INSTITUTO OSWALDO CRUZ

Doutorado em Biologia Celular e Molecular

Interação *Trypanosoma cruzi*-célula hospedeira: danos moleculares e celulares e o efeito de amiodarona na recuperação de cardiomiócitos infectados

DANIEL ADESSE PEDRA MARTINS

Rio de Janeiro
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Ministério da Saúde

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Celular e Molecular

Orientação: Dra. Maria de Nazareth S. L. Meirelles
Dra. Luciana Ribeiro Garzoni
Prof. Dr. Herbert B. Tanowitz (Orientador Colaborador)

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“É preciso estar atento e forte,
Não temos tempo de temer a morte”

“Se eu tivesse mais alma pra dar, eu daria.
Isso pra mim é viver.”

(Caetano Veloso)

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Esta tese é composta de quatro publicações, três artigos publicados em revistas indexadas e um capítulo de livro. Outros três trabalhos foram publicados durante o curso de Doutorado em Biologia Celular e Molecular:

1) *Trypanosoma cruzi* induces changes in cardiac connexin43 expression. **Adesse D**, Garzoni LR, Huang H, Tanowitz HB, de Nazareth Meirelles M, Spray DC. *Microbes Infect*. 2008. 10(1):21-8.

2) Fibrosis and hypertrophy induced by *Trypanosoma cruzi* in a three-dimensional cardiomyocyte-culture system. Garzoni LR, **Adesse D**, Soares MJ, Rossi MI, Borojevic R, de Meirelles Mde N. *J Infect Dis*. 2008. 15;197(6):906-15.

3) Dissecting coronary angiogenesis: 3D co-culture of cardiomyocytes with endothelial or mesenchymal cells. Garzoni LR, Rossi MI, de Barros AP, Guarani V, Keramidas M, Balottin LB, **Adesse D**, Takiya CM, Manso PP, Otazú IB, Meirelles Mde N, Borojevic R. Exp Cell Res. 2009. 15;315(19):3406-18.

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Neste período o aluno participou de seis congressos internacionais e ganhou cinco prêmios por apresentação de trabalhos ou *travel grants*.

RESUMO

A doença de Chagas é causada pelo protozoário *Trypanosoma cruzi* sendo a principal causa de morbi-mortalidade entre as cardiopatias na América Latina, onde é endêmica. No presente trabalho nós utilizamos modelos experimentais tais como cultivo celular e infecção de camundongos albinos para avaliar aspectos relacionados à patogênese desta doença. Primeiramente utilizamos a técnica de microarranjos de oligonucleotídeos para determinar as diferentes assinaturas transcriptômicas induzidas por quatro diferentes cepas de *T. cruzi* em uma linhagem de células musculares. Neste trabalho, verificamos que os genes alterados significativamente nas células infectadas ($p<0,05$) foram diferentes para cada cepa e apenas 21 sofreram a mesma alteração nas quatro cepas estudadas. No entanto, mioblastos apresentaram assinaturas transcriptômicas únicas após infecção pelas quatro cepas de *T. cruzi* utilizadas. Estes resultados indicam que a infecção com diferentes cepas do parasito modula vias similares, porém não idênticas, nas células hospedeiras. Em seguida analisamos o impacto da infecção de cardiomiócitos murinos na expressão de caveolina-3 (Cav-3), proteína formadora das cavéolas, importantes para a manutenção da homeostase de cálcio intracelular e envolvida em processos de hipertrofia cardíaca. A infecção *in vitro* e *in vivo* por *T. cruzi* induziu redução significativa ($p<0,05$) nos níveis de Cav-3. Esta redução foi acompanhada pela ativação da quinase regulada por sinal extracelular (ERK), majoritariamente envolvida no processo de remodelamento e hipertrofia cardíacos, sugerindo a participação desta via de sinalização na patogênese da doença de Chagas. Por último, avaliamos a capacidade tripanocida do composto antiarrítmico amiodarona em culturas de cardiomiócitos infectadas. Este composto apresentou atividade seletiva anti-*T. cruzi*, promovendo danos ultra-estruturais às formas amastigotas intracelulares e a recuperação da célula hospedeira, incluindo restabelecimento do citoesqueleto de actina e junções comunicantes, além da contratilidade espontânea das culturas. O conjunto de dados gerado com esta tese traz importantes avanços para o entendimento do estabelecimento da doença de Chagas cardíaca e novas opções para o tratamento etiológico desta doença negligenciada.

