela análise por imunofluorescência do tecido cardíaco, onde é possível ver uma diminuição da expressão de PD-L1 (Figura 11E e F).

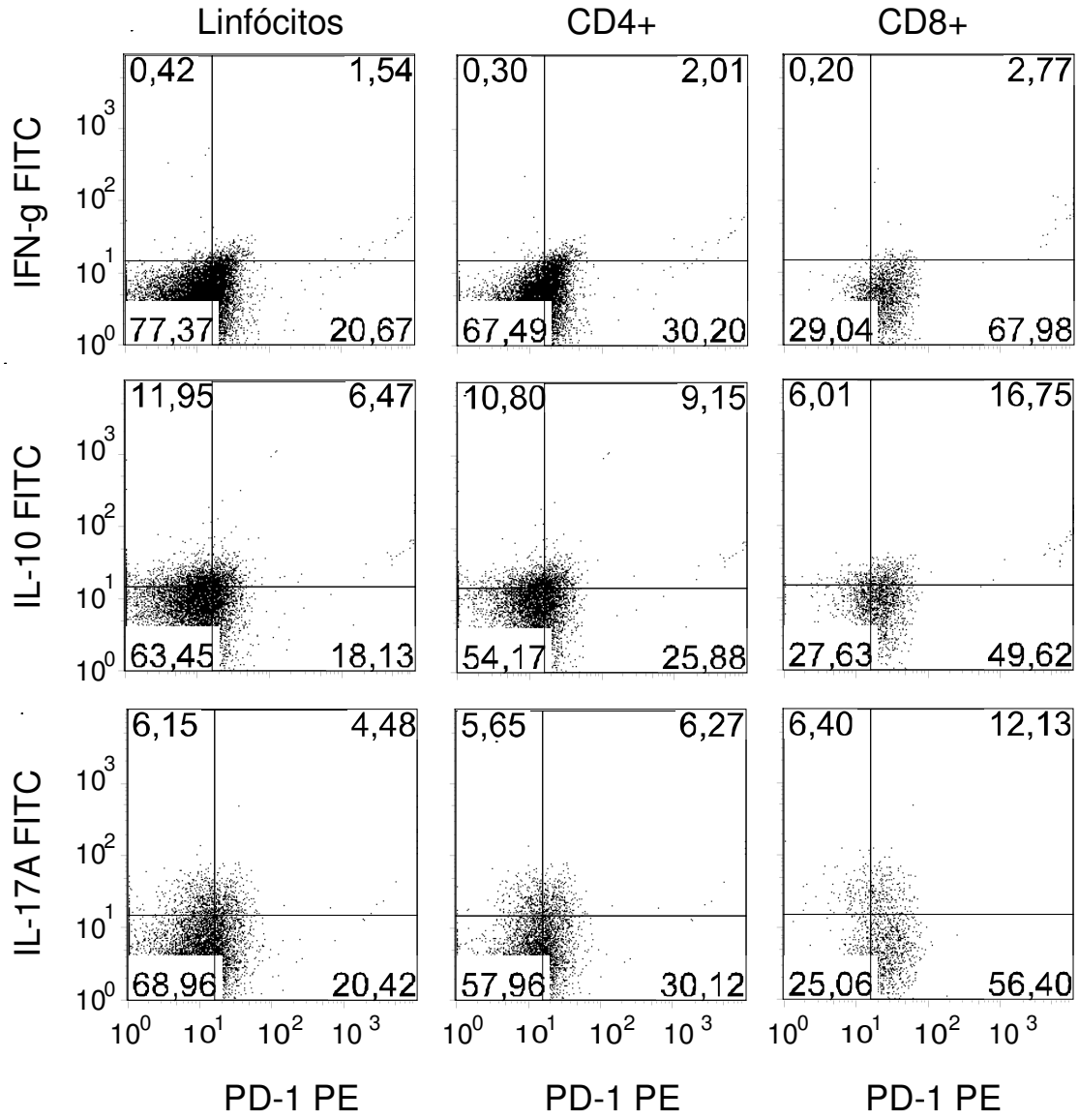

Figura 10. Detecção de citocinas intracelulares e sua relação com a expressão de PD-1 na superfície de linfócitos do tecido cardíaco de camundongos infectados com *T. cruzi*. A figura mostra as frequências de expressão de PD-1 na superfície (eixo X) *vs* a expressão intracelular de citocinas (eixo Y) dentro das *gates* de linfócitos totais (coluna da esquerda) células $CD4^+$(coluna central) ou $CD8^+$(coluna da direita) isoladas do tecido cardíaco de camundongos 14 dias após serem infectados com *T. cruzi* . A figura é representativa de dois experimentos independentes, com 3 animais.

Esses resultados indicam que no cardiomiócito, uma diminuição da expressão de PD-L1 poderia favorecer uma perda da tolerância periférica durante o desenvolvimento da resposta imune efetora no tecido miocárdico, o que levaria a dano tecidual colateral ou eventualmente autoimunidade, em concordância com a miocardite aumentada após o bloqueio desta molécula, como mostrado anteriormente. Além disso, é sabido que existe durante a infecção por *T cruzi*, a perda da tolerância frente a antígenos cardíacos "crípticos" do miocárdio como a troponina I, o que leva ao desenvolvimento de miocardite autoimune em camundongos deficientes desta via de sinalização.

Presença de autoanticorpos anti-Troponina I cardíaca durante a fase aguda da infecção

Baseados nos resultados apresentados, nós suspeitamos que a sinalização via PD-1 estaria favorecendo o controle da resposta imune frente a antígenos próprios liberados incidentalmente durante a lise de cardiomiócitos (como a troponina I). Isso limitaria à geração de uma resposta imune exacerbada que, junto com a resposta inflamatória presente no miocárdio, estaria sendo responsável por um dano extensivo no tecido infectado.

A troponina I é uma proteína que faz parte da maquinaria contráctil do cardiomiócito. Essa proteína é presente com exclusividade no miocárdio e existem evidências de que em situações inflamatórias do tecido cardíaco, ela é liberada e pode desencadear autoimunidade. Ainda, sabe-se que PD-1 é crucial no controle desta autoimunidade. Para testar a hipótese de que camundongos infectados com *T. cruzi* apresentam anticorpos autorreativos frente a troponina

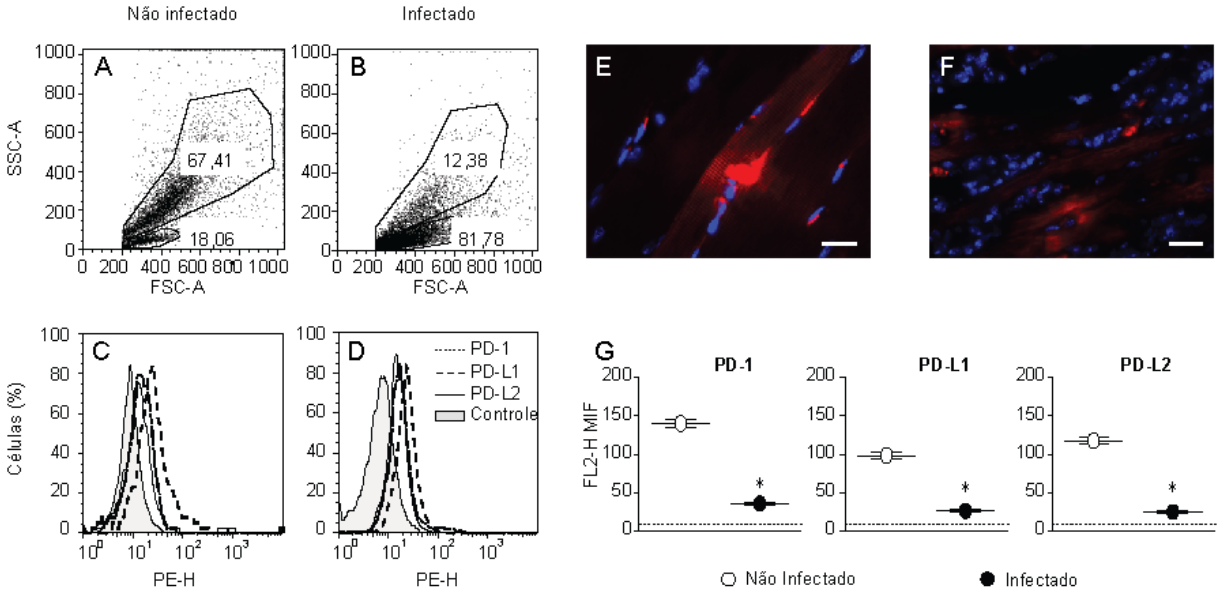

Figura 11. Expressão de PD-1 e ligantes em cardiomiócitos de animais infectados ou não com *T. cruzi*. **A** e **B** mostram o tamanho e a granularidade de células retiradas dos corações de animais não infectados (**A**) ou 20 dias após a infecção (**B**). Em **C** e **D** são mostrados os histogramas da fluorescência para PD-1 (linha pontilhada), PD-L1 (linha tracejada), PD-L2 (linha sólida) ou isotipo controle (histograma cinza). Em **E** e **F**: microfotografias representativas da imunofluorescência para PD-L1 em tecido cardíaco de camundongos naive (**E**) ou infectados (**F**). Tamanho da barra da escala: 25 µm.**G**: média da intensidade da fluorescência (MIF) dos histogramas representados em **C** e **D**. Os asteriscos representam significância estatística ($p<0,05$) no teste t-*student*.

I, realizamos um ELISA indireto para detecção de IgG total anti-troponina I cardíaca no soro de animais normais ou infectados, nos dias 14º e 20º após a infecção. Os resultados mostraram que camundongos infectados apresentam altos níveis de autoanticorpos do tipo IgG no soro frente a

troponina cardíaca. Esses autoanticorpos estão presentes já no 14º dia após a infecção e aumentam no dia 21º (Figura 12). Ainda, esses dados foram confirmados por *immunobloting* dos soros de animais infectados, os quais reconhecem uma proteína de aproximadamente 26 kDa no extrato de tecido cardíaco normal e que também reconhecem a proteína recombinante, que corresponde à banda detectada no extrato de tecido cardíaco (Figura 12 H).

Esse aumento nos níveis de autoanticorpo frente a troponina I cardíaca estava em associação com o aparecimento do infiltrado inflamatório no coração, como mostra a histologia cardíaca (Figura 12 A-C, E, F). Esse evento ocorre tipicamente após o controle da proliferação do parasito na circulação (Figura 12 D).

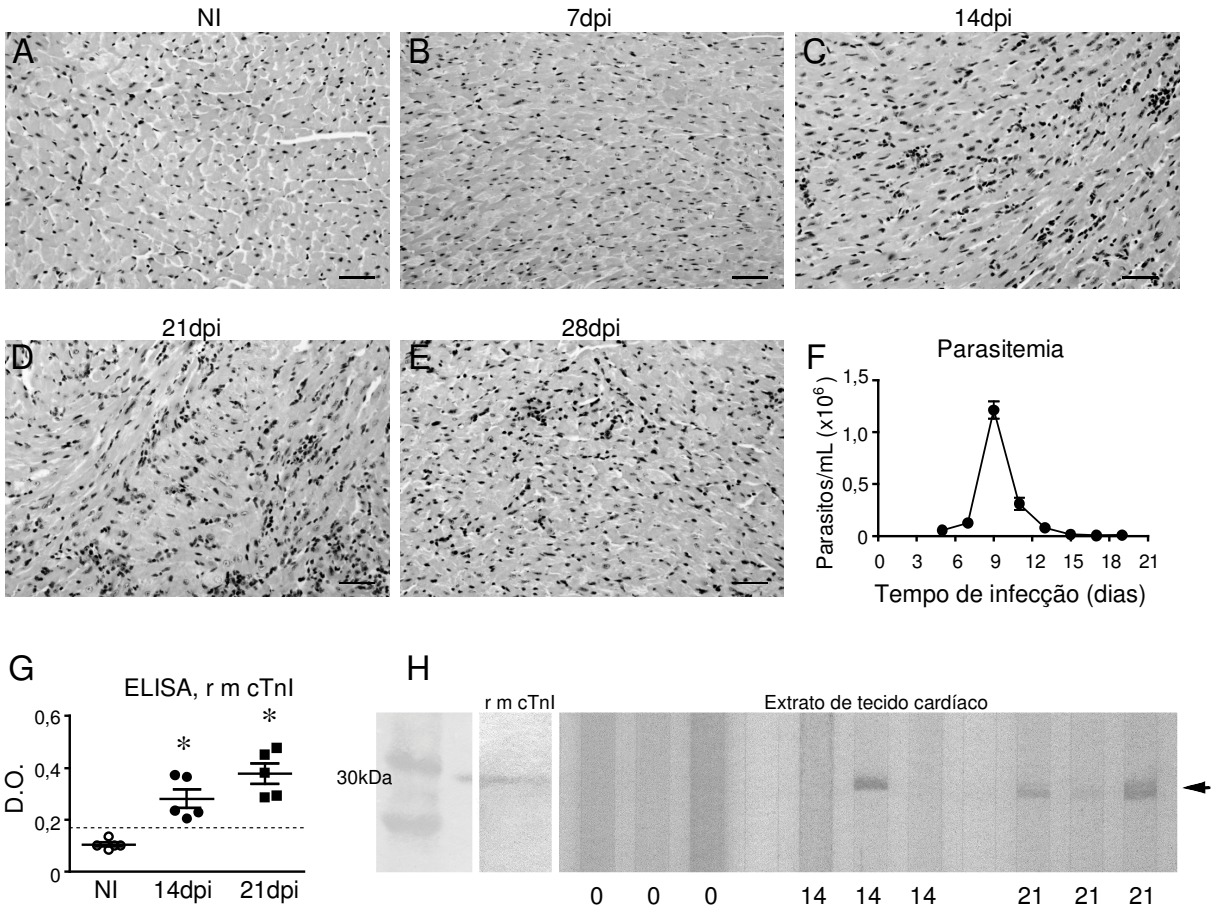

Figura 12. Evidência de anticorpos reativos frente a Troponina I cardíaca durante a fase aguda da infecção por *T. cruzi.* **A**, **B**, **C**, **D**, **E**: Microfotografias representativas da histologia cardíaca de camundongos normais (NI) ou infectados com *T. cruzi*. As barras nas microfotografias representam 10µm. **F**: avaliação da carga parasitária circulante ao longo da fase aguda da infecção (n=5). **G**: Detecção de IgG anti-troponina I cardíaca murina por ELISA no soro de camundongos normais NI ou 14 ou 21 dias após a infecção com *T. cruzi* (n=8). A linha tracejada representa a média dos animais não infectados, mais 2,5 vezes o valor do desvio padrão. **H**: *Immunobloting* para detecção de IgG anti-troponina I cardíaca recombinante (rmcTnI), usando o soro de animais *naive* (0) ou com 14 ou 21 dias após a infecção e extrato de tecido cardíaco normal. rmcTnI representa o "*pool*" dos soros de animais infectados com 21 dias frente à proteína recombinante murina (n=3). Os dados são representativos de dois experimentos independentes. Os asteriscos indicam significância estatística ($p<0,05$) na ANOVA de uma via seguida de Newman-keuls.

Esses dados constituem novas evidências de que existe uma resposta imune frente a um antígeno exclusivo do coração logo no inicio da infecção, ainda durante a fase aguda.

Efeito do bloqueio de PD-1 e de seus ligantes na resposta linfoproliferativa in vitro

Uma vez que encontramos inflamação aumentada no miocárdio de animais tratados com anticorpos bloqueadores de PD-1 e PD-L1 e sabendo de estudos anteriores que PD-1 regula a proliferação de linfócitos (SUGITA, USUI *et al.*, 2009), o próximo passo no estudo foi verificar o efeito do bloqueio destas moléculas *in vitro*. Para este propósito, as células obtidas dos baços de camundongos C57BL/6 normais ou infectados com *T. cruzi* (14º dia de infecção), foram marcadas com CFSE e cultivadas na presença ou não de anti-CD3.

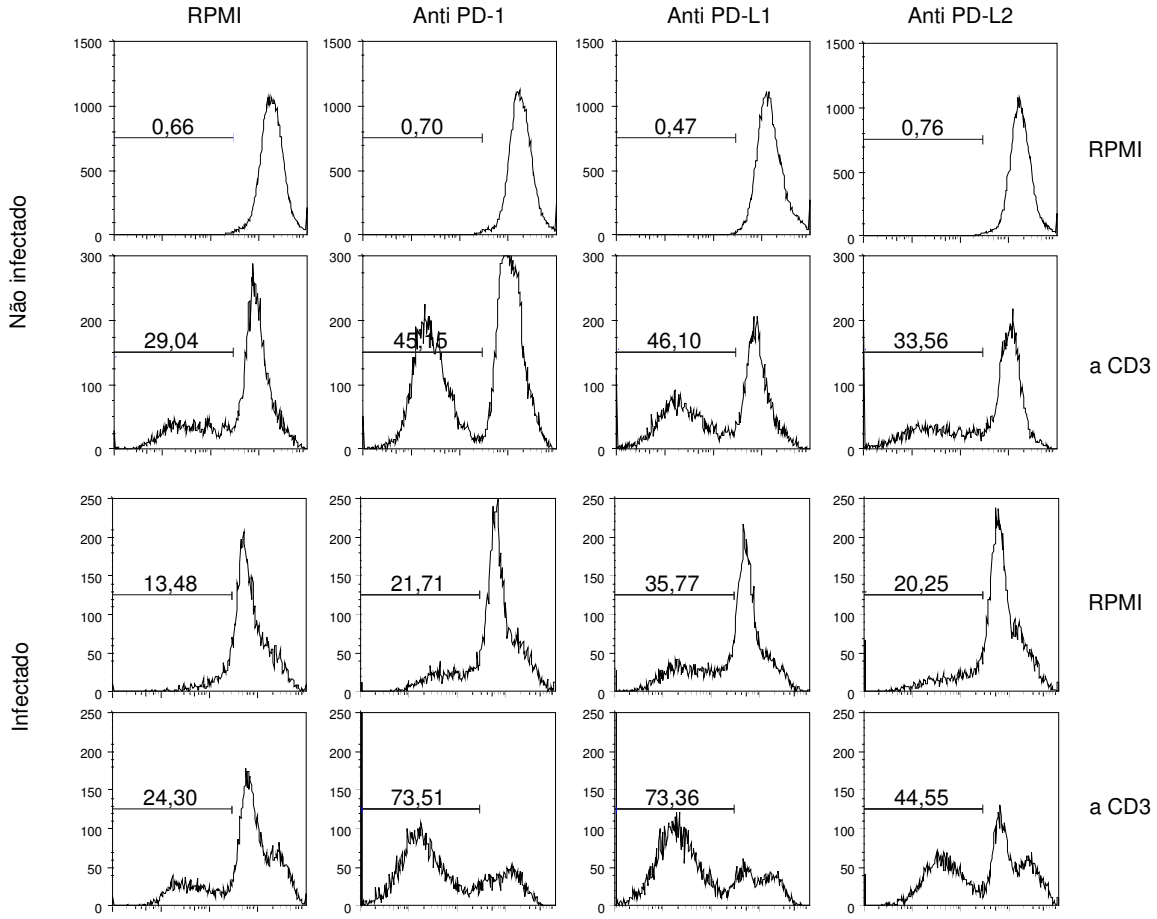

Figura 13. Efeito do bloqueio de PD-1, PD-L1 ou PD-L2 na proliferação de linfócitos provenientes do baço de camundongos infectados ou não com *T. cruzi*. Os baços foram extraídos de camundongos sadios ou 14 dias após a infecção. Essas células foram marcadas com CFSE e incubadas na presença de cada um dos anticorpos ou meio de cultura, na presença ou não de anti-CD3 (OKT3) e a proliferação celular foi avaliada. A figura é representativa do resultado obtido em 3 experimentos realizados com 3 animais por grupo. O valor expresso na região esquerda do histograma representa a frequência de células presentes dentro da *gate* de células em divisão.

Adicionalmente as células foram ou não tratadas com anti-PD-1, anti-PD-L1 ou anti-PD-L2 durante 72 horas. Após este período, a proliferação celular foi analisada em cada caso mediante citometria de fluxo. Os resultados mostraram ausência de proliferação nas células provindas de animais sadios cultivadas somente com meio de cultura e inclusive se estas eram cultivadas na presença de anticorpos bloqueadores de PD-1 ou ligantes (Figura 13). Diferentemente, as células de animais infectados apresentaram uma baixa proliferação na ausência de qualquer estímulo, que aumentou após o bloqueio de PD-L1 e em menor grau na presença dos outros dois anticorpos. Além disso, as células provindas de animais infectados apresentaram uma resposta proliferativa muito maior à estimulação com anti-$CD3^+$somente na presença de anti-PD-1 ou anti-PD-L1 (73,51 e 73,36%, respectivamente, Figura 13). Estes resultados demonstram que o bloqueio de PD-1 e de PD-L1, mas não de PD-L2 induz aumento da resposta proliferativa em células do baço de camundongos infectados por *T. cruzi.*

Efeito do bloqueio de PD-1 ou de seus ligantes na inflamação miocárdica

Desde que existe modulação da expressão das moléculas da via PD-1 durante a infecção por *T. cruzi*, avaliamos o efeito *in vivo* do bloqueio destas sobre a patogenia da infecção. Para tal fim, foram usados anticorpos monoclonais específicos para cada molécula, administrados por via i.p. durante as duas primeiras semanas da infecção. Os resultados mostraram que o bloqueio de PD-1 e, principalmente de PD-L1, leva a um aumento significativo da inflamação miocárdica, se comparada à observada no grupo de animais que receberam IgG de rato normal. De maneira interessante, isso não ocorre em outros tecidos afetados pela infecção, como o músculo esquelético (quadríceps) e o fígado (Figura 14).

A quantificação da miocardite revela que existe um aumento significativo na intensidade do infiltrado inflamatório no miocárdio em animais infectados e tratados com anti-PD-1 e anti-PD-L1, mas não com anti-PD-L2, se comparados com o tecido cardíaco de animais que receberam IgG de rato normal (Figura 15 F).

Efeito do bloqueio de PD-1 ou de seus ligantes na produção de quimiocinas e citocinas no tecido cardíaco de camundongos infectados

A produção das quimiocinas MIP-1α, RANTES e MCP-1 no tecido cardíaco é um fator determinante na migração de células inflamatórias durante a infecção por *T. cruzi* (TEIXEIRA, GAZZINELLI *et al.*, 2002). Visto que os animais tratados com anticorpos bloqueadores da via de PD-1 apresentam uma maior reação inflamatória no miocárdio, nós avaliamos a produção destas quimiocinas no coração dos animais nos dias 14º, 20º 25º após a infecção.

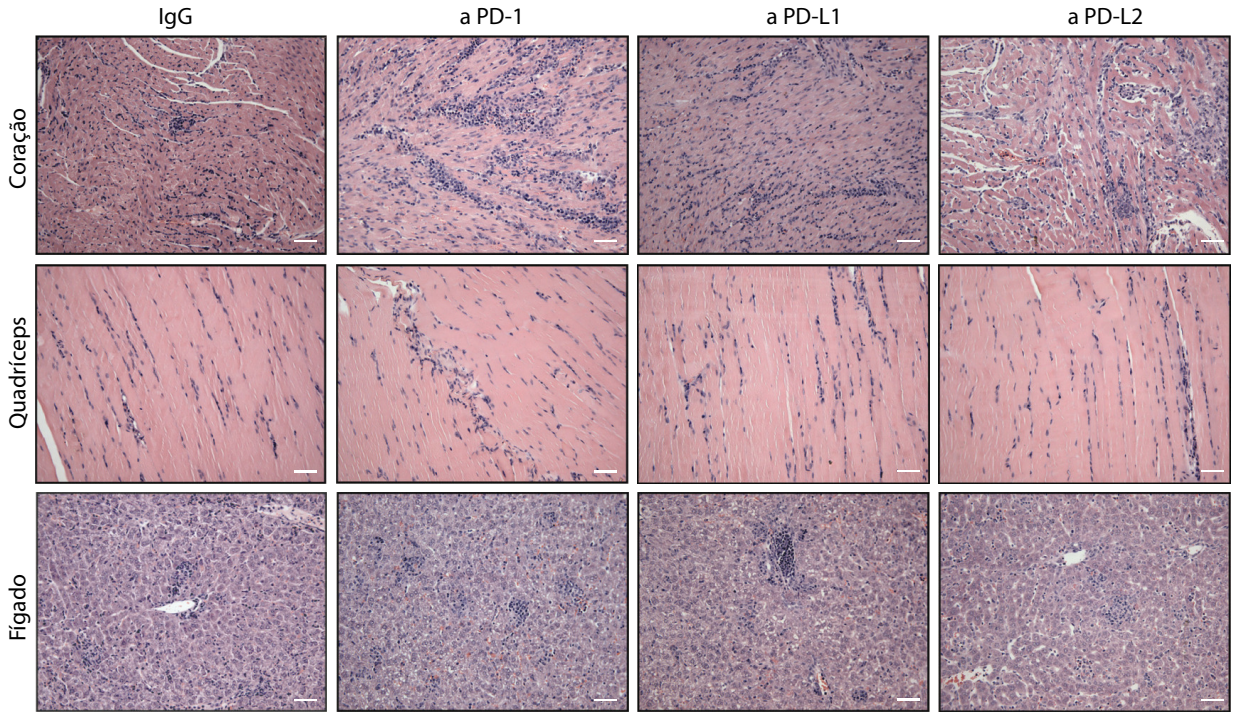

Figura 14. Efeito do tratamento *in vivo* com anticorpos bloqueadores de PD-1 e ligantes. A figura mostra microfotografias representativas da histologia de coração, músculo esquelético e fígado, de animais tratados com IgG de rato, anti-PD-1, anti-PD-L1, ou anti-PD-L2, no dia 20 após a infecção com *T. cruzi*. Os resultados mostrados são representativos de dois experimentos independentes com 3 a 5 animais por grupo. Barra: 20µm.

Os resultados mostraram correlação entre o curso do infiltrado celular no miocárdio e os níveis detectados das quimiocinas MIP1-α (Figura 16 D, E, F) e MCP1 (Figura 16 G, H, I), mas não de RANTES (Figura 16 A , B, C). Nos camundongos controle. Ainda, dos períodos avaliados, o ponto que apresentou a maior produção destas quimiocinas no tecido cardíaco dos animais controle foi no 14º dia após a infecção (Figura 16 B, F, H).

Nos camundongos que receberam anti-PD-1, foi detectada produção significativamente aumentada de RANTES, MIP1-α e MCP1 no 14º dia após a infecção (Figura 16 A, D, G). Já no 20º dia da

infecção, somente RANTES e MCP1 estavam aumentados significativamente nesses camundongos (Figura 16 B, H). O tratamento com anti-PD-L1 induziu aumento de RANTES e MCP1, mas não de MIP1-α no 14º dia após a infecção (Figura 16 A, D, G). No 20º dia após a infecção, de maneira interessante, os níveis de todas as três quimiocinas diminuíram significativamente no tecido cardíaco desses animais (Figura 16 B, F, H).No caso dos animais tratados com anti-PD-L2, somente apresentaram aumento significativo de MCP1 no 14º dia da infecção (Figura 16 G). Já no 20º dia após a infecção, esses animais apresentaram níveis menores de MIP1-a e MCP1, mas não de RANTES quando comparados aos animais controle (Figura 16 B, F, H).

No dia 25º após a infecção, não foi possível detectar diferenças significativas na produção de quimiocinas no tecido cardíaco entre os grupos (Figura 16 C, F, I). Esses resultados suportam o fato de infiltrado inflamatório aumentado encontrado principalmente nos camundongos tratados com anti-PD-1 e anti-PD-L1, mas não com anti-PD-L2.

Desde que esse bloqueio tem um efeito sobre a intensidade da resposta inflamatória miocárdica, é de se esperar que estes animais apresentem maior produção de citocinas proinflamatórias como TNF-α e IFN-γ no tecido cardíaco. Para examinar tal possibilidade, dosamos essas citocinas no tecido cardíaco dos animais correspondentes a cada grupo. Os resultados permitiram verificar que há, de fato, uma produção aumentada destas citocinas no miocárdio de animais tratados com anti-PD-1 nos dias 14º e 20º após a infecção (Figura 17 A, B, D, E). Porém, não foi possível verificar um aumento significativo de tais citocinas nos grupos de animais tratados com anti-PD-L1 ou anti-PD-L2 nesses mesmos períodos (Figura 17 A, B, D, E). Já no 25º dia da infecção, não houve diferenças significativas entre os grupos, na produção desses mediadores. Em conjunto, esses dados permitem afirmar que a via de sinalização por PD-1 participa no controle da resposta inflamatória induzida por *T. cruzi* no coração, uma vez que o seu bloqueio exacerba tal resposta.

Efeito do bloqueio de PD-1 ou de seus ligantes no parasitismo tecidual e circulante e na sobrevida de camundongos infectados

Para avaliar a capacidade de camundongos tratados em controlar a proliferação do parasito, a parasitemia, o parasitismo tecidual e a mortalidade foram avaliadas em animais tratados com os

anticorpos (Figura 18). Contrário ao esperado, os camundongos tratados com anti-PD-1 apresentaram um pico significativamente maior de parasitemia no 9º dia após a infecção quando comparados com o grupo controle.

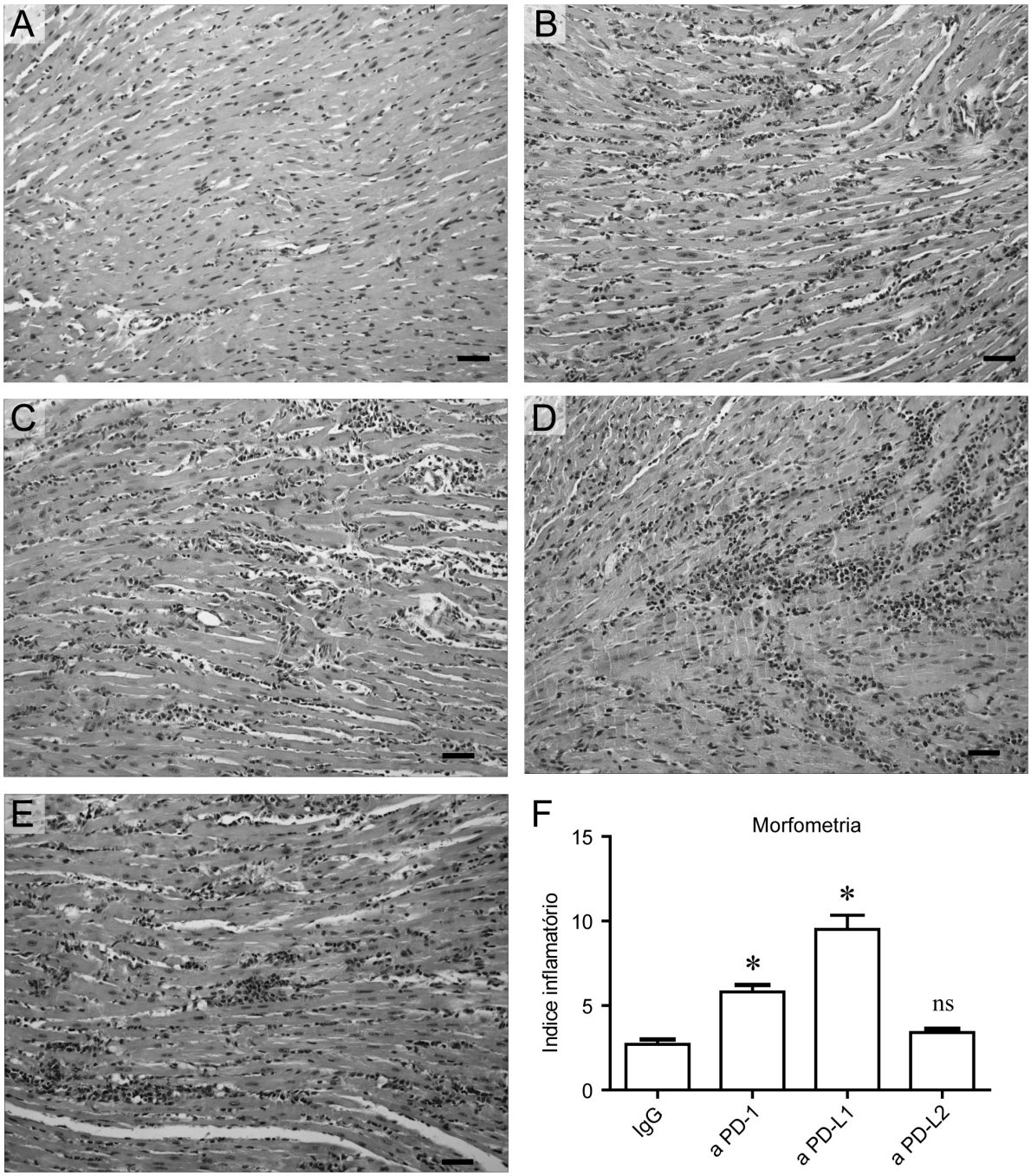

Figura 15. Quantificação do infiltrado inflamatório miocárdico em camundongos tratados com anticorpos monoclonais anti-PD-1, anti-PD-L1 ou anti-PD-L2. A figura mostra microfotografias representativas da histologia do tecido cardíaco: **A**, tecido cardíaco normal; **B** a **E**, tecido cardíaco de animais no 20º dia primeiro após a infecção com *T. cruzi.* **B**, tratados com IgG de rato; **C**, tratados com anti-PD-1; **D**, tratados com anti-PD-L1; **E**, tratados com anti-PD-L2. F: Análise morfométrica de 50 microfotografias por animal, em 3 animais diferentes. As barras indicam a média do índice inflamatório e o erro padrão da média. O índice inflamatório indica a proporção da área da fotografia (200x) correspondente à inflamação. A barra preta nas fotos corresponde a 40 µm. Os asteriscos indicam significância estatística ($p < 0,05$) na ANOVA de uma via seguida por teste de Newman-Keuls. ns: não significativo.

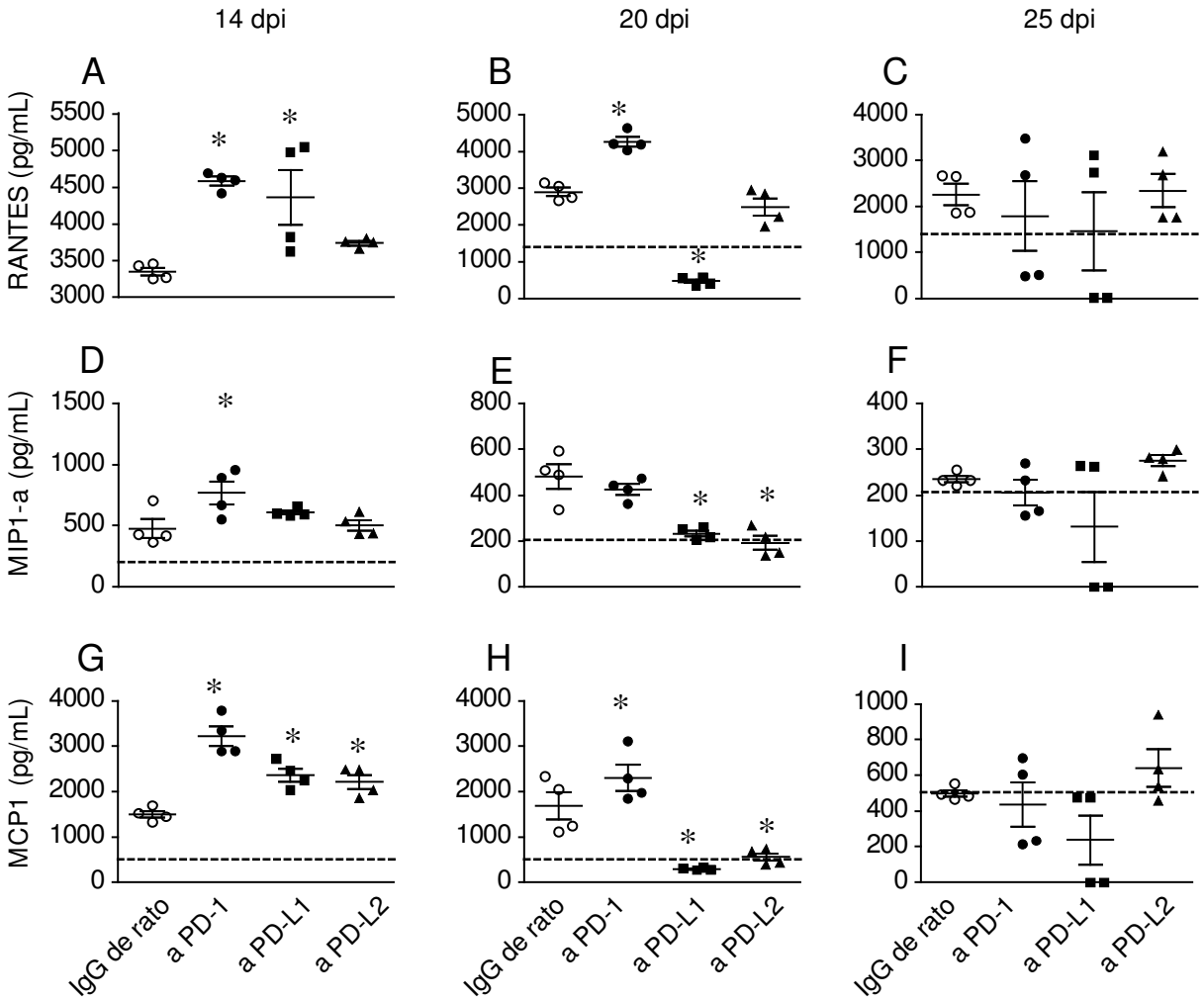

Figura 16. Efeito de bloqueio de PD-1 ou de seus ligantes sobre a produção de quimiocinas no tecido cardíaco de camundongos infectados com *T. cruzi*. A figura mostra a média e o erro padrão da média dos níveis de cada quimiocina encontrados nos tecidos cardíacos de camundongos C57/BL6 infectados com *T. cruzi* e submetidos aos diversos tratamentos. O tecido cardíaco foi analisado por ELISA para RANTES, MIP1-α e MCP1 nos dias 14, 20 e 25 após a infecção. Dados representativos de 2 experimentos independentes (n=4). *: p<0,05, diferente de IgG. A linha tracejada indica os níveis das quimiocinas em corações de animais não infectados.

Contudo, essa diferença foi transitória e os animais conseguiram controlar o parasitismo circulante (Figura 18A). Inclusive, o parasitismo tecidual foi menor que no grupo controle, quando avaliado por PCR em tempo real (Figura 18 B). Apesar disso, estes camundongos também apresentaram uma sobrevida significativamente diminuída quando comparados ao grupo que recebeu IgG (Figura 18 C). Esses resultados indicam que o bloqueio de PD-1 induz um aumento transiente da parasitemia, apesar de levar a um aumento da capacidade de controlar o parasitismo tecidual. A mortalidade aumentada nesses camundongos sugere uma perda dos mecanismos de controle do dano tecidual no miocárdico desses animais.

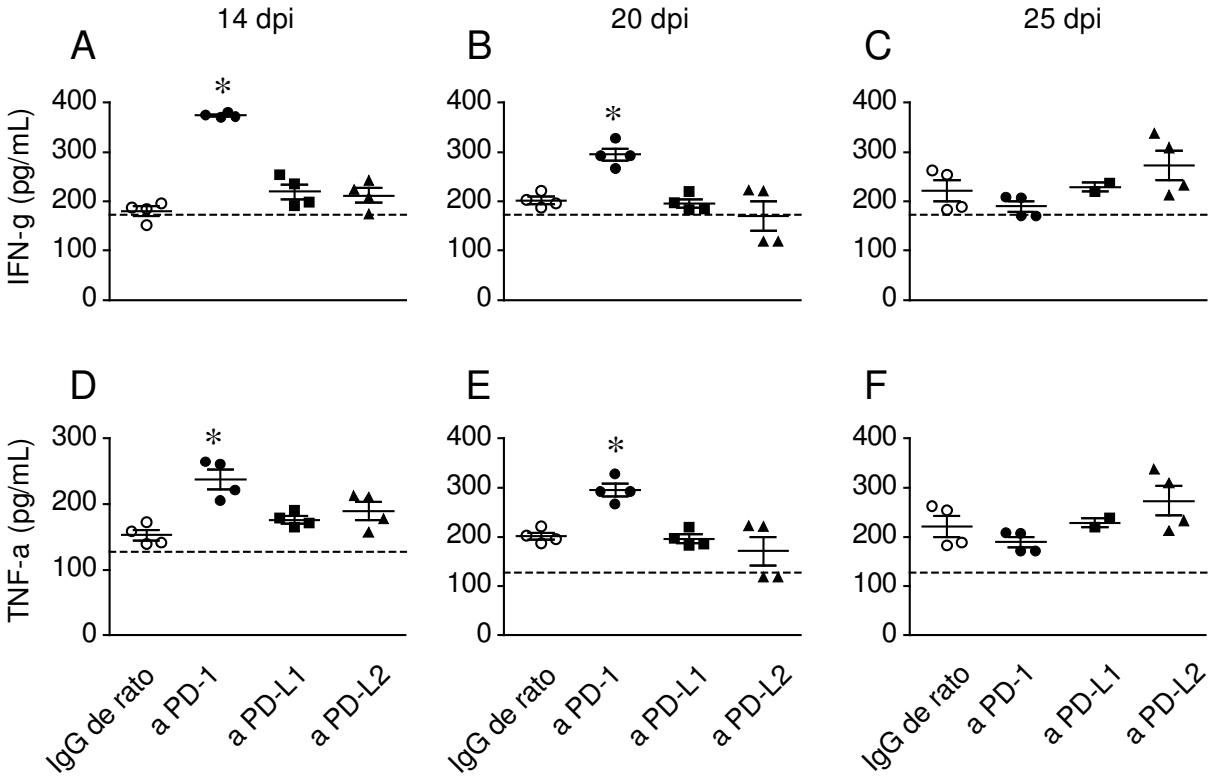

Figura 17. Efeito de bloqueio de PD-1 ou de seus ligantes sobre a produção de citocinas proinflamatórias no tecido cardíaco de camundongos infectados com *T. cruzi*. A figura mostra a media e o erro padrão dos níveis de cada citocina encontrados nos tecidos cardíacos de camundongos C57/BL6 infectados com *T. cruzi* e submetidos aos diversos tratamentos. O tecido cardíaco foi analisado por ELISA para TNF-α e IFN-γ, nos dias 14, 20 e 25 após a infecção. Dados representativos de 2 experimentos independentes (n=4). *: p<0,05, diferente de IgG. A linha tracejada indica os níveis das citocinas em corações de animais não infectados.

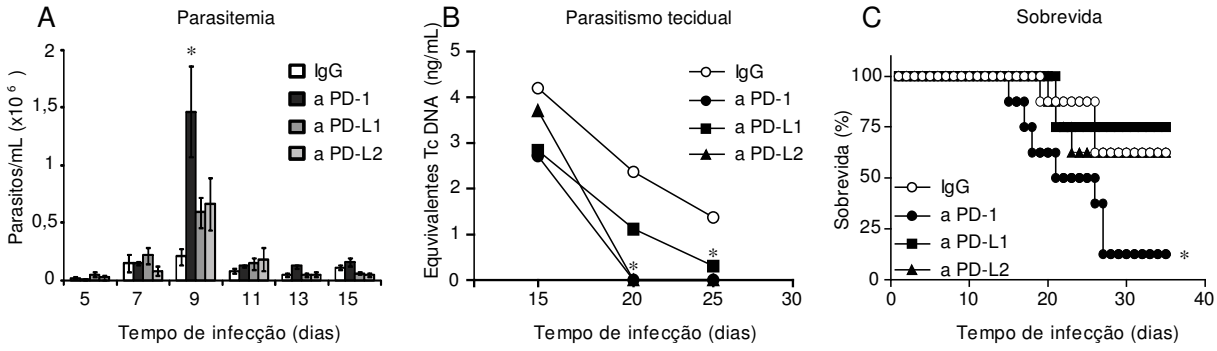

Figura 18. Efeito do tratamento *in vivo* com anticorpos bloqueadores de PD-1 e ligantes. Camundongos C57/BL6 foram infectados com *T. cruzi* e a parasitemia (**A**), o parasitismo miocárdico (**B**) e a sobrevida (**C**) foram avaliadas. Os animais distribuídos em grupos diferentes receberam cada um dos seguintes tratamentos: IgG de rato normal,anti-PD-1,anti-PD-L1 ou anti-PD-L2. A figura é representativa de 2 experimentos independentes (n=8). Os asteriscos indicam p<0,05 quando comparado ao grupo que recebeu IgG em cada caso (**A** e **B**, ANOVA seguida de Newman-Keuls; **C**, *qui* quadrado).

Efeito do bloqueio de PD-1 ou de seus ligantes na produção de NO em camundongos infectados

Em concordância com os níveis reduzidos de parasitismo no tecido cardíaco nos animais que receberam os anticorpos bloqueadores de PD-1 ou de seus ligantes, a análise de imunohistoquímica e morfometria permitiu verificar que havia níveis significativamente maiores da enzima sintase induzível de NO (NOS2). Isso sugere que há uma produção de NO aumentada nos tecidos cardíacos de animais tratados. A figura 19 mostra o resultado da análise morfométrica do tecido cardíaco de camundongos pertencentes a cada grupo experimental quanto à extensão da presença de NOS2 por imunohistoquímica. Em concordância com os resultados anteriores, o bloqueio de todas as três moléculas induziu aumento da presença de NOS2 no tecido cardíaco no 14º dia. Já no 20º dia, não houve diferenças significativas entre os grupos (Figura 19).

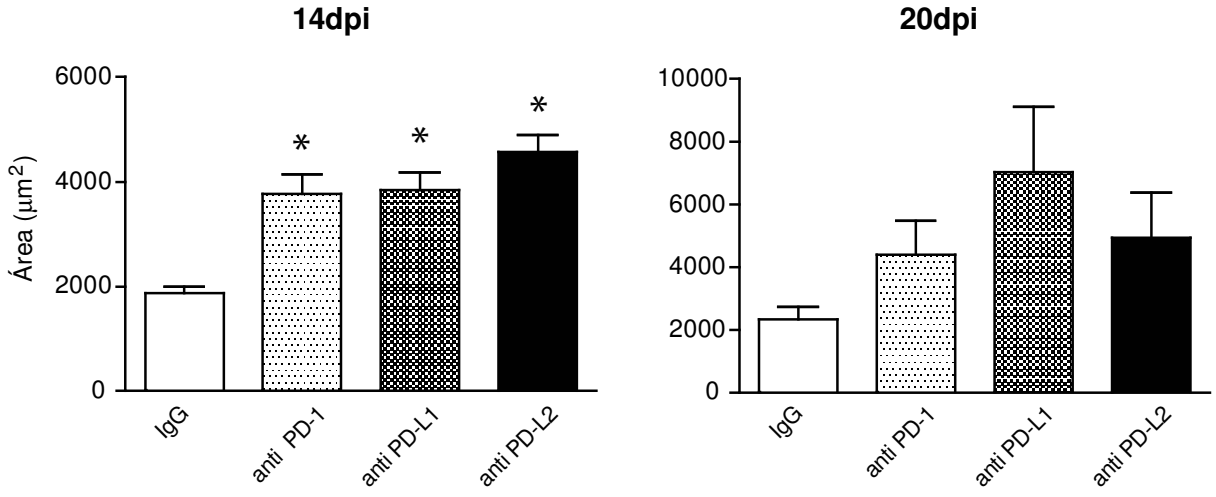

Figura 19. Morfometria da expressão de NOS2 no tecido cardíaco de camundongos infectados com *T. cruzi* e tratados com anticorpos bloqueadores de PD-1 e seus ligantes. A figura mostra a extensão da detecção de NOS2 no tecido cardíaco por imunohistoquímica (n=3). Os camundongos foram infectados com *T. cruzi* e tratados com anticorpos bloqueadores de PD-1, PD-L1 ou PD-L2 em dias alternados durante os primeiros 14 dias. * $p<0,05$ comparado com o controle (IgG).

Adicionalmente, os níveis circulantes de nitrito e nitrato foram analisados, através da reação de Griess, em soro de animais pertencentes a cada grupo experimental nos dias 14º, 20º ou 25º após a infecção (Figura 20). Os resultados mostraram que os animais tratados com anti-PD-1 não apresentaram níveis diferentes destes mediadores na circulação no 14º ou 20º dia após a infecção, quando comparados aos do grupo controle. Porém, essa produção foi diminuída no 25º dia após a infecção. No caso dos animais que receberam os anticorpos bloqueadores dos dois ligantes, estes

apresentaram redução significativa de nitrito e nitrato quando comparados ao grupo controle nos dias 14º e 25º após a infecção. Um fato interessante é o aumento observado na produção de nitrito e nitrato no 20º dia após a infecção no grupo de animais tratados com anti-PD-L1, já que os outros grupos não apresentaram aumento na produção de NO em nenhum dos períodos avaliados. A redução dos níveis de nitrato e nitrito no 14º no soro dos animais tratados com anti-PD-L1 e anti-PD-L2 sugere que essas moléculas estão relacionadas com a produção sistêmica destes mediadores.

