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Curso de Pós-Graduação em Patologia

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Tese de Doutorado

TERAPIA CELULAR EM CAMUNDONGOS COM CARDIOPATIA CHAGÁSICA CRÔNICA: MECANISMOS ENVOLVIDOS NA MELHORA DA MIOCARDITE CHAGÁSICA CRÔNICA EXPERIMENTAL

ALUNO: Ricardo Santana de Lima

Salvador, 2010

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RESUMO

TERAPIA CELULAR EM CAMUNDONGOS COM CARDIOPATIA CHAGÁSICA CRÔNICA: MECANISMOS ENVOLVIDOS NA MELHORA DA MIOCARDITE CHAGÁSICA CRÔNICA EXPERIMENTAL. **RICARDO SANTANA DE LIMA**. **[INTRODUÇÃO]** A cardiomiopatia chagásica crônica é uma das principais causas de insuficiência cardíaca nos países latino-americanos. Cerca de 30% dos indivíduos infectados pelo *Trypanosoma cruzi* desenvolvem essa forma grave e sintomática da doença, caracterizada pela presença de uma resposta inflamatória intensa seguida de fibrose no coração. Foi demonstrado previamente que o transplante de células da medula óssea (BMC) melhora a inflamação e a fibrose em corações de camundongos chagásicos crônicos. Neste trabalho nós investigamos alterações da expressão gênica no coração de camundongos chagásicos crônicos submetidos ou não à terapia BMC. **[MÉTODOS]** Camundongos C57BL/6 cronicamente infectados com *T. cruzi* (6 meses) foram transplantados com células mononucleares da medula óssea (BMC) ou solução salina intravenosamente (i.v.) e sacrificados após 2 meses. RNA foi extraído de corações de animais controles normais e chagásicos. Análise de microarranjos de DNA foi realizada utilizando uma matriz de 27.400 cDNAs. Imunofluorescência e análises morfométricas foram realizadas em secções dos corações. **[RESULTADOS]** Foram encontradas alterações significativas na expressão de ~12% dos genes amostrados. Genes com expressão aumentada nos corações chagásicos foram associados com as respostas imune-inflamatória (quimiocinas, moléculas de adesão, catepsinas e moléculas de MHC) e fibrose (componentes da matriz extracelular, lisil oxidase e Timp1). Quando corações chagásicos de animais tratados com BMC foram comparados com o de animais normais, cerca de 90% das alterações gênicas não foram encontradas. Muitos dos genes com expressão modulada pelo tratamento com BMC foram relacionados à inflamação e fibrose. Imunofluorescência e análise morfométrica confirmaram os efeitos moduladores da terapia com BMC no padrão de resposta inflamatória e na expressão de moléculas de adesão. Nossos resultados demonstram um importante efeito imunomodulador do BMC e indicam fatores potencialmente relevantes envolvidos na patogênese da doença, que podem constituir novos alvos terapêuticos para esta doença. Para investigar a contribuição natural das células da medula óssea nas lesões no coração e músculo esquelético durante a infecção aguda pelo *T. cruzi*, camundongos quiméricos foram gerados através do transplante de células da medula óssea GFP^+ em camundongos receptores do tipo selvagem letalmente irradiados que foram infectados com *T. cruzi* um mês após o transplante. A migração de células derivadas da medula óssea para o coração e músculo esquelético foi vista durante e após a fase aguda da infecção, uma vez que o infiltrado inflamatório era composto por células GFP^+. Cardiomiócitos e células endoteliais GFP^+ foram encontrados nas secções de coração de camundongos quiméricos infectados. Além disso, um grande número de fibras GFP^+ foi observado no músculo esquelético de camundongos quiméricos em diferentes momentos após a infecção por *T. cruzi*. **[CONCLUSÃO]** Nossos resultados reforçam o papel das células derivadas da medula óssea na regeneração tecidual em lesões provocadas pela infecção por *T. cruzi*. **[PALAVRAS-CHAVE]** Terapia celular; doença de Chagas; cardiopatia; transplante; células de medula óssea; camundongo.

ABSTRACT

CELL THERAPY IN MICE WITH CHRONIC CHAGASIC CARDIOMYOPATHY: MECHANISM INVOLVED IN THE IMPROVEMENT OF EXPERIMENTAL CHRONIC CHAGASIC MYOCARDITIS. **RICARDO SANTANA DE LIMA. [INTRODUCTION]** Chronic chagasic cardiomyopathy is a leading cause of heart failure in Latin American countries. About 30% of *Trypanosoma cruzi*-infected individuals develop this severe symptomatic form of the disease, characterized by intense inflammatory response followed by fibrosis in the heart. We have previously shown that bone marrow cell (BMC) transplantation improves inflammation and fibrosis in hearts of mice with chronic Chagas' disease. Here we investigated alterations of gene expression in the hearts of chronic chagasic mice submitted or not to BMC therapy. **[METHODS]** C57Bl/6 mice chronically infected with *T. cruzi* (6 months) were transplanted with bone marrow mononuclear cells (BMC) or saline solution intravenously (i.v.) and sacrificed 2 months later. RNA was extracted from the hearts of normal controls and chagasic mice. DNA microarray analysis was performed using an array of 27,400 mouse cDNAs. Immunofluorescence and morphometric analyses were performed in heart sections. **[RESULTS]** We found significant alterations in expression of ~12% of the sampled genes. Extensive upregulations in chagasic hearts were associated with immune-inflammatory responses (chemokines, adhesion molecules, cathepsins and MHC molecules) and fibrosis (extracellular matrix components, lysyl oxidase and Timp1). When BMC-treated chagasic hearts were compared to normal mice, about 90% of the alterations were not found. Many of the genes with expression modulated by BMC were related to inflammation and fibrosis. Immunofluorescence and morphometric analyses confirmed the modulatory effects of BMC therapy in the pattern of inflammatory response and expression of adhesion molecules. Our results demonstrate an important immunomodulatory effect of BMC and indicate potentially relevant factors involved in the pathogenesis of the disease which may provide new therapeutic targets. To investigate the natural contribution of bone marrow cells on lesions in the heart and striated muscle during acute *T. cruzi* infection, chimeric mice were generated by transplanting GFP^+ bone marrow cells into lethally irradiated wild-type recipient mice and infected with *T. cruzi* one month after transplantation. Migration of bone marrow-derived cells to the heart and striated muscle was seen during and after the acute phase of infection, since the inflammatory infiltrate was composed by GFP^+ cells. GFP^+ cardiomyocytes and endothelial cells were found in the heart sections of chimeric chagasic mice. In addition, a large number of GFP^+ myofibers were seen in the striated muscle of chimeric mice at different time points after infection. **[CONCLUSION]** Our results reinforce the role of bone marrow-derived cells in tissue regeneration in lesions caused by *T. cruzi* infection. **[KEYWORDS]** Celular therapy; Chagas' disease; cardiopathy; transplant; bone marrow cell; mice.

SUMÁRIO

LISTA DE ABREVIATURAS

FITC	Isotiocianato de fluoresceína (Fluorescein isothiocyanate)
G-CSF	Fator estimulador de colônia de granulócito (Granulocyte colony stimulator factor)
GFP	Proteína verde fluorescente (Green protein fluorescence)
ANP	Peptídeo Natriurético Atrial (Atrial natriuretic peptide)
OMS	Organização mundial de saúde (World health organization)
PCR	Reação da polimerase em cadeia (Polymerase chain reaction)
qPCR	Reação da polimerase em cadeia em tempo real (Real Time Polymerase chain reaction)
SCF	Fator de célula-tronco (Stem cell factor)
TGF-β	Fator de crescimento tumoral-β (Tumoral growth factor-β)
TMO	Transplante de medula óssea (Bone marrow transplant)
TNF-α	Fator de necrose tumoral-α (Tumor necrosis factor-α)
IC	Insuficiência cardíaca (Cardiac insufficiency)
DNA	Ácido desoxiribonucleico (Desoxirribonucleic Acid)
RNA	Ácido ribonucléico (Ribonucleic Acid)
DTH	Reação de hipersensibilidade tardia (Delayed test hypersensitivity)
SDF-1	Fator derivado de célula estromal 1 (Stromal cell-derived factor-1)
SCA	Antígeno de célula-tronco (Stem cell antigen)
MMP	Metaloproteinase (Matrix metalloproteinase)
TIMP	Inibidor tecidual de Metaloproteinase (Tissue inhibitor of metalloproteinases)
MDR	Resistente a múltiplas drogas (Mult-drugs resistence)

IGF	Fator de crescimento do tipo insulina (Insulin Growth Factor)
HGF	Fator de crescimento de Hepatócito (Hepatocyte Growth Factor)
FGF	Fator de crescimento de fibroblasto (Fibroblast growth factor)
VEGF	Fator de crescimento de endotélio vascular (Vascular endothelial growth factor)
Ang-1	Angiopoietina-1 (angiopoietin-1)
IL-	Interleucina (Interleucin)
PDGF	Fator de crescimento derivado de Plaquetas (Platelet-derived growth factor)
MCP-	Proteína quimiotática de monócitos (Monocyte chemotactic protein)
CD-	Grupo de diferenciação (Cluster of differentiation)
BMC	Células de medula óssea (Boné marrow cells)
iv	Via Intravenosa (Intravenous route)
MHC	Complexo de histocompatibilidade principal (Major histocompatibility complex)
SLPI	Inibidor de protease secretada por leucócitos (Secretory leukocyte protease inhibitor)
ET-1	Endotelina-1 (Endotelin-1)
LAMP	Proteínas de membrana associada a lisossomos (lisossomal-associate membrane protein)
PA	Ativador de plasminogênio (Plasminogen activator)
PCNA	Antígeno nuclear de proliferação celular (Proliferating cell nuclear antigen)

1- INTRODUÇÃO

1.1 - Doença de Chagas

A doença de Chagas, causada pelo parasito flagelado *Trypanosoma cruzi*, foi descrita primeiramente em 1909 pelo pesquisador brasileiro Carlos Chagas. A doença, que ocorre em todo México, Américas Central e Sul, continua representando uma séria ameaça para a saúde. A prevalência global da infecção humana pelo *Trypanosoma cruzi* foi estimada em 16-18 milhões de casos, segundo a Organização Mundial de Saúde (OMS, 2004). Somente na América Latina estima-se que estejam quase que a totalidade dos casos estimados pela OMS, com 15-16 milhões de indivíduos infectados e cerca de 75-90 milhões de pessoas, em risco de contrair a infecção (SCHOFIELD *et al*, 2006; COURA, 2009). Sabe-se que em torno de 30% das pessoas infectadas com o parasito vai avançar para a forma crônica cardíaca e/ou digestiva (OMS, 2004). Além disso, estima-se que aproximadamente 676.000 pessoas por ano tornem-se incapacitadas de desenvolver suas atividades de trabalho por conta desta doença (MOREL, 2000). A morbi-mortalidade associada à doença de Chagas na América Latina tem um impacto maior do que a associada com a malária, a esquistossomose ou a leishmaniose (URBINA, 2003; MOREL, 2000). Nos países do Cone Sul existem iniciativas e programas que diminuíram drasticamente as taxas de transmissão vetorial do *T. cruzi* (DIAS *et al,* 2002). No entanto, a transmissão não foi completamente erradicada em áreas endêmicas. Além disso, os indivíduos já infectados e que cursarão para a forma crônica da doença não têm um tratamento adequado na atualidade (DIAS *et al,* 2002). Grandes avanços têm sido alcançados no controle da transmissão da doença pelas vias transfusional e vetorial nas últimas décadas, particularmente nos países da América

Latina, onde se reduziu o número de pessoas sob risco de contrair a doença de aproximadamente 100 milhões para 40 milhões de pessoas (MOREL, 2000; OMS, 2004). Porém, o tratamento das pessoas já infectadas, sobretudo dos 30% destes indivíduos que desenvolverão a fase crônica da doença, ainda permanece um grande desafio (URBINA, 2003). Portanto, é necessário o desenvolvimento de novas tecnologias e/ou estratégias terapêuticas para o tratamento da doença de Chagas e de outras doenças, ditas negligenciadas que acometem principalmente os países em desenvolvimento (TARLETON, 2007).

1.2 - O *Trypanosoma cruzi*

O *Trypanosoma cruzi* é um parasito pertencente à ordem Kinetoplastida e à família Trypanosomatidae. Estudos biológicos, bioquímicos e moleculares têm demonstrado que o *T. cruzi* é um táxon muito heterogêneo. O primeiro método experimental que demonstrou a extensa variabilidade genética do *T. cruzi* foi a análise de variantes eletroforéticas de enzimas celulares (isoenzimas) (MACEDO *et al*, 2004). Mais tarde, a análise de uma região conservada do DNA ribossomal do parasito, revelou a presença de dois grupos de *T. cruzi*, mas que somente o sub-grupo *T. cruzi* tipo II, que infecta alguns animais domésticos e que vivem no ambiente peridomiciliar e o homem, parece estar associado às manifestações cardíacas e gastrointestinais crônicas no Brasil, pois foram identificados em biópsias de tecidos como coração, cólon e esôfago de toda uma amostra de 25 pacientes com as formas crônicas da doença (FREITAS *et al*, 2005). No ano seguinte, outro trabalho isolou através de biópsia endomiocárdica de um paciente em estágio final da cardiopatia chagásica crônica, *T. cruzi* tipo I, considerado parasito encontrado em animais silvestres, corroborando outros trabalhos que mostram esta linhagem de

parasito do tipo I em indivíduos infectados na Venezuela e na amazônia brasileira e que ressaltam a importância da observação da variabilidade dos indivíduos desta espécie como potenciais causadores da cardiopatia chagásica crônica (TEIXEIRA *et al,* 2006). Esta variabilidade intra-específica é um dos aspectos mais investigados e interessantes sobre o *T. cruzi.* Análise de diferentes linhagens isoladas demonstrou um grande polimorfismo genético dentro desta categoria de indivíduos, reforçando a idéia de uma estrutura populacional basicamente clonal para a espécie (BUSCAGLIA & DI NOIA, 2003; MACEDO, *et al*, 2004).

O parasito é encontrado sob três formas em ambientes distintos: formas epimastigotas de *T. cruzi* são encontradas no trato digestivo do triatomíneo vetor, e se diferenciam a partir de tripomastigotas presentes no sangue ingerido pelo inseto hematófago quando este se alimenta em um hospedeiro infectado. As formas tripomastigotas metacíclicas são as formas infectantes, presentes nas regiões posteriores do intestino do triatomíneo, e são eliminadas junto com as fezes do inseto. Os tripomastigotas metacíclicos penetram no organismo, invadem as células do hospedeiro e se diferenciam em formas amastigotas. Estes se multiplicam no citoplasma da célula do hospedeiro mamífero e se diferenciam em tripomastigotas, rompendo as células e atingindo a circulação do hospedeiro, podendo invadir outras células, ou serem ingeridos pelo inseto vetor durante seu repasto sanguíneo e iniciar um novo ciclo (ARAUJO-JORGE & PIRMEZ, 2000).

O *T. cruzi* é um dos parasitos de maior e mais bem-sucedida distribuição na natureza, sendo capaz de infectar oito ordens de mamíferos, incluindo animais selvagens, domésticos e mesmo o homem. O parasito é também capaz de infectar diferentes tipos celulares do hospedeiro, incluindo células do baço, fígado,

linfonodos, embora tenha uma preferência por células musculares esqueléticas e cardíacas (BRENER *et al,* 2000; COURA *et al,* 2009).

1.3 - Infecção por *T. cruzi*

A doença de Chagas é transmitida para seres humanos por um inseto conhecido popularmente como "barbeiro", pelo hábito de picar a face de pessoas adormecidas. Este vetor está distribuído geograficamente do México até o sul da Argentina (OMS, 2004) e pertence à família reduviidae, subfamília triatominae. São conhecidas 130 espécies de triatomíneos, agrupadas em 17 gêneros. Os gêneros mais importantes do ponto de vista de transmissão da doença são os gêneros *Triatoma* com 69 espécies, *Panstrogylus*, com 13 espécies, e *Rhodnius*, também com 13 espécies. (SANTOS-MALLET, 2000; CARCANVALLO *et al*, 2001). A destruição do habitat natural dos triatomíneos vetores pelo homem provocou a invasão por estes insetos de áreas peridomiciliares, facilitando a transmissão da doença (OMS, 2004).

Os modos de transmissão da doença de Chagas são: vetorial, através das fezes contaminadas com formas tripomastigotas que são depositadas próximo ao local da picada do triatomíneo no momento do seu repasto sanguíneo; por via transfusional; e menos freqüentemente, por via congênita, em transplante de órgãos ou por aleitamento materno (ARAUJO-JORGE & PIRMEZ, 2000; GÜRTLER *et al,* 2003; YOUNG *et al,* 2007).

A infecção progride em duas fases: aguda e crônica. A fase aguda da doença é transitória, geralmente sem manifestações clínicas ou com manifestações clínicas de pequena relevância, e caracteriza-se pela presença de formas tripomastigotas do parasito no sangue periférico e formas amastigotas multiplicando-se dentro de vários tipos celulares no hospedeiro. No homem, esta fase regride espontaneamente após

cerca de 12 meses, com diminuição dos parasitos do sangue e tecidos (KOBERLE, 1968; MARIN-NETO *et al,* 1999). A maioria dos indivíduos infectados permanece livre de qualquer expressão clínica, radiológica ou eletrocardiográfica por um longo período de tempo ou até por toda a vida, na forma crônica indeterminada, com um bom prognóstico de vida. Estudos mostram que em torno de 70% dos indivíduos chagásicos estudados permaneceram na forma indeterminada da doença durante dez anos de acompanhamento, e que praticamente 50% da população global de indivíduos infectados nunca apresentará qualquer manifestação clínica da doença (MARIN-NETO *et al,* 1999). Cerca de 30% dos indivíduos infectados pelo *T. cruzi* apresentarão manifestações clínicas cardíacas e/ou digestivas após um período que pode variar de alguns anos até décadas após a infecção aguda, representando a forma crônica sintomática da doença de Chagas (ANDRADE, 1983; MARIN-NETO *et al,* 1999).

1.4 - A cardiopatia chagásica crônica

A cardiomiopatia crônica ocorre em 25% dos casos, e resulta da destruição de fibras cardíacas por processos inflamatórios focais ocorridos ao longo dos anos (ANDRADE, 1983). Esta fase é caracterizada pela mionecrose, miocitólise e intensa fibrose intersticial, resultado da destruição de fibras cardíacas por processos inflamatórios focais ocorridos ao longo dos anos (COURA *et al,* 2007). É freqüente o aparecimento de aneurisma ventricular apical, as reações inflamatórias observadas na cardiopatia chagásica crônica têm um aspecto semelhante ao observado em reações de hipersensibilidade tardia, com infiltrado inflamatório composto principalmente por células mononucleares (ANDRADE, 1983; MARIN-NETO *et al,* 1999, BENVENUTI & GUTIERREZ, 2007).

Clinicamente, a doença manifesta-se sob a forma de insuficiência cardíaca e/ou arritmias e cursa, na maioria dos casos, com alterações da contratilidade, da condução atrioventricular ou intraventricular e arritmias ventriculares, decorrentes da destruição das fibras do sistema de condução cardíaco por processo inflamatório, constituindo causa freqüente de invalidez e de morte súbita na América Latina (MENDOZA *et al,* 1986; ANDRADE, 1999; DE CARVALHO *et al*, 1994; COURA *et al,* 2007). Os sintomas e sinais físicos presentes na forma crônica da cardiopatia chagásica derivam de três eventos essenciais, que podem coexistir no mesmo paciente: insuficiência cardíaca (IC), arritmias cardíacas e tromboembolismo sistêmico e/ou pulmonar (RASSI JR *et al,* 2009). A IC geralmente tem padrão biventricular. Bloqueio de ramo direito, hipertensão pulmonar, extra-sístoles, bloqueio átrio-ventricular e batimentos ventriculares ectópicos são algumas das alterações observadas. Embolia sistêmica ou pulmonar, originada das próprias câmaras cardíacas ou de trombose venosa profunda precipitada por débito cardíaco baixo cronicamente, constitui complicação freqüente e grave da cardiopatia chagásica (MARIN-NETO *et al,* 1999; RASSI JR *et al,* 2009).

Os fatores que determinam a progressão da forma assintomática para a cardiopatia chagásica ainda são desconhecidos. Uma característica marcante da miocardite chagásica crônica é o fato de haver pouco parasitismo tecidual nesta fase da doença. Alguns estudos têm demonstrado a falta de correlação entre a presença de parasitos ou antígenos do parasito e inflamação, em indivíduos com infecção crônica (BARBOSA JR & ANDRADE, 1984; JONES *et al,* 1993; PALOMINO *et al,* 2000). Análises de fragmentos de corações humanos não demonstraram uma correlação entre a intensidade de inflamação e o parasitismo, mesmo com o uso de técnicas sensíveis como PCR e imunohistoquímica (PALOMINO *et al,* 2000;

OLIVAREZ-VILLAGOMEZ *et al,* 1998). Outros trabalhos, no entanto, mostram que a presença do parasito é fundamental para induzir e manter a resposta imune do indivíduo gerando o quadro crônico da doença. Alguns autores, usando métodos mais robustos como PCR e imunohistoquímica, são capazes de localizar antígenos parasitários ou DNA do *T. cruzi* em lesões crônicas dos pacientes; os tratamentos que diminuam a carga parasitária tendem a diminuir as manifestações clínicas; re-infecções e exposição contínua ao parasito provocam uma piora da gravidade da doença em modelos experimentais e em casos humanos. Todos estes fatores levam a crer que o parasito tem um papel crucial na patogênese da cardiopatia chagásica crônica (HIGUCHI *et al,* 2003; KIERSZENBAUM, 2005).

A ausência de parasitos circulantes e a sua escassez no músculo cardíaco, além da presença de um processo inflamatório disseminado no miocárdio, geraram diversas hipóteses para explicar a etiologia da miocardite chagásica crônica. Doença vascular coronariana, destruição do gânglio do sistema nervoso autônomo, persistência do parasito e auto-imunidade contra antígenos cardíacos têm sido descritas como fatores associados à patologia (MORRIS *et al,* 1990; DE CARVALHO *et al,* 1994; SOARES *et al,* 2001; ENGMAN *et al*, 2002; KIERSZENBAUM, 2005). A autoimunidade é discutida por alguns autores que mostram uma reação cruzada entre antígenos do parasito e do hospedeiro (RASSI JR *et al,* 2009). A imunização de camundongos com extrato de *T. cruzi* mesmo sem a presença de parasitos vivos nos animais gerou forte resposta de hipersensibilidade tardia (DTH) específica contra miosina cardíaca in vitro (LEON *et al,* 2004). Outro trabalho mostra a ação de uma proteína do *T. cruzi*, a proteína ribosomal P2β, que tem homologia com proteínas humanas que atuam nos receptores β1-adrenérgicos ou M2-colinérgicos e provocam alterações eletrocardiográficas vistas na patogênese da doença (MAHLER

et al, 2004). Outro trabalho mostra que, induzindo tolerância imunológica com antígenos cardíacos em camundongos e depois os infectando com *Trypanosoma cruzi* da cepa Colombiana, os animais desenvolvem uma miocardite crônica menos intensa do que aqueles animais somente infectados (PONTES-DE-CARVALHO *et al,* 2002).

Sendo assim, é cada vez mais evidente que a etiologia da patogênese da cardiopatia chagásica crônica é multi-fatorial e envolve certamente a presença do parasito ou de seus antígenos, distúrbios vasculares e de condução elétrica no coração, destruição de gânglios do sistema nervoso autônomo e autoimunidade (KIERSZENBAUM, 2005). Portanto, mesmo que a transmissão da doença fosse erradicada hoje, como já ocorre em alguns estados brasileiros, 25% dos indivíduos infectados ainda assim desenvolveriam a patologia em seu estágio crônico (MONCAYO, 2003). Até o momento não existe nenhum tratamento eficaz para pacientes com cardiopatia chagásica crônica além do transplante cardíaco, nos casos mais graves. Os diversos tratamentos com drogas, como o benzonidazol, trazem uma série de complicações para os pacientes e não erradicam o parasito na fase crônica (ANDRADE *et al,* 1996; CANÇADO, 1999; CALDAS *et al,* 2008). Desta forma, o estudo de terapias alternativas e os mecanismos de ação capazes de interferir em um quadro já instalado da doença, ou de prevenir o desenvolvimento da mesma são de grande importância.

1.5 - Quimioterápicos

Os fármacos utilizados para o tratamento da doença de Chagas são compostos nitroheterocíclicos como nitrofuran e nifurtimox (Lampit ®, Bayer) e o benzonidazol, um derivado de nitroimidazol (Rochagan ®, Radanil ®, Roche), ambos com atividade anti-*T. cruzi* descoberta empiricamente há mais de três décadas (ANDRADE *et al,* 1996). O nifurtimox atua através da redução de grupos nitros, produzindo metabólitos de oxigênio reduzidos altamente tóxicos. O benzonidazol atua por mecanismos de estresse redutivo que envolve modificações covalentes de macromoléculas. O *T. cruzi* é deficiente na detoxificação de metabólitos de oxigênio, particularmente o peróxido de hidrogênio (DO CAMPO, 1990). Esses tratamentos têm uma alta eficácia na fase aguda da doença de Chagas, com uma cura parasitológica de mais de 80% dos pacientes tratados.

A cura parasitológica é definida como sendo a negatividade para todos os testes sorológicos e parasitológicos (CANÇADO, 1999). Nos pacientes com a forma crônica da doença, o tratamento não é eficiente com nenhuma das duas drogas, que demonstram nesta fase uma atividade antiparasitária baixa, com cura parasitológica de menos que 20% dos pacientes tratados (CANÇADO, 1999). Outro estudo demonstrou apenas 10% de cura nos pacientes com a forma crônica tratados com benzonidazol e 6% naqueles tratados com o nifurtimox, ressaltando o fato de os resultados terapêuticos poderem variar em países diferentes e/ou em função das diferentes cepas do *Trypanosoma cruzi* ou mesmo diferentes genótipos dos indivíduos infectados (ANDRADE *et al,* 1996; CALDAS *et al,* 2008; CALDAS *et al,* 2008a). Além disso, sérias reações adversas foram documentadas em pacientes adultos tratados com benzonidazol, incluindo dermopatia alérgica generalizada, neuropatia periférica e granulocitopenia (ANDRADE *et al,* 1996). Recentemente,

Caldas e colaboradores demonstraram que o tratamento com benzonidazol reduz a parasitemia nas fases aguda e crônica da doença, porém este fato não está correlacionado com a diminuição das lesões observadas cronicamente no coração dos animais infectados e tratados (CALDAS *et al*, 2008).

1.6- Células-tronco

1.6.1- Definição

Têm sido descritas na literatura células com capacidade de auto-renovação e de diferenciação em um ou mais tipos celulares especializados, sendo estas células denominadas células-tronco. Elas estão presentes desde o desenvolvimento embrionário até o adulto e podem ser encontradas em diversos órgãos e tecidos, tais como a medula óssea, pele, músculo, coração, cérebro, fígado, cordão umbilical e tecido adiposo, entre outros (BISHOP *et al,* 2002; POULSOM *et al,* 2002; HUGHES, 2002; GOLDRING *et al,* 2002; BONNET, 2002; BARRY *et al,* 2004; CAPLAN, 2007). Estes conhecimentos têm gerado um potencial de utilização destas células na medicina regenerativa, não só para a regeneração de tecidos, principalmente em doenças crônicos degenerativas, como também em terapia gênica pela manipulação genética de células somáticas e reprogramação das mesmas tornando-as pluripotentes, tratando doenças genéticas até então não tratáveis (FUCHS *et al,* 2000; KORBLING & ESTROV, 2003; KORBLING *et al,* 2003; MAVILIO & FERRARI, 2008; SHI, 2009).

1.6.2- Características

Existem duas teorias para explicar o comportamento das células-tronco. A primeira postula que a célula-tronco, em um determinado estágio de desenvolvimento, se divide assimetricamente dando origem a células chamadas progenitoras, com um potencial de proliferação menor, e que a partir destas células se originam células diferenciadas em resposta a estímulos ambientais. A outra teoria pressupõe que existam populações de células-tronco assimétricas e que, a depender do nicho ou do microambiente onde essas células se encontrem, elas se diferenciam em tipos celulares com diferentes características (WATT *et al,* 2000).

A manutenção do comportamento das células-tronco depende de reguladores autônomos próprios, intrínsecos das células, que são modulados por sinais externos ou do microambiente. O controle intrínseco inclui proteínas responsáveis pela regulação de fatores nucleares envolvidos na expressão gênica e nas modificações cromossomais da célula (BONNET, 2002). A interação entre o receptor tirosina quinase c-Kit e seu ligante, o fator de célula-tronco (SCF), regula a proliferação, adesão, migração, diferenciação e ativação funcional de células-tronco hematopoiéticas (SHARMA *et al,* 2006). Mutações no gene do c-Kit que promovem sua ativação constitutiva aumentam a taxa de proliferação das células hematopoiéticas. De forma semelhante, o aumento da expressão do fator de transcrição NF-Ya induz a ativação do promotor HOXB4 que está relacionado à função de divisão celular e auto-renovação das células-tronco (SHARMA *et al,* 2006).

Microambiente ou nicho são locais no tecido ou órgão onde fatores, como por exemplo citocinas ou fatores de crescimento e de proliferação celular, são secretados pelas células, onde há contato célula-célula, ou interação da célula com

a matriz extracelular mediada por moléculas de adesão (KIROUAC *et al*, 2009; WATT *et al,* 2000). O conceito de nicho como microambiente especializado onde as células-tronco habitam foi proposto inicialmente no âmbito da hematologia, há mais de 30 anos, para estudo da hematopoiese, usando células obtidas de modelos experimentais de invertebrados, tais como a *Drosophila melanogaster* (SCADDEN, 2006). Os estudos realizados sugerem que existam tipos celulares diferentes em um mesmo ambiente que criam uma estrutura tridimensional onde as células-tronco residem e interagem entre si e com as demais células ou com a matriz extracelular, regulando suas funções, tais como divisão, adesão e proliferação celular (SCADDEN, 2006; KIROUAC *et al,* 2009).

Estes microambientes são importantes para a dinâmica de populações e de comportamento das células-tronco e das células de uma maneira geral (KIROUAC *et al*, 2009). A interação das células-tronco hematopoiéticas com o seu microambiente particular é crítico para a manutenção das propriedades características destas células que envolvem adesão, sobrevivência e divisão celular. (ARAI *et al,* 2004; ARAI *et al,* 2005). Por exemplo, as células-tronco hematopoiéticas expressam o receptor tirosina kinase Tie-2 que, ao interagir com a angiopoietina-1, induz adesão forte destas células com as células estromais da medula óssea, e isto é suficiente para a manutenção desta população presente no interior da medula, cumprindo seu papel na hematopoiese a longo prazo (ARAI *et al,* 2005). Outro exemplo em que o microambiente influencia a função celular mostra que a depleção da medula óssea de camundongos induz a superprodução do fator derivado de célula estromal-1 (SDF-1), que aumenta a expressão de metaloproteinase-9 (MMP-9), induzindo a liberação do fator solúvel de célula-tronco (SCF ou sKitL), o que resulta em um

recrutamento de células-tronco e de progenitoras c-Kit$^+$ (HEISSIG *et al,* 2002; SHARMA *et al,* 2006).

As células-tronco são caracterizadas pela presença ou mesmo ausência de alguns marcadores de superfície como c-Kit, SCA-1, AC133, CD34, CD105, CD90 dentre outros e em vários trabalhos foi demonstrado que muitas destas populações celulares que estão presentes nos tecidos são passiveis de serem isoladas e purificadas, sendo usadas como fontes de células para a terapia celular (JACKSON *et al,* 2001; ORLIC *et al,* 2001; HUNG *et al,* 2002; POULSOM, R. *et al.* 2002; GUO *et al,* 2003; STAMM *et al,* 2003; YANMING *et al*, 2007). Não existe até o momento um marcador único para células-tronco de medula óssea. Estas células são caracterizadas por um conjunto de marcadores de superfície que possibilitam a identificação, o isolamento e a purificação destas populações na medula óssea (POULSOM *et al.,* 2002; GUO *et al,* 2003). Alguns trabalhos demonstraram que várias populações celulares estão presentes na medula óssea e vêm sendo utilizadas em terapias celulares. Células Lin$^-$ e c-kit$^+$ foram usadas em terapias cardíacas após lesão isquêmica (JACKSON *et al,* 2001; ORLIC *et al,* 2001). Uma população celular obtida a partir de medula óssea de camundongo, denominada de "side population", é Sca-1$^+$ e CD34$^-$, e contém células-tronco hematopoiéticas. Existem alguns marcadores que caracterizam as populações de células de medula humana utilizadas em terapias celulares. Uma sub-população de células da medula óssea humana caracterizada como AC133$^+$ e CD34$^-$ tem alto potencial de induzir angiogênese. O AC133 é tido como um marcador de células-tronco humanas (STAMM *et al,* 2003).

Uma população de células denominadas "size-sieved stem cells", isoladas de medula óssea humana, consideradas como células mesenquimais, é caracterizada

por não expressar CD34, AC133, CD62L e expressar CD90 (um marcador de timócitos e linfócitos T periféricos), integrinas CD29 e CD51 e os receptores de matriz extracelular CD44 e CD105 (HUNG *et al,* 2002; JERKIC *et al,* 2002; MAJUMDAR *et al,* 2003; BARRY *et al,* 2004).

1.6.3- Células-tronco de medula óssea

A medula óssea tem sido estudada como um órgão composto por tecido hematopoiético, sustentado por um estroma (BIANCO *et al,* 2001). É um local que contêm um material esponjoso, onde se encontra gordura, sangue, tecido conjuntivo, vasos sanguíneos e pequenos segmentos de osso, os ossos trabeculares (HAM *et al,* 2006). Evidências apontam para a presença de microambientes diferentes na medula óssea, onde diversas linhagens e fenótipos celulares, que os compõe, não só coexistem, mas também cooperam funcionalmente uns com os outros. Dentre as células-tronco residentes na medula óssea, podemos destacar as células hematopoiéticas e as células mesenquimais (BIANCO *et al,* 2001; HAM *et al,* 2006; KIROUAC *et al,* 2009). Primeiramente estudadas pelo seu papel crítico na formação do ambiente hematopoiético, as células-tronco hematopoiéticas foram bastante investigadas. Já as células estromais ou mesenquimais da medula óssea foram descritas mais recentemente como sendo as células-tronco progenitoras de tecidos esqueléticos, e tem sido demonstrado que estas células possuem um potencial de diferenciação em tecido nervoso e muscular, apesar de representarem uma população bastante rara na medula (BIANCO *et al,* 2001; HAM *et al,* 2006).