ABSTRACT

Chagas' disease is caused by the protozoan *Trypanosoma cruzi* and is the main cause of morbidity and mortality by heart problems in endemic countries in Latin America. In the present work we employed as experimental models cell cultures and murine experimental infection to evaluate aspects related to the pathogenesis of this disease. In the first part we determined through oligonucleotide microarrays the transcriptomic signatures induced in a myoblast cell line by four reference *T. cruzi* strains. We observed that the significantly altered ($p<0.05$) genes differed for each strain studied and only 21 suffered changes in the same direction in all four strains. However, infected myoblasts presented proportional alterations in the the overall transcriptome. These results indicate that the infection with distinct strains modulate similar, but not identical, pathways in the host cell. Next we analyzed the impact of *T. cruzi* infection on cardiac caveolin-3 (Cav-3) expression, which has an important role in the maintenance of intracellular calcium homeostasis and is also involved in cardiac hypertrophy pathways. Both *in vitro* and *in vivo* infection induced a significant reduction of Cav-3 levels. This reduction was followed by the activation of extracellular signal regulated kinase (ERK), which plays a major role in cardiac remodeling and hypertrophy, suggesting that this pathway may be involved in the pathogenesis of Chagas' heart disease. Finally we tested the tripanocidal potential of the anti-arrhythmic drug amiodarone in infected cultures of cardiac myocytes. This compound displayed a selective anti-*T. cruzi* activity, inducing ultrastructural alterations in intracellular amastigotes and promoted host cell recovery with actin cytoskeleton and gap junction reassembly, followed by restoration of the spontaneous contractility. The data generated in this work bring important advances into the understanding of Chagas heart disease establishment and also a new option for the etiological treatment of this neglected disease.

LISTA DE ABREVIATURAS

AIDS: Ssíndrome da Iimunodeficiência Aadquirida

CCL3: quimiocina do tipo CC- ligante-3 (MIP1α)

CCL5: quimiocina do tipo CC ligante-5 (RANTES)

ERK: quinase regulada por sinal extracelular

GPI: glicosilfosfatidilinositol

IFN-γ: γgama-interferon

IgG: imunoglobulina G

IgM: imunoglobulina M

IL-12: interleucina-12

IL-4: interleucina-4

IL-6: interleucina-6

IP3: fofatidilinositol 1,4,5-trifosfato

KDa: quilodálton

MAPK: proteína quinase ativada por mitógeno

MMP9: metalopeptidase de matriz-9

mRNA: ácido ribonucléico mensageiro

PCR: reação em cadeia da polimerase

PDGF: fator de crescimento derivado de plaquetas

PKB: proteína quinase B

PKC: proteína quinase C

TGF-β: fator de crescimento de transformação-β

TIMP-1: inibidor de metalopeptidase de tecido-1

TNF-α: fator de necrose tumoral-α

VEGF: fator de crescimento de endotélio vascular

ÍNDICE

INTRODUÇÃO

1. A doença de Chagas

1.1. Aspectos epidemiológicos

A doença de Chagas ou tripanossomíase americana (CID 10: B57) é uma doença endêmica na América Latina e atualmente atinge 9 a 12 milhões de pessoas (de Souza, 2007). A doença é causada por formas infectivas do protozoário *Trypanosoma cruzi*, um parasito intracelular obrigatório, da família *Trypanosomatidae* que é transmitido por triatomíneos. Carlos Chagas observou pela primeira vez a presença de protozoários flagelados no trato intestinal de insetos barbeiros (Panstrogylus megistus) e também em mamíferos, incluindo humanos que apresentavam estes flagelados em seu sangue periférico (Chagas, 1909).

Existem mais de 120 espécies conhecidas de vetores para o *T. cruzi*, sendo as mais importantes: *Triatoma infestans* (Argentina, Bolívia, Chile, Peru e Uruguai), *Rhodnius prolixus* (Colômbia, México, Venezuela e América Central) e *Triatoma dimidiata* (Equador, México e América Central). A transmissão da doença pelo *T. infestans* no Brasil, seu principal vetor, foi decretada erradicada no 15° Encontro da Comissão Intergovernamental do Cone Sul contra doença de Chagas (*Intergovernment Commission of the Southern Cone Initiative against Chagas' disease*) em 2006 (Schofield et al., 2006). A transmissão também foi eliminada no Uruguai (1997) e Chile (1999) e em algumas áreas da Argentina, Bolívia, Paraguai e América Central. Apesar desta vitória, ainda há preocupação com outros possíveis vetores e reservatórios para o *T. cruzi* como: (a) *Panstrongylus megistus* infectados que já foram encontrados em residências no estado do Rio de Janeiro (Lisboa et al., 2004); (b) marsupiais (*Didelphis marsupialis*) que podem ser reservatórios de formas tripomastigota e epimastigota (Deane et al., 1984; Pinho et al., 2000); e (c) os surtos de infecção de alimentos contaminados com fezes de *Triatomas* ou com o próprio triatomíneo, cada vez mais freqüentes no Brasil (Benchimol Barbosa, 2006).