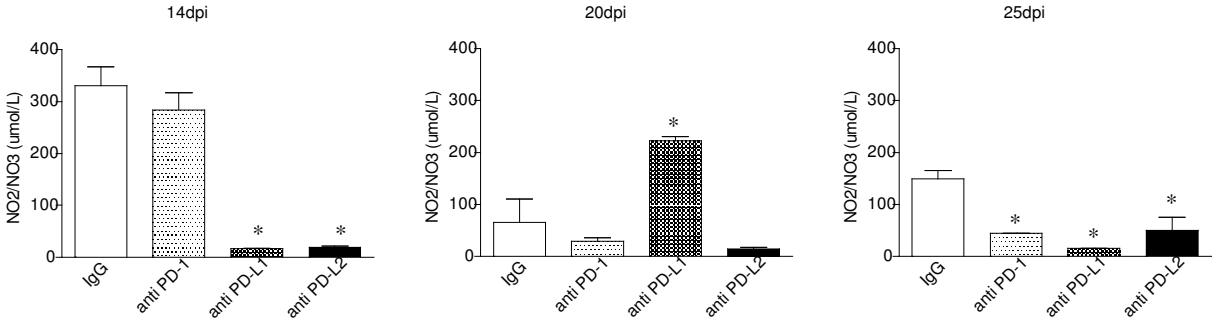

Figura 20. Níveis de óxido nítrico em soro de camundongos infectados com *T. cruzi* e tratados com anticorpos bloqueadores de PD-1 ou dos seus ligantes. A dosagem de NO foi realizada em amostras de soro retiradas nos dias 14, 20 e 25º após a infecção. A figura mostra em cada caso a média e o erro padrão da média, dos níveis séricos de nitrito e nitrato obtidos em 3-5 animais por grupo. Dados representativos de dois experimentos independentes. O asterisco indica significância estatística ($p < 0,05$) na ANOVA de uma via, seguida de Newman-keuls.

Efeitos da deficiência de PD-1 na resistência de macrófagos à infecção por T. cruzi *in vitro*

Uma vez que determinamos que macrófagos expressam PD-1 na presença do parasito *in vitro* e *in vivo* (Figura 6) e que não foi possível determinar claramente o efeito do bloqueio de PD-1 através do uso de anticorpos, experimentos foram conduzidos para avaliar a capacidade microbicida de macrófagos provenientes de camundongos geneticamente PD-$1^{-/-}$ e compará-la à de macrófagos provindos de camundongos normais. Para isso, macrófagos peritoneais foram extraídos dos dois tipos de animais e postos em cultura, sob estimulação com concentrações crescentes de IFN-γ. Após 48 horas de cultura, as células e o sobrenadante foram analisados para verificar o parasitismo intracelular e a produção de nitrito, respectivamente.

Para nossa surpresa, os macrófagos PD-$1^{-/-}$ apresentam uma resposta extremamente eficiente na produção de nitrito (Figura 21 A), que é um mediador extremamente importante na morte do parasito e consequentemente no controle do parasitismo intracelular (Figura 21 B-D). Esta resposta é significativamente maior quando comparada à apresentada por células de camundongos selvagens.

Ainda, a contagem de parasitos intracelulares demonstrou uma quantidade significativamente menor de parasitos por célula nos macrófagos PD-1$^{-/-}$ (1,19 contra 5,17 parasitos por célula, Figura 21 B). É interessante ressaltar o fato de que mesmo na ausência de IFN-γ, essas células conseguem sobreviver à infecção *in vitro*, controlando a proliferação do parasito (dados não apresentados).

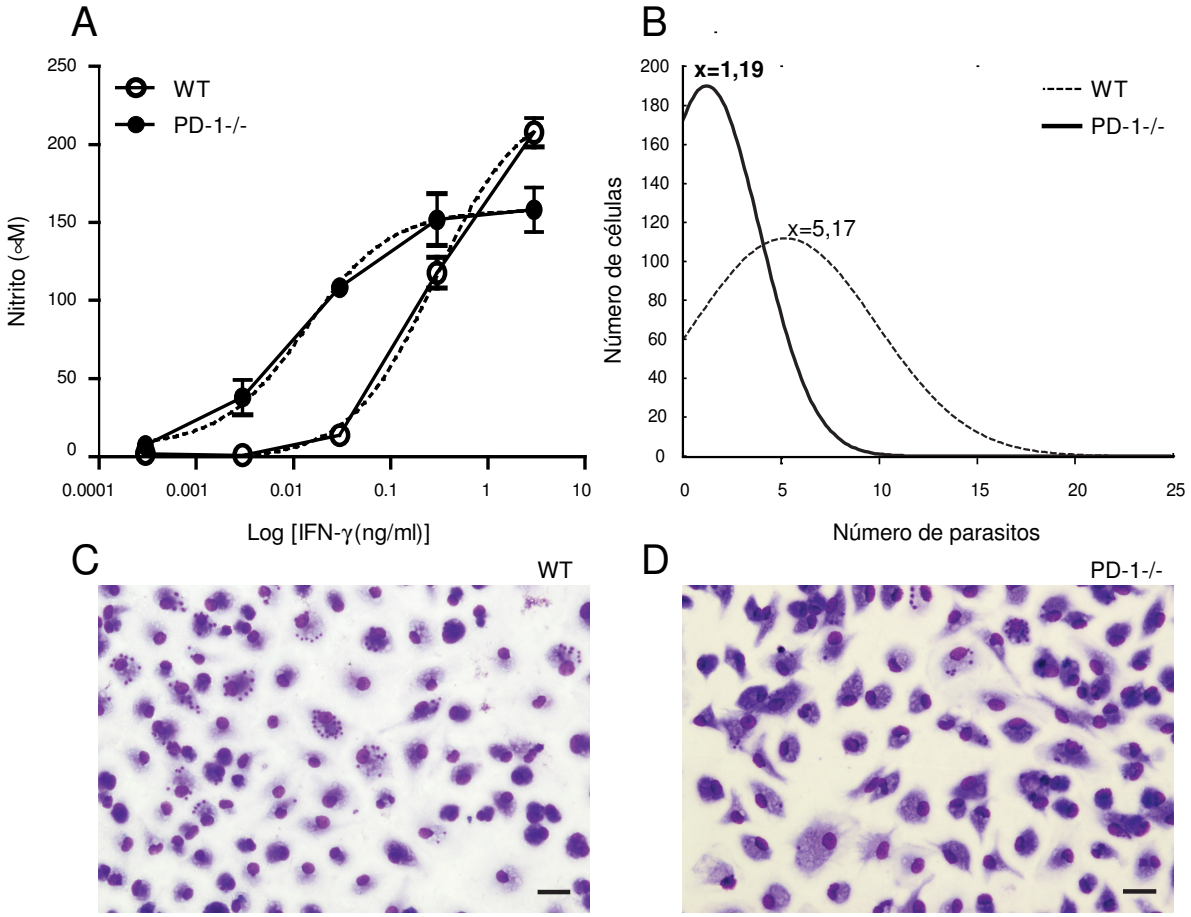

Figura **21. Macrófagos provenientes de camundongos PD-1$^{-/-}$ são mais resistentes à infecção por *T. cruzi in vitro***. Macrófagos peritoneais de camundongos WT ou PD-1$^{-/-}$ foram incubados por 48h na presença de tripomastigotas de *T. cruzi* (na razão de 5 parasitos por célula), adicionando IFN-γ em diferentes concentrações. **A**. Dose-resposta da produção de nitrito por macrófagos estimulados com diferentes concentrações de IFN-γ. A linha tracejada indica a curva ideal de dose-resposta em cada caso. **B**. Histograma da distribuição de frequência do número de parasitos por célula. **C** e **D**, Microfotografias representativas da cultura de macrófagos WT (**C**) e PD-1$^{-/-}$ (**D**). A barra representa 10 um. As figuras **B** - **D** representam o ponto da maior concentração de IFN-γ usada para estimular os macrófagos. O asterisco representa diferença estatisticamente significativa na ANOVA de duas vias seguida do teste Newman-Keuls (**A**), ou no teste t-*student* (**B**).

Efeito da deficiência de PD-1 na parasitemia e os níveis de NO em camundongos infectados

Com o objetivo de avaliar o efeito *in vivo* da deficiência de PD-1 sobre a capacidade do hospedeiro em controlar a proliferação do parasito, animais C57BL/6 normais ou PD-1$^{-/-}$ foram infectados com

dois inóculos diferentes de *T. cruzi* e a parasitemia foi avaliada durante a fase aguda. Os resultados demonstraram um aumento da capacidade tripanocida *in vivo* nos camundongos PD-$1^{-/-}$, se comparados aos animais selvagens (Figura 22 A,B). Contudo, quando os níveis de nitrito/nitrato circulantes foram analisados, estes animais apresentam redução significativa deste mediador na circulação (Figura 22 C, D). Isso constitui um achado extremamente interessante e sugere que neste caso, os camundongos PD-$1^{-/-}$ apresentam requerimentos menores de NO para conseguir controlar a proliferação do parasito.

Efeito da deficiência de PD-1 na resposta linfoproliferativa induzida por T. cruzi

Visto que os camundongos PD-$1^{-/-}$ são mais eficientes em controlar a proliferação do parasito e que isto ocorre mesmo na presença de níveis menores de NO, o foco do estudo foi voltado para verificar se havia proliferação aumentada de células efetoras da resposta imune. Para avaliar esta hipótese, inicialmente foi estudada a resposta linfoproliferativa sistêmica em animais selvagens ou PD-$1^{-/-}$.

A análise macroscópica do baço permitiu verificar que a deficiência de PD-1 induz uma esplenomegalia exagerada, se comparada com aquela existente em animais selvagens (Figura 23). Sendo que o tamanho dos baços de animais PD-$1^{-/-}$ e infectados tinha em média o dobro do tamanho dos baços de animais selvagens.

Efeito da deficiência de PD-1 nas subpopulações de linfócitos durante a infecção

Uma vez que os animais PD-$1^{-/-}$ apresentaram aumentada linfoproliferação em resposta à infecção por *T. cruzi*, as principais subpopulações de linfócitos foram analisadas com o objetivo de entender o aumento da resistência ao parasitismo circulante, que havia sido previamente evidenciada nestes animais. A análise de citometria foi realizada para verificar a frequência de células $CD3^+$, $CD19^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD25^+$ e $CD4^+FoxP3^+$ (Figura 24). Os resultados mostraram aumento significativo da frequência de células B (Figura 24 C) e diminuição significativa na frequência de células $CD4^+CD25^+$ e $CD4^+FoxP3^+$ (Figura 24 F,H). Esses resultados sugerem uma diminuição nos mecanismos reguladores da resposta efetora e aumento da resposta humoral em camundongos PD-$1^{-/-}$ infectados com *T. cruzi*, quando comparados aos animais selvagens.

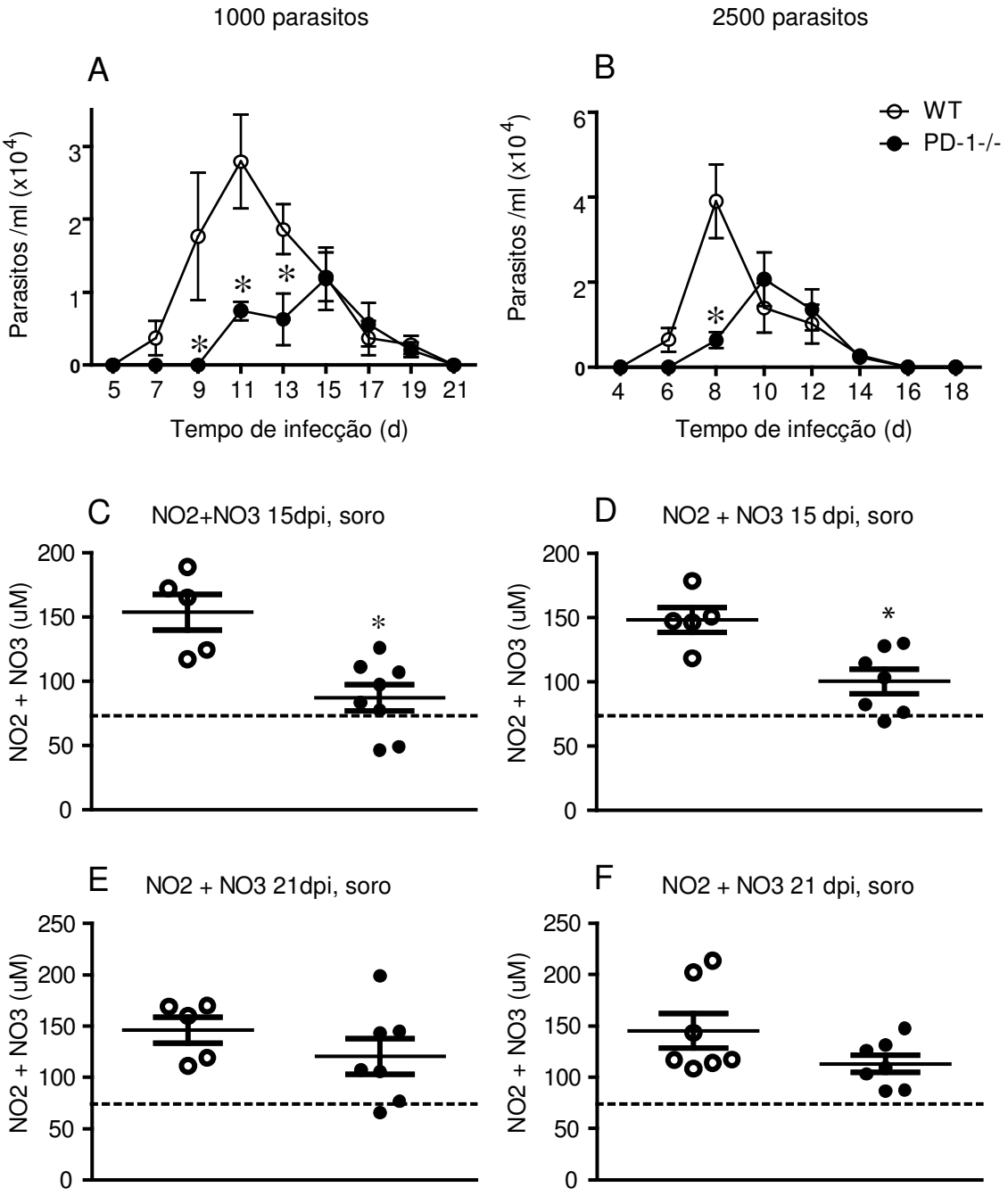

Figura 22. A deficiência de PD-1 induz aumento da resistência à *T. cruzi in vivo*, apesar de diminuição da produção de NO. Camundongos C57Bl/6 ou PD-$1^{-/-}$ foram infectados com a quantidade especificada de parasitos (esquerda: 1000; direita: 2500 parasitos i.p.). A parasitemia (**A**, **B**) foi avaliada durante as primeiras 3 semanas de infecção. Os níveis séricos de NO2NO3 foram avaliados nos dias 15 (**C**, **D**) e 21 (**E**, **F**) após a infecção. Os asteriscos indicam significância estatística ($p<0,05$) na ANOVA de duas vias (**A**, **B**) ou no teste t-*student* (**C**-**F**)

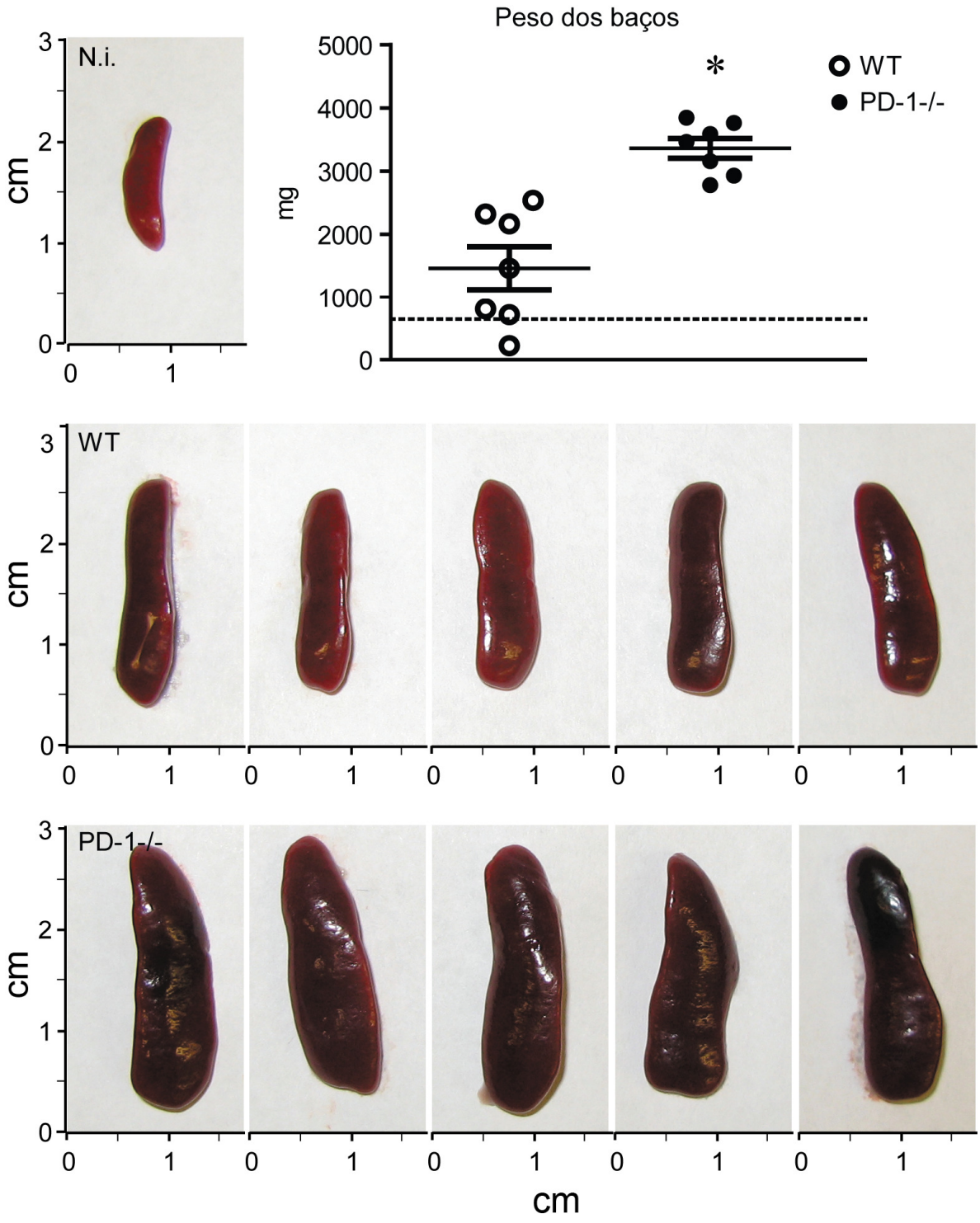

Figura 23. Efeito da deficiência de PD-1 na esplenomegalia induzida pela infecção com *T. cruzi*. Camundongos C57BL/6 normais (wt, circulo branco) ou PD-1$^{-/-}$ (PD-1$^{-/-}$, circulo preto) foram infectados por via i.p. com 1000 formas de *T. cruzi*. Os baços de 5-7 animais foram coletados no dia décimo quinto após a infecção e o tamanho e peso dos órgãos foram avaliados. A figura mostra os dados representativos de dois experimentos independentes. O asterisco indica p< 0,05 no teste t-*student*.

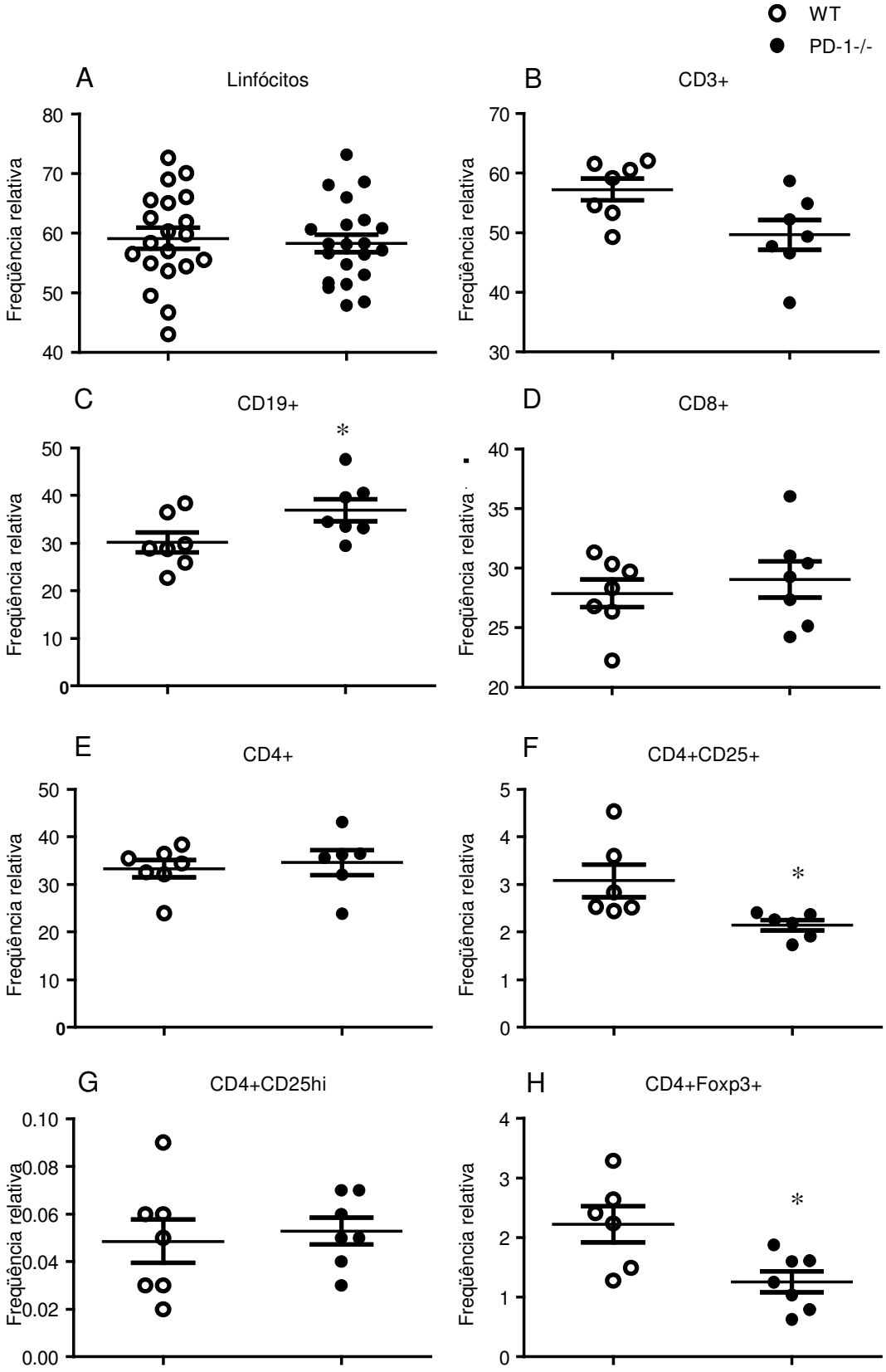

Figura 24. Frequência de subpopulações de linfócitos no baço de camundongos normais ou PD-1$^{-/-}$. As células foram retiradas do baço de camundongos C57BL/6 normais (wt, circulo branco) ou PD-1$^{-/-}$ (PD-1$^{-/-}$, circulo preto) no dia 21º após a infecção por *T. cruzi*. A expressão dos marcadores para as diferentes subpopulações de linfócitos indicadas foi avaliada por citometria de fluxo. A figura mostra os dados representativos de dois experimentos independentes. O asterisco indica p< 0,05 no teste t-*student*.

Efeito da deficiência de PD-1 na frequência de linfócitos apoptóticos durante a infecção

Visto que a resposta imune que controla o parasitismo circulante é mais eficiente em camundongos PD-$1^{-/-}$ e que a resistência está associada com reduzidos níveis de NO sem alteração na frequência de células $CD4^+$ ou $CD8^+$ totais, o próximo objetivo foi avaliar se a deficiência de PD-1 afeta a indução de apoptose em linfócitos $CD4^+$ e $CD8^+$ durante a infecção por *T. cruzi.* Para isso, as células foram obtidas do baço de camundongos selvagens ou PD-$1^{-/-}$ infectados com *T. cruzi*. Foi realizada analise de citometria de fluxo nestas células e os resultados demonstraram que existe uma frequência significativamente diminuída de células $CD4^+$ e $CD8^+$ que se encontram em apoptose (Figura 25).

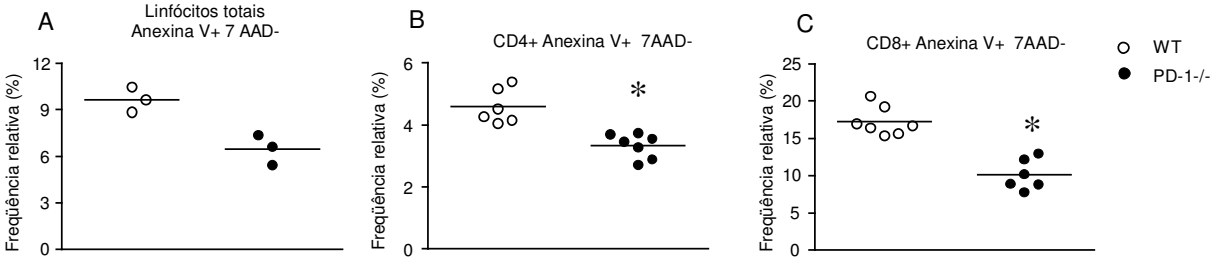

Figura 25. Frequência de apoptose em células $CD4^+$e $CD8^+$de camundongos normais ou PD-$1^{-/-}$. A figura mostra a média e dispersão da frequência relativa (porcentagem) de células positivas para anexina V e negativas para 7AAD, dentro da *gate* de linfócitos totais (**A**), ou em células $CD4^+$(**B**) ou $CD8^+$(**C**) . As células foram extraídas do baço de camundongos C57BL/6 normais (wt, circulo branco) ou PD-$1^{-/-}$ (PD-$1^{-/-}$, circulo preto) no 21º dia após a infecção por *T. cruzi*. O asterisco indica significância estatística ($p< 0,05$) no teste t-*student*.

Efeito da deficiência de PD-1 na produção de citocinas no soro de camundongos infectados

IFN-γ e TNF-α são citocinas cruciais na geração da resposta imune protetora frente a *T. cruzi*, já IL-10 é importante no controle dessa resposta, entre outros mecanismos reguladores. Para determinar se o aumento da capacidade microbicida evidenciada em animais deficientes de de PD-1 estava relacionado com diferenças na produção desta citocinas, os soros foram coletados de animais selvagens ou PD-$1^{-/-}$ e foi realizada a determinação dos níveis circulantes destas citocinas. Os resultados mostraram que a deficiência de PD-1 leva a uma diminuição significativa nos níveis de IFN-γ no 15º dia após a infecção, se comparados aos níveis encontrados nos soros de animais selvagens (Figura 26 A).

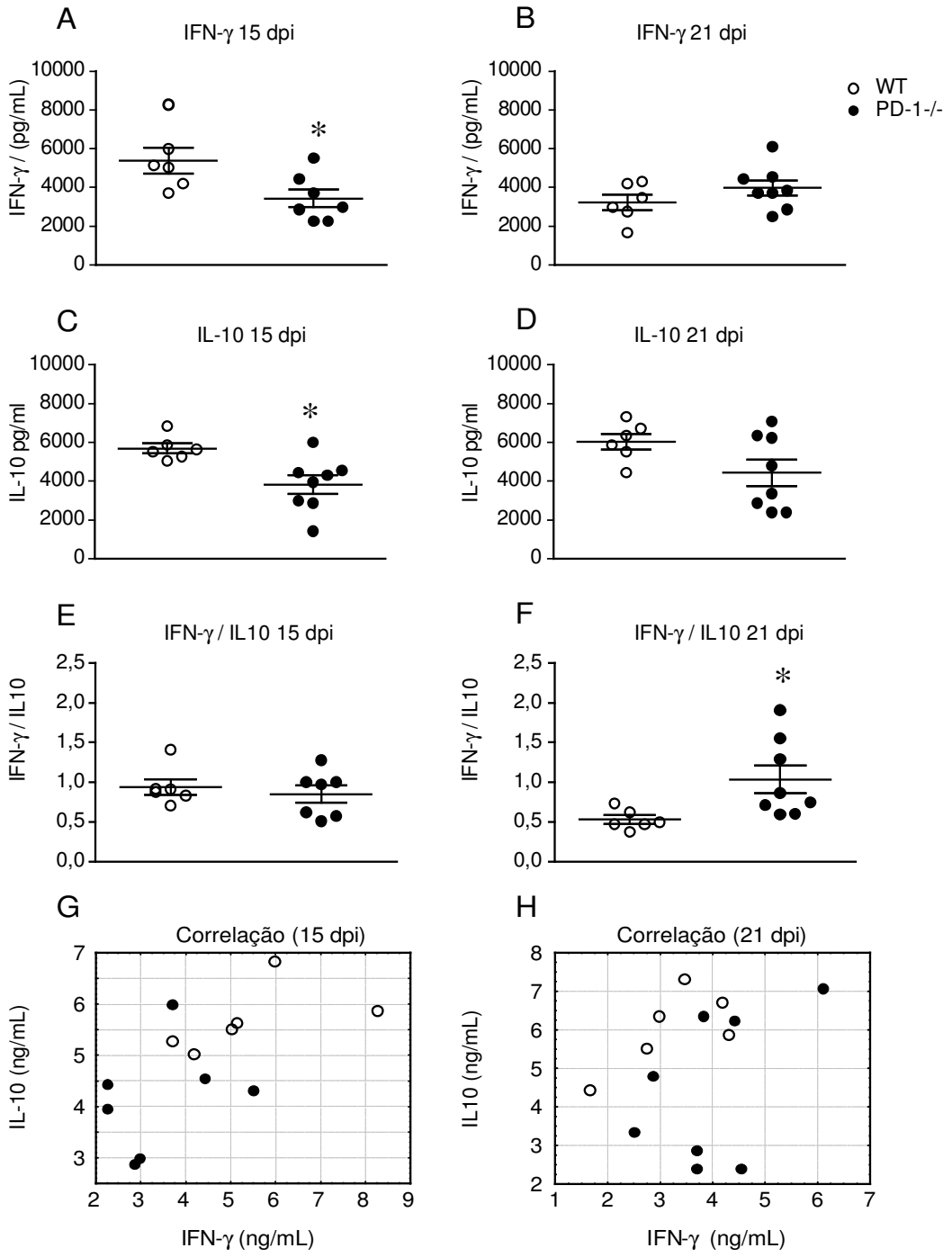

Figura 26 Níveis de IFN-γ e IL-10 em soro de camundongos C57BL/6 selvagens ou PD-1$^{-/-}$ após a infecção por *T. cruzi*. Os animais (6-8 por grupo) foram infectados com 1000 formas tripomastigotas de *T. cruzi* e após 15 ou 21 dias, os soros foram coletados e os níveis de IFN-γ ou IL-10 foram determinados por ELISA. A figura mostra o resultado da determinação dos níveis de IFN-γ (**A,B**), IL-1º (**C,D**) e a razão IFN-γ/IL-10 (**D,E**) bem como o gráfico de correlação (**G,H**) para estas citocinas. O asterisco indica p <0,05 no teste t-*student*. Resultado representativo de dois experimentos independentes.

Além disso, os níveis de IL-10 presentes na circulação dos animais PD-1$^{-/-}$ também foram significativamente inferiores aos detectados em animais selvagens (Figura 26 C). Já no 21º dia da infecção, não foram detectadas diferenças na produção destas citocinas entre os grupos (Figura 26 B, D). Contudo, quando analisada a razão da produção de IFN-γ pela produção de IL-10 em cada

indivíduo, foi verificado que no 21º dia, há aumento significativo dessa razão apenas nos camundongos deficientes de PD-1 (Figura 26 F, H), indicando uma produção proporcionalmente maior de IFN-γ nestes camundongos. Isso não foi observado no 15º dia após a infecção (Figura 26 E, G).

No caso da produção de TNF-α, os resultados mostraram uma diferença extremamente significativa nos níveis circulantes desta citocina apenas no 21º dia após a infecção (Figura 27B). Essa diferença não foi evidenciada no 15º dia após a infecção.

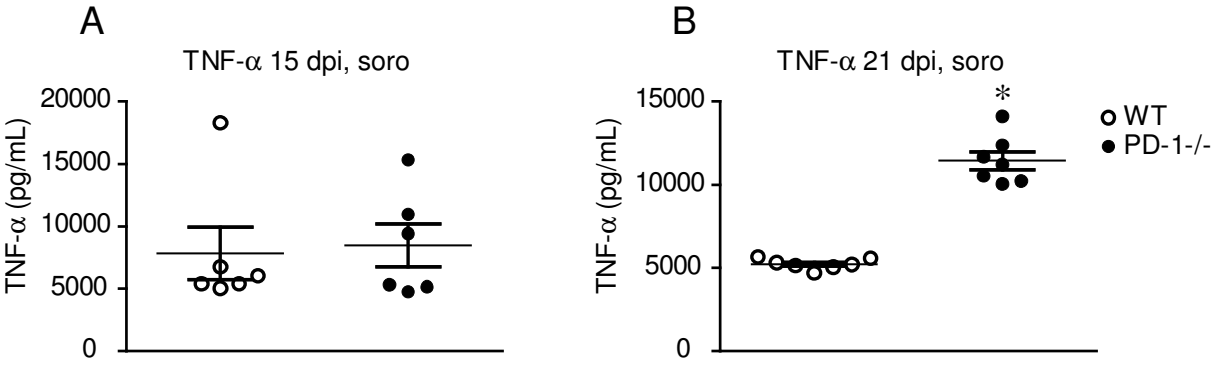

Figura 27. Níveis de TNF-α em soro de camundongos C57BL/6 selvagens ou PD-1$^{-/-}$. Os animais (6-8 por grupo) foram infectados com 1000 formas tripomastigotas de *T. cruzi* e após 15 ou 21 dias, os soros foram coletados e os níveis de TNF-α foram determinados por ELISA. A figura mostra o resultado da determinação dos níveis da citocina. O asterisco indica p <0,05 no teste t-*student*. Resultado representativo de dois experimentos independentes.

Como IL-12 é uma citocina importante durante a fase inicial do desenvolvimento da resposta imune a *T. cruzi* e está envolvida na amplificação da resposta imune inata, bem como na ativação de mecanismos efetores da resposta imune adaptativa, nós avaliamos os níveis dessa citocina no soro de camundongos C57BL/6 normais ou PD-1$^{-/-}$. De forma interessante, os animais PD-1$^{-/-}$ não apresentaram diferença nos níveis desta citocina no 10º dia após a infecção (Figura 28 A), mas houve aumento significativo de IL-12 p40 no soro no 15º dia da infecção (Figura 28 B). No 21º dia da infecção, esses níveis diminuíram significativamente (Figura 28 C).

Em conjunto, os dados da produção de citocinas em animais PD-1$^{-/-}$ sugerem que sua maior capacidade parasiticida pode estar relacionada com o aumento na produção de citocinas sabidamente relacionadas com o controle do parasitismo circulante, como são TNF-α e IL-12. Ainda, isso ocorre na presença de níveis reduzidos de IFN-γ e IL-10, duas citocinas relacionadas principalmente com a efetuação da resposta adaptativa.

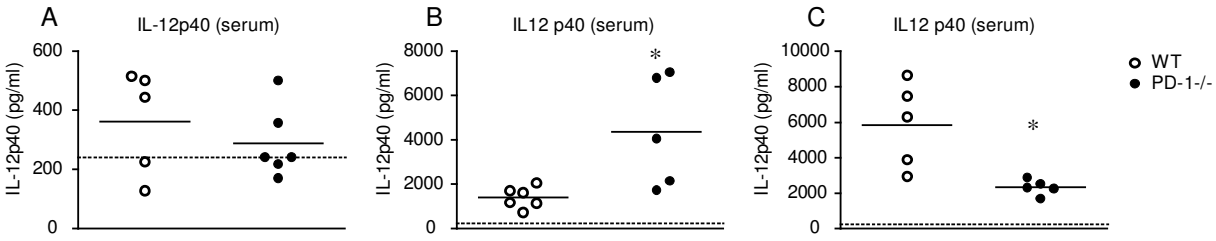

Figura 28. Níveis de IL-12 p40 em soro de camundongos C57BL/6 selvagens ou PD-1$^{-/-}$. Os animais (6-8 por grupo) foram infectados com 1000 formas tripomastigotas de *T. cruzi* e após 10 (**A**), 15 (**B**) ou 21 dias (**C**) os soros foram coletados e os níveis de IL-12 p40 foram determinados por ELISA. A figura mostra o resultado da determinação dos níveis de cada citocina. A linha tracejada indica a media os níveis detectados em animais não infectados. O asterisco indica p <0,05 no teste t-*student*. Resultado representativo de dois experimentos independentes.

Efeito da deficiência de PD-1 na atividade de arginase no soro de camundongos infectados

Durante a infecção por *T. cruzi*, uma alta produção de NO é observada, o que está amplamente correlacionado com o grau de patologia. Contudo, NO é também crucial no controle da proliferação do parasito. Esta produção aumentada de NO é regulada principalmente através do metabolismo da arginina, precursor deste mediador.

A enzima arginase compete com a NOS2 pelo substrato L-arginina, gerando L-ornitina e uréia. Além de inibir a formação de NO, alguns metabólitos produzidos a partir da L-ornitina favorecem o crescimento do parasito (PELUFFO, PIACENZA *et al.*, 2004). Consequentemente, a atividade da arginase é um fator favorecedor da proliferação do parasito, além de inibir a produção de NO.

Desde que os animais PD-1$^{-/-}$ apresentam reduzidos níveis séricos de NO quando comparados aos camundongos selvagens e que a enzima arginase controla a produção de NO, foi mensurada a atividade da enzima arginase em amostras de soro. Os resultados mostram baixos níveis de atividade de arginase em amostras de animais selvagens e PD-1$^{-/-}$, nos dia 10º e 17º após a infecção (Figura 29A, B). Já no dia 23º após a infecção, os animais selvagens apresentam um aumento significativo da atividade desta enzima, o que não acontece em camundongos PD-1$^{-/-}$ (Figura 29C). Isso pode sugerir que nos animais deficientes de PD-1 há uma reduzida regulação da atividade de NOS2 quando comparados aos camundongos selvagens, provávelmente em relação com o fato de essa atividade ser menor, como observado pelos baixos níves de NO circulantes.

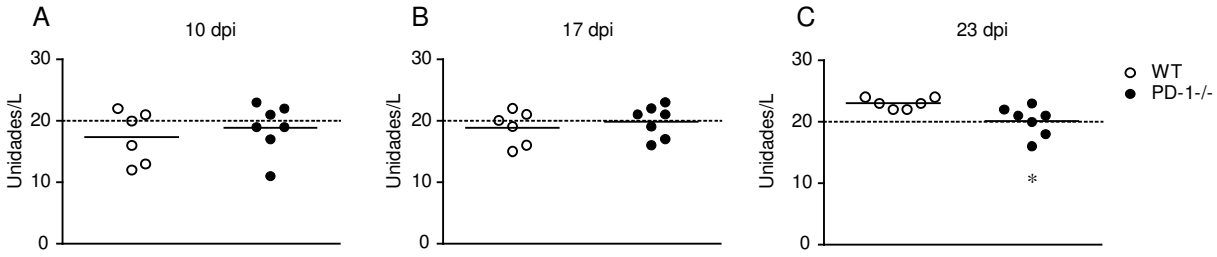

Figura 29. Atividade de arginase no soro de camundongos C57BL/6 normais (WT) ou PD-1$^{-/-}$ (PD-1$^{-/-}$) durante a infecção por *T. cruzi*. Os camundongos foram infectados por via i.p. com 1000 formas tripomastigotas de *T. cruzi* e após o tempo indicado em cada gráfico, a atividade da arginase foi determinada em amostras de soro. A figura mostra o resultado da determinação da atividade da enzima nos camundongos WT ou PD-1 $^{-/-}$. A linha tracejada representa a atividade encontrada em amostras de soro de animais não infectados. O asterisco representa diferença significativa (p<0,05) no teste t-*student*.

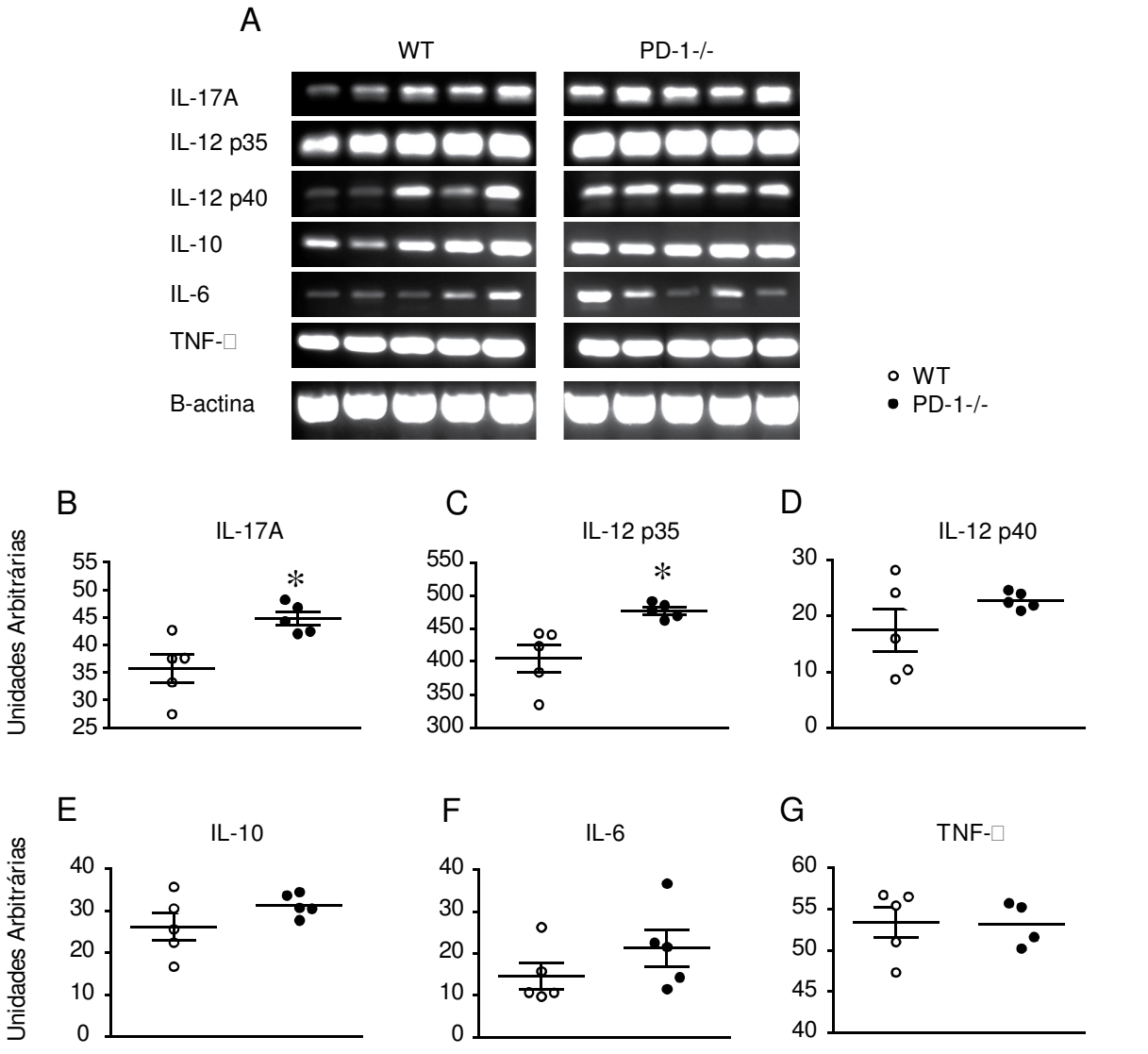

Figura 30. RT-PCR do tecido cardíaco de animais C57BL/6 normais ou PD-1$^{-/-}$. O RNA foi extraído de amostras de tecido cardíaco obtidas de camundongos infectados com *T. cruzi* no 21º dia após a infecção. A partir do RNA, foi sintetizado o cDNA e quantidades iguais dele foram usadas para a RT-PCR para cada um dos genes listados. Em **A** o resultado da eletroforese em gel de agarose em cada caso é mostrado. **B-G** mostram o resultado da densitometria para cada um dos genes das citocinas. Resultado representativo de dois experimentos independentes com 5-8 camundongos por grupo. Os asteriscos representam diferença estatisticamente significativa (p<0,05) no teste t-*student*.

Efeito da deficiência de PD-1 nos níveis de transcritos para citocinas no tecido cardíaco de camundongos infectados

Para avaliar os níveis de transcritos para citocinas no tecido, foi realizado o PCR a partir de amostras de cDNA, preparado através de RT-PCR com amostras de RNA extraído do coração de animais selvagens ou PD-$1^{-/-}$ no dia 21º da infecção. Os resultados mostraram aumento significativo nos níveis de transcritos para IL-17A e IL-12p35 (Figura 30 A, B, C), mas não para IL-12 p40, IL-10, IL-6 ou TNF-α (Figura 30 A, D-G). Esses resultados sugerem uma produção aumentada dessas citocinas, relacionadas principalmente com a resposta imune inata, no tecido cardíaco de animais PD-$1^{-/-}$.

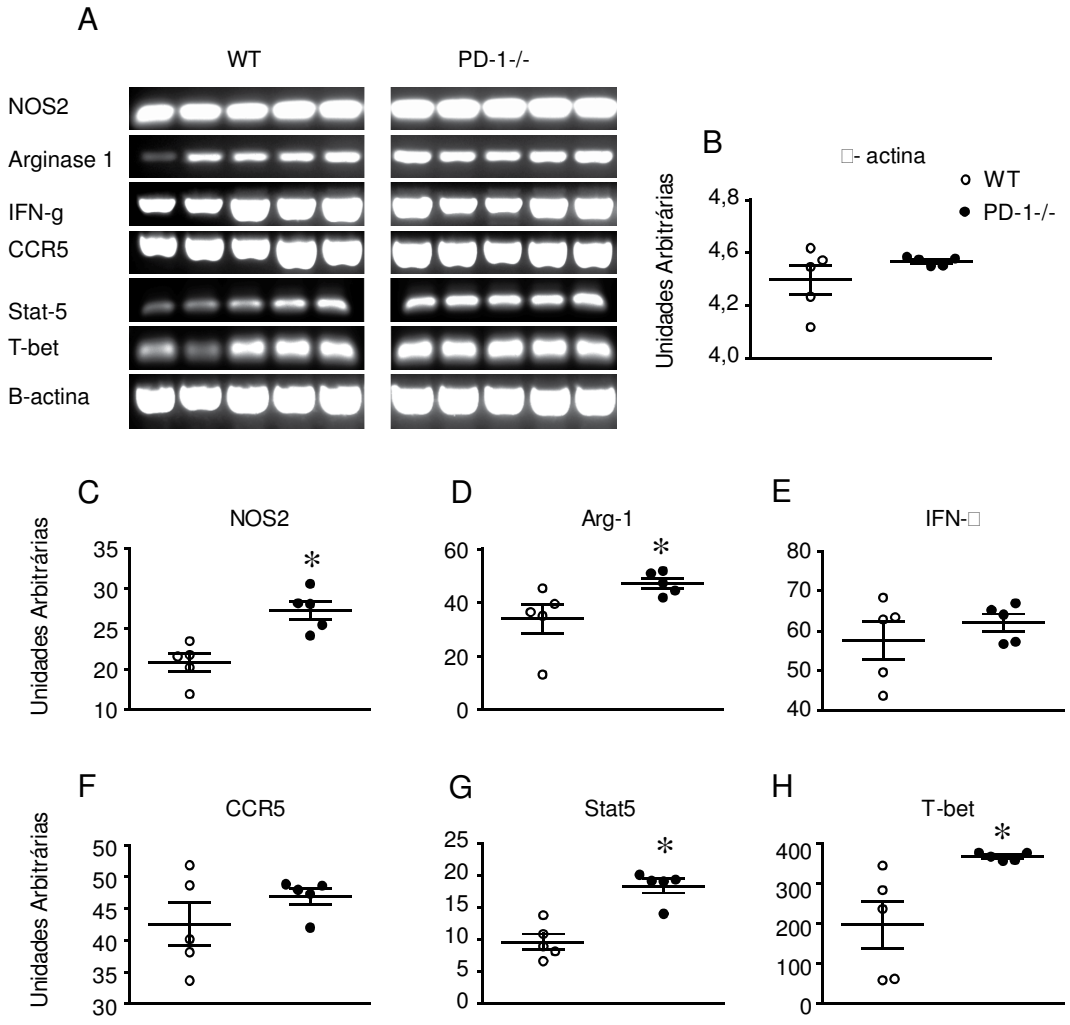

Figura 31. RT-PCR do tecido cardíaco de animais C57BL/6 normais ou PD-$1^{-/-}$. O RNA foi extraído de amostras de tecido cardíaco obtidas de camundongos infectados com *T. cruzi* no 21º dia após a infecção. A partir do RNA, foi sintetizado o cDNA e quantidades iguais dele foram usadas para a RT-PCR para cada um dos genes listados. Em **A** o resultado da eletroforese em gel de agarose em cada caso é mostrado. **B-G** mostram o resultado da densitometria para cada gene. Resultado representativo de dois experimentos independentes com 5-8 camundongos por grupo. Os asteriscos representam diferença estatisticamente significativa ($p<0,05$) no teste t-*student*.