Trabalhos publicados nos últimos anos demonstraram que células da medula óssea, além de células de outras fontes, mesmo aquelas presentes no próprio músculo cardíaco (BELTRAMI *et al,* 2001; BELTRAMI *et al,* 2003), são capazes de

se diferenciar e regenerar não só o tecido cardíaco formando cardiomiócitos, mas também outras células como células endoteliais, células musculares lisas e fibroblastos, ou ainda outros tecidos, como por exemplo, osso (KON *et al,* 2000; KAJSTURA *et al,* 2008) e músculo, onde células oriundas da medula óssea injetadas em animais migram para o local da lesão e participam da regeneração das fibras musculares lesadas (FERRARI *et al,* 1998). Células derivadas do estroma da medula óssea, cultivadas na ausência de células hematopoiéticas e estímulos de diferenciação, mantêm uma população celular morfologicamente homogênea que exibe um fenótipo de células progenitoras mesenquimais, com capacidade de proliferar e de se diferenciar em precursores de osteoblastos, condroblastos, adipócitos e mioblastos (CONGET & MINGUELL, 1999).

Vários trabalhos demonstraram o efeito da terapia celular em cardiopatias. Células embrionárias, células mononucleares de medula óssea, mioblastos esqueléticos, células mesenquimmais e progenitores endoteliais já foram utilizados na terapia celular de patologias cardíacas (MAZHARI & HARE, 2007). Em modelos experimentais de cardiopatia, vários trabalhos têm demonstrado o papel promissor das células de medula óssea na promoção de neovascularização, formação de cardiomiócitos e melhora funcional do coração dos animais tratados. Transplantes de células da medula óssea originaram progenitores endoteliais responsáveis por neovascularização no miocárdio de animais infartados (ASAHARA *et al,* 1999). O transplante de células da medula óssea próximo à área da lesão em camundongos levou à regeneração do miocárdio infartado através de neovascularização e proliferação de cardiomiócitos (ORLIC *et al,* 2001; JACKSON *et al,* 2001). O transplante autólogo de células da medula óssea restaurou a função cardíaca de ratos induzindo angiogênese, e observou-se diferenciação das células

transplantadas em cardiomiócitos (TOMITA *et al,* 1999). A mobilização de células da medula óssea com SCF e G-CSF para periferia reduz a mortalidade e induz o reparo do miocárdio de camundongos infartados (ORLIC *et al,* 2001a).

Em humanos, a injeção de células tronco autólogas da medula óssea nas bordas da lesão do coração de seis pacientes melhorou a função ventricular, bem como a perfusão no tecido cardíaco após infarto do miocárdio. Estas células eram positivas para o marcador de superfície CD133 (STAMM *et al,* 2003). O transplante das células de medula óssea foi capaz de restaurar a função do coração de pacientes com infarto agudo do miocárdio. Houve regeneração no miocárdio infartado após transplante autólogo intracoronariano de células mononucleares da medula óssea em humanos (STRAUER *et al,* 2002). Angioblastos formados a partir de células da medula óssea foram capazes de promover neovascularização e melhorar a função cardíaca, inibindo a apoptose de cardiomiócitos e diminuindo o remodelamento cardíaco (KOCHER *et al*, 2001). Nas cardiopatias crônicas, também observaram-se efeitos benéficos da terapia celular. O transplante autólogo intracardíaco de células mononucleares de medula óssea em oito pacientes com cardiopatia isquêmica grave promoveu a melhora da perfusão no miocárdio e melhora funcional na região isquêmica três meses após o transplante (TSE *et al,* 2003).

Um grupo de 14 pacientes com infarto crônico do miocárdio e insuficiência cardíaca grave recebeu células mononucleares da medula óssea, via cateterismo, nas bordas da lesão cardíaca. De um modo geral, houve uma melhora significativa nas funções cardíacas e na qualidade de vida dos pacientes (PERIN *et al*, 2003).

Há evidências de que existam precursores circulantes, provavelmente com origem na medula óssea, que migram naturalmente para os tecidos. Cardiomiócitos

diferenciados a partir de células de medula óssea foram encontrados em biópsias de coração de quatro pacientes do sexo feminino submetidas a transplante de medula óssea de doadores do sexo masculino, evidenciados pela observação do cromossomo Y nestas células (DEB *et al,* 2003). Células com cromossomo Y foram encontradas também no fígado e no músculo esquelético destes pacientes, reforçando a idéia da pluripotência das células-tronco de medula óssea (DEB *et al,* 2003). Não se sabe o que levou à migração destas células, pois não havia evidências de inflamação no coração de nenhuma das pacientes. Porém, não se pode descartar a possibilidade de uma modulação resultante de um regime de imunossupressão em que eram mantidas as pacientes. No entanto, este fato indica que as células da medula óssea podem contribuir para a formação de miócitos cardíacos (DEB *et al,* 2003). O contrário também pode ser visto no coração transplantado de doadoras para receptores do sexo masculino, onde foram observadas células de origem do receptor, evidenciando a migração celular e a diferenciação em miócitos e células de arteríolas coronarianas e de capilares (QUAINI *et al,* 2002). Em biópsias de coração de pacientes transplantados, observou-se cardiomiócitos diferenciados a partir de células de origem não cardíaca com interação célula-célula, demonstrada pela presença de conexina 43, uma proteína integral de membrana constituinte das junções comunicantes tipo "gap junctions" (MÜLLER *et al,* 2002).

1.6.4- Células-tronco cardíacas

As células cardíacas, estudadas em alguns trabalhos, detêm características de célula-tronco e células progenitoras cardíacas. Nos últimos anos, grupos de pesquisadores isolaram e caracterizaram células-tronco do coração de indivíduos adultos com potencial para terapia celular (BELTRAMI *et al,* 2003; STEELE *et al,* 2005; DAWN *et al,* 2005). Pesquisadores mostraram a presença de uma sub-população de células indiferenciadas, precursoras cardíacas chamadas de "LIM-homeodomain transcription factor islet-1^{+}" (isl1^{+}) em regiões das paredes dos átrios, no septo intra-atrial e no ventrículo direito de animais. Estas células fazem parte da embriogênese cardíaca e são conservadas em diferentes espécies como o camundongo, o rato e o homem. Seu número diminui muito ao longo do desenvolvimento do animal e cerca de 500 a 600 destas células podem ser encontradas em algumas regiões do coração em ratos de 1 a 5 dias de idade. Além disso, as células foram capazes de gerar novos cardiomiócitos *in vitro* (LAUGWITZ *et al,* 2005). Outro trabalho mostrou a possibilidade de isolamento e expansão de células indiferenciadas, que crescem em aglomerados denominados de "cardiosferas" de biópsias de coração humano de pacientes submetidos à cirurgia cardíaca ou de corações de camundongos. Estes grupos de células são homogêneos e possuem marcadores característicos de células-tronco ou progenitores endoteliais como c-Kit, SCA-1, flk-1, CD31 e CD34, têm capacidade de auto-renovação e de diferenciação em miócitos e vasos, passando a expressar antígenos como cadeia pesada de miosina cardíaca, troponina cardíaca I e peptídeo natriurético atrial (ANP) (MESSINA *et al,* 2004).

A célula-tronco cardíaca é auto-renovável e multipotente, podendo dar origem às três principais linhagens de células que compõe o miocárdio: os miócitos, as

células endoteliais e as células de músculo liso. Já a célula progenitora cardíaca é uma célula imatura pré-determinada a diferenciar-se em uma das três linhagens citadas anteriormente (TORELLA *et al,* 2005). Entre outras características, as células-tronco cardíacas correspondem a linhagens celulares indiferenciadas que expressam antígenos de superfície de células-tronco em combinações variadas, como o MDR-1, que caracteriza as células que expressam o transportador de membrana Abcg2, que foi inicialmente caracterizado com base na resistência a multidrogas (MARTIN *et al,* 2004). Daí a nomenclatura do marcador MDR-1 (*resistence multi drugs*), que pode caracterizar fenotipicamente esta *Side population* cardíaca, a qual é capaz de proliferação e diferenciação *in vitro* (MARTIN *et al,* 2004).

O C-kit, uma proteína da família das tirosina kinases também é a molécula de superfície expressa pelas células-tronco cardíacas (ORLIC, 2001; TORELLA *et al,* 2005). Outra molécula de superfície expressa pelas células-tronco cardíacas é o Sca-1(Stem Cell Antigen -1). O Sca-1 é um membro da família da Ly-6 e foi primeiro relatado como marcador de superfície das células-tronco hematopoiéticas da medula óssea que têm capacidade de diferenciação e, além disso, podem fazer fusão tanto com cardiomiócitos adjacentes quando transplantadas quanto com outros tipos celulares como células endoteliais ou fibroblastos cardíacos in vitro, entrando em ciclo de divisão celular e mantendo as características de cardiomiócitos (OH *et al,* 2003; MATSUURA *et al,* 2004). Além destes marcadores, as células-tronco cardíacas adultas possuem receptor para o fator de crescimento ligado a insulina-1 (IGF-1), sintetizam e secretam o fator de crescimento de hepatócito (HGF) e o próprio IGF-1, ambos envolvidos na migração, sobrevivência e proliferação destas

células no miocárdio infartado, promovendo regeneração e melhora da função ventricular nos animais tratados com IGF-1 e HGF (URBANEK *et al,* 2005).

Estes achados revelam que o coração possui diferentes populações de célula-tronco e é provável que estas células estejam envolvidas na formação de novos miócitos, substituindo as células que foram perdidas em lesões cardíacas (BARILE *et al,* 2007; MAZHARI *et al*, 2007).

1.6.5- Terapia celular na cardiopatia chagásica

Estudos desenvolvidos em modelos animais de infecção pelo *T. cruzi*, sugerem que o transplante de células derivadas da medula óssea melhora parâmetros importantes e relevantes para o estabelecimento da doença de Chagas na sua fase cardíaca crônica. Além disso, sugerem que esta poderá ser uma nova estratégia terapêutica para os pacientes chagásicos. (SOARES *et al,* 2004; SOARES & DOS SANTOS, 2009; CAMPOS DE CARVALHO *et al,* 2009; LIMA *et al,* 2009).

No modelo murino de cardiopatia chagásica, as células mononucleares de medula óssea transplantadas migram para o coração e são capazes de induzir uma redução da inflamação e da fibrose no tecido cardíaco dos animais transplantados, bem como de induzir um aumento do número de células inflamatórias em apoptose nestes animais (SOARES *et al,* 2004). Imagens de ressonância magnética revelaram que o transplante de células mononucleares de medula óssea foi capaz de prevenir e melhorar a dilatação ventricular direita induzida pela infecção, corroborando com os dados de melhora na avaliação histopatológica mostrados por Soares e colaboradores em 2004 (GOLDENBERG *et al,* 2008). Outro trabalho mostrou que a injeção de células mesenquimais derivadas da medula óssea, e de mioblastos esqueléticos co-cultivados previamente, no ventrículo esquerdo de ratos

cronicamente infectados pelo *T. cuzi*, melhorou a função cardíaca dos animais, aumentando a fração de ejeção e diminuindo os volumes sistólicos e diastólicos finais dos mesmos (GUARITA-SOUZA *et al,* 2006). Este trabalho mostra também que, apesar da injeção local, as células migram para outras áreas de lesão no músculo cardíaco. Este fato é muito importante na cardiopatia de etiologia chagásica, onde vários pontos do coração podem e são afetados durante o processo patológico (GUARITA-SOUZA *et al,* 2006). Não só a injeção das células, mas também o tratamento de animais chagásicos crônicos com G-CSF, foi capaz de diminuir o processo inflamatório e a fibrose, bem como melhorar os distúrbios de condutibilidade no coração dos animais tratados, deixando-os mais estáveis. Outros parâmetros, como distância percorrida em esteira, tempo de exercício, consumo de O_2, produção de CO_2 e taxa de troca respiratória, também foram melhores no grupo tratado com G-CSF. O tratamento parece conferir uma melhora não só estrutural como também no desempenho funcional dos animais tratados (MACAMBIRA *et al,* 2009). Baseado nos estudos em animais, os pesquisadores desenvolveram estratégias para a abordagem de pacientes que apresentavam a cardiopatia chagásica crônica (SOARES and DOS SANTOS, 2007; CAMPOS DE CARVALHO *et al,* 2009; LIMA *et al,* 2009).

Iniciando com ensaios em pacientes, o primeiro relato de caso de terapia celular em paciente chagásico foi publicado (VILAS-BOAS *et al,* 2004). Neste paciente foram injetadas células mononucleares de medula óssea autólogas por via coronariana através de cateter de angioplastia. O paciente não apresentou nenhuma alteração elétrica nem arritmias, nem alterações bioquímicas ou hematológicas durante o procedimento e mudanças de alguns aspectos funcionais foram observados como aumento da fração de ejeção, diminuição dos diâmetros

ventriculares e melhora na classe funcional e no escore de qualidade de vida de Minnesota, critérios qualitativos que avaliam a gravidade da doença no paciente (VILAS-BOAS *et al,* 2004). Baseado nestas observações iniciais, um ensaio clínico de fase I foi realizado em 28 pacientes, nos quais foram injetadas células mononucleares de medula óssea, como descrita no trabalho anterior. Além de injeção de células, os pacientes também receberam injeções diárias de G-CSF, para mobilização das células da medula óssea para a periferia. Houve três óbitos não relacionados à terapia. Não houve nenhuma alteração dos marcadores de necrose miocárdica 24 horas após o procedimento, nem alterações eletrocardiográficas sugestivas de isquemia ou infarto (VILAS-BOAS *et al,* 2006). Houve melhora significativa da fração de ejeção e dos critérios qualitativos de classe funcional e de qualidade de vida de Minnesota, bem como do teste de distância percorrida em seis minutos, quando comparado aos dados obtidos dos pacientes antes do tratamento (VILAS-BOAS *et al,* 2006). Em um relato de caso foi colocado que pesquisadores injetaram células mononucleares de medula óssea marcadas com um contraste por via coronariana, como nos estudos anteriores, em um paciente em estágio final de cardiomiopatia decorrente da doença de Chagas e observaram a movimentação das células após a injeção, relatando que as mesmas estavam concentradas em algumas áreas do coração do paciente, provavelmente relacionadas a áreas de fibrose no músculo cardíaco do paciente, 2 e 6 horas após a injeção das células (JACOB *et al,* 2007). Estes resultados embasaram a proposta de um ensaio clinico de fase II, multicêntrico, duplo cego, randomizado e controlado, com um grupo em que os pacientes não receberiam as células (grupo placebo), denominado de MiHeart Study (Multicenter randomized trial of cell therapy in cardiopathies), que reuniu quatro ensaios independentes em patologias cardíacas específicas e avaliou

o efeito da injeção das células da medula óssea em pacientes com doença cardíaca isquêmica aguda e crônica, pacientes chagásicos e cardiomiopatia dilatada (TURA *et al,* 2007). Os dados deste ensaio ainda estão sendo avaliados.

Todos estes trabalhos sugerem uma nova modalidade terapêutica para a cardiopatia chagásica, baseada na terapia celular. Ainda faltam ser esclarecidos alguns aspectos muito importantes, como qual ou quais os tipos celulares a serem usados. Qual ou quais os mecanismos responsáveis pelos resultados obtidos? E como podemos aperfeiçoar a ação da terapia celular? São questionamentos ainda pouco esclarecidos que começam a ser explorados (SOARES *et al,* 2007; SOARES and DOS SANTOS, 2009; CAMPOS DE CARVALHO *et al,* 2009; LIMA *et al,* 2009).

1.6.6- Regeneração tecidual e mecanismos de ação das células-tronco

A capacidade de regeneração dos tecidos é uma função essencial do corpo humano, bem como de todos os organismos. Um exemplo clássico é a regeneração da pele que em todo o momento é lesada (ROSENTHAL, 2003). Apesar de até recentemente se postular que alguns órgãos, como por exemplo, o coração e o cérebro, não têm nenhuma capacidade de regeneração, é crescente o número de evidências contra este postulado.

No coração, alguns estudos demonstraram a capacidade de replicação de miócitos após infarto do miocárdio em humanos e em modelos experimentais (ANVERSA *et al*, 2007; ANVERSA & NADAL-GINARD, 2002). Miócitos cardíacos humanos foram encontrados em divisão após infarto do miocárdio, o que pode ser devido à divisão de cardiomiócitos residentes no coração ou devido à mobilização de células-tronco a partir da circulação para a área do infarto (BELTRAMI *et al,* 2001; MAZHARI *et al*, 2007). De fato, o coração é um órgão cuja capacidade de auto-

regeneração é muito pequena, e requer tratamentos capazes de promover regeneração e revascularização que são críticas para a recuperação do miocárdio lesado (ROSENTHAL, 2001).

As modalidades terapêuticas convencionais para o tratamento de estágios avançados de danos cardíacos são limitadas. A habilidade em regenerar áreas com danos isquêmicos no miocárdio ainda são um grande desafio no tratamento de doenças cardiovasculares (HUGHES, 2002). No entanto, trabalhos publicados nos últimos anos demonstraram que a terapia celular tem se mostrado promissora na melhora da função cardíaca (MAZHARI *et al*, 2007). Apesar de vários estudos terem obtido resultados satisfatórios, os mecanismos de recrutamento, crescimento, migração e diferenciação das células-tronco, circulantes, presentes no órgão ou no tecido ou mesmo transplantadas para o local da lesão, ainda não foram bem esclarecidos. O microambiente estabelecido no tecido ou órgão lesado, a produção de quimiocinas e citocinas e a expressão de algumas moléculas podem ter um papel importante no recrutamento das células-tronco (KORBLING & ESTROV, 2003; KORBLING *et al,* 2003). O melhor entendimento dos mecanismos pelos quais as células atuam que resultam nos efeitos observados nos trabalhos deve ser alvo de novos estudos. Com base nestes questionamentos, algumas hipóteses têm sido colocadas em discussão. Uma delas é o efeito direto das células na regeneração do tecido lesionado por transdiferenciação ou fusão celular (ORLIC *et al,* 2001; ALVAREZ-DOLANO *et al,* 2003; MURRY *et al,* 2004). Para estes autores, as células atuam mediante sua capacidade de diferenciação em tipos celulares ou pela fusão com as células do tecido, mostrando uma característica que define as células-tronco, que é a plasticidade celular. Mais recentemente, pesquisadores têm trabalhado com a hipótese de ação das células mediante um efeito parácrino, ou seja, através da

produção e secreção de fatores de crescimento celular, fatores angiogênicos, citocinas e quimiocinas, modulando assim a proliferação celular, neovascularização, morte celular, fibrose e inflamação, dentre outras funções, permitindo a melhora da função cardíaca bem como da lesão tecidual (LI *et al,* 2009; GNECCHI *et al,* 2005; KINNAIRD *et al,* 2004). Fatores como FGF, VEGF, angiopoietina-1 (Ang-1), IL-1β, TNF-α, SDF-1 e IGF são produzidos por células-tronco, frente ao estímulo da lesão em modelos de isquemia cardíaca, e medeiam a angiogênese (BURCHFIELD *et al,* 2008). O perfil de expressão gênica das células no microambiente da lesão também se altera frente a um agente causador de lesão celular, promovendo um nicho adequado para o reconhecimento, migração e ação das células transplantadas. Células-tronco mesenquimais em cultura aumentam a expressão de genes relacionados com a síntese de IL-1, IL-6, TGF-b, FGF, VEGF, bem como a secreção das proteínas MCP-1, MMP-9, PDGF, VEGF, ativador de plasminogênio (PA), dentre outros, frente a uma situação de hipóxia (BURCHFIELD *et al,* 2008; KINNAIRD *et al,* 2004). A injeção intravenosa de células estromais multipotentes humanas em camundongos imunodeficientes infartados diminuiu a fibrose na área da lesão cardíaca e foi observado um aumento de expressão de genes relacionados ao balanço entre a formação e degradação das proteínas da matriz como a metaloproteinase 2 (MMP-2) e os inibidores TIMP-1 e TIMP-2, sugerindo que estes fatores tenham efeito no remodelamento da matriz extracelular (ISO *et al,* 2007). Com relação a inflamação, células mesenquimais injetadas no modelo murino de infarto do miocárdio parecem modular a produção de fatores pró-inflamatórios como as citocinas TNF-α, IL-1β e IL-6, que têm um envolvimento na gênese do processo inflamatório, bem como inibem a expressão da proteína quimiotática de monócitos (MCP-1). No modelo de infarto do miocárdio, o infiltrado de linfócitos T parece ter

ação citotóxica sobre os cardiomiócitos (VARDA-BLOOM, 2000), além de interferirem no processo de remodelamento e fibrose no músculo cardíaco, pela interação e aumento da expressão de pró-colágenos produzidos por fibroblastos cardíacos (Yu *et al,* 2005). O transplante de células-tronco mesenquimais parece modular esta resposta, protegendo o animal contra a perda de células cardíacas, além de modular o efeito do remodelamento ventricular pós-infarto (VARDA-BLOOM, 2000; Yu *et al,* 2005). Tendo em vista este cenário, parece que as células-tronco têm um papel imunomodulador evidente nos modelos citados anteriormente (BURCHFIELD *et al,* 2008).

A melhor compreensão das características do microambiente cardíaco de animais chagásicos, comparada com aquelas de animais normais ou chagásicos transplantados com células, bem como a análise dos resultados dos estudos apresentados na literatura a respeito do papel das células da medula óssea, tornará possível aumentar o conhecimento a respeito da capacidade de migração e regeneração e modulação do ambiente cardíaco lesado que contribui para uma melhora nos parâmetros de inflamação e fibrose observados em estudos anteriores no modelo experimental de cardiopatia chagásica crônica. Assim, algumas hipóteses sobre qual o papel da terapia celular no modelo experimental da doença de Chagas poderão ser sugeridas, aperfeiçoando o conhecimento a cerca do mecanismo de ação das células.

2- JUSTIFICATIVA

A doença de Chagas ainda é considerada um problema de saúde pública com uma prevalência de infecção humana de 15-16 milhões de casos e uma estimativa de 75-90 milhões de pessoas sob o risco de infecção (COURA, 2007). A cardiomiopatia chagásica crônica deverá acometer aproximadamente 5 milhões de indivíduos, o que equivale a 25-30% do total de infectados. Além disso, a identificação dos fatores envolvidos na patogênese das lesões cardíacas crônicas decorrentes da moléstia de Chagas é de grande interesse para o desenvolvimento de novas estratégias para o tratamento dos pacientes chagásicos, uma vez que estes indivíduos não têm uma perspectiva longa de vida nem tão pouco uma forma adequada de terapia de tal estágio da doença, a não ser o transplante cardíaco, que tem alto custo e não é definitivo para a melhora do estado de saúde do paciente (CALDAS *et al,* 2008; RASSI JR *et al,* 2009). Os mecanismos que levam ao desenvolvimento da cardiopatia chagásica crônica ainda estão sendo investigados, mas acredita-se que se trata de uma patogênese multifatorial (KIERSZENBAUM, 2005; BILATE & CUNHA-NETO, 2008; RASSI JR. *et al*, 2009).

A medula óssea tem participação fundamental na emigração de células para os sítios de lesão, na instalação de processos inflamatórios encontrados nos tecidos, bem como na mediação da recuperação dos tecidos afetados (SOARES et al, 2004; HAN *et al*, 2006; HARDISON *et al,* 2006). Células mononucleares de medula óssea transplantadas em animais chagásicos crônicos foram capazes de reduzir o processo inflamatório e a fibrose nos animais tratados, mostrando a possibilidade de utilização destas células no tratamento da doença (SOARES et al, 2004). O melhor entendimento das alterações mediadas pelas células da medula óssea no âmbito da cardiopatia chagásica poderá contribuir para o desenvolvimento de novas terapias para esta doença, com base na utilização de células e/ou fatores celulares.

3- OBJETIVOS

3.1- OBJETIVO GERAL:

Investigar quais os possíveis mecanismos que estão envolvidos na regeneração do miocárdio no transplante de células da medula óssea em animal chagásico, utilizando o modelo experimental de camundongos infectados por *Trypanosoma cruzi* da cepa Colombiana.

3.2- OBJETIVOS ESPECÍFICOS:

- Caracterizar o microambiente cardíaco de animais não infectados e infectados com *T. cruzi,* comparado o nível de expressão de genes possivelmente envolvidos na patogênese da doença de Chagas.
- Observar a participação das células da medula óssea na regeneração tecidual após a infecção pelo *T. cruzi* em animais quiméricos.
- Avaliar o perfil de citocinas, quimiocinas e metaloproteases produzido no tecido cardíaco dos animais transplantado com células de medula óssea, comparando com o perfil produzido por animais não transplantados e por animais não infectados.
- Elaborar uma hipótese sobre o possível papel das células da medula óssea na terapia celular de animais chagásicos.

4- MANUSCRITO I

Título: Gene expression changes associated with myocarditis and fibrosis in hearts of mice with chronic chagasic cardiomyopathy.

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Abstract: INTRODUCTION: Chronic chagasic cardiomyopathy is a leading cause of heart failure in Latin American countries. About 30% of Trypanosoma cruzi-infected individuals develop this severe symptomatic form of the disease, characterized by intense inflammatory response accompanied by fibrosis in the heart. **METHODS:** We performed an extensive microarray analysis of hearts from a mouse model of this disease. **RESULTS:** Were determined significant alterations in expression of ~12% of the sampled genes. Extensive upregulations were associated with immune-inflammatory responses (chemokines, adhesion molecules, cathepsins and MHC molecules) and fibrosis (extracellular matrix components, lysyl oxidase and Timp1). **CONCLUSION:** Our results indicate potentially relevant factors involved in the pathogenesis of the disease that may provide new therapeutic targets in chronic Chagas' disease. **KEYWORDS:** Chagas disease; cardiomyopathy; microarray; gene expression; mice.

MAJOR ARTICLE

Gene Expression Changes Associated with Myocarditis and Fibrosis in Hearts of Mice with Chronic Chagasic Cardiomyopathy

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Chronic chagasic cardiomyopathy is a leading cause of heart failure in Latin American countries. About 30% of *Trypanosoma cruzi*–infected individuals develop this severe symptomatic form of the disease, characterized by intense inflammatory response accompanied by fibrosis in the heart. We performed an extensive microarray analysis of hearts from a mouse model of this disease and identified significant alterations in expression of ~12% of the sampled genes. Extensive up-regulations were associated with immune-inflammatory responses (chemokines, adhesion molecules, cathepsins, and major histocompatibility complex molecules) and fibrosis (extracellular matrix components, lysyl oxidase, and tissue inhibitor of metalloproteinase 1). Our results indicate potentially relevant factors involved in the pathogenesis of the disease that may provide new therapeutic targets in chronic Chagas disease.

Chagas disease, caused by infection with the protozoan *Trypanosoma cruzi*, is still a major health problem in Latin America, where it affects 16–18 million people [1]. The most common chronic form, chagasic cardiomyopathy (CCM), is a fatal disease for which there is no effective treatment available other than heart transplantation. CCM is characterized by focal or disseminated inflammation causing myocytolysis, necrosis, and progressive fibrosis [2–4].

The pathological basis of CCM is multifactorial [5, 6]. It may in part result from inflammatory responses to *T. cruzi* antigen, to cardiac autoantigens, or to both types of antigens [7]. A prominent role of parasite antigens in this pathology has been supported by the demonstration that a decrease in parasite load caused a reduction in myocarditis and cardiac disturbances in mice chronically infected with *T. cruzi* [8].

The identification of factors involved in the establishment of chronic heart lesions is of great interest for the development of new therapeutic strategies for patients with this fatal disease. In this study we performed a DNA microarray analysis to identify alterations in gene expression in the myocardium of mice chronically infected with the Colombian strain of *T. cruzi*, compared with uninfected counterparts. Our results indicate a profound effect on expression of a number of genes related to inflammation and fibrosis in the hearts of mice with CCM.

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METHODS

Trypomastigotes of Colombian *T. cruzi* strain [9] were obtained from culture supernatants of infected LCC-MK2 cells. C57Bl/6 male and female mice were infected by intraperitoneal injection of *T. cruzi* trypomastigotes. Parasitemia was evaluated at various times after infection by counting the number of trypomastigotes in peripheral blood aliquots. Animals were raised and maintained at the Gonçalo Moniz Research Center/Fundação Oswaldo Cruz (FIOCRUZ) and provided with rodent diet and water ad libitum. Animals were handled according to the National Institutes of Health guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

Mice were killed after 8 months of infection, and their hearts removed and fixed in 10% buffered formalin. Morphometric analyses were performed in hematoxylin-eosin– or Sirius red–stained heart sections captured using a digital camera adapted to a BX41 microscope (Olympus). Images were analyzed using Image-Pro Program software (version 5.0; Media Cybernetics).

Frozen heart sections were used for detection of CD4, CD8, CD11b, intercellular adhesion molecule 1 (ICAM-1), and major histocompatibility complex (MHC) class II expression by immunofluorescence, using specific antibodies (BD Biosciences) followed by streptavidin (Alexa Fluor 568; Molecular Probes). The myocardium was stained with phalloidin (Molecular Probes) or an anti–cardiac myosin antibody (Sigma). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Vectashield HardSet mounting medium with DAPI H-1500; Vector Laboratories). Sections were analyzed using a BX61 microscope equipped with epifluorescence and appropriate filters (Olympus) and a system to enhance the fluorescence resolution (OptiGrid; Thales Optem).

Stromal cell–derived factor 1 (SDF-1), tumor necrosis factor (TNF) α, and interferon (IFN) γ concentrations were measured in total heart extracts. Heart proteins were extracted from 100 mg tissue/mL phosphate-buffered saline, to which 0.4 mol/L sodium chloride, 0.05% Tween 20, and protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L benzethonium chloride, 10 mmol/L ethylenediaminetetraacetic acid, and 20-KI aprotinin A/100 mL) were added. The samples were centrifuged for 10 min at 3000 *g*, and the supernatant was kept frozen at −70°C. Cytokine levels were estimated using commercially available enzyme-linked immunosorbent assay kits for mouse SDF-1, TNF-α, and IFN-γ (R&D Systems), according to the manufacturer's instructions. Reaction was revealed after incubation with streptavidin–horseradish peroxidase conjugate, followed by detection using 3,3′,5,5′-tetramethylbenzidine peroxidase substrate and reading at 450 nm.

Hearts of normal and *T. cruzi*–infected mice were extracted and quickly frozen in liquid nitrogen for 5 min. The material was ground, and RNA extraction was performed using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. After addition of 1 U/μL DNase I (Invitrogen), the complementary DNA (cDNA) was obtained using SuperScript II Reverse Transcriptase (Invitrogen) in a final volume of 30 μL. Reaction cycles were performed on an Eppendorf Mastercycler gradient for 1 h (42°C for 60 min; 70°C for 15 min). Polymerase chain reaction (PCR) amplification was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primers and TaqMan probe for *Timp1*, the glyceraldehyde 3-phosphate dehydrogenase control reference gene, were designed and synthesized according to Assay-by-Design (Applied Biosystems). Quantitative data were analyzed using Sequence Detection System software (version 1.0; Applied Biosystems). PCRs were carried out in a total volume of 25 mL, according to the manufacturer's instructions. The standard curves of the target and reference genes showed similar results for efficacy (>90%). The relative quantification was given by the ratio between the mean values of the target gene and the reference gene (*Gapdh*) in each sample. The relative amount of PCR product generated from each primer set was determined on the basis of the cycle threshold (Ct) value. The relative quantification was calculated by the $2^{-\Delta\Delta Ct}$ method (Ct, fluorescence threshold value; ΔCt, the Ct of the target gene minus the Ct of the reference gene; $\Delta\Delta$Ct, the infected sample ΔCt minus the reference sample ΔCt).

Total RNA (20 μg) extracted from each of the 4 control and 4 infected hearts was reverse transcribed into cDNA incorporating fluorescent Alexa Fluor_647 or Alexa Fluor_555–aha-dUTPs (Invitrogen), by means of the SuperScript Plus Direct cDNA Labeling System (Invitrogen). Differently labeled biological replicas were cohybridized overnight at 50°C with MO30N mouse oligonucleotide arrays spotted with 32,620 70mer Operon oligonucleotides (Duke Microarray Facility; version 3.0.1) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL8938) using the "multiple yellow" strategy described elsewhere [10]. In this strategy, differently labeled biological replicas are cohybridized with the array. Thus, we have hybridized 2 arrays with samples from 4 control hearts and 2 other arrays with samples from 4 infected hearts. After washing (0.1% sodium dodecyl sulfate and 1% saline-sodium citrate) to remove the nonhybridized cDNAs, each array was scanned with an Axon 4000B dual-laser scanner (MDS Analytical Technologies) and images were primarily analyzed with GenePix Pro software (version 6.0; Axon Instruments). Locally corrupted or saturated spots, as well as those for which the foreground median fluorescence did not exceed twice the median local background fluorescence in 1 sample, were eliminated from analysis in all samples.

Microarray data were processed as described in our other studies [10–12]. In brief, we used a normalization algorithm that alternates intrachip and interchip normalization of the net

fluorescence (ie, background-subtracted foreground) signals of the validated spots until the residual error is <5% in subsequent steps. Intrachip normalization balances the averages of net fluorescence values in the 2 channels within each pin domain (subset of spots printed by the same pin), corrects the intensity-dependent bias (usually referred as Lowess normalization), and forces the standard distribution (mean, 0; standard deviation, 1) of $\log_2$ ratios (scale normalization) for net fluorescent values in the 2 channels for each array. Interchip normalization assigns a ratio between the corrected net fluorescence of each valid spot and the average net fluorescence of all valid spots in both control (C1, C2, C3, and C4) and infected (I1, I2, I3, and I4) samples. The spots probing the same gene were organized into redundancy groups, and their background-subtracted fluorescence was replaced by a weighted average value. A gene was considered significantly up- or down-regulated in the comparison between 4 infected and 4 control hearts if the absolute fold change was >1.5 and the P value was <.05 (Student's heteroscedastic t test of equality of the mean distributions, with Bonferroni-type adjustment for redundancy groups). GenMAPP [13] and MAPPFinder software (http://www.genmapp.org) and databases were used to identify the most affected gene ontology categories.