Efeito da deficiência de PD-1 nos níveis de transcritos para NOS2, ARG1, IFN-γ , Stat-5 e t-bet no tecido cardíaco de camundongos infectados

Para verificar se os animais PD-1$^{-/-}$ apresentavam um aumento de fatores relacionados com a resposta de padrão Th1, foi realizado também PCR para NOS2, Arginase 1 (Arg-1), IFN-γ, CCR5, Stat5 e T-bet (Figura 31). Os resultados mostraram aumento significativo dos transcritos para NOS2, Stat5 e T-bet (Figura 31 C, G, H), fatores relacionados com a resposta imune do tipo Th1. Além disso, os transcritos para Arg-1 estavam aumentados significativamente no tecido cardíaco desses animais (Figura 31 D).

Contudo, não houve diferenças significativas quanto aos níveis de transcritos para IFN-γ e CCR5, que também são produzidos no contexto de um processo inflamatório do tipo Th1 (Figura 31 E, F). A figura 31 B mostra a quantificação por densitometria do resultado da PCR para β-actina nas amostras.

Esses resultados sugerem que a deficiência de PD-1 induz aumento significativo dos fatores de transcrição T-bet e Stat5 em animais infectados por *T. cruzi*, o que pode estar favorecendo um aumento do processo inflamatório no tecido cardíaco desses animais.

Efeito da deficiência de PD-1 nos níveis de transcritos para SOCS-1, SOCS-2, SOCS-3, ATG5 e Beclin-1 no tecido cardíaco de camundongos infectados

Uma vez que não foi verificado aumento nos transcritos para citocinas cruciais na geração de uma resposta imune adaptativa nos animais PD-1$^{-/-}$ (como IFN-γ e IL-10) e que eles apresentam um melhor controle do parasitismo (Figura 22), avaliamos se havia diferença nos níveis de transcritos para genes envolvidos na supressão da sinalização por citocinas (SOCS-1, SOCS-2 e SOCS-3). Os resultados mostram que os animais PD-1$^{-/-}$ apresentam aumento significativo nos transcritos para SOCS-1 (Figura 32 A) e SOCS-3 (Figura 32 C), mas não para SOCS-2 (Figura 32 B), quando comparados aos animais do grupo controle, provavelmente em associação com a maior produção das citocinas IL-12 p35 e IL-17 e do fator de transcrição T-bet.

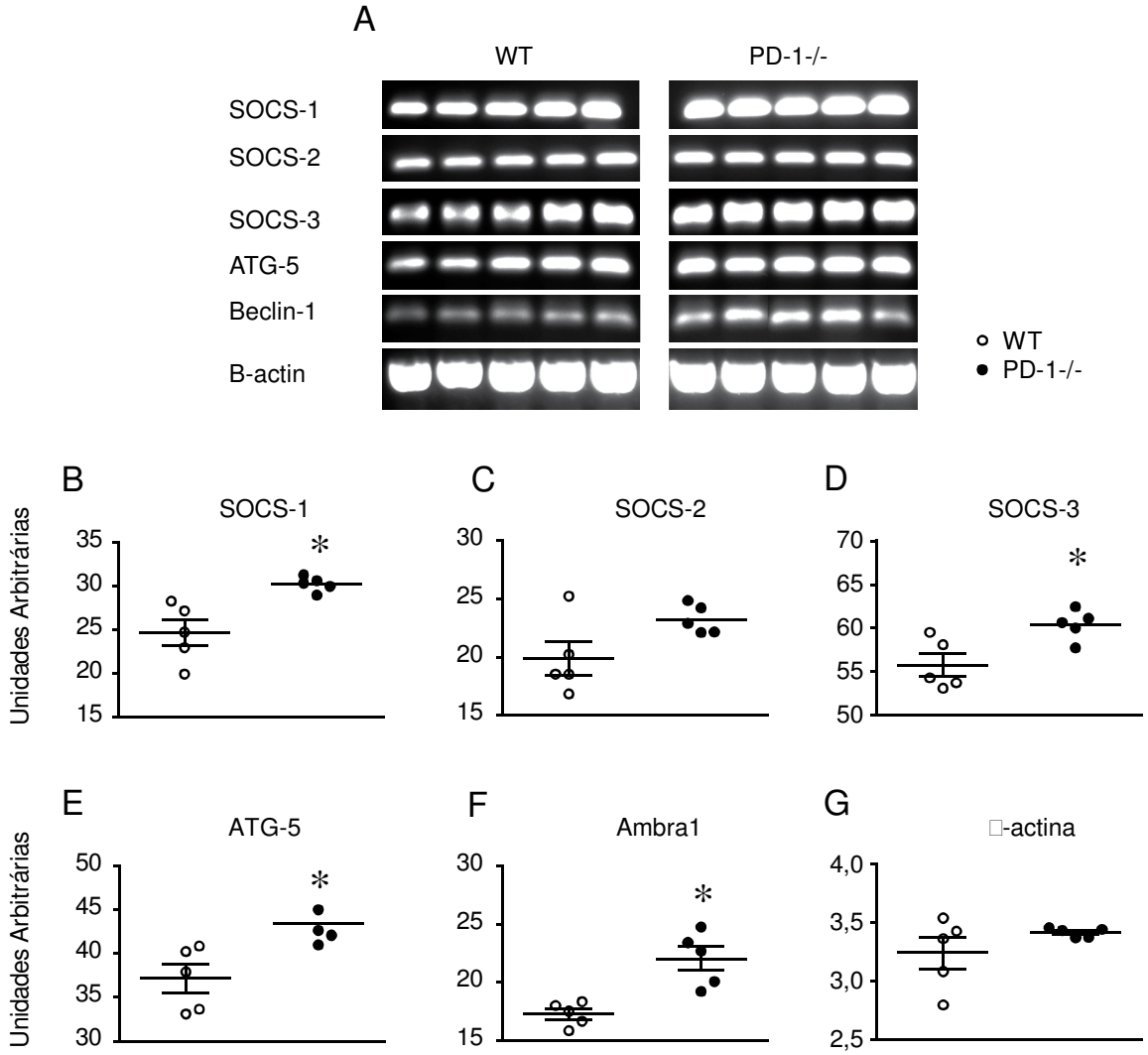

Figura 32. RT-PCR do tecido cardíaco de animais C57BL/6 normais ou PD-1$^{-/-}$. O RNA foi extraído de amostras de tecido cardíaco obtidas de camundongos infectados com *T. cruzi* no 21º dia após a infecção. A partir do RNA, foi sintetizado o cDNA e quantidades iguais dele foram usadas para a RT-PCR para cada um dos genes listados. Em **A** o resultado da eletroforese em gel de agarose em cada caso é mostrado. **B-G** mostram o resultado da densitometria para cada gene. Resultado representativo de dois experimentos independentes com 5-8 camundongos por grupo. Os asteriscos representam diferença estatisticamente significativa ($p<0,05$) no teste t-*student*.

De maneira interessante, apesar de que não foi possível verificar diferenças no parasitismo tecidual nesses animais quando comparados aos animais controle (dados não apresentados), encontramos aumento significativo de transcritos de genes envolvidos na autofagia, como ATG-5 e Beclin-1 (Figura 32 E, F). Sabe-se que alguns protozoários induzem bloqueio dessas moléculas como mecanismo de escape do fagossomo (COLOMBO, 2007). Assim, é possível afirmar que os animais PD-1$^{-/-}$ apresentam níveis aumentados de 2 fatores envolvidos com o controle do parasitismo intracelular, o que pode estar relacionado com os resultados observados com macrófagos em cultura (Figura 21).

Efeito da deficiência de PD-1 sobre o infiltrado inflamatório miocárdico durante a infecção

Para confirmar o aumento do infiltrado inflamatório observado após o tratamento com anticorpos bloqueadores de PD-1 e de seu ligante PD-L1, foi realizada a citometria do tecido cardíaco no 21º dia após a infecção. A análise das células retiradas dos corações de camundongos C57BL/6 normais ou PD-$1^{-/-}$ permitiu verificar um aumento significativo da quantidade de células infiltrando o tecido cardíaco (Figura 33). Esse aumento foi verificado no número total de linfócitos, células $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$, monócitos e macrófagos ($F480^{+}$) (Figura A-G). Ainda, esses dados foram confirmados por imunofluorescência do tecido para detecção de CD8.

Um dos mecanismos pelos quais PD-1 regula a proliferação de linfócitos é através da regulação da via de sinalização Stat5, ativada pela citocina IL-2 nessas células. Para verificar se essas células $CD8^{+}$ presentes em tecido cardíaco de camundongos PD-$1^{-/-}$ apresentavam níveis aumentados de fosforilação de Stat5, foi realizada a imunofluorescência com anticorpo específico para a proteína fosforilada O resultado comprovou que a deficiência de PD-1, induz aumento da expressão de pStat5 no tecido cardíaco de camundongos infectados por *T. cruzi* (Figura 33 J,K). Esses resultados confirmam a participação de PD-1 na regulação da resposta inflamatória nesse tecido.

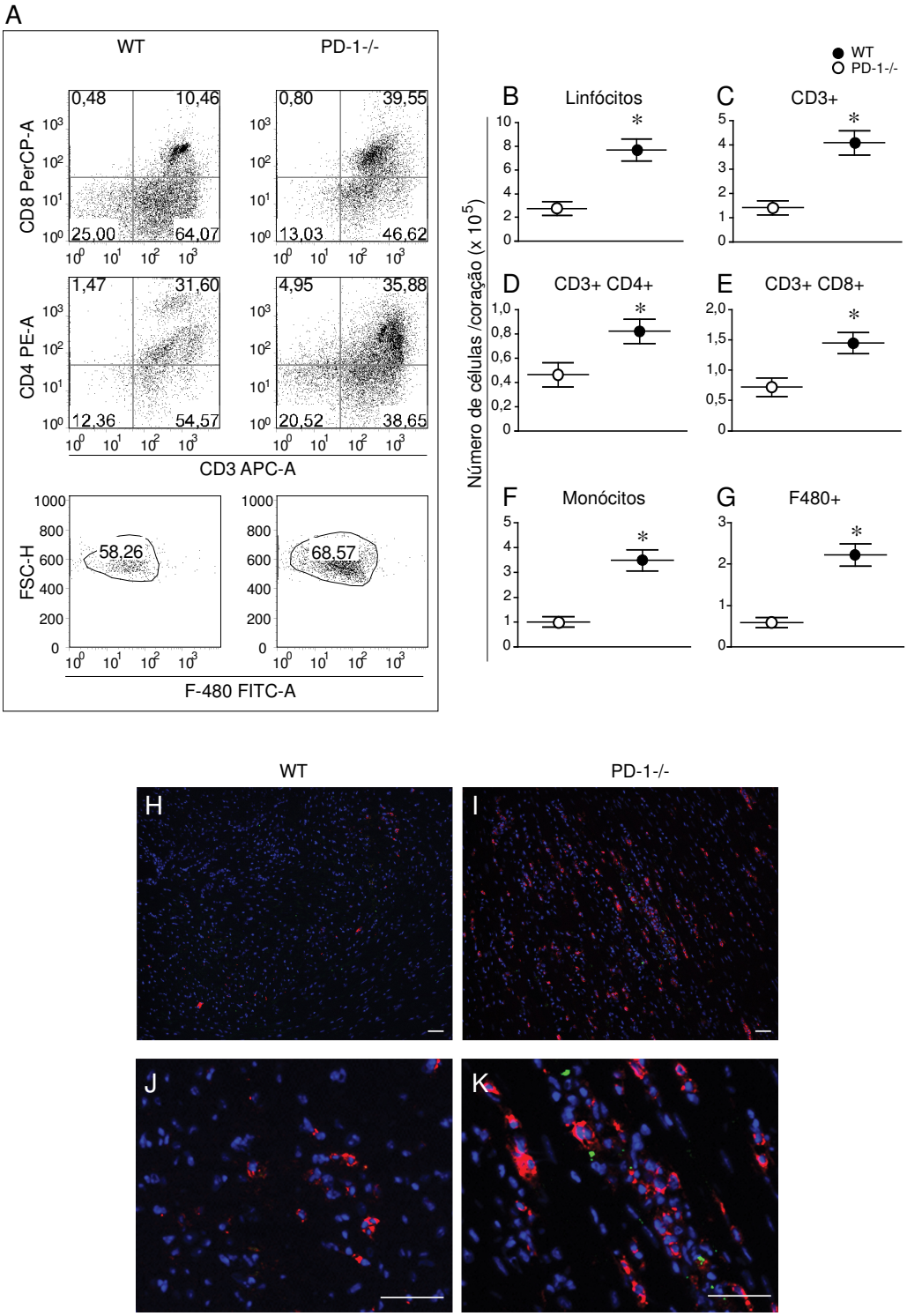

Figura 33. Quantificação e fenotipagem do infiltrado celular no miocárdio durante a infecção por *T. cruzi* em camundongos C57BL/6 normais ou PD-1$^{-/-}$. Análise de citometria de fluxo foi realizada em células extraídas do coração de camundongos no 21º dia da infecção. **A-G**, quantificação dos subtipos de leucócitos isolados do tecido cardíaco. **H,I**, microfotografias representativas da imunofluorescência para detecção de CD8 (vermelho) e pStat5 (verde) no tecido cardíaco. **J,K**, mostram em maior aumento essa detecção. As linhas brancas nas microfotografias representam 20 mm. Os asteriscos indicam significância estatística ($p<0,05$) no teste t-*student*.

Discussão

Os resultados obtidos neste estudo permitem estabelecer claramente que o co-receptor inibidor PD-1 participa no controle do processo inflamatório no coração de camundongos infectados por *T. cruzi*. O bloqueio da sinalização de PD-1 com anticorpos monoclonais induziu aumento significativo da inflamação miocárdica, quando comparada à inflamação observada no miocárdio dos camundongos que receberam IgG de rato. Tal participação depende preferencialmente da interação de PD-1 com o seu ligante PD-L1, mais do que com o PD-L2, visto que o aumento da inflamação foi mais intenso nos animais que receberam anticorpos anti-PD-1 ou anti-PD-L1.

PD-1 exerce um papel inibidor como molécula acessória na ativação de linfócitos durante o processo de apresentação antigênica (ZHA, BLANK *et al.*, 2004), razão pela qual tem sido associado à manutenção da tolerância imunológica frente a autoantígenos, determinando seu papel crucial durante o desenvolvimento de respostas autoimunes (KOBAYASHI, KAWANO *et al.*, 2005; OKAZAKI e HONJO, 2006), ou na evasão da resposta imune por parte de tumores (HE, ZHANG *et al.*, 2004; GHEBEH, BARHOUSH *et al.*, 2008) e patógenos intracelulares como vírus (KEIR, BUTTE *et al.*, 2008; LUKENS, CRUISE *et al.*, 2008). De fato, há um aumento dos transcritos de PD-1 e da expressão de seu ligante PD-L1 no tecido cardíaco após a infecção, coincidindo com o período de máxima inflamação (nos dias 14º e 20º após a infecção). A expressão destas moléculas nas células inflamatórias que migram para o coração de camundongos infectados, bem como no próprio tecido cardíaco também foi verificada.

A modulação da expressão de PD-L1 observada em células cardíacas indica que essas células também participam ativamente na regulação deste processo, como é o caso da expressão de PD-L1 em células não imunológicas durante processos inflamatórios órgão-específicos (MAZANET e HUGHES, 2002).

A presença desses ligantes principalmente na região de interação de células T e B, no baço sugere sua participação no processo de apresentação antigênica. Nós também evidenciamos que há modulação da expressão de PD-1 e seus ligantes praticamente em todas as populações de linfócitos analisadas durante a infecção, o que pode indicar um papel importante dessa via de sinalização no controle da resposta imune ao parasito. No 20º dia da infecção, houve redução da expressão de PD-1

em células do baço de animais infectados, possivelmente em associação com o controle do parasitismo circulante no período avaliado, ou com reorganização estrutural decorrente do processo de ativação dos linfócitos. Recentemente, uma nova subpopulação de células T *helper* caracterizada pela expressão de PD-1tem sido descrita. Essas células têm sido denominadas T *helper* foliculares (TFH), estão presentes nos folículos de células B e desempenham um papel crucial na indução da resposta imune mediada por essas células (CHTANOVA, TANGYE *et al.*, 2004). Apesar de não termos encontrado expressão significativa de PD-1 no interior dos folículos de células B em animais sadios ou infectados, nós evidenciamos aumento significativo da frequência dessas células nos baços de camundongos PD-$1^{-/-}$. Isso pode estar relacionado com a expressão já descrita de PD-1 nesse tipo celular (FINGER, PU *et al.*, 1997), indicando que PD-1 exerce um papel regulador na resposta imune humoral. Contudo, nós não avaliamos essa reposta e o efeito dessa regulação precisa ser estudado durante a infecção experimental por *T. cruzi*.

Nós mostramos que camundongos PD-$1^{-/-}$ apresentam uma intensa esplenomegalia, com diminuição da freqüência de células Treg e aumento substancial de células B. Assim, podemos sugerir que PD-1 participa na manutenção da homeostase linfocitária durante a infecção. Uma das principais características da resposta imune durante a infecção por *T. cruzi* em humanos e camundongos é a indução de uma grande expansão de linfócitos, envolvendo todas as subpopulações de linfócitos T e B (MINOPRIO, COUTINHO *et al.*, 1986; MINOPRIO, EISEN *et al.*, 1986). O processo de linfoproliferação excessiva é persistente (D'IMPERIO LIMA, EISEN *et al.*, 1986) e caracterizado pela ativação policlonal em massa (MINOPRIO, BURLEN *et al.*, 1988; WEBSTER, ONIONS *et al.*, 1997). Esse processo necessariamente leva a um detrimento da capacidade homeostática do sistema imune durante a infecção.

Neste estudo, a especificidade clonal dos linfócitos em camundongos PD-$1^{-/-}$ e a correlação das células expressando PD-1 com o seu estado de ativação não foram avaliadas e constituem questões necessárias de serem respondidas futuramente. A análise de clones específicos de células $CD3^{+}CD8^{+}$ que respondem a antígenos de *T. cruzi* e/ou próprios, assim como de células B produtoras de autoanticorpos nesses camundongos permitiria verificar a participação do PD-1 no mecanismo que mantém a tolerância humoral frente a autoantígenos no coração, por exemplo.

A apoptose é uma forma de morte celular programada, através de um processo cuidadosamente regulado, que tem papel fundamental no desenvolvimento de tecidos e na organogênese (WHITE, 1996), bem como na patogênese de diferentes doenças (THOMPSON, 1995). *T. cruzi* induz apoptose em linfócitos $CD3^+CD4^+$ e $CD3^+CD8^+$ do baço (LOPES, DA VEIGA *et al.*, 1995), que pode ser devida à intensa ativação policlonal que ocorre durante a fase aguda da infecção, uma vez que a ativação de linfócitos via TCR desencadeia processos que culminam com a apoptose dessas células (DOSREIS, FONSECA *et al.*, 1995). No presente estudo, nós mostramos que a deficiência de PD-1 induz diminuição da apoptose em linfócitos do baço, o que pode estar relacionado com maior tempo de sobrevida dessas células favorecendo um melhor controle do parasitismo. Porém, a redução da taxa de apoptose nessas células pode desencadear fenômenos de autoimunidade, uma vez que células persistentemente ativadas poderiam levar a dano tecidual colateral.

A apoptose de linfócitos pode ser regulada através das moléculas coestimuladoras. De fato, A sinalização via CD28 aumenta a sobrevida dessas células principalmente por induzir aumento da produção de IL-2 junto com aumento da expressão de fatores intrínsecos que inibem a apoptose como Bcl-xL. Em contraste, a sinalização via CTLA-4 leva à morte de células previamente ativadas.

Durante a infecção por *T. cruzi* há participação das moléculas coestimuladoras. De fato, estudos anteriores evidenciaram a modulação da expressão de CD28 e CTLA-4 em linfócitos T ativados (DUTRA, MARTINS-FILHO *et al.*, 1996; GRISOTTO, D'IMPERIO LIMA *et al.*, 2001; MIYAHIRA, KATAE *et al.*, 2003; GRAEFE, JACOBS *et al.*, 2004; SOUZA, ROCHA *et al.*, 2007). Além disso, o bloqueio de CD28 ou CTLA-4, resulta em maior suscetibilidade e resistência à infecção, respectivamente, (MARTINS, CAMPANELLI *et al.*, 2004; MARTINS, TADOKORO *et al.*, 2004).

Neste trabalho nós mostramos evidências da indução da expressão da molécula co-inibidora PD-1 e de seu ligante PD-L1 em células da resposta imune inata como CD e macrófagos. Esses achados coincidem com as recentes publicações mostrando que *T. cruzi* é capaz de induzir CD tolerogênicas, que possuem baixa capacidade em ativar células T (PONCINI, ALBA SOTO *et al.*, 2008).

As CD delineiam a resposta de células T e determinam os desfechos iniciais, intermediários e tardios do reconhecimento imunológico. Elas podem facilitar a estimulação da resposta imune ou induzir tolerância. Esses desenlaces são determinados por sinais iniciais na ativação das células

dendríticas, como os desencadeados pelo reconhecimento específico de ligantes de receptores de tipo toll (TLR, "*toll-like receptors*"). De acordo com um estudo recente, sabe-se que a estimulação de CD através de ligantes de TLR3 como poly (I:C) induz uma redução na proliferação de células $CD3^+CD4^+$ *in vitro,* limitando a resposta adaptativa. Quando as células $CD3^+CD4^+$ foram cultivadas na presença de CD pré-incubadas com LPS, houve um rápido aumento de expressão de CD69 (marcador de ativação de linfócitos). Por outro lado, as células $CD4^+$ incubadas com CD expostas a poly (I:C) não apresentaram tal fenótipo de ativação e mostraram-se marcadamente suprimidas. Essa deficiência na proliferação das células $CD3^+CD4^+$foi dependente de contato, independente de IFN-α e foi resolvida pela adição de IL-2 recombinante à cultura, indicando a indução de anergia neste contexto. Essa anergia estava relacionada com a expressão de PD-1 nas células $CD3^+CD4^+$ e de PD-L1 nas células dendríticas, visto que a inibição dessas moléculas conseguiu restabelecer a proliferação dos linfócitos $CD3^+CD4^+$ (GROSCHEL, PIGGOTT *et al.*, 2008). CD aumentam a expressão dos ligantes de PD-1 quando ativadas (HOCHWELLER e ANDERTON, 2005). Como descrito, estes ligantes desempenham um papel crucial na geração de anergia e na regulação da resposta imune. De fato, *T. cruzi* é capaz de manipular ativamente a expressão de moléculas coestimuladoras na superfície de células dendríticas, tornando-as tolerogênicas (PONCINI, SOTO *et al.*, 2008), um mecanismo de evasão que tem sido relatado para outros patógenos intracelulares (FREEMAN, WHERRY *et al.*, 2006)

Neste estudo nós mostramos a indução da expressão de ligantes de PD-1 em CD por *T. cruzi*. Além disso, mostramos que o bloqueio desta sinalização leva a uma diminuição da carga parasitária no coração, apesar de um aumento transiente do parasitismo circulante. É altamente provável que *T. cruzi* module a expressão de PD-L1 e PD-L2 em outros tipos celulares que são alvos do parasitismo durante a infecção, como células musculares. Nós mostramos que as células do miocárdio apresentam expressão de PD-L1, a qual diminui após a infecção.

Além das células dendríticas, os macrófagos exercem funções importantes na resposta imune inata e na indução de uma resposta imune adaptativa protetora, como apresentação antigênica, produção de citocinas, fagocitose, migração e produção de espécies reativas de oxigênio e nitrogênio (GUTIERREZ, MARIANO *et al.*, 2007).

Nós mostramos que *T. cruzi* é capaz de induzir em macrófagos a expressão de PD-1, uma molécula relacionada com a regulação da resposta imune adaptativa e mais recentemente, da resposta imune inata. Ainda, nós mostramos que a deficiência de PD-1 leva a aumento da capacidade tripanocida de macrófagos, devido a uma ativação mais eficiente dessas células por IFN-γ, levando a um aumento da produção de NO *in vitro*. Esses resultados indicam que o PD-1 estaria controlando a sinalização intracelular por IFN-γ e provavelmente por TNF-α, necessários na ativação da produção de NO por fagócitos. Contudo, a atividade da arginase circulante está aumentada em períodos tardios da fase aguda da infecção e há aumento nos níveis dos transcritos para a enzima arginase 1 e NOS2 no miocárdio, em paralelo com a diminuição da produção de NO no soro, sugerindo que os mecanismos de regulação dessas duas enzimas são diferencialmente modulados pela infecção nesses animais.

Existem subpopulações de macrófagos que possuem capacidades distintas de gerar uma resposta imune frente a parasitos. Os macrófagos são classicamente ativados (M1) através da sinalização de duas citocinas: IFN-γ e TNF-α. A primeira citocina é produzida principalmente por células $CD3^+CD4^+$ do tipo Th1 ou de células $CD3^+CD8^+$. Já o TNF-α pode ser induzido na presença de produtos microbianos que agem como ligantes de receptores tipo t*oll* (TLR). Macrófagos ativados no microambiente citado produzem altas quantidades de NO, aumentam a expressão de MHC de classe II e a expressão de moléculas coestimuladoras como CD86. Além disso, essas células produzem altas quantidades das citocinas IL-1, IL-6 e TNF-α, que são cruciais na indução do processo inflamatório. Nós encontramos níveis aumentados de TNF-α no soro de animais $PD\text{-}1^{-/-}$, junto com uma produção aumentada de NO em macrófagos após incubação com IFN-γ

T. cruzi possui múltiplas estratégias que lhe permitem evadir a resposta imune gerada por macrófagos. Uma delas envolve o metabolismo da L-arginina, substrato crucial na síntese de NO pela NOS2. A cruzipaina é uma enzima de *T. cruzi* capaz de induzir diretamente aumento da atividade da arginase, levando á inibição competitiva da síntese de NO e ao favorecimento do crescimento do parasito (STEMPIN, GIORDANENGO *et al.*, 2002; STEMPIN, TANOS *et al.*, 2004). Esse aumento da atividade da arginase ocorre fisiologicamente quando os macrófagos são ativados no contexto de uma resposta de tipo Th2, com predomínio das citocinas IL-4 e IL-10 ou da citocina TGF-β, envolvida em mecanismos reguladores da resposta imune (RIBEIRO-GOMES, LOPES *et al.*, 2007). Assim, o

aumento da atividade da arginase constitui um mecanismo fisiológico de controle da atividade de macrófagos explorado ativamente por *T. cruzi*. O aumento observado nos níveis de transcritos para NOS2 e arginase 1 sugere que existem mecanismos postranscripcionais modulando a produção de NO nesses animais, visto que eles apresentam reduzidos níveis deste mediador na circulação. NO é um mediador importante da regulação da resposta imune. Porém, esse mediador é também crucial na destruição intracelular do parasito por fagócitos (VESPA, CUNHA *et al.*, 1994).

Neste trabalho também encontramos aumento dos níveis de transcritos para proteínas envolvidas no processo de autofagia: atg5 e Beclin-1 em tecido cardíaco de camundongos PD-1$^{-/-}$, o que pode estar em associação com maior a capacidade parasiticida observada nesses animais. *T. cruzi* pode evadir o processo de fagocitose para escapar da resposta imune (ANDREWS, 2002). Contudo, quando o parasito cai no ambiente intracitoplasmático, pode ser capturado pelos mecanismos de autofagia, um processo catabólico que envolve a degradação de componentes citoplasmáticos através da maquinaria lisossômica (COLOMBO, 2007). De fato, recentemente foi demonstrado que *T. cruzi* pode ativar essas vias, numa forma dependente de Atg5 e Beclin-1, duas proteínas envolvidas na formação do autofagossomo (ROMANO, ARBOIT *et al.*, 2009).

Apesar de não terem sido esclarecidos os mecanismos pelos quais essa regulação da resposta imune inata acontece, nós suspeitamos que o co-receptor PD-1 esteja agindo nas células fagocíticas de maneira análoga a como age nas células T. Assim, nós hipotetizamos que o receptor esteja acoplado de alguma maneira aos mecanismos reguladores da sinalização intracelular induzida pela ligação de citocinas pro inflamatórias aos seus receptores.

Neste estudo, foi possível estabelecer o curso da expressão de PD-1, PD-L1 e PD-L2 em células CD3$^+$CD4$^+$e CD3$^+$CD8$^+$presentes no baço de camundongos ao longo da fase aguda da infecção por *T. cruzi*. Esse estudo cinético permite correlacionar a expressão destas moléculas com outros parâmetros que têm sido bem estudados neste modelo, como o parasitismo circulante. De fato, há modulação da expressão dessas moléculas em linfócitos ao longo da fase aguda. Ainda, essas moléculas apresentaram expressões diferenciais nos subtipos de células T durante a infecção.

Foi observado que no inicio da infecção há um aumento transiente da expressão de PD-1 em células CD4$^+$ do baço até o 15º dia da infecção, onde o controle do parasitismo começa a ser alcançado

e o processo inflamatório no coração é desenvolvido. Após isso, os níveis de expressão de PD-1 retornam ao normal nas células CD4. Isso está em concordância com o papel da carga antigênica na função dos ligantes de PD-1 (LATCHMAN, WOOD *et al.*, 2001). Já em células $CD3^+CD4^+$da circulação há uma queda da expressão de PD-1 e PD-L1 no terceiro dia após a infecção, para aumentar posteriormente, inclusive acima dos níveis basais. É muito provável que isso seja uma conseqüência da ativação excessiva nessas células, levando a exaustão da sua capacidade efetora, como tem sido demonstrado com as células $CD3^+CD8^+$. A expressão de PD-L2 em células $CD3^+CD4^+$do sangue sofre uma diminuição no dia 14º, para aumentar posteriormente.

No caso do PD-L1, há uma redução drástica na sua expressão no 5º dia após a infecção, período no qual a presença do parasito começa a ser detectada na circulação. Após este período, a expressão de PD-L1 em células $CD4^+$ é mantida abaixo dos níveis normais. Esses dados sugerem fortemente que a proliferação exacerbada de linfócitos, na qual as células $CD4^+$ efetoras participam, pode estar associada com baixa expressão de PD-L1 nesse período, necessária para controlar a proliferação de linfócitos T (BROWN, DORFMAN *et al.*, 2003; SUGITA, USUI *et al.*, 2009) , como confirmado nos nossos experimentos de proliferação. Ainda, nas células $CD3^+CD4^+$ é verificado um aumento gradativo da frequência de expressão de PD-L2, que atinge níveis muito altos após o 20º dia da infecção, sugerindo sua participação ativa na regulação da resposta imune quando o parasitismo tem sido controlado. A nosso entender, a expressão desse ligante em células $CD3^+CD4^+$ não foi descrita em outro modelo e pouco se sabe sobre o mecanismo de sinalização intracelular que poderia ativar nas células $CD3^+CD4^+$. Contudo, a sua capacidade em ligar a PD-1, expresso em outras células T, sugere que possa existir uma interação entre as células T através dessa via. O significado fisiológico dessa possível interação é desconhecido e precisa ser estudado em detalhes.

A importância das células $CD3^+CD8^+$ na resistência à infecção por *T. cruzi* tem sido amplamente relatada (DOSREIS, 1997). Tais células predominam nos infiltrados inflamatórios em tecidos de hospedeiros infectados (HIGUCHI MDE, GUTIERREZ *et al.*, 1993; REIS, JONES *et al.*, 1993; SUN e TARLETON, 1993; HIGUCHI MDE, BENVENUTI *et al.*, 2003), têm atividade citotóxica *in vitro* sobre células infectadas (WIZEL, PALMIERI *et al.*, 1998) e na ausência das mesmas há aumento do parasitismo, menor resposta inflamatória e maior mortalidade (TARLETON, KOLLER *et al.*, 1992;

TARLETON, SUN *et al.*, 1994). Contudo, as células $CD3^+CD8^+$ encontradas em indivíduos infectados são pouco respondedoras frente a peptídeos do parasito (LAUCELLA, POSTAN *et al.*, 2004), indicando incapacidade em proteger adequadamente o hospedeiro.

A expressão de PD-1 em células $CD3^+CD8^+$ tem sido estudada em modelos de infecções virais crônicas, tumores e outros modelos de patógenos intracelulares onde foi determinado que PD-1 pode atuar no sentido de favorecer a evasão da resposta imune adaptativa por esses patógenos (BLANK e MACKENSEN, 2007). Diferente de células $CD3^+CD8^+$ recentemente ativadas, que possuem potente capacidade proliferativa e proteção frente a infecções virais e parasitárias crônicas, as células $CD3^+CD8^+$ exauridas apresentam alta expressão de PD-1 e perda da capacidade responsiva frente a vírus e tumores (KAUFMANN e WALKER, 2008).

Durante a infecção por *T. cruzi*, uma grande proporção de células $CD8^+$ apresentam características fenotípicas próprias do estado de exaustão, provavelmente devido à ativação policlonal induzida pelo parasito (ALBAREDA, LAUCELLA *et al.*, 2006). Neste estudo foi demonstrado que há modulação da expressão das moléculas PD-1, PD-L1 e PD-L2 por células $CD3^+CD8^+$ durante a fase aguda da infecção por *T. cruzi*. A frequência da expressão de PD-1 nestas células no baço é baixa (menos de 5%) e sofre poucas alterações no inicio da infecção. Contudo, numa fase tardia há diminuição significativa de sua expressão.

Estudos anteriores mostraram que a inibição da interação entre PD-1 e seus ligantes resulta em melhora das funções efetoras dessas células T exauridas, restabelecendo sua capacidade citotóxica e a produção de citocinas. Isso sugere que o estado de exaustão induzido pela ativação persistente dessas células é um processo reversível (BARBER, WHERRY *et al.*, 2006; FREEMAN, WHERRY *et al.*, 2006). Contudo, abordagens terapêuticas desse tipo poderiam levar ao desenvolvimento de uma resposta imune não controlada, em detrimento da sobrevida do hospedeiro, como aconteceu nos nossos experimentos com anticorpos bloqueadores de PD-1.

O sistema de regulação via PD-1 é importante para manutenção de tolerância periférica, aceitação de transplantes e evasão da resposta imune por tumores (DONG, STROME *et al.*, 2002; GAO, DEMIRCI e LI, 2003; GAO, DEMIRCI, STROM *et al.*, 2003; STROME, DONG *et al.*, 2003). A perda da tolerância frente a antígenos próprios do miocárdio pode explicar a imunopatologia de

pacientes chagásicos, os quais desenvolvem anticorpos autorreativos contra componentes do coração, inicialmente denominados como anticorpos anti-EVI (endotélio, vasos e interstício)(COSSIO, DIEZ *et al.*, 1974). PD-1 é crucial na manutenção dessa tolerância imunológica, uma vez que camundongos PD-1$^{-/-}$ desenvolvem anticorpos anti-troponina I (OKAZAKI, TANAKA *et al.*, 2003), resultando no aparecimento de um quadro de cardiomiopatia dilatada, muito similar ao observado em indivíduos infectados com *T. cruzi*. Não há duvidas que exista um paralelo entre estes dois fatos, levando-nos a supor que possívelmente anticorpos contra componentes cardíacos, produzidos devido a uma perda da tolerância, poderiam predispor para o dano cardíaco encontrado em aproximadamente 30% dos pacientes infectados.

Troponina I é uma proteína do sistema contráctil que faz parte do complexo troponina de miócitos. Esta molécula se liga à actina presente em mioligamentos finos e sua função é a de manter fixo o complexo troponina-tropomiosina. Existem três subunidades de troponina I, sendo sua presença restrita a alguns tecidos: TnI1 e TnI2, em músculo esquelético; e a TnI3 (cTnI), exclusiva de músculo cardíaco. Essa especificidade a converteu em um marcador diagnóstico de extrema sensibilidade na detecção de danos miocárdicos mínimos, permitindo o diagnóstico precoce de infarto miocárdico (ANTMAN, TANASIJEVIC *et al.*, 1996). Além disso, é mais fácil induzir autoimunidade frente à troponina I cardíaca do que frente à outras proteínas cardíacas (GOSER, ANDRASSY *et al.*, 2006). Dados recentes demonstram um papel central do PD-1 na manutenção da tolerância frente a este antígeno em camundongos (OKAZAKI, TANAKA *et al.*, 2003).

A presença de anticorpos anti-troponina I, demonstrada neste estudo no soro de camundongos infectados com *T. cruzi,* pode ter importância na patogênese da cardiopatia induzida por este parasito. Contudo, essa participação ainda necessita ser confirmada. A pesar que neste estudo não é mostrado um vínculo direto entre a sinalização por PD-1 e a existência de tal autorreatividade, nem a relevância fisiopatológica da presença desses autoanticorpos, nós mostramos que existem altos níveis deste anticorpo na circulação de camundongos C57BL/6 ainda durante a fase aguda da infecção. Tais autoanticorpos têm sido descritos como cruciais na patogenia da miocardite autoimune experimental induzida pelo coxsackievirus B3 (LATVA-HIRVELA, KYTO *et al.*, 2009). Ainda, foi demonstrado por outros autores, que a imunização com troponina I cardíaca e não com troponina T, é capaz de

induzir uma forte resposta inflamatória miocárdica, com a presença de autoimunidade frente à miosina. A intensidade dessa resposta autoimune foi dependente do *background* genético do hospedeiro, indicando que diferenças nos mecanismos homeostáticos da resposta imune possam estar envolvidos no desenlace final após a exposição do sistema imune a esse autoantígeno cardíaco (KAYA, KATUS *et al.*, 2009). Esses dados sugerem que a análise de polimorfismos genéticos de PD-1 e suas possíveis associações com formas clínicas mais agressivas da doença de Chagas, poderia permitir a detecção precoce de pessoas com maior risco de um agravamento das manifestações clínicas e um desenlace catastrófico da infecção.

Estudos anteriores têm demonstrado a existência de imunidade humoral (LEON, GODSEL *et al.*, 2001) e celular (GIRONES, CARRASCO-MARIN *et al.*, 2007) frente a vários autoantígenos presentes no coração (ENGMAN e LEON, 2002) porém, não exclusivos do miocárdio, como a miosina (RIZZO, CUNHA-NETO *et al.*, 1989), durante a infecção por *T. cruzi*. Contudo estudos posteriores falharam em demonstrar um papel patogênico crucial desses anticorpos na geração da miocardite Chagásica (LEON, WANG *et al.*, 2003). No entanto, há modulação da resposta imunológica frente a esses antígenos após o tratamento com agentes antiparasitários (HYLAND, LEON *et al.*, 2007). A presença destes autoanticorpos na circulação de animais infectados (e não nos animais sadios) certamente é um fator relevante, provavelmente agravante da miocardite induzida pelo parasito, durante a fase aguda e muito provavelmente também na fase crônica. Contudo, a evidência definitiva da relevância patogênica destes anticorpos virá de experimentos induzindo eficientemente tolerância imunológica frente a este antígeno previamente à infecção experimental, de maneira análoga aos experimentos realizados anteriormente por outros pesquisadores com miosina (LEON, WANG *et al.*, 2003), uma proteína cardíaca que apresenta mimetismo com antígenos do parasito (CUNHA-NETO, BILATE *et al.*, 2006). É provável que os animais tolerizados frente à troponina I cardíaca apresentem redução na patologia cardíaca ou controle da mesma. Ainda, é provável que esse controle dependa da expressão de PD-1 e PD-L1. Esses experimentos estão sendo realizados atualmente no nosso laboratório.

Assim, nós propomos que durante o dano miocárdico devido à inflamação, há liberação de antígenos próprios, como a troponina I. O desencadeamento de uma resposta imune prejudicial contra

esse tipo de antígenos estaria sendo evitado, entre outros mecanismos, pela expressão de PD-L1 nas células musculares. No entanto, devido à intensa ativação dos leucócitos presentes no tecido, junto com a diminuição na expressão deste ligante, as condições favoreceriam um fenômeno de perda da tolerância.

A análise da produção das citocinas por linfócitos presentes no sangue dos camundongos infectados mostrou que há um predomínio de células expressando PD-1 produzindo citocinas e que linfócitos $CD4^+$ expressam IFN-γ de maneira predominante, enquanto que as células $CD8^+$ são as principais produtoras de IL-17 entre as células analisadas. Ainda, nos mostramos que isso também acontece em linfócitos retirados do coração. Esses resultados acrescentam novos dados sobre o fenótipo das células produtoras de citocinas durante a infecção, aos reportados por estudos anteriores, que mostraram um predomínio de células produtoras de IFN-γ e TNF-α no tecido cardíaco de animais infectados (CUNHA-NETO, RIZZO *et al.*, 1998) e sugerem um papel da expressão de PD-1 na imunopatologia da infecção por *T. cruzi*.

A pesar de mostrar que a deficiência de PD-1 ou o seu bloqueio com anticorpos induzem aumento da resposta inflamatória, somente o tratamento com anticorpos permitiu verificar a correlação dessa resposta aumentada, com a diminuição da carga parasitária no tecido. No caso dos animais $PD\text{-}1^{-/-}$, não foi possível observar diferenças no parasitismo tecidual. Esse fenômeno poderia ser explicado pelo efeito do anticorpo, já que foi usada a molécula da imunoglobulina inteira para tratar os animais.

A imunopatogênese da miocardite produzida durante a infecção por *T cruzi* continua tendo muitos pontos a serem esclarecidos. A perda da tolerância periférica a antígenos cardíacos é uma das hipóteses estudadas, sendo que o mecanismo de como estaria sendo induzida essa alteração não é conhecido. A ativação dos linfócitos é um processo altamente regulado e o resultado final (ativação ou anergia) depende de uma grande serie de fatores, incluindo o balanço entre a expressão das moléculas estimuladoras e inibidoras e um dos principais determinantes. Entre esses fatores, a presença de receptores inibitórios (como PD-1) nos linfócitos, assim como de ligantes para esses receptores (PD-L1 e PD-L2) nas APCs são especialmente importantes. PD-1 é um receptor inibitório de linfócitos, que está associado a controle da tolerância periférica, particularmente no tecido cardíaco, pois ocorre desenvolvimento de miocardite autoimune na sua ausência (NISHIMURA, OKAZAKI *et al.*, 2001). A

presença de PD-1 aumentado na inflamação cardíaca pode ser o resultado de um mecanismo de manutenção da tolerância periférica, que de alguma maneira resulta afetado, diante do desenvolvimento de miocardite induzida por *T. cruzi*.

Conclusões

O presente estudo mostrou que o co-receptor PD-1 e seus ligantes são expressos em células da imunidade inata e adaptativa durante a fase aguda da infecção experimental por *T. cruzi*. Essa via de sinalização participa na regulação da resposta imune no miocárdio por diversos mecanismos, sendo os principais a produção de NO por fagócitos, a indução de apoptose de linfócitos e a manutenção de células Treg. Finalmente, evidenciamos a perda da tolerância frente a um antígeno próprio do miocárdio: a troponina I, a qual sabe-se que é governada pela expressão de PD-1.

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Anexos

The up-regulation of negative co-receptor PD-1 in the heart-infiltrating lymphocytes controls *Trypanosoma cruzi*-induced myocarditis.

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***Trypanosoma cruzi* infection is characterized by intense myocarditis which is poorly regulated and causes severe cardiac dysfunction and chronic cardiomyopathy. During activation of T lymphocytes, co-receptors expressed on T cells surface can trigger a stimulatory or inhibitory signal after binding to their ligands in APC cells. The development of protective immune response largely depends on adequate balance in positive and negative signaling through these molecules. *T.cruzi* is able to modulate the expression of co-receptors in lymphocytes after infection. A recent described negative T cell signaling pathway mediated by the PD-1 co-receptor and its ligands: PD-L1 and PD-L2 is associated with impaired T cell function and persistent infections. We aimed to study the role of PD-1 signaling during *T.cruzi*-induced acute myocarditis in mice. Flow cytometry assays showed that PD-1 and its ligands are strongly up-regulated in lymphocytes and APC in response to *T.cruzi* infection *in vivo* and *in vitro*. Interestingly, the lymphocytes infiltrating the myocardium also exhibited a high expression of these molecules. Signaling through this co-receptor is required for the control of myocardial inflammation during the acute phase of *T.cruzi* infection, as demonstrated by increased inflammatory cardiac response in mice treated with blocking antibodies against PD-1 and PD-L1 and to a lesser extent PD-L2, compared to the control group (rat IgG). Transiently increased parasitemia but no effect on tissue parasitism was also observed after the PD-1 blockade. These results demonstrate the participation of PD-1 signaling pathway in the control of acute myocarditis induced by *T.cruzi*. The implications of this novel regulatory mechanism for immune pathogenesis of Chagas disease are discussed.**

Keywords: Infections- Parasitic-Protozoan, Costimulation, Inflammation

Introduction

Chagas disease, the most important cause of acquired cardiomyopathy in Latin America, results as a consequence of the intricate interaction between the human immune system and the hemoflagellate prokaryote *Trypanosoma cruzi*. In the natural infection, the flagellated forms in the feces of infected haematophagous insects of the *Triatominae* subfamily, invade the host through skin lesions or intact mucosa.

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The parasite then proliferates intracellularly and disseminates systemically from the site of inoculation, causing an inflammatory reaction of variable intensity, along with splenomegaly, cardiac parasitism and myocarditis, which is largely associated with morbidity. Most frequently, a chronic asymptomatic infection is established, which eventually leads to dilated cardiomyopathy and heart failure as well as esophageal or intestinal dilatations (1).

The mechanisms underlying this silent and relentless infection and heart pathology remain elusive despite several decades of research. However, it is well established that a T-cell-mediated immune response is essential to control the parasite replication during the acute phase of the infection (2). The cytokines IFN-γ, IL-12 and TNF-α strengthen the activation of innate and adaptive effector immune responses, resulting in a more efficient killing of the parasite, and leading to a strong inflammatory response in several tissues where parasites replicate, including the myocardium.

On the other hand, the cytokines IL-10 and TGF-β counter regulate the inflammatory process and indirectly favor parasite persistence within infected host cells because they are potent inhibitors of NO production and other IFN-γ and IL-12 mediated cell activation processes (3, 4).

The extent of this regulation seems to be crucial for the final outcome of the illness, since patients with the indeterminate (asymptomatic) form of the disease appear to have a more controlled immune response (5) than patients with advanced stages of infection.

The intensity and efficiency of a protective immune response can be determined by the balanced expression of co-stimulatory and co-inhibitory molecules during priming of T cells by antigen presenting cells (APC).

CD28 and ICOS are co-stimulatory receptors, while CTLA-4 and PD-1 exert a negative function to prevent excessive T cell activation (6).

Programmed death receptor-1 (PD-1) is a recently described member of the CD28 family mainly expressed on activated T, B and myeloid lineage cells (7). It signalizes through two ligands: PD-L1 (8) and PD-L2 (9), which are expressed by an upward gamma of cell types including myeloid, lymphoid and non-lymphoid cells. Engagement of PD-1 with any of its two ligands inhibits the activation of T cells and the production of cytokines, especially IL-2 and IFN-□ (6). Mice deficient in PD-1 can suffer spontaneous autoimmune disorders including autoimmune dilated cardiomyopathy (10). In addition, the blockade of PD-1 engagement accelerates autoimmune disorders (11, 12) and graft-versus-host disease (13), suggesting that PD-1 is critical for the maintenance of peripheral tolerance (14). PD-L1 expression has also been demonstrated as a mechanism for immune evasion by viruses (15) and tumors. Recent evidence suggests that avoiding signaling through co-inhibitory molecules could constitute promising immunotherapeutic strategies in anti-viral and anti-tumor cellular immunity (16). Indeed, the blockade of the PD-1 pathway is helpful to antiviral and anti-tumoral cellular immunity (15, 16). Moreover, the treatment with anti-CTLA-4 antibodies improves the cellular immune response against T.cruzi (17). It seems therefore reasonable to **hypothesize that** PD-1 may participate in the cell mediated immune response and in the maintenance of cardiac tolerance during an infection with T.cruzi. Here we show that this infection induces increased expression of PD-1 signaling molecules by immune system cells, and that this signaling pathway is involved in the control of acute myocarditis, as its inhibition leads to increased cardiac inflammation.

Materials and Methods

Mice, antibodies and treatments. C57BL/6 mice aged 6-8 weeks, obtained from the local animal facilities (FMRP-USP,) were treated with anti-PD-1 (RPM1-14), anti-PD-L1 (MIH5), anti-PD-L2 (TY25) or normal rat IgG starting from 48 hours before infection and lasting for two weeks. During this period, i.p. injections containing 250 micrograms of antibody were administered to mice every 72 hours. Four to five hearts from mice at days 14, 20 and 25 days após a infecção were collected for histology, immunohistochemistry, PCR, and ELISA studies. Non-infected age-matched mice were used as controls. For survival studies, two independent (anti-PD-1-treated and rat IgG-treated) groups of 8 animals were followed until 35 days post-infection. Mice were cared for according to the local guidelines on ethics in animal experiments.

Parasites and experimental infection. Mice were infected (i.p.) with 1,000 bloodstream forms of T.cruzi (Y strain) obtained from intermediary strain-matched mice. Parasitemia levels were evaluated in 5 µl of blood drawn from the tail. Before infection of intermediary mice, parasites were grown and purified from the monkey kidney fibroblast cell line LLC-MK2 (ATCC).

Histological analysis. Quantification of heart tissue inflammation was assessed by counting inflammatory cells stereologically in four representative nonconsecutive hematoxylin-eosin stained sections (thickness of 5 µm) per organ (n=3) at days 14, 20 and 25 post-infection. A Zeiss Integrationsplatte II eyepiece reticule (Oberkochen, Germany), along with an Olympus BHS microscope (magnification of ×400) were used, as previously described (18).

Immunohistochemistry. Hearts of 4 infected mice were removed, embedded in tissue-freezing medium Tissue-Tek (Sakura Finetek, Torrance, CA, USA), and stored in liquid N2. Serial 5-7 µm-thick sections were fixed in cold acetone and subjected to immunoperoxidase staining using antibodies against PD-1 (RPMI-14), PD-L1 (MIH5) and PD-L2 (TY25). Antibodies against PD-1 (RPMI-14), PD-L1 (MIH5) and PD-L2 (TY25) were produced in the labs of Dr T. Honjo and M. Azuma.

Cytokines/chemokines production measurement. The concentrations of cytokines and chemokines in heart homogenates or serum were measured by ELISA. The following ELISA sets were used: IFN-γ (BD Biosciences, San Jose, CA), TNF-α (R&D Systems, Minneapolis MN), MCP-1, MIP-1α and RANTES (All Peprotech, Rocky Hill, NJ), according to manufacturers' instructions. The reaction was revealed with peroxidase-conjugated streptavidin (Sigma) followed by the substrate mixture containing hydrogen peroxide and tetramethyl benzidine (TMB, Kirkegaard & Perry Laboratorie, Marylan) as a chromogen.

RNA extraction. Total RNA was extracted from homogenates of ventricular tissues of infected mice using the Trizol reagent (Invitrogen, Carlsbad, CA). Briefly, each organ was homogenized in Trizol, followed by addition of 0.2 ml of chloroform and

centrifugation at 12000 x g for 15 minutes. RNA was isolated from supernatants using the SV Total RNA Isolation System kit (Promega, Fitchburg, Madison). The purified RNA was eluted in 50µL of RNAse-free water, quantified in a spectrophotometer Biomate 3 (Thermo Spectronic, Waltham, MA), and its integrity evaluated in agarose 1.5 % gel.

gene	Primer	
β-actin	sense	AGC TGC GTT TTA CAC CCT TT
	antisense	AAG CCA TGC CAA TGT TGT CT
PD-1 mouse	sense	TTC AGG TTT ACC ACA AGC TGG
	antisense	TGA CAA TAG GAA ACC GGG AA
PD-L1 mouse	sense	GCT GAA GT CAA TGC CCC ATA
	antisense	TCC ACG GAA ATT CTC TGG TTG
PD-L2 mouse	sense	TTG TCG GTG TGA TTG GCT TC
	antisense	AAA AGG CAG CAC ACA GTT GC

Table1 Sequences of primers used in real-time PCR

cDNA synthesis, and real-time PCR. Complementary DNA (cDNA) was synthesized using 2 µg of RNA through a reverse transcriptase reaction, using ImProm-II reagents (Promega, Fitchburg, Madison), in a PTC 100 thermal cycler (MJ Research, Watertown, MA). The conditions of the reaction were as follows: 5 minutes at 70° C and 1 hour at 42° C, followed by refrigeration at 4° C. The total volume of the reaction was 25 µL, which was diluted 8 fold, reaching a total volume of 200 µL. Real-time PCRs were performed using the Platinum SYBR Green qPCR SuperMix UDG with ROX reagents (Invitrogen, Carlsbad, CA), from 5µL of diluted cDNA. cDNA samples obtained from mice belonging to different groups (not infected and infected at various time points) were amplified in the 7000 Sequence Detection Systems device (Applied Biosystems, Foster City, CA) using forward and reverse primers (sequences are listed in Table 1) that we designed with the Primer Express software (Applied Biosystems, Foster city, CA), according to nucleotide sequences available in the GenBank database. The expression of each mRNA was normalized to a constitutive mRNA (β-actin) by the ΔΔCt method as previously described (19).

Isolation and cytometry of inflammatory cells from cardiac tissues. Hearts collected from 5 mice at day 20 após a infecção were minced, pooled and incubated for 1h at 37°C with RPMI-1640, supplemented with $NaHCO_3$, penicillin/streptomycin/gentamycin and 0.05g/ml of liberase blendzyme CI (Roche, Basel). The organs were processed in a Medimachine (BD Biosciences), in PBS containing BSA 0.01%. After tissue digestion and washes, cell viability was assessed by trypan blue exclusion, counted in a hemocytometer and stained with the optimal dilution of each fluorescent labeled antibody. Phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against CD3, CD4, CD8, PD-1, PD-L1, PD-L2 and respective isotype controls were employed (BD Biosciences). Fluorocytometric analysis was performed in a FACScan apparatus along with the Cellquest software (both from BD Biosciences) and FloJo software (Tree Star, Ashland, OR).

Lymphocyte proliferation assays: Analysis of lymphocytes proliferation was performed by carboxy-fluorescein diacetate succinimidyl-ester (CFSE) staining. In brief, spleen derived leukocytes (1×10^7 cells/ml) were stained with 5 µmol/L CFSE (5 minutes, 37°C, darkness). Staining was stopped by adding complete culture medium, the cells were centrifuged (5 minutes, 300 ×g). Cell suspension was adjusted to $5x10^6$ cells/ml, and plated in a 96 well culture plate (Nunc) at 200ul/well, and then the cells treated with 1 microgram per well of each antibody and stimulated with ConA (2ug/ml) or *T. cruzi* antigen (10ng/ml) or leave with medium alone for 72h. Data analysis was performed by flow cytometer on a FACsCanto II apparatus (BD) using FACsDiva (BD) and FlowJo software (Tree Star) by setting a gate on the live cells on side scatter versus forward scatter dot plot and determining the expression of CFSE.

In vitro antigen presentation assays: The ability of totally differentiated bone marrow derived dendritic cells (BMDC, near 95% expressing CD11c on surface) to stimulate lymphocyte proliferation was evaluated by incubating both cells at a ratio of 5 lymphocytes to 1 DC in the presence of the same stimuli as in the proliferation assay. DCs were pre-incubated for 24h with anti PD-L1 or anti PD-L2, and lymphocytes were pre-incubated with antiPD-1, each case in separate wells. After 72h of culture under these conditions, the production of IFN-g was measured in supernatants by ELISA.

Statistic analysis. Data are expressed as the mean ± SEM. ANOVA followed by Student's t-test was used to determine the statistical significance of the observed differences between treated and control groups. The Kaplan-Meier method was used to compare survival times of the study groups. Differences were considered statistically significant when $p<0.05$. All analyses were performed using the PRISM 3.0 program (GraphPad Software, San Diego, CA).

Results

The infection with *T.cruzi* induces increased expression of PD-1 and its ligands in the heart. The expression of mRNA for PD-1 and its ligands were analyzed in cardiac tissue at days 14 and 20 após a infecção The results showed significantly increased levels of PD-1 mRNA on both examined time points (5 and ~3 fold, respectively). Regarding the levels of transcripts for PD-L1 and PD-L2, only those for the former was found to be significantly augmented on

day 20 após a infecção (Figure 1A). We further confirmed the presence of PD-1 and ligands by an immunohistochemistry of cardiac tissues. The results revealed marked expression of PD-1 only in cardiac tissue from infected mice. Marked expressions of PD-1, PD-L1and PD-L2 were observed in infected individuals at day 14 após a infecção, although a weak expression was also observed in normal mice. Furthermore, the expression of PD-1 and PD-L1 were largely in association with inflammatory reaction, which is more intense by day 14 and 20 após a infecção However, no differences were noticeable in the intensity of staining at these two após a infecção time points. These show that *T.cruzi* infection induces the expression of PD-1 and its ligands in the cardiac tissue (Figure 1B).

Modulation of PD-1 signaling pathway by *T.cruzi* infection. We next asked if *T.cruzi* infection up-regulates the expression of PD-1 and its ligands in blood leukocytes and spleen cells *in vivo*. The results showed that among PD-1 family molecules, PD-L1 is the one which showed the greatest expression level after infection with *T.cruzi* in spleen lymphocytes (Figure 2A). The infection leads to a gradual reduction in the expression of PD-L1 in circulating $CD4^+$T lymphocytes and the frequency of cells expressing this molecule on the surface is increased along the first three weeks of infection. PD-1 and PD-L2 were also modulated in lymphocytes and macrophages from the spleen, and the patterns of expression for these two molecules were similar along the observed periods of time in the spleen. Interestingly, PD-1 and PD-L2 were strongly up-regulated by day 7 após a infecção, where the most prominent pathological issue is systemic parasite proliferation.

On blood cells, a transient reduction was observed in the frequency of expression of PD-1 and ligands in both $CD4^+$and $CD8^+$T lymphocytes at a very early phase of the infection (3 d.após a infecção) which increased later on day 21 após a infecção (Figure 2B) These results demonstrated for the first time, that *T.cruzi* is able to modulate the levels of expression of a signaling pathway which is known to inhibit the activation of effector T cells and that have been involved in the immune evasion by tumors and viruses (20).

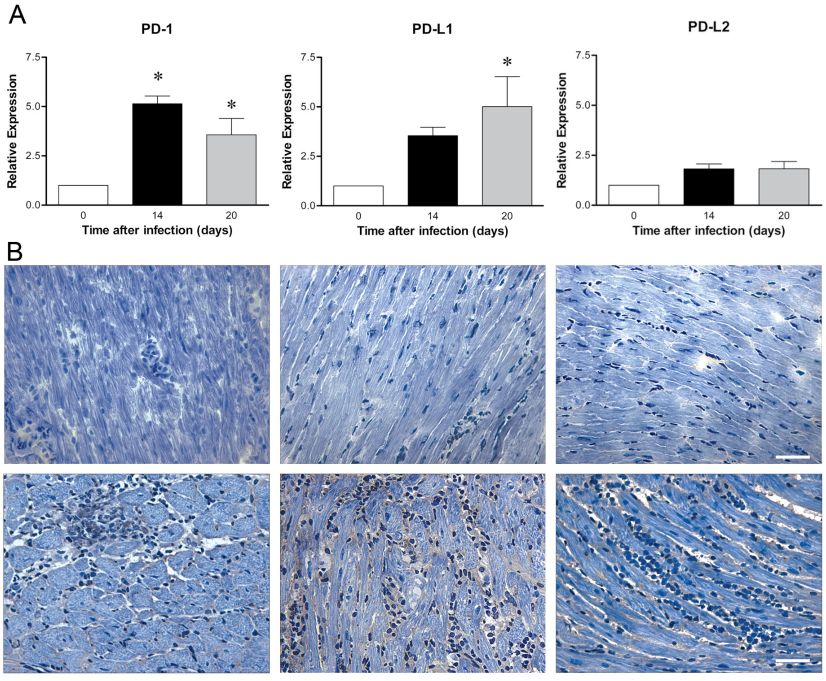

Figure 1. Expression of PD-1, PD-L1 and PD-L2 in the cardiac tissue during acute *T.cruzi* infection. The mRNA transcripts levels for PD-1, PD-L1 and PD-L2 (A) were measured by real-time-PCR in cardiac tissue from control mice (NI) or at day 14 or 20 p.i. In B, representative microphotographs of the immunohistochemistry for detection of PD-1 and both ligands in heart tissue from not infected mice (up) and 14 d.p.i. (down). Scale bar = 50 um. Data are representative from 3 independent experiments.

Heart-infiltrating $CD4^+$and $CD8^+$cells express PD-1. Since altered PD-1 signaling plays a pathogenic role in the loss of peripheral tolerance, particularly in the heart, we focused our study on the cardiac tissue of infected mice. Therefore, the expression of PD-1 was determined in $CD4^+$and $CD8^+$T cells infiltrating the heart tissue on day 20 após a infecção (Figure 3). The results showed that 88.03% and 99.34% of $CD4^+$T cells express PD-1 and PD-L1. The frequency of $CD8^+$T cells expressing PD-1 and PD-L1 was 98.62% and 98.62% respectively, while the frequency of PD-L2 positive $CD8^+$and in $CD4^+$T cells was 79.29% *vs.* 49.68%, respectively. These results clearly show that PD-1 and ligands are expressed in lymphocytes found in the heart of *T. cruzi*-infected mice.

PD-1 blockade induces increased acute myocarditis and reduced survival in mice infected with *T.cruzi* infection. Aiming to test if PD-1 signaling is indeed involved in the maintenance of cardiac tissue tolerance during *T.cruzi* infection *in vivo*, we treated *T.cruzi* infected mice with blocking antibodies against each one of the PD-1 related molecules (PD-1, PD-L1 and PD-L2), and studied the heart histopathology and the local production of cytokines and chemokines. The results showed that the blockade of PD-1 receptor (and to a lesser extent of its ligands) leads to increased myocarditis scores (figure 4A and B). This increased inflammation was observed neither in the skeletal muscle nor in the hepatic tissues. The quantification of inflammatory scores also demonstrated that PD-1 and PD-L1 blockade increased the cardiac inflammation on day 14 após a infecção (Figure 4B), while all treatments induced increased expression of iNOS in the cardiac tissue observable at the same time point (Figure 4C). These results suggest a role for the PD-1 pathway in regulating the inflammatory response at the myocardium during *T.cruzi* infection.

To further explore if the treatments induce increased myocarditis we assayed for detection of the levels of the pro-inflammatory cytokine TNF-α and the chemokines MIP-1α, RANTES and MCP-1 in heart homogenates from mice belonging to each experimental group. Increased levels of TNF-α, MIP-1α, RANTES, and MCP-1 were found at day 14 após a infecção in the cardiac tissues of animals that received the anti-PD-1 antibody (Figure 5). Furthermore, the levels of RANTES were still increased at day 20 após a infecção (data not shown). Also of note is that the expression of mRNA for CCR5 which is a receptor for these chemo-attractants involved in the Th-1 biased immune response was higher in the group that received anti-PD-L1 treatment (data not shown). These data demonstrate that PD-1 signaling pathway is one of the factors involved in the regulation of the Th-1 biased immune response observed in the cardiac tissue during the acute phase of the infection with *T.cruzi.*

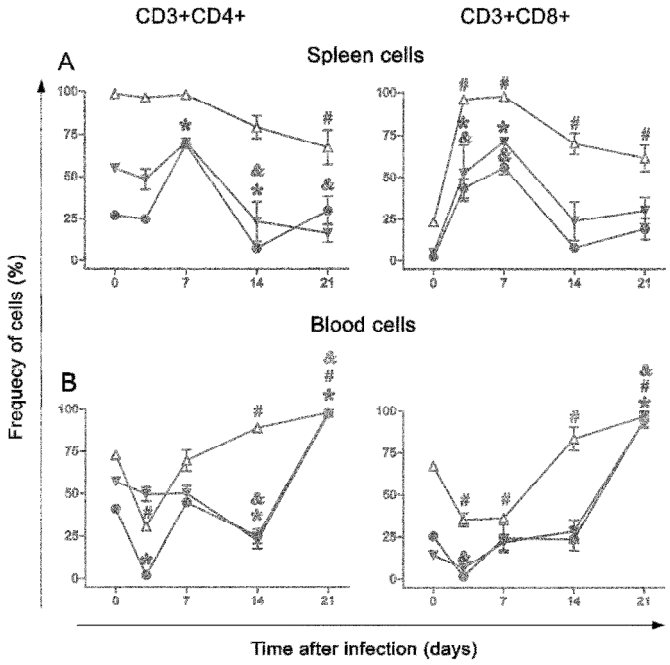

Figure 2. Expression of PD-1, PD-L1 and PD-L2 in the surface of lymphocytes isolated from spleen (A) and blood (B) of *naive* mice or after various p.i. times with *T.cruzi.* Cytometry analyses were performed within the gate of lymphocytes. The graphs represent the relative frequency +/- SEM for each marker (PD-1: ●, PD-L1: Δ, PD-L2:▼) in the indicated cell population (n=3). Symbols indicate $p < 0.05$ compared to 0.

PD-1 pathway is involved in the mechanism that mediates host resistance to the acute phase of *T.cruzi* infection.

The increased myocardial inflammation observed in *T.cruzi* infected mice after the blockade of PD-1 signaling molecules suggests that it could be also involved in the resistance to T.cruzi infection. To address this issue, we measured the production of the cytokines IFN-□ (Figure 6A), TNF-□ (Figure 6B), as well as the levels of nitric oxide (NO, not shown) in the serum of mice belonging to each experimental group. These molecules are known to be important for the control of the proliferation of the parasite by immune cells (21). In agreement with the increased levels of pro-inflammatory factors observed in cardiac tissues after the blockade of PD-1, the results showed that mice treated with anti-PD-1 produce higher levels of IFN-γ and TNF-α than the control mice ($p < 0.05$, Figure 6A and B) at day 14 após a infecção Furthermore, this increased production of IFN-γ (but not of TNF-α) was maintained at day 20 após a infecção These results point to a role for PD-1 in the

control of the systemic inflammatory response during this infection. To determinate the role of PD-1 in the control of parasite proliferation and resistance to the acute phase of the infection, we studied the parasitemia and mortality of mice belonging to each experimental group. Surprisingly, the results demonstrated that despite having a significantly increased peak of parasitemia at day 9 após a infecção when compared to the control group

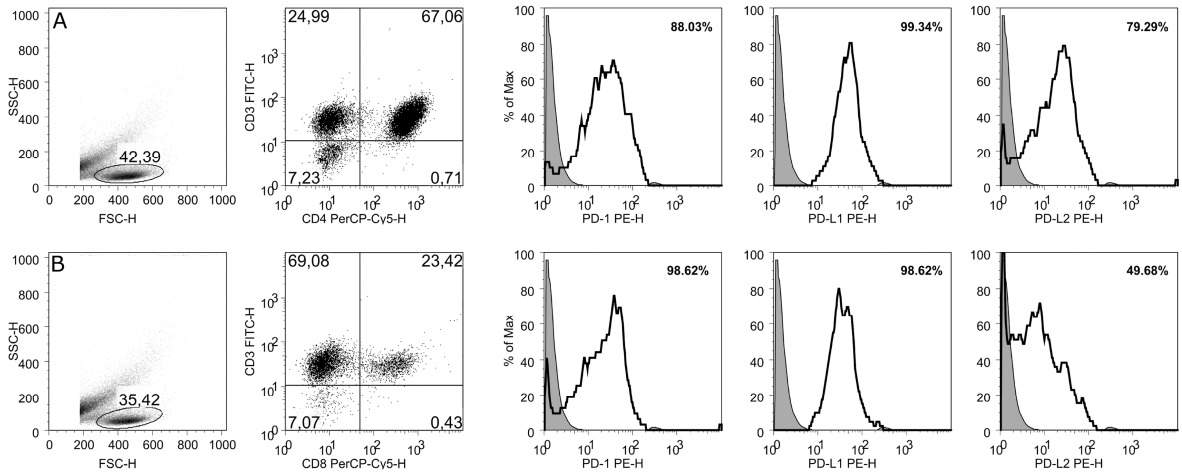

Figure 3. Expression of PD-1, PD-L1 and PD-L2 in TCD4$^+$(A) and CD8$^+$(B) lymphocytes infiltrating cardiac tissue of mice infected with *T.cruzi*. The expression of PD-1, PD-L1, and PD-L2 in CD3CD4$^+$or CD3CD8$^+$T cells isolated from heart tissues of infected mice were assessed by flow cytometry. Numeric values in each region of histograms represent the frequency of positive cells for each marker (%) the gray histograms represents control iso-type. Data are representative from 3 independent experiments.

($p<0.05$, figure 6C), the group of mice treated with anti-PD-1 were comparable to the other groups in controlling the parasite burden by day 17 após a infecção However, these mice also exhibited a significantly decreased survival rate when compared to the control group ($p< 0.05$ Figure 6D), as they started to die by day 15 após a infecção, and by day 35 após a infecção more than 90% of them had succumbed, while in the control group the survival rate at this date is more than 55% ($p< 0.05$ Figure 6D). These findings are in accordance with that of parasitism of the cardiac tissues (not shown) and suggest that during *T.cruzi* infection, PD-1 has a role in the control of myocardial inflammation **PD-1 Blockade induces increased proliferative response and cytokines production by lymphocytes**. To definitively demonstrate if PD-1 blockade induces increased inflammation by increasing lymphocyte proliferation and cytokine production, CFSE stained naïve or primed spleen cells were analyzed for proliferation after being incubated with anti PD-1, anti PD-L1 or anti PD-L2. In accordance with the existent data published by other authors, our results showed that the blockade of PD-1 and ligands lead to increased lymphocyte proliferation of naïve cells (Figure 7A and B) and in primed cells after stimulation with *T. cruzi* antigen (Figure 7C). Moreover, the blockade of PD1, PD-L1 or PD-L2 in an in vitro antigen presentation assay induced an increased production of IFN-□ by primed cells after additional stimulation with T. cruzi antigen (Figure 8). These data constitute definitive evidence that the blockade of PD-1 as well as its ligands induces increased immune response by lymphocytes.

but not in the response against the parasite itself.

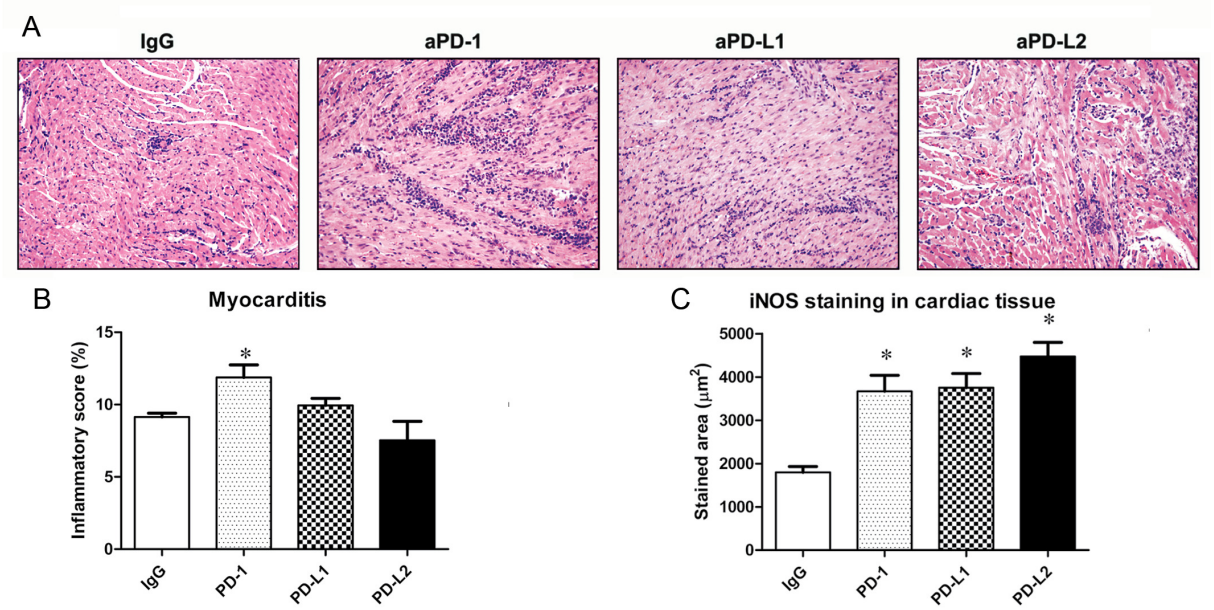

Figure 4. Morphometric analysis of the inflammatory response of *T.cruzi* infected mice after treatment with blocking antibodies against PD-1, PD-L1 and ligands. The histopathology of myocardium (A) of infected mice were studied by conventional HE staining. B represents the measurement of inflammatory infiltrate on cardiac tissues on day 14 após a infecção C shows the morphometric analysis of the extent of immune reactivity for iNOS in the cardiac tissues of mice belonging to each experimental group. (n=3).* p<0,05 compared to control group (IgG). Data are representative from 3 independent experiments.

Discussion

In this study we show that PD-1 and its ligands are effectively involved in the pathogenesis of T.cruzi infection in mice. Specifically, we demonstrate that infection with *T.cruzi* is able to up-regulate the expression of PD-1 by $CD8^{+}$T cells migrating to the myocardium during the acute phase of this systemic infection. This signaling pathway plays an inhibitory role in myocardial inflammation, without participating substantially in resistance to parasite proliferation.

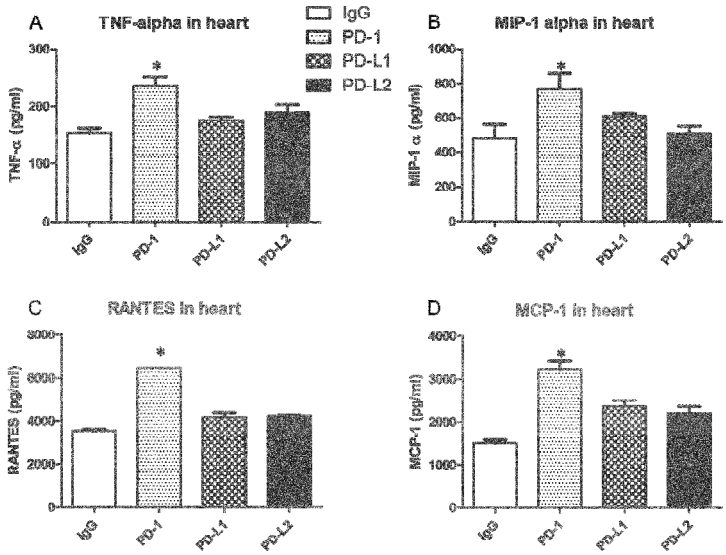

Figure 5 Production of cytokines and chemokines in inflamed myocardium after treatment with blocking antibodies against PD-1, PD-L1 and ligands. Levels of the cytokine TNF-□ (A), and the chemokines MIP-1□ (B), RANTES (C) and MCP-1 (D), measured by ELISA on heart homogenates of mice belonging to each experimental group at day 14 após a infecção (n=3).* p<0,05 compared to control group (IgG). Data are representative from 3 independent experiments.

One of the mechanisms by which the inflammatory response induced by *T.cruzi* can be controlled is through regulating the activation of effector T cell responses. Two signals are required for an optimal T cell response. The first one is mediated by the engagement of T cell receptor (TCR) to MHC-antigen complex in antigen-presenting cells (APC), and the second one is through T cell co-receptors.

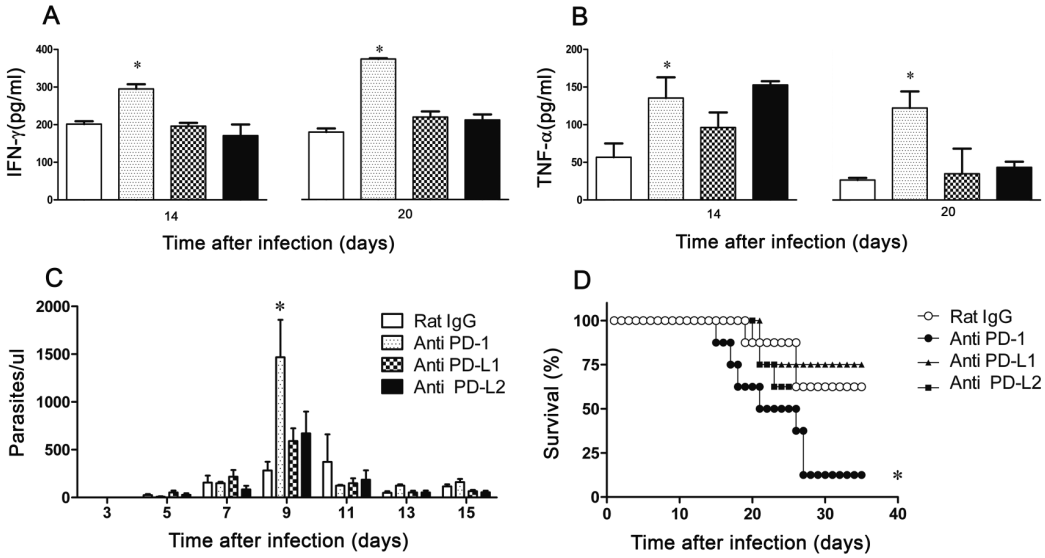

Figure 6. Effects of the blockade of PD-1 and ligands on the production of cytokines and resistance to infection of *T.cruzi*-infected mice after treatment with blocking antibodies against PD-1, PD-L1 and ligands. Levels of the cytokines IFN-[illegible] and TNF-[illegible] (B), measured by ELISA on sera of mice belonging to each experimental group at days 14 and 20 após a infecção (n=3). C shows the parasitemia profile of mice belonging to each experimental group (n=5). D, survival study. * p<0,05 compared to control group (IgG). Data are representative from 3 independent experiments.

Co-receptors can trigger a stimulatory or inhibitory signal. CD28 is a co-stimulatory molecule which plays a critical role for efficient $CD4^+$T cell activation in response to *T.cruzi* infection in mice (22, 23). *T.cruzi* induces increased expression of the co-stimulatory ligands CD80 and CD86 in macrophages.

However, a recent study showed that the parasite is also able to exert immune evasion by down-regulating these co-stimulatory ligands and MHC molecules in dendritic cells, which is a key cell type during T cell activation (24). In addition, it has been proposed that differential expression levels of these co-stimulator molecules induced by the parasite can dictate the intensity of the inflammatory response, leading to different clinical forms of the disease (24, 25). These data demonstrate that *T.cruzi* does modulate the expression of co-stimulators, which may dictate the intensity of the immune response against the parasite at the level of antigen presentation, where dendritic cells play a key role.

In addition to positive co-stimulators, the increased expression of mRNA for PD-1 and its ligands, as well as the presence of the proteins by immunohistochemistry that we had confirmed in infected but not in control cardiac tissue clearly indicate that this signaling pathway is involved in the modulation of the inflammatory response against this parasite. In fact, it has been demonstrated that *T.cruzi* has the ability to induce the expression of negative co-receptors and ligands in T cells and monocytes. For example, the negative co-stimulator CTLA-4 is up-regulated in lymphocytes after infection with *T.cruzi in vivo* and *in vitro*, and the blockade of this inhibitory signaling pathway lead to increased inflammation and decreased tissue parasitism (17). Up-regulation of the co-inhibitor PD-1 in T cells constitutes a novel additional mechanism by which *T.cruzi* could evade the effector T cell response.

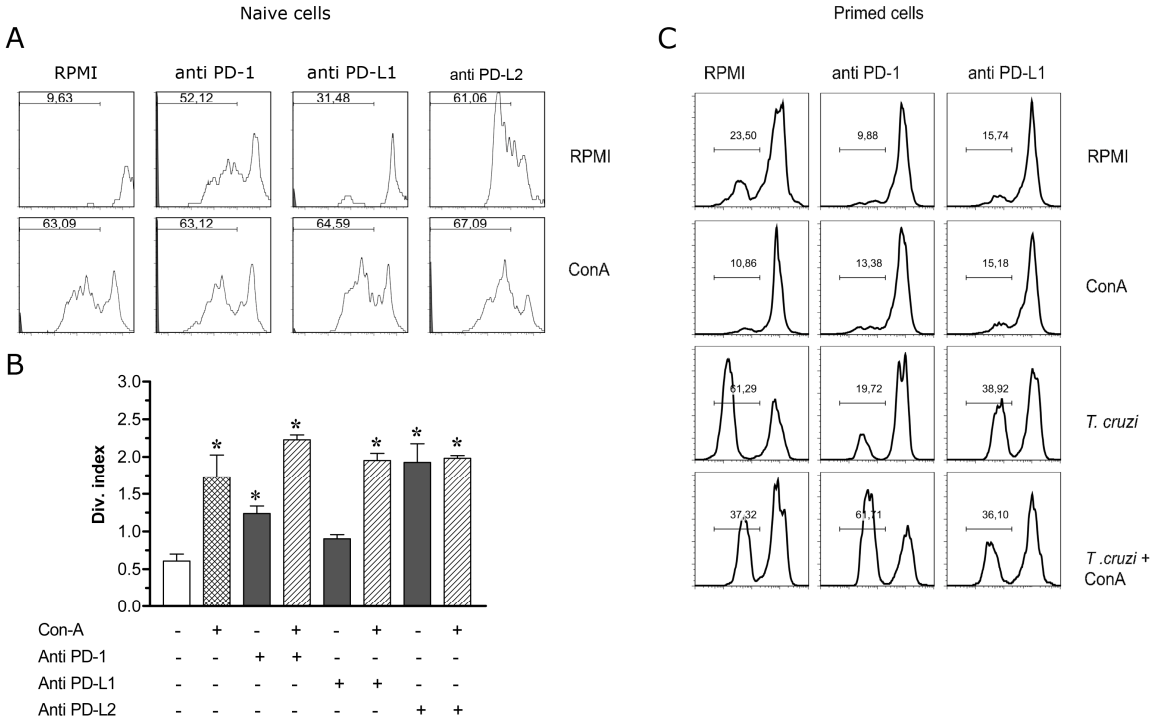

Figure 7. Effect of the blockade of PD-1 pathway on proliferation of spleen derived lymphocytes. CFSE stained naïve (A and B) or *T. cruzi*-primed cells (C) were incubated with the indicated stimuli during 72h in the presence of each blocking antibody or medium alone. * p<0,05 (n=3). Data are representative from 2 independent experiments.

The modulation of the expression of PD-1, PD-L1 and to a lesser extend PD-L2 by circulating and spleen cells *in vivo* became clear by the time course cytometry study performed on T cells from these tissues. In all cases the most elevated expression frequency was that of PD-L1, which is known to participate in the immune evasion by other microorganisms (20). We also demonstrate that $TCD4^+$and $TCD8^+$cells migrating into cardiac tissues express high levels of PD-1 and related molecules. Previous reports showed that PD-1 and its ligands are induced in immune cells late after activation, having been considered as markers of cell exhaustion during chronic infections and tumor immune evasion (20). The T cells that are present at the myocardium are certainly activated cells, as they migrate in response to the parasite's presence at the myocardium (26) and they are known to exhibit a phenotype of activated cells, producing massive amounts of cytokines, predominantly of type 1 response (27). This constitutes a novel mechanism by which the immune system regulates the inflammatory response in cardiac tissues during this parasitic infection.

An intriguing issue is how, despite such increased expression of these negative co-receptors the normal host still develops intense myocardial inflammation and controls the parasite burden. In fact, we believe that it is the balanced expressions and signaling through positive and negative co-receptors are what determine the final outcome in terms of the intensity and type of effector response. The pathogenic role of collateral destruction of cardiac tissue during this inflammatory reaction is also undeniable. It is mediated by cellular and soluble components of immune response, which is poorly regulated by classic immune regulatory mechanisms. For instance, regulatory cells do not play a role in the modulation of this inflammatory response (28, 29). Certainly, the effector lymphocytes that are initially recruited in response to the presence of the parasite in the myocardium must have a specificity directed toward parasite antigens. However, the antigenic specificity of T cells present at myocardium during this infection has been largely discussed, and it has been suggested that these cells may present an altered tolerance, leading to self-damage in an autoimmune fashion. These could be due to activated T cells being directed to self-antigens through cross-reactivity between host and parasite (30-33), or because an altered peripheral tolerance induced by the pathogen.

Thus, it is possible that the myocardial inflammatory response occurs as a consequence of an altered balance in the expression profile of PD-L1 by the cardiomyocytes themselves. It could involve some not yet described mechanism of altered tolerance, as PD-L1 is known to preserve specifically the tolerance to cardiac troponine I, which is a protein that is exclusively expressed in cardiomyocytes.

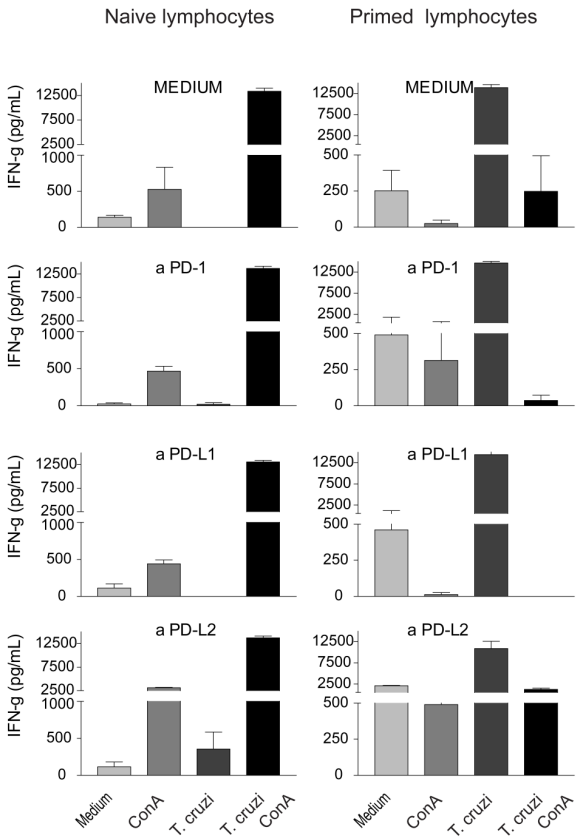

Figure8. Effect of the blockade of PD-1 pathway on IFN-γ production in in vitro antigen presentation assay. Levels of IFN-γ produced after co-culturing BMDC with naïve or primed spleen-derived lymphocytes at the ratio of 5 lymphocytes to each DC for 72h in the presence of the indicated stimuli. DC were previously incubated for 24 h with anti PD-L1 or anti PD-L2 antibodies, while in the case of PD-1 were the lymphocytes pre-incubated with the antibody. The production of IFN-γ was measured by ELISA of supernatant. * $p<0,05$ (n=3). Data are representative from 2 independent experiments.

Nevertheless, the regulatory role for PD-1 in acute myocarditis was demonstrated in this study by the treatment of mice with blocking antibodies against PD-1, PD-L1 or PD-L2. These treatments induced increased inflammation that was more remarkable in the cardiac but not in other tissues where the presence of the parasites has been described during this infection. In addition, it was demonstrated that the blockade of PD-1 and PD-L1 were more effective in inducing such worsened myocarditis. This data is in agreement with that from previous studies involving intracellular pathogens and suggest that PD-L1 have a more remarkable immune regulatory role than PD-L2 during this parasitic infection. However, further studies using knockout mice are required in order to determine the role of these signaling molecules during the chronic phase of infection, which is not easily tested by blocking antibodies.

The increased histological inflammatory score after blockade of PD-1 was confirmed by increased expression of iNOS in cardiac tissues, as well as increased levels of the proinflammatory cytokine TNF-a and chemokines MIP-1a, RANTES and MCP-1. Furthermore, we observed increased levels of mRNA for the Th-1 transcription factor T-bet, as well as for the chemokine receptor CCR5 in mice receiving the anti-PD-1 or anti-PD-L1 antibodies (data not shown). These data support the regulatory role for PD-1 signaling, mainly through the PD-L1 ligand in the infected myocardium during the acute phase of *T.cruzi* infection.

An interesting issue is the fact that these mice exhibited increased myocarditis but have not significantly altered tissue parasitism. Besides of a transient increased parasitemia in mice receiving anti-PD-1, no significant alterations were observed in systemic or cardiac (not shown) parasitism curves in mice after any blocking treatment. Indeed, anti-PD-1 treated mice were able to control the parasitemia burden, indicating that PD-1 could has a stimulatory role in the expanding phase of immune response, perhaps by acting in other ligand not yet described, or perhaps due to the effect of other regulatory mechanisms, mainly CTLA-4. Further studies designed in a way to isolate the PD-1 pathway (i.e. by using mice deficient in other negative co-receptors like CTL-A4) should rule out whether or not the parasite exploits the PD-1 signaling pathway as a mechanism of immune evasion.

However, PD-1 signaling is important for the survival of the infected host, as mice receiving the anti-PD-1 treatment die earlier than the control group or the mice receiving other treatments. We hypothesized that the increased mortality rate of these mice could be associated with the uncontrolled, intense myocardial inflammation observed as a consequence of the PD-1 blockade. This worsened response appears to be specific to the myocardial tissue, as not significant alterations were observed in other tissues. One possibility is that PD-1 blocking could cause increased lymphoproliferative response, which is in fact demonstrated *in vitro* in figures 7 and 8. However, it is possible that *in vivo*, the magnitude of this rise could be masked by the expression of the ligands by other non-immune cells, mainly in cardiac tissues.

In conclusion, our data demonstrated that PD-1 and ligands participate in *T.cruzi* induced myocarditis, and that they have a role in the regulation of the inflammatory immune response, suggesting that the understanding of the role of this signaling pathway on *T.cruzi* driven T cells could allow a more detailed knowledge of the pathogenesis of Chagas heart disease.

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Disclosures

he authors have no financial conflict of interest.

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The role of parasite persistence in pathogenesis of Chagas heart disease

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SUMMARY

Chagas disease (CD) is caused by the infection with the protozoan haemoflagellate Trypanosoma cruzi. *This disease is still a great menace to public health, and is largely neglected as it affects mostly the poorest populations of Latin America. Nonetheless, there are neither effective diagnostic markers nor therapeutic options to accurately detect and efficiently cure this chronic infection. In spite of the great advances in the knowledge of the biology of natural transmission, as well as the immunobiology of the host–parasite interaction, the understanding of the pathogenesis of CD remains largely elusive. In the recent decades, a controversy in the research community has developed about the relevance of parasite persistence or autoimmune phenomena in the development of chronic cardiac pathology. One of the most notable aspects of chronic CD is the progressive deterioration of cardiac function, derived mostly from structural derangement, as a consequence of the intense inflammatory process. Here we review the evidence supporting the multifactorial nature of Chagas heart disease comprising pathogen persistence and altered host immunoregulatory mechanisms.*

3***Keywords*** *xxxx,xxxxx*

INTRODUCTION

Trypanosoma cruzi is an intracellular protozoan which causes Chagas disease (CD). Endemic to several regions in Latin America, this disease persists as the major infectious heart disease in the world (1,2). It is estimated that around 75 million people live in risk areas (3) and 13 million people are currently infected in Central and South America. The global incidence of the disease is considered to be 300 000 new cases per year.

Natural transmission of the disease occurs through faeces of the vector (a haematophagous bug belonging to the subfamily Triatominae, family Reduviidae), deposited near a skin lesion or mucosa (80–90%), via organ transplantation or blood transfusion (5–20%) or congenital transmission (0·5–8%) (4). Majority of infections occur during early childhood, and around 30% infected people develop chronic cardiac involvement, usually after decades of asymptomatic infection (5).

The interaction between human beings and vectors is favoured by several factors. For example, human invasion of the natural habitat where Triatominae insects are endemic, deforestation and even climatic phenomena can con-
4tribute to domestication of novel species of transmitter haematophages (6,7). These phenomena tend to occur in the poorest countries of Latin America.

The South Cone Initiative against CD, carried out as an attempt to control the transmission of the disease by eradicating the insects from residential settings, has had an impact on the natural transmission of the disease. As a result, it is estimated that the local annual incidence has fallen from ~800 000 new cases in the 1980s to ~200 000 today, and in some of these countries the disease was declared to be 'controlled' (8,9).

The maintenance of this trend depends on continued surveillance and interventions where necessary. However, CD is still a serious menace to public health in various countries (10,11). In fact, CD has been considered as one

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of the most neglected infectious diseases (1,12,13) mostly due to deficiencies in epidemiologic control, the persistence of some endemic foci, sporadic disease outbreaks, transmission through transplantation or by oral route, along with the absence of diagnostic and prognostic markers and the limited efficacy of the anti-parasitic drugs.

As mentioned above, the currently available therapeutic options for CD are limited. Most of the therapeutic measures are aimed at treating the consequences of disease such as cardiac failure. Specific antimicrobial therapy [trypanocidal therapy (TT), which consists mainly of nitrofurans and imidazoles], when established early during the acute phase of CD, can prevent progression to the chronic phase in 50–70% of the cases (14). Although a study showed reduced parasitism, inflammation and conduction disturbances after benznidazole administration to mice chronically infected with *T. cruzi* (15), there is inconsistent clinical evidence supporting the use of TT in chronic chagasic cardiomyopathy (CCC) or in the indeterminate phase of the disease (16,17), mainly because most of the cases are detected at advanced stages of the disease, and because of the scarcity of the parasite at these stages. However, ongoing studies are exploring if TT does offer therapeutic advantage in chronic patients (15,18).

During the acute phase of the infection, an exacerbated immune response is commonly observed in the myocardium of infected individuals, leading to collateral damage which in extreme cases may lead to a systemic inflammatory response and death. At advanced stages of the disease, additional pathological changes appear, including the development of conduction disturbances, dysautonomia, cardiomegaly, fibrosis, thinning of the ventricular wall and microvascular damage. The main challenge in the understanding of immunopathology during *T. cruzi*-induced myocarditis is why, despite a robust immune response in the myocardium during the acute phase of infection, the parasite is not completely eliminated from tissues (19), being able to persist chronically.

HISTORIC REMARKS

Almost a century ago, Carlos Chagas described the general pathologic features of American trypanosomiasis, characterized its main clinical features and described the aetiological agent and major form of transmission. Such level of comprehension reached by a single researcher in the pathogenesis of a disease constitutes an exceptional event in the history of medicine.

By then, Chagas was established in Lassance and involved in governmental public health attempts to eradicate malaria and yellow fever from Rio de Janeiro, where both diseases were prevalent. While he was working there, a local railway engineer told him about the existence of a haematophagous bug which dropped from the ceilings of huts onto the faces of people while they were asleep. They were known as 'barbeiros' (barbers) or 'chupança' (kissing bugs). Chagas began to speculate that these bugs might act as hosts for a microorganism and identified in them a flagellate parasites. He soon demonstrated that this previously unrecognized parasite (later named *Trypanosoma cruzi*) was able to infect mammals (20).

Chagas searched for evidence of animal infection with this parasite and found it in the bloodstream of a domestic cat. Shortly afterwards, he was asked to see a 2-year-old girl named Berenice who owned the infected cat, and who fell ill with fever. He was not only able to demonstrate the same organism in her blood in the acute phase of the illness, but also noted that it was cleared as she recovered.

In the next few years he described the most important chronic features of the disease, including the cardiac, gastrointestinal and neurological manifestations, which were endemic in the region. In 1911, he described the congenital transmission of the infection. He received many honours and awards for this work (21).

After almost two decades of being forgotten, several remarkable discoveries were made on the epidemiology of CD. Of note, the Argentinean researcher Salvador Mazza suggested in 1936 that the disease could be acquired through transfusions (22), and then Dias and Pellegrino described in 1947 that the burden of the disease could be controlled by elimination of the vector using insecticides (23). However, it was only in the early 1990s that the real magnitude of the disease was recognized, leading to the formulation of public health policies to exclude infected blood donors and to eradicate the vector from the houses. As mentioned before, these strategies have proved to be extremely efficient in the control of disease burden. [For a detailed description on the history of CD, see Ref. (24).]

ACUTE IMMUNE RESPONSE IN THE MYOCARDIUM

Currently, there is no doubt that chagasic cardiomyopathy (CC) is the result of an inflammatory process. Indeed, one of the pathologic hallmarks of CC is the presence of a large number of inflammatory cells in the myocardium. Such infiltration of immune cells can be a response to the cardiac tropism of the parasite, or as a consequence of altered immunological tolerance; this remains controversial (25) but certainly depends on the genetic background of the host, as some people never develop heart disease despite infection.

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After invading a mammalian host, the replication of *T. cruzi* occurs within the cytoplasm of different cell types including macrophages, fibroblasts, skeletal and heart muscle cells, neuronal and epithelial cells. Particularly, the parasite has a tropism to myocardial cells and forms nests, a pathological feature of the acute disease (Figure 1). Immediately after parasitism, the recruitment of leucocytes to the tissue is triggered. The migration of immune cells depends on the local production of cytokines and chemokines, as well as the upregulation of expression of their receptors and adhesion molecules (26,27). In addition, these inflammatory cells have to cross the extracellular matrix, in a process that is orchestrated by the production and activity of extracellular matrix metalloproteinases (MMP) (28). We recently demonstrated that *T. cruzi* induces increased MMP activities in the cardiac tissue, and that this increased activity is associated with cellular inflammation as well as mortality during the acute phase of the experimental infection (29).

Host resistance during experimental CD is dependent on both innate and acquired immunity, requiring the combined effects of a number of cell types, including NK cells (30), $CD8^+$ (31,32) and $CD4^+$ (33,34) T cells, as well as antibodies produced by B cells (35).

Cytokines play key roles in regulating both parasite replication and immune responses in infected animals. It has been demonstrated that the cytokines IFN-γ and TNF-α are involved in the protective response to *T. cruzi* (36–40). IFN-γ is synthesized shortly after infection, mainly by NK cells, in response to IL-12 and TNF-α (41). However, the $CD4^+$ and $CD8^+$ T cells also produce IFN-γ during *T. cruzi* infection (34). In concert with TNF-α, IFN-γ leads to the activation of inducible nitric oxide synthase (iNOS) (40,42), the enzyme that catalyses nitric oxide (NO) synthesis by macrophages and inhibits parasite replication(40,43).

A predominant Th1 reaction is central to the control of *T. cruzi.* The treatment of *T. cruzi*-infected mice with an mAb to IFN-γ results in exacerbation of parasitaemia and mortality (44–46). In contrast, the regulatory cytokines IL-4, IL-10 and TGF-β have been associated with susceptibility to *T. cruzi* infection by inhibiting the effects of IFN-γ and macrophage activation (47,48).