Morphometric, quantitative reverse-transcription PCR, and cytokine data were analyzed using Student's t test. Differences were considered significant at $P < .05$.

RESULTS

CCM caused by chronic infection with Colombian strain* T. cruzi *in C57Bl/6 mice. On infection with 1000 trypomastigote forms of Colombian strain *T. cruzi*, C57Bl/6 mice develop blood parasitemia peaking at ~35 days after infection (Figure 1*A*). The mortality rate reached 28.5% during the first 100 days (Figure 1*B*) and ~31.4% after 8 months of infection. Progressive myocarditis accompanied by fibrosis occurs after the acute phase of infection. At 8 months of infection, heart sections from chagasic mice revealed a multifocal inflammatory response composed mainly of mononuclear cells (Figure 1*C* and 1*E*) and exhibited areas of fibrosis (Figure 1*D* and 1*F*).

Global gene expression analysis. Microarray data from this experiment have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17363). When control hearts from C57Bl/6 mice were compared with those from age- and sex-matched mice chronically infected with the Colombian strain of *T. cruzi*, genes differentially expressed were detected. Spots corresponding to 14,356 unigenes satisfied the criteria of adequate quantitation for all 8 RNA samples. Of these, 1221 (8.5%) were significantly up-regulated in the chagasic hearts and 494 (3.4%) were significantly down-regulated (>50% difference; $P < .05$). A list of all genes that were found to be differentially expressed is presented in Table 1, and subsets of the genes showing higher fold change in expression ratio are considered below.

Pathways of proteins encoded by genes that were significantly affected by parasitic infection were determined using GenMAPP software (http://www.genmapp.org), in which significance is assessed by whether regulated genes are disproportionately represented within a gene ontology term. Pathways of genes significantly up-regulated in infected hearts ($P < .05$) are listed in Table 2 and prominently include immune response and related terms (eg, inflammatory response, intracellular signaling cascade, and chemokine and cytokine receptor activity). Results of the GenMAPP analysis of these altered genes are shown in Figure 2*A*. In addition, up-regulated pathways include phosphate transport, cell proliferation, and actin binding (eg, Arp2/3 protein complex and actin filament organization, cytoskeleton, and membrane ruffling). These genes related to the actin cytoskeleton are illustrated in Figure 2*B*. In addition to these well-represented pathways, smaller pathways showed prominent perturbation, including genes involved in cardiac differentiation (*Tgfb2* and *Itgb1*) and regulation of action potential (*Gnaq*, *Hexa*, *Hab1*, and *Cd9*).

Pathways containing an overrepresentation of down-regulated genes (Table 2) included mitochondrion, enzymatic activity of several types, and tyrosine kinase signaling. Genes down-regulated in less extensive pathways included negative regulation of notch plus bone morphometric protein signaling (*Htra1* and *Twsg1*) and regulation of vascular endothelial growth factor receptor signaling (*Flt1*).

Mice chronically infected with the Colombian strain of *T. cruzi* have intense myocarditis (Figure 1*E*). The inflammatory infiltrate is mainly composed by mononuclear cells, including $CD4^+$ and $CD8^+$ T lymphocytes (Figure 3*A* and 3*B*) and macrophages (Figure 3*C*). The analysis of genes that were up-regulated ≥5-fold in the arrays showed alterations in a number of genes related to inflammation and immune responses. Genes coding for the macrophage cell surface marker CD68 and the lymphocyte antigens CD38 and CD52 had their expression increased in chronic chagasic hearts (Table 3), a finding compatible with the presence of these cells in the inflammatory infiltrate.

Up-regulation of genes coding for chemoattractant factors Ccl2, Ccl7, Ccl8, and Ccl12 was observed (Table 3). Immunocytochemistry confirmed that the levels of Ccl12 (SDF-1) in hearts of chronically chagasic mice were increased in comparison with those of normal mice (Figure 4*A*). In addition, the expression of phospholipase A2, group VII (platelet-activating factor [PAF] acetylhydrolase), and complement factor B genes were highly increased (47.6- and 42.5-fold, respectively) by chronic infection (Table 3).

The expression of genes coding for adhesion molecules, such as galectin-3, P-selectin ligand (CD162), integrin β3 (CD61),

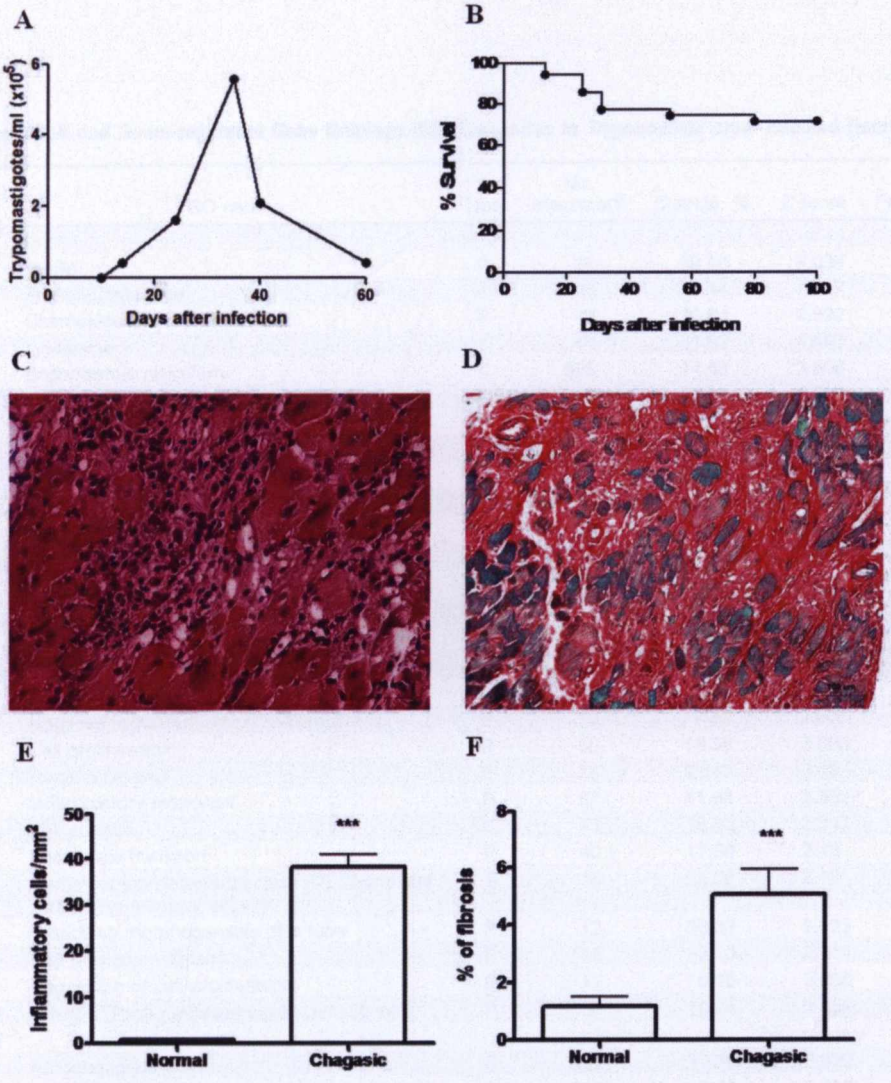

Figure 1. Infection of C57Bl/6 mice with Colombian strain *Trypanosoma cruzi*. Mice ($n = 35$) were infected with 1000 Colombian strain trypomastigotes. *A* and *B*, Parasitemia (*A*) and mortality (*B*) evaluated during the acute phase of infection. Data in panel A represent medians for individual parasitemia. *C* and *D*, Inflammation (*C*) and fibrosis (*D*) evidenced in heart sections from mice 8 months after infection, stained with hematoxylin-eosin and Sirius red. *E* and *F*, Morphometric quantification of inflammatory cells (*E*) and fibrosis area (*F*) in heart sections from normal mice ($n = 4$) and chagasic mice ($n = 9$; 8 months after infection with *T. cruzi*). Bars represent means ± standard errors of the mean. ***$P < .05$.

and ICAM-1 (CD54), was increased in hearts of chagasic mice (Table 3). Immunostaining revealed that ICAM-1 is virtually absent in control hearts, but in hearts of infected mice it is found mainly in inflammatory and endothelial cells (Figure 3*D* and 3*E*). The expression of genes coding for several cathepsins, proteases important in lysosomal degradation, was also up-regulated (Table 3). Of special interest is cathepsin S, which mediates degradation of the invariant Ii chain in antigen-presenting cells [13]. The expression of genes coding for MHC class II molecules IEb and IAa were highly altered. MHC class II molecules were observed to be highly expressed in cells of the inflammatory infiltrate in infected hearts (Figure 3*F* and 3*G*). In addition, the expression of genes encoding 2 proteasome subunits was also up-regulated (Table 3).

Cytokine-associated genes were differentially expressed in hearts of chagasic mice (Table 3). Of special interest is up-regulation of genes associated with 2 cytokines related to the severe form of chronic CCM [14, 15], IFN-γ (*Igtp*, *Ifi30*, *Ifi47*, *Irf1*, and *Irf5*) and TNF-α (*Tnfaip2*, *Tnfrsf1b*, and *Litaf*). Although regulation of genes encoding IFN-γ and TNF-α could not be analyzed in this microarray data set owing to technical problems, the protein levels of both cytokines were increased

Table 1. Genes Found to Be Differentially Regulated

This table is available in its entirety in the online version of the *Journal of Infectious Diseases*

Table 2. Up-regulated and Down-regulated Gene Ontology (GO) Categories in *Trypanosoma cruzi*–Infected Hearts

GOID	GO name	Type	No. measured[a]	Change, %	*Z* score	Permuted *P*
Up-regulated						
1726	Ruffle	C	21	38.10	5.039	<.001
6955	Immune response	P	108	19.44	4.817	<.001
8009	Chemokine activity	F	19	36.84	4.592	<.001
5764	Lysosome	C	44	31.82	4.487	<.001
5783	Endoplasmic reticulum	C	305	14.43	3.806	<.001
7242	Intracellular signaling cascade	P	169	12.43	3.302	<.001
6935	Chemotaxis	P	31	25.81	3.254	.004
30036	Actin cytoskeleton organization and biogenesis	P	32	12.50	3.232	.002
4180	Carboxypeptidase activity	F	11	27.27	3.22	.007
16798	Hydrolase activity, acting on glycosyl bonds	F	25	28.00	3.172	.004
15629	Actin cytoskeleton	C	40	10.00	3.09	.007
5938	Cell cortex	C	10	20.00	3.035	.01
7015	Actin filament organization	P	15	26.67	2.914	.015
5279	Amino acid–polyamine transporter activity	F	11	18.18	2.91	.016
42552	Myelination	P	13	23.08	2.806	.024
8285	Negative regulation of cell proliferation	P	45	15.56	2.67	.015
8284	Positive regulation of cell proliferation	P	56	16.07	2.669	.014
45596	Negative regulation on cell differentiation	P	13	7.69	2.603	.014
8283	Cell proliferation	P	55	16.36	2.588	.012
8201	Heparin binding	F	28	21.43	2.585	.019
6954	Inflammatory response	P	67	11.94	2.582	.008
9986	Cell surface	C	43	18.60	2.347	.026
6817	Phosphate transport	P	40	17.50	2.18	.039
4896	Hematopoietin/interferon class (D200 domain) cytokine receptor activity	F	16	25.00	2.18	.048
48754	Branching morphogenesis of a tube	P	12	33.33	2.123	.048
45165	Cell fate commitment	P	16	12.50	2.113	.038
42127	Regulation of cell proliferation	P	17	0.00	2.055	.045
7264	Small GTPase-mediated signal transduction	P	83	10.84	2.055	.05
Down-regulated						
5739	Mitochondrion	C	351	17.38	13.839	<.001
5737	Cytoplasm	C	613	2.61	5.628	<.001
3824	Catalytic activity	F	104	5.77	5.241	<.001
16491	Oxidoreductase activity	F	208	6.73	4.835	<.001
3954	NADH dehydrogenase activity	F	10	30.00	4.599	.005
50660	FAD binding	F	34	17.65	4.541	<.001
30170	Pyridoxal phosphate binding	F	31	16.13	3.871	.002
5777	Peroxisome	C	51	13.73	3.839	.002
6118	Electron transport	P	189	7.41	3.727	<.001
9055	Electron carrier activity	F	60	8.33	3.349	.003
5975	Carbohydrate metabolic process	P	81	6.17	3.169	.001
16874	Ligase activity	F	121	8.26	3.123	.005
8483	Transaminase activity	F	10	20.00	2.947	.017
7169	Transmembrane receptor protein tyrosine kinase signaling pathway	P	30	13.33	2.801	.012
6631	Fatty acid metabolic process	P	33	12.12	2.735	.013
166	Nucleotide binding	F	715	3.78	2.698	.007
9058	Biosynthetic process	P	31	12.90	2.511	.009
7050	Cell cycle arrest	P	24	12.50	2.43	.034
8152	Metabolic process	P	288	8.68	2.319	.025
6629	Lipid metabolic process	P	87	8.05	2.041	.043
16740	Transferase activity	F	558	4.84	1.995	.044

NOTE. C, cellular location; F, molecular function; FAD, flavin adenine dinucleotide; GOID, GO identification no.; NADH, nicotinamide adenine dinucleotide, reduced; P, biological process.

[a] No. of genes analyzed in that GOID.

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases*.

Figure 2. Genes found to be altered within the category of immune response and related terms from the GenMAPP database (Gladstone Institute, University of California, San Francisco).

in the hearts of chagasic mice compared with uninfected controls (Figure 4*B* and 4*C*). The expression of genes coding for surface receptors, such as C3a receptor 1, Fc receptors for immunoglobulin E (high affinity) and G (low affinity), and Toll-like receptor 2, was also elevated in chagasic hearts (Table 3).

Fibrosis is characteristic of hearts in chronically chagasic mice (Figure 1*F*), and there was marked up-regulation of genes related to synthesis of extracellular matrix components (Table 3). In addition, the gene expression of lysyl oxidase, an enzyme that promotes the cross-linking of collagen fibers, was increased (Table 3). The tissue inhibitor of metalloproteinase 1 (TIMP-1), an inhibitor of collagen degradation, was also up-regulated in chronic chagasic hearts (Table 3). Quantitative real-time PCR analysis confirmed a significant overexpression in *Timp1* in hearts of chronically chagasic mice compared with normal controls (Figure 4*D*).

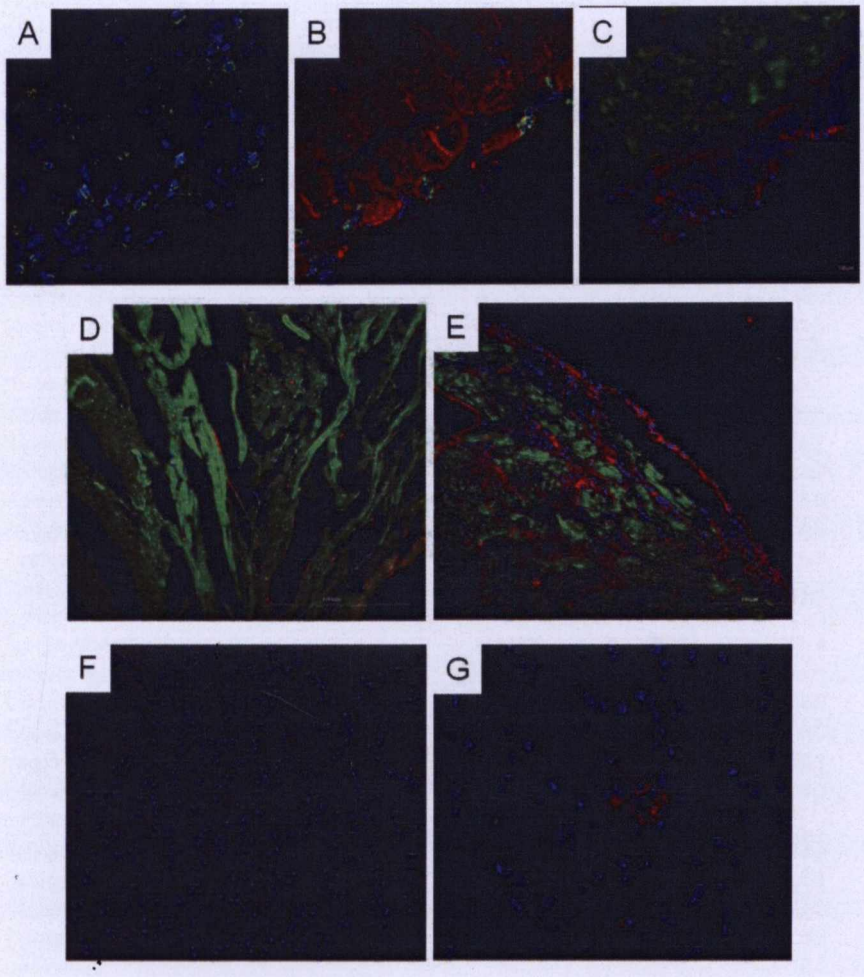

Figure 3. Analysis of heart sections from *Trypanosoma cruzi*–infected mice. Hearts from uninfected controls (*D* and *F*) and chronically chagasic mice (*A–C*, *E*, and *G*) were compared. *A*, Presence of $CD4^+$ cells (*green*) in infected myocardium. *B*, Section stained with anti-CD8 antibody (*green*) and phalloidin (*green*). *C*, Presence of $CD11b^+$ cells (*red*) in the inflammatory infiltrate and phalloidin staining (*green*) reveal proximity of macrophages to cardiac myocytes. *D* and *E*, Control (*D*) and infected (*E*) sections stained with an anti–intercellular adhesion molecule 1 antibody (*red*) and phalloidin (*green*), revealing up-regulation of this protein in the chagasic heart. *F* and *G*, Control (*F*) and infected (*G*) sections stained with an anti–major histocompatibility complex (MHC) II (Ia/Ie) antibody (*red*), showing the presence of MHC-II–expressing cells in the inflammatory infiltrate of chagasic hearts. All sections were stained with 4,6-diamidino-2-phenylindole for nuclear visualization (*blue*).

Table 3. Selected Up-regulated (>5-Fold) Genes

Gene name	Symbol	Fold regulation
Cytokine-related genes		
Chemokine (C-C motif) ligand 2	*Ccl2*	26.5
Chemokine (C-C motif) ligand 7	*Ccl7/MCP3*	16.2
Chemokine (C-C motif) ligand 8	*Ccl8*	50.6
Chemokine (C-C motif) receptor 5	*Ccr5*	12.1
Chemokine (C-X-C motif) ligand 12	*Cxcl12/SDF1*	5.0
IFN-γ–induced GTPase	*Igtp*	12.4
IFN-γ–inducible protein 30	*Ifi30*	11.9
IFN-γ–inducible protein 47	*Ifi47*	11.1
IFN regulatory factor 1	*Irf1*	7.7
IFN regulatory factor 5	*Irf5*	11.1
IL-10 receptor, α chain	*IL-10ra*	7.9
IL-18 binding protein	*IL-18bp*	6.6
IL-4 receptor, α chain precursor	*IL-4Rα*	9.2
LPS-induced TNF	*Litaf*	9.0
TNF-α–induced protein 2	*Tnfaip2*	6.2
TNF receptor superfamily, member 1b	*Tnfrsf1b*	9.4
TNF-α–induced protein 8–like	*Tnf p8l*	8.9
Immune response–related genes		
CD38 antigen	*Cd38*	7.0
CD52 antigen	*Cd52/B7*	21.6
CD68 antigen	*Cd68*	8.9
Complement component 4B	*C4b*	6.0
Complement factor B	*Cfb*	42.5
Fc receptor, IgE, high affinity I, gamma polypeptide	*Fcer1g*	17.6
Fc receptor, IgG, low affinity III	*Fcgr3*	9.1
Histocompatibility 2, class II antigen A, α	*H2-Aa*	38.5
Histocompatibility 2, class II, locus Mb1	*H2-DMb1*	12.7
Histocompatibility 2, Q region locus 7	*H2-Q7*	23.7
Histocompatibility 2, T region locus 10	*H2-T10*	5.0
Semaphorin 4A	*SemaA4*	8.9
T cell–specific GTPase	*Tgtp*	5.7
T cell, immune regulator 1, ATPase, H^+ transporting, lysosomal V0 protein A3	*Tcirg1, TIRC7*	9.4
Toll-like receptor 2	*Tlr2*	5.4
Cell adhesion		
Galectin-3	*Gal3*	36.9
Integrin β3	*Itgb3*	6.0
Integrin β1–binding protein 3	*Itgbp3*	36.2
Intercellular adhesion molecule	*Icam-1/Mala2*	6.7
Enzymes		
Cathepsin C	*Ctsc*	12.5
Cathepsin H	*Ctsh*	8.1
Cathepsin S	*Ctss*	47.5
Cathepsin Z	*Ctsz*	8.3
Lysozyme 1	*Lyz1*	8.7
Lysoyme 2	*Lys2*	7.0
Lysyl oxidase	*Lox*	5.3
Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	*Pla2g7*	47.6
Proteasome (prosome, macropain) subunit, β type 10	*Psmb10*	6.56
Proteasome (prosome, macropain) subunit, β type 8 (large multifunctional peptidase 7)	*Psmb8*	8.1
Matrix metalloproteinase 14	*Mmp14*	10.8

Table 3. ***(Continued.)***

Gene name	Symbol	Fold regulation
ECM-related genes		
α3 Type IX collagen	*Col9a3*	7.3
ECM protein 1	*Ecm1*	5.5
Microfibrillar-associated protein 5	*Mfap5*	8.0
Procollagen, type I, α2	*Col1a2*	6.0
TIMP-1	*Timp1*	49.6
TGF-β induced	*Tgfbi*	15.4

NOTE. ECM, extracellular matrix; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

DISCUSSION

The factors responsible for the establishment of the symptomatic form of chronic Chagas heart disease are still not fully understood. However, it is likely that the damage sustained by the myocardium is derived from parasite as well as host factors [7]. Here we have identified, by microarray analysis, a number of genes up-regulated in hearts of chronically chagasic mice that probably play a role in modulating inflammation and fibrosis in this phase of infection and thus may represent targets for therapeutic intervention in this disease.

A significant proportion of cells in the inflammatory infiltrate found in hearts of chronically chagasic mice are macrophages, as shown here by the expression of CD11b and up-regulation of CD68 gene expression. These cells are found in close contact with myofibers and may directly contribute to their damage through the secretion of TNF-α. In addition, macrophages are in close contact with T lymphocytes in the inflammatory foci presenting antigens by MHC II molecules to $CD4^+$ T lymphocytes, which secrete IFN-γ, increasing the cytotoxic potential of macrophages as well as of $CD8^+$ T cells present in the inflammatory foci. Interestingly, we found expression of the Toll-like receptor 2 gene (*Tlr2*) up-regulated in hearts of chronically chagasic mice. *T. cruzi* molecules, such as glycosylphosphatidylinositol anchors and glycoinositolphospholipids, activate macrophages to produce interleukin 12, TNF-α, and nitric oxide [16]. Thus, the residual parasitism found in the chronic infection probably contributes directly to the maintenance of TNF-α levels and indirectly to the maintenance of IFN-γ levels (through interleukin 12 production) in the hearts of chronically chagasic mice.

Although a growing body of evidence indicates that TNF-α contributes to the pathogenesis of heart failure [17], other reports have suggested beneficial effects of this cytokine in the heart [18]. Cardiac myocytes express both TNF-α receptors, type 1 (TNFR1) and type 2 (TNFR2) [19], which mediate its functions. TNFR1 seems to mediate the majority of the deleterious effects of TNF-α, such as TNF-α–induced cell death [20]. In contrast, activation of TNFR2 appears to exert protective effects against cardiac myocyte damage and apoptosis [21–23]. The strong up-regulation of the TNFR2 gene (*Tnfrsf1b*) in the hearts of chronically chagasic mice indicates that this receptor may contribute to the low number of apoptotic cardiac myocytes found during this phase of infection [24].

PLA2G7, another molecule secreted by monocytes and macrophages and found to be up-regulated in our study, degrades PAF, a lipid mediator that activates various cell types and promotes inflammation. Mice lacking PAF receptor (PAFR) have increased inflammation and parasitism in their hearts during acute infection [25]. This may be due to decreased parasite uptake and macrophage activation in the absence of PAFR activation, because PAF has been shown to mediate nitric oxide production and resistance to *T. cruzi* infection in mice [26]. In our model of chronic chagasic myocarditis, the role of PAF degradation by PLA2G7 is unknown, but the reduction in PAF accumulation may be related to the progressive damage of the myocardium, because PAF was shown to have a cardioprotective effect in isolated hearts [27].

A number of molecules involved in the recruitment of inflammatory cells to the heart of chagasic mice, including adhesion molecules and chemoattractant factors, were found to be up-regulated in our study. ICAM-1 expression in heart and endothelial cells was also increased in chagasic hearts, as described elsewhere [28, 29]. TNF-α increases the adhesiveness of endothelium for leukocytes and induces ICAM-1 expression [30]. Thus, the proinflammatory cytokines produced at the inflamed heart may be promoting the maintenance of inflammation by increasing the expression of ICAM-1. In agreement with the present study, overexpression of galectin-3 has been reported in *T. cruzi*–infected mice [31]. Galectin-3 binds to extracellular matrix components and was shown to participate in the adhesion of the parasite to coronary artery smooth muscle cells [32].

In the present study we found that chemokine genes encoding for CCL2, CCL8, and CCL7 (monocyte chemoattractant protein [MCP] 1, 2, and 3, respectively) are up-regulated in hearts of chronically chagasic mice. A number of studies have shown

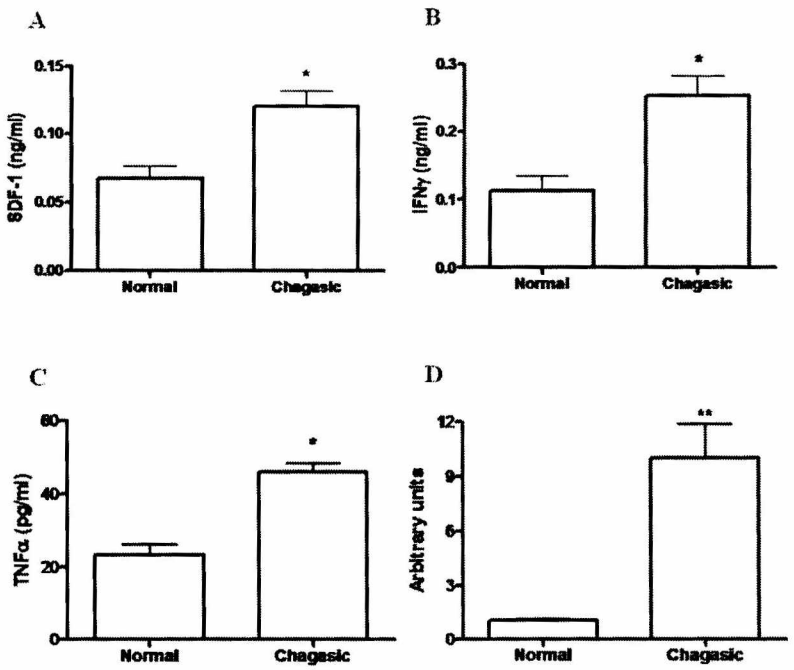

Figure 4. Increased production of stromal cell–derived factor 1 (SDF-1), interferon (IFN) γ, and tumor necrosis factor (TNF) α and transcript levels of *Timp1* in hearts of chronically chagasic mice. *A–C*, Levels of SDF-1 (*A*), IFN-γ (*B*), and TNF-α (*C*) identified in heart homogenates of normal mice ($n = 4$) and chagasic mice ($n = 9$; 8 months after infection with *Trypanosoma cruzi*), by enzyme-linked immunosorbent assay. $^{*}P < .05$ and $^{**}P < .01$. *D*, *Timp1* analyzed by quantitative real-time reverse-transcription polymerase chain reaction, using complementary DNA samples prepared from messenger RNA extracted from the hearts of normal ($n = 5$) or chronically chagasic ($n = 5$) mice. Data represent means ± standard errors of the mean for values obtained from individual mice.

that *T. cruzi* infection stimulates the production of chemokines by macrophages as well as by cardiomyocytes [33–35]. These chemokines are known to recruit monocytes and T lymphocytes. Other studies have demonstrated an association between the expression of MCP-1 and MCP-2 in the heart and both myocarditis and heart dysfunction [36, 37], suggesting a role of these cytokines in the maintenance of chagasic myocarditis.

CCR5 is a receptor for several chemokines of the CC family, including CCL3, CCL4, and CCL5, known to be up-regulated by infection with the Colombian strain of *T. cruzi* [38], and also for CCL8. Hearts of CCR5-deficient mice infected with *T. cruzi* have reduced migration of T cells. Because this receptor is predominantly expressed on the surface of Th1 cells [39], and a type 1 response with production of IFN-γ is associated with severity of CCM, CCR5 may play an important role in the pathogenesis of chronic chagasic myocarditis, as described in a model of autoimmune myocarditis [40]. In fact, treatment of chagasic mice with a selective CCR1 and CCR5 antagonist (Met-RANTES) decreased heart inflammation and fibrosis [41]. A positive correlation between severity of cardiomyopathy and the presence of $CCR5^{+}$ IFN-γ^{+} T cells was found in patients with chronic Chagas disease [14].

We found that CXCL12 (SDF-1) expression is increased in hearts of chronically chagasic mice. SDF-1 is a potent chemoattractant factor for lymphocytes [42], and therefore its expression may be relevant for the maintenance of immune-mediated heart destruction during the chronic phase of infection. Conversely, because this chemokine is also a stem cell recruitment factor, its increased expression may contribute to tissue regeneration of the damaged myocardium, as reported elsewhere in a model of myocardial ischemia [43]. In addition, MCP-3 (CCL7) was recently shown to be a mesenchymal stem cell homing factor for the myocardium [44]. Thus, the increased expression of these chemokines indicates that migration of stem cells can be promoted in chronic chagasic myocarditis by the presence of stem cell chemoattractant factors such as SDF-1 and MCP-3. In fact, we have shown that intravenously injected bone marrow cells migrate to the hearts of chronically chagasic mice [45].

The myocardial interstitial collagen matrix surrounds and supports cardiac myocytes and the coronary microcirculation, and its integrity is critical for the proper function of the heart. Thus, alterations in the collagen matrix will disrupt myocardial mechanical properties and ventricular function [46]. In Chagas dis-

ease, as a consequence of the sustained inflammatory process found in the myocardium during the chronic phase of infection, fibrosis is evident and contributes to cardiac remodeling. We also observed alterations in genes related to extracellular matrix deposition, such as extracellular matrix components and *Timp1*. Plasma concentrations of TIMP-1 are significantly elevated in patients with terminal heart failure compared with healthy controls [47], suggesting that this metalloproteinase inhibitor may also play a role in the evolution of heart failure in chronic Chagas disease. In addition, lysyl oxidase was increased in chagasic hearts. This enzyme promotes the cross-linking of collagen fibers, irreversibly altering the structure and function of the extracellular matrix proteins, causing dysfunction of the cardiomyocytes and, consequently, of the heart [46]; it therefore probably plays an important role in the evolution of fibrosis in chronic chagasic hearts.

To understand the delicate balance of multiple factors involved in the pathogenesis of Chagas disease is a complex task. Microarray approaches have been used before in mouse models of *T. cruzi* infection (C3H/HeN mice infected with *T. cruzi* Sylvio X10/4 strain [47] and C57Bl/6.129sv mice infected with *T. cruzi* Brazil strain [48, 49], as well as in hearts of chronically chagasic patients [50]. Using the model of infection with Colombian *T. cruzi* strain, we have identified new potentially important genes that may serve as a basis for therapeutic interventions in chronic Chagas heart disease.

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5- MANUSCRITO II

Título: Therapy with bone marrow cells reverses gene expression alterations in hearts of mice with chronic chagasic cardiomyopathy

A ser submetido.

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Abstract: INTRODUCTION: Chronic chagasic cardiomyopathy is one of the leading causes of heart failure in Latin American countries. An intense inflammatory response accompanied by fibrosis is found in hearts of chagasic patients. We have previously shown that bone marrow mononuclear cell (BMC) transplantation improves inflammation, fibrosis and ventricular diameter in hearts of mice with chronic Chagas' disease. Here we investigated alterations of gene expression in the hearts of chronic chagasic mice submitted or not to BMC therapy. **METHODS:** C57Bl/6 mice chronically infected with *T. cruzi* (6 months) were transplanted with BMC or saline i.v. and sacrificed 2 months later. RNA was extracted from the hearts of normal controls, chagasic and BMC transplanted mice and microarray analysis was performed using MO30k oligonucleotide arrays. **RESULTS:** Out of the 9390 unigenes quantified in all samples, 1702 had their expression altered in chronic chagasic hearts compared to those of normal mice. Major categories of significantly upregulated genes were related to inflammation, fibrosis and immune responses, while genes involved in mitochrondrion function were downregulated. When BMC-treated chagasic hearts were compared to infected mice, 1631 (96%) of the alterations detected in infected hearts were not found, although an additional 109 genes were altered by treatment, indicating a remarkable 84% transcriptomic recovery. Immunofluorescence and morphometric analyses confirmed the effects of BMC therapy in the pattern of inflammatory-immune response and expression of adhesion molecules. **CONCLUSION:** Our results demonstrate important immunomodulatory effects of BMC therapy in chagasic cardiomyopathy and indicate potentially relevant factors involved in the pathogenesis of the disease that may provide new therapeutic targets. Moreover, the novel use of global gene expression profiling and the measure of transcriptomic recovery efficacy offer unbiased quantitative assessment of treatment outcome. **KEYWORDS:** Chagas' disease; cardiomyopathy; cell therapy; microarray; immunomodulation; adhesion molecules

Therapy with bone marrow cells reverses gene expression alterations in hearts of mice with chronic chagasic cardiomyopathy

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Abstract

Chronic chagasic cardiomyopathy is one of the leading causes of heart failure in Latin American countries. An intense inflammatory response accompanied by fibrosis is found in hearts of chagasic patients. We have previously shown that bone marrow mononuclear cell (BMC) transplantation improves inflammation, fibrosis and ventricular diameter in hearts of mice with chronic Chagas' disease. Here we investigated alterations of gene expression in the hearts of chronic chagasic mice submitted or not to BMC therapy. C57Bl/6 mice chronically infected with *T. cruzi* (6 months) were transplanted with BMC or saline i.v. and sacrificed 2 months later. RNA was extracted from the hearts of normal controls, chagasic and BMC transplanted mice and microarray analysis was performed using MO30k oligonucleotide arrays. Out of the 9390 unigenes quantified in all samples, 1702 had their expression altered in chronic chagasic hearts compared to those of normal mice. Major categories of significantly upregulated genes were related to inflammation, fibrosis and immune responses, while genes involved in mitochrondrion function were downregulated. When BMC-treated chagasic hearts were compared to infected mice, 1631 (96%) of the alterations detected in infected hearts were not found, although an additional 109 genes were altered by treatment, indicating a remarkable 84% transcriptomic recovery. Immunofluorescence and morphometric analyses confirmed the effects of BMC therapy in the pattern of inflammatory-immune response and expression of adhesion molecules. Our results demonstrate important immunomodulatory effects of BMC therapy in chagasic cardiomyopathy and indicate potentially relevant factors involved in the pathogenesis of the disease that may provide new therapeutic targets. Moreover, the novel use of global gene expression profiling and the measure of transcriptomic recovery efficacy offer unbiased quantitative assessment of treatment outcome.

Keywords: Chagas' disease; cardiomyopathy; cell therapy; microarray; immunomodulation; adhesion molecules

Introduction

The discovery of stem cells capable of differentiating into specialized cell types, such as cardiomyocytes and endothelial cells, has opened new avenues for the treatment of degenerative and traumatic disorders, including heart failure (Goldenberg et al, 2008). Patients with Chagas disease, one of the main causes of heart failure in Latin American countries, could possibly benefit from a stem cell-based therapy aiming to ameliorate the heart function deteriorated by the chronic infection with *Trypanosoma cruzi* (Soares et al, 2007). Currently, heart transplantation is the only efficient therapy for chagasic patients with heart failure, a procedure limited by the scarcity of donated organs and complications when performed in *T. cruzi*-infected patients due to the risk of a resurgence of parasitemia upon administration of immunosuppressive drugs (Campos et al, 2008). Therefore, instead of replacing the hearts of chagasic heart failure individuals, the perspective of repairing the heart with the patient's own cells presents an attractive option that should be investigated.

Chronic chagasic cardiomyopathy (CCM) is a progressive disease characterized by focal or disseminated inflammation causing myocytolysis, necrosis, and progressive deposition of collagen (Andrade, 1999). Transplantation of bone marrow cells (BMC) was reported to reduce inflammation and fibrosis in the hearts (Soares et al, 2004). The decrease in inflammatory cells correlated with an increase in apoptotic cells in the inflammatory infiltrate. A regression of right ventricular dilation, typically observed in a specific chagasic mouse model, was observed in mice treated with BMC, as detected by magnetic resonance analysis (Goldenberg et al, 2008). In addition, administration of granulocyte-colony stimulating factor (G-CSF), a cytokine capable of mobilizing bone marrow cells to the peripheral blood, to chagasic mice caused a decrease in inflammation and fibrosis and improvement of cardiopulmonary function (Macambira et al, 2009).

The identification of the mechanisms by which bone marrow cells promote improvement of cardiac damage in infected mice may contribute to the development of more efficient therapies for chagasic patients. We have previously shown that a number of genes related to inflammation and fibrosis are altered in hearts of chagasic mice compared to

normal controls (Soares et al, 2010). In this study we have performed cDNA microarray analyses as an unbiased approach to evaluate the efficacy of bone marrow transplantation to restore the normal transcriptome in the myocardium of mice chronically infected with *T. cruzi.* Our results show a marked transcriptomic recovery caused by the bone marrow cell therapy in the hearts of mice with CCC.

Materials and methods

Animals

Three to four week-old female and male C57Bl/6 mice were used for *T. cruzi* infection. All animals, weighing 20-23 g, were raised and maintained at the Gonçalo Moniz Research Center/FIOCRUZ in rooms with controlled temperature (22 ± 2°C) and humidity (55 ± 10%) and continuous air flow. Animals were housed in a 12 h light/12 h dark cycle (6 am-6 pm) and provided with rodent diet and water *ad libitum*. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described had prior approval from the local animal ethics committees (Einstein College of Medicine and Fiocruz, Bahia).

Infection with Trypanosoma cruzi

Trypomastigotes of the myotropic Colombian *T. cruzi* strain (Federici *et al*, 1964) were obtained from culture supernatants of infected LCC-MK2 cells. Infection of C57BL/6 mice was performed by intraperitoneal injection of 1000 *T. cruzi* trypomastigotes in saline. Parasitemia of infected mice was evaluated at various times after infection by counting the number of trypomastigotes in peripheral blood aliquots.

Bone marrow cell (BMC) transplantation

BMC obtained from femurs and tibiae of C57BL/6 mice were used in transplantation experiments (Soares et al, 2004: Goldenberg et al, 2008). Briefly, BMC were purified by centrifugation in Ficoll gradient at 1000 g for 15 minutes (Histopaque 1119 and 1077, 1:1; Sigma, St. Louis, MO). After two washings in RPMI medium (Sigma), the cells were

resuspended in saline, filtered over nylon wool, and injected intravenously in chagasic mice ($3x10^6$ cells/mouse). Non-transplanted control mice received intravenous injections of the same volume (200 µl) of saline.

Morphometric analysis

Groups of mice were sacrificed after 8 months of infection and hearts removed and fixed in 10% buffered formalin. Heart sections were analyzed by light microscopy after paraffin embedding, followed by standard hematoxylin/eosin staining. Inflammatory cells infiltrating heart tissue were counted using a digital morphometric evaluation system. Images were digitized using a color digital video camera adapted to a microscope. The images were analyzed using the Image Pro Program (Media Cybernetics, San Diego, CA, USA), such that the inflammatory cells were counted and integrated with respect to area. Ten fields per section were counted in 5-10 sections per heart. The percentage of fibrosis was determined using Sirius red-stained heart sections and the Image Pro Plus v.7.0 Software to integrate the areas.

Immunofluorescence

Formalin-fixed paraffin embedded hearts were sectioned and 4 µm-thick sections were used for detection of galectin 3 and syndecan 4 expression by immunofluorescence. First, a heat-induced antigen retrieval step in citrate buffer (pH=6.0) was performed. Then, sections were incubated overnight with the following primary antibodies: anti-galectin 3, 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-syndecan 4, 1:50 (Santa Cruz Biotechnology) or anti-vWF, 1:100 (Dako, Glostrup, Denmark). On the following day, sections were incubated with Alexa fluor 633 conjugated Phalloidin, 1:50, mixed with one of the secondary antibodies Alexa fluor 488-conjugated anti-goat IgG, 1:200 or Alexa fluor 488-conjugated anti-rabbit IgG, 1:200 (Molecular Probes, Carlsbad, CA, USA) for 1 hour. Nuclei were stained with 4,6-diamidino-2-phenylindole (VectaShield Hard Set mounting medium with DAPI H-1500; Vector Laboratories, Burlingame, CA). The presence of fluorescent cells

was determined by observation on a IX61 confocal microscope (Olympus). Approximately 10 random fields per animal were captured using a 40x objective. Morphometric analyses were performed using the Image Pro Plus software.

DNA microarray and data analysis

We compared RNA samples extracted from whole hearts of 4 control, 4 chagasic and 4 BMC-treated chagasic mice by analyzing hybridization to microarrays printed by Duke University (http://www.ncbi.nlm.nih.gove/geo/query/acc.cgi?acc=GPL8938) spotted with MO30k mouse Operon version 3.0 70-mer oligonucleotides. The hybridization protocol (see Soares et al, 2010), the slide type and the scanner settings were uniform throughout the entire experiment to minimize the technical noise. Briefly, 20 μg total RNA extracted in Trizol from each of the twelve samples (individual hearts) was reverse transcribed in the presence of fluorescent Alexa Fluor® 555- and Alexa Fluor®647-aha-dUTPs (Invitrogen, Carlsbad, CA) to obtain labeled cDNA. Red and green labeled samples of biological replicas were then co-hybridized ("multiple yellow" strategy: Iacobas et al, 2006) overnight at 50° C. After washing (0.1% SDS and 1% SSC) to remove the non-hybridized cDNA, each array was scanned at 630V (635 nm) and 580V (532 nm) with GenePix 4100B scanner (Axon Instruments, Union City, CA) and images were primarily analyzed with GenePixPro 6.0 (Molecular Devices, Sunnyvale, CA). Microarray data were processed as described previously (Soares et al, 2010). A gene was considered as significantly up- or down-regulated when comparing four hearts from one condition to those from another if the absolute fold change was >1.5x and the p-vlaue of the Sutdent"s heteroscedastic t-test of equality of the means of the distributions with a Bonferroni-type adjustment for each redundancy group (set of spots probing the same gene) was <0.05. GenMapp (Dahlquist et al, 2002) and MappFinder (www.genmapp.org) software and associated databases were used to identify the most affected GO (Gene Ontology) categories. In order to determine whether genes differentially expressed in untreated and treated infected hearts with respect to uninfected hearts were disproportionately affected in specific pathways, we used

GenMAPP and MappFinder software (Gladstone Institute; San Francisco: www.genmapp.org) to provide the statistics of affected GO categories. GO sets with fewer than 10 analyzed members were excluded from this analysis.

The novel parameter transcriptomic recovery efficacy (TRE) was computed as:

$$TRE = \frac{DX + UX - XD - XU}{DX + UX + XD + XU + DD + DU + UD + UU} \times 100\%,$$

where: D, U, X indicate whether the gene was down-, up- or not-regulated in the untreated (first position) or treated (second position) infected hearts.

Statistical analysis

Morphometric and qRT-PCR data were analyzed using Student's *t* test or ANOVA. Differences were considered significant when $p < 0.05$.

Results

Injection of BMC in mice with CCM reduces inflammation and fibrosis

Mice infected with the Colombian strain of *T. cruzi* develop a progressive myocarditis accompanied by fibrosis during the chronic phase of infection. Treatment with BMC caused a decrease in the number of inflammatory cells (Fig. 1A) and percentage of fibrosis (Fig. 1B) compared to saline-treated controls. An intense multifocal inflammatory response composed mainly by mononuclear cells, causing myocytolysis, was seen in sections of saline-treated chagasic mice (Fig. 1C). Smaller and less frequent foci of inflammatory cells were found in heart sections of chagasic mice treated with BMC (Fig. 1D).

We examined transcriptomic changes in the infected mouse hearts, defining as significant those genes whose mean expression level changed by more than 1.5 fold and that were different at the 0.05 level of significance when the variation among the four biological replicas was considered. At eight months of infection, 9390 genes were quantifiable on all arrays (from uninfected controls, infected and not treated and BMC treated infected hearts); of these, 480 (5.12%) were downregulated and 1222 (13.01%) were

upregulated in the infected not treated hearts. GenMapp analysis was used to determine whether regulated genes encoding proteins performing specific functions were differentially affected. Of the upregulated genes, most prominent Gene Ontogeny (GO) terms that corresponded to these pathways were immune system process/immune response, chemokine receptor binding and chemokine activity, including inflammatory response and chemotaxis [genes *CCl12, CCl6, CCl7, Ccl8* (50-fold upregulated), *CCl9* (40-fold upregulated) and *Cxcl1* (data not shown). Downregulated genes in the infected hearts were most prominently associated with mitochondria (cofactor binding, TCA cycle, glycoloysis, oxidative phsophorylation, coenzyme biosynthesis, oxidoreductase acrtivity, electron transport, NADH, RNA biosynthesis), although other affected pathways included axon guidance receptor (*Ephb3, Q8C419), negative regulation of BMP signaling (Htra1, Twsg1, FLt1*), transmembrane receptor tyrosine kinase activity (*Erbb3, Ephb3, Q8C419*), cell cycle arrest (*Ak1, Cdkn1b, Cdkn1c, Sesn1*) and multidrug transport *(Slc47a1*).

In the infected hearts of mice treated with bone marrow cells, we found that expression of 103 genes (1.21% of the quantified genes) were upregulated compared to controls, and 77 (0.91%) were downregulated. Most prominent categories of upregulated genes were those associated with angiogenesis and blood vessel development (*Casp8, Col18a1, Dicer1, Myh9, Pdgfa, Tnsf12a, Cxcl12, Fgf8, Serpinf1, Stqab1, Btg1, Notch1*) and endothelial cell development (*Vezf1*), thiamin transport and folate carrier activity (*Slc19a2*) and vitamin biosynthesis (*Itgb1bp3*), cyclin binding (*Cdkn1a*) and purine catabolism (*Entpd2*). Downregulated gene categories included nuclear transport (*Rac1, Ran, Akt1*), cofactor metabolic process (*Ak1, Ndufs1, Ldhd, Acss1, Idh3a, Sdha, Sacla2, Snclg2, Coq2, Park1*) and isomerase activity (*Flbp10, Hsd367, Lgals3bp*), calcium ion binding (*Eef2k, Fkbp10, Frem2*), and cell cycle arrest (*Ak1, Cdkn1b, CDkn1c, Sesn1*). The remarkable restoration of the control gene expression pattern by BMC therapy of infected mice is summarized in the form of a Venn diagrams in Figure 2 and is quantitated by the TRE score, calculated as follows:

$$TRE = \frac{DX + UX - XD - XU}{DX + UX + XD + XU + DD + DU + UD + UU} \times 100\% \quad ;$$

$$TRE = \frac{454+1166+62+46}{454+1166+62+46+39+0+1+31} \text{ x } 100\% = 84\%$$

Note that only 70 genes (of 1702) retained their up- or downregulation in the chagasic heart following bone marrow cell therapy and only a single gene was switched between up- and down-regulated.

Alterations in the expression pattern of galectin 3 and syndecan-4 in hearts of mice with CCC after BMC transplantation

The expression of two proteins encoded by genes upregulated by *T. cruzi* infection and modulated by BMC therapy was investigated by confocal analysis. Galectin 3 was found highly expressed in macrophages of the inflammatory infiltrate in chagasic hearts, when compared to non-infected controls (Figs. 3A and 4A and C). Upon BMC treatment, the number of galectin 3 positive cells was significantly reduced in heart sections of chagasic mice (Figs. 3A and 4E). Syndecan 4, a heparan-sulfate proteoglycan that regulates cell-matrix interactions and is present in focal adhesions, was found highly expressed in endothelial cells in chagasic hearts compared to normal mice (Figs. 3B and 4B and D). Heart sections of BMC-treated mice showed a significant reduction in the intensity of syndecan 4 staining and in the number of syndecan 4^+ blood vessels (Figs. 3B and C and 4F). In contrast, the total number of blood vessels were similar in the three groups, as indicated by staining with antibodies against von Willebrand Factor (Fig. 3D).

Discussion

Stem cell transplantation has become an attractive therapeutic possibility for patients with cardiac diseases, including CCC. The demonstration that BMC transplantation has beneficial effects in hearts of mice with CCC has raised several questions, and the determination of the molecular and cellular mechanisms by which these cells exert their action may contribute to the development of new therapeutic strategies for CCC based on

cell transplantation and/or cell factors. Here we performed a microarray analysis in an attempt to understand some of the mechanisms involved in the activity of BMC in the mouse model of CCC. Gene expression analysis was carried out comparing hearts of mice with CCC treated or not with BMC and non-infected controls. We have found that most of the genes altered by infection are modulated by BMC therapy, indicating a dramatic effect of these cells in the mechanisms of pathogenesis of the disease.

Previously we have shown that a significant number of genes with expression altered in the heart by infection with the Colombian *T. cruzi* strain are related to inflammation and fibrosis (Soares et al, 2010). As expected, since BMC therapy causes a significant decrease in inflammation and fibrosis, we found that most of these upregulated genes in CCC have their expression in the heart decreased after cell therapy.

The decrease in inflammation observed two months after BMC therapy was previously associated to an increased number of apoptotic cells compared to untreated chagasic controls (Soares et al, 2004). In contrast to the high number of inflammatory cells undergoing apoptosis during the indeterminate form (Andrade, 1999), a reduced number (about 0.5%) of cells in the inflammatory infiltrate were found undergoing apoptosis in hearts of individuals with CCC (Rossi and Souza, 1999). We found several genes related to apoptosis altered by *T. cruzi* infection and modulated by BMC therapy. Analysis such as the one used in this study will detect global alterations in gene expression, representing all cell types present in the heart, including cardiomyocytes and inflammatory cells. Thus, additional studies are needed to clarify which mechanisms are involved in the prevention of apoptosis of inflammatory cells in CCC, as well as those promoting apoptosis after BMC therapy.

The inflammatory infiltrate present in hearts of mice with CCC is mainly composed by mononuclear cells found adhered to myofibers, many of them in process of myocytolysis (Rossi *et al*, 1984). Macrophages are one of the main populations found in the inflammatory site, and are highly activated by IFNγ and TNFα, two cytokines produced in the hearts of chagasic mice (Soares et al, 2010). Here we found that the expression of galectin 3 (also known as Mac-2), a member of a large family of lectins that are highly conserved throughout

animal evolution, upregulated in chagasic mice, is modulated by BMC therapy. By immunofluorescence analysis, we showed that this molecule is expressed mainly in macrophages within the inflammatory infiltrate in the hearts of chagasic mice. A previous study has demonstrated, in a model of hypertrophied heart in rats, that galectin-3 was the most overexpressed gene in failing versus functionally compensated hearts (Sharma et al, 2004). Galectin-3 colocalized with activated myocardial macrophages, and treatment of rats with recombinant galectin-3 induced cardiac fibroblast proliferation, collagen production, cyclin D1 expression, and left ventricular dysfunction (Sharma et al, 2004). In addition, galectin 3 is known to play important roles in inflammatory responses, including suppression of T-cell apoptosis (Rabinovich et al, 2002) and its expression is induced by IFNγ in macrophages found in inflammatory infiltrates in the heart (Reifenberg et al, 2007). Altogether, these data suggest an important role of galectin 3 in the pathogenesis of CCC, and indicate this molecule as a target for development of new treatments for CCC. In fact, galectin 3 production has recently been pointed out as a novel marker of heart failure in patients (Lok et al, 2010).

Another molecule with its gene expression modulated by BMC therapy was syndecan-4, a heparin sulfate-carrying cell surface protein expressed by a number of different cell types, including endothelial cells, smooth muscle cells, and cardiac myocytes that participates in processes of cell signaling, adhesion and migration (Nikkari et al, 1994; Li et al, 1997). Little is known about the regulation of syndecan-4, but it has been reported to increase after various forms of tissue injury including vascular wall injury (Nikkari et al, 1994), or myocardial infarction (Li et al, 1997). Syndecan-4 expression has been shown to be increased with migration of blood-derived macrophages after myocardial infarction (Li et al, 1997). Zhang et al (1999) have shown that TNFα is the principal factor produced by the ischemic myocytes responsible for induction of endothelial cell syndecan-4 expression. To our knowledge, this is the first study investigating the expression of syndecan 4 in Chagas disease. The fact that TNF-α levels are increased in chronic chagasic hearts (Soares et al, 2010) may explain the finding of enhanced expression of syndecan 4 observed herein.

Furthermore, the modulation of the chronic inflammatory response in chagasic hearts induced by BMC therapy may explain the reduction of syndecan 4 expression found in BMC-treated animals.

A correlation between intensity of expression of syndecan 4 in endothelial cells with inflammatory infiltrates was found, but this was not due to an increase in the number of vWF^+ blood vessels. The microarray analysis suggested an increase in angiogenesis in hearts of chronic chagasic mice after BMC therapy, but this was not confirmed by the immunohistochemistry analysis. This apparent disparity may be explained by the fact that the genes upregulated after BMC therapy participate in a number of other biological processes in addition to those related to blood vessel formation, such as apoptosis (*Casp 8*, reviewed in Zhao et al, 2010), cell recruitment (*Cxcl12*, reviewed in Pelus et al, 2002) and phagocytosis (*Myh9*, reviewed in Berg et al, 2001).

In conclusion, a global gene expression analysis indicated a potent modulatory effect of BMC in the hearts of mice with CCC, probably mediated by a paracrine effect through the secretion of soluble mediators. The identification of such factors may lead to the association or the replacement of cell therapy by these cellular hormones in order to achieve the desired repair to the damaged chagasic heart.

We previously commented on the prominent upregulation of immune response genes in the chagasic heart and validated substantial numbers of these gene products using ELISA, real-time PCR, and confocal microscopy (Soares et al, 2010). The identification of mitochondria-associated genes as one of the pathways that are most downregulated in the infected heart is entirely consistent with reports by Garg and others indicating that mitochondrial function is a prominent target of infection in acute and chronic states (Garg et al, 2003; Gupta et al, 2009; Wen et al, 2006).

Gene expression profiling with microarrays has been widely used to characterize tissue and cell responses to various stimuli, to identify disease biomarkers and to reveal components and cellular and molecular interplay within and between gene regulatory networks. The application of this unbiased high throughput method to compare a pathological

situation with changes occurring after a therapeutic regimen here revealed a striking degree of recovery of control gene expression status in infected mice treated with bone marrow cells. As we previously showed in a study examining structural and physiological parameters of hearts of chagasic mice, the bone marrow cell therapy not only prevents further damage over time but also reverses the damage that was present before the stem cells were injected (Goldenberg et al, 2008).

Acknowledgements

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Legends to figures

Figure 1: BMC therapy decreases inflammation and fibrosis in hearts of C57Bl/6 mice chronically infected with Colombian strain *T. cruzi*. Mice were infected with 1000 Colombian strain *T. cruzi* trypomastigotes. Inflammation (**A**) and fibrosis (**B**) were quantified in heart sections of normal mice, 8 months after infection injected with saline (Saline) or with bone marrow cells (BMC), stained with H&E and Sirius red. Bars represent the means±S.E.M. of 5-8 animals/group. *, $P<0.05$; ***, $P<0.001$. Heart sections of chagasic mice injected with saline (**C**) or with BMC (**D**), stained with H&E.

Figure 2: Venn diagram indicating efficacy of BMC therapy. Of 9390 genes whose expression was quantifed on every array, indicated numbers were significantly up- and down-regulated in infected hearts and following BMC therapy. Note the very large number of genes whose expression was rescued to normal levels by BMC and the small number of newly regulated genes (side effect of therapy).

Figure 3: Quantification of galectin 3, syndecan 4 and vWF. Expression of galectin 3 (A), syndecan 4 (B and C) and vWF (D) were quantified by immunohistochemistry and morphometric analysis in heart sections of normal mice, mice 8 months after infection injected with saline (Saline) or with bone marrow cells (BMC). Bars represent the means±S.E.M. of 3 animals/group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 4: Expression of galectin 3 and syndecan 4 in hearts of chronic chagasic mice treated with BMC. Heart sections of normal mice (**A** and **B**), of mice injected with saline (**C** and **D**) or with bone marrow cells (**E** and **F**). Sections were stained (red) with anti-galectin 3 (**A**, **C**, and **E**) or anti-syndecan 4 (**B**, **D**, and **F**). Nuclei (blue) were stained with DAPI.

Figure 1

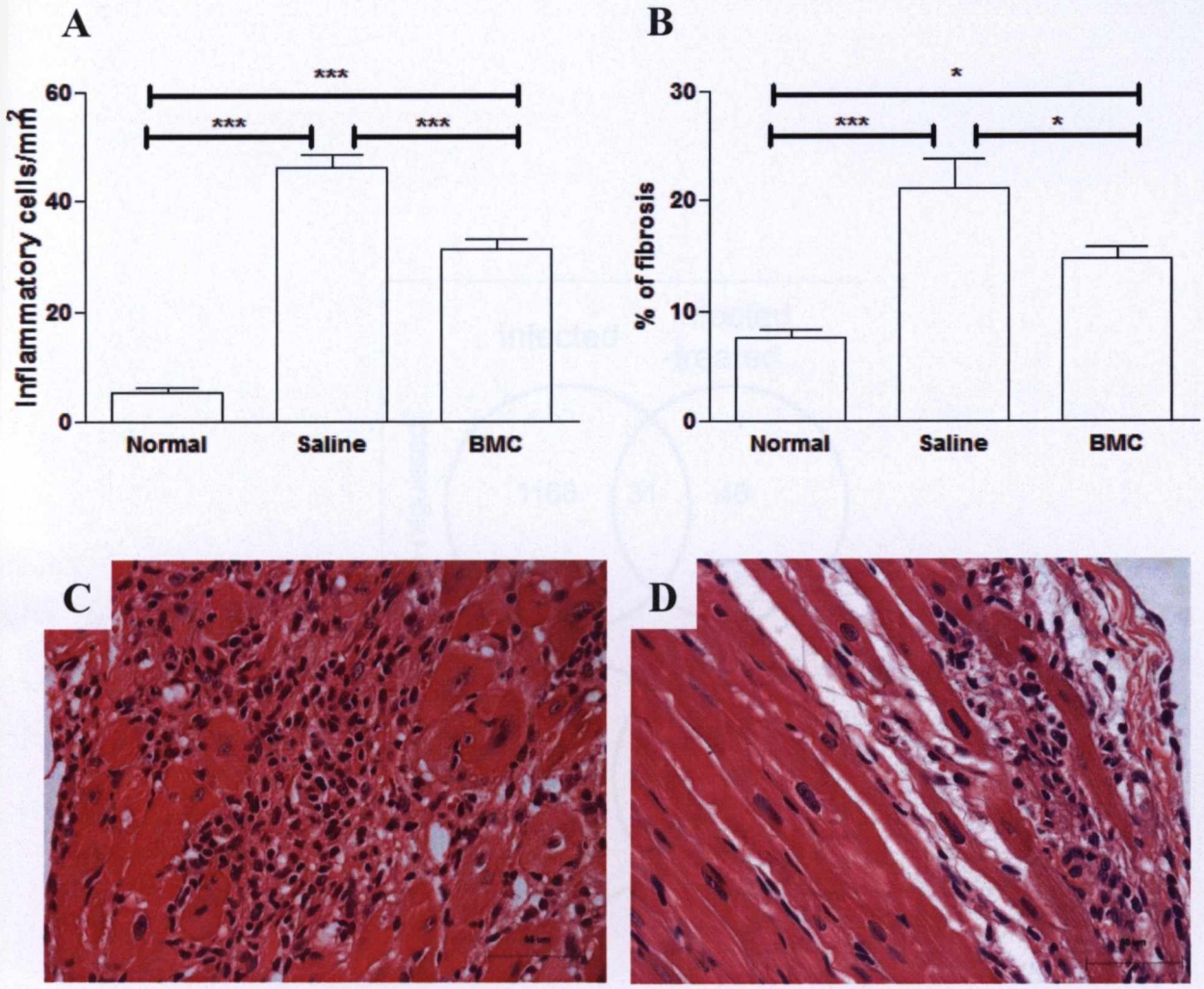


Figure 2

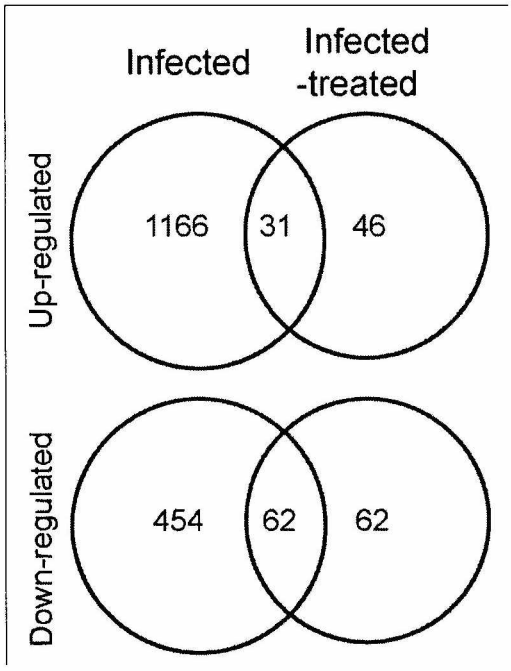

Infected
Infected -treated
Up-regulated
1166
31
46
Down-regulated
454
62
62

Figure 3

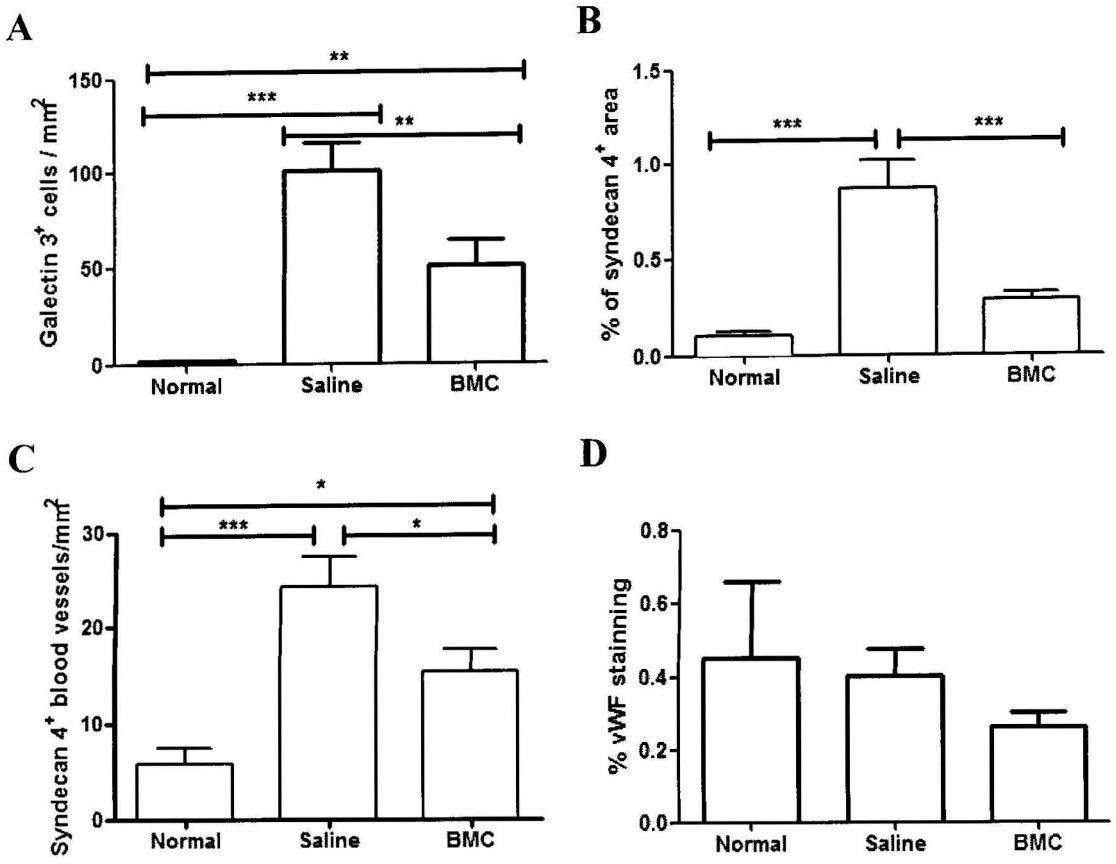
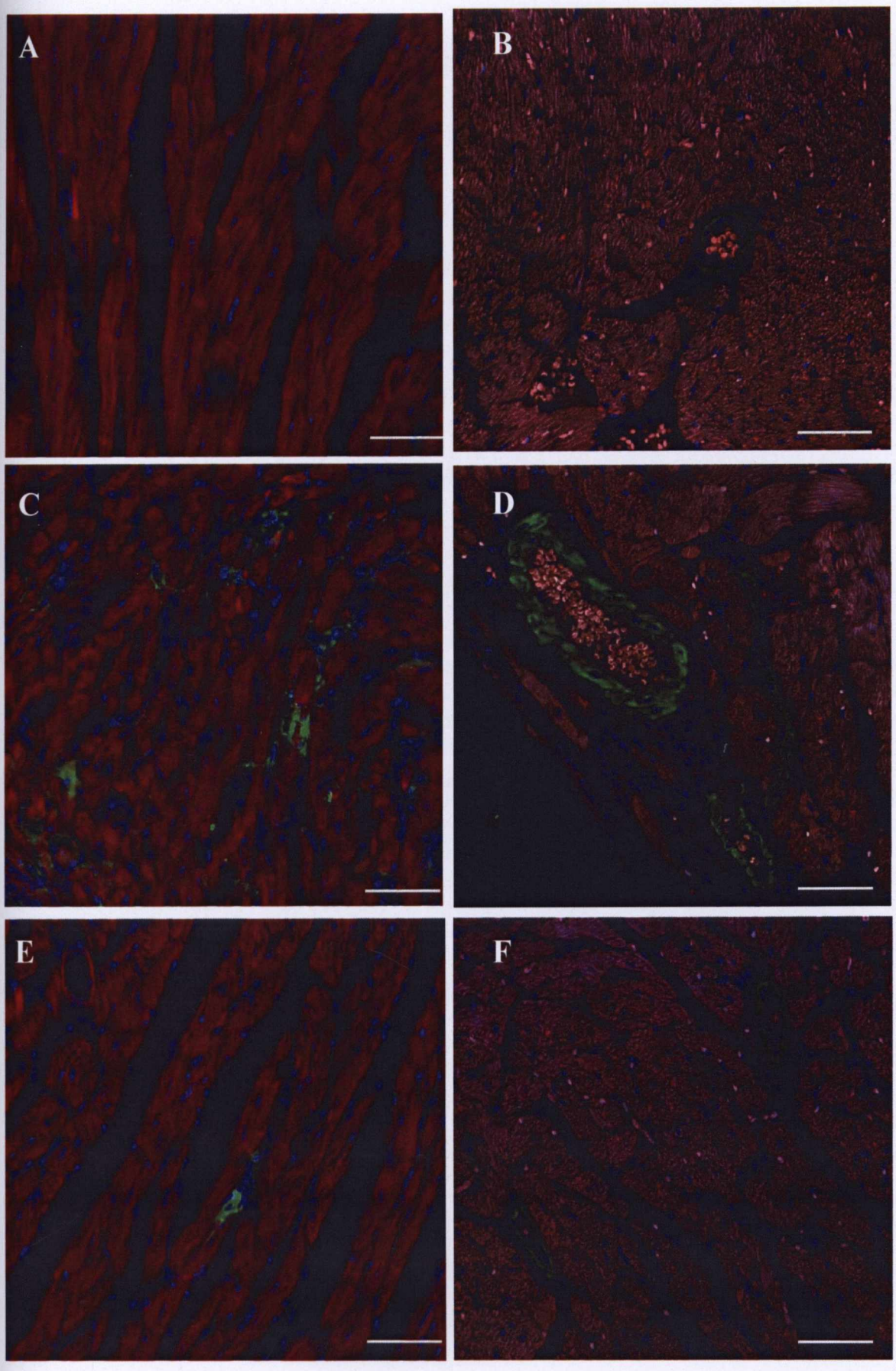

A
Galectin 3+ cells / mm2
150
100
50
0
**
***
**
Normal
Saline
BMC
B
% of syndecan 4+ area
1.5
1.0
0.5
0.0
***
***
Normal
Saline
BMC
C
Syndecan 4+ blood vessels/mm2
30
20
10
0
*
***
*
Normal
Saline
BMC
D
% vWF stainning
0.8
0.6
0.4
0.2
0.0
Normal
Saline
BMC

Figure 4



6- MANUSCRITO III

Título: Contribution of bone marrow cells in tissue regeneration after *Trypanosoma cruzi*-infection

A ser submetido.