Among the factors characterizing the immune response elicited by *T. cruzi* infection, it has been described that chagasic patients have increased frequencies of activated lymphocytes in the blood (49). *Trypanosoma cruzi*-activated leucocytes produce large amounts of IFN-γ, IL-12 and TNF-α (42,47,48,50), as well as NO (51) and extracellular MMP (29). Additionally, they exhibit low production of anti-inflammatory cytokines like IL-4 or IL-10 (52,53). It is noteworthy that overproduction of cytokines and

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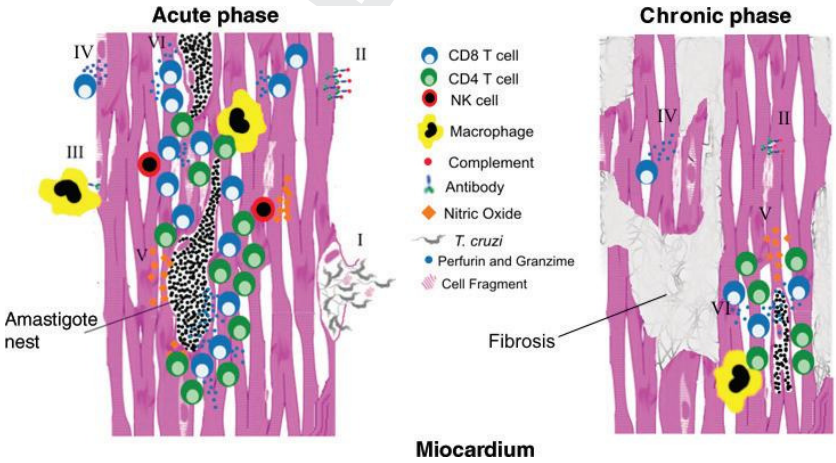

Figure 1 Immunopathogenesis of Chagas heart disease. The tissue damage in Chagas heart disease starts with multiplication of *Trypanosoma cruzi* which leads to cardiomyocyte destruction (I). This starts a specific immune response against the parasite but also involves the release of cell components leading to bystander activation, whereby immune responses are generated against host cellular components (I). This bystander activation and molecular mimicry between parasite and host antigens generate autoantibodies (II and III) and autoreactive T cells (IV). The autoantibodies can generate lesions mediating complement activity (II) and opzonizing tissue for macrophage activation (III). Autoreactive $CD8^+$ T cells recognize self-antigens and destroy cardiac tissue (IV). Cytolytic T lymphocytes not only destroy infected cells but also contribute to the destruction of adjacent cells (VI). Tissue injury leads to TNF-α production, resulting in nitric oxide production. *Trypanosoma cruzi* infection also induces nitric oxide production directly, which is responsible for the generation of extensive cardiac damage derived from oxidative stress (V). The immunopathological process continues during the chronic phase of infection. Despite lower parasite levels at this stage small and progressive intrafascicular inflammatory focuses continue to develop for years, ending in fibrosis and the development of dilated cardiomyopathy.

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enhanced expression of chemokine receptors induce a massive migration of inflammatory cells to the myocardium and other organs (27,36,53–56). From these data, it is possible to infer that a host imbalance in immune response, along with the parasite's ability to trigger a polyclonal immune stimulation, does play a central role in the maintenance of such persistent lymphocyte activation (57,58).

In addition, the acute and chronic phases of *T. cruzi* infection are characterized by extensive polyclonal activation of T and B lymphocytes (59). Several studies have demonstrated that $CD8^+$ cells are a predominant cell population in the cardiac tissue of chronic chagasic patients (60–62). Besides their cytolytic activities, $CD8^+$ T cells also contribute to immune responses to *T. cruzi* and immunopathogenesis (63) by producing cytokines such as IFN-γ and IL-10 (50,64).

Regulatory T cells

The recent identification of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) as a natural mechanism of control of the immune response has stimulated research interest and the development of therapeutic strategies for autoimmune and inflammatory diseases. The presence of Tregs in an inflammatory site is generally regarded as contributing to pathogen persistence, because it maintains an attenuated immune response against infectious agents (65).

These cells modulate the immune response, suppressing the proliferation of co-cultured $CD4^+CD25^-$ T cells and inhibiting both induction and effector functions of autoreactive T cells (65) through co-inhibitory molecules (CTLA-4) and cytokines (IL-10, TGF-β, IL-35) (66,67). TGF-β has been implicated as a mediator of immunosuppression, inhibiting IL-2 receptor induction (68), thymocyte proliferation (69), B-cell proliferation and differentiation (70), IFN-γ-induced class II antigen expression (71), cytotoxic lymphocyte generation and lymphokine activated killer cells (72).

Recent studies suggest that Tregs may not be important in the modulation of the immune response during experimental *T. cruzi* infection. Despite migrating to the myocardium of the infected host, they may not participate actively in the control of the inflammatory response, as the blockade of the high-affinity IL-2 receptor CD25 (one of the molecular markers of Treg) did not significantly alter the local or systemic inflammatory response (73–75).

However, it has recently been demonstrated that the approach of blocking CD25 is not sufficient to rule out a role for Treg cells. In fact, we confirmed a limited role for CD25 signalling in controlling the inflammatory response during this protozoan infection. However, our data also suggest that agonistic signalling through glucocorticoid-induced tumour necrosis factor receptor (GITR, which regulates Treg activities) induces increased heart inflammation, parasite replication and host resistance against the infection. These data suggest that Treg cells are involved in the immunological balance during *T. cruzi* infection (74). Tregs are also able to migrate to the site of cardiac inflammatory lesions triggered by *T. cruzi*, and can suppress effector function of $CD4^+$ and $CD8^+$ T cells during infection (73,75,76). In addition, the cytokine TGF-β, which is produced by these cells, is involved in the pathogenesis of collagen deposition and fibrosis in the chronic phase of CD (77). Thus, similar to other parasitic agents (65), *T. cruzi* may evade the immune system and persist in host tissues by inducing an endogenous population of Tregs or exploiting host immunoregulatory mechanisms.

Th17 cells

For years, immune responses in inflammation were explained based on a dichotomy of cytokines that are produced. However, the Th1–Th2 paradigm has been reconsidered, as a novel lineage of effector $CD4^+$ T helper lymphocytes, Th17, which produces IL-17A and IL-17F, IL-21, IL-22 and TNF-α, has been described (78). Differentiation of naïve T cells into Th17 may be mediated by the combined effect of the transcriptional factors RORγt and RORα, which are dependent on activation of the signal transducer and activator of transcription STAT-3 and require the cytokines IL-1β, IL-6, IL-21, TGF-β, as well as the expression of the chemokine receptor CCR6 (79,80). IL-17 has pro-inflammatory properties and acts by inducing fibroblasts, endothelial cells, macrophages and epithelial cells to produce a range of inflammatory mediators, such as GM-CSF, IL-1, IL-6, TNF-α, iNOS, metalloproteinases and chemokines (CXCL1, CXCL2, CXCL8, CXCL10), leading to the recruitment of neutrophils and inflammation (81–83).

T-helper-17 cells have been linked to the pathogenesis of several inflammatory and autoimmune diseases such as multiple sclerosis, psoriasis, rheumatoid arthritis, colitis, autoimmune encephalitis (84) and some infectious diseases including schistosomiasis (85), toxoplasmosis and tuberculosis (86). Beyond Th17 cells, IL-17 is also produced by other cells types including $CD8^+$ T cells, γδ T cells, neutrophils, monocytes and NK cells (87).

We have recently evaluated the role of IL-17 during *T. cruzi* infection. *Trypanosoma cruzi*-infected mice were treated with anti-mouse IL-17 mAb, which resulted in premature mortality, reduction in cardiac parasitism, enhanced production of TNF-α, IFN-γ, chemokine and chemokine receptors, expression of type 1 response and

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increase in cardiac inflammatory infiltrates compared with control animals (unpublished data). Our results indicate that IL-17 may be important in the control of cardiac inflammation by playing a negative feedback role on production of TNF-α, IFN-γ and chemokines during experimental *T. cruzi* infection, modulating the cardiac immune pathology of CD.

Chemokines

Chemokines also play important roles in immune modulation, leucocyte activation, co-stimulation and differentiation during innate and adaptive immune responses (88–90). Human and murine macrophages as well as cardiomyocytes produce CCL-2, CCL-3 and CCL-5 after being infected with *T. cruzi* and respond to these chemokines *in vitro* (91) by increasing *T. cruzi* uptake, enhancing NO production and controlling parasite replication (56,92). The cytokines IFN-γ and TNF-α are essential for the production of CCL-5 and CCL-3, respectively (36).

Thus, it is possible that IFN-γ-induced chemokines produced in the cardiac tissue of *T. cruzi*-infected individuals could also participate in the control of parasitism. Indeed, one study has demonstrated the differential co-expression of chemokine receptors (CCR2, CCR3, CCR5, CXCR3 and CXCR4) and intracellular cytokines (IL-4, IL-10, TNF-α and IFN-γ) on $CD4^+$ and $CD8^+$ peripheral T cells from individuals with indeterminate (IND) or cardiac (CARD) clinical forms of CD after *in vitro* stimulation with *T. cruzi* antigens(53).

In addition, it has been observed that the percentage of $CD4^+$ and $CD8^+$ T cells co-expressing CCR5 and IFN-γ, CXCR3 and IFN-γ, and CXCR3 and TNF-α are higher in individuals classified with the cardiac than that with the indeterminate form of the disease. On the other hand, the percentage of $CD4^+$ or $CD8^+$ T cells co-expressing CCR3 and IL-10 or co-expressing CCR3 and IL-4 were lower in CARD individuals than in IND individuals (53). CCR5 and CXCR3 are important immunological markers of Th1 responses, while CCR3 and CCR4 are associated with Th2 responses (93). These results indicate that a *T. cruzi*-exacerbated specific type 1 immune response developed by CARD chagasic patients is associated with the development of heart pathology.

The strong inflammatory activity of *T. cruzi* activated lymphocytes seems largely harmful rather than beneficial to the host. In order to minimize collateral damage from excessive inflammation, the initial migration of cells into the heart tissue should be controlled. However, the extent of this inflammatory response should also act to prevent parasite persistence (56). Thus, it is possible that in the absence of such adequate immune response regulation, the extent of myocardial inflammatory damage would be higher, as demonstrated by high levels of IFN-γ observed in patients with CC (50). Strategies targeting natural regulatory mechanisms in addition to antimicrobial treatment may be beneficial but have been not explored in research of chemotherapy against *T. cruzi* (94).

Cytokines

Various cell types and soluble molecules have been shown to participate in the control of infection as well as in the induction of pathogenesis during *T. cruzi* infection. Among them, the study of the profile of cytokines that are produced during infection may constitute a valuable key to the understanding of the immunopathological mechanisms involved in CC and controlling the immune response (54,95).

Some studies have demonstrated that peripheral lymphocytes as well as mononuclear cells infiltrating the heart tissue of patients with CC produce significantly more IFN-γ, TNF-α and IL-6, and less IL-4 and IL-10, than blood cells from infected asymptomatic individuals (50,96,97). In addition, peripheral mononuclear cells from patients with the indeterminate form produce high levels of IFN-γ and TNF-α associated with high IL-10 levels (53,97–100). On the other hand, other studies failed to demonstrate a correlation between production of inflammatory or anti-inflammatory cytokines and the clinical signs of CD (101). Thus, further studies are required to thoroughly establish if there is a correlation between the different cytokines present in cardiac tissue, lymphocyte culture or in serum during *T. cruzi* infection, with the outcome in terms of intensity of myocardial damage.

Immunoglobulins

During the sub-acute stages of experimental (102,103) and human (104) *T. cruzi* infection, the number of immunoglobulin-secreting cells in the spleen and peripheral lymph nodes is very high and the majority of activated B cells secrete nonspecific antibodies. These responses may lead to the potential expansion of self-specific clones that might be responsible for the killing of parasitized and nonparasitized cells.

In this phase of experimental mice infection, the B-cell polyclonal proliferation is characterized by a typical isotypic profile, IgG2a, and IgG2b in the spleen and lymph nodes (59). The molecular mimicry between parasite proteins and several self-antigens has been widely described. For example, autoantibodies against cardiac myosin, tubulin, actin, ribosomal P protein, keratin, β-adrenergic and muscarin receptors, myoglobulin, thyroglobulin,

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myelin and Cha antigen (105–109) have been detected in infected individuals. Recently, anti-beta (1)-adrenoceptor autoantibodies were found in sera of patients with idiopathic dilated cardiomyopathy. This finding could link the pathogenesis of chagasic dilated cardiomyopathy to the existence of these antibodies (110). However, the mechanisms implicated in the generation of such autoantibodies, as well as their role in cardiac pathology, remain to be established.

Antibodies are not only involved in the resistance to *T. cruzi* infection by opsonization, but may also mediate tissue destruction induced by complement (Figure 1). Several studies have indicated the importance of antibodies for host survival and parasite clearance (35,111,112). Indeed, mice deficient in B lymphocytes are hyper-susceptible to *T. cruzi* infection, demonstrating premature mortality rates and increasing parasitaemia (113).

The biological properties of IgG antibodies may be diverse, and have an important effect on the natural history of CD. The production of different IgG isotype subclasses is controlled by distinct cytokines. In chronic chagasic patients, IgG1 and IgG2 form about 90% of the IgG produced (114). Th1 cytokines (IL-12, IFN-γ, TNF-α) are responsible for the production of IgG1 and IgG3 isotype subclasses, while Th2 cytokines (IL-4 and IL-10) stimulate IgG2 production (115,116). IgG1 mediates lysis binding to complement C1q protein and macrophage phagocytosis, while IgG2 mediates immunity by nonphagocytic effector cells (117).

It has not been clearly defined whether a correlation between the different clinical forms of CD and the levels of IgG isotype subclasses exists. Several authors have tried to demonstrate the correlation between IgG isotype subclasses and the severity of the different clinical forms of CD in patient groups (118–120) and in experimental models (102,121). While some studies detected no differences between immunoglobulin levels among individuals with different clinical manifestations (118,120), others observed higher levels of IgG2 antibodies in the sera of patients with cardiac and gastrointestinal manifestations of disease (119,122).

IMMUNE EVASION STRATEGIES OF *TRYPANOSOMA CRUZI*

Trypanosoma cruzi has several strategies to evade the immune system, via induction of TGF-β production by immune cells, induction of lymphocyte apoptosis, transfer of sialidase to the cellular membrane, antigenic competition, acquired tolerance, as well as the possible blocking role of soluble antigens or circulating immune complexes. The release of excretory–secretory products which have potent immunosuppressive activity is a direct evasion mechanism.

Apoptosis in immune cells can be induced by factors such as the CD95 receptor ligand system (Fas–Fas-L) (123) and by cytokines such as TNF-α and IFN-γ (124). *Trypanosoma cruzi* induces apoptosis of lymphocytes by several mechanisms. For example, the high levels of NO induced *in vitro* and *in vivo* by *T. cruzi* lead to apoptotic cell death in many different cell types (125–127).

We showed that the induction of apoptosis by Fas–Fas-L interaction in *T. cruzi*-infected mice is dependent on NO and IFN-γ (126). Another strategy used by *T. cruzi* to induce lymphocyte apoptosis is mediated by trans-sialidase (TS). This enzyme transfers sialic acid from the microenvironment to the parasite's mucins which entirely cover the parasite surface (128). Sialylated mucins are involved in parasite protection against components of the complement cascade. TS from *T. cruzi* not only acts on the parasite surface but is also shed into the milieu, being detected in the blood during the infection. The enzyme persists in blood because of the presence of a C-terminal region named shed acute-phase antigen (SAPA) (129,130).

Trypanosoma cruzi strains that produce and secrete higher amounts of TS and induce 100% mortality in mice induce thymic involution and thymocyte depletion in infected mice (131). Natural or recombinant TS injected into naive mice induce apoptosis in the thymus and in lymph nodes (132–134). *Trypanosoma cruzi* TS induces polyclonal lymphocyte activation and subsequent hypergammaglobulinaemia. It is noteworthy, however, that this polyclonal lymphocytic response appears to be important for host survival, as sensitization of mice with small doses of *T. cruzi* TS renders the mice highly susceptible to *T. cruzi* infection (135).

Sialylated mucins also confer on the parasite the ability to specifically inhibit some components of the innate immune system, such as the complement-mediated lysis. Trypomastigote forms of *T. cruzi* are resistant to complement-mediated lysis in the absence of lytic antibodies, whereas epimastigotes are sensitive to lysis via the alternative complement pathway (136,137). It was demonstrated that antibodies to a 160-kDa protein correlate with the capacity of the sera to support complement-mediated lysis of trypomastigotes (138).

The *T. cruzi* gp160 restricts complement activation by binding the complement component C3b and inhibiting C3 convertase formation. The protein is anchored in the parasite membrane via a glycosyl phosphatidylinositol linkage, similar to the human complement regulatory protein, decay-accelerating factor (139). Moreover, epimastigotes transfected with a *T. cruzi* expression vector carrying the trypomastigote complement regulatory

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protein (GP160) acquired resistance to lysis (140). *Trypanosoma cruzi* calreticulin (TcCRT) was also shown to inhibit the human complement system when binding to the collagenous portion of C1q and to inhibit the generation of classical pathway convertases and membrane attack complexes. Moreover, antibodies against TcCRT inhibit the phagocytic activity of macrophages (141).

The surface of trypanosomatid parasites contains large amounts of glycosylphosphatidylinositol (GPI), which occurs either as GPI anchors for glycoproteins and polysaccharides or as free GIPLs that contain the identical core structure of GPI (142). GPI-anchored mucin from the *T. cruzi* membrane is capable of binding to the macrophage cell surface, altering activation of human macrophage and dendritic cells (143). The administration of C10 antigen (mucin-like molecule from *T. cruzi* membrane) reduced the secretion of TNF-α and IL-I2 by macrophage in a dose-dependent manner (143).

By contrast, *T. cruzi* GPI induces inflammatory responses by Toll-like receptor (TLR) 4 activation and resistance to *T. cruzi* infection (144). TLRs function as pattern-recognition receptors in mammals and play an essential role in the recognition of microbial components (145). However, $TLR2^{-/-}$ mice infected with *T. cruzi* produce higher levels of pro-inflammatory cytokines and nitric oxide than wild-type mice. Thus, TLR2 has an important immunoregulatory role preventing excessive activation of innate immunity and uncontrolled production of pro-inflammatory cytokine (146,147), suggesting that recognition of *T. cruzi* via TLR2 may be related to immune evasion strategies of the parasite.

MECHANISMS OF CHAGASIC CARDIOMYOPATHY: PARASITE PERSISTENCE AND AUTOIMMUNITY

The complexity of this parasitic disease is better comprehended when observed from an evolutionary viewpoint. *Trypanosoma* is a very versatile genus, as it is able to parasitize a wide variety of animals, from reptiles to mammals (148). *Trypanosoma cruzi* has been a constant threat to humans for millennia (149). Both parasite and host must have evolved together during this ancient relationship, otherwise, one of the species would be extinct.

Chagas heart disease is essentially an infectious myocarditis (150). During the acute phase of the infection, there is no doubt that the inflammatory response is directed towards the parasite, which is present in large amounts in the myocardium. The opinion of the authors is that the extension of this acute damage to myocardial tissue, as well as the quality of the immune regulatory response, is related to the presence and extent of chronic cardiomyopathy. However, there are some individuals who develop an intense myocarditis but remain asymptomatic.

No evidence exists for an association between grade of acute myocarditis and different clinical outcomes, mainly because detection of individuals undergoing the acute phase is extremely rare. In addition, the experimental models fail to mimic the chronic phase of the infection (i.e. the infrequent development of dilated cardiomyopathy even in chronic-resistant mice strains). Thus, the study of the pathological basis of the chronic phase of the disease represents additional complexities that are derived from the nature and evolution of the immune response, the presence of the parasite and the host's cardiac physiology.

From an immunologist's perspective, the main controversial point in CD has been the specificity of the immune response raised in the myocardium. Two main hypotheses have been proposed, centred on two mechanisms wrongly proposed to be mutually exclusive: parasite persistence or autoimmunity (151). The authors believe that both these phenomena co-exist in CD. Thus, both immune evasion strategies of the parasite and immune homeostasis defects of the host that are determined mainly by genetic features are important for the development of the clinical forms of CD. Our opinion is that in the chronic phase of the disease, it is too limited to state that inflammation is the unique factor leading to pathogenesis. The healing and physiological adaptations of the heart after myocardial injury are crucial and determined by several host factors. The mechanisms of parasite persistence and the quality of the host immune response are both crucial in determining the extent of tissue damage (152).

The autoimmunity hypothesis arose from studies reporting that a strong inflammatory response can be found in advanced stages of the disease, even in the absence of parasites (153–155). Moreover, several experimental approaches unquestionably demonstrated the existence of autoreactive phenomena of cellular and humoral nature in infected individuals (108,155–158). Lymphocytes from infected mice and from chagasic patients are able to recognize self-epitopes (108,157). However, it has been difficult to demonstrate the pathophysiological relevance of this autoreactivity (159), as has been the case in several other autoimmune diseases triggered by infections.

The hypothesis of parasite persistence emerged as a potential mechanism to explain the chronic tissue damage following studies demonstrating genetic material from the pathogen in tissues of infected animals (160–163), where other techniques were not able to detect the presence of the microorganism (61,152). The parasite may also play an active role in the induction of cardiac pathology during CD (60,164). In fact, some evidence points a role for inherent parasite factors, such as biological and genetic

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variability among *T. cruzi* populations, in the different outcomes of the disease (165–167). A positive correlation between inflammation and the presence of *T. cruzi* in the myocardium, as well as the absence of *T. cruzi* in preserved areas of cardiac tissue, constitutes additional evidence that *T. cruzi* is able to induce myocardial damage directly (164,168).

Thus, the genetic characteristics of both host and microbe do determine not only the host resistance to the parasite itself, but also the adaptability of the host to the altered myocardial physiology, including the fibrotic response. Evolutionarily speaking, we may say that CD is the cost that humans have paid to survive *T. cruzi*.

THE CHRONIC EXTRACELLULAR MATRIX REMODELLING

One of the most notable aspects of chronic CD is the progressive deterioration of cardiac function, derived mostly from structural derangement (169), as a consequence of the intense inflammatory process (Figure 1). This structural damage can be perpetuated by some factors derived from the parasite and from the host itself (54,170). Other disease features like dysautonomia (171) and microvascular derangements caused directly by the parasite can also add further complexity to the pathophysiology of CD.

Cardiac extracellular matrix (CECM) actively participates in the process of migration of inflammatory cells (28,172), as some of its components need to be transiently broken in order to establish the inflammatory infiltrate in the tissue. Matrix metalloproteinases are essential for the local rupture of CECM during inflammation and tissue repair. Nonetheless, MMP are also able to modify the molecular structure of soluble inflammatory mediators such as cytokines and chemokines, thus acting as modulators of their activities. These enzymes are particularly active in CECM in response to several stimuli, ranging from ischaemia to inflammation. Increased MMP activity (mostly of MMP9) has been reported during the progression of cardiac failure because of other aetiologies (28). While participation of MMPs in acute cardiac inflammation has been demonstrated (29), their role in remodelling of myocardial structure during the chronic phase of the disease remains an avenue for investigation.

CONCLUDING REMARKS

As in other complex pathologies, a global perspective on this disease is important to gain understanding of the role of some concepts in its pathophysiology. Thus, CD could be considered as the cumulative contribution of diverse mechanisms which vary in their intensity among individuals depending on their genetic background. These mechanisms are triggered immediately upon infection and involve all components of the immune response, the tissue regenerative response and cardiac physiological responses. The immunological mechanisms are the focus of this review. The tissue regenerative response involves mainly the extracellular matrix remodelling. Among cardiac physiological responses we would mention the electrophysiological dysfunctions which may be the consequence of parasite invasion of cardiac cells or due to collateral damage from free nitrogen and oxygen radical species.

In our opinion, the presence of the parasite within the myocardium is important in triggering and maintaining the immunopathogenic process during CD. However, we also believe that a unbalanced immune homeostasis can trigger parallel autoimmune phenomena which amplify the immune response, thus worsening the outcome of the disease.

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The effects of nitric oxide on the immune system during *Trypanosoma cruzi* infection

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Trypanosoma cruzi *infection triggers substantial production of nitric oxide (NO), which has been shown to have protective and toxic effects on the host's immune system. Sensing of trypomastigotes by phagocytes activates the inducible NO-synthase (NOS2) pathway, which produces NO and is largely responsible for macrophage-mediated killing of* T. cruzi. *NO is also responsible for modulating virtually all steps of innate and adaptive immunity. However, NO can also cause oxidative stress, which is especially damaging to the host due to increased tissue damage. The cytokines IFN-γ and TNF-α, as well as chemokines, are strong inducers of NOS2 and are produced in large amounts during* T. cruzi *acute infection. Conversely, TGF-β and IL-10 negatively regulate NO production. Here we discuss the recent evidence describing the mechanisms by which NO is able to exert its antimicrobial and immune regulatory effects, the mechanisms involved in the oxidative stress response during infection and the implications of NO for the development of therapeutic strategies against* T. cruzi.

Key words: *Trypanosoma cruzi* - nitric oxide - myocarditis - cytokines - regulatory T cells

Nitric oxide (NO) and the respiratory cycle: the beginnings of oxidative stress

Nitrogen monoxide, also called NO, is a low-molecular weight radical (30 kDa) that performs multiple biologic activities. The biological importance of this ubiquitous intra- and intercellular signalling molecule was first described in the early 1980s as being part of the endothelial derived relaxing factors (Furchgott & Zawadzki 1980). NO was named "Molecule of the Year" in 1992 by the journal *Science* and, later that decade, studies were conducted to demonstrate its cardinal mechanism of action on vascular smooth muscle cells (Murad 1986). These studies made it clear that generation of NO by endothelial cells causes smooth muscle relaxation through activation of guanylate cyclase by nitrosation of its heme group. This work resulted in the Nobel Prize in Physiology and Medicine being conferred to Murad, Furchgott and Ignarro, in 1998 (Murad 1986).

It is hypothesised that NO may have originated in metazoans as an ancient mechanism of first-line defence against intracellular pathogens. This theory has been confirmed by the wide occurrence of the enzyme responsible for NO production, NO-synthase (NOS2), in several species, ranging from invertebrates (Ribeiro et al. 1993) to mammals and non-mammalian vertebrates. In mammals, NO production is upregulated in response to infection by a wide range of unicellular organisms such as bacteria, yeast and parasites (i.e., *Trypanosoma cruzi*) (Cardoni et al. 1990). Evidently, evolutionary diversity has induced NO synthesis to be performed in response to different kinds of stress stimuli.

Under homeostatic conditions, NO is produced at low concentrations from constitutive NOS2 and acts as an intracellular messenger and a cytoprotective (antioxidant) factor. Indeed, overexpression of NOS3 blocks the exocytosis of inflammatory mediators by endothelial cells, thus preventing blood vessel inflammation. Conversely, exposure to inflammatory stimuli leads to the production of substantial amounts of NO in a variety of cell types, as well as modifications of the cellular microenvironment, which by its turn upregulates NO effects. These effects are a consequence of the formation of dinitrogen trioxide and peroxynitrite at sites of simultaneous superoxide formation, as occurs in phagocytes (Chen & Deen 2001).

The old paradigm stating that NO is a mere "unspecific" cytostatic mediator of defence has been challenged by the recent discovery that NO has a large variety of effects on the biology of leukocytes. These effects can be direct or indirect and can influence several physiological processes, ranging from DNA transcription and replication to protein synthesis and secretion (Marnett et al. 2003). Under physiological conditions, NO mediates homeostatic anti-inflammatory reactions, such as inhibition of neutrophil adhesion (Dal Secco et al. 2006), cyclooxygenase activity (Gilroy 2005), cytokine production (Livonesi et al. 2009), osteoclast bone resorption (Fukada et al. 2008), among others, in order to prevent autoimmunity.

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The broad spectrum of effects performed by NO can be exerted through two main mechanisms: the activation of guanylate cyclase (which can be soluble in the cytosol or coupled to the cell membrane) (Poulos 2006) or through its interaction with the major cellular source of superoxide anion, the NO/Cytochrome C oxidase, which is found in mitochondria.

The guanylate cyclase-dependent effects of NO mainly affect the vascular tonus thereby affecting the inflammatory reaction. Other effects pertaining to mitochondrial functions involve the respiratory burst (Ghafourifar & Cadenas 2005). Mitochondria can produce NO through its own Ca^{2+}-sensitive synthase (mitochondrial, mtNOS). This enzyme regulates mitochondrial oxygen consumption and transmembrane potential via a reversible reaction with cytochrome C oxidase. The intramitochondrial reaction of NO with superoxide anion yields peroxynitrite, which irreversibly modifies susceptible targets within the mitochondria, inducing oxidative and/or nitrative stress.

In addition to their primary role in the production of energy (ATP), mitochondria generate reactive oxygen species (ROS) that can directly or indirectly affect the NO response (Poderoso 2009). Since NO and ONOO- can inhibit cellular respiration at the level of cytochrome C oxidase and complexes I-III, respectively, it has been suggested that mitochondrial function can influence the balance between apoptosis and necrosis induced by NO (Lizasoain et al. 1996). In addition, NO can stimulate the biogenesis of new mitochondria in a guanosine 3',5'-monophosphate (cGMP)-dependent manner (Nisoli et al. 2003).

These findings are of particular relevance for *T. cruzi* infection, since it has been described that *T. cruzi* causes an energetic impairment in myocardial mitochondria, without altering the organelle ultra structure (Uyemura et al. 1995). Hence, it is possible that *T. cruzi* can control the central machinery responsible for energetic metabolism in the host in order to access metabolites that are crucial to its proliferation (Schwarcz de Tarlovsky et al. 1995, Baez et al. 2008). This possibility is crucial and warrants further research in order to understand the mechanisms that induce oxidative stress during *T. cruzi* infection.

Iron-proteins constitute a predominant scavenger mechanism of NO (Angelo et al. 2008, Richardson & Lok 2008). As iron is mainly provided by the heme group, it constitutes an additional link between the functions of NO and the respiratory cycle (Chung et al. 2008). Oxygen drives the conversion of nitrosylhemoglobin in the "tense" structure (or partially nitrosylated, deoxy) to S-nitrosohemoglobin in the "relaxed" structure (or ligand-bound, oxy). In the absence of oxygen, nitroxyl anion (NO-) is liberated in a reaction which produces methemoglobin. The yields of both S-nitrosohemoglobin and methemoglobin are dependent on the NO/Hb ratio. These recently discovered reactions have provided new insights into the origin of S-nitrosothiols, methemoglobin and its related valence hybrids.

Mechanistic re-examination of the interactions of NO with other heme proteins containing allosteric thiol sites may be warranted (Gow & Stamler 1998). In addition, it is well established that, in the Haber-Weiss reaction (a reaction that generates hydroxyl radicals [•OH] from hydrogen peroxide and superoxide [$\bullet O_2^-$]), iron has a catalytic role, which leads to the propagation of damaging ROS. Thus, NO appears to be involved in cellular defence against iron-mediated ROS generation, mainly by the induction of cellular iron removal (Larrainzar et al. 2008, Trujillo et al. 2008). The role of these mechanisms in the pathogenesis of *T. cruzi*-induced myocarditis is currently unknown.

NO and the immune response

As previously stated, one of the most important functions of NO in the immune system is in antimicrobial defence (De Groote & Fang 1995, Fang 1997, Nathan & Shiloh 2000). Reactive oxygen and nitrogen species derived from NO are essential for protection against various intracellular pathogens including viruses, bacteria, fungi and protozoans. More specifically, NO has been demonstrated to protect against infection from *T. cruzi* (Figs 1, 2) and other protozoa as *Toxoplasma gondii, Leishmania major, Leishmania donovani, Plasmodium sp* and *Schistosoma mansoni* (Adams et al. 1990, Vespa et al. 1994, Wynn et al. 1994, James 1995, Stenger et al. 1996, Murray & Nathan 1999, Brunet 2001). Furthermore, the killing activity of NO has also been shown to be effective in host defence against tumour cells (Huerta et al. 2008) and alloantigens (Shi et al. 2008).

NO is perhaps the most important among the group of early mediators produced by cells of the innate immune system. Phagocytes constitute the first line of microbial defence and they function by sensing the presence of different types of infectious agents (Carneiro-Sampaio & Coutinho 2007) through pattern recognition receptors, including Toll-like receptors (TLRs) and the most recently described NOD- (NLRs) and RIG-like receptors. These receptors recognise multiple microbial patterns; therefore, they are critical for triggering the production of inflammatory mediators and essential for activation of the adaptive immune response (Schnare et al. 2001, Kanneganti et al. 2007, Underhill 2007). In fact, several antigens derived from intracellular parasites can be recognized by innate immune receptors on macrophages, triggering NOS2 activity (Xie et al. 1992, MacMicking et al. 1997).

NOS2 is produced by antigen-presenting cells (APC) during antigen processing and presentation to T cells and it can modulate various functions of APCs. It can inhibit the expression of major histocompatibility complex class II molecules in activated macrophages and, at high concentrations, may also inhibit IL-12 synthesis, thus contributing to the desensitization of macrophages after exposure to inflammatory stimuli (van der Veen 2001). Indeed, NO induces transcription of IL-12 p40, but not of IL-12 p35, in human macrophages (Salvucci et al. 1998). The IL-12 p40 homodimer is an antagonist for IL-12 and this antagonism might be at least partially responsible for the reduced Th1 reactivity in the presence of NO (Pahan et al. 2001). However, a new report has indicated that the IL-12 p40 homodimer can also induce

NO production by microglia (Jana et al. 2009), revealing the complex functions of NO in innate immunity.

Furthermore, NO affects the immune profile of Th1 cells, as mice with a disrupted NOS2 gene exhibit enhanced Th1 activity, which in turn, can affect the Th1/Th2 balance (Singh et al. 2000). It has been shown that high amounts of NO prevent apoptosis and, given that Th1 cells are more susceptible to apoptosis than Th2 cells, this represents an additional regulatory mechanism of the Th1/Th2 balance (Xiao et al. 2008).

NO can also affect immune responses through its ability to regulate S-nitrosylation of several components of the apoptotic machinery (Okuda et al. 1996, Melino et al. 1997, Johann et al. 2007, Shibata et al. 2007). Apoptosis is an important process in lymphocyte homeostasis and maturation in the thymus, as well as in lymphocyte proliferation in the periphery. Decreased S-nitrosylation of caspase-3 increases its intracellular enzymatic activity. In addition, Fas-mediated activation of caspase-3 is induced not only by cleavage of the zymogen to its active subunits, but also by denitrosylation of its active thiol site. The regulation of apoptosis by NO has an obvious impact on the strength of effector immune responses.

The cytoprotective properties of low/intermediate levels of NO may limit tissue damage during inflammation (Cattell & Jansen 1995, Okuda et al. 1996, Niedbala et al. 1999, De Gouw et al. 2001). Interestingly, NO significantly increases the proliferation, division and viability of regulatory T cells (Sakaguchi 2004), a lymphocyte subset which has been shown to be involved in acute experimental *T. cruzi* infection (Mariano et al. 2008). Indeed, regulatory T cells induced by NO stimulation (NO-Treg) are as efficient as natural Tregs in suppressing the differentiation of different effector lymphocyte subsets (Niedbala et al. 1999, Packard & Khan 2003). Furthermore, exposure of murine lymphocytes to NO suppresses IL-2 transcription, reducing clonal expansion and indirectly favouring a Th2 response (Taylor-Robinson et al. 1994).

Other important feedback mechanisms mediated by NO, which prevent dysregulated immune responses, include downregulation of cell adhesion and migration, which unchecked, would result in serious and overwhelming inflammatory injury (Biffl et al. 1996, Hokari et al. 1998, Staykova et al. 2003, Dal Secco et al. 2006). Of note, inactivation of P-selectin expression by NO, which affects leukocyte adherence, may also preferentially affect Th1 cell migration (van Wely et al. 1998).

Upon stimulation by cytokines or bacterial lipopolysaccharide (LPS), endothelial cells exhibit increased expression of ICAM-1 in vitro, which contributes to the transmigration of all classes of leukocytes, but mainly neutrophils (Biffl et al. 1996). In addition, NOS2 deficiency or inhibition of NOS or sGC by pharmacological inhibitors, leads to enhanced LPS-induced ICAM-1 expression on mesenteric microcirculation (Dal Secco et al. 2006).

The migration of inflammatory cells may also be affected by the chemical modifications of matrix metalloproteinases (MMPs) (Ridnour et al. 2007). Recently, it was demonstrated that the activities of MMP-2 and MMP-9 are increased during acute myocarditis in experimental *T. cruzi* infection and that the inhibition of these enzymes leads to reduced myocarditis and improved survival in mice (Gutierrez et al. 2008). Accordingly, MMPs are activated in inflammatory or ischemic/reperfusion conditions (Gu et al. 2002).

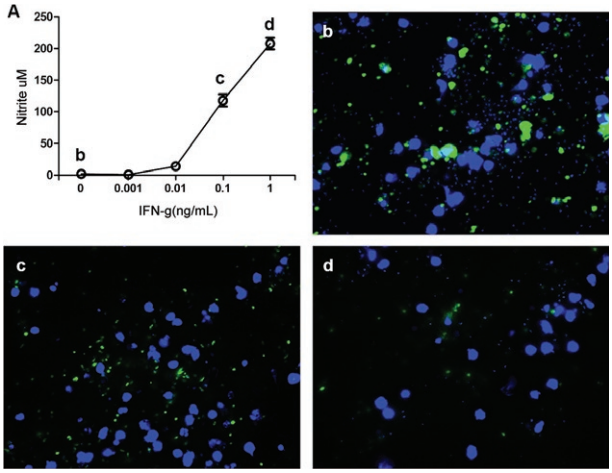

Fig. 1: production of nitric oxide by macrophages correlates with the intracellular killing of *Trypanosoma cruzi*. Peritoneal murine macrophages were infected with *T. cruzi* and cultured for 48 h in the presence of recombinant IFN-γ as indicated on the X axis of A. Then, the levels of nitrite were measured in the supernatants by Griess reagent method (A); b, c, d: microphotographs show the parasites (labeled with CFSE before infection) when the cells where not stimulated with IFN-g (b) or with 0.1 ng/mL (c) or 1 ng/mL (d) of recombinant IFN-γ. Note the reduction in the parasite staining as concentration of IFN-γ increases in the culture.

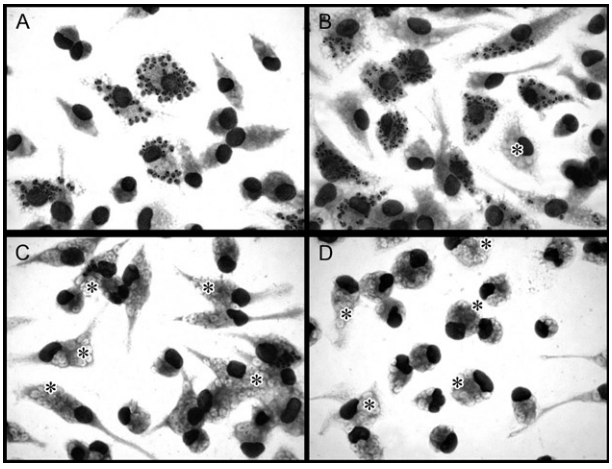

Fig. 2: IFN-γ induces macrophage activation and intracellular killing of *Trypanosoma cruzi*. Peritoneal murine macrophages were cultured for 48 h in the presence of *T. cruzi* and medium alone (A, B) or supplemented with 0.1 ng/mL of recombinant IFN-γ (C,D). Observe in C and D the absence of intracellular parasites and, instead of that, empty vacuoles can be noted (asterisks).

NO may also affect lymphocyte migration by altering cell motility. In vitro, NO induces actin polarization in T cells, inhibiting their trans-endothelial migration in a p70S6 kinase-independent manner (Staykova et al. 2003). Moreover, NO may also inhibit the expression of integrins, such as CD11a/CD18, in neutrophils (Banick et al. 1997, Grisham et al. 1998). Since NOS2 is involved in peroxynitrite-dependent tyrosine nitration (Sato et al. 2000, Yeh et al. 2007), it also regulates chemokine production and affects the inflammatory response mediated by IP-10, MCP-1, MIP-1a and MIP-2, and IL-8 (Mach et al. 1999, Pfeilschifter et al. 2001).

NO also participates in the maintenance of inflammatory diseases (such as arthritis, ulcerative colitis and Crohn's disease) and in the pathogenesis of *T. cruzi*-induced myocarditis (Silva et al. 2003, Machado et al. 2008). Indeed, several classic inflammatory symptoms, for example erythema and vascular leakiness, are related to the production of NO and can be reverted by NOS inhibition (Cuzzocrea et al. 2002). In chronic immune responses to intracellular pathogens, NO is reported to play a regulatory role and may promote parasite persistence. For these reasons, it is suggested that NO is cytostatic rather than cytotoxic for parasites (Klotz et al. 1995).

The dual role of NO during *T. cruzi* infection

Intracellular protozoans have infected vertebrates since ancient times and are usually able to establish chronic infection. A spontaneous cure is uncommon in these diseases, suggesting that potent mechanisms have been developed by these pathogens in order to evade immune detection or destruction. Among these keystone mechanisms, which attest to their remarkable strength, is the capacity of *T. gondii*, *T. cruzi* and *Leishmania* spp. to invade and replicate within many different cell types (Leiriao et al. 2004, Denkers & Butcher 2005, Gregory & Olivier 2005).

Infection with *T. cruzi* in humans can lead to the development of Chagas disease, the clinical features and evolution of which are determined by a combination of parasite factors (i.e. tissue tropism and evasion mechanisms), mode of inoculation (i.e. the mode of contamination or transmission and the size of the inoculum), as well as by host-derived factors (i.e. exacerbated immune response) (Coura 2007).

During *T. cruzi* infection, NO can directly or indirectly modulate the effector leukocyte machinery through diverse mechanisms. This process involves microbicidal effects derived from toxic-free radicals (peroxinitrite and superoxide) generated after NO production, as well as regulation/enhancement of the inflammatory response induced during this type of infection, a dual role in the immunity that is usually observed for NO. This well-known immune duality is usually dependent on concentration and, once dysregulated, may lead to host cell toxicity, autoimmunity or parasite persistence due to immune evasion, all of which can lead to pathology (FR Gutierrez et al. 2009, unpublished observations).

NO is involved in the control of *T. cruzi*-induced parasitemia and directly kills the parasite in vitro (Vespa et al. 1994) (Figs 1, 2). NO affects *T. cruzi* by chemically modifying cysteine-containing proteins and/or by binding to metalloproteins that mediate crucial metabolic processes. Recently, it was reported that NO or NO donors can inhibit the catalytic activity of cruzipain, the major papain-like cysteine proteinase in *T. cruzi*. Analogous to a similar protein in *Plasmodium,* this dose-dependent effect was attributed to S-nitrosylation of Cys25, a catalytic residue present in the active site of cruzipain (Venturini et al. 2000).

The strength of NO toxicity is dependent on the sensitivity of the parasite, which differs among parasite strains and according to the physiological microenvironment. NO has been demonstrated to be the principal effector molecule involved in macrophage-mediated killing of *T. cruzi* amastigotes (Nathan & Shiloh 2000, Colasanti et al. 2002, Silva et al. 2003). Contradictory evidence suggests that susceptible mouse strains display increased macrophage activation after contact with the parasite, which may be due to the fact that, in these animals, infection with *T. cruzi* induces an overwhelming production of both NO and $\bullet O_2^-$ (Russo et al. 1989, Cardoni et al. 1990, Arantes et al. 2004).

An additional mechanism by which NO can affect the metabolism of *T. cruzi* is through the reduction of available growth factors. For example, iron is an important growth factor for *T. cruzi* (Ciccarelli et al. 2007). NO induces nitrosilation of the heme group from haemoglobin, haematin or haemin, the main sources of iron. The main target of oxidative stress during *T. cruzi* infection is the erythrocyte, as it is the major principle site of the antioxidant chemical machinery. The nature and extent of oxidative injury depends on three factors: (i) the induction of NOS2 and, thus production of NO in response to infection (Alvarez et al. 2004); (ii) the oxidative stress generated outside of the erythrocyte, particularly phagocyte-derived $\bullet O_2^-$ and (iii) the rate of reaction between NO and either haemoglobin or $\bullet O_2^-$. The imbalanced counteraction of the oxidative response leads to haematological disorders (i.e., anaemia), which are observed in the acute phase of *T. cruzi* infection (Malvezi et al. 2004).

Oxidative stress is also observed in myocarditis during experimental *T. cruzi* infection. As myocarditis progresses, a substantial decline in cardiac mtDNA content (54-60%) and mitochondria-encoded transcripts (50-65%) indicate that alterations in mtDNA contribute to the quantitative deficiencies in respiratory chain activity of infected individuals (Vyatkina et al. 2004). In fact, during chagasic cardiomyopathy, mitochondrial dysfunction occurs as a consequence of intense oxidative stress (Wen et al. 2006) and is evidenced by deficiencies in respiratory chain complexes (CI-CV) (Garg 2005).

As previously suggested, the accuracy of initial pathogen recognition by the immune system is crucial for the production of NO in order to mount an appropriate immune response. TLRs can sense the presence of *T. cruzi* (Campos & Gazzinelli 2004), however, because it is an intracellular protozoan, *T. cruzi* has an extremely complex antigenic repertoire (Buscaglia et al. 2006). This

makes it difficult to determine the exact mechanism by which the large diversity of cell-surface molecules on *T. cruzi* are recognised by the innate immune system (Tarleton 2007). Although other molecules may be involved, it is known that innate recognition of glycophosphatidylinositol-anchored mucin-like glycoproteins from *T. cruzi* are potent inducers of NO biosynthesis by IFN-γ-activated macrophages (Camargo et al. 1997).

Early after infection, IL-12 is required for the induction and maintenance of IFN-γ production by innate and adaptive immune cells (Silva et al. 1998). IFN-γ production by Th1 effector cells has consistently been implicated in the pathogenesis of Chagas disease and is an important factor for maintaining *T. cruzi*-mediated pathology. During acute experimental *T. cruzi* infection in mice, the parasite induces a profound suppression of the lymphoproliferative response to mitogens and *T. cruzi* antigens. This process is largely mediated by increased NO synthesis and decreased IL-2 production (Abrahamsohn & Coffman 1995). Our group demonstrated that NO induces apoptosis of cells from BALB/c mice acutely infected by *T. cruzi*. Splenocytes from infected mice displayed reduced viability and elevated levels of spontaneous apoptosis after 48 h in culture. Inhibition of NO production, by the addition of the L-arginine analogue NG-monomethyl-L-arginine or the addition of monoclonal antibodies (mAbs) against IFN-γ or TNF-α partially restored viability and decreased apoptosis of splenocytes from infected mice (Martins et al. 1998). In addition, the production of IL-17 has recently been implicated in mediating regulatory responses against *T. cruzi* (Monteiro et al. 2007). Of note, IL-17 markedly augments NOS2 mRNA and subsequent NO production. Additionally, *T. cruzi* infection induces the expression of chemokines (MIG, IP-10, RANTES, MIP) and adhesion molecules at sites of $CD4^+$ and $CD8^+$ T cell infiltration (Teixeira et al. 2002). Cytokines and NO can modulate the production of chemokines and adhesion molecules in vivo and in vitro, influencing the course of infection (Savino et al. 2007, Machado et al. 2008). Chemokine receptors are also involved in cellular activation during parasitic infections and this G-protein-coupled signalling pathway is implicated in NO production as well (Benevides et al. 2008).

One mechanism by which the innate immune response can affect the activation of T cells is through the macrophage-mediated reduction of available L-arginine in the microenvironment. The levels of this metabolite depend on the cytokine milieu. For example, macrophages stimulated with IL-4 and IL-13 (but not IFN-γ) up-regulate arginase I and the L-arginine receptor CAT-2B, thus inducing a rapid reduction in the concentrations of L-arginine; this, in turn, down-modulates the expression of CD3ε in T lymphocytes reducing their activation (Rodriguez et al. 2003). Arginine is also required for the synthesis of NO, thus this can constitute a feedback mechanism to regulate the immune system. Recently, recognition of intracellular pathogens by TLRs has been implicated in the downregulation of NO production, through increased arginase I activity, in a STAT6-independent manner, which favours parasite growth and survival (El Kasmi et al. 2008).

As one of the most successful parasitic protozoans, *T. cruzi* has evolved active strategies to evade host defences (Eckmann et al. 2000). Interestingly, epimastigote forms of *T. cruzi* synthesise their own NO through a partially characterized NOS enzyme which displays regulatory and immunochemical properties resembling those of endogenous NOS1 (Pereira et al. 1999, Goldstein et al. 2000, Piacenza et al. 2001).

Furthermore, *T. cruzi* can also exploit the removal of apoptotic cells by professional phagocytes, which is an important mechanism by which some pathogen-induced cell alterations are ultimately detected and which is involved in the recycling of cellular constituents. Uptake of apoptotic cells does not induce an inflammatory response. Accordingly, macrophages upregulate arginase II after phagocytosis of apoptotic cells, which regulates NO production by NOS2 (Freire-de-Lima et al. 2000, Johann et al. 2007). Additionally, L-arginine, the substrate for NO production, can inhibit the programmed cell death of epimastigotes, either by NOS2-dependent production or by the activity of arginine decarboxylase, which produces polyamines that support parasite proliferation (Paveto et al. 1995).

Implications of NO in therapeutic treatment against Chagas disease

The current pharmacological agents available to treat Chagas disease include benznidazole (Rochagan and Rodanil; Roche, Brazil) and nifurtimox (Lampit; Bayer, Germany). These drugs are relatively effective in the acute and sub-chronic stages of Chagas disease (Russomando et al. 1998, Sosa Estani et al. 1998, Cançado 2002, Altclas et al. 2005). However, both drugs have significant side effects, including anorexia, vomiting, peripheral polyneuropathy and allergic dermopathy (Rassi et al. 1999). Moreover, several parasite strains are resistant to these treatments, even during the acute phase of the disease (Filardi & Brener 1987, Galvao et al. 1993, Urbina 1999). The rate of cure observed in patients with these drugs is 50-70% during the acute phase and 0-20% during the chronic phase (Guedes et al. 2006). This situation is severely aggravated by the absence of a diagnostic standard, which makes the parameters for a cure, in order to evaluate the outcome of trypanocidal therapies, debatable. Thus, there is an imperative requirement for the development of novel, safe therapeutic agents to treat Chagas disease.

As stated before, parasite elimination largely depends on the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-12, as they act in concert to activate macrophages to kill the intracellular parasite through the production of NO and its derived nitrogen and oxygen radicals (Aliberti et al. 1999, 2001, Machado et al. 2000). Studies using experimental models of acute *T. cruzi* infection have demonstrated that the anti-parasitic activity of benznidazole involves the participation of these cytokines (Michailowsky et al. 1998, Molina et al. 2000), as well as covalent modifications of macromol-

ecules by nitroreducer intermediates (reductive stress). Conversely, nifurtimox acts by reducing the nitro group to unstable nitro anion radicals, which, in turn, react to produce highly toxic reduced oxygen metabolites (superoxide anion and hydrogen peroxide) (Docampo 1990).

NO donor compounds have low toxicity in vitro and in vivo and are stable in aqueous media in the presence of oxygen and NO released by reducing agents that are present in the host inflammatory microenvironment (Bogdan 2001, Silva et al. 2007). These donor compounds have recently emerged as an interesting and important alternative treatment to experimental *T. cruzi* infection (Silva et al. 2007). We recently reported that a series of ruthenium nitrosyls, *trans*-$[Ru^{II}(NO^{+})(NH_3)_4L]X_3$, L: imidazole "{imidazole in complex with nitrogen [imN] or imidazole in complex with carbon [imC], pyridine [py], L-histidine [L-hist], sulphite [SO_3^{2-}], pyrazine [pz], nicotinamide [nic], 4-picoline [4-pic], triethylphosphite [$(P(OEt)_3)$], isonicotinamide [isn], isonicotinic acid [ina], X = BF_4^-, Cl^- or PF_6^-} and $[Ru^{II}(NO^{+})(Hedta)]$ display trypanocidal activity against the Y strain of *T. cruzi*. Such compounds were efficient in reducing parasitemia, cardiac inflammation and also allowed increased survival of infected mice (Silva et al. 2007). We also showed that the new and more potent NO donor, *trans*-$\{RuCl[(15)aneN_4]NO\}^{2+}$ complex [$(15)aneN_4$ = 1,4,8,12-tetraazacyclopentadecane, a macrocyclic quadridentate amine ligand] induced parasitological cure in a therapeutic schedule that involved a 20-day treatment of mice infected with the Y strain of *T. cruzi*. We evaluated the parasitological cure of mice treated with *trans*-$\{RuCl[(15)aneN_4]NO\}^{2+}$ and compared it to treatment with benznidazole or treatment with both drugs. Benznidazole or *trans*-$\{RuCl[(15)aneN_4]NO\}^{2+}$ administrated alone resulted in a 40% and 20% parasitological cure, respectively. However, when administered together, 80% of the treated animals were considered cured. These findings were associated with reduced or absent cardiac damage during the acute phase of *T. cruzi* infection (PMM Guedes et al., unpublished observations).

These studies provide evidence that NO donors help to improve the efficacy of current trypanocidal drugs, reducing the time of treatment and preventing adverse reactions. Hence, administration of NO donors and other drugs in conjunction can constitute a promissory therapeutic avenue that could be explored as a new alternative for the treatment of Chagas disease.

In conclusion, NO is essential for host survival during acute experimental *T. cruzi* infection. Its production is rapidly triggered in cells of the innate immune system, after the parasite is detected, and later by adaptive immune cells. A delicate, yet not completely understood, interplay exists between the components of the immune response and the concentration of NO.

Vast scientific evidence shows that NO can exert its effects on the immune response either directly or through the activity of its derivatives (mainly oxygen and nitrogen reactive species), which are able to induce structural modifications in cytokines and chemokines, thus altering their biological activities. In the same manner, NO can affect the biology of *T. cruzi* by direct toxicity, by affecting essential metabolites, or by enhancing the immune response against the parasite.

Nonetheless, this broad spectrum of activity of NO can also be responsible for extensive damage to the tissues of infected hosts and for manifestation of the disease. These data have led investigators to propose NO as a crucial target for the immunotherapy of this infectious disease. However, additional studies are required to further understand the multiple roles of NO and to establish the risks and benefits of such therapeutic approaches during parasitic infection in patients.

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MAJOR ARTICLE

Increased Activities of Cardiac Matrix Metalloproteinases Matrix Metalloproteinase (MMP)–2 and MMP-9 Are Associated with Mortality during the Acute Phase of Experimental *Trypanosoma cruzi* Infection

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The strong inflammatory reaction that occurs in the heart during the acute phase of *Trypanosoma cruzi* infection is modulated by cytokines and chemokines produced by leukocytes and cardiomyocytes. Matrix metalloproteinases (MMPs) have recently emerged as modulators of cardiovascular inflammation. In the present study we investigated the role of MMP-2 and MMP-9 in *T. cruzi*–induced myocarditis, by use of immunohistochemical analysis, gelatin zymography, enzyme-linked immunosorbent assay, and real-time polymerase chain reaction to analyze the cardiac tissues of *T. cruzi*–infected C57BL/6 mice. Increased transcripts levels, immunoreactivity, and enzymatic activity for MMP-2 and MMP-9 were observed by day 14 after infection. Mice treated with an MMP inhibitor showed significantly decreased heart inflammation, delayed peak in parasitemia, and improved survival rates, compared with the control group. Reduced levels of cardiac tumor necrosis factor–α, interferon-γ, serum nitrite, and serum nitrate were also observed in the treated group. These results suggest an important role for MMPs in the induction of *T. cruzi*–induced acute myocarditis.

Chagasic cardiomyopathy, triggered by infection with *Trypanosoma cruzi,* is one of the most important causes of acquired heart disease in Latin America. It is the most frequent and severe manifestation of Chagas disease and affects approximately 25%–30% of *T. cruzi*–infected patients [1]. Various investigations have attributed chagasic cardiomyopathy to parasite persistence, autoimmunity, microvascular alterations, or neurogenic disturbances [1]. Despite these observations, the pathogenesis of this condition is not completely understood. One protein family that may contribute to the manifestations of chagasic cardiomyopathy is the matrix metalloproteinases (MMPs).

MMPs comprise a vast class of zinc-dependent endopeptidases that are divided into families according to their substrates [2]. They are known to control tissue remodeling by regulating extracellular components during several homeostatic and pathologic processes [2, 3]. Increased levels of various MMPs (collagenases, stromelysins, and gelatinases) have been associated with inflammatory diseases of connective tissues. The actions of collagenases MMP-2 and MMP-9 are involved in regulation of the inflammatory response in several circumstances, including the direct cleavage of immune system proteins [4]. Additionally, a direct pathogenic role has

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been demonstrated for MMP-2, which causes heart disease by cleaving intracellular proteins, such as troponin I and myosin light chain 1, during oxidative stress [5–7]. As a result, it has become clear that gelatinases are crucial factors in the pathogenesis of inflammation, autoimmune diseases, and cancer. Both MMP-2 and MMP-9 are regulated by the tissue inhibitors of MMPs (TIMPs) [2]. An altered balance between MMPs and TIMPs contributes to a number of cardiovascular pathologies, including viral myocarditis, ischemia and reperfusion injury, and heart failure [5, 8, 9].

During acute *T. cruzi*–induced myocarditis, a diffuse infiltration of T cells and macrophages is observed [10], accomplished by cellular migration through the endothelial and basement membranes, as well as the connective tissues, to reach their final targets (e.g., an infected cell or an opsonized pathogen). In other models of cardiovascular pathology, the immune cell infiltration is largely dependent on the cleavage of extracellular matrix by MMPs. In the acute phase of *T. cruzi* infection, myocardial inflammatory infiltrate produces a significant tissue injury, which may cause acute morbidity and mortality [1, 11] and lead to chronic alterations in cardiac structure (e.g., collagen deposition and fibrosis) [1, 10, 12, 13]. Moreover, chagasic cardiomyopathy frequently leads to a progressive depression of myocardial contractile function and ventricular dilatation, inducing heart failure [1].

The acute infiltration of immune cells in *T. cruzi*–associated myocarditis is induced by a Th1-biased immune response [14]. Mice that are genetically deficient in or treated with monoclonal antibodies against the Th1 cytokines interferon (IFN)–γ, interleukin (IL)–12, and tumor necrosis factor (TNF)–α have reduced heart inflammation during *T. cruzi*–induced myocarditis [15–17]. Conversely, the Th2 cytokines IL-10, transforming growth factor–β, and IL-4 downregulate the immune response and prevent potential tissue damage to the host [15, 18, 19]. Interestingly, MMP-2 and MMP-9 have been shown to be important modulators of immune responses. For instance, these gelatinases cleave and modulate the activities of several chemokines and cytokines [2, 20, 21]. Several factors that stimulate the immune system are also able to activate or induce MMP activity [22, 23]. Thus, in *T. cruzi*–induced myocarditis, MMPs may regulate immune functions by proteolysis, thereby acting as a switch factor and catalyst in both innate and adaptive immunity [20, 21]. In the current study we investigated the participation of MMP-2 and MMP-9 during acute experimentally induced *T. cruzi* infection, in which myocarditis is an important factor for mortality. Collectively, our findings suggest that *T. cruzi* infection leads to increased levels of MMP-2 and MMP-9 and that its inhibition reduces myocarditis and improves survival during the acute phase of infection. We hypothesized that MMP-2 and MMP-9 contribute to the myocarditis induced by *T. cruzi,* by favoring the infiltration of immune cells and modulating the immune response.

MATERIALS AND METHODS

Animals. C57BL/6 female mice 6–8 weeks old (8–10 per group) were cared for according to institutional ethical guidelines. Four to 5 animals from each group were euthanized at several time points after infection, and their hearts were collected for ELISA, histology, immunohistochemistry, polymerase chain reaction (PCR), and zymography studies. Noninfected, age-matched mice were used as controls. For survival studies, 2 independent groups (a doxycycline-treated group and a control group) of 8 animals were followed up until 35 days after infection.

Parasites and experimental infection. Mice were infected intraperitoneally with 1×10^3 blood trypomastigote forms of *T. cruzi* (Y strain). Parasitemia levels were evaluated in 5 μL of blood obtained from the tail vein. Trypomastigote forms of parasites were grown in the monkey kidney fibroblast cell line (LLC-MK2).

Doxycycline preparation and treatment. Animals were orally treated with doxycycline for MMP inhibition, as described elsewhere [24]. Doxycycline solution was prepared in distilled water, using Vibramycin (Pfizer). Mice were treated with 30 mg/kg once daily, as calculated on the basis of the daily average water intake of C57BL/6 mice. The drinking water was placed in light-shielded bottles and changed every 24 h. Treatment started 48 h before infection and was continued until day 14 after infection.

Histological analysis. To determine the percentage of inflammation of cardiac tissue, total mononuclear inflammatory cells were counted in 50 microscopic fields in $\geq$4 representative, nonconsecutive hematoxylin-eosin–stained sections (5 μm thick) per organ from 3 mice per group at day 20 after infection. Sections were examined with a Zeiss Integrationsplatte II eyepiece reticule, used with an Olympus BHS microscope at a final magnification of $\times$ 400.

Gelatin zymography. The gelatinolytic activities of MMPs were examined by gelatin zymography of cardiac tissue homogenate, as described elsewhere [8]. In brief, 30 μg of protein from heart homogenate were electrophoresed through an 8% polyacrylamide gel copolymerized with gelatin (2 mg/mL, type A from porcine skin; Sigma). Supernatant of HT1080 cells (ATCC) was used as a standard to normalize activities between gels. The gels were washed with 2.5% Triton X-100 and incubated for 24 h at 37°C in activation buffer (50 mmol/L Tris-HCl, 150 mmol/L sodium chloride, 5 mmol/L calcium chloride, and 0.05% sodium azide). After incubation, the gels were stained with 0.05% Coomassie brilliant blue (G-250; Sigma). Gelatinolytic activities were detected as transparent bands against the dark blue background. Zymograms were digitally scanned, and band intensities were quantified using SigmaGel software (version 1.0; Jandel) and expressed as a ratio to the internal standard. To confirm that the quantified gelatinolytic proteinase activities were specific for

Table 1. Sequences of the primers used for real-time polymerase chain reaction.

Name	Forward (5'→3')	Reverse (5'→3')
β-Actin	AGC TGC GTT TTA CAC CCT TT	AAG CCA TGC CAA TGT TGT CT
T-bet	CCC CTG TCC AGT CAG TAA CTT	CTT CTC TGT TTG GCT GGC T
GATA-3	AGG AGT CTC CAA GTG TGC GAA	TTG GAA TGC AGA CAC CAC CT
MMP-2	CGG AGA TCT GCA AAC AGG ACA	CGC CAA ATA AAC CGG TCC TT
MMP-9	GCG TGT CTG GAG ATT CGA CTT	TAT CCA CGC GAA TGA CGC T
TIMP-1	CTA TCC CTT GCA AAC TGG AGA	ACC TGA TCC GTC CAC AAA CA
TIMP-2	TTC ACG CTA GGT TGA TTC TGC C	GGC CGG CTA CAC AGT CTT ACA A
TIMP-3	TCC TAA TAT GGC GCT CCT GAT C	ACA GCC TAC ACA TGG CAC ATG A
Trypanosoma cruzi kDNA	GCT CTT GCC CAC AMG GGT GC	CCA AGC AGC GGA TAG TTC AGG

NOTE. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP.

MMPs, either *o*-phenanthroline (100 μmol/L) or GM6001 (10 μmol/L) was added to incubation buffer, abolishing all gelatinolytic activities.

Measurement of nitric oxide production. Nitrite concentrations in serum samples from treated or control mice at 14 and 20 days after infection were determined by the Griess method. In this assay, 0.1 mL of reductase-treated serum was mixed with 0.1 mL of Griess reagent in a multiwell plate, and the absorbance was read at 550 ηm 10 min later. Nitrite concentrations were determined by reference to a standard curve of sodium nitrite (1–200 μmol/L).

Measurement of cytokine production. Cytokine concentrations were measured in heart homogenates by use of ELISA. The ELISA sets were IFN-γ (BD OptEIA; BD Biosciences), TNF-α (DuoSet; R&D), and IL-10 (BD OptEIA), and procedures were undertaken in accordance with the manufacturers'

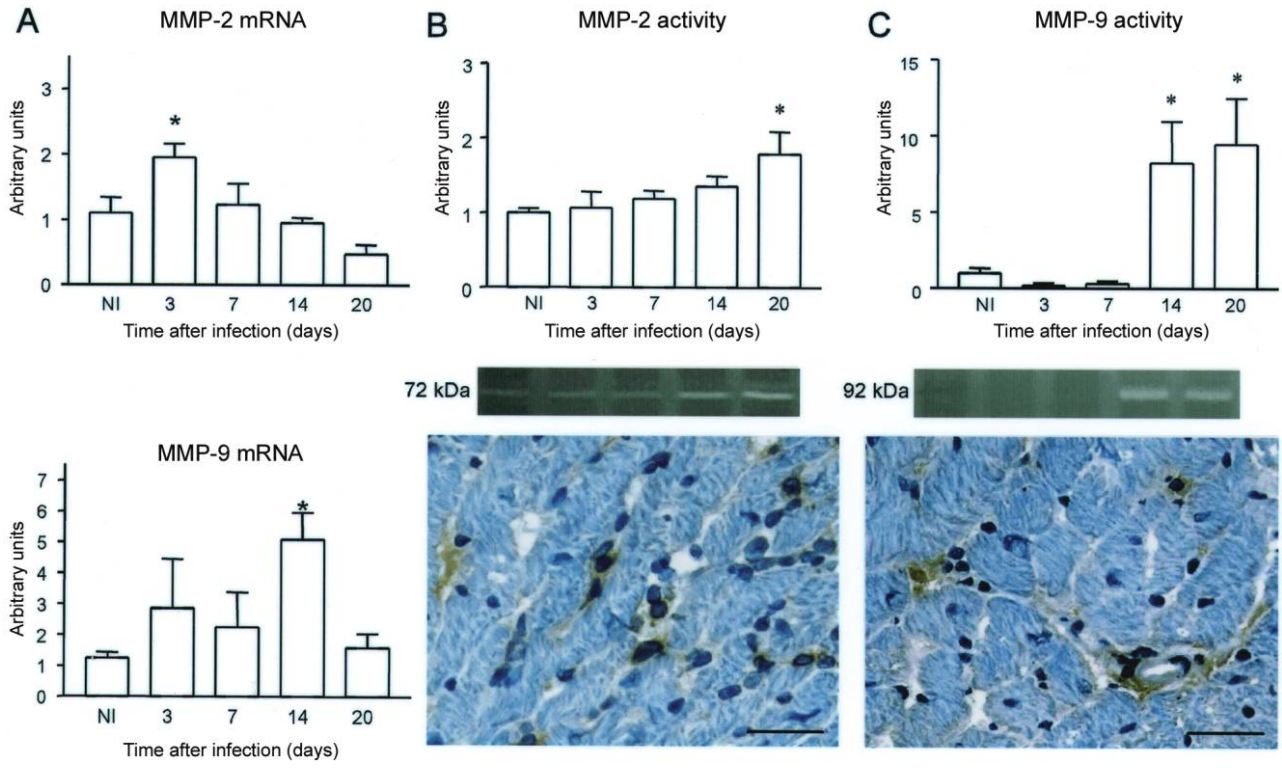

Figure 1. Matrix metalloproteinase (MMP)–2 and MMP-9 in heart tissue during acute *Trypanosoma cruzi* infection. Heart tissues from noninfected (NI) mice or from *T. cruzi*–infected mice were subjected to real-time polymerase chain reaction (PCR), gelatin zymography *(A, B [upper panel],* and *C [upper panel]),* and immunohistochemistry analysis *(B* and *C, lower panels)* to detect the presence and activity of MMP-2 or MMP-9 after infection. The photomicrographs in the lower panels of *B* and *C* are from 20 days after infection. The results shown are representative of 2 independent experiments performed with 5–9 mice per group. For real-time PCR, the expression shown is relative to that of the NI group, represented by a value of 1 in the scale (arbitrary units). $*P < .05$, compared with mice in the NI group.

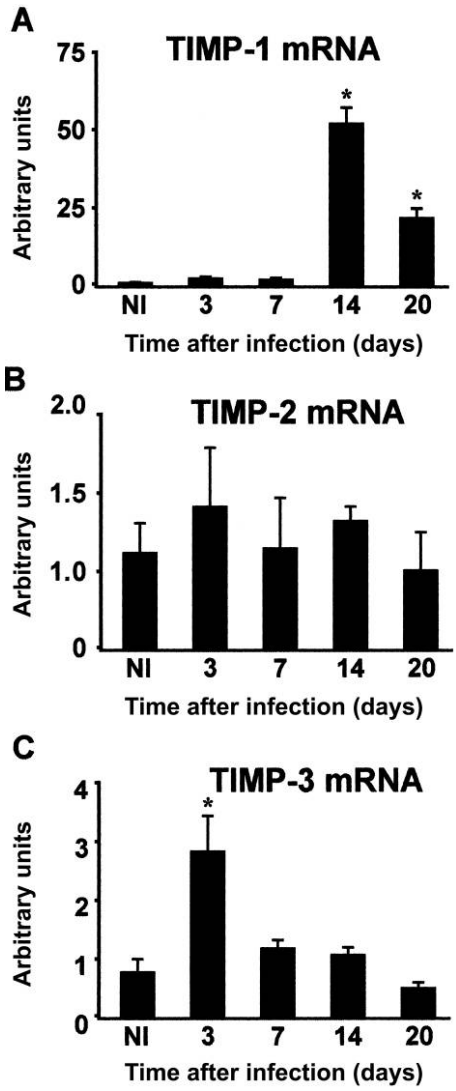

Figure 2. The mRNA expression for tissue inhibitors of matrix metalloproteinases (TIMPs) in the heart during acute *Trypanosoma cruzi* infection. Heart tissues from noninfected (NI) and infected mice at various time points after infection were subjected to real-time polymerase chain reaction for TIMP-1, TIMP-2, and TIMP-3. The results shown are relative to the expression in the NI group, represented by a value of 1 in the scale (arbitrary units). Data are representative of 2 independent experiments performed with 3 mice per time point after infection. $*P < .05$, compared with mice in the NI group.

instructions. The reaction was detected by peroxidase-conjugated streptavidin followed by a substrate mixture that contained hydrogen peroxide and ABTS (Sigma) as a chromogen.

Immunohistochemistry. Mice belonging to each group were euthanized at day 20 after infection. The hearts were removed, embedded in tissue-freezing medium (Tissue-Tek; Miles Laboratories), and stored in liquid nitrogen. Serial sections 5–7 μm thick were fixed in cold acetone and subjected to immunoperoxidase staining using antibodies against MMP-2 and MMP-9 (Santa Cruz) along with rabbit anti–*T. cruzi* serum, all diluted 1:200. Sections of spleen were used as positive controls.

RNA extraction, cDNA synthesis, and real-time PCR. Total RNA (or DNA in the case of *T. cruzi* kDNA assays) was extracted from homogenates of ventricular tissues at different time points during infection, as in gelatin zymography. After addition of TRIzol reagent (Invitrogen) (1 mL per sample), tissues were macerated, and RNA and/or DNA was purified from the homogenate using the SV Total RNA/DNA Isolation System kit (Promega), in accordance with the manufacturer's instructions. The purified RNA was eluted in 50 μL of RNAse-free water, quantified in a spectrophotometer (BioMate 3; Thermo Spectronic), and evaluated for quality in an agarose 1.5% formaldehyde gel, with visualization of only the 18S and 28S bands corresponding to ribosomal RNA. Complementary DNA (cDNA) was synthesized using 2 μg of RNA via a reverse-transcriptase reaction with ImProm-II reagents (Promega), in accordance with the manufacturer's instructions, in a thermal cycler (PTC-100; MJ Research). Reaction conditions were as follows: 5 min at 70°C and 1 h at 42°C, followed by refrigeration at

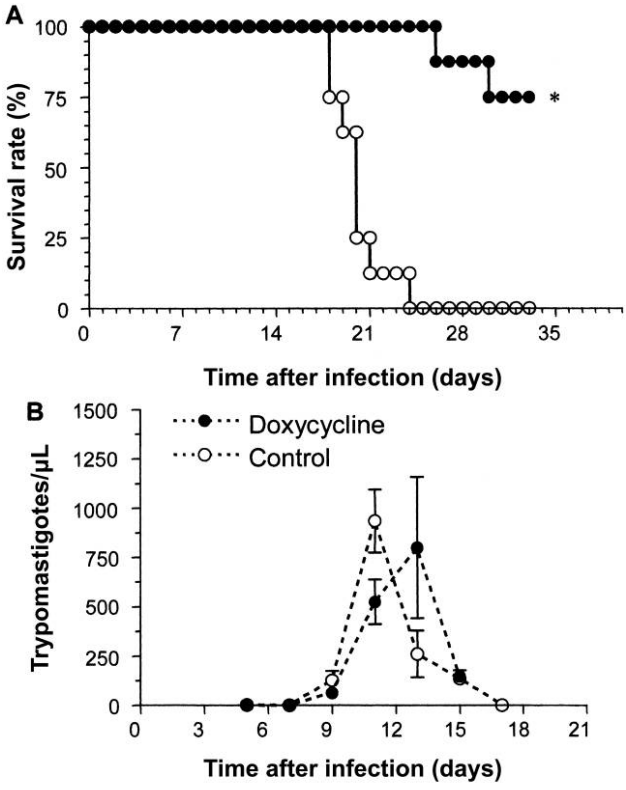

Figure 3. Effect of doxycycline treatment on mortality and parasitemia in *Trypanosoma cruzi*–infected mice. Survival rate *(A)* and parasitemia level *(B)* were evaluated in mice infected with *T. cruzi* and treated *(filled circles)* or not treated *(open circles)* with doxycycline. Data shown are representative of 2 independent experiments performed with 8 mice per group for each experiment. $*P < .05$, compared with control mice.

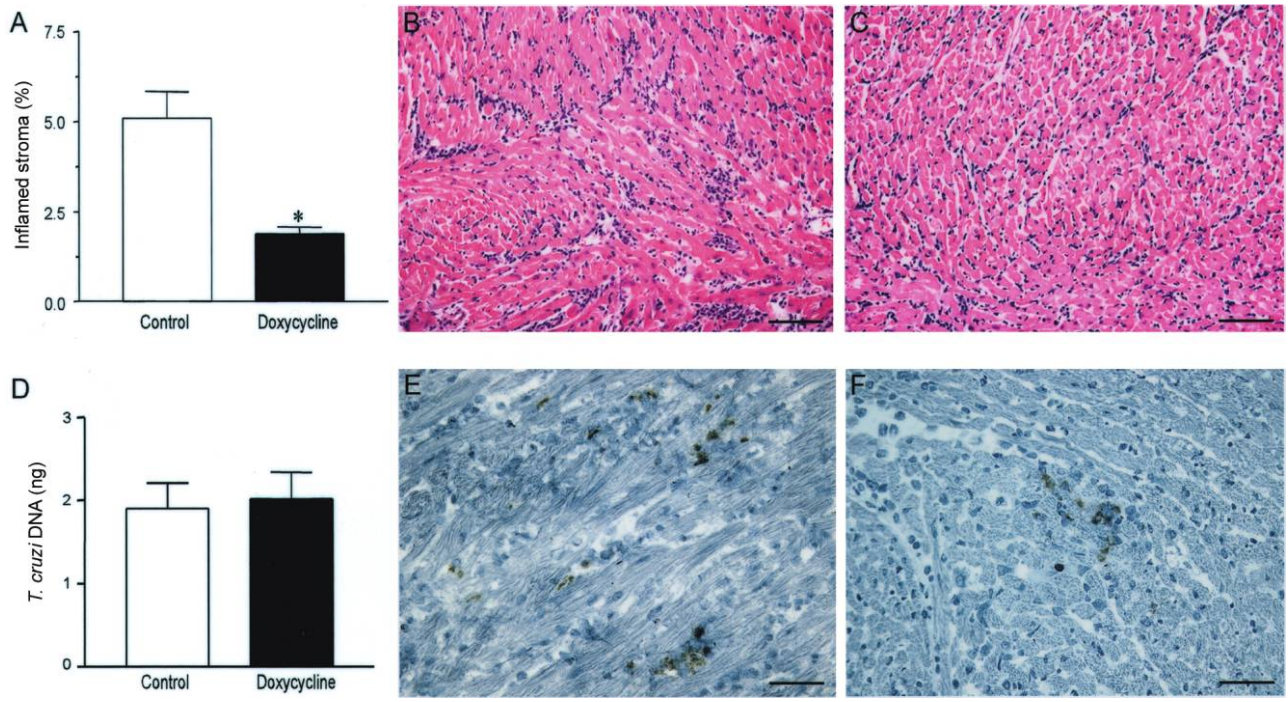

Figure 4. Effect of doxycycline on myocarditis and tissue parasitism in *Trypanosoma cruzi*–infected mice. Histological analysis of sections of hearts from mice at day 20 after infection with *T. cruzi* was performed by morphometry *(A)* to quantify the intensity of inflammatory reactions in mice treated with doxycycline or vehicle (control). Fifty microscopic fields (final magnification, × 400) were analyzed in ⩾4 nonconsecutive slides per heart for 4 mice per group. Photomicrographs of histological results in the control group *(B)* and the doxycycline group *(C)* (final magnification, × 200). *D, T. cruzi* kDNA was quantified by real-time polymerase chain reaction, and the results are the means for *T. cruzi* DNA in 100 ng of total DNA extracted from the hearts of mice on day 20 after infection. Photomicrographs of immunohistochemical staining performed to detect *T. cruzi* antigens in the control group *(E)* and the doxycycline group *(F)* (final magnification, × 400). $^*P < .05$, compared with control group.

4°C. Real-time PCR reactions were performed using the Platinum SYBR Green qPCR SuperMix-UDG with ROX reagents (Invitrogen), with 5 μL of diluted cDNA from mice hearts. The mRNAs for MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, T-bet, and GATA-3 were amplified in the 7000 Sequence Detection Systems device (Applied Biosystems). Primers used for quantitative real-time PCR reactions were synthesized using primer express software (Applied Biosystems) and nucleotide sequences present in the GenBank database (sequences listed in table 1). Each mRNA was normalized to a constitutive mRNA (β-actin) with the ΔCt (cycle threshold) method, as described elsewhere [25], except for the kinetics assays, for which normalization was not assessed owing to progressive increase in β-actin expression along with infection (not shown). For these samples, the results were calculated from Ct values. For real-time PCR involving *T. cruzi* kDNA detection, a standard curve was constructed by use of serial dilutions of DNA extracted from samples with known concentration of trypomastigotes, as described elsewhere [26].

Statistical analysis. Data were expressed as means ± standard errors of the means. The Student *t* test was used to analyze the statistical significance of the observed differences in treated assays, compared with control assays. In time course studies, 1-way analysis of variance was used, followed by Tukey-Kramer post hoc analysis. The Kaplan-Meier method was used to compare survival curves for the groups studied. Differences were considered significant at $P < .05$. All analyses were performed with Prism software (version 3.0; GraphPad).

RESULTS

Expression and activity of gelatinases (MMP-9 and MMP-2) in heart tissue during the acute phase of* T. cruzi *infection. In accordance with previously published data [12], all *T. cruzi*–infected mice exhibited a diffuse and intense myocarditis that started within 2 weeks after infection and became more intense after 3 weeks of infection. We aimed to determine whether this inflammation was associated with increased expression of MMP-2 or MMP-9. Analysis of immunohistochemistry revealed the expression of MMP-2 and MMP-9 in heart tissue on day 20 after infection (figure 1). MMP-2 and MMP-9 were associated with leukocyte infiltration, and MMP-9 was also seen in the vascular wall (figure 1*B* and 1*C*). Such immunoreactivity was not observed in the heart tissue of noninfected mice.

To investigate whether the augmented expression of MMPs was related to increased enzymatic activity, gelatin zymography was carried out on cardiac tissue extracts from mice on days 3, 7, 14, and 20 after infection and on tissue extracts obtained from

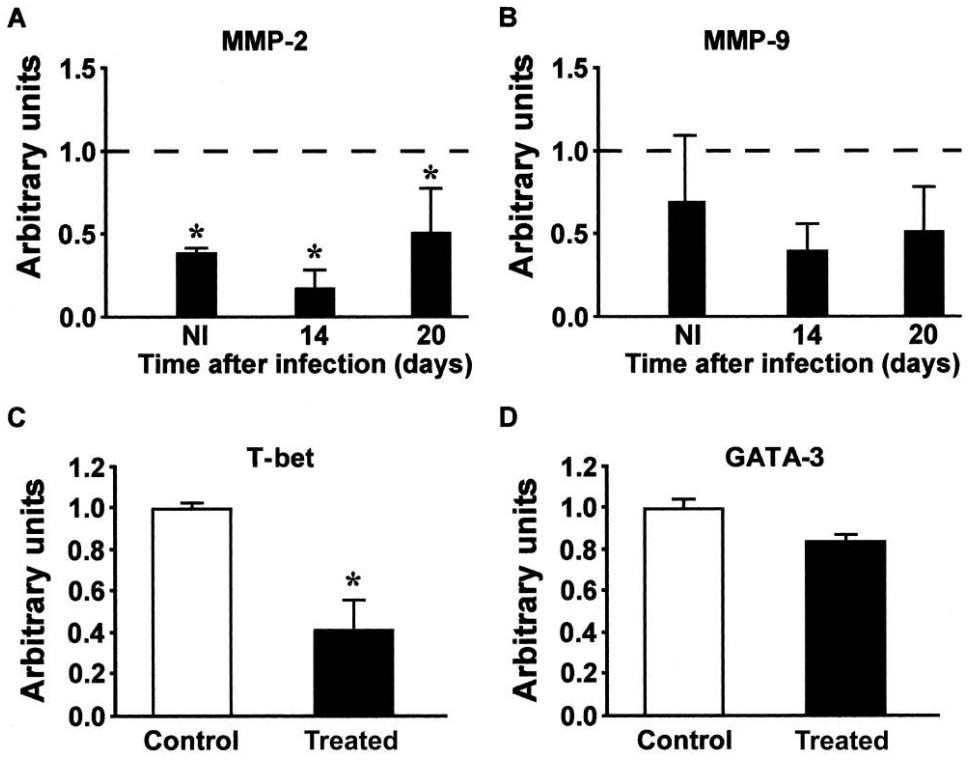

Figure 5. Effect of doxycycline treatment on cardiac tissue mRNA levels for matrix metalloproteinase (MMP)–2, MMP-9, T-bet, and GATA-3 during acute *Trypanosoma cruzi* infection. The levels of mRNA for MMP-2 *(A)* and MMP-9 *(B)* were assessed in heart tissue samples from infected mice on days 14 and 20 after infection and in samples from noninfected (NI) mice; both groups received doxycycline. Data are presented as relative to the levels in each control (untreated) group, which was normalized to 1 in the scales *(dashed lines)*. On day 20 after infection, the levels of mRNA for transcription factors T-bet *(C)* and GATA-3 *(D)* were determined in heart tissue of infected mice treated with doxycycline *(filled bars)* and control (untreated) mice *(open bars)*. Data are means ± standard errors of the mean for 4 mice per group and are representative of 2 independent experiments. $^*P < .05$, compared with control group.

noninfected mice. The 72-kDa MMP-2 enzymatic activity gradually increased along the observed time points and became significantly increased by day 20 (figure 1*B*). MMP-9 activity was virtually undetectable in noninfected and infected mice at days 3 and 7 after infection. A significant increase in 92-kDa MMP-9 activity was detected at days 14 and 20 after infection (figure 1*C*). This increase in the activity of MMP-2 and MMP-9 coincides with the intense myocarditis observed by days 14 and 20 after infection. The levels of mRNA for MMPs in heart tissue homogenates, determined by real-time PCR, showed a significant increase in MMP-2 at day 3 after infection, with a return to baseline thereafter (figure 1*A*). In accordance with the data from immunohistochemistry experiments, increased mRNA for MMP-9 was observed by day 14 after infection (figure 1*B*).

Because TIMPs are important regulators of the activity of MMPs, we next assayed TIMP expression during the acute phase of infection. We found that the expression of mRNA for TIMP-1 was significantly elevated by days 14 and 20 after infection (figure 2*A*). Conversely, TIMP-2 mRNA expression was not substantially affected (figure 2*B*), and TIMP-3 was significantly increased only on the third day of infection (figure 2*C*).

Effects of MMP inhibition on survival, heart inflammation, and mRNA levels of MMPs and transcription factors in* T. cruzi*–infected mice. The increased expression and activity of cardiac MMP-9 during infection with *T. cruzi* suggested a role for this MMP in the pathogenesis of myocarditis. To investigate this possibility, *T. cruzi*– infected mice were treated with doxycycline at a dose previously shown to inhibit MMPs [24]. We found a significantly improved survival rate in the group of mice treated with doxycycline (75% survival in treated mice vs. 0% survival in control mice by day 25 after infection; $P < .001$) (figure 3*A*). Although doxycycline induced a delayed peak in parasitemia level (days 11–13), these findings were not significantly different from those in untreated mice (figure 3*B*). In addition, the inflammatory index (percentage of inflamed tissue) was also determined for doxycycline-treated and control mice. Histological analysis showed that the doxycycline-treated animals had decreased heart inflammation, compared with untreated mice (figure 4). Moreover, doxycycline (at doses of 10, 20, and 40 μg/mL) was not able to kill parasites or affect their replication when added to the infected macrophages in vitro. The effects of doxycycline on the mRNA levels of MMPs were

studied by real-time PCR. Doxycycline produced a 50% reduction in the expression of MMP-2 mRNA in hearts from noninfected mice on days 14 and 20 after infection (figure 5*A*, and figure 5*B*). Interestingly, as reported elsewhere [27], the treatment of mice significantly reduced MMP-2 but not MMP-9 mRNA levels. The levels of mRNA for Th1-inducer transcription factor T-bet (figure 5*C*) were also diminished after treatment, but not the levels for Th2-inducer GATA-3 (figure 5*D*). These results suggest that MMPs are not directly involved in the control of parasite burden but are probably involved in the mechanisms that generate cardiac inflammation.

Effect of MMP inhibitor on production of NO_2^-, NO_3^-, IFN-γ, and TNF-α. Because the nitric oxide (NO) system and a Th1-biased response are also involved in *T. cruzi*–induced myocarditis, we quantified NO_2^- and NO_3^- in serum and cytokines in heart homogenates from vehicle-treated and doxycycline-treated mice. In the group treated with doxycycline, the systemic concentration of NO_2^- and NO_3^- was reduced on day 14 after infection. On day 20, a total of 6 days after the end of doxycycline treatment, a significant increase in NO_2^- and NO_3^- was noted. Reductions in cardiac levels of IFN-γ and TNF-α were also observed at both time points in doxycycline-treated mice. In contrast, no alterations were observed in the myocardial levels of IL-10 (figure 6). These data are in agreement with the reduced myocarditis found in mice treated with doxycycline.

DISCUSSION

In this study we showed that the expression and activity of MMP-2 and MMP-9 are upregulated in cardiac tissue during the acute phase of *T. cruzi* infection and that they are detected in association with inflammatory cells infiltrating the myocardium. Moreover, the MMP inhibitor doxycycline reduces cardiac inflammation and prevents death in infected mice.

Large increases in MMP-9 mRNA level, protein content, and enzymatic activity were noted after *T. cruzi* infection. A significant increase in MMP-9 mRNA levels was first observed at day 14 after infection, in parallel with increases in MMP-9 protein content and activity. Although subtle changes may be occurring in myocardial cells, immunohistochemistry results show that infiltrating inflammatory cells are a major source of MMP-9. Foci of MMP-9 were also seen in the myocardial vasculature, suggesting a potential role for this enzyme in *T. cruzi*–induced vasculitis, which is consistent with the important role of MMP-9 in cellular infiltration [28] and suggests that it may contribute to observed lesions in the heart tissue. In fact, the increased activity of MMP-9 contributes to viral myocarditis, myocardial ischemia and reperfusion injury, and heart failure [5, 8, 9]. Also, the late decrease in MMP-9 mRNA levels observed by day 20 possibly reflects the establishment of inflammation control mechanisms that opposed MP-9 expression.

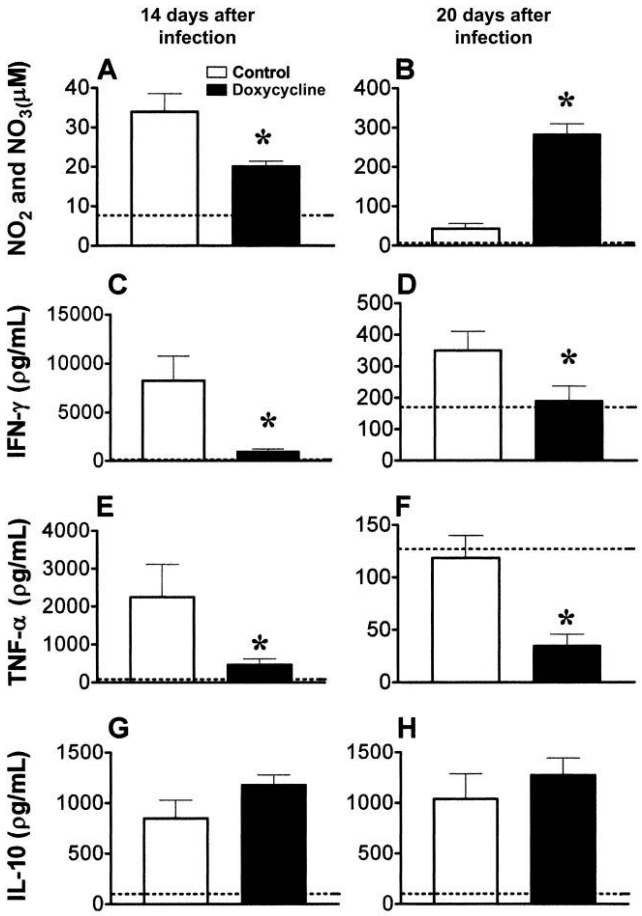

Figure 6. Effect of doxycycline treatment on nitric oxide, interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and interleukin (IL)–10 production in *Trypanosoma cruzi*–infected mice. The levels of NO_2^- and NO_3^- in serum samples *(A, B)* and the levels of cytokines IFN-γ *(C, D)*, TNF-α *(E, F)*, and IL-10 *(G, H)* in heart tissue were examined in samples from infected mice treated *(filled bars)* or not treated *(open bars)* with doxycycline, on days 14 and 20 after infection. *Dotted lines,* cytokine levels in samples obtained from noninfected mice. Results are the mean levels of cytokines and NO_2 and NO_3 detected in the samples of 4 mice per group on days 14 and 20 after infection and are each representative of 2 independent experiments. $^*P < .05$, compared with mice in the untreated control group.

Regarding MMP-2, it is known to be constitutively expressed in a wide variety of tissues, including myocardium. With PCR, higher levels of mRNA for MMP-2 at the beginning of the infection (day 3) was detected. Increases in MMP-2 protein content noted by immunohistochemical analysis at day 20 after infection paralleled the increases in global MMP-2 activity measured by zymography. No direct correlation was found between mRNA levels and protein expression of MMP-2, probably because MMPs are regulated at several points after transcription [2]. However, the detection of MMP-2 and MMP-9 coincided with higher enzymatic activity and with the intensity of the inflammatory infiltrate, as previously shown in a model of virally induced myocarditis [9, 29].

TIMPs are known to act as key local regulators of MMP activities. We found increased levels of mRNA for TIMP-1 (>50-fold by day 14 after infection) and TIMP-3, whereas TIMP-2 mRNA levels were not affected by the infection. This increase in cardiac TIMP transcripts may reflect a response to the strongly inflammatory stimuli produced during *T. cruzi*–induced myocarditis. Because TIMPs counterregulate MMP activities, they may prevent collateral injury during inflammation. Indeed, overexpression of TIMP-1 by gene therapy prevents heart remodeling and fibrosis in ischemia-induced cardiomyopathy [30]. In *T. cruzi* infection, however, this response appears insufficient to avoid myocardial damage, because severe myocarditis continues to be observed. In addition, TIMP-1 expression has also been associated with the induction of collagen deposition, favoring fibrosis [22]. Thus, overexpression of TIMPs may contribute to the pathogenesis of the chronic phase of *T. cruzi* infection, in which an exacerbated fibrotic response is a hallmark of heart disease. Future investigations should examine the possible role of TIMPs in chronic chagasic cardiomyopathy.

Pharmacological inhibition of MMPs has proved effective in limiting tissue damage after inflammation in various types of cardiac injury [31]. Doxycycline is a member of the tetracycline class of antibiotics and one of the most potent inhibitors of MMP activity [24, 32, 33]. To establish the role of MMPs in vivo, we treated *T. cruzi*–infected mice with doxycycline, at a dose known to reduce MMP activity and expression [27]. We found that doxycycline treatment increased the survival of *T. cruzi*–infected mice and reduced myocardial inflammation, but did not alter levels of parasitemia or tissue parasitism. The direct toxic or inhibitory effect of doxycycline on the parasite in vitro was excluded. Its beneficial effects could therefore be explained by several possible mechanisms, including (1) the inhibition of tissue infiltration by inflammatory cells, (2) effects on NO production, and (3) effects on cytokine expression. Although these mechanisms are not entirely dependent on MMP activity, the activation of MMPs by inflammatory mediators and cytokines is essential for tissue incursion of migratory inflammatory cells [34, 35]. Thus, influx of inflammatory cells in cardiac tissue during the acute phase of *T. cruzi* infection is likely to be facilitated by MMP activity. This hypothesis is supported by the increased expression and activity of MMP observed in the hearts of infected mice, as well as by the diminished number of cardiac-infiltrating inflammatory cells in mice treated with the MMP inhibitor doxycycline.

In addition to the reduction in the number of myocardial inflammatory cells, treatment with doxycycline resulted in less production of NO, probably because of inhibition of inducible NO synthase expression [36]. NO is an important effector molecule produced during *T. cruzi* infection [37], mainly through the generation of reactive species of oxygen and nitrogen (e.g., peroxynitrite) by macrophages [38]. However, excessive production of NO can also be responsible for myocardial tissue destruction [38, 39]. Therefore, treatment with doxycycline reduces NO and peroxynitrite synthesis, which could prevent parasite-induced cardiac lesions. In turn, less peroxynitrite results in less activation of MMP-2 and MMP-9 [40]. Doxycycline treatment was also associated with a significant reduction in the levels of IFN-γ and TNF-α, Th1 cytokines normally found in the myocardium of infected mice [41]. The reduction in these cytokines could be related to the reduced levels of the transcription factor T-bet, an important inducer of the Th1 cytokines [42], usually expressed in high amounts during *T. cruzi* infection. These changes in both the NO pathway and proinflammatory cytokines in the heart may be due to a reduced migration of inflammatory cells to the cardiac tissues, secondary to diminished MMP levels, or due to a direct transcriptional or posttranscriptional [43–45] effect of doxycycline that could affect the production of IFN-γ and TNF-α. Therefore, the reduction of NO and TNF-α, both related to myocardial dysfunction [39, 46], may also help increase survival in doxycycline-treated mice. Interestingly, doxycycline delayed the time to peak parasitism levels but did not affect the parasite load in the heart tissue, indicating that it had no effect on host cell invasion. Furthermore, experiments that used macrophages infected with the parasite revealed that doxycycline did not have a direct parasiticidal effect, nor did it change the ability of macrophages to kill *T. cruzi*. Taken together, our data suggest that doxycycline modulates cardiac inflammation without modulating parasite proliferation. This is of particular interest, because it counters the generally held belief that the development of myocarditis is required for control of *T. cruzi* growth in vivo. Future studies will determine the effect of doxycycline on the cardiac extracellular matrix and sarcomeric proteins during the chronic phase of the disease and the potential benefit of MMP inhibition therapy in human Chagas disease.

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Tick saliva induces regulatory dendritic cells: MAP-kinases and Toll-like receptor-2 expression as potential targets

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Ticks (Acari: Ixodidae) are a bloodsucking ectoparasitic arthropods of human and veterinary medical importance. Tick saliva has been shown to contain a wide range of bioactive molecules with vasodilatory, antihemostatic, and immunomodulatory activities. We have previously demonstrated that saliva from Rhipicephalus sanguineus ticks inhibits the maturation of dendritic cells (DCs) stimulated with LPS. Here we examined the mechanism of this immune subversion, evaluating the effect of tick saliva on Toll-like receptor (TLR) -4 signalling pathway in bone marrow-derived DCs. We demonstrated that R. sanguineus tick saliva impairs maturation of DCs stimulated with LPS, a TLR-4 ligand, leading to increased production of interleukin-10 and reduced synthesis of IL-12p70 and TNF-α. The immunomodulatory effect of the tick saliva on the production of pro-inflammatory cytokines by DCs stimulated with LPS was associated with the observation that tick saliva inhibits activation of the ERK 1/2 and p38 MAP kinases. These effects were independent of the expression of TLR-4 on the surface of DCs. Additionally, saliva-treated DCs also presented a similar pattern of cytokine modulation in response to other TLR ligands. Since the recent literature reports that several parasites evade immune responses through TLR-2-mediated production of IL-10, we evaluated the effect of tick saliva on the percentage of TLR-2 DCs stimulated with the TLR-2 ligand lipoteicoic acid (LTA). The data showed that the population of DCs expressing TLR-2 was significantly increased in DCs treated with LTA plus saliva. In addition, tick saliva alone increased the expression of TLR-2 in a dose- and time-dependent manner. Our data suggest that tick saliva induces regulatory DCs, which secrete IL-10 and low levels of IL-12 and TNF-α when stimulated by TLR ligands. Such regulatory DCs are associated with expression of TLR-2 and inhibition of ERK and p38, which promotes the production of IL-10 and thus down-modulates the host's immune response, possibly favouring susceptibility to tick infestations.