Ordem dos autores: Ricardo Santana de Lima; Bruno Solano de Freitas Souza; Carine Machado Azevedo; Juliana Fraga Vasconcelos; Elisalva Teixeira Guimarães; Milena Pereira Botelho Soares; Ricardo Ribeiro dos Santos.

Abstract: INTRODUCTION: Acute infection by *Trypanosoma cruzi*, the etiological agent of Chagas disease, causes an intense inflammatory reaction triggered by the presence of parasites in several tissues, including the myocardium. Although this inflammation causes damage in the myocardium of intensity not seen in any other diseases, a regenerative process takes place after the control of parasitemia by the organism. We have previously shown that transplantation of bone marrow cells to mice with chronic Chagas disease ameliorates the lesions in the heart. Here we investigated the natural contribution of bone marrow cells in the repair of lesions in the heart and striated muscle during acute *T. cruzi* infection. **METHODS:** Chimeric mice were generated by transplanting GFP^+ bone marrow cells into lethally irradiated wild-type recipient mice and infected with a myotropic *T. cruzi* strain one month after transplantation. **RESULTS:** Migration of bone marrow-derived cells to the heart and striated muscle was seen during and after the acute phase of infection, since the inflammatory infiltrate was composed by GFP^+ cells. GFP^+ cardiomyocytes and endothelial cells were found in the heart sections of chimeric chagasic mice. In addition, a large number of GFP^+ myofibers were seen in the striated muscle of chimeric mice at different time points after infection. No GFP^+ myofibers were found positive for PCNA, a marker of cell proliferation. **CONCLUSION:** Our results reinforce the role of bone marrow-derived cells in the tissue regeneration and suggest the use of mouse models of *T. cruzi* infection to investigate the mechanisms involved in tissue regeneration. **KEYWORDS:** Chagas disease; bone marrow cells; chimeric mice; myocytes; tissue regeneration.

Contribution of bone marrow cells in tissue regeneration after *Trypanosoma cruzi*-infection

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Abstract

Acute infection by *Trypanosoma cruzi*, the etiological agent of Chagas' disease, causes an intense inflammatory reaction triggered by the presence of parasites in several tissues, including the myocardium. Although this inflammation causes damage in the myocardium of intensity not seen in any other diseases, a regenerative process takes place after the control of parasitemia by the organism. We have previously shown that transplantation of bone marrow cells to mice with chronic Chagas disease ameliorates the lesions in the heart. Here we investigated the natural contribution of bone marrow cells in the repair of lesions in the heart and striated muscle during acute *T. cruzi* infection. Chimeric mice were generated by transplanting GFP^+ bone marrow cells into lethally irradiated wild-type recipient mice and infected with a myotropic *T. cruzi* strain one month after transplantation. Migration of bone marrow-derived cells to the heart and striated muscle was seen during and after the acute phase of infection, since the inflammatory infiltrate was composed by GFP^+ cells. GFP^+ cardiomyocytes and endothelial cells were found in the heart sections of chimeric chagasic mice. In addition, a large number of GFP^+ myofibers were seen in the striated muscle of chimeric mice at different time points after infection. No GFP^+ myofibers were found positive for PCNA, a marker of cell proliferation. Our results reinforce the role of bone marrow-derived cells in the tissue regeneration and suggest the use of mouse models of *T. cruzi* infection to investigate the mechanisms involved in tissue regeneration.

Keywords: Chagas disease; Bone marrow cells; Chimeric mice; Myocytes; Tissue regeneration

Introduction

Chagas' disease is a zoonosis caused by the flagellate parasite *Trypanosoma cruzi*. The disease occurs in Mexico, Central and South America and still represents a major public health problem (Schofield *et al*, 2006). The prevalence of human infection by *T. cruzi* was estimated at 15-16 million cases and about 75-90 million people are at risk of infection in the Latin America (Dias and Coura, 1997, p.33; Coura *et al*, 2009).

The infection progresses in two consecutive phases: acute and chronic (Köberle, 1968). The acute phase of the disease is transient, usually without clinical signs, or with symptoms of minor relevance, and is characterized by the presence of trypomastigote forms of the parasite in the peripheral blood and amastigotes multiplying within several cell types in the host (Köberle, 1968; Andrade, 1983; Rassi *et al*, 2000, p.231). An intense inflammatory reaction is triggered by the presence of parasites in tissues, such as the myocardium, causing a destruction of unparalleled proportions in the myocardium (Andrade, 1983; Andrade, 2000, p.201-202). However, after the control of parasitemia, a regenerative process takes place in the affected organs. In humans, the acute phase regresses spontaneously after about 12 months, with reduction of parasites in the blood and tissues (Andrade, 1983; Andrade, 2000, p.201-202). About 70% of infected individuals remain free of clinical symptoms, radiological or electrocardiogram for lifetime, considered the indeterminate form of the disease. Only about 30% of the individuals infected by *T. cruzi* develop the symptomatic form of the disease, for which there is no effective treatment (Andrade, 1983).

The bone marrow is one of the best studied sources of stem cells (Bianco *et al*, 2001; Orlic *et al*, 2001a). A number of studies have shown that bone marrow-derived stem cells migrate to various organs, such as striated muscle and heart (Orlic *et al*, 2001b; Soares *et al*, 2004; Goldenberg *et al*, 2008), and may contribute to the formation of new specialized cells. In a previous study we demonstrated that transplanted bone marrow cells migrate to the heart of mice with chronic infection by *Trypanosoma cruzi*, the causative agent of Chagas disease (Soares *et al*, 2004). By using bone marrow cells from GFP-transgenic donors, we observed some GFP^+ cardiomyocytes in the hearts of chronic chagasic mice after transplantation (Soares *et al*, 2004; Soares *et al*, 2007). In the present work we sought to investigate the contribution of bone marrow-derived cells in the regenerative process that occurs naturally in the hearts and muscles of *T. cruzi*-infected mice during and early after the acute phase of infection, by using bone marrow chimeric mice generated using GFP transgenic mice as bone marrow cell donors.

Materials and Methods

Mice and chimeric mice

Six to eight weeks old female C57BL/6 mice were used as recipients for the production of chimeric animals. Four weeks old EGFP transgenic male C57BL/6 mice were used as bone marrow cells donors for reconstitution of irradiated mice. All mice were raised and maintained at the animal facilities at the Gonçalo Moniz Research Center, FIOCRUZ/BA, and provided with rodent food and water *ad libitum.*

Generation of chimeric mice

C57BL/6 female mice were irradiated with 6 Gy for depletion of the bone marrow cells in a ^{137}Cesium source irradiator (CisBio International, France). Bone marrow cells were obtained from femurs and tibiae from male EGFP transgenic mice and used to reconstitute irradiated mice. The mononuclear cells were purified by centrifugation in Ficoll gradient at 1000 *g* for 15 minutes (Histopaque 1119 and 1077, 1:1; Sigma, St. Louis, MO). After two washings in DMEM medium (Sigma), the cells were filtered over nylon wool, resuspended in saline, and injected 1 x 10^7cells/mouse in a dose of 200 µl intravenously in all irradiated mice. After 30 days of recovery, animals were infected with *T. cruzi*. Non infected chimeras were used as controls.

Parasites and infection

Trypomastigotes of the myotropic Colombian *T. cruzi* strain (Federici *et al*, 1964) were obtained from culture supernatants of infected LCC-MK2 cells. Infection of chimeric mice was performed by intraperitoneal injection of 100 *T. cruzi* trypomastigotes in saline. Parasitemia of infected mice was evaluated at various times after infection by counting the number of trypomastigotes in peripheral blood aliquots. Twenty-eight days after infection the animals were treated daily for one week with 40 mg/kg of benzonidazole diluted in saline for control of the parasitemia.

Morphometric analysis

Groups of animals were sacrificed 33, 66 and 192 days after infection and different organs were removed and fixed in 10% buffered formalin. Tissue sections were analyzed by light microscopy after paraffin embedding, followed by standard hematoxylin/eosin staining. Inflammatory cells infiltrating heart and striated tissues

were counted using a digital morphometric evaluation system. Images were digitalized using a color digital video camera adapted to a microscope. The images were analyzed using the Image Pro Program (Media Cybernetics, San Diego, CA, USA), so that the inflammatory cells were counted and integrated with respect to area. Ten fields per section were counted in 5-10 sections per heart.

Immunofluorescence analysis

Ten-μM frozen sections or 5 μM paraffin-embedded sections of hearts, livers, spleen and skeletal muscle were obtained and used for detection of GFP^+ cells. The following primary antibodies were used: chicken anti-GFP (1:400, Aves Labs, Tigard, Oregon, USA), rabbit anti-myosin (1:200, Sigma Aldrich, St. Louis, MO, USA), rabbit anti-von Willebrand Factor (1:50, Zymed Laboratories, San Francisco, CA, USA) and mouse anti-PCNA (1:200, Dako, Denmark) biotinylated with Dako Ark kit. Secondary antibodies, anti-chicken Alexa Fluor 488 conjugated (Molecular Probes, Carlsbad, CA, USA) and anti-rabbit Alexa Fluor 568 conjugated (Molecular Probes), were used. Some sections was used phalloidin Alexa Fluor 633 conjugated (Molecular Probes), an actin marker. For biotinylated anti-PCNA stained sections, we used streptavidin Alexa Fluor 568-conjugated (Molecular Probes). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA). Images were collected using a confocal microscope FluoView 1000 (Olympus, Tokyo, Japan).

Flow cytometry analysis

The presence of GFP^+ cells was confirmed in blood samples of irradiated and reconstituted mice at different times after transplantation, by flow cytometry analysis. Blood samples obtained from wild-type and GFP transgenic C57BL/6 mice were

used as negative and positive controls, respectively. The acquisition and analysis were done using a FACScalibur cytometer (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected.

Statistical analysis

Results were expressed as means ± S.E.M. of 3-4 mice per group. Statistical comparisons between groups were performed by analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test, using GraphPad InStat program (Software Inc., San Diego, CA, USA). Results were considered to be statistically significant when $P < 0.05$.

Results

Infection of bone marrow chimeric mice with Colombian T. cruzi *strain.*

To investigate the fate of bone marrow cells after *T. cruzi* infection, we generated bone marrow chimeric mice by transplanting GFP^+ bone marrow cells into C57BL/6 lethally irradiated recipients. Flow cytometry analysis demonstrated that 83 to 100% of the cells circulating in the blood of the chimeric animals one month after reconstitution and during infection were GFP^+ (Figure 1). Chimeric mice infected with 100 trypomastigotes of Colombian *T. cruzi* strain, became more susceptible than wild-type C57BL/6 mice, being unable to control the parasitemia and dying within 60 days of infection. Therefore, we treated infected chimeric mice with benznidazole on the 28^{th} day of infection, with a daily dose of 40 mg/kg orally for one week. A decrease of parasitemia levels in these animals was observed a few days after the beginning of treatment with benznidazole (Figure 2A).

The numbers of inflammatory cells in the heart and skeletal muscles were quantified. As shown in figure 2B, the number of inflammatory cells in the heart tissue is higher around the peak of parasitemia (33 days after infection), decreasing significantly after 192 days after infection. In contrast, the number of inflammatory cells in skeletal muscle remained high from the 33th to the 192th day of infection (Figure 2C). Parasite nests can be found in the heart (Figure 3A), as well as in skeletal muscle (Figure 4A) 33 and 60 days after infection.

Presence of GFP$^+$ cells in the heart and skeletal muscle of T. cruzi*-infected chimeric mice*

Heart sections of chimeric mice sacrificed after different times after infection had a massive influx of GFP$^+$ inflammatory cells (Figure 3B). In all time points analyzed, some GFP$^+$ cardiomyofibers were seen (Figures 3C-E). The GFP$^+$ cardiomyocytes were positively stained with an anti-myosin antibody (Figure 3C), and were seen as a group of adjacent cells or individual cells in the myocardium. In contrast, heart sections of uninfected control chimeric mice had occasional or no GFP$^+$ cells (Figures 3F and G). The GFP$^+$ cells found in the hearts of uninfected chimeric mice did not have morphology characteristic of cardiomyocytes, and did not express myosin.

GFP$^+$ cells also composed the intense inflammatory infiltrates found in section of skeletal muscle from chimeric mice (Figure 4B). A significant number of GFP$^+$ myofibers were found in all time points analyzed (Figures 4A-E). These GFP$^+$ cells also expressed myosin (Figures 4C-E). GFP$^+$ myofibers in skeletal muscle sections obtained from uninfected control mice were not found, even in those sacrificed at the 192th day (Figures 4F and G).

Some GFP^+ cells present in the inflammatory infiltrates of cardiac and skeletal muscle were positively stained for PCNA, a marker of cell proliferation (Figures 5A and B). However, myofibers expressing GFP and PCNA were not found in any sections from heart or skeletal muscle analyzed.

In addition to myofibers, GFP^+ cells were found within endothelial cells in blood vessels of hearts from chagasic (Figures 5C and D), but not from uninfected chimeric mice (Figure 5E). Some of these GFP^+ cells were also positive for von Willebrand factor, an endothelial cell marker (Figure 5D). GFP^+ cells were also found in other organs of chimeric chagasic mice, such as liver (Figure 5F) and spleen (data not shown).

Discussion

Stem cell-based therapies offer a new frontier for the treatment of chronic degenerative diseases, including muscular and cardiac diseases. Bone marrow is an easily accessible source of stem cells and therefore its potential therapeutic applications have been intensely investigated. We have previously shown that therapy with bone marrow mononuclear cells contributed to regeneration in chronic phase of Chagas disease (Soares *et al*, 2004). It is clear that there is a lack of basic knowledge to allow the efficient use of these cells or cell populations purified from the bone marrow, as well as to determine the diseases in which a therapy based on bone marrow cells will be beneficial. We bring here a model of Chagas disease in which the contribution of bone marrow-derived cells, as well as the role of inflammatory mediators, can be investigated in lesions of multiple organs and tissues.

Acute infection by *Trypanosoma cruzi* causes intense tissue damage in several organs, but an intense tissue regeneration process occurs when parasitemia

is controlled by the immune response (Andrade, 1983; Rassi *et al*, 2000, p.233-239). Little is known, however, about the mechanisms by which this tissue repair occurs in Chagas disease. The understanding of the cells and molecules naturally involved in tissue repair may open new avenues for the development of new therapies. In this report we showed that bone marrow derived cells not only migrate to, but also contribute to the formation of new resident cells in the heart and skeletal muscle up to the chronic phase of infection.

Previous studies reported the presence of bone marrow derived-myocytes after acute myotoxic injury (Corbel *et al*, 2003). It has been suggested that these cells are the product of fusion between damaged myofibers and bone marrow-derived cells (Rudnicki, 2003). In these studies, the frequency of bone marrow-derived myofibers was reported to be very low, even in the presence of muscle injury, where increased frequencies are found (Corbel *et al*, 2003). It was previously shown that the numbers of bone marrow-derived cells are increased in Duchenne muscle dystrophy experimental model, probably due to selective advantage (Dezawa *et al*, 2005). In the present study, we show that persistent inflammation leads to increased number of bone marrow-derived cells compared to those previously reported in acute injury experimental models. Furthermore, we observed different intensity of fluorescence emission between the population of GFP^+ myocytes, which can be the result of fusion with different numbers of bone marrow-derived cells. Therefore, it is possible that the number of bone marrow derived-cells is proportional to the degree of the inflammatory process found in each mouse.

In *T. cruzi*-infected mice, different degrees and patterns of inflammation are seen depending on the genetic background of the host (Marinho *et al*, 2004). Here we used the myotropic Colombian strain of *T. cruzi* (Federici *et al*, 1964), which

causes intense inflammation in the heart and skeletal muscles. The inflammation in chimeric mice was persistent, lasting for over 6 months of infection. The presence of a persistent and intense inflammation may be a relevant factor for stem cell. In fact, the production of SDF-1, a chemokine known to recruit stem cells through ligations to its receptor on the cell surface CXCR4 (Vandervelde *et al*, 2005; Wojakowski *et al*, 2004; Mieno *et al*, 2006), was found in the inflamed hearts of chagasic mice (Soares *et al*, in press).

The inflammatory infiltrate found in *T. cruzi*-infected mice is mainly composed by mononuclear cells. In fact, in addition to SDF-1, other chemokines, such as MCP 1, 2, and 3, are also expressed in the hearts of chagasic mice (Soares *et al*, in press) and may recruit monocytes and lymphocytes abundantly found in the inflammatory infiltrates in Chagas' disease. It has been recently shown that some monocyte subpopulations can differentiate into specialized cell types, such as endothelial cells, neurons, or cardiomyocytes (Kuwana et al, 2006; Kodama *et al*, 2005; Kodama *et al*, 2006). It has been reported that some monocyte subpopulations display common features to mesenchymal stem cells (Kuwana *et al*, 2003). Thus, it is possible that monocytes are a sub-population participating in the generation of GFP^+ myocytes and endothelial cells observed in our study.

It is now known that skeletal muscle regeneration is a dynamic process that occurs with the contribution of different stem cells sources, including skeletal muscle side population cells, bone marrow-derived cells, mesoangioblasts and pericytes. These cells can contribute to the satellite cell niche and generate myofibers. This process may finally contribute to the generation of bone marrow-derived myocytes (Otto *et al*, 2009), such as the one observed herein.

In conclusion, we have demonstrated that bone marrow cells actively participate in the regeneration process that occurs naturally in damaged skeletal muscles and hearts in an experimental model of Chagas' disease. These observations give support to the potential benefits of bone marrow cell therapy in chronic phase of Chagas disease, in order to increase a regeneration process that naturally occurs.

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Figure legends

Figure 1: Generation of GFP$^+$ bone marrow chimeric mice. Letally irradiated wild-type C57BL/6 mice were transplanted with bone marrow obtained from GFP transgenic mice. Flow cytometry analyses of blood samples collected from a wild-type C57BL/6 mouse (**A**), a GFP transgenic mouse (**B**), and a bone marrow chimeric mouse 33 days after *T. cruzi* infection (**C**).

Figure 2: Infection of bone marrow chimeric mice with *Trypanosoma cruzi*. One month after transplantation, chimeric mice were infected with 100 Colombian strain *T. cruzi* and treated with benznidazole 28 days later. **A**, Blood parasitemia of *T. cruzi*-infected chimeric mice. Heart (**B**) and Striated muscle (**C**), Quantification of inflammation in chimeric mice at various times after infection. Data represent the means±S.E.M. of 3-4 mice per group. ***, $P<0.001$.

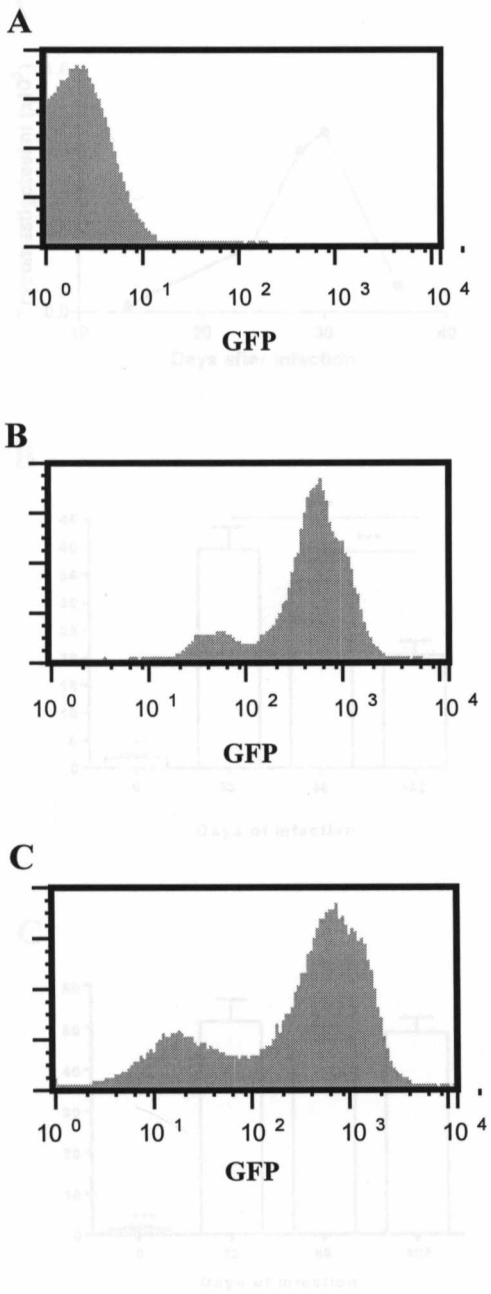
Figure 3: Presence of GFP$^+$ cells in the hearts of chagasic chimeric mice. Hearts of chimeric mice sacrificed at different time points after infection with *T. cruzi* were analyzed by immunofluorescence microscopy. **A**, Parasite nest within the myocardium, stained in red using an anti-*T. cruzi* antibody. **B**, Myocarditis composed by GFP$^+$ inflammatory cells (green). **C-E**, GFP$^+$ (green) myosin$^+$ (red) cardiomyocytes were found in heart sections of mice after 33 (**C**), 60 (**D**), and 192 (**E**) days of infection. **F and G**, Heart sections of uninfected chimeric mice. A rare GFP$^+$ cell is shown in (**G**). Nuclei were stained with DAPI (blue). Images were taken in a confocal microscope.

Figure 4: Presence of GFP$^+$ cells in striated muscle of chagasic chimeric mice. Striated muscle of chimeric mice sacrificed at different time points after infection with *T. cruzi* were analyzed by immunofluorescence microscopy. **A**, Parasite nest within a GFP$^+$ myofiber (arrow). **B**, Presence of GFP$^+$ inflammatory infiltrate (green). **C-E**,

GFP$^+$ (green) myosin$^+$ (red) myofibers were found in striated muscle sections collected from mice after 33 (**C**), 60 (**D**), and 192 (**E**) days of infection. **F**, Striated muscle section of an uninfected chimeric mouse stained with anti-myosin antibody (red). Nuclei were stained with DAPI (blue). Images were taken in a confocal microscope.

Figure 5: Characterization of GFP$^+$ cells in different organs of T. *cruzi*-infected chimeric mice. Presence of some GFP$^+$ (green) proliferating cells (PCNA; red) in the inflammatory infiltrates of heart (**A**) and striated muscle (**B**) tissue after 33 days of infection. GFP+ cells (green) were seen associated with blood vessels in the hearts of mice 33 (**C**) and 60 (**D**) days after infection, but not in uninfected control chimeric mice (**E**). In red, staining for von Willebrand factor. Liver section (**F**) of a chimeric mouse sacrificed 33 days after *T. cruzi* infection showing the presence of GFP$^+$ cells (green). Nuclei were stained with DAPI (blue). Images were taken in a confocal microscope.

Figure 1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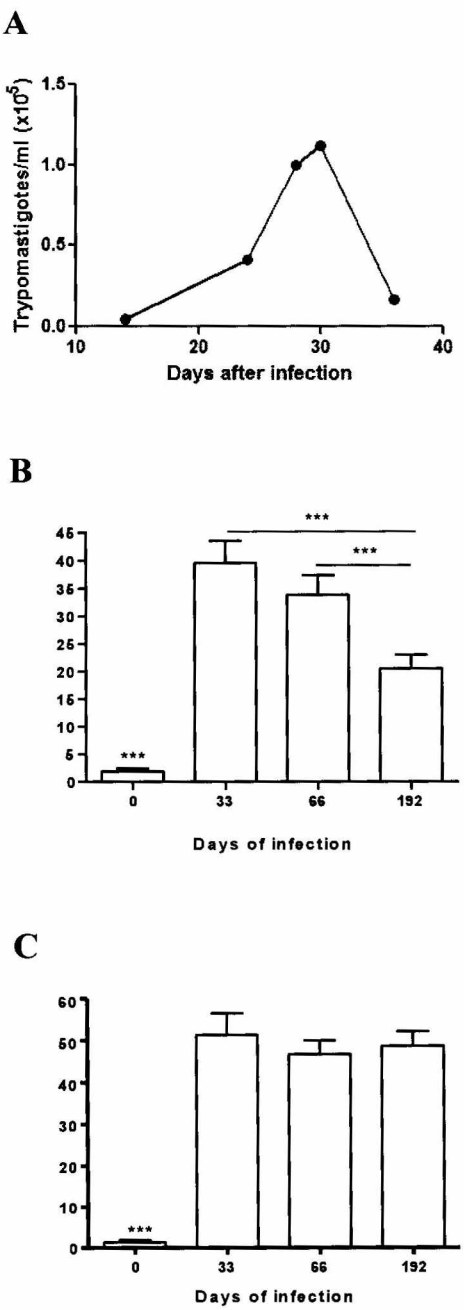
GFP

Figure 2


A
Trypomastigotes/ml (x10^5)
1.5
1.0
0.5
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10
20
30
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Days after infection
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45
40
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25
20
15
10
5
0
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Figure 3

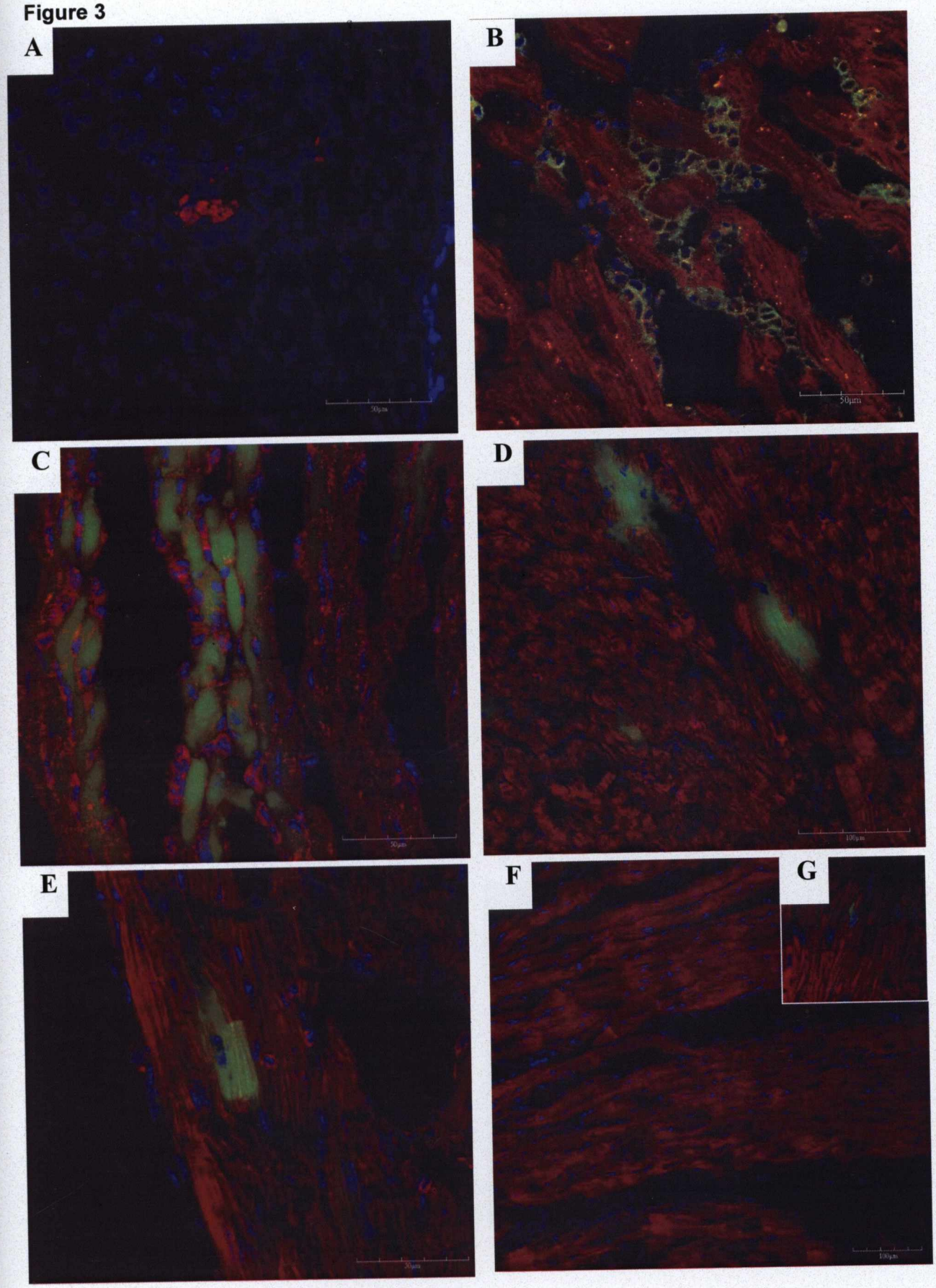


Figure 4

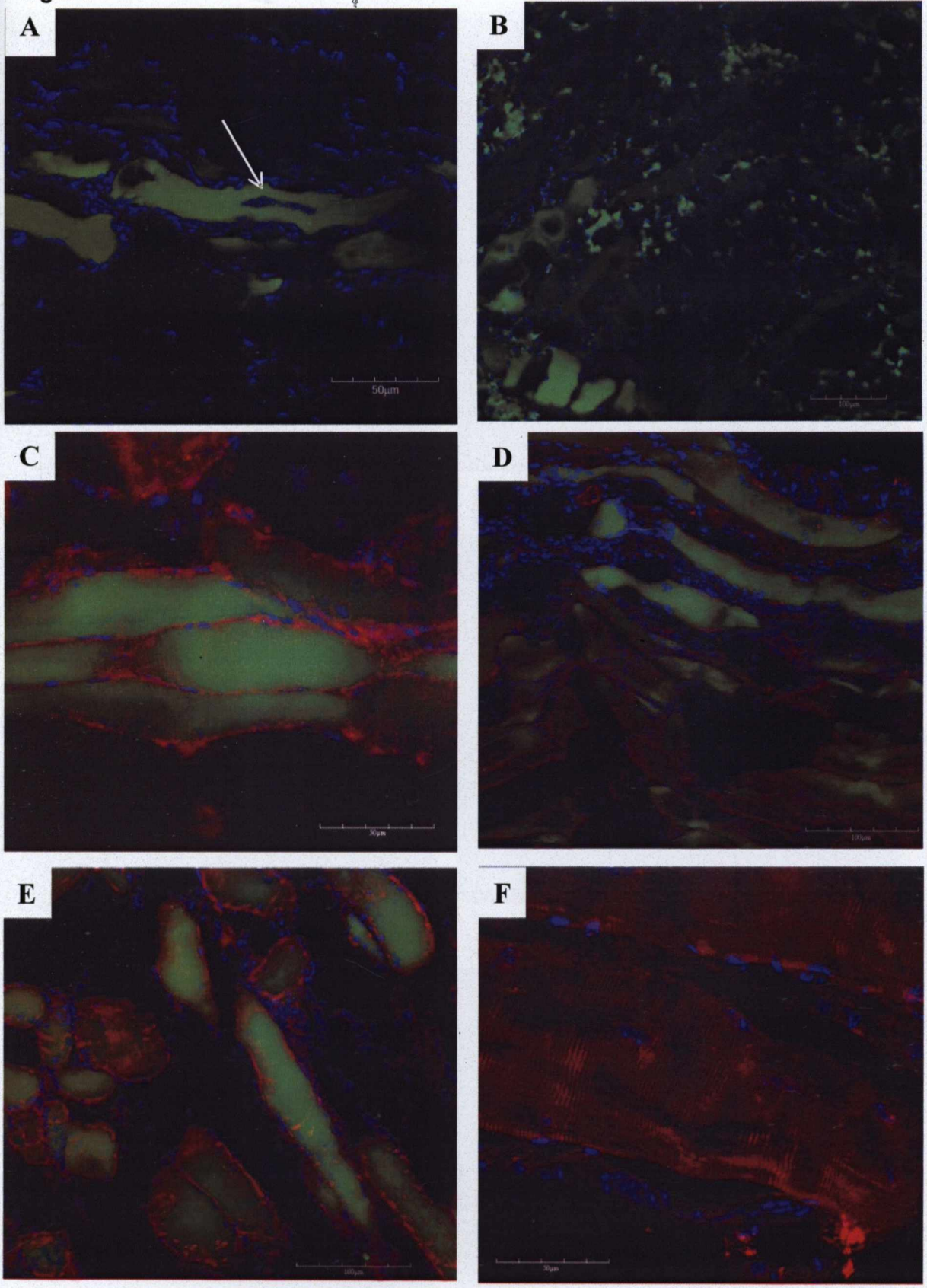
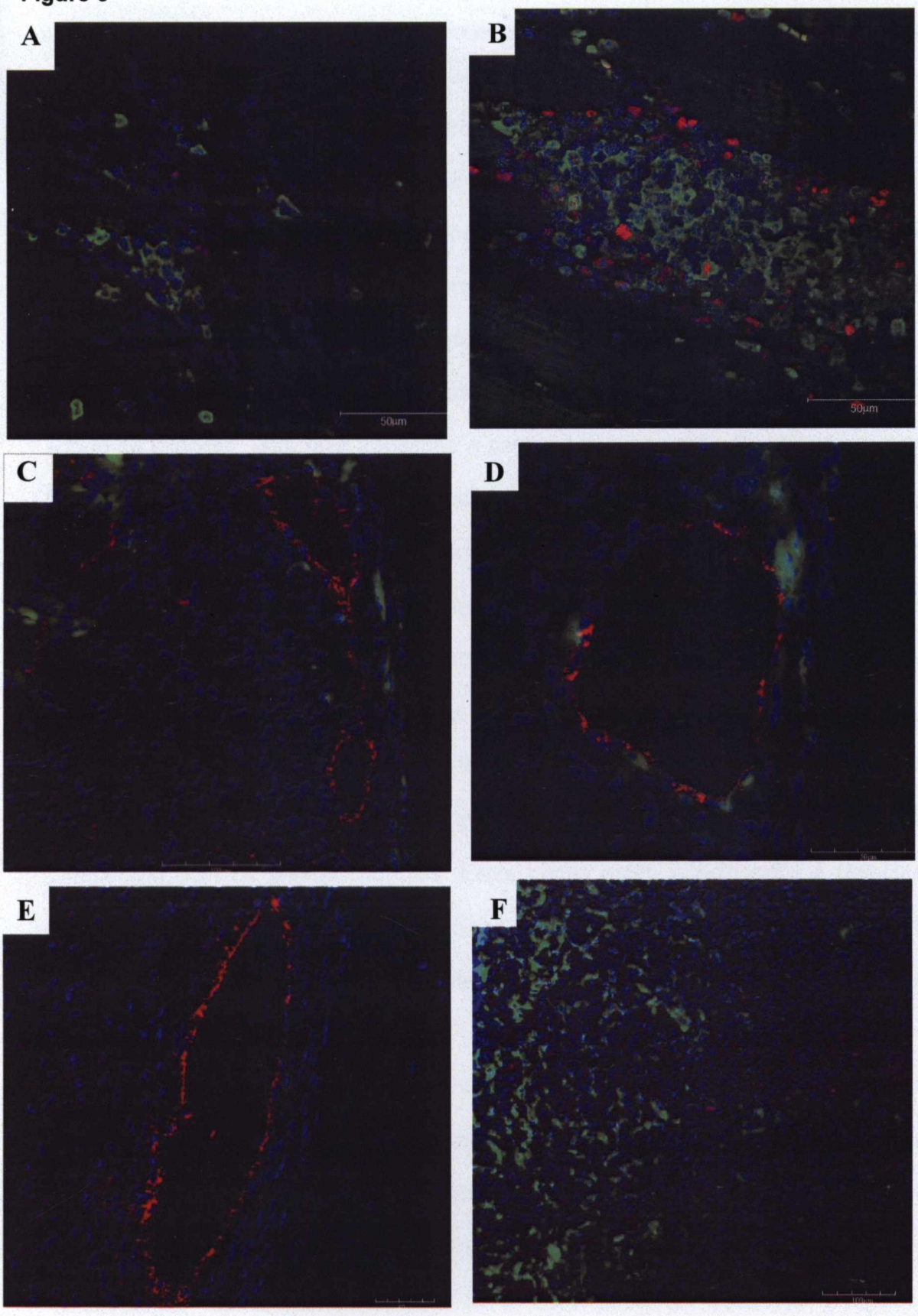


Figure 5



7- DISCUSSÃO

Alguns autores têm proposto que o perfil de expressão gênica no modelo murino de cardiopatia chagásica crônica difere daquele do animal normal (MUKHERJEE *et al,* 2003). Outro trabalho demonstra que as mudanças marcantes de expressão estão possivelmente associadas às fases da infecção. A avaliação do perfil de expressão gênica de camundongos infectados com a cepa *Brazil* de *T. cruzi* indicou alterações entre 30-60 dias pós-infecção, depois entre 60-90 dias e, por último, no período entre 90-180 dias pós-infecção, representando assim um período agudo de infecção, uma fase transitória a partir do segundo mês de infecção e uma fase tardia, semelhante àquela dita fase crônica da doença (MUKHERJEE *et al,* 2008). Os autores demonstraram alterações em genes importantes no processo inflamatório, bem como na adesão celular ao endotélio, fatores de transcrição, sinalização e transporte intracelular, dentre outros, em animais chagásicos crônicos quando comparado a animais não infectados (MUKHERJEE *et al,* 2003; MUKHERJEE *et al,* 2008). No nosso trabalho, aproximadamente 12% dos genes avaliados tiveram expressão alterada nos corações de animais chagásicos, quando comparado com os animais normais. Estes genes estão relacionados à resposta imune, sinalização intracelular, quimiocinas, receptores de citocinas, proliferação e adesão celular que, dentre outros fatores, apresentaram níveis de expressão diferentes.