Keywords: Dendritic cells; tick saliva; Toll-like receptors; Rhipicephalus sanguineus; MAP kinases

Introduction

Rhipicephalus sanguineus, known as the brown dog tick, is found world-wide and transmits tick-borne diseases such as spotted and boutonneuse fever and ehrlichiosis in man, and babesiosis and ehrlichiosis in dogs (Flechtmann, 1973; Walker et al., 2000; Demma et al., 2006). These ticks have evolved mechanisms to impair the host immune response while successfully taking their blood meal. Upon attachment, ticks inoculate their saliva, which contains a great repertoire of compounds that facilitate its attachment, the blood-feeding process and transmission of tick-borne pathogens.

These tick saliva components include vasodilators, and anti-inflammatory, antihemostatic and immunosuppressive molecules (Ribeiro, 1995, Bowman et al., 1997; Valenzuela, 2004). Indeed, saliva of different tick species inhibits the function of neutrophils, hampers the complement system and activity of natural killer (NK) cells and macrophages, diminishes the production of cytokines, such as interleukin (IL)-12 and interferon-γ (IFN-γ), decreases T-cell proliferation and modulates chemokine activity and antigen-presenting cells, such as dendritic cells (DCs) (Ribeiro et al., 1990; Ribeiro, 1987; Kubes et al., 1994; Ramachandra and Wikel, 1992; 1995; Urioste et al., 1994; Ferreira and Silva, 1998; Hajnická et al., 2001, Cavassani et al., 2005, Vancová et al., 2007; Oliveira et al., 2008).

Recent studies have shown that saliva from several tick species modulates different steps of the biology of the dendritic cell. Saliva from R. sanguineus ticks inhibits the chemotactic function of MIP-1α and selectively impairs chemotaxis of immature dendritic cells by down-regulating cell-surface CCR5 (Oliveira et al., 2008), possibly due to a chemokine-binding protein named Evasin 1 (Frauenschuh et al., 2007). In addition, the inhibitory effects of saliva from Rhipicephalus sanguineus and Ixodes ricinus ticks upon differentiation, migration and antigen

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presentation by DCs has also been described (Cavassani et al., 2005; Skallová et al., 2008). In spite of this wealth of information, most of the mechanisms by which tick saliva delivers these effects have not been evaluated so far.

DCs are professional antigen-presenting cells that play a crucial role in determining adaptive immunity. Maturation of DCs begins when exogenous danger signals, such as pathogen-associated molecular patterns (PAMPs), bind to the appropriate Toll-like receptor (TLR) and trigger or suppress the immune response. This process occurs through defined mitogen-activated protein kinase (MAPK)-signalling pathways. Moreover, these events are also associated with the expression of TLRs on the surface of DCs'.
We demonstrated previously that saliva from R. sanguineus inhibits the maturation of DCs stimulated with LPS (TLR–4 ligand) (Cavassani et al., 2005).

Thus, the aim of this study was to investigate the mechanism of this immune subversion by evaluating if R. sanguineus tick saliva modulates expression of TLRs and MAPK-signalling pathways in DCs. Data presented herein provide a mechanistic insight for the contribution of TLR-2 and MAPK in saliva-induced modulation of the host inflammatory/immune response.

Materials and methods

Animals
Experimental animals C57BL/6 mice (6–8 weeks of age) and mongrel dogs (1–3 years old) were bred and maintained under standard pathogen-free conditions in the animal facilities of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto-SP, Brazil. All experiments were evaluated and approved by the Experimental Animal Ethics Committee (CETEA) of the School of Medicine of Ribeirão Preto (USP) and are in line with the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences.

Saliva collection
R. sanguineus ticks were laboratory-reared, as previously described by Ferreira & Silva, (1998). All ticks used for infestations were 1-3- month-old adults. To obtain engorged ticks for saliva collection, dogs (n=10) were infested with 70 pairs of adult R. sanguineus ticks restricted in plastic feeding chambers fixed to their backs. The saliva-collection procedure was performed using fully engorged female ticks (after 5-7 days of feeding) by inoculation of 10-15 µl of a 0.2% (v/v) solution of dopamine in phosphate-buffered saline (PBS), pH 7.4, using a 12.7 X 0.33 mm needle (Becton-Dickinson, Franklyn Lakes, NJ).

Saliva was harvested by using a micropipette, kept on ice, pooled, centrifuged through a 0.22 µm pore filter (Costar-Corning Inc., Cambridge, MA) and stored at -20oC for further use. Six pools of saliva were used for the experiments and each saliva pool consisted of material harvested from 100 to 200 female ticks. The saliva protein concentration was determined by using a bicinchoninic acid solution (Procedure TPRO-562; Sigma Chemical Co., St Louis, MO). The protein concentrations of the saliva's pools were very similar and found to be 815.6 µg/mL in media. Saliva was not diluted before added to the cell culture wells. In most experiments the final dilution of saliva in each well was 1:20, which corresponds to 40.7 µg of saliva protein /mL, with exception of the dose-dependent assays, where the final dilution is shown in the text and legends.

Antibodies and flow cytometric analysis
For cell staining, fluorescein isothiocyanate (FITC) or phycoerythrin (PE) –conjugated antibodies against murine CD11c, CD40, CD80, CD86, MHC II, TLR-2 and TLR-4 were used. TLR-2 (6C2) and TLR-4 (UT41) were purchased from eBioscience (San Diego, CA), while CD11c (HL3) was acquired from BD Biosciences (San Jose, CA) and CD40 (1C10), CD80 (1G10), CD86 (GL1), and MHC II (NIMR4) were purchased from Southern Biotechnologies (Birmingham, AL). Data acquisition was performed by using a FACscan flow cytometer with cellquest software (both Becton-Dickinson Immunocytometry Systems Inc., San Jose, CA). Appropriate isotype-matched irrelevant mAbs served as negative controls for each molecule and for each stimulus analyzed.

Results were expressed as the relative frequency (%) or the mean fluorescence intensity (MFI) obtained with specific antibodies within the studied gates. For the Western Blotting assays, rabbit polyclonal antibodies recognizing either the unphosphorylated forms of anti-extracellular signal-regulated kinase (ERK 1/2) or p38, or the double phosphorylated (Thr-202/Tyr-204) ERK 1/2 or (Thr-180/Tyr-182) p38 were used. The latter antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of bone marrow (BM)-derived DCs
BM-derived DCs were generated, as previously described by Lutz et al., (1999) with some modifications. Briefly, femurs and tibias were flushed with RPMI-1640 (Gibco-BRL Life Technologies, Grand Island, NY) to release the BM cells that were cultured in 24-well-culture plates in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 µg/mL of penicillin, 100 µg/mL of

streptomycin, 5 X 10-5 M 2-mercaptoethanol (all from Sigma) plus murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (30 ng/mL) and IL-4 (10 ng/mL) (Peprotech, Rocky Hill, NJ). On days 3 and 6 the supernatants were gently removed and replaced with the same volume of supplemented medium. On day 9, the non-adherent cells were removed and analyzed by flow cytometry using DCs surface markers, given that more than 80% of cells expressed CD11c.

Measurement of the expression of DC surface markers

To evaluate the effect of tick saliva on activation of antigen presenting cells and TLR surface expression, DCs were exposed for 24 hours to tick saliva (1:20) in the presence of TLR-2 and TLR-4 ligands (lipopolysacaride, LPS and lipoteicoic acid, LTA, respectively). After incubation, culture media was carefully collected and stored at –20°C for subsequent cytokine measurements and the DCs were collected for flow cytometric analysis. After incubating with anti-CD16/32 (Fc block) for 35 minutes on ice, DCs were washed with PBS and cultured with the following antibodies: FITC-conjugated anti-CD11c or -CD40 mAb, PE-conjugated anti-MHC II, -CD80, -CD86, TLR-2 or –TLR-4 antibodies for 45 min. After washing twice in PBS, cytometric and fluorescence data were acquired. In addition, the effect of tick saliva alone (1:20) on TLR-2 and TLR-4 expression was evaluated in 16, 24 and 48 hours after incubation.

Cytokine assays

Nine-day cultured BM-derived DCs were gently collected, washed twice, and resuspended at 106 cells/mL in complete medium. Cells were seeded at 106 cells/well in 24-well cluster plates (Costar, Corning Glass) and incubated with medium, saliva (1:20), TLR-4 (LPS, 1 μg/mL), TLR-2 (LTA, 10 μg/mL), TLR-3 (Poly I:C, 5 μg/mL), TLR-5 (flagellin, 1.5 μg/mL), TLR-9 (CPG-ODN 1826, 10 μg/mL) ligands with or without tick saliva (1:20). Measurements of IL-12p40, IL-12p70, TNF-α, IL-1, IL-10 and IL-6 were performed using specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). BD OptEIA ELISA sets were used according to manufacturer's instructions (BD Biosciences). None of the tested samples were thawed more than once.

Determination of phosphorylated forms of ERK and p38 MAPKs by Western Blot analysis

Activated forms of ERK 1/2 (p42/44) and p38 MAPKs were detected by Western Blot analysis using antibodies to the phosphorylated forms of these kinases. Extracts from untreated cells (controls) and from cells cultured with saliva, LPS or LPS plus saliva (15, 30 or 60 min of incubation) were made by sonication in ice-cold RIPA buffer (150 mM NaCl, 0.1 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) containing a mixture of protease inhibitors and the phosphatase inhibitor sodium orthovanadate (Sigma, 1 mM). For Western Blot analysis, 30 μg cell extract proteins were electrophoresed in a SDS polyacrylamide gel 12% and electro blotted onto a nitrocellulose filter. Blots were blocked overnight at 4oC in PBS containing 0.2% Tween 20 and 5% low fat milk. They were then incubated overnight at 4oC with rabbit polyclonal antibodies recognizing either the phosphorylated or unphosphorylated forms of ERK or p38. Blots were then washed five times and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Groove, PA). After further washing, the blots were developed with the Enhanced Chemiluminescence's Detection ECL-kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL). Bands of ERK MAPK or p38 MAPK were visualized after exposing the blots to a Kodak RX film.

Statistics

Significant differences between saliva-treated and control groups were determined by analysis of variance (ANOVA) followed by post-hoc analysis with the Tukey-Kramer test (INSTAT software; GraphPad, San Diego, CA).

Results

Saliva from R. sanguineus ticks induces production of interleukin-10 and inhibits production of IL-12p70 and TNF-α in DCs stimulated with LPS

In order to carry out their main role of initiating a primary immune response, immature DCs must be stimulated for maturation and a widely used stimulus that induces maturation of these cells is bacterial LPS. To evaluate the effect of tick saliva on LPS-matured DCs, we exposed BM-derived DCs to LPS (1 μg/mL) for 24 h in the presence or absence of tick saliva (1:20), and measured the production of IL-12 p40 and p70, IL-10, IL-1, IL-6 and TNF-α by ELISA. As shown in figure 1, saliva led to a decrease of 36.1, 63.6 and 26.2% in the production of IL-12 p40, IL-12p70 and TNF-α, respectively, by DCs stimulated with LPS ($P<0.05$ and $P<0.01$, respectively, compared to LPS only). As previously published (Harizi et al., 2002), anti-inflammatory cytokines such as IL-10 are also produced in low quantities by DCs stimulated with LPS. However, when tick saliva was added, the effect of LPS was enhanced by 72.9% ($P<0.01$) (Fig. 1D). Interestingly, tick saliva did not modulate the production of the LPS-induced IL-1 and IL-6 cytokines (Fig. 1E and F). DCs cultured in the presence of saliva alone did not present a significant

difference in the production of any cytokine, when compared to immature DCs cultured with medium only (Fig. 1). To eliminate the possibility that saliva had contaminating LPS, which could affect cytokine production, we quantified the level of LPS in saliva by using the LAL (limulus amoebocyte lysate) assay (Sigma).

The LAL assay demonstrated that the amount of tick saliva used in the cell culture assays (1:20) contained only 0.006 ng/mL of LPS. Moreover, stimulation of DCs with this quantity of LPS did not induce increased levels of inflammatory or anti-inflammatory

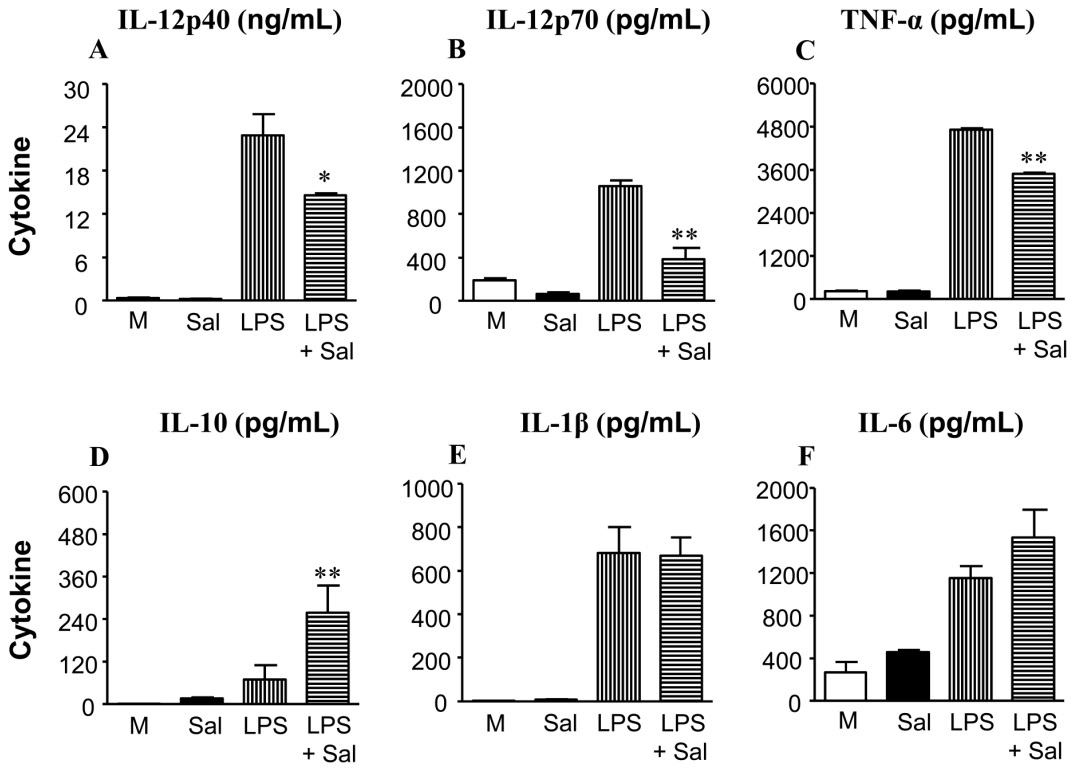

Figure. 1. Tick saliva induces interleukin-10 and inhibits IL-12 and TNF-α production in DCs stimulated with LPS. BM-derived cells (2.5 × 106/well) from C57BL/6 mice were differentiated for 9 days in the presence of GM-CSF (30 ng/mL) and IL-4 (10 ng/mL). On day 9, when ≈ 80% of the cells were CD11c, maturation was induced by the addition of LPS (1 µg/mL) in the presence or absence of saliva (1:20). In addition, these cells were cultured with saliva alone (1:20) or with medium. After 24 h of incubation, the supernatants were collected and assayed by ELISA for IL-12p40 (A), IL-12p70 (B), TNF-α (C), IL-10 (D), IL-1β (E) and IL-6 (F) (*: P <0.05 and **: P <0.01 respectively, compared to LPS alone). Results are presented as the mean of duplicate cultures ± SD. The figure is representative of three independent experiments.

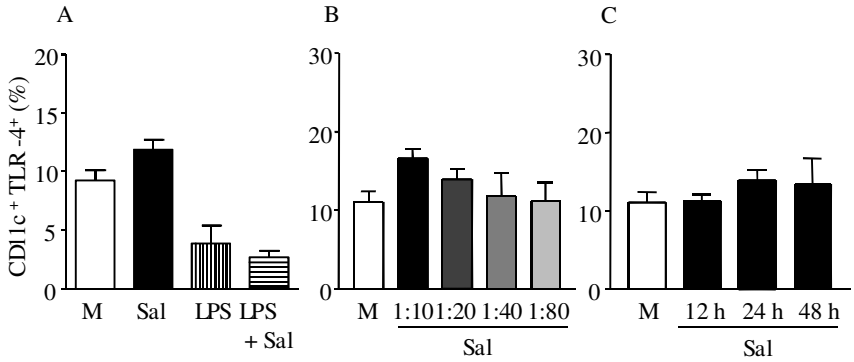

Figure. 2. Tick saliva does not modulate the percentage of CD11cTLR-4 DCs stimulated with LPS. BM-derived cells (2.5 × 106/well) from C57BL/6 mice were differentiated for 9 days in the presence of GM-CSF (30 ng/mL) and IL-4 (10 ng/mL). On day 9, when ≈ 80% of the cells were CD11c, DCs were exposed to LPS (1 μg/mL) in the presence or absence of saliva (1:20) for 24 h (A). In addition, DCs were exposed to different saliva concentrations (1:10, 1:20, 1:40 and 1:80) (B) or different periods of time with saliva 1:20 (16, 24 and 48 h) (C). Cells were then harvested, labeled with TLR-4 monoclonal antibody and analyzed by flow cytometry for the expression of this receptor gated on CD11c cells. Results are expressed as the bar graphs of the percentage of CD11c TLR-4 cells ± SD. The results shown are representative of one of three independent experiments.

cytokines, neither did it induce expression of co-stimulatory molecules related with DCs maturation (data not shown).

Tick saliva does not impair the effect of LPS upon expression of TLR-4 by DCs

Knowing that saliva-treated DCs present a diminished cytokine response to LPS (a ligand that works through TLR-4), we sought to address whether tick saliva could have a direct effect upon expression of TLR. As demonstrated in figure 2, tick saliva did not alter the expression of TLR-4 on the surface of DCs stimulated with LPS, even after 48 h. In addition, tick saliva alone also did not present any effect (Fig. 2).

LPS-induced p38 and ERK phosphorylation is reduced in saliva-treated DCs

Activation of the MAPK signalling pathway is an important event underlying maturation of DCs (Rescigno et al., 1998). Furthermore, stimulation of DCs with LPS has been shown to activate MAPK signalling pathways in DCs (Arrighi et al., 2001; An et al., 2002). To gain insight into the signalling events induced by saliva, we determined whether two components of the MAPK pathway, p38 and ERK, might be involved in these pathways in DCs. BM-DCs were treated with medium, saliva (1:20), LPS (1 μg/mL) or LPS plus saliva (1:20), as described in the materials and methods section. As expected, LPS induced the phosphorylation of ERK and p38 MAPKs (Fig. 3).

However, the treatment of LPS plus saliva significantly inhibited the LPS-induced p38 (reduction of 60.5% in 30 min) and ERK phosphorylation (reduction of 43.1% and 16.1% in 30 and 60 min, respectively). In addition, saliva alone did not induce significant phosphorylation of ERK and p38 MAP kinases in the different points analyzed (Fig. 3 and data not shown). These results indicate that tick saliva possibly inhibits LPS-induced maturation by suppressing the MAPKs signal pathway.

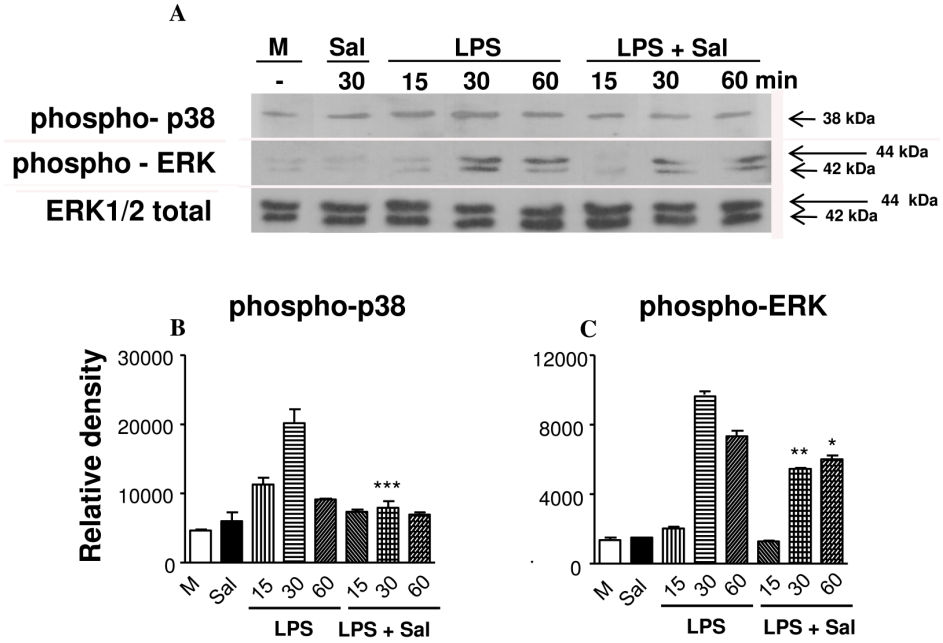

Figure. 3. Tick saliva decreased the activation of MAPKs in DCs stimulated with LPS. BM- derived cells (2.5 x 106/well) from C57BL/6 mice were differentiated for 9 days in the presence of GM-CSF (30 ng/mL) and IL-4 (10 ng/mL). On day 9, when ≈ 80% of the cells were CD11c, DCs were exposed to medium, saliva (1:20), LPS (1 μg/mL) or LPS plus saliva in different points (15, 30 and 60 min). The cells lysates were prepared and blotted with anti-phospho-ERK1/2, anti-ERK1/2 and anti-phospho-p38 antibodies (A). The degree of staining of the Western Blot p-p38 and ERK1/2 signal was measured by quantitative analysis of overlapping pixels by using Image J version 1.32j software (NIH, Bethesda, MD, USA) (B and C respectively). Results of the relative density ± SD (***: P <0.001, **: P <0.01 *: P <0.05 respectively, compared to LPS 30 and LPS 60 min) are shown. The results shown are representative of one of two independent experiments.

Effect of tick saliva on DCs stimulated with TLR-2, -3, -5 and -9 ligands

LPS, which activates TLR-4, one of the 13 known mammalian TLRs, is the most commonly used ligand for the maturation of DCs in vitro. As we have demonstrated before, R. sanguineus tick saliva inhibits production of cytokines and expression of specific surface markers of LPS-matured DCs (Fig. 1 and Cavassani et al., 2005). Nevertheless, the effect of R. sanguineus tick saliva on maturation of DCs by other TLR ligands, such as LTA, Poly I:C, flagellin and CpG-DNA (respectively, ligands for TLR-2, TLR-3, TLR-5 and TLR-9) remains unclear. To evaluate the effect of these TLR ligands on maturation of DCs we exposed BM-derived DCs to LTA (10 μg/mL), Poly I:C (5 μg/mL), flagellin (1.5 μg/mL) and CpG-ODN-1826 (10 μg/mL) for 24 h in the presence or absence of tick saliva (1:20), and measured the secretion of cytokines (IL-12p70, IL-10, IL-1, IL-6 and TNF-α) by ELISA and markers of cell co-stimulation by flow cytometry. Purified TLR agonists LTA, Poly I:C, flagellin or CpG-ODN induced different levels of IL-12p70, IL-1β, IL-6 and TNF-α on DCs (Fig. 4A). When saliva was added to the culture, a significant ($P<0.05$ and $P<0.01$) inhibition of LTA- or Poly I:C-induced production of IL-12p70 by DCs was observed (reduction of 50.1% and 58.3%, respectively), whereas CpG-induced production of IL-12p70 was only marginally inhibited (Fig. 4A). In addition, saliva strongly up-regulated TLR ligand-induced IL-10 cytokine production by DCs stimulated with LTA and Poly I:C (more than a 9.5 and 5.3-fold increase, respectively), but it induced only slight production of IL-6 cytokine in DCs stimulated with TLR-3 and TLR-5 ligands (Fig. 4B and D). As for LPS, saliva significantly inhibited TNF-α production (reduction of 29.4%) in DCs matured with CpG-ODN, but no differences were observed in DCs stimulated with LTA, Poly I:C or flagellin (Fig. 4C). Concerning co-stimulatory molecules, tick saliva did not interfere with expression of CD40 and CD80 but inhibited that of CD86 on DCs stimulated with all TLR ligands tested (data not shown).

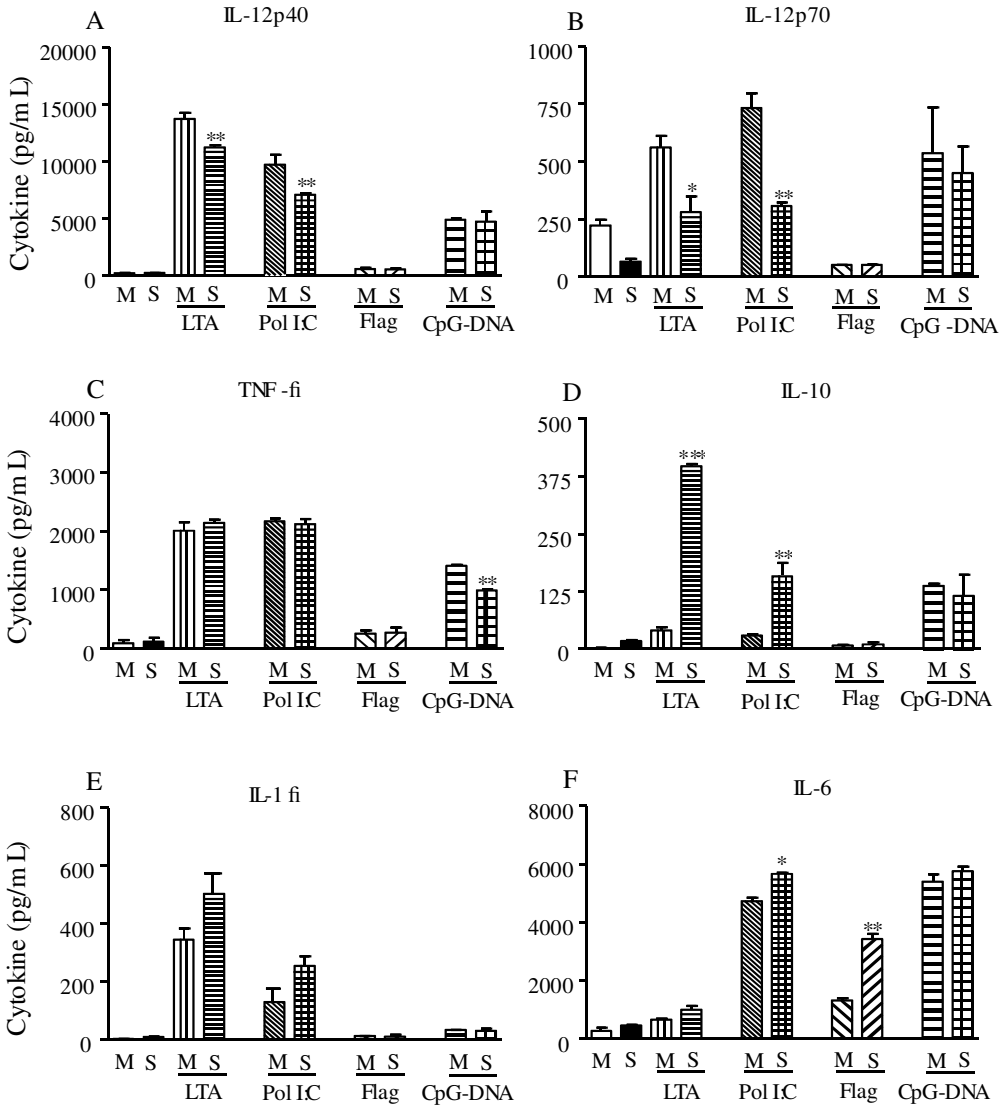

Figure. 4. Effect of tick saliva on DCs stimulated with TLR-2, -3, -5 and -9 ligands. BM-derived cells (2.5 × 106/well) from C57BL/6 mice were differentiated for 9 days in the presence of GM-CSF (30 ng/mL) and IL-4 (10 ng/mL). On day 9, when ≈ 80% of the cells were CD11c, maturation was induced by the addition of lipoteicoic acid (LTA), Poly I:C (Pol I:C), flagellin (Flag) and CpG-ODN-1826 (CpG-DNA) in the presence or absence of saliva (S) (1:20). In addition, these cells were cultured with saliva alone (S) (1:20) or with medium (M). After 24 h of incubation, the supernatants were collected and assayed by specific ELISA for IL-12p40 (A), IL-12p70 (B), TNF-α (C), IL-10 (D), IL-1β (E) and IL-6 (F) (*: P <0.05, **: P <0.01 and ***: P <0.001 respectively, compared to LTA, Poly I:C, flagellin or CpG-ODN treated DCs). Results are presented as the mean of duplicate cultures ± SD. The figure is representative of three independent experiments.

Tick saliva induces the expression of TLR-2 of DCs in a dose and time-dependent manner

TLR-2 has been widely implicated in immune evasion by several parasites (Sing et al., 2002; Netea et al., 2004; Ferreira et al., 2007). Therefore, we evaluated the effect of tick saliva on expression of TLR-2 receptors on DCs stimulated or not with LTA (TLR-2 ligand). Tick saliva increased significantly the frequency of CD11c/TLR-2 DCs (increase in 29.1%) in LTA-treated DCs. Moreover, addition of saliva alone to the cell culture almost doubled the percentage of CD11c/TLR-2 DCs compared to control cells (Fig. 5A). This increase happened in a time and concentration dependent manner. Up-regulation of TLR-2 was only marginally detectable at the concentration of 1:40, and reached the higher levels at a concentration of 1:10, when it increased the number of DCs expressing TLR-2 by 48.8% when compared to medium only (Fig. 5B). The enhancement of TLR-2 expression was already observed by 16 h (62.0% compared with medium only) and remained significant after 24 h (Fig. 5C).

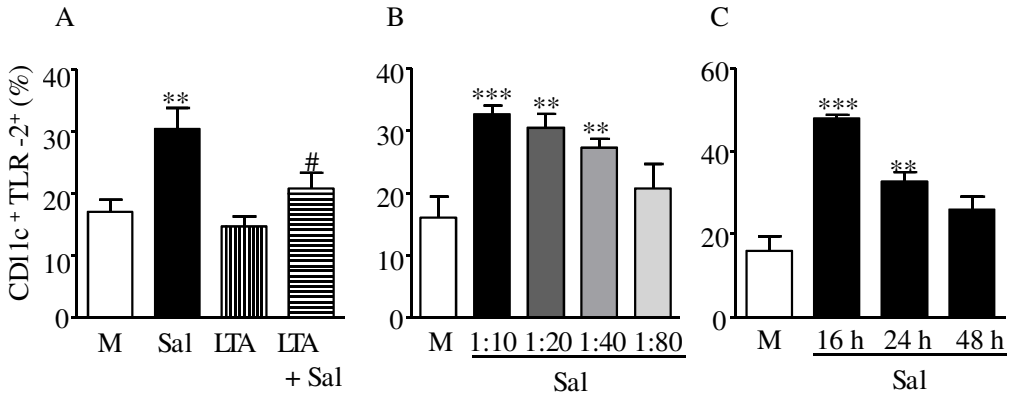

Figure. 5. Tick saliva up-regulates the expression of TLR-2 on the surface of DCs in a time and concentration-dependent manner. BM-derived cells (2.5 × 106/well) from C57BL/6 mice were differentiated for 9 days in the presence of GM-CSF (30 ng/mL) and IL-4. On day 9, when ≈ 80% of the cells were CD11c, DCs were exposed to LTA in the presence or absence of saliva (Sal) for 24 h (A). In addition, DCs were exposed to saliva only, in different concentrations (saliva 1:10, 1:20, 1:40 e 1:80) (B) or periods of time with saliva 1:20 (16, 24 and 48 h) (C). Cells were then harvested, labeled with TLR-2 monoclonal antibody and analyzed by flow cytometry for the expression of this receptor gated on CD11c cells. Results are expressed as the bar graphs of the percentage of CD11c TLR-2 cells ± SD (#: P <0.05 compared to LTA, **: P <0.01 and ***: P <0.00.1 respectively, compared to medium). The results shown are representative of one of three independent experiments.

Discussion

DCs are potent antigen-presenting cells with the ability to initiate both innate and antigen-specific adaptive immunity. Hence, these cells have been the focus of extensive investigations inveterinary and biomedical studies. Located in tissues like the skin, DCs act as immune sentinels for infectious agents and inflammatory products of pathogens. Immature DCs can capture antigens in peripheral tissues and undergo maturation processes to stimulate naive T cells in secondary lymphoid organs.

Here we demonstrate how saliva from R. sanguineus ticks modulates the phenotypic and functional maturation of DCs through a signalling pathway used by the TLRs. Our results show that R. sanguineus tick saliva impairs maturation of DCs stimulated with LPS (a TLR-4 ligand), leading to increased interleukin-10 production and reduced synthesis of IL-12p70 and TNF-α. This profile of cytokine production was accompanied by inhibition of the expression of co-stimulatory molecules CD40, CD80 and CD86 in DCs (data not shown). These results are of great interest since IL-12 and TNF-α can improve functional maturation of DCs and can selectively stimulate their capacity to induce the development of T helper 1 type responses, while IL-10 is widely known to be an immunosuppressive cytokine (Trinchieri, 1995; D'Andrea et al., 1993; Filippi and von Herrath, 2008). Our observations are in accordance with data showing that saliva from the R. sanguineus tick inhibits IL-12 production and CD40, CD80 and CD86 expression by murine BM–derived DCs in vitro and prevents the polarization of naïve T cells (Cassavani et al., 2005). Additionally, R. sanguineus tick saliva also modulated cytokine production by splenocytes stimulated with Trypanosoma cruzi, inducing IL-10 and inhibiting IL-12 cytokine production (Ferreira and Silva, 1998; 1999). Saliva from I. scapularis ticks also was shown to inhibit IL-12 and TNF-α production by murine BM–derived DCs, as well as to decrease the expression of some co-stimulatory molecules (Sá-Nunes et al., 2007). These authors suggest that prostaglandin E2 (PGE2) present in I. scapularis saliva is the major in vitro inhibitor of maturation and function of DCs. Very recently, it was also demonstrated that the administration of I. ricinus tick saliva in vivo significantly inhibited maturation and early migration of DCs from inflamed skin to draining lymph nodes, and decreased the capacity of lymph node DCs to present soluble antigens to specific T cells (Skallová et al., 2008).

An alternate mechanism used by ticks to inhibit TLR-4 stimulation could be the production of decoy receptors (molecules that bind to a ligand, inhibiting it from binding to its normal receptor) in saliva. It has been shown that mites produce an allergen protein, Der p 2, which has similarity with MD-2, an LPS-binding protein with a ML domain essential for the recognition of LPS by TLR-4 (Keber et al. 2005). Also, I. ricinus and Rhipicephalus (boophilus) microplus ticks seem to produce similar compounds, since salivary glands cDNA libraries present clones with similar sequences to ML domain-containing proteins (Rudenko et al., 2005; I.K.F. de Miranda Santos, unpublished data). Future studies must be

done to test this possibility.

The response of immune cells to PAMPs is correlated to some extent with the level of expression of TLRs. It has been shown that TLR4 over-expression amplifies the host response to LPS (Bihl et al., 2003). Conversely, a decline in expression and function of TLR may account for the increased susceptibility to infections and poor adaptive immune responses in aging (Renshaw et al., 2002; Boehmer et al., 2004). One example is that down-regulation of TLR-1, 2, 4, and 9 was shown to be an important mechanism of immune evasion in filarial infections (Babu et al., 2005).
To assess the contribution of TLR-4 on the regulation of cytokine production and co-stimulatory molecule expression in LPS-treated DCs, we examined the expression of TLR-4 on DCs surface.

Our data demonstrate that tick saliva does not inhibit maturation of LPS-treated DCs by modulation of TLR-4 surface expression. Saliva possibly can operate through other cell receptors, or can even hamper some intracellular signalling pathway.

The molecular basis of the intracellular signal transduction pathway inhibited by tick saliva is not understood, mainly due to the lack of the identification of specific cell surface receptors that bind tick saliva, as well as the mechanisms how this saliva could alter cytoplasm signaling pathways induced by established stimulus (i.e. LPS). MAPKs have received increased attention as target molecules for mature DC-related modulation (Agrawal et al., 2003; Nakahara et al., 2006). It has been reported that activation of the MAPK pathways may cause induction of phase II detoxifying enzymes, and blockage of MAPK pathways may inhibit AP-1- and/or NF-κB-mediated gene expression (Ninomiya-Tsuji et al., 1999). MAPK pathway consists of a three-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (ERK, JNK, and p38), resulting in the activation of NF-κB, cellular growth and survival and production of cytokines. In summary, the stimulation of TLRs leads to the activation of several MAPK pathways (Agrawal et al., 2003). In this study we demonstrate for the first time that tick saliva impairs maturation of murine BM-derived DCs probably by the inhibition of phosphorylation of ERK and p38 MAP kinases. As expected, LPS induced the phosphorylation of ERK and p38 MAP kinases, however, the addition of saliva significantly inhibited the LPS-induced ERK 1/2 and p38 phosphorylation. Many findings are compatible with the proposition that modulation of MAPK pathway by parasites or their products may help them to subvert the host's immune responses. For example, Leishmania donovani infection down-regulates TLR-induced production of IL-12p40 and activates production of IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathway (Privé and Descoteaux, 2000; Chandra and Naik, 2008). Well characterized compounds also can impair the function of DCs by interfering with MAPK. Rosmarinic acid down-regulates the LPS-induced production of MCP-1 and MIP-1α chemokines via the MAPK pathway in BM-DCs (Kim et al., 2008). Parthenolide, an anti-inflammatory drug, inhibits LPS- but not TNF-alpha-induced maturation of human monocyte-derived DCs by inhibiting the p38 kinase pathway (Uchi et al., 2002). Moreover, a recent work showed that D. variabilis tick salivary gland extract and saliva regulates fibroblast migration by suppressing ERK signalling (Kramer et al., 2008).

In order to test the possibility that saliva modulates different TLRs, other than TLR4, we stimulated DCs with LTA, Poly I:C, flagellin and CpG-DNA (TLR2, TLR3, TLR5 and TLR9 ligands, respectively) in the presence or absence of tick saliva. Saliva enhanced significantly the production of IL-10 by LTA and Poly I:C-stimulated DCs, while it reduced synthesis of IL-12p70.

These results demonstrate that tick saliva is capable to subvert the immune response mediated by different PAMPs. Furthermore, ticks may evade the host protection by multiple mechanisms, since different antigens can induce diverse patterns of immune responses. Our findings with R. sanguineus tick saliva are in accordance with other studies that show that I. scapularis and I. ricinus tick saliva can modulate activation of DCs by different TLR-ligands (Sá-Nunes et al., 2007; Skallová et al., 2008).

Given that stimulation of TLR-2 results in production of IL-10 (Pulendran, 2005), our results suggest a possible mechanism to explain a finding in common for various species of ticks, namely that they induce production of IL-10 (Ferreira and Silva, 1999; Schoeler et al., 1999; Mejri et al., 2001). Other parasites/microbes like Schistosoma mansoni, Yersinia enterocolitica, Candida albicans, Bifidobacterium breve, Paracoccidiodes brasiliensis and L. donovani exploit TLR-2–mediated release of IL-10 to induce immunosuppression (van der Kleij et al., 2002; Sing et al., 2002; Netea et al., 2004; Hoarau et al., 2006; Ferreira et al., 2007; Chandra and Naik, 2008).

Our experiments also showed that tick saliva alone increased, in a time and concentration-dependent manner, the percentage of CD11c DCs expressing TLR2 on their surface, possibly leading DCs towards a more suppressive pattern. Furthermore, tick saliva up-regulated expression of TLR-2 molecules on the

surface of DCs. Similar results have been found for the fungus P. brasiliensis, which induced high expression of the gene for TLR-2 and production of IL-10 in susceptible, but not resistant mice after infection (Ferreira et al., 2007).

To our surprise, tick saliva also induced expressive production of IL-6 by DCs stimulated with TLR-3 and TLR-5 ligands. IL-6, a pleiotropic cytokine produced in response to a wide range of inflammatory stimuli, including intracellular infection, is usually considered to be a pro-inflammatory factor (Diehl and Rincon, 2002; Kishimoto, 2005). However, IL-6 has also been reported to down-regulate inflammatory mechanisms and impair macrophage activation and antimicrobial effects to Mycobacterium avium and Toxoplasma gondii (Bermudez et al., 1992; Beaman et al., 1994). In addition, Murray (2008) showed that there was an accelerated control of visceral L. donovani infection in IL-6-deficient mice. Interestingly, IL-6 also directs the differentiation of T helper type 2, but not type 1 cells (Rincón et al., 1997). This mechanism could explain why successive infestations of mice with R. sanguineus polarize the immune response towards a type 2 response (Ferreira and Silva, 1999; Mejri et al., 2001; Skallová et al., 2008). Based on these contributions, we suggest that increased secretion of IL-6 and IL-10 by DCs in response to saliva may be part of a negative feedback regulatory mechanism that may limit the inflammatory/immune response to ticks. In summary, our results demonstrate that R. sanguineus tick saliva suppresses TLR-stimulated production of IL-12p70, while at the same time it increases production of IL-10 by DCs.

This immunomodulatory effect probably is related to the fact that tick saliva modulates the TLR-stimulated MAPK pathway by suppressing MAPK p38 and ERK1/2 phosphorylation. Moreover, tick saliva induces in a dose and time-dependent manner the expression of TLR-2 in BM-derived DCs. The increase in TLR-2 expression could be a mechanism used by ticks to induce suppressive DCs, once TLR-2 activation may stimulate high production of IL-10. These observations suggest that ticks possibly have evolved survival strategies that subvert the host pro-inflammatory/immune response through TLRs.

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Nitric oxide donor *trans*-[RuCl([15]aneN$_4$)NO]^2 as a possible therapeutic approach for Chagas disease

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Running title: NO donor therapy for Chagas' disease

Background and purpose: Benznidazole is the therapy currently available for clinical treatment of Chagas disease. However, many strains of *T. cruzi* parasites are naturally resistant. In addition, the effectiveness of this drug has not been demonstrated during the chronic phase of disease (at which the most cases are detected). Nitric oxide (NO) is produced by activated macrophages and is crucial to the intracellular killing of parasites. Here we investigated the *in vitro* and *in vivo* activities of trans-[RuCl([15]aneN$_4$)NO]^2 (NO donor) against partially drug-resistant *T. cruzi* Y strain. **Experimental approach:** Mice were treated in the acute phase of Chagas disease and the anti-*T. cruzi* activity evaluated by parasitemia, survival rate, cardiac parasitism, myocarditis and cure rate.
Key results: We showed that the NO donor *trans*-[RuCl([15]aneN$_4$)NO]^2 is more potent than benznidazole *in vitro* and *in vivo,* since it induced 100% of trypanocidal activity at a 100 times lower dose. The treatment of mice using trans-[RuCl([15]aneN$_4$)NO]^2 at 3,33 µmol/Kg/day by 20 consecutive days suppressed the parasitemia, protected 100% of the animals from mortality, and reduced inflammation and cardiac parasitism. Bz and NO donor when administrated alone generated 40% and 30% of parasitological cure, respectively. However, when administrated together, 80% of treated animals were considered cured. The cured animals showed absence of myocarditis and normalization of cytokine production in the sera. In addition, no *in vitro* toxicity was observed at the tested doses.
Conclusions and implications: These findings make *trans*-[RuCl([15]aneN$_4$)NO]^2 a promissory drug for testing in human Chagas' disease therapeutics.
Key words: NO donors, *trans*-[RuCl([15]aneN$_4$)NO]^2, benznidazole, *Trypanosoma cruzi*, Chagas' disease.

Introduction

Trypanosoma cruzi is an intracellular protozoan able to cause human disease and constitutes a major cause of heart disease in Latin America. Chagas disease affect 13 million people in Central and South Americas, and 75 million are at risk of infection (WHO, 2005). Moreover, in non endemic countries as Australia, Canada, Spain, and the United States, transmission through transfusions and transplants represents a public health concern (Schmunis, 2007). The current anti-parasitic chemotherapy with Bz (Rochagan and Rodanil; Roche, Brazil) or nifurtimox (Lampit; Bayer, Germany) is relatively efficacious in the acute and sub-chronic stages of Chagas disease (Altclas *et al.*, 2005; Cancado, 2002; Russomando *et al.*, 1998; Sosa Estani *et al.*, 1998). The adequacy of anti-parasitic treatment for patients in the indeterminate and chronic stages of the disease has not been confirmed. Some non-randomized studies suggested that trypanocidal treatment was associated with negativation of serologic tests and prevention of clinical and ECG worsening (Fabbro De Suasnabar *et al.*, 2000; Lauria-Pires *et al.*, 2000; Viotti *et al.*, 1994; Viotti *et al.*, 2006), while others yielded inconclusive results (Lauria-Pires *et al.*, 2000). However, the treatment with Bz is unsatisfactory in the chronic phase of the disease. Therefore the development of new drugs is necessary.

Parasite elimination largely depends on the production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-12, as they act in concert to activate macrophages to kill the intracellular parasite through the production of nitric oxide (NO) and its derived nitrogen and oxygen radicals (Aliberti *et al.*, 1999; Aliberti *et al.*, 2001; Machado *et al.*, 2000). Studies using experimental models of the acute infection have demonstrated that the anti-parasitic activity of benznidazole involves the participation of these cytokines (Michailowsky *et al.*, 1998; Molina *et al.*, 2000), as well as covalent modifications of macromolecules by nitro reducers intermediates (reductive stress) (Docampo, 1990). Conversely, nifurtimox acts via the reduction of the nitro group to unstable nitro anion radicals, which in turn react to produce highly toxic reduced oxygen metabolites

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(superoxide anion and hydrogen peroxide) (Docampo, 1990).

However, both drugs havesignificant side effects, including anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy (Rassi *et al.*, 1999). Moreover, several parasite strains do not respond to these treatments, even during the acute phase of the disease (Filardi *et al.*, 1987; Galvao *et al.*, 1993; Urbina, 1999). The rate of cure observed in patients with these drugs is 50-70% in acute phase and 0-20% during chronic phase (Guedes *et al.*, 2006). This situation is severely aggravated by the absence of a diagnostic gold standard, which turns debatable all the cure parameters used for evaluating the outcome of trypanocidal therapies. Thus, there is an imperative requirement for the development of novel therapeutic agents for Chagas disease.

Ruthenium NO donors compounds have recently emerged as an interesting and important alternative treatment to *T. cruzi* experimental infection (Silva *et al.*, 2007). These compounds showed *in vitro* and *in vivo* low toxicities and stability in aqueous media in the presence of oxygen and NO released by reducing agents present in the host environment (Bogdan, 2001; Silva *et al.*, 2007). Our group recently reported the trypanocidal activity *in vitro* and *in vivo* of a series of ruthenium nitrosyls, *trans*-$[Ru^{II}(NO)(NH_3)_4L]X_3$, L=imidazole (imidazole coordinated by nitrogen (imN) or imidazole coordinated by carbon (imC), pyridine (py), L-histidine (L-hist), sulphite (SO_3^{2-}), pyrazine (pz), nicotinamide (nic), 4-picoline (4-pic), triethylphosphite ($[P(OEt)_3]$), isonicotinamide (isn), isonicotinic acid (ina), X=BF_4^-, Cl^- or PF_6^-, and $[Ru^{II}(NO)(Hedta)]$ against the Y strain of *T. cruzi*. Such compounds were efficient in reducing the parasitemia, cardiac inflammation and also allowed total survival of treated infected mice (Silva *et al.*, 2007). However, the therapeutic schedule was used only for 15 days of treatment and it did not focus on the evaluation of parasitological cure. Here we used the new and more potent NO donor, *trans*-$[RuCl([15]aneN_4)NO]^2$ complex, ($[15]aneN_4$ = 1,4,8,12-tetraazacyclopentadecane, a macrocyclic quadridentate amine ligand) in a therapeutic schedule of 20 days of treatment in mice infected with the Y strain of *T. cruzi.* We evaluated the parasitological cure of mice treated with *trans*-$[RuCl([15]aneN_4)NO]^2$, and compared it to benznidazole, or the association of both drugs. Benznidazole and *trans*-$[RuCl([15]aneN_4)NO]^2$ administrated alone generated 40% and 20% of parasitological cure, respectively. However, when administered together, 80% of treated animals were considered cured. This study provides evidence that NO donors could be helpful to improve the efficacy of current trypanocidal drugs, reducing the time of treatment and thus, preventing the adverse reactions. These finding were associated with reduced or absent cardiac damage during the acute phase of *T. cruzi* infection. Thus, association of *trans*-$[RuCl([15]aneN_4)NO]^2$ can constitute a promissory therapeutic avenue that could be explored as an alternative for the treatment of Chagas' disease.

Material and methods

Parasite and experimental infection

The Y strain of *T. cruzi* was used in all experiments (Silva *et al.*, 1953). Mice were infected by intra peritoneal route with 1×10^3 blood trypomastigotes forms of *T. cruzi.* Culture trypomastigotes were obtained from a fibroblast cell line (LLC-MK_2) epimastigotes forms were obtained at the exponential phase of growth in LIT (Liver Infusion Tryptose) medium.

Drugs

Benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide, Roche Company) and *trans*-$[RuCl([15]aneN_4)NO]^2$ (look further for synthesis procedure) were used. Ascorbic acid was used as reducing agent to *trans*-$[RuCl([15]aneN_4)NO]^2$ conduced NO release.