Algumas das moléculas presentes na matriz extracelular e moléculas de adesão cuja expressão está aumentada nos animais chagásicos podem explicar a migração e a intensa presença de células inflamatórias na cardiopatia chagásica (MUKHERJEE *et al,* 2003). O CD44 é uma proteína envolvida na interação/adesão célula-célula, célula-matriz extracelular e está super-expressa no chagásico

(MUKHERJEE *et al,* 2003). Moléculas de adesão celular e proteínas de interação com a matriz extracelular importantes, tais como galectina-3, CD162, CD61 e ICAM-1, estão também com a expressão aumentada nos animais infectados crônicos. Este cenário produz um microambiente muito favorável ao recrutamento, migração e adesão das células inflamatórias nos sítios de lesão no miocárdio e músculo esqueléticos dos animais chagásicos.

O processo inflamatório na cardiopatia chagásica crônica é intenso, principalmente mediado por células T CD4 e CD8 positivas e macrófagos (DOS REIS *et al,* 2005). As análises de expressão mostram alterações na expressão gênica do CD68, uma glicoproteína expressa por monócitos e macrófagos, da família das proteínas de membrana associada a lisossomos (LAMP), que está envolvida na fagocitose de células mortas, ativação e recrutamento de macrófagos no sítio da inflamação, e está super-expressa nos animais chagásicos. Além do CD68, CD38 e CD52 estão também super-expressos nos animais infectados. O CD38 é uma glicoproteína que está presente tanto no núcleo quanto na membrana da célula e parece estar envolvido na diferenciação de células hematopoiéticas humanas, tendo papel no controle da homeostase nuclear de cálcio (ORCIANI *et al*, 2008). O CD52, uma glicoproteína presente nos linfócitos e principalmente no espermatozóide, não tem uma função claramente definida, mas parece estar envolvido na ativação do complemento, mediando citotoxicidade celular e pode ser usado para depleção dos linfócitos em indivíduos com doenças linfoproliferativas (LAPALOMBELLA *et al*, 2008). O grande número de linfócitos no processo inflamatório pode explicar a super-expressão desta molécula encontrada em nosso estudo. Estes dados corroboram o importante papel destas células na gênese do processo inflamatório na doença de Chagas.

Algumas quimiocinas como Ccl2 (MCP-1), Ccl7 (MCP-3), Ccl8 (MCP-2), também conhecidas como proteínas quimioatratoras de monócitos tipos I, II e III e Ccl12, também estão expressas em níveis aumentados. Os níveis de Ccl12 (SDF-1), TNF-α e IFN-γ, foram maiores quando dosados por ELISA do sobrenadante do extrato de coração dos animais chagásicos crônicos quando comparados com os animais normais. Estas citocinas estão muito relacionadas ao processo inflamatório e estão aumentadas em pacientes com insuficiência cardíaca grave ou mesmo na cardiomiopatia chagásica (GOMES *et al,* 2005; TALVANI *et al*, 2004). O SDF tem papel no recrutamento de linfócitos e provavelmente seu aumento deve sustentar a migração destas células no sítio da inflamação durante a fase crônica da doença, porém tem sido mostrado um papel importante desta molécula no recrutamento de células-tronco para o ambiente lesionado, o que pode favorecer a regeneração do tecido (ASKARY *et al*, 2003). Além disso, alguns trabalhos mostram a atividade de SDF-1, pela interação com o seu receptor CXCR4 presente em células-tronco desde a embriogênese até o indivíduo adulto (RATAJCZAK *et al*, 2006). Ccl2 e Ccl7 têm uma expressão correlacionada e estão envolvidas no recrutamento de macrófagos e células T na infecção pelo *T. cruzi*, e foram observadas em níveis marcantemente elevados na fase aguda da doença e, na fase crônica, níveis elevados de CCL-2 e TNF-α estão relacionados à cardiopatia (TEIXEIRA *et al,* 2002; TALVANI *et al*, 2004; HARDISON *et al,* 2006). A Ccl8 tem uma ação mais ampla sobre o recrutamento de células, atuando também sobre células como mastócitos, eosinófilos e basófilos em processos de resposta alérgica, além de atuar nas células NK, células T, monócitos/macrófagos (COILLIE *et al*, 1997).

Moléculas do complexo de histocompatibilidade principal (MHC) classe II principalmente H2-IAa e H2-IEb, também estão super-expressas nos chagásicos,

corroborando com outro trabalho que mostra a super expressão destas moléculas nos animais infectados com a cepa *Brazil* de *T. cruzi*, estas moléculas estão altamente expressas nas células que compõe o infiltrado inflamatório na cardiopatia chagásica (MUKHERJEE *et al,* 2008).

O inibidor tecidual de metaloproteinase tipo I (TIMP I) teve, em nosso trabalho, expressão elevada. Foi observado que esta molécula apresenta níveis séricos aumentados em pacientes em estágios terminais de insuficiência cardíaca (MILTING *et al*, 2008). O TIMP-1 é uma molécula envolvida na síntese de componentes da matriz-extracelular e inibição da degradação de colágeno e madeia a função de reabsorção óssea pela atividade de osteoclastos. Alta expressão de TIMP-1 está associada ao aumento de expressão de marcadores de ativação e sobrevivência das células e na resistência à morte celular por apoptose e conseqüentemente proliferação celular (BAKER *et al*, 2002). A lisil-oxidase é uma enzima relacionada à interação e deposição da matriz extracelular e estabilização das fibras colágenas (LUCERO & KAGAN, 2006). Como a fibrose é uma característica marcante da cardiopatia crônica, estas observações podem sugerir a participação destas moléculas na evolução da fibrose observada na cardiopatia crônica de etiologia chagásica.

A medula óssea também tem um papel na gênese da cardiopatia chagásica da fase aguda a fase crônica da doença. Desde a sua definição, na hematopoiese até os trabalhos mais recentes que utilizam células da medula óssea na terapia celular, este órgão tem se mostrado fundamental, seja no fornecimento das células que conduzem todo o processo inflamatório, como as células T, macrófagos, dentre outras, seja no abastecimento da área da lesão com células indiferenciadas capazes de gerar novas células cardíacas, vasos sanguíneos e modular o microambiente de

lesão (BIANCO *et al,* 2001; CALABRESE *et al*, 2003; HAM *et al,* 2006; DOS REIS *et al*, 2005; SOARES *et al*, 2004; CARDILLO *et al*, 2007).

Com o intuito de avaliar qual o papel da terapia celular ou transplante de células-tronco utilizando células de medula óssea na cardiopatia chagásica crônica, camundongos C57BL/6 foram infectados com *T. cruzi* da cepa Colombiana sendo transplantados, seis meses após a infecção, com células mononucleares de medula óssea (BMC). Foi observado o nível de inflamação e fibrose e o perfil de expressão gênica no coração dos animais tratados com as células de medula óssea comparando com o de animais somente infectados e animais normais. Cerca de 90% das alterações da expressão gênica observadas nos animais chagásicos crônicos não aparecem na análise dos animais tratados com BMC. Outros trabalhos na literatura mostram que o transplante de células da medula óssea é capaz de melhorar uma série de alterações cardíacas seja pelo papel das células na regeneração tecidual, ou pela função mais recentemente demonstrada de modulação do microambiente lesado (ORLIC ET AL, 2001; SOARES *et al,* 2004; MAZHARI & HARE, 2007; KAJSTURA *et al,* 2008). Esta última hipótese tem sido postulada como o efeito parácrino das células-tronco (LI *et al,* 2009; GNECCHI *et al,* 2005). Fatores de crescimento, quimiocinas, citocinas e vias de transdução de sinal intracelular podem ser produzidas e/ou ativadas mediante a ativação e/ou interação com as células transplantadas.

O perfil de expressão gênica de um microambiente frente a um agente causador de lesão pode se modificar e apontar a direção para onde as células de medula devem se deslocar. Em uma situação de hipóxia, células mesenquimais aumentaram a expressão de genes como TGF-β, VEGF, FGF e os níveis das proteínas MCP-1, MMP-9, dentre outros, também foram aumentados. Isso mostra a

capacidade de estímulo a migração, proliferação e modulação da produção de fibrose, bem como na formação de novos vasos sanguíneos para suprimento da área lesada (BURCHFIELD *et al,* 2008; KINNAIRD *et al,* 2004). A expressão gênica foi aumentada para TIMP-1 e 2 e MMP-2 em modelo de camundongos infartados transplantados com células mesenquimais humanas, certamente modulando o balanço entre a formação e degradação da fibrose decorrente do infarto induzido nos animais (ISO *et al,* 2007). No nosso trabalho, os níveis de expressão das proteínas galectina-3 e syndecan-4 foram modulados no grupo de animais infectados e tratados com BMC. Galectina-3 tem alta expressão no coração dos animais chagásicos, enquanto que sua expressão foi diminuída nos animais do grupo BMC. A galectina está envolvida na resposta inflamatória, ela tem um papel fundamental na estabilização do complexo TCR das células T com as células apresentadoras de antígenos e esta interação define a ativação ou tolerância das células T específicas em resposta a um patógeno (RABINOVICH and TOSCANO, 2009). Esta é a característica das células-tronco da medula óssea de modular o microambiente através da produção ou mesmo estimulação local de fatores quimiotáticos, citocinas, fatores de crescimento celular em uma tentativa de regulação parácrina do órgão lesado (LI *et al,* 2009).

Uma boa estratégia definida a partir destas observações foi a capacidade de aumentar a freqüência das células-tronco na periferia, otimizando sua capacidade de agir na lesão chagásica. Sendo assim, as alterações que foram observadas na expressão de determinados genes quando se comparou grupos de animais transplantados com BMC, com aqueles animais somente infectados ou mesmo animais normais, devem estar associadas às mudanças no microambiente cardíaco destes animais. Estas mudanças estão, portanto, relacionadas ao recruttamento das

células para o local da lesão. A terapia celular modula este microambiente e consequentemente melhora aspectos importantes para o estabelecimento da patologia como inflamação e fibrose que reduzem após a injeção das células.

Para observar o comportamento natural das células de medula óssea frente à infecção por *T. cruzi*, camundongos C57BL/6 selvagens foram irradiados letalmente e depois foram reconstituídos pela injeção endovenosa de células mononucleares de medula óssea oriundas de animais transgênicos para a proteína verde fluorescente (GFP), gerando assim camundongos quiméricos em que as únicas células fluorescentes presentes neste animal tinham origem na medula óssea que fora transplantada. Trinta dias após a transferência das células, os animais apresentavam mais de 90% da composição da medula óssea GFP+, mostrando o alto grau de reconstituição deste órgão do animal. Após este período, os animais foram infectados com formas tripomastigotas de *T. cruzi* da cepa Colombiana e avaliados por microscopia de fluorescência quanto a presença de componentes GFP^+ fora do ambiente medular. De fato, as células foram encontradas participando da formação dos infiltrados inflamatórios no músculo cardíaco e esquelético dos animais chagásicos, e também eram marcadas para PCNA, que indica proliferação celular, fator de Von-Willebrand e miosina, mostrando a capacidade destas células de produzirem células específicas como células endoteliais, cardiomiócitos e fibras musculares esqueléticas. Estes achados são sugestivos de uma regeneração, induzida naturalmente no animal, modulada tanto pelos fatores que foram alterados decorrentes da infecção quanto pela ação das células oriundas da medula óssea do próprio animal.

Outros trabalhos já relatavam a participação de células de origem na medula óssea na formação de novos miócitos após lesão miotóxica (CORBEL *et al*, 2003),

após infarto induzido em animais (ORLIC *et al*, 2001) ou mesmo na cardiopatia chagásica (SOARES *et al*, 2004). As células também estavam presentes em outros órgãos, como fígado e baço mostrando que estas circularam e permaneceram em alguns órgãos no animal, acompanhando os locais onde os eventos relacionados à patologia estão acontecendo. Trabalhos semelhantes que vem sendo desenvolvidos mobilizando as células da medula óssea para a periferia. Sabe-se que a mobilização destas células oriundas da medula óssea tem um papel na melhora da cardiopatia de etiologia chagásica.

Não só a injeção das células, mas também o tratamento de animais chagásicos crônicos com G-CSF, foi capaz de diminuir o processo inflamatório e a fibrose bem como melhorar os distúrbios de condutibilidade no coração dos animais tratados, deixando-os mais estáveis (MACAMBIRA *et al,* 2009). Outros parâmetros fisiológicos, também foram melhores no grupo tratado com G-CSF. O tratamento parece conferir uma melhora tanto estrutural quanto no desempenho funcional dos animais tratados (MACAMBIRA *et al,* 2009). Estes dados mostram que há uma participação das células de medula óssea na gênese do processo inflamatório, mas também na tentativa de regeneração própria do músculo cardíaco e esquelético, uma vez que o G-CSF é um potente agente quimiotático de células-tronco presentes na medula óssea (PELUS, 2008). Este tratamento deve, portanto, aumentar a concentração destas células na periferia, facilitando a migração das mesmas para a área da lesão.

Nossos dados mostram que naturalmente estas células podem migrar para a área lesada, diminuindo os efeitos deletérios da infecção, no entanto não sendo suficientes para controlar os distúrbios que acometem o indivíduo chagásico de forma definitiva. Novos trabalhos devem ser conduzidos considerando estes

achados, na tentativa de aperfeiçoar a terapia celular, levando-se em consideração as características das células e a capacidade que estas têm de modular situações de descontrole fisiológico quando ocorre a agressão tecidual presente na doença de Chagas.

8- CONCLUSÕES

- Há mudanças na expressão gênica nos corações de camundongos C57BL/6 cronicamente infectados pelo *Trypanosoma cruzi* da cepa Colombiana em relação a controles não-infectados, sendo um grande número dos genes super-expressos envolvidos na resposta imune como quimiocinas, moléculas de adesão celular, bem como genes relacionados à fibrose como componentes da matriz-extracelular e TIMP-1.
- As alterações observadas corroboram os dados da literatura estando relacionadas à gênese da cardiomiopatia chagásica crônica e montam um cenário para a migração e ativação das células que compõe o processo inflamatório presente no coração dos animais.
- No modelo experimental de infecção pelo *T. cruzi* ocorre uma diminuição da inflamação e da fibrose no grupo tratado com BMC, que pode ser mediada tanto pela ação direta das células quanto pelo seu efeito em modular o ambiente da lesão. Há poucas células GFP^+ presentes nas lesões, para a grande redução de inflamação e fibrose observada, portanto não é o efeito direto das células o único mecanismo de ação neste modelo. Além disso, no grupo chagásico tratado com BMC, a maioria das alterações na expressão gênica dos chagásicos não tratados é revertida. Indicando também um efeito modulador das células transplantadas.
- As células de medula óssea que migram para o coração e músculo esquelético compõem o infiltrado inflamatório nestes órgãos dos animais e podem contribuir para a formação de novos cardiomiócitos, fibras musculares e vasos sanguíneos. Portanto, há uma participação tanto no fornecimento das

células que medeiam o processo de lesão quanto naquelas que podem ajudar a diminuir ou modular as lesões decorrentes da infecção.

- A hipótese gerada a partir dos dados observados é a de que as células derivadas da medula óssea têm um papel na terapia de animais chagásicos, pelo fato de ao migrarem para as áreas de lesão estas células geram novas unidades celulares específicas, como miócitos e vasos, além de modular o microambiente cardíaco, revertendo alterações induzidas durante doença de Chagas.

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10- ANEXOS

Artigos publicados durante o período de doutoramento.

REVISTA BRASILEIRA DE HEMATOLOGIA E HEMOTERAPIA

Revisão / Review

Terapia celular na doença de Chagas

Cell therapy in Chagas' disease

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A doença de Chagas, que ocorre no México e nas Américas Central e do Sul, continua representando um grave problema de saúde pública. A prevalência global da infecção humana pelo Trypanosoma cruzi foi estimada em 16-18 milhões de casos no ano de 2005, sendo corrigida para aproximadamente 28 milhões de pessoas no ano de 2007, segundo a Organização Mundial de Saúde. A cardiopatia chagásica crônica é a forma mais comum de cardiomiopatia nas Américas Central e do Sul e a principal causa de morte por doença cardiovascular em áreas endêmicas. Até o momento não existe nenhum tratamento eficiente para esta doença a não ser o tratamento farmacológico ou o transplante cardíaco nos indivíduos que desenvolvem um quadro mais grave da doença. Trabalhos atuais têm mostrado o uso de células-tronco de várias origens em modelos animais e humanos de doenças do coração, como infarto do miocárdio, destacando uma melhora em aspectos como neovascularização, regeneração do músculo cardíaco, aumento da fração de ejeção e melhora na qualidade de vida dos indivíduos tratados. Estes dados induziram os pesquisadores a investigar os efeitos terapêuticos do transplante de células mononucleares de medula óssea em um modelo murino e em indivíduos chagásicos crônicos. Esta revisão tem por objetivo mostrar os trabalhos realizados usando a terapia celular na cardiopatia chagásica crônica. Rev. Bras. Hematol. Hemoter. 2009;31(Supl.1):87-92.

Palavras-chave: *Doença de Chagas; cardiopatia; células-tronco; medula óssea; terapia celular.*

Introdução

A doença de Chagas, que ocorre em todo o México, Américas Central e Sul, continua representando uma séria ameaça para a saúde. A prevalência global da infecção humana pelo *Trypanosoma cruzi* foi estimada em 16-18 milhões de casos no ano de 2005, sendo corrigida para aproximadamente 28 milhões de pessoas no ano de 2007 segundo a Organização Mundial de Saúde.[1] Cerca de 120 milhões de pessoas, 25% dos habitantes da América Latina, estão em risco de contrair infecção, e em torno de 30% das pessoas infectadas com o parasita vão avançar para a forma crônica cardíaca e/ou digestiva, com uma alta morbimortalidade.[1]

A cardiomiopatia chagásica crônica pode resultar em arritmias cardíacas, aneurisma apical, insuficiência cardíaca congestiva, tromboembolismo e morte súbita cardíaca; é a forma mais comum de cardiomiopatia nas Américas Central e do Sul e a principal causa de morte por doença cardiovascular em áreas endêmicas.[2]

Nos países do Cone Sul existem iniciativas e programas que diminuiram drasticamente as taxas de transmissão vetorial do *T. cruzi*.[3] No entanto, a transmissão não foi completamen-

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te erradicada em áreas endêmicas. Além disso, os indivíduos já infectados e que cursarão para a forma crônica da doença não têm um tratamento adequado na atualidade. Os tratamentos com benzonidazol ou nifurtimox causam uma série de efeitos colaterais, exigem longos períodos de tratamento e não têm uma eficiência claramente demonstrada na fase crônica da doença de Chagas.[4-6] Portanto, é necessário o desenvolvimento de novas tecnologias e/ou estratégias terapêuticas para o tratamento da doença de Chagas e de outras doenças, ditas negligenciadas, que acometem principalmente os países em desenvolvimento.[7]

Tendo como base este panorama e as descobertas na chamada medicina regenerativa, que usa células-tronco e terapia celular para o reparo de lesões crônico-degenerativas, investigou-se a possibilidade de intervenção nas manifestações apresentadas na cardiopatia de etiologia chagásica com o intuito de se observarem quais os possíveis efeitos que estas novas metodologias poderiam trazer para o campo da pesquisa no tratamento da doença de Chagas.[8]

A terapia celular permanece em evidência mundial pelo fato de que esta estratégia se propõe a estabelecer condições de melhora ou manutenção da qualidade de vida de indivíduos acometidos por patologias que ainda não têm uma condição de tratamento adequada, que são extremamente debilitantes e que constituem as principais causas de morbimortalidade no mundo, tais como doenças cardíacas, diabetes melito, câncer, doenças neurológicas, todas com características, em geral, degenerativas.

As cardiopatias têm sido alvo das terapias celulares desde o início das pesquisas, principalmente por se tratarem da maior causa de morte em todo o mundo.[9,19] Sendo assim, acredita-se que esta seja a área onde as terapias celulares encontram-se mais avançadas e com uma boa possibilidade de implantação nos serviços de saúde, desde que sejam realizados estudos mais bem controlados para comprovação da eficácia do tratamento.[10,11]

O infarto agudo do miocárdio é a causa de morte mais importante nos países desenvolvidos. No Brasil, os grandes focos nas cardiomiopatias são a cardiomiopatia chagásica e isquêmica. Elas produzem e impõem um ônus socioeconômico extremamente grande para o sistema de saúde do País.[12,13]

Dentre outras causas, a grande prevalência das cardiopatias pode ser explicada pela pouca capacidade regenerativa dos miócitos cardíacos perdidos pelos processos patológicos que acometem o coração e pela permanência dos processos de agressão que continuam atuando no músculo cardíaco. Além disso, o reparo, com intensa deposição de fibrose, que acompanha na maioria das vezes a lesão cardíaca, tem também um papel fundamental na perda da qualidade funcional deste órgão.

Trabalhos na literatura têm demonstrado o potencial da terapia celular em cardiopatias em protocolos experimentais e em ensaios clínicos iniciais em humanos.[14-18] Diversos trabalhos têm usado células de origens distintas para terapia celular, como células da medula óssea (mesenquimais ou hematopoéticas), mioblastos esqueléticos, ou, ainda, células que supostamente residem no próprio miocárdio.[19-24]

Experimentalmente tem sido demonstrada a melhora funcional em animais infartados e transplantados com células obtidas a partir da medula óssea.[25] Diminuição da área de fibrose, formação de novos cardiomiócitos e neovascularização são alguns dos achados importantes observados nos trabalhos em terapia celular na cardiologia.[26,27] Mais recentemente, estudos clínicos também demostraram estes benefícios associados à melhora da função cardíaca.[28,29] A mobilização das células da medula óssea pode representar uma alternativa à coleta e injeção destas. A mobilização com fatores de crescimento como G-CSF mostrou-se eficaz na melhora funcional e na sobrevivência de animais infartados.[30]

Este panorama tem estimulado novas perspectivas de trabalho, desenvolvimento de projetos de pesquisa e tratamento para as doenças cardíacas nos últimos anos.[19] Na doença de Chagas, o primeiro trabalho que se propôs a avaliar o potencial da terapia celular começou a ser desenvolvido no ano de 2002. Neste trabalho utilizou-se o modelo murino, bastante utilizado em estudos experimentais sobre a doença de Chagas. Camundongos foram infectados com a cepa colombiana de *Trypanosoma cruzi*, que é um modelo bem estabelecido para a cardiopatia chagásica crônica. Nos animais infectados observa-se, após um tempo médio de seis a oito meses de infecção, um intenso infiltrado inflamatório, difuso e com apresentação de áreas de fibrose evidenciando um processo de cicatrização pós-inflamação e destruição de fibras cardíacas.[31] Soares *et al.* realizaram a injeção de células mononucleares obtidas a partir da medula óssea de animais normais ou chagásicos em animais cronicamente infectados pelo *T. cruzi* para observação histológica e quantificação da inflamação e fibrose, podendo-se então avaliar o potencial da terapia celular na cardiopatia de etiologia chagásica. Os resultados mostraram que, a partir de um mês após a injeção das células, houve uma redução da inflamação e da fibrose no grupo de animais chagásicos tratados em comparação ao grupo que não recebeu células. Dois meses após a injeção esta diminuição é ainda maior e se mantém até seis meses depois do transplante celular.[31]

O trabalho mostra que houve migração de células para o tecido cardíaco dos animais chagásicos quatro dias após a injeção e com 15 dias as células se assemelham morfologicamente a fibras cardíacas e apresentam marcação específica de miosina cardíaca.[31] Ao injetar, nos animais chagásicos, células obtidas a partir da medula óssea de animais também chagásicos, os autores observam os mesmos resultados acima descritos e discutem a importância deste experimento, pois simulam o transplante autólogo também mostrado por Stamm *et al.*, em 2003.[32] O transplante autólogo foi então proposto e realizado em pacientes posteriormente. Com os resultados deste trabalho experimental, os autores propõem um estudo piloto em pacientes com o intuito de observar a

segurança da técnica e a possibilidade da realização do método em pacientes chagásicos crônicos.

A proposta foi aprovada pelo Conep (Comitê Nacional de Ética em Pesquisa), tendo sido realizado o primeiro transplante de células mononucleares de medula óssea em paciente e publicada como relato de caso em artigo no ano de 2004.[33] Neste trabalho, os autores descrevem o perfil do paciente, relatam sobre a sua limitação funcional grave, revelam seu esquema de tratamento farmacológico a fim de evidenciar o grau de severidade do quadro patológico, classificando-o como classe funcional III, em uma escala de valor máximo IV, no esquema de classificação internacional estabelecido pela New York Heart Association (NYHA). Foi também avaliado o escore de qualidade de vida de Minnesota, obtido através de questionário aplicado ao paciente.

Sob sedação, as células usadas foram obtidas a partir da punção da medula óssea do osso ilíaco, a suspensão celular foi passada em um sistema de filtragem, para remoção de espículas ósseas e pequenos coágulos e, em seguida, foram submetidas a um gradiente de ficol para obtenção das células mononucleares. Cerca de 2,4 x 10^8 células foram injetadas no sistema coronariano direito e esquerdo, através de cateter de angioplastia. Foram injetados 10 mL da suspensão na artéria coronária descendente anterior, lentamente, em 10 min, 5 mL na artéria circunflexa e 5 mL na artéria coronária direita. Doença arterial coronariana foi excluída através de coronariografia.[33]

O paciente não apresentou nenhuma alteração elétrica nem arritmias durante o procedimento e seus sinais vitais permaneceram estáveis durante o desenvolvimento do protocolo. Não foram observadas alterações bioquímicas nem hematológicas nem nos marcadores indicativos de necrose miocárdica, e quatro dias após o procedimento o paciente recebeu alta hospitalar, ficando sob uso das mesmas medicações usadas anteriormente.[33] Alguns achados indicaram uma melhora funcional, como a diminuição dos diâmetros ventriculares, o aumento de fração de ejeção, classe funcional da NYHA e escore de qualidade de vida de Minnesota.[33]

A doença de Chagas possui alguns aspectos particulares dentre as cardiopatias. De caráter inflamatório bem evidente, onde há uma intensa produção de citocinas e fatores quimioatratores, este ambiente pode favorecer a terapia, pois as células injetadas seriam atraídas para o órgão lesionado com maior eficiência. Não se observou nenhum efeito adverso relacionado à infusão celular. No trabalho experimental foi observado que as células injetadas migram para o coração e se diferenciam em cardiomiócitos.

Um ensaio clínico de fase I, com 28 pacientes, foi realizado com o objetivo de avaliar os efeitos do transplante de células mononucleares de medula óssea em pacientes chagásicos.[34] Vinte e cinco dias após o transplante das células, os pacientes também receberam diariamente injeções subcutâneas de G-CSF (Granulokine®) na dose de 5 g/kg durante cinco dias, para mobilização das células-tronco da medula óssea para o sangue periférico. Neste trabalho não foram observadas complicações diretamente ligadas ao procedimento. Três pacientes evoluíram para óbito durante o segundo mês de acompanhamento, um deles por morte súbita, outro com insuficiência respiratória e hemorragia pulmonar e o terceiro com insuficiência cardíaca em estado terminal, porém nenhum dos óbitos teve associação direta com o transplante das células.[34]

Os autores não detectaram nenhuma mudança significativa nos níveis dos marcadores de necrose miocárdica 24 horas após a injeção das células, nem mudanças eletrocardiográficas sugestivas de isquemia ou infarto.[34] O número de batimentos ventriculares prematuros em 24 horas tendeu a aumentar, contudo, sem o aumento de episódios de taquicardia ventricular.[34] Houve constatação de uma melhora significativa na fração de ejeção, na classificação dos pacientes segundo os critérios da NYHA, no escore de qualidade de vida de Minnesota e no teste de distância percorrida em seis minutos quando comparados valores dos pacientes antes e após a terapia celular. Um aumento dos níveis séricos de sódio foi observado no período entre o 3° e o 14° dia após o transplante e se manteve durante o primeiro e o segundo mês de acompanhamento dos pacientes.[34] Os autores concluem que a realização de um ensaio clínico controlado e randomizado, bem como o acompanhamento dos pacientes por um maior período de tempo, corroborando com os achados obtidos neste trabalho, podem sugerir uma nova modalidade terapêutica para os pacientes chagásicos. Estes resultados geraram informação suficiente e contribuiram para a realização de um ensaio clínico de fase II, multicêntrico, duplo-cego, randomizado, controlado com grupo placebo, que visa avaliar a eficiência do transplante autólogo de células mononucleares da medula óssea em quatro tipos diferentes de cardiopatias.[35]

O ensaio clínico MiHeart Study (*Multicenter randomized trial of cell therapy in cardiopathies*) reúne quatro ensaios independentes nas patologias específicas e avaliou o efeito da injeção das células em pacientes com doença cardíaca isquêmica aguda e crônica, pacientes chagásicos e cardiomiopatia dilatada. Para cada patologia serão incluídos 300 pacientes, dos quais metade receberá a terapia celular e a outra metade tratamento placebo (solução salina com 5% de soro autólogo).[35] Os pacientes serão acompanhados por seis e doze meses após o transplante das células. O desfecho primário é a medida da fração de ejeção e a hipótese do trabalho é a de que os pacientes que receberem células mononucleares de medula óssea autólogas terão um aumento de 5% na fração de ejeção do ventrículo esquerdo quando comparados ao grupo controle, avaliados pelo método de Simpson. Este estudo ainda está em andamento no momento.