Synthesis of the ruthenium compound (trans-$[RuCl([15]aneN_4)NO]^2$)

The synthesis of *trans*-$[RuCl([15]aneN_4)NO]^2$ was prepared in accordance with the procedure published by Bonaventura et al. (Bonaventura *et al.*, 2004). Chemical structure of *trans*-$[RuCl([15]aneN_4)NO]^2$ is represented in Fig. 1.

Nitric oxide measurement in bone marrow-derived macrophages (BMDM)

Bone marrow-derived macrophages (BMDM) were generated from bone marrow stem cells cultured as previously described (Celada *et al.*, 1984). Then, the cells were loaded with the selective NO fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA) (10 µM) for 30 minutes, at room temperature (Rodrigues *et al.*, 2008). The membrane permeable DAF-2 DA readly enters the cells and is subsequently hydrolyzed by cytosolic esterases releasing free DAF-2, which does not leak into the medium. At physiological pH, DAF-2 is relatively non-fluorescent; however, in the presence of NO and oxygen, it forms DAF-2 triazole (DAF-2T), a fluorescent product. This approach allows the direct visualization and semi-quantitative analysis of the basal NO availability at the cell level. The dye excess was removed by washing out the dye with a bath solution. The cytosolic NO concentration was assessed by a confocal scanning laser microscope (Leica TCS SP5). DAF-2T fluorescence was excited with the 488 nm line of an argon ion laser and the emitted fluorescence was measured at 515 nm. Time course software was used to capture images of the cells at 2-second intervals in the Live Data Mode acquisition. By applying the LSCM computer software, the intensities of the intracellular maximum or minimum fluorescence were measured. From these data, the basal fluorescence intensity (FI) value (Basal) was registered at time zero of the experiment of the experiment. The final FI value (15-ane) was registered at 300 s after *trans*-$[RuCl([15]aneN_4)NO]^2$) (1 mM) addition to the medium.

Culture of RAW 264.7 macrophages and luciferase assays

The murine RAW 264.7 Luc macrophage, bearing the luciferase vector inserted in the NF-κB promoter (pNF-κB-Luc), was routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin with incubation at 37°C in a humidified atmosphere of 5% CO_2. For luciferase reporter assays, RAW 264.7 macrophages (5×10^5 cells/mL) were grown in 24-well plates to 60-70% confluence. After cultured for 24 h, cells were stimulated with LPS (1 µg/ml) following addition of *trans*-$[RuCl([15]aneN_4)NO]^2$ (1.0, 0.5 and 0.1 mM), AA (1.0, 0.5 and 0.1 mM), and Bz (1.0 mM) to the growth medium. Cells were treated at different drug concentrations and kept overnight under incubation conditions. Cells were harvested, and extracted in lysis buffer (TNT) and the luciferase activities in the cell lysates determined using the Dual Luciferase Reporter assay system (Promega). Data were then expressed as a ratio of light units.

Surface molecule expression and cytotoxicity of treated macrophage

The expressions of co-stimulatory molecules (CD40, CD80, CD86) and MHC-II were assessed in bone marrow-derived macrophages (BMDM) after being incubated during 24 h with *trans*-$[RuCl([15]aneN_4)NO]^2$. Surface staining of untreated- and drug treated macrophages was performed, in each case, by cell incubation with specific monoclonal antibodies PE- or FITC-labeled anti-CD40, anti-CD86, anti-CD86, anti-MHC-II (BD-Pharmingen, San Diego, CA). For cytotoxicity assays, triplicates of BMDM wereincubated with *trans*-$[RuCl([15]aneN_4)NO]^2$ (0.1, 0.5 and 1.0 mM) and/or ascorbic acid (in the same concentrations), respectively, diluted in PBS, at 37°C, in a humidified atmosphere of 5% CO_2 for 24 h. Bz (1.0 mM) was used as the reference trypanocidal drug, and Tween 20 at 5.0, 0.5 and 0.005% was used as death positive control in cell. Cells were harvested, incubated with propidium iodide at 50 µg/mL and data acquired after 15 min. Data acquisition was performed using a FACSorter, apparatus (Becton-Dickinson Immunocytometry System Inc., San Jose, CA, USA) Multivariate data analysis was performed in the FlowJo software (Ashland, Oregon, USA).

In vivo lethal dose of trans-$[RuCl([15]aneN_4)NO]^2$

For determination of the half-lethal dose (LD_{50}) *in vivo*, three Swiss mice (30 g) per group were injected by intra-peritoneal route with *trans*-$[RuCl([15]aneN_4)NO]^2$ and ascorbic acid plus *trans*-$[RuCl([15]aneN_4)NO]^2$ diluted in PBS in the dosages of 83, 250, 750, 2000 and 6000 µmol/Kg, respectively, and the animal survival observed during 48 hours, as previously described (Silva *et al.*, 2007).

Trypanocidal activity of trans-$[RuCl([15]aneN_4)NO]^2$ in vitro

In vitro anti-proliferative and trypanocidal activities of *trans*-$[RuCl([15]aneN_4)NO]^2$ were initially evaluated against epimastigotes and trypomastigotes forms of *T. cruzi* Y strain, respectively, as previously described (Silva *et al.*, 2007). Trypomastigotes and epimastigotes cultures were re-suspended to 6.5×10^6 parasites/ml in RPMI 10% fetal bovine serum (FBS) and LIT, respectively. Triplicates were treated with the *trans*-$[RuCl([15]aneN_4)NO]^2$ (0.1, 0.5 and 1.0 mM) and/or ascorbic acid (in the same concentrations) diluted in PBS, at 37°C, 5% CO_2. Benznidazole (1.0 mM) (Roche) were used as the reference trypanocidal drug (positive control). Parasite viability was subsequently tested by determining the number of motile forms, according to Brener (Brener, 1962b). The concentration of compound corresponding to 50% anti-proliferative or trypanocidal activities after 24 h of incubation were expressed as IC_{50epi} (inhibitory concentration on epimastigotes forms) and IC_{50try} (inhibitory concentration on trypomastigotes forms), respectively. The same protocol was used to determine trypanocidal activity of the *aqua* complex *trans*-$[RuCl([15]aneN_4)H_2O]$.

The intracellular action of the drugs was also determined on amastigotes forms. Subconfluent monolayers of Vero cells (American Type Culture Collection CLL-81) were plated at 1.25×10^4 cells in RPMI (GIBCO, Grand Island, New York, US) supplemented with 5% FBS (Nutricell, Campinas, São Paulo, Brasil) in each well of a eight-well chamber slide (chamber slide, Nunc Inc. Illinois, USA), at 37°C, 5% CO_2, humidified atmosphere 24 h prior to infection. Cell subconfluent monolayers were then infected with tissue culture trypomastigote forms (12.5×10^4 parasites/well) for 24 h. After infection, chamber slides were washed twice with cold PBS in order to lose extracellular adhered parasites and cells were re-incubated in RPMI 5% FBS. Triplicates were treated according as described above. Triplicates were treated according described above. Media was removed 24h post infection and chamber stained by Giemsa, according to the previously described (Guedes *et al.*, 2007). The percent of infected cells was determined by random examination of slides and counting a minimum of 500 cells under the microscope using high magnification (400 ×).

Treatment of mice with NO donor and benznidazole

Female Swiss mice 6-8 weeks old were infected with 1.0×10^3, blood trypomastigotes per animal. Mice used in the experiments belonged to the animal stock of the Department of Biochemistry and immunology, Medicine School of Ribeirão Preto, University of São Paulo, São Paulo, Brazil. Animals were housed in temperature-controlled rooms (22-25°C) and received water and food *ad libitum* in the animal facilities. All protocols using animals were approved by the Ethics Committee on Animal Research of the University of São Paulo. Eight experimental groups of 10 Swiss mice weighting 25 to 30

g were used. Treatment started soon after detection of parasitemia, occurring on day 4 post-inoculation. Bz was administered orally at 385 µmol/kg of body weight per day (equivalent to 100 mg/kg) in a suspension made with 4% arabic gum, for 20 consecutive days. *Trans*-$[RuCl([15]aneN_4)NO]^2$ and AA were administered by intraperitoneal route (3.33 µmol/kg/day) diluted in PBS, for 20 consecutive days. The groups received following treatments: Group 1: PBS, Group 2: AA, Group 3: BZ, Group 4: *trans*-$[RuCl([15]aneN_4)NO]^2$, Group 5: *trans*-$[RuCl([15]aneN_4)NO]^2$ plus BZ, Group 6: *trans*-$[RuCl([15]aneN_4)NO]^2$ plus AA, Group 7: *trans*-$[RuCl([15]aneN_4)NO]^2$ plus BZ plus AA, Group 8: not infected and not treated. Ten animals from each group were euthanized at the end of the treatment (25 days post-infection) for quantification of heart inflammation and cytokine production. Another 10 animals were used for parasitemia, mortality and parasitological cure evaluation. Evaluation of parasitemia was performed as previously described (Brener, 1962a).

Cytokine quantification (Enzyme-linked immunosorbent assay)

The levels of cytokines were determined by ELISA of sera samples obtained on day 25 após a infecção, using ELISA sets for IL-10, IFN-γ and TNF-α (all R&D Duoset, R&D, Minneapolis, MN, USA), according to manufacturers' instructions. The reaction was detected by peroxidase-conjugated streptavidin followed by a substrate mixture containing hydrogen peroxide and ABTS (Sigma Aldrich, St. Louis, MO, USA) as a chromogen.

Nitrite measurement in the sera of treated animals

Nitrite production in the sera of noninfected and infected mice were measured by accumulation of nitrite, nitric oxide's metabolite, in sera collected in the 25 day on day 25 of the infection (thelast day of treatment), as described previously (Green *et al.*, 1981). Briefly, 0.05 ml of Griess reagent (0.1% naphthyl ethylenediamine and 1% sulfanilamide in 2.5% phosphoric acid (prepared with reagents from Sigma) was added to 0.05 ml of sera, and absorbance was read at 540 nm using an automated plate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Evaluation of parasitological cure

To verify the occurrence of parasitological cure, a battery of three independent tests was performed thirty days after the end of treatment, including parasitological (hemoculture and real time PCR) and serological assays (ELISA). The parasitological cure was based on a negative result in both parasitological and serological methods.

The **hemoculture** was performed 30 days after the end of mice treatment (55 days post-infection). Animals were bled from the orbital venous sinus and 400 µL of blood was collected and divided into two tubes containing 3 mL of LIT medium. Tubes were incubated at 28 °C for 90 days and examined monthly for parasite detection (Caldas *et al.*, 2008). **Real time PCR** was performed using the Platinum® SYBR® Green qPCR SuperMix UDG with ROX system (Invitrogen, Carlsbad, CA, USA). 2µg of DNA was used plus 25 pmol of primers S_{35} (5'AAATAATGTACGGG (T/G)GAGATGCATGA3') and S_{36} (5'GGGTTCGATTGGGGTTGGTGT3') (OPERON Technologies, INC). Samples were amplified for 40 cycles in a 7000 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) generating a product of 330pb. ELISA and flow cytometry were performed with sera obtained on the 1st month after treatment. ELISA plates were coated with *T. cruzi* antigen prepared from alkaline extraction of the *T. cruzi* Y strain at the exponential phase of growth in LIT medium. Anti-mouse IgG-peroxidase conjugated antibody (Sigma Chemical Co.) was used. The mean absorbance for 10 negative control samples plus two standard deviations were used as the cut-off to discriminate between positive and negative results.

Quantification of myocardial inflammation

Myocarditis was performed as previously described (Guedes *et al.*, 2007), total nucleus number cells were counted in fifty microscopic fields in at least four representatives, nonconsecutive HE stained sections (thickness of 5 µm) of each mouse. Sections were examined with a Zeiss Integrationsplatte II eyepiece (Zeiss Co, Oberkochen, Germany) reticule, used with an Olympus BHS microscope (Olympus, Miami, FL, USA) at a final magnification of ×400.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Student's *t* test was used to analyze the statistical significance of the differences observed between infected vs. control assays. In time course studies, two-way ANOVA was used followed by Tukey-Kramer *post-hoc* analysis. The Kaplan-Meier method was used to compare survival curves of the studied groups. Differences were considered statistically significant when $P < 0.05$. All analyses were performed using the PRISM 3.0 software (GraphPad, San Diego, CA, USA).

Results

Trans-$[RuCl([15]aneN_4)NO]^2$ has low toxicity and high lethal dose

Benznidazole, the drug available for clinical treatment of Chagas disease, is used in high dosages and long therapeutic schedule, with significant side effects. We determined toxicity of new NO donor *trans*-$[RuCl([15]aneN_4)NO]^2$ as compared to benznidazole using bone marrow-derived macrophages (BMM). Ascorbic acid, NO donor and benznidazole at doses of 1.0, 0.5 and 0.1 mM did not show *in vitro* cytotoxicity (Figure 2). The lethal dose 50 (LD_{50}) of *trans*-$[RuCl([15]aneN_4)NO]^2$ for

mouse was approximately 2000 µmol/Kg (lethal dose between 2000 to 6000 µmol/Kg).

Therefore, we tested whether NO donor enhances the intracellular NO concentration, thus being active against intracellular forms of *T. cruzi.* We observed that macrophages cultured in the presence of *trans*-$[RuCl([15]aneN_4)NO]^2$ exhibited enhanced intracellular concentrations of NO (Figure 3). To verify if it ~~is~~ was due to the direct activation of macrophages by *trans*-$[RuCl([15]aneN_4)NO]^2$ or by its biological reducing byproducts, BMDM macrophages were cultured in the presence of the NO donor and the expressions of co-stimulatory- CD40, CD80, CD86 and MHC-II molecules were analyzed. The addition of drugs (NO donor, ascorbic acid and benznidazole) did not enhance the expression of co-stimulatory molecules in macrophages (Supplementary Figure 1A). This absence of direct activation of macrophages by NO donor was confirmed by using murine RAW macrophage-like cells stably expressing a NF-κB luciferase reporter.

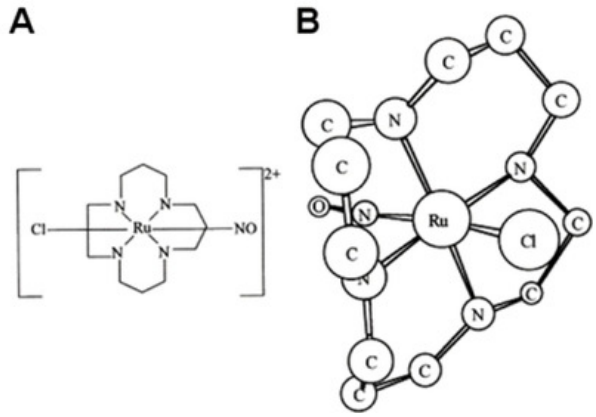

Figure 1: (A) Chemical structure of *trans*-$[RuCl([15]aneN_4)NO]^2$; (B) Chemical 3Dδ calculated *trans*-$[RuCl([15]aneN_4)NO]^2$ structure (hydrogen atoms are omitted).

After being cultured with the drugs, the luciferase bearing reporter cells did not show enhanced luciferase expression (Supplementary Figure 1B) These results showed that the enhancement of concentration of NO in macrophages did not result from macrophage activation by the drugs, but it is the result of the NO release by *trans*-$[RuCl([15]aneN_4)NO]^2$.

Trans-$[RuCl([15]aneN_4)NO]^2$has potent in vitro trypanocidal activity

After verifying the safety of *trans*-$[RuCl([15]aneN_4)NO]^2$ through its low *in vitro* toxicity and wide therapeutic window, we decided to evaluate its ability to inhibit all life cycle stages of *T. cruzi*. Thus, *in vitro* anti-proliferative activity against epimastigotes was assessed. The benznidazole showed 100% of antiproliferative effect at 1.0 mM concentration. The NO donor ministrated alone showed almost 10-20% of activity against epimastigotes forms of *T. cruzi.* The nitric oxide releasing activity of the NO donor depends on the presence of reducing agents in the medium. Thus, we added the NO donor together with AA and the NO donor showed 100% activity against epimastigotes forms, similar to Bz at the concentration of 1.0 mM (Figure 4A). Interestingly, against trypomastigotes, the forms of *T. cruzi* present in the blood of vertebrates hosts, the *trans*-$[RuCl([15]aneN_4)NO]^2$ was more efficient at concentrations 100 fold lower than that of benznidazole (Figure 4B). At a 1.0 mM concentration, Bz killed 53% of trypomastigotes forms after 24 hours of incubation, and the *trans*-$[RuCl([15]aneN_4)NO]^2$ plus ascorbic acid at 0.1, 0.5 and 1.0 mM killed 100% of trypomastigotes forms after 8 hours (Figure 4B). While, Bz was required at 10 mM (data not showed) to kill 100% of parasites. As observed against epimastigotes forms, the action of the NO donor against trypomastigotes forms also depended on the presence of reducing agents.

Moreover, after NO release by the *trans*-$[RuCl([15]aneN_4)NO]^2$, the drug becomes an *aqua* complex/*trans*-$[RuCl([15]aneN_4)H_2O]$, and we demonstrated here, that this compound has also trypanocidal activity. For this, *aqua* complex was incubated with trypomastigotes and epimastigotes forms of *T. cruzi,* killing about 20% of trypomastigotes forms and suppressed 30% of epimastigotes proliferation through all the concentration range used (0.1, 0.5 and 1.0 mM) (Figure 4C and 4D) fluorescence in macrophage cells were recorded before (Medium) and 100 s, 200 s and 300 s post-1.0 mM addition of *trans*-$[RuCl([15]aneN_4)NO]^2$. Note the steadily increasing number of diffusely-fluorescing cells from time zero till time 300 s (intracellular concentration of NO) post- NO donor addition.

The NO donor probably has a synergic effect between NO release and the nucleus of the metallic compound. Furthermore, in cultured Vero cells the *trans*-$[RuCl([15]aneN_4)NO]^2$ was also more efficient than Bz against the intracellular amastigote form, when added together with the reducing agent (Figure 4E and 4F).

The Vero cells were infected with *T. cruzi* and after parasite internalization the culture were treated with drugs. The NO donor did not show significant effect against amastigotes forms of the parasite when added alone to the culture. The rate of infected cells treated with 1.0 mM of benznidazole and *trans*-$[RuCl([15]aneN_4)NO]^2$ plus ascorbic acid was 14.33±4.2 and 1.83±0.5, respectively. Taken together, these results indicate that trypanocidal effect of *trans*-$[RuCl([15]aneN_4)NO]^2$ in the presence of reducing agent is higher than benznidazole *in vitro*.

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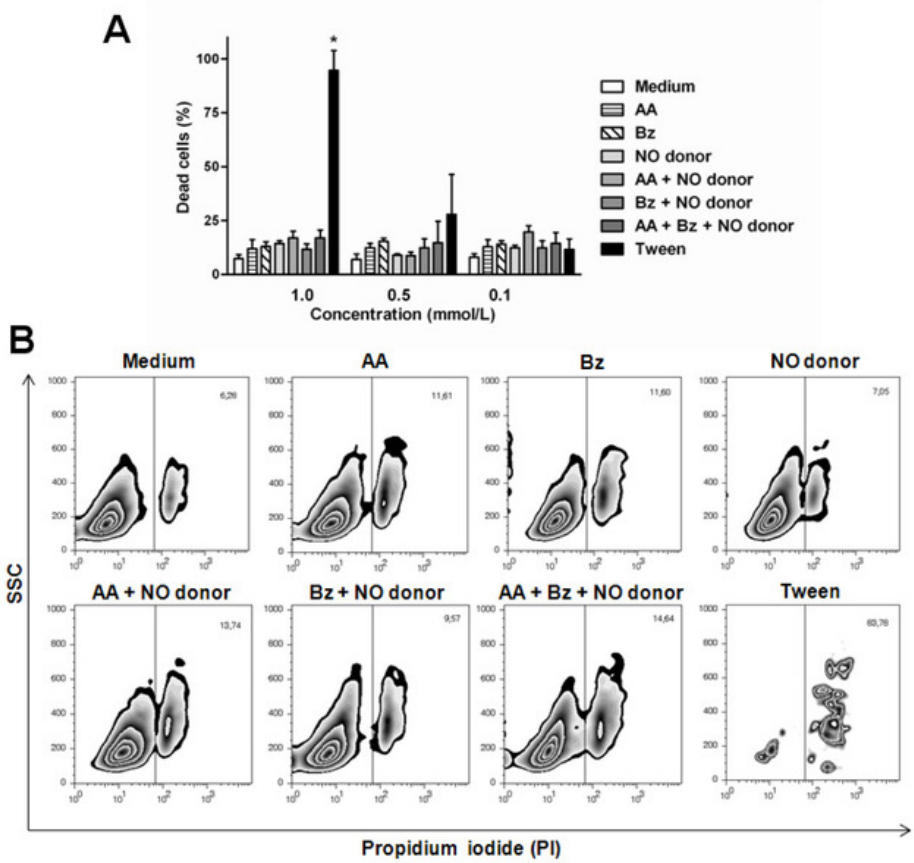

Figure 2: *Trans*-[RuCl([15]aneN$_4$)NO]^2 has no *in vitro* toxicity in culture of bone marrow macrophages. The in vitro toxicity assay of NO donor *trans*-[RuCl([15]aneN$_4$)NO]^2 (0.1, 0.5 and 1.0 mM) dosed alone and/or: ascorbic acid (0.1, 0.5 and 1.0 mM), benznidazole (1.0 mM) as well as the NO donor plus ascorbic acid plus benznidazole (same concentrations), diluted in PBS, in bone marrow-derived macrophages (BMDM) after 24h under incubation conditions, is presented. Tween 20 (5.0, 0.5 and 0.005%) was used as positive control of death cell. After being collected cells were incubated with propidium iodide at 50 µg/mL and data collected after 15 min. Data acquisition was performed in a FACSorter apparatus. Multivariate data analysis was done using the FlowJo software. (A) Frequency of macrophage death in the presence of different concentrations of the compounds tested. (B) representative plots of the BMDM death. Note the undamaging action of *trans*-[RuCl([15]aneN$_4$)NO]^2 on the BMDM target cells. Data represent means ± standard error of the mean and are representative of three independent experiments. SSC, Side-scattered light; PBS, phosphate buffered saline. * Significant difference ($P < 0.05$).

NO-donor suppresses the parasitemia, mortality and myocarditis

The control untreated animals and the ascorbic acid treated animals showed similar curves of parasitemia, the parasitemia peak was observed at 9th day of infection. The parasitemia was suppressed between the second and fourth day of treatment in all treated animals with NO donor and benznidazole when each drug was administrated alone. However, the association of both drugs suppressed the parasitemia in the first day of treatment. All control (untreated) animals showed significantly higher parasitemia levels and patent periods ($P < 0.001$) than animals treated with NO donor and/or Benznidazole (Figure 5A). Diversely as occurred *in vitro*, the treatment of mice with the NO donor alone or together with AA generated similar results. This fact was due to the presence of reducing agents in the blood and host tissues.

A survival rate of 100% of the *T. cruzi*- infected and *trans*-[RuCl([15]aneN$_4$)NO]^2 plus Bz treated animals was observed until six month after the treatment, in all the therapeutic schemes used. In contrast, untreated controls and animals treated with ascorbic acid showed 0% and 10% of survival, respectively (Figure 5B).

The cardiac inflammation was evaluated at the end of the treatment (25 days post infection/d.após a infecção). When administrated separately, benznidazole and NO donor were not able to full clearance of cardiac inflammation (Figure 5C and 5D).

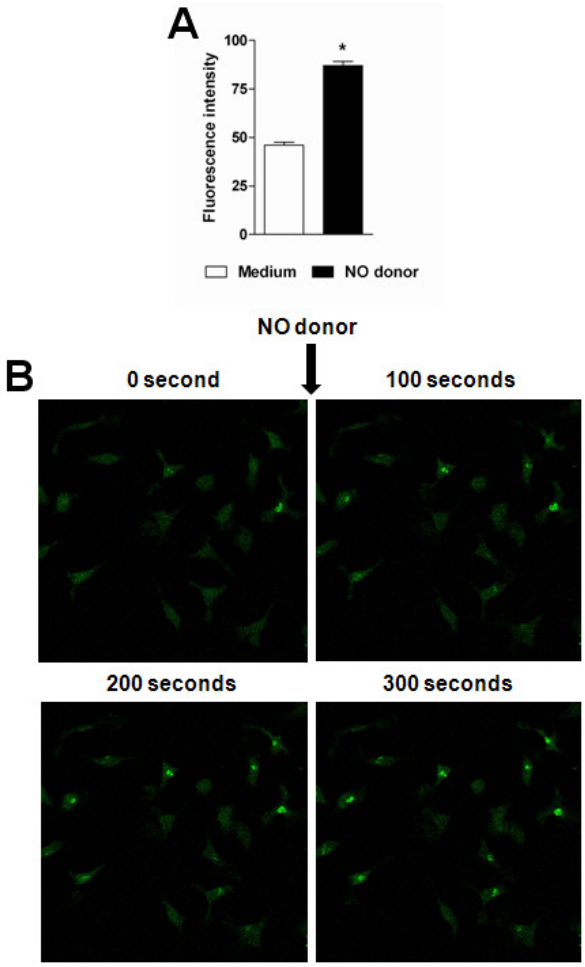

Figure 3. *Trans*-[RuCl([15]aneN$_4$)NO]^2 enhances cytosolic NO concentration in macrophage cells from mice. Treated- and untreated *trans*-[RuCl([15]aneN$_4$)NO]^2 (1.0 mM, for 100 s, 200 s and 300 s) BMDM were pre-incubated in DAF-2DA (10 μM) for 30 min at room temperature and imaged using a fluorescent light. (A) Significant enhancement of the DAF-2DA fluorescent green staining 300 seconds after NO donor addition as compared to the control culture (Medium). Bars represent the mean ± SEM of 5 different samples. *denotes statistical difference in the fluorescence intensity, $P <$ 0.001 (B) Serial confocal images from BMDM in culture medium (Control) and NO donor- treated macrophages. The microphotographies representative of

Benznidazole or *trans*-[RuCl([15]aneN$_4$)NO]^2 ministrated alone showed significantly lower levels of myocardial inflammation (P<0.05) than control animals treated with ascorbic acid or untreated animals. Interestingly, benznidazole and *trans*-[RuCl([15]aneN$_4$)NO]^2 administered together eliminated myocarditis of mice (Figure 5C and 5D), regardless of being associated or not with ascorbic acid. Ascorbic acid was used as reducing agent assisting NO release mainly *in vitro*. But during mice treatment, the presence of natural reducing agents in the physiological environment eliminates the requirement of its use.

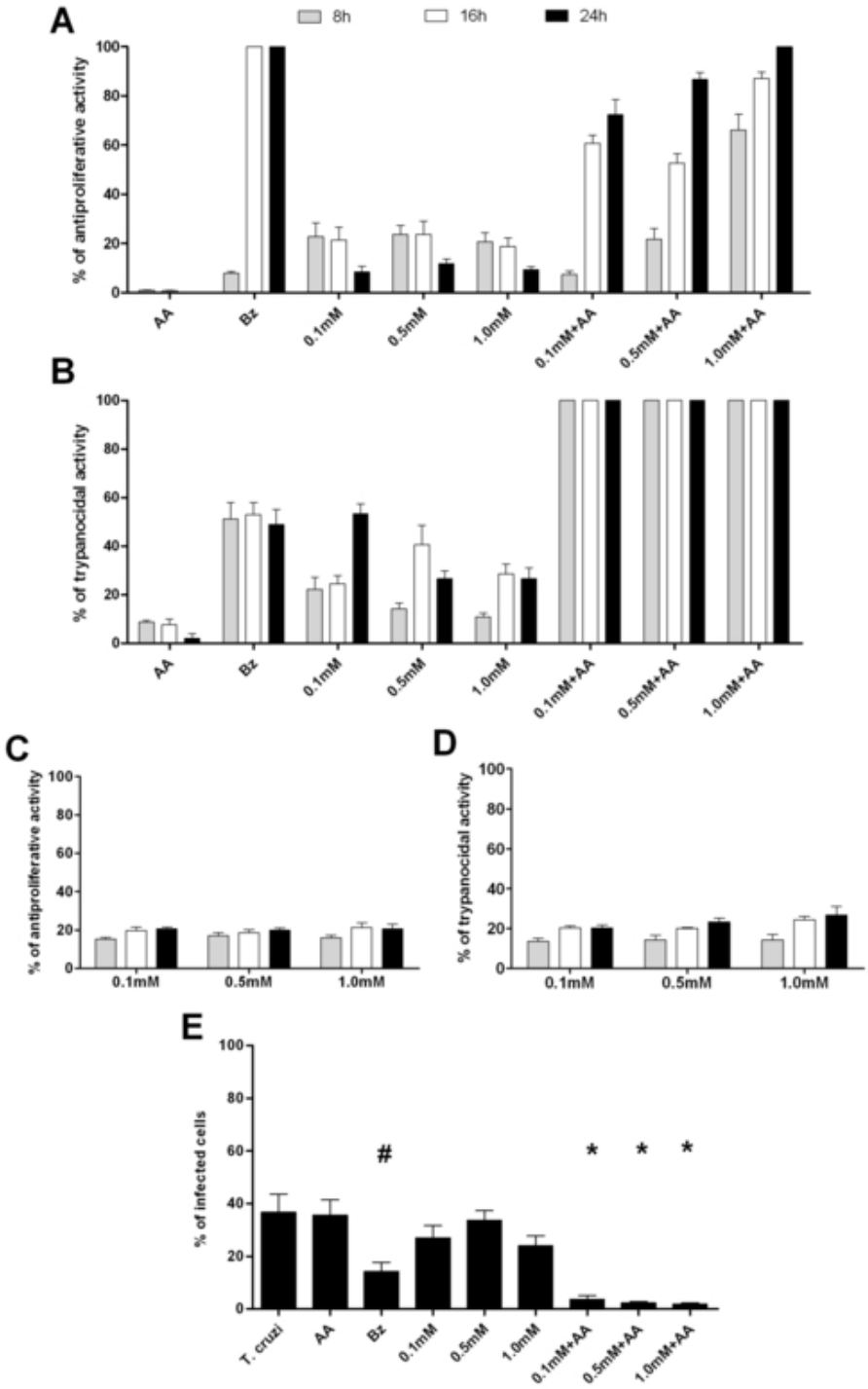

Figure 4: *Trans*-[RuCl([15]aneN$_4$)NO]^2 shows potent trypanocidal activity *in vitro*. The percentages of the antiproliferative and trypanocidal activities of the NO donor *trans*-[RuCl([15]aneN$_4$)NO]^2 (0.1, 0.5 and 1.0 mM) on *T. cruzi* Y strain: epimastigotes (A) and trypomastigotes (B) forms in culture media (LIT and RPMI 10% BSF, respectively) after being dosed alone or in a therapeutic schedule combined with/or: ascorbic acid (0.1, 0.5 and 1.0 mM), benznidazole (1.0 mM), and also the NO donor plus ascorbic acid plus benznidazole are shown, as well as the percentages of the antiproliferative (C) and trypanocidal (D) activities of the aqua compound/*trans*-[RuCl([15]aneN$_4$)H$_2$O] (0.1, 0.5 and 1.0 mM) administered alone. Antiproliferative and trypanocidal activities of *trans*-[RuCl([15]aneN$_4$)NO]^2 on epimastigotes and trypomastigotes forms of *T. cruzi* were determined through parasite viability counting at 8h, 12h, and 24h post-treatment. The same for the aqua compound/*trans*-[RuCl([15]aneN$_4$)H$_2$O]. The same concentrations described above for intracellular activity of *trans*-[RuCl([15]aneN$_4$)NO]^2 were also used against amastigotes forms (E); cultures were stained by Giemsa. Note the strikingly effect of the NO donor on trypomastigotes forms, present in the blood of the vertebrate host, when dosed

together with ascorbic acid: this formulation revealed to be more efficient against blood trypomastigotes at concentrations 100-fold lowers than benznidazole (Figure 4B), the drug currently used in the treatment of Chagas' disease. Furthermore, the secondary product originated after NO release from the NO donor, the aqua complex/ *trans*-[RuCl([15]aneN$_4$)H$_2$O], also has trypanocidal activity (Fig. 4C and Fig. 4D). Each point is the mean ± standard error of the mean and is representative of three independent experiments with similar results, n=10). Statistically significant differences from control (unpaired t test, $P < 0.05$) are denoted by *, #.
185x283mm (72 x 72 DPI)

Two month after each treatment, the animals were separated in cured or not cured groups according to the parasitological and serological tests. The cured animals treated with *trans*-[RuCl([15]aneN$_4$)NO]^2 and/or BZ did not show cardiac inflammation, and the animals not cured showed a significantly decreased cardiac inflammation ($P<0.05$), when compared to those treated with ascorbic acid (data not show). At this time point, it was not possible to compare the treated group to those infected and not treated animals because all untreated mice succumbed to infection. In a similar way the ascorbic acid- treated animals succumbed to infection, and only one survival remained one month after treatment. The reduced observed myocarditis at the end of the treatment correlated with the poor production of NO and cytokines (IL-10, IFN-γ and TNF-α) detected in sera of all treated animals (Supplementary Figure 2a and 2b). As observed in the cardiac inflammatory process 25 days post-infection (d.p.i), the nitrite and inflammatory cytokine production were high in the infected and not treated animals, and in the ascorbic acid- treated mice, when compared to the animals treated with *trans*-[RuCl([15]aneN$_4$)NO]^2 and/or Bz (Supplementary Figure 2a and 2b). The parasitological cure was correlated with the absence of myocarditis and normalization of seric cytokine and NO production in the sera of animals where *trans*-[RuCl([15]aneN$_4$)NO]^2 and Bz were administered together (Figure 5 and Supplementary Figure 2a and 2b).

The ratio of parasitological cure was verified by three independent criteria: hemoculture, Real time PCR and the presence of specific IgG anti-*T. cruzi* antibodies, detected by ELISA.

In mice treated with ascorbic acid (group 1), benznidazole (group 2), *trans*-[RuCl([15]aneN$_4$)NO]^2 (group 3), *trans*-[RuCl([15]aneN$_4$)NO]^2 plus benznidazole (group 4), ascorbic acid added *trans*-[RuCl([15]aneN$_4$)NO]^2 (group 5) and ascorbic acid plus *trans*-[RuCl([15]aneN$_4$)NO]^2 plus benznidazole (group 6) parasitological and serological tests were negative for 0%, 40%, 30%, 80%, 20% and 80% of animals, respectively. Animals with 80% negativation of the parasitological and serological tests were considered cured (Table 1). Seric levels of *T. cruzi*-specific immunoglobulin G antibodies besides parasite DNA levels in the animal groups treated with the NO donor plus Bz are absent or remarkably low, falling in the same region as the data from the uninfected control group (Supplementary Figure 3). Similarly to the untreated control group, death occurred to mice treated with ascorbic acid during the acute phase of the infection. Just one survived until the period of the cure rate evaluation.

Overall, these results indicate the treated, but not cured animals had a reduction in cardiac parasitism along with cardiac damage, showing the importance of treatment, even when the therapeutic cure was not totally achieved.

Discussion and conclusions

In this study we observed that the treatment with the new NO donor *trans*-[RuCl([15]aneN$_4$)NO]^2 reduced parasitic load, cardiac inflammation, and generated 100% of survival in mice during the acute phase of the experimental infection with a highly virulent strain of *T. cruzi*. Of note, this treatment was able to induce the parasitological cure of 40% in mice. Moreover, the association with benznidazole enhanced the rate of parasitological cure up to 80%.

Currently available antiparasitic agents for etiological treatment of Chagas' disease include benznidazole and nifurtimox.

The outcomes obtained with both drugs are diverse, and their cure rates depend on the stage of the disease, as well as on the sensibility of the parasite strain, which varies geographically.

Nonetheless, adverse effects as anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy are commonly observed, leading to high rates of discontinuation of treatment (Filardi *et al.*, 1987; Guedes *et al.*, 2006; Sosa Estani *et al.*, 1999). Despite of being helpful during the acute infection, the benefits of antiparasitic therapies during the chronic infection have not been systematically evaluated. Noteworthy, most cases are detected in the chronic phase of the disease, when cardiovascular and gastrointestinal commitments can be irreversible, so the only available therapeutic option is surgery. Thus, the development of novel therapeutic approaches is highly important for the treatment of Chagas' disease patients. At the moment, a multicentre, randomized double-blinded clinical trial is being developed to test if administration of benznidazole to patients with non acute forms of the disease will prevent the chronic cardiovascular damage (Marin-Neto *et al.*, 2008). Nitric oxide (NO) plays multiple important roles in the immune response, including antiviral, antimicrobial, immunostimulatory, immunosuppressive, cytotoxic and cytoprotective effects. The analysis of iNOS$^{-/-}$ mice unequivocally demonstrates that most of the above described effects are mediated by iNOS-derived NO (Bogdan *et al.*, 2000a; Bogdan *et al.*, 2000b; Nathan *et al.*, 2000).

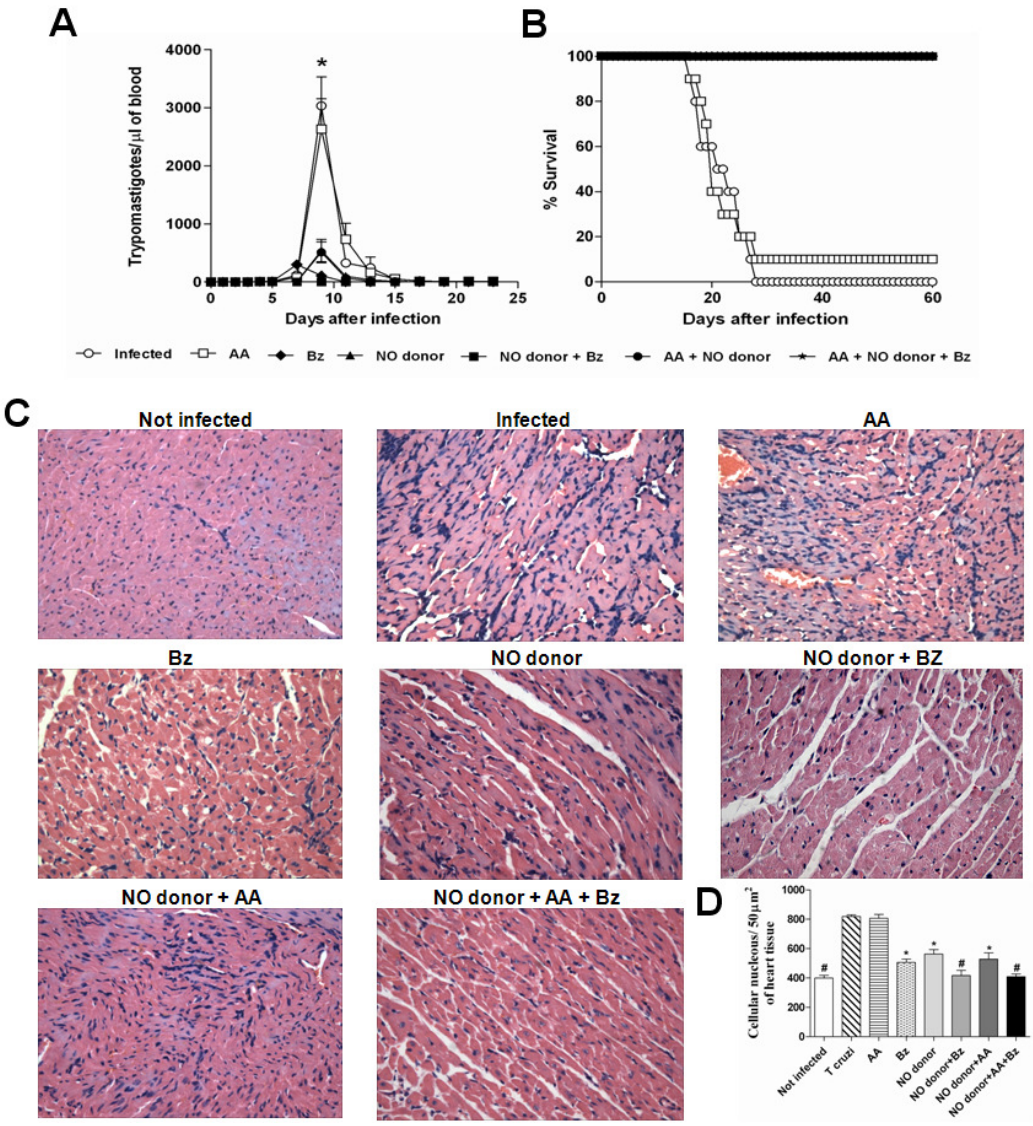

Figure 5: The treatment with *trans*-[RuCl([15]aneN$_4$)NO]^2 suppresses parasitemia, mortality and myocarditis of mice infected with *T. cruzi*. Swiss mice infected with 1 × 10^3 blood trypomastigotes of *T. cruzi* Y strain and treated with the following therapeutic schedule: ascorbic acid (3.33 µmol/Kg/day) alone, benznidazole (385 µmol/kg/day) alone, the NO donor *trans*-[RuCl([15]aneN$_4$)NO]^2 (3.33 µmol/Kg/day) alone, NO donor (3.33 µmol/Kg/day) plus benznidazole (385 µmol/kg/day), NO donor (3.33 µmol/Kg/day) in combination with ascorbic acid (3.33 µmol/Kg/day), and the NO donor (3.33 µmol/Kg/day) in association with ascorbic acid (3.33 µmol/Kg/day) and benznidazole (385 µmol/kg/day). Treatment started on day five post-infection, being applied daily throughout 20 consecutive days. Control group received PBS. Parasitemia (A) and survival (B) were evaluated daily over a period of 60 days post-infection. Representative sections of cardiac tissue (C) 25 days after infection (the last day of treatment). Original magnification microphotographs, X 400. Quantification of cellular nucleus in 50 µm^2 of heart tissue (D) of non-treated and treated animals. Note the synergic effect of the NO donor with benznidazole in eliminating myocarditis in *T. cruzi*- infected mice (Fig. 5C and Fig. 5D) regardless of being associated or not with ascorbic acid. Each point is the mean ± standard error of the mean and is representative of three independent experiments with similar results, n=10). Statistically significant differences from control (unpaired t test, $P < 0.05$) are denoted by *, #. For experimental details refer to the legend in Table 1.

NO is produced by activated macrophages and iscrucial to the intracellular killing of parasites, including *T. cruzi* (Martins *et al.*, 2001). Many protozoa are able to inhibit NO production, thus evading the immune system. *T. cruzi* induces apoptosis in lymphocytes, and regulation of iNOS mediated by cell-cell contact has been seen in apoptotic lymphocytes (Freire-de-Lima *et al.*, 2000). Uptake of apoptotic cells drives the growth of *T. cruzi* within macrophages. The uptake of apoptotic, but not necrotic lymphocytes by macrophages through fibronectin receptor and CD36 leads to down-regulated expression of iNOS and thus, shifts L-arginine metabolism toward the arginase pathway. This leads to increased ornithine and putrescine production, which are known growth factors for *T. cruzi*.

These effects result from the induction of endogenous TGF-b, immune system evasion (Freire-de-Lima *et al.*, 2000). Thus, as NO produced within phagocytes is crucial to parasite killing, therapeutic approaches using NO releasing compounds could improve the protective immune response, being promissory agents to test against this intracellular pathogen.

Indeed, the treatment of *T. cruzi*-infected immunosuppressed mice with benznidazole generates lower survival and rate cure than the same treatment in immunocompetent animals, indicating that the protector effects of benznidazole are dependent on immune system integrity, particularly the process of macrophage activation, NO production and parasite destruction (Molina *et al.*, 2000; Silva *et al.*, 1995). Recently, our group demonstrated that NO donors administered at low concentrations are capable of killing *T. cruzi* (Silva *et al.*, 2007). However, the rate of parasitological cure of these compounds has not been determined. Moreover, the NO donor used in the present study showed better effects against *T. cruzi* than those previously tested by our group, probably because of the longer period of NO release(Bonaventura *et al.*, 2004) and synergic activity of *aqua* compounds formed by *trans*-$[RuCl([15]aneN_4)NO]^2$. The NO releasing activity of *trans*-$[RuCl([15]aneN_4)NO]^2$lasts for 2 hours, thus the administrating schedule of *trans*-$[RuCl([15]aneN_4)NO]^2$ during two or three times a day improve its pharmacodynamics properties. Based on the results reported herein it will be possible to perform studies aiming to increase the releasing period of NO.

Here we demonstrated that *trans*-$[RuCl([15]aneN_4)NO]^2$ is harmless, as it did not show *in vitro* toxicity to bone marrow macrophages. Likewise the cytosolic NO concentration within macrophages in culture was remarkably enhanced after addition of *trans*-$[RuCl([15]aneN_4)NO]^2$ to the culture medium. In addition, the therapeutic dosage used *in vivo* was lower than its LD_{50} (3.33 µmol/Kg *vs.* 2000 to 6000 µmol/Kg). Even when used at lower concentrations, *trans*-$[RuCl([15]aneN_4)NO]^2$ was more efficient than benznidazole in killing epimastigotes, trypomastigotes and amastigotes forms of *T. cruzi*. We observed 100% of survival in mice treated with 3.33 µmol/Kg/day of *trans*-$[RuCl([15]aneN_4)NO]^2$ alone or together with 385 µmol/kg/day of benznidazole, while death occurred for all infected and not treated not treated animals. However, the NO donors that we have tested against *T. cruzi* are not bioequivalents, since the survival rates of mice treated with Ru(NO)imN or Ru(NO)isn at the same molar dose were 40% and 60%, respectively (Silva *et al.*, 2007).

These results demonstrate that the *trans*-$[RuCl([15]aneN_4)NO]^2$ and those NO donors previously tested are more potent and safer than Bz. Our results are promising in that the NO donor *trans*-$[RuCl([15]aneN_4)NO]^2$ displays characteristics that fulfill the essential criteria to meet the product profile for antiparasitic drugs specified by the World Health Organization in conjunction with those responsible for control programs in the affected countries.

In fact, *trans*-$[RuCl([15]aneN_4)NO]^2$ was shown here to be active *in vivo* against *T. cruzi* at the dose of 3.33 µmol/kg, exceeding in this way, a hundred times the activity of the standard drug Bz (385 µmol/kg) currently in use against Chagas' disease.

Table 1: NO donor induce parasitological cure of experimentally Chagas disease.
The effect of the NO donor (*trans*-$[RuCl([15]aneN_4)NO]^2$) dosed alone and/or: ascorbic acid (AA), benznidazole (Bz) as wellas the NO donor plus ascorbic acid plus benznidazole on mice infected with *T. cruzi* Y strain is presented[a].

Therapeutic schedule[a]	Positive results/Test[b]			N° of positive tests post-treatment/ Total (%)	Cure Rate (%)
	N° of positive ELISA/Total	N°of positive Hemoculture/Total	N° of positive Real Time PCR/Total		
Ascorbic Acid[c] (AA)	1	1	1	1/1 (100)	0
Benznidazole	6	5	6	6/10 (60)	40
NO donor	7	6	7	7/10 (70)	30
NO donor Bz	2	1	2	2/10 (20)	80
NO donor AA	8	4	8	8/10 (80)	20
NO donor AA Bz	2	2	2	2/10 (20)	80

[a]Swiss female mice, 28-30 g, were infected with 1 x 10^3 blood trypomastigotes of *T.cruzi* Y strain. Treatment started (soon) after detection of patent parasitemia, on day 5post-infection proceeding daily over a period of 20 days. The nitric oxide donor t*rans*-[RuCl([15]aneN_4)NO]^2, and the ascorbic acid (AA) were administered by intraperitoneal route at 3.33 µmol/kg/day. Benznidazole (Bz) was administered by oral route at 385 µmol/kg. The infected control groupreceived PBS. Data are expressed as the mean ± standard error of the mean and are representative of three independent experiments with similar results, n = 10. PBS, phosphate-buffered saline.
[b] Results are expressed as the number of positive ELISA, hemoculture, and real time PCR tests. 10 mice were used per condition, as well as for the not infected- and *T. cruzi* infected controls.
[c]Only one animal remained alive until the period of the cure rate evaluation in the control group. No survivals were observed in the PBS, untreated control group.

The cardiac inflammatory process was decreased at the end of the treatment with *trans*-[RuCl([15]aneN_4)NO]^2 and/or with benznidazole (26 d.após a infecção). A less intense reduction of myocarditis is observed with other NO donors and benznidazole (Andrade *et al.*, 1991; Segura *et al.*, 1994; Silva *et al.*, 2007). However, when the NO donor and Bz were administered together, the myocarditis was absent at the end of the treatment. Moreover, the animals treated and considered not cured showed a reduced cardiac inflammation when compared to the control (animals treated with AA alone). These animals also showed decreased parasitism. The presence of parasite is thought to be important to the genesis of cardiac lesions, hence a specific therapy could reduce the myocardial damage and morbidity during acute and chronic phases of the human disease (Coura *et al.*, 1997). Literature data also show a reversibility of cardiac fibrosis in mice chronically infected with *T. cruzi*, under specific chemotherapy (Andrade *et al.*, 1991).

The new drugs for Chagas' disease treatment should be able to improve the quality of life of the infected people, reducing chronic morbidity, and consequently the expenditure of government health systems as well as precocious retirement in developing countries. In conclusion, our results indicate that *trans*-[RuCl([15]aneN_4)NO]^2 has *in vitro* and *in vivo* trypanocidal activity and is capable of inducing radical parasitological cure in the mice model of acute infection. Furthermore, the compound can be used in high dosages without toxicity and thus represents a potentially useful candidate for the treatment of human Chagas' disease.

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