Com base nos resultados apresentados, a grande questão que se quer discutir é como as células transplantadas

Tabela 1: Resumo dos estudos com terapia celular em doença de Chagas

Fase do estudo	Descrição	Principais resultados	Referência
Pré-clínico	Tratamento com células mononucleares de medula óssea em camundongos com cardiopatia chagásica crônica	- Redução da inflamação e da fibrose nos animais tratados - Migração das células para o tecido cardíaco dos animais tratados	Soares *et al.* 2004
Pré-clínico	Transplante autólogo de células-tronco mesenquimais cocultivadas com mioblastos esqueléticos em um modelo de doença de Chagas em ratos	- Melhora da função cardíaca - Aumento da neovascularização	Guarita-Souza *et al.* 2006
Clínico	Transplante autólogo de células mononucleares de medula óssea em paciente com insuficiência cardíaca de etiologia chagásica - relato de caso	- Ausência de eventos adversos - Melhora da fração de ejeção, da classe funcional (NYHA) e do escore de qualidade de vida	Vilas-Boas *et al.* 2004
Clínico	Transplante autólogo de células mononucleares de medula óssea em pacientes com insuficiência cardíaca de etiologia chagásica; Ensaio clínico de fase II - 28 pacientes	- Melhora da fração de ejeção, da classe funcional (NYHA) e do escore de qualidade de vida - Aumento da distância percorrida em 6 minutos no teste do corredor e aumento dos níveis séricos de sódio	Vilas-Boas *et al.* 2006
Clínico	Transplante autólogo de células mononucleares de medula óssea em pacientes com insuficiência cardíaca de etiologia chagásica; Ensaio clínico de fase III (MiHeart Study) - 300 pacientes	Em andamento	Tura *et al.* 2007

podem atuar na melhora da função cardíaca (Tabela 1). A transdiferenciação das células-tronco tem sido questionada, embora alguns trabalhos na literatura tenham demonstrado esta capacidade das células em se diferenciar em células especializadas.[29,30] No modelo experimental de cardiopatia chagásica, as células de medula óssea migraram para o coração e apresentaram um fenótipo de cardiomiócitos.[31] Não se pode, no entanto, descartar a hipótese de fusão celular, pois alguns trabalhos têm demonstrado este fenômeno em terapias celulares.[36,37] Na doença de Chagas, os processos crônicos de inflamação e fibrose presentes e persistentes que agridem e geram as alterações funcionais e de condução elétrica deste órgão devem criar um microambiente diferente do ambiente cardíaco normal. Este é um grande foco das pesquisas com células-tronco. É a possibilidade de atuação das células através de efeitos parácrinos, onde o microambiente cardíaco, certamente alterado no indivíduo chagásico, sinaliza a região ou o órgão lesionado atraindo as células para o local da lesão. Estas células poderiam então modular a produção de fatores presentes neste ambiente, gerando um ambiente propício para a reestruturação tecidual a partir das próprias células do tecido.[38-42]

É possível também que células-tronco de outras origens sejam mais eficazes na regeneração das lesões cardíacas na doença de Chagas. Neste sentido, no modelo murino de cardiopatia chagásica, células-tronco obtidas a partir de polpa do dente de leite humano causaram a diminuição de inflamação e fibrose (dados não publicados). As células humanas provavelmente não se incorporaram ao miocárdio de camundongo, fazendo conexões intercelulares e regeneraram o músculo destes animais. Porém, ao migrarem para o tecido cardíaco danificado, estas células podem ter modulado algum efeito local estimulando a regeneração e a diminuição dos parâmetros avaliados como inflamação e fibrose. Citocinas, quimiocinas, mataloproteinases e seus inibidores são algumas das moléculas alvo que devem ser investigadas, a fim de se avaliar o mecanismo de atuação das células transplantadas no animal chagásico. Com isso poderá ser gerada uma nova modalidade terapêutica mais eficiente para esta enfermidade que acomete um grande número de pessoas e é causa importante de morte dos indivíduos nos países endêmicos para esta doença.

Abstract

Chagas' disease occurs throughout Mexico, Central and South America and still represents a serious threat to public health. The overall prevalence of infection by Trypanosoma cruzi was estimated at 16-18 million cases in 2005 and updated to approximately 28 million people in 2007, according to the World Health Organization. Chronic Chagas heart disease is the most common form of cardiomyopathy in Central and South America and one of the leading causes of death from cardiovascular disease in endemic areas. So far, there is no effective treatment for this disease except for heart

transplantation in individuals who develop a more severe form the disease. Recent works have shown that the use of stem cells from various sources tested in animal models and in human heart diseases, including myocardial infarction, have resulted in an improvement in aspects such as vascularization, regeneration of the heart muscle, in ejection fraction and in quality of life of individuals treated. These data led researchers to investigate the effects of transplantation of bone marrow mononuclear cells in murine models and chronic chagasic individuals. The present review aims to show the work carried out using cell therapy in chronic Chagas heart disease. Rev. Bras. Hematol. Hemoter. 2009;31 (Supl.1):87-92.

***Key words**: Chagas' disease; cardiomyopathy; stem cells; bone marrow; cellular therapy.*

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Granulocyte colony-stimulating factor treatment in chronic Chagas disease: preservation and improvement of cardiac structure and function

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ABSTRACT **This study investigates the effects of granulocyte colony-stimulating factor (G-CSF) therapy in experimental chronic chagasic cardiomyopathy. Chagas disease is one of the leading causes of heart failure in Latin America and remains without an effective treatment other than cardiac transplantation. C57BL/6 mice were infected with 10^3 trypomastigotes of *Trypanosoma cruzi*, and chronic chagasic mice were treated with G-CSF or saline (control). Evaluations following treatment were functional, immunological, and histopathological. Comparing hearts of G-CSF-treated mice showed reduced inflammation and fibrosis compared to saline-treated chagasic mice. G-CSF treatment did not alter the parasite load but caused an increase in the number of apoptotic inflammatory cells in the heart. Cardiac conductance disturbances in all infected animals improved or remained stable due to the G-CSF treatment, whereas all of the saline-treated mice deteriorated. The distance run on a treadmill and the exercise time were significantly greater in G-CSF-treated mice when compared to chagasic controls, as well as oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), and respiratory exchange ration (RER) during exercise. Administration of G-CSF in experimental cardiac ischemia had beneficial effects on cardiac structure, which were well correlated with improvements in cardiac function and whole animal performance.—Macambira, S. G., Vasconcelos, J. F., Costa, C. R. S., Klein, W., Lima, R. S., Guimarães, P., Vidal, D. T. A., Mendez, L. C., Ribeiro-dos-Santos, R., Soares, M. B. P. Granulocyte colony-stimulating factor treatment in chronic Chagas disease: preservation and improvement of cardiac structure and function. *FASEB J.* 23, 000–000 (2009). www.fasebj.org**

Key Words: chagasic cardiomyopathy · inflammation · arrhythmias · treadmill performance

CHAGAS DISEASE, CAUSED BY *Trypanosoma cruzi* infection, is one of the main causes of death due to heart failure in Latin American countries. About 25% of chagasic individuals develop a chronic chagasic cardiomyopathy (CChC), the most severe form of disease. The chemotherapy used in chagasic patients is highly toxic and has limited efficacy, especially in the chronic disease. The unique definitive treatment for CChC aggravated by severe heart failure is heart transplantation.

Studies establishing therapies to restore the cardiac function using stem cells or the administration of growth factors have been developed. Bone marrow stem cells (BMSCs) differentiate into cardiomyocytes and endothelial cells and may participate in the regeneration of cardiac lesions (1–4). Granulocyte colony-stimulating factor (G-CSF) increased the number of peripheral granulocytes (5) and induced the mobilization of BMSCs to the periphery (6). This property and regenerative capacity of BMSCs justify the efforts to prove the efficacy of this therapy.

The beneficial effects of G-CSF in the treatment of cardiac ischemia lesions have been shown (7–9). The therapeutic use of G-CSF is attractive because it is already employed in clinical practice, has mild side effects, and is a less invasive treatment than bone marrow aspiration and cell transplantation. We have previously shown that therapy with BMSCs decreases heart inflammation and fibrosis in experimental CChC (10). Here, we investigated the effects of G-CSF on cardiac alterations in a model of CChC.

MATERIALS AND METHODS

Animals

Two-month-old male C57BL/6 mice, raised and maintained in the animal facilities at the Gonçalo Moniz

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Research Center (Fiocruz, Rio de Janeiro, Brazil) were used in the experiments, and were provided with rodent diet and water *ad libitum*. All animals were sacrificed under anesthesia by intraperitoneal injection of xylazine at 10 mg/kg body wt and ketamine at 100 mg/kg body wt, and handled according the National Institutes of Health guidelines for ethical use of laboratory animals.

T. cruzi infection and treatment with G-CSF

Mice were infected by intraperitoneal injection of 1000 trypomastigote forms of Colombian strain *T. cruzi* (11) obtained by *in vitro* infection of LCC-MK2 cell line. Parasitemia was evaluated at different time points after infection by counting the number of trypomastigotes in peripheral blood aliquots (12). Groups of chronic chagasic mice (6 mo after infection) were treated with human recombinant G-CSF (Granulokine 30; Hoffman la Roche, Switzerland) with 200 μg/kg/d during 5 consecutive days with 3 cycles of administration or with 5% glucose saline solution in the same regimen.

Histopathological analysis

Hearts from G-CSF-treated mice and untreated controls were removed and fixed in buffered 10% formalin. Sections of paraffin-embedded tissue were stained by standard hematoxylin-and-eosin (H&E) and Sirius red staining for evaluation of inflammation and fibrosis, respectively, by optical microscopy. Images were digitized using a color digital video camera (CoolSnap, Montreal, QC, Canada) adapted to a BX41 microscope (Olympus, Tokyo, Japan). The images were analyzed using Image Pro 5.0 (Media Cybernetics, San Diego, CA, USA), to integrate the number of inflammatory cells counted by area. Ten fields per heart were counted from every mouse of each group.

Parasite quantification by immunofluorescence analysis

Frozen heart sections (5 μm thick) were prepared in a cryostat in poly-L-lysine-coated slides and fixed with cold acetone. Sections were incubated with PBS 5% BSA for 30 min, followed by overnight incubation with rat serum anti-*T. cruzi* (1:400). After washing with PBS, sections were incubated for 1 h with FITC-conjugated rabbit anti-rat IgG 1:100 (Sigma, St. Louis, MO, USA). Sections were washed 3 times, counterstained with Evans blue, and mounted with Vectashield (Vector, Burlingame, CA, USA). Images were digitized using a color digital video camera (DP-70; Olympus) adapted to an AX-70 microscope (Olympus). The images were analyzed using Image Pro, and the numbers of parasite foci were counted (10 fields/heart, 5 mice/group) and integrated by area.

Stroma-derived factor-1 (SDF-1) assessment in total-protein heart extracts

Heart proteins were extracted at 100 mg of tissue/ml of PBS to which 0.4 M NaCl, 0.05% Tween 20 and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 ml) were added. The samples were centrifuged for 10 min at 3000 *g*, and the supernatant was frozen at −70°C for later quantification. SDF-1 levels were estimated using a commercially available Immunoassay ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's guidelines.

Apoptosis assay

Apoptosis in heart sections fixed using 4% formaldehyde was evaluated by terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, performed using *in situ* DeadEnd Colorimetric TUNEL System kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Images were digitized, and quantitative analysis was performed using Image Pro. Results were expressed as the number of positive cells per square millimeter of 10 sections from 4 animals/group. The positive controls were nuclease-treated slides.

ECG analysis

Electrocardiography was performed using the Bio Amp PowerLab System (PowerLab 2/20; ADInstruments, Castle Hill, NSW, Australia), recording the bipolar lead I. All animals were anesthetized by intraperitoneal injection of xylazine at 10 mg/kg body weight and ketamine at 100 mg/kg body weight to obtain the records. All data were acquired for computer analysis using Chart 5 for Windows (PowerLab). Records were bandpass filtered (1 to 100 Hz) to minimize environmental signal disturbances. The sampling rate was 1 kHz. The ECG analysis included heart rate, PR interval, P wave duration, QT interval, QTc, and arrhythmias. Wave durations (ms) and heart rate were calculated automatically by the software after the cursors were placed. The QTc was calculated as the ratio of QT interval by square roots of RR interval.

Treadmill

A motor-driven treadmill chamber for one animal (LE 8700; Panlab, Barcelona, Spain) was used to exercise the animals. The speed of the treadmill and the intensity of the shock (mA) were controlled by a potentiometer (LE 8700 treadmill control; Panlab). Room air was pumped into the chamber at a controlled flow rate (700 ml/min) by a chamber air supplier (Oxylet LE 400; Panlab). Outflow was directed to an oxygen and carbon dioxide analyzer (Oxylet 00; Panlab) to measure consumption of oxygen ($\dot{V}O_2$), production of carbon dioxide ($\dot{V}CO_2$), and the respiratory exchange ratio (RER). The mean room temperature was maintained at 21 ± 1°C. After an adaptation period of 40 min in the treadmill chamber, the mice exercised at 5 different velocities (7.2, 14.4, 21.6, 28.8 and 36.0 m/min), with increasing velocity after 10 min of exercise at a given speed. Velocity was increased until the animal could no longer sustain a given speed and remained >10 s on an electrified stainless-steel grid, which provided an electrical stimulus to keep the mice running. After reaching exhaustion, animals were left undisturbed in the treadmill chamber for 30 min. $\dot{V}O_2$ and $\dot{V}CO_2$ were determined using the program Metabolism for 2 channel PowerLab (ADInstruments), analyzing the last 5 min recorded at rest and at each speed. During recovery, $\dot{V}O_2$ and $\dot{V}CO_2$ were determined during a 3-min period following immediately after cessation of exercise (recovery 1; exhaustion) and at the end of the 30-min recovery period (recovery 2). Subsequently, RER was calculated. Total running distance and running time were recorded. To determine peak oxygen consumption, we measured the greatest value in oxygen consumption shown by each mouse during exercise.

Statistical analyses

All continuous variables are presented as means ± SE. Morphometric and cytokine levels were analyzed using 1-way

ANOVA, followed by Newman-Keuls multiple-comparison test. Cardiopulmonary parameters were analyzed using Student's *t*-test and Mann Whitney *U* test with Prism 3.0 (GraphPad Software, San Diego, CA, USA). Degree of severity was analyzed using a 2-way ANOVA, followed by a Bonferroni post-test. Treadmill data were analyzed applying a repeated-measures 1-way ANOVA, followed by an all-pairwise multiple comparison procedure (Student-Newman-Keuls method) using Prism 3.0. All differences were considered significant at values of $P \leq 0.05$.

RESULTS

Decreased myocarditis and fibrosis after G-CSF treatment

A marked decrease in the number of inflammatory cells was observed 2 mo after G-CSF treatment, compared with saline-treated chagasic mice (**Fig. 1*A*, *C***). Morphometric analysis showed a significant reduction in the number of inflammatory cells after G-CSF treatment (**Fig. 2*A***). In addition, hearts of G-CSF-treated mice had a reduced area of fibrosis (Fig. 1*B*, *D*), statistically different from saline-treated mice (Fig. 2*B*). The number of inflammatory cells undergoing apoptosis was ~7-fold greater in hearts of G-CSF-treated mice compared to those of saline-treated mice (Fig. 2*C*; $P<0.0001$). The levels of the chemokine SDF-1 (CXCL2) in hearts of saline-treated, but not of G-CSF-treated mice, were significantly higher than those of normal mice (Fig. 2*D*). Hearts from both saline- and G-CSF-treated mice had similar numbers of parasite foci 2 mo after therapy (2.4 ± 0.4 and 2.0 ± 0.6 parasite foci/mm^2, respectively; $P<0.05$).

G-CSF treatment ameliorates cardiac electrogenesis in chronic chagasic mice

All infected mice showed severe cardiac conduction disturbances in ECG records, such as AV blockage, intraventricular conduction disturbances, and abnormal cardiac rhythm 6 mo after infection, compared to normal mice (**Fig. 3**). In saline-treated mice ($n=7$), none improved cardiac function, and 5 became worse after therapy. In contrast, in the G-CSF-treated group ($n=6$), 2 animals improved cardiac conduction (**Fig. 4**), and 4 had no alterations (Fig. 3).

G-CSF treatment improves exercise capacity

Among the saline-treated infected mice, only 2 of 6 were able to keep up with a belt speed of up to 14.4 m/min. The other 4 untreated infected mice were not able to run on the treadmill. All animals in the G-CSF-treated group ($n=7$) and in the normal control group ($n=10$) were able to exercise on the treadmill. All of the G-CSF-treated infected mice sustained locomotion at a speed of 14.4 m/min, but 3 of 7 animals were able to perform at 21.6 m/min. These differences resulted in significantly greater running times and distances covered by G-CSF-treated mice when compared to saline-treated mice, although G-CSF-treated mice still performed at lower capacity than normal mice (**Fig. 5**).

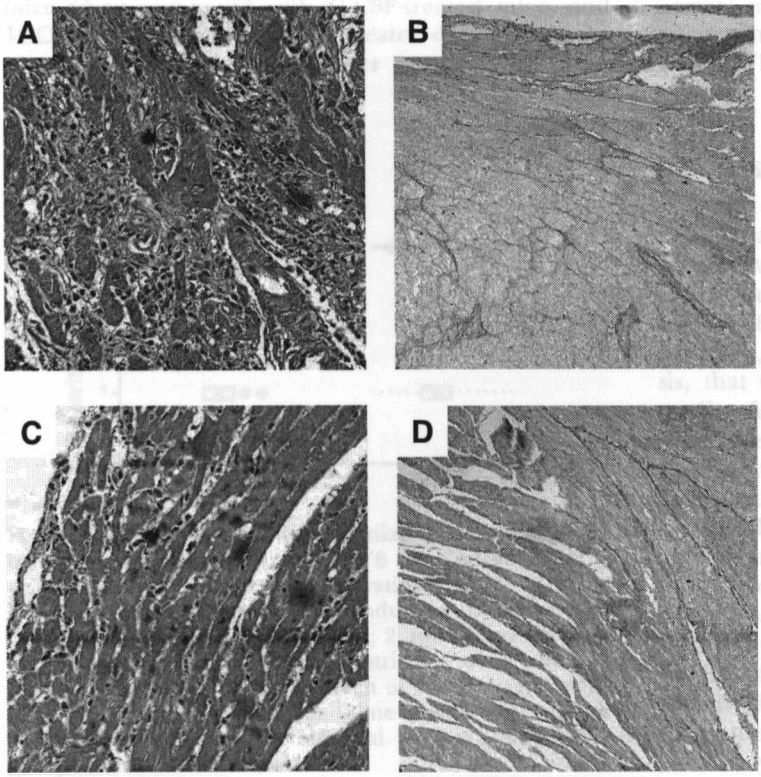

Figure 1. Histopathological analysis in heart sections of *T. cruzi*-infected mice. Heart sections of saline-treated (*A*, *C*) or G-CSF-treated (*B*, *D*) *T. cruzi*-infected mice were analyzed 2 mo after therapy. Staining: H&E (*A*, *B*; ×400); Sirius red (*C*, *D*; ×200).

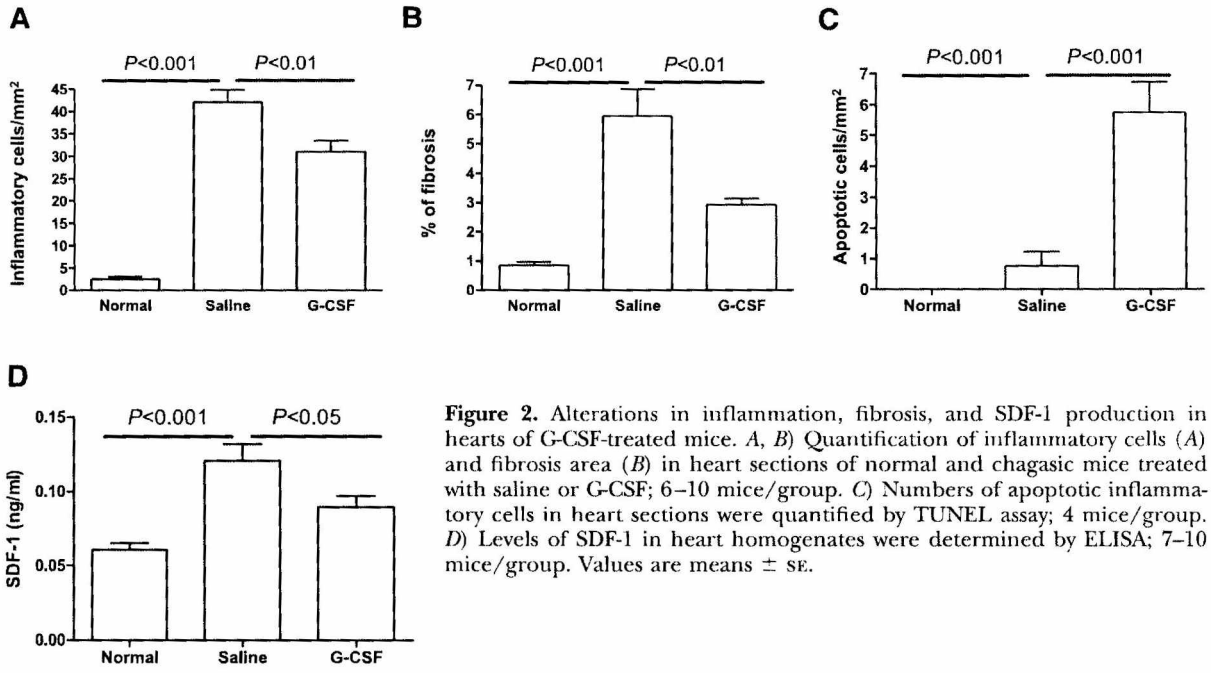

Figure 2. Alterations in inflammation, fibrosis, and SDF-1 production in hearts of G-CSF-treated mice. *A, B*) Quantification of inflammatory cells (*A*) and fibrosis area (*B*) in heart sections of normal and chagasic mice treated with saline or G-CSF; 6–10 mice/group. *C*) Numbers of apoptotic inflammatory cells in heart sections were quantified by TUNEL assay; 4 mice/group. *D*) Levels of SDF-1 in heart homogenates were determined by ELISA; 7–10 mice/group. Values are means ± SE.

Under resting conditions, the respiratory exchange ratio was significantly different between each group, whereas $\dot{V}O_2$ and $\dot{V}CO_2$ showed no differences (**Fig. 6**). During exercise stages 1 and 2, $\dot{V}CO_2$ was significantly greater in the normal mice compared with G-CSF-treated mice, and respiratory exchange ratio was significantly greater in normal mice at exercise stage 2 when compared with the G-CSF group. Shortly after exercise, $\dot{V}O_2$ and $\dot{V}CO_2$ were significantly greater in normal mice when compared with G-CSF-treated mice, and $\dot{V}CO_2$ remained significantly greater during recovery when compared with both other groups. Within the normal mice, $\dot{V}O_2$, $\dot{V}CO_2$, and RER rose significantly above resting values during exercise, reaching greatest $\dot{V}O_2$ shortly after cessation of exercise, with decreasing respiratory variables during the recovery phase. The G-CSF-treated mice showed no significant elevation of $\dot{V}O_2$ and $\dot{V}CO_2$ during exercise when compared with resting values, but $\dot{V}O_2$ and $\dot{V}CO_2$ shortly after exercise were significantly greater than the values measured during the other stages. Peak oxygen consumption of normal mice (8068 ml O_2/min/kg) was significantly greater than that of the G-CSF-treated group (6561 ml O_2/min/kg).

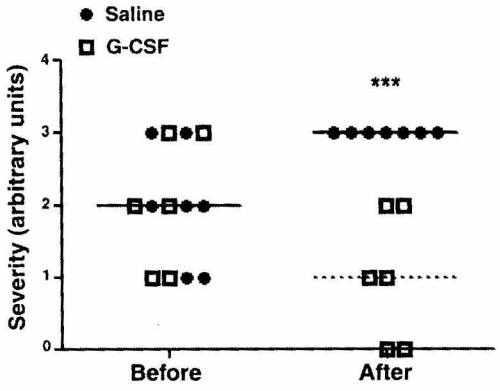

Figure 3. Graph representing cardiac conduction disturbances in arbitrary units in C57BL/6 mice infected with *T. cruzi* before and 2 mo after administration of saline or G-CSF. Degree of severity: 0, no cardiac conduction disturbances; 1, first-degree atrium-ventricular block; 2, intraventricular conduction disturbance; 3, atrium-ventricular dissociation; 4, death. Dashed line indicates median of G-CSF treated mice; solid line indicates median in saline-treated mice. Before treatment, both medians are identical. ***$P < 0.001$ between groups after treatment with G-CSF.

DISCUSSION

G-CSF is a cytokine known to improve cardiac function and recovery in models of ischemic disease (7, 13, 14). In this study, we demonstrated that repeated administration of G-CSF induces beneficial effects on cardiac structure, such as reduction of inflammation and fibrosis, that were well correlated with improvements in cardiac function in an experimental model of CChC that closely resembles the human disease (15, 16). One may question the low number of infected animals used in this study, and consequently the significance of our results. However, the fact that all of the G-CSF-treated animals improved their performance on the treadmill, when compared with saline-treated mice, shows that G-CSF has a profound effect on chronic chagasic mice. Regarding the cardiac conduction disturbances studied here, 6 of 6 infected animals showed improvements or remained stable due to the G-CSF treatment, whereas all of the saline-treated mice deteriorated; even mice with only a slight first-degree AV blockage evolved

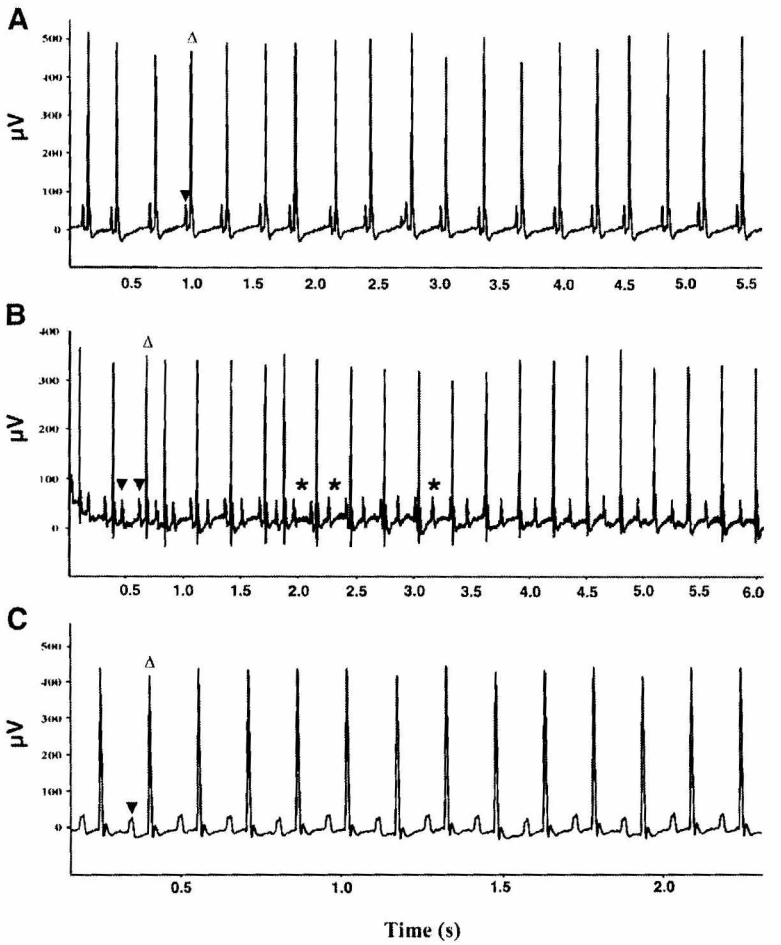

Figure 4. Reversion of cardiac disturbance after G-CSF treatment. ECG of a mouse before infection (*A*); 6 mo after infection, before G-CSF treatment (*B*); and 2 mo after G-CSF treatment (*C*). ▼, P wave; △, QRS complex; *, A-V dissociation.

toward an AV dissociation. G-CSF-treated mice, on the other hand, improved cardiac conductance and never surpassed a first-degree AV blockage. These facts, taken together, indicate that G-CSF has a significant therapeutic effect on mice with chronic Chagas disease.

One of the main features of CChC is the presence of prominent inflammation with participation of an autoimmune component (17–19) that causes destruction of myofibers and fibrosis deposition. We have recently shown that autologous BMC transplant modulates the myocarditis in experimental CChC, an effect associated with apoptosis of inflammatory cells (10). In the current study, we also found that the decreased inflammation after G-CSF therapy correlated with an increase in apoptosis of inflammatory cells. Thus, the benefits of G-CSF therapy may result, in part, from regulation of pathological immune responses. In fact, recent reports have demonstrated that G-CSF inhibits T cells by stimulating apoptosis (20, 21).

Heart SDF-1 levels were increased in CChC. The modulation of heart inflammation by G-CSF therapy also correlated with a reduction in SDF-1 in chagasic hearts. This chemokine promotes the recruitment of inflammatory cells, including T cells, and of stem cells, which express its receptor CXCR4 (22), and may play a role in other inflammatory processes in the heart (23, 24). The fact that G-CSF mobilizes stem/precursor cells to the periphery suggests that these cells migrate to the inflamed myocardium and contribute to tissue regeneration, since it has been shown before that mobilized BMSCs repair the damaged myocardium (25). In fact, we have previously found that transplanted BMSCs migrate to and differentiate into cardiomyocytes (10).

Sugano *et al.* (13) described an accelerated healing process due to increased reparative collagen synthesis in affected areas after G-CSF administration, in an experimental myocardial infarction. A reduction in fibrosis was observed after a long-term treatment using low doses of G-CSF after myocardial infarct (26). Here, we also found a significant reduction in heart fibrosis after G-CSF treatment.

Another feature of chronic Chagas disease is the scarce parasitism found in this phase of infection. Several studies have demonstrated that *T. cruzi* parasites or antigens can be found, although rarely, in individuals with chronic infection (27, 28). Although the inflammatory response was modulated after G-CSF therapy, the residual parasite load in our model was not affected by this treatment.

It has been proposed that the main effect of G-CSF after severe cardiac injury is to induce the proliferation of cardiac stem cells rather than BMSC migration and

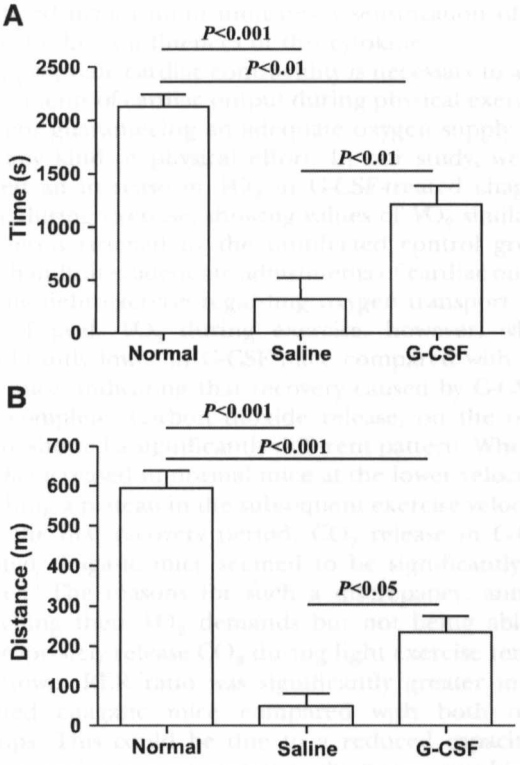

Figure 5. G-CSF treatment improves the capacity of infected mice to perform exercise. Time (*A*) and distance run (*B*) on a motorized treadmill by normal (noninfected) mice (n=10) and chronic chagasic mice treated with saline (n=2) or with G-CSF (n=7). Data are means ± SE. Note that running time and distance of saline-treated mice are highly influenced by 2 of the 6 animals tested that were able to exercise on the treadmill, whereas the other 4 saline-treated animals were not able to run on the treadmill.

proliferation. Kanellakis *et al.* (29) demonstrated, in a model of acute myocardial infarction, that G-CSF/SCF therapy improved cardiac function, increasing the number of blood vessels and cells of the cardiomyogenic lineage. However, differently from previous studies (30, 31), they demonstrated that these cells were of myocardial rather than of bone marrow origin. They also provided evidence that the effects were due to G-CSF alone, since the addition of SCF to G-CSF provided little additional benefit at the functional level. Brunner *et al.* (32) demonstrated that treatment with G-CSF after myocardial infarct reduces the migratory capacity of bone marrow cells into ischemic tissue, but increases the number of resident cardiac cells.

In our study, we showed that G-GSF administration can avoid the aggravation of cardiac disturbances associated with chronic Chagas disease and, more important, seems to reverse some of the severe pathologies associated with CChC. On the other hand, the untreated group aggravated the cardiac abnormalities. These results were in agreement with other preclinical studies that investigated the antiarrhythmic effects of G-CSF and showed an increase in protein expression levels of β-catenin and connexin 43 (33, 34). β-Catenin is necessary to attach many proteins to the cell membrane, including proteins related to gap junction formation. Conexin 43 is responsible for cell-cell communication, and thereby allows the appropriate conduction of cardiac impulses through the whole heart, avoiding arrhythmias. The expression of this protein is reduced by *T. cruzi* infection (35) and may contribute to the arrhythmias. Thus, the beneficial effects of G-CSF treatment on cardiac electrogenesis may be related to an increase of conexin 43 expression. Kuhlmann *et al.* (33) proposed that the enhanced expression of G-CSF receptor in cardiomyocytes and other cell types of the

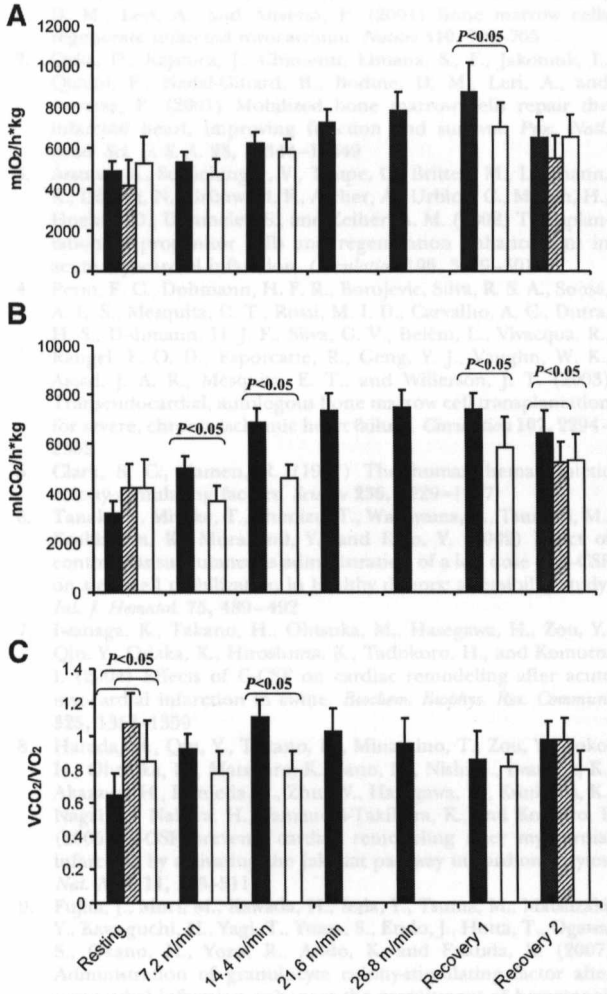

Figure 6. Cardiopulmonary function amelioration during physical effort after G-CSF administration. Oxygen consumption (*A*), carbon dioxide release (*B*), and respiratory exchange ratio (*C*) in normal mice (solid columns), saline-treated chagasic mice (dashed columns), and G-CSF-treated chagasic mice (open columns) during resting conditions, exercising at 4 different velocities (7.2, 14.4, 21.6, and 28.8 m/min) on a motorized treadmill, immediately (recovery 1) and 30 min after exercise (recovery 2). Data are means ± SD; 6–10 mice/group. Note that only 2 of 6 saline-treated infected animals were able to run at a velocity of 14.4 m/min; therefore, no data are given for saline-treated infected mice during exercise.

infarcted myocardium indicates a sensitization of the heart to direct influences of this cytokine.

Appropriate cardiac contractility is necessary to allow adjustments of cardiac output during physical exertion, thereby guaranteeing an adequate oxygen supply during any kind of physical effort. In our study, we observed an increase in $\dot{V}O_2$ in G-CSF-treated chagasic mice during exercise, showing values of $\dot{V}O_2$ similar to the levels reached by the uninfected control group, which indicates adequate adjustments of cardiac output during light exercise regarding oxygen transport. Values of peak $\dot{V}O_2$ during exercise, however, where significantly lower in G-CSF mice compared with normal mice, indicating that recovery caused by G-CSF is not complete. Carbon dioxide release, on the other hand, showed a significantly different pattern. Whereas $\dot{V}CO_2$ increased in normal mice at the lower velocities, reaching a plateau in the subsequent exercise velocities and the first recovery period, CO_2 release in G-CSF-treated chagasic mice seemed to be significantly impaired. The reasons for such a discrepancy, animals matching their $\dot{V}O_2$ demands but not being able to appropriately release CO_2 during light exercise remain unknown. RER ratio was significantly greater in untreated chagasic mice compared with both other groups. This could be due to a reduced capacity of chagasic mice to carry oxygen to the tissues, resulting in elevated anaerobic metabolism and increased release of CO_2. The performance improvement of G-CSF-treated mice can be attributed solely to the beneficial effect of G-CSF on cardiac structure, improving cardiac efficiency.

Li *et al.* (36) showed that administration of G-CSF in experimental chronic heart failure improved the myocardium contractility by avoiding the systolic and diastolic dysfunction through the changes in the geometry of the infarcted heart to short and thick, induced hypertrophy among surviving cardiomyocytes, and reduced myocardial fibrosis. These effects could be explained by a direct effect of G-CSF on cardiomyocytes that could lead to the activation of an intracellular signal, since the expression of G-CSF receptor was confirmed in failing hearts and was up-regulated by G-CSF treatment (8, 13). Our results are in agreement with Li *et al.* (36), as G-CSF administration caused a reduction in functional impairment and partial recovery of heart structure.

Chronic heart failure remains a leading cause of mortality due to the absence of an efficient therapy that avoids structural and electrical cardiac remodeling. The only option for these patients remains heart transplantation. Besides limitations of available donated organs, in the specific case of Chagas disease, the use of immunosupressive drugs following heart transplantation can affect the latent parasitism. On the basis of the beneficial effects of G-CSF shown in the present study, we conclude that this may be a promising therapy for the treatment of patients with heart failure due to Chagas disease. An ongoing phase I/II clinical trial of G-CSF therapy with multiple administrations in chagasic patients (37) may indicate the benefits of this therapy in humans. FJ

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The MHC Gene Region of Murine Hosts Influences the Differential Tissue Tropism of Infecting *Trypanosoma cruzi* Strains

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Abstract

We have previously demonstrated that both parasite genetic variability and host genetic background were important in determining the differential tissue distribution of the Col1.7G2 and JG *T. cruzi* monoclonal strains after artificial infections in mice. We observed that the JG strain was most prevalent in hearts of mouse lineages with the MHC haplotype *H-2*^{d} (BALB/c and DBA2), while Col1.7G2 was predominant in hearts from C57BL/6 mice, which have the *H-2*^{b} haplotype. To assess whether the MHC gene region indeed influenced tissue tropism of *T. cruzi*, we used the same two parasite strains to infect C57BL/6 (*H-2*^{b}) and C57BLKS/J (*H-2*^{d}) mice; the latter strain results from the introgression of DBA2 MHC region into the C57BL/6 background. We also performed *ex vivo* infections of cardiac explants from four congenic mice lineages with the *H-2*^{b} and *H-2*^{d} haplotypes arranged in two different genetic backgrounds: C57BLKS/J (*H-2*^{d}) versus C57BL/6 (*H-2*^{b}) and BALB/c (*H-2*^{d}) versus BALB/B10-*H2*^{b} (*H-2*^{b}). In agreement with our former observations, Col1.7G2 was predominant in hearts from C57BL/6 mice (*H-2*^{b}), but we observed a clear predominance of the JG strain in hearts from C57BLKS/J animals (*H-2*^{d}). In the *ex vivo* experiments Col1.7G2 also prevailed in explants from *H-2*^{b} animals while no predominance of any of the strains was observed in *H-2*^{d} mice explants, regardless of the genetic background. These observations clearly demonstrate that the MHC region influences the differential tissue distribution pattern of infecting *T. cruzi* strains, which by its turn may be in a human infection the determinant for the clinical forms of the Chagas disease.

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Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, has a variable clinical course, ranging from asymptomatic to severe chronic cardiac and/or gastrointestinal disease. The mechanisms involved in this wide range of clinical manifestations of Chagas disease are still not completely understood, but certainly both parasite and host-associated aspects are important [1]. Using the sensitive DNA profiling technique, LSSP-PCR (Low-Stringency single Specific Primer PCR), we previously showedthat hearts of BALB/c mice doubly infected with JG and Col1.7G2 *T. cruzi* populations were mainly colonized by JG strain, while Col1.7G2 was preponderant in the rectum and all other analyzed tissues [2]. This provided strong and direct evidence that parasite genetic variability was involved in the differential tissue tropism and pathology of the infection.

The influence of host genetic factors in this process was revealed subsequently using different mouse strains, by the demonstration that the patterns of parasite tissue distribution were similar for BALB/c and DBA-2 mice, but different for C57BL/6 mice [3]. Since BALB/c and DBA-2 lineages share the MHC haplotype (*H-2*^{d}) and the C57BL/6 lineage has a different haplotype (*H-2*^{b}), we hypothesized that the host MHC gene region might influence the differential tissue distribution of *T. cruzi* strains in these mice. The murine MHC gene region spans approximately 4 Mb of chromosome 17 (23.0 cM, cytoband B–C) and contains 3 major classes of highly polymorphic gene sets: class I (*H-2*-K, *H-2*-D, Q, *H-2*-T18 genes), class II (*H-2*-I genes), and class III (*H-2*-S genes) [4]. These genes are involved in many immunological processes, including graft rejection, immune response, antigen presentation and complement component (http://www.informatics.jax.org).

We have tested the influence of host MHC gene region on differential tissue distribution of *T. cruzi* strains by studying four congenic mice lineages with two different *H-2* haplotypes arranged in two different genetic backgrounds: C57BLKS/J (*H-2*^{d}) versus C57BL/6 (*H-2*^{b}); BALB/c (*H-2*^{d}) versus BALB/B10-*H2*^{b} (*H-2*^{b}). Our data showed irrevocably both *in vivo* and *ex vivo* that predominance of one or the other strain of *T. cruzi* (JG or Col1.7G2) in mice heart tissue was dependent on the MHC gene region background, where *H-2*^{b} haplotype selected for Col1.7G2 clone while *H-2*^{d} haplotype selected for JG monoclonal population. These observations strongly suggest there is a significant role

of host MHC and/or associated genes in the differential rates of growth and tissue distribution pattern of *T. cruzi* strains.

Materials and Methods

All procedures for animal manipulation and experiments are in accordance with the COBEA, the Brazilian institution that regulates animal experimentation.

Congenic mice

Male mice (5–6 weeks old) were used in these experiments. Two strains, C57BLKS/J (*H-2*^{d}) - Stock Number: 000662 - resulting from the introgression of the DBA2 MHC into the C57BL/6 background and BALB/B10-*H2*^{b} – also named C.B10 *H-2* Stock Number: 001952 - in which the BALB/cLilMcdJ MHC region was introgressed into the C57BL/10J MHC gene region, were obtained directly from Jackson Laboratories. C57BL/6 (*H-2*b) mice were obtained from "Centro de Bioterismo" of ICB / UFMG and BALB/c (*H-2*^{d}) were obtained from CPqGM, FIOCRUZ/BA.

Parasites

For artificial infections we used two different populations of *T. cruzi*: Col1.7G2 (*T. cruzi* I - Zymodeme 1, rDNA group 2, miniexon group 2, mitochondrial haplotype A) and JG (*T. cruzi* II - Zymodeme 2, rDNA group 1, miniexon group 1, mitochondrial haplotype C) originally isolated from patients with distinct forms of Chagas' disease. The JG strain, isolated from the blood of a patient with megaesophagus, was previously typed at eight different microsatellite loci[5] and did not show more than two alleles in any of them, indicating that it is monoclonal (data not shown). Col1.7G2 was cloned from the Colombian strain, which was originally cultured from the blood of a chronic cardiac patient [6]. Infective trypomastigote forms were obtained from blood of infected Swiss mice and diluted to 50 parasites/100 µl of sterile PBS for infection of mice. For infection of heart explants, *T. cruzi* infective trypomastigotes were prepared from the supernatant of LLCMK2 cell cultures infected with each parasite lineage.

Experimental infections in mice

For infections in mice we used the same protocol described by Andrade [2]. Briefly, mice were intraperitoneally inoculated with a mixture of both parasites (50+50). All infections were done in duplicates at different days.

Six months after infection, corresponding to the chronic phase, animals were killed and samples from the heart and rectum were collected. Age-matched animals were used as controls. Two fragments taken from each organ were washed exhaustively in isotonic saline and stored in ethanol at −20°C. Tissue samples were subjected to alkaline lysis[2] and used directly in the PCR after 10-fold dilution in double-distilled water.

Cardiac murine explants

Hearts from the four mouse lineages were aseptically removed and sliced at 0.5 mm width in a Tissue Chopper (McIlwain MTC/2 – The Mickle Laboratory engineering Co. LTD.). Two or three slices totaling approximately an area of 10 mm^2 were exhaustively washed with PBS buffer and deposited over a thin layer of 2% bovine gelatin in DMEM media supplemented with 10% FBS and 50 µg/ml gentamycin in individual wells of a 24-well culture plate, and covered with 2 ml of the same media without gelatin. After 2 h of incubation at 37°C in a 5% CO_2 chamber, 5×10^5 trypomastigotes of the JG strain and/or Col1.7G2 clone were added. All wells were washed 24 h later with sterile PBS and fresh media was added to eliminate non-internalized parasites. For PCR analysis, tissue slices were rinsed and collected at 24, 96 and 120 h, submitted to the alkaline lysis protocol[2] and used directly in the PCR after 10-fold dilution in double-distilled water.

Detection and characterization of parasites by LSSP-PCR of infected tissues

Detection of parasites from each tissue or explant sample was performed by specific PCR amplification of a fragment (about 330 bp) corresponding to the four-variable region of the *T. cruzi* kinetoplast DNA minicircle, as described previously [2]. The PCR products were visualized in a 6% polyacrylamide gel electrophoresis and silver stained as previously described [7]. Characterization of the parasites from the positive tissues by LSSP-PCR was performed as described earlier [8]. Briefly, kDNA amplicons were subjected to electrophoresis in 1.5% agarose gel (1.0% agarose, 0.5% agarose low melting point), punctured from the gel, diluted 10 times in double distilled water, and submitted to a second step of low stringency amplification, using a single primer (S35G: 5′-ATGTACGGGGA-GATGCATGA-3′). The LSSP-PCR products were also visualized after a 6% polyacrylamide gel electrophoresis and silver staining.

Semi-quantitative data from LSSP profiles

For a semi-quantitative analysis, the proportions of the *T. cruzi* strains in each tissue of the doubly infected animals or cardiac explant samples were estimated in a fluorescent automated DNA Sequencer (ALF, Pharmacia Biotech) as described before [2]. For this, the LSSP-PCR was carried out using a fluorescein-labeled S35G primer. The PCR products were then subjected to a 6% polyacrylamide gel electrophoresis under denaturing conditions (8 M urea) and the data obtained were analyzed using the AlleleLinks software (Pharmacia Biotech). The area under specific peaks of each population was calculated and used to evaluate approximately the relative proportions of the JG strain and Col1.7G2 clone by reference to a standard curve. It was demonstrated that LSSP-PCR profiles of equal mixtures of the two strains were the sum of the individual profiles where the specific peak areas were related to the proportions of each population (25:75; 50:50; and 75:25) [3].

Two-sample Student t test was applied to validate differences between values obtained from murine cardiac explants.

Detection and characterization of parasites in infected tissues by real-time PCR

The characterization of the parasites in infected mice explants was also done by real-time PCR of the D7 region of the rRNA 24S gene. For that, five nanograms of parasite DNA or 5µl of the product of the alkaline lyses obtained from culture explants were used as template in a two-round PCR assay, as described previously [9]. Briefly, samples were first submitted to a PCR using two primers: D75 = 5′– CAGATCTTGGTTGGCGTAG–3′ and D72 = 5′– TTTTCAGAATGGCCGAACAGT–3′). Two microliters of these PCR products were used as template in the second PCR round performed in a real time PCR apparatus (ABI7900 - Applied Biosystems), using the primers: D71 (5′-AAGGTGCGTCGACAGTGTGG-3′) and D72. Since Col1.7G2 and JG D7 regions presented amplicons with TM of 81.5 and 78.2°C, respectively, they are easily distinguishable by two distinct peaks in the melting curves obtained from the real time apparatus.

Results

In order to test the influence of the *H-2* murine region in the *T. cruzi* tissue tropism, we utilized initially two different clonal isolates

of parasite to infect C57BL/6 ($H\text{-}2^b$) and C57BLKS/J ($H\text{-}2^d$) mice. Data were collected from mice after six months infection, to simulate the chronic phase of Chagas' disease. The characterization and semi-quantitative assessment of the infecting *T. cruzi* population by LSSP-PCR in different organs of mice simultaneously infected with both JG and Col1.7G2 were used to analyze infection and tropism.

The molecular characterization of the parasites in mice co-infected with JG and Col1.7G2 showed no specific differential distribution of *T. cruzi* in the recta of C57BL/6 and C57BLKS/J (Fig 1). However, a completely different result was observed for the hearts of these mice. The tissue distribution of the two *T. cruzi* populations showed a complete correlation with its genetic background. A high proportion of Col1.7G2 (99% of infecting population; n = 8) in relation to JG was always obtained in the hearts from C57BL/6, while in the hearts from C57BLKS/J mice, the proportion of Col1.7G2 was reduced to basal levels (5%; n = 8) (Fig. 1).

The influence of the humoral immune response on the tissue distribution pattern of *T. cruzi* was assessed by using the same parasite populations to infect simultaneously cardiac explants from four mice lineages. These mice differed from each other by interchanging their *H-2* haplotypes in two different genetic backgrounds. The relative amount of each infecting parasite was initially assessed using the LSSP-PCR technique. Two parameters were analyzed: the rate of *T. cruzi* cell invasion, evaluated after 24 h of parasite incubation with the host cell, and parasite intracellular growth, analyzed four (96 h) and five days (120 h) post-infection. No difference in the relative amount of JG and Col1.7G2 was observed after 24 h of infection in all mouse lineages (Fig. 2a). In addition, no difference in intracellular growth of both *T. cruzi* populations was observed in heart explants from C57BLKS/J and BALB/c, which share the $H\text{-}2^d$ haplotype. However a clear predominance of the Col1.7G2 clone was observed in explants from mice with $H\text{-}2^b$ haplotype (C57BL/6 and BALB/B10) after 96 h or 120 h respectively (Fig. 2a). Similar results were also seen by using real time PCR of the D7 rDNA alleles of *T. cruzi* where a clear selection of Col1.7G2 was observed during parasite growth in $H2^b$ haplotype cardiac explants (Fig. 2b).

Discussion

One of the most intriguing characteristics of Chagas disease is its broad range of clinical manifestations. Although the exact causes of this variability remain to be elicited, data from our group have demonstrated that, in mice, the MHC gene region could be involved with the distribution of different *T. cruzi* strains into different tissues [3]. The role of MHC haplotypes at different levels of the interaction of *T. cruzi* and hosts has been discussed by many authors and its supposed influence ranges from the humoral response against *T. cruzi*[10] and induction of antigen presentation to $CD8^+$ T cells[11] through modulation of the expression of MHC class II molecules by the parasite [12]. While some authors saw none or few statistically significant influence of MHC locus on development of the different clinical forms of Chagas disease in patients from Brazil [13], others identified a correlation with either seropositivity or the development and severity of the clinical manifestations in humans, as well as the infection in animal models. Borrás et al. (2006), studying patients from Argentina showed a statistically significant correlation of HLA and the level of serologically positive individuals for *T. cruzi* [14]. Although not statistically significant they also found a trend for the development of the Chagas cardiomyopathy. Other studies with Mexican chagasic patients suggested that some MHC alleles could be associated with the clinical forms of chronic Chagas disease especially with the higher risk of heart disease development [15]. Furthermore molecular class II MHC typing among chagasic patients has allowed the identification of putative MHC susceptibility genes in *T. cruzi* seropositive individuals with cardiac disease, as compared with non symptomatic individuals [16]. In the murine model, there are also indications that the MHC region modulates tissue damage, host survival[17] and the susceptibility/

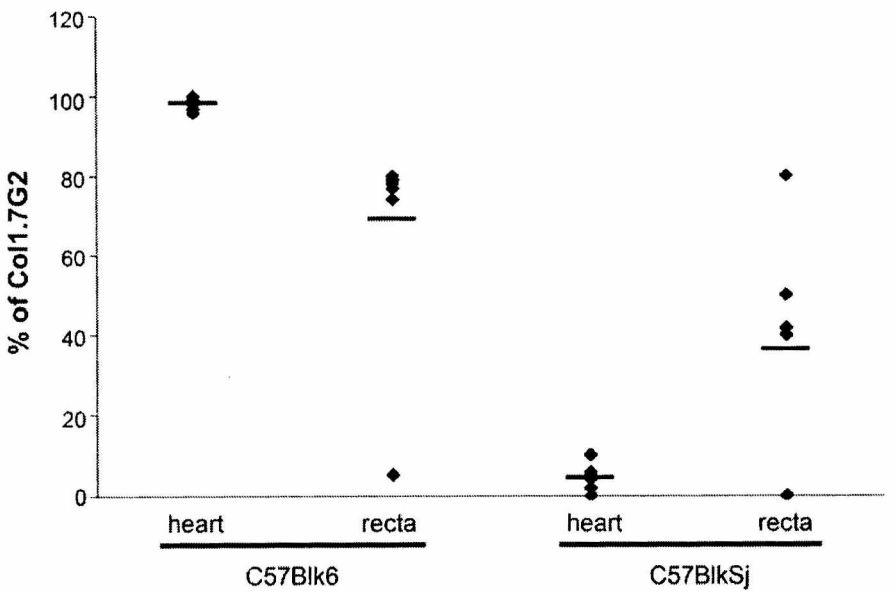

Figure 1. Relative percentage of the Col1.7G2 clone of *T. cruzi* in tissues of C57Blk6 ($H2^b$) and C57BlkSj ($H2^d$) after six months of double infection with Col1.7G2 and JG strains, using the LSSP-PCR technique. Each point indicates data from individual mice and the bar represents the median value.
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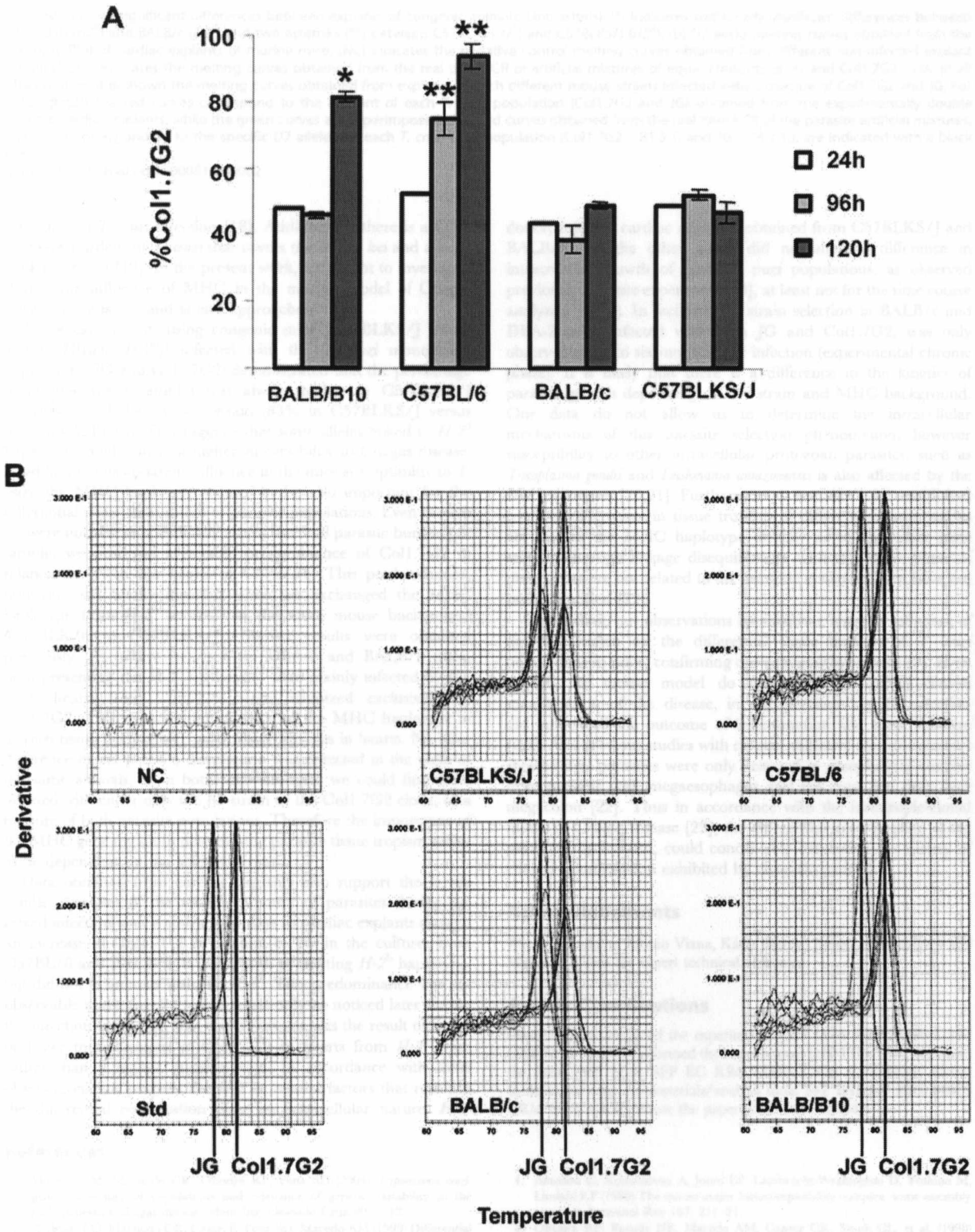

Figure 2. Quantitative analyses of the percentage of Col1.7G2 and/or JG after intracellular development in distinct murine cardiac explants exposed to equal mixture of trypomastigote forms of each population. (a) Relative percentage of the Col1.7G2 clone using the LSSP-PCR technique, 24, 96 and 120 h post-infection in murine cardiac explants. P values above the bars were obtained by the two-sample Student t

test and indicate significant differences between explants of congenic animals. One asterisk (*) indicates statistically significant differences between BALB/B10 ($H2^b$) and BALB/c ($H2^d$) and two asterisks (**) between C57BL/6 ($H2^b$) and C57BLKS/J ($H2^d$). (b) D7 allele melting curves obtained from the real time PCR of cardiac explants of murine mice. (NC) indicates the negative control melting curves obtained from different non-infected explant samples; (Std) indicates the melting curves obtained from the real time PCR of artificial mixtures of equal amounts of JG and Col1.7G2 DNA. In all other graphs, it is shown the melting curves obtained from explants of each different mouse strains infected with a mixture of Col1.7G2 and JG. For these graphs the red curves correspond to the amount of each *T. cruzi* population (Col1.7G2 and JG) obtained from the experimentally double infected cardiac explants, while the green curves are superimposed standard curves obtained from the real time PCR of the parasite artificial mixtures. The peaks corresponding to the specific D7 alleles for each *T. cruzi* DNA population (Col1.7G2 – 81.5°C and JG – 78.2°C), are indicated with a black line.

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resistance to *T. cruzi* infection [18]. Additionally, there is a QTL for tissue burdens of *T. cruzi* that covers the MHC *loci* and a large flanking region [19]. In the present work, we sought to investigate further the influence of MHC in the murine model of Chagas disease using *in vivo* and *in vitro* approaches.

In vivo experiments using congenic mice [C57BLKS/J ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$)] infected with the *T. cruzi* monoclonal populations (JG and Col1.7G2) demonstrated that the percentage of positive tissue samples was always higher in C57BLKS/J independent of the parasite strain (83% in C57BLKS/J versus 57% in C57BL/6). This suggests that some alleles linked to $H\text{-}2^d$ haplotype could lead to a higher susceptibility to Chagas disease. In addition to its apparent influence in the mice susceptibility to *T. cruzi*, the MHC haplotype seemed to be also important for the differential tissue distribution of parasite populations. Even though we were not able to specifically access the total parasite burden per sample, we observed a notable predominance of Col1.7G2 in relation to JG in the hearts of C57BL/6. This predominance, however, was totally inverted when we exchanged the MHC haplotype from $H\text{-}2^b$ to $H\text{-}2^d$ in the same mouse background (C57BLK/6 to C57BLKS/J). Similar results were obtained previously [3], where hearts from DBA-2 and BALB/c mice, both presenting the $H\text{-}2^d$ haplotype, were mainly infected by JG, while hearts from C57BL/6 were colonized exclusively by Col1.7G2. This apparent interference of the MHC haplotype in *T. cruzi* tissue tropism was particularly obvious in hearts. No clear difference in the parasite distribution was detected in the recta of the same animals, as in both mice lineages, we could find recta infected with either only the JG strain or the Col1.7G2 clone, or a mixture of both parasite populations. Therefore the importance of the MHC gene region in determining parasite tissue tropism seems to be dependent on the infected organ.

Data obtained from cardiac explants also support the *in vivo* results. Analyses of the relative amount of parasites during the mixed infection over a course of 5 days in cardiac explants showed an increase of Col1.7G2 in relation to JG in the cultures from C57BL/6 and BALB/B10 mice, both presenting $H\text{-}2^b$ haplotype, but did not in animals with the $H\text{-}2^d$. This predominance was not observable in the first 24 h, but could only be noticed later during the infection suggesting that this difference was the result of better or faster multiplication of Col1.7G2 in hearts from $H\text{-}2^b$ mice rather than a higher invasion rate. In accordance with these observations, we propose the *H-2* or related factors that result in the differential colonization have an intracellular nature. $H\text{-}2^d$ doubly infected cardiac explants (obtained from C57BLKS/J and BALB/c), on the other hand, did not show a difference in intracellular growth of both *T. cruzi* populations, as observed previously in *in vivo* experiments [3], at least not for the time course analyzed (96 hs). In fact parasite strain selection in BALB/c and DBA-2 mice infected with both JG and Col1.7G2, was only observed three to six months post infection (experimental chronic phase). It is likely that there is a difference in the kinetics of parasite growth depending on the strain and MHC background. Our data do not allow us to determine the intracellular mechanisms of this parasite selection phenomenon, however susceptibility to other intracellular protozoan parasites, such as *Toxoplasma gondii* and *Leishmania amazonensis* is also affected by the *H-2* haplotypes [20,21]. Furthermore, it remains to be established whether differences in tissue tropism of different parasite strains are due to the MHC haplotype directly or to any other gene selected through linkage disequilibrium with this locus. Some of these genes are not related to the immune system or even have not been identified [22].

Concluding, our observations demonstrate a strong influence of the *H-2* region on the differential tissue tropism of *T. cruzi* populations in mice, confirming our previous hypothesis [3]. Even though the mouse model do not reproduce actual clinical manifestation of the disease, in our previous studies, selection did influenced the outcome of inflammation and tissue damage [2,3]. Also previous studies with chronic digestive chagasic patients showed that parasites were only detected in oesophageal samples from patients with megacesophagus and not from the ones with megacolon [22]. Thus in accordance with the histotropic-clonal model of Chagas disease [23], the differential tissue tropism of the parasites, by its turn, could conceivably determine the variety of clinical manifestation exhibited by chagasic patients.

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Author Contributions

Conceived and designed the experiments: JMF LOA RRS MBPS CRM GRF SDJP AMM. Performed the experiments: JMF LOA RSL. Analyzed the data: JMF LOA SFP EC RRS MBPS CRM GRF SDJP AMM. Contributed reagents/materials/analysis tools: SFP RSL EC RRS MBPS CRM GRF AMM. Wrote the paper: JMF LOA AMM.

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Characterization of cardiopulmonary function and cardiac muscarinic and adrenergic receptor density adaptation in C57BL/6 mice with chronic *Trypanosoma cruzi* infection

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SUMMARY

Circulating antibodies in chagasic patients interact with myocardial β adrenergic and muscarinic cholinergic receptors, triggering intracellular signals that alter cardiac function along the course of the disease. However, until now, experimental data in models of chronically infected chagasic mice linking the effects on myocardial β adrenergic and muscarinic receptors to cardiopulmonary dysfunction is lacking. Thus, we studied C57BL/6 mice 8 months after intraperitoneal injection of 100 trypomastigote forms of the Colombian strain of *T. cruzi*. Uninfected mice, matched in age, were used as controls. Histopathological analyses (inflammation and fibrosis) and radio-ligand binding assays for estimation of muscarinic and adrenergic receptor density were performed in myocardium tissue samples. When compared to controls, infected mice had electrical conduction disturbances, diastolic dysfunction, lower O_2 consumption and anaerobic threshold. In addition, hearts of chronic chagasic mice had intense inflammation and fibrosis, and decreased β adrenergic and increased muscarinic receptor densities than normal controls. Our data suggest that chronic *T. cruzi* infection causes alterations in cardiac receptor density and fibrosis deposition which can be associated with cardiac conduction abnormalities, diastolic dysfunction and lower exercise capacity, associating for the first time all these functional and histopathological alterations in chagasic mice.

Key words: Chagas' disease, cardiopulmonary function, cardiac receptors, mouse model.

INTRODUCTION

Chagas' disease, caused by a haemoflagellate protozoan parasite *Trypanosoma cruzi*, is one of the most common determinants of congestive heart failure and sudden death in Latin America, where it represents a serious health problem, affecting millions of persons (WHO, 1995). Chagas' disease is a complex illness that can evolve in different consecutive phases. The first corresponds to an acute phase occurring after the parasite infection and characterized by intense parasitism and blood parasitaemia. After this, an asymptomatic or indeterminate period, marked by the absence of clinical symptoms is observed. Finally, months or decades after the primary infection, some of the infected individuals enter the chronic phase, which is characterized by chronic myocarditis and the so-called mega syndromes, affecting the gastro-intestinal tract. Less than 10% of chronic patients present gastrointestinal abnormalities, such as pathological dilations of organs from the digestive tract (Dias, 1989; Dias and Coura, 1997). Approximately 30% of the infected individuals show heart involvement leading to heart failure. Furthermore, the chronic chagasic cardiomyopathy (CChC) is characterized by intense myocarditis and multiple arrhythmias, such as ventricular premature beats, complete right bundle branch block, left anterior fascicular block and atrioventricular block (Koerbele, 1968; Rassi *et al.* 1992).

The mechanism responsible for the development of cardiomyopathy is still controversial and several hypotheses have been proposed. One hypothesis is based on an immune response directed against *T. cruzi* antigens at sites of parasite persistence, leading to a pathogenic inflammatory process (Tarleton, 2001; Higuchi *et al.* 1997). Another hypothesis is that CChC is the result of an autoimmune process triggered in some individuals by *T. cruzi* infection (Cunha-Neto *et al.* 1995; Engman and Leon, 2002). The autoimmunity hypothesis is

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Synthesis, docking, and in vitro activity of thiosemicarbazones, aminoacyl-thiosemicarbazides and acyl-thiazolidones against *Trypanosoma cruzi*

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Abstract—A novel series of thiosemicarbazone and aminoacyl-thiazolidones derivatives were synthesized. Their structure suggests that these compounds could have anti-*Trypanosoma cruzi* activity. Biological evaluation indicates that some of these compounds are able to inhibit the growth of *T. cruzi* in concentrations non-cytotoxic to mammalian cells. Docking studies were carried out in order to investigate the binding pattern of these compounds for the *T. cruzi* cruzain (TCC) protein, and these showed a significant correlation with experimental data.

1. Introduction

Chagas' disease is a serious health problem that affects around 20 million people in Central and South Americas. The protozoan *Trypanosoma cruzi* is the causative agent of this disease. Current therapy is based on nifurtimox and benznidazole, drugs capable of eliminating parasitaemia and reducing serological titers in the acute phase of infection but not effective for all *T. cruzi* strains, especially in the chronic phase of infection. In addition, these drugs may cause serious adverse side effects due to their high toxicity.[1–4]

To develop new drugs to combat parasitic infections, research is often directed toward key differences between the metabolism of the mammal and the parasite. For this reason, cruzain (aka cruzipain) and trypanothione reductase (TR) are specific targets in the search for novel and selective inhibitors and subversive substrates.[1,5]

Cruzain is the major cysteine protease of *T. cruzi*, and is released at all life cycle stages of the parasite, but delivered to different cellular compartments at each stage. This enzyme is essential for replication of the intracellular parasite and appears to have potential for new antitrypanosomal chemotherapy.[5]

The forms of *T. cruzi* present in the human host are the bloodstream trypomastigote and the intracellular replicative amastigote. The epimastigote form, an obligate mammalian intracellular stage, has been confirmed recently.[6] Since vaccinations against trypanosomatic infections are still under development, the need for new drugs is indisputable.

The trypanocidal activity of several aromatic and heterocyclic hydrazones and acyl-hydrazine-hydrazone has been reported.[2–4] In addition, some derivatives with

Keywords: Thiosemicarbazones; Acyl-hydrazones; Aminoacyl-thiazolidones; Anti-*Trypanosoma cruzi* compounds; Docking studies.
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