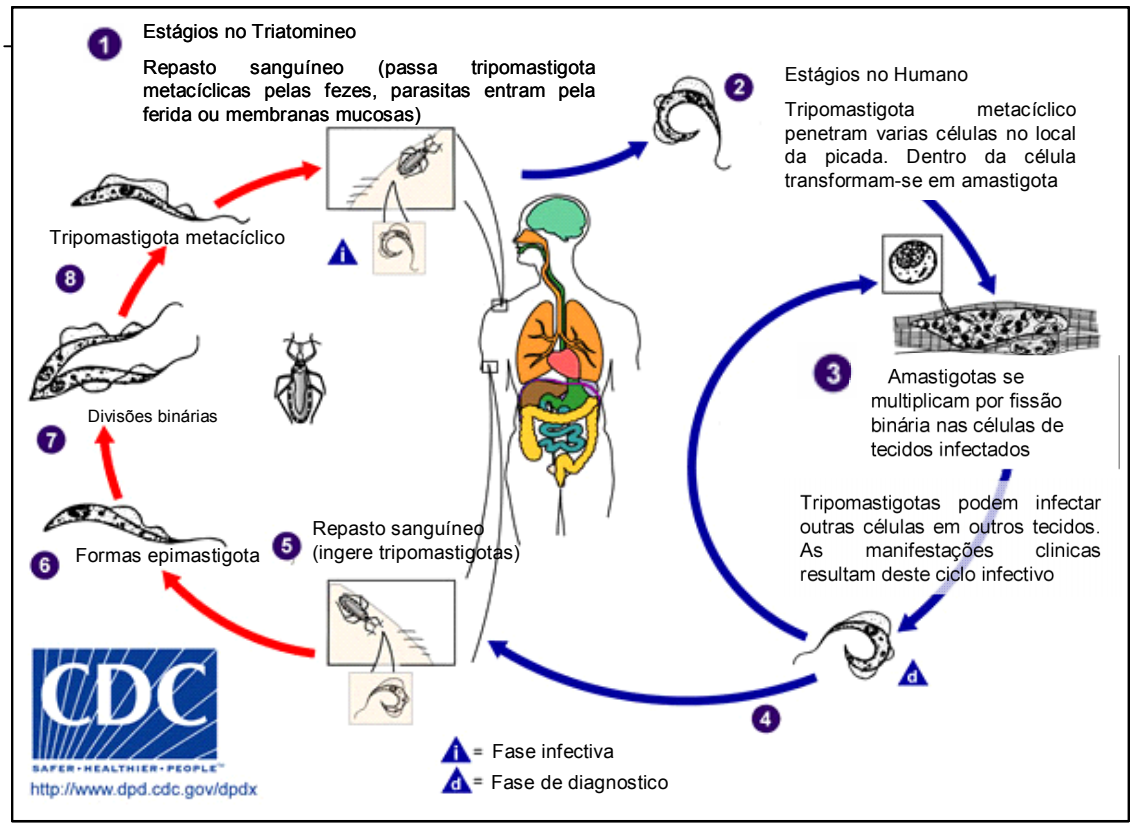


Figura 1: Esquema do ciclo de transmissão do *Trypanosoma cruzi*. As setas azuis correspondem aos estágios da presença do parasita no hospedeiro vertebrado (humano) e as vermelhas, no hospedeiro invertebrado. Fonte: Centro de Controle de Doenças (Center for Disease Control). www.dpd.cdc.gov/dpdx

Formas epimastigotas de *T. cruzi* se desenvolvem no lúmen intestinal do inseto vetor, elas são não-infectivas, e se multiplicam extracelularmente por divisão binária A diferenciação para forma infectiva, tripomastigota metacíclica, se dá na porção final do reto do inseto, num processo chamado metaciclogênese, iniciado quando epimastigotas aderem à derme por uma zona de adesão do flagelo (Rocha et al., 2006). Durante o repasto sanguíneo o barbeiro deixa no local da picada suas fezes, que contêm as formas metacíclicas. Estas formas penetram a pele do hospedeiro vertebrado, através de lesão ocorridda durante a picada. Após sete a dez dias de incubação, pode-se desenvolver no local um edema periorbital local chamado chagoma de inoculação (Mazza, 1940) ou um edema ocular bipalpebral ou unipalpebral chamado sinal de Romaña, provavelmente resultante de uma resposta imunológica localizada dependente de IFN-γ (Chessler et al., 2009). O ciclo de transmissão do parasito é completado quando um vetor invertebrado alimenta-se de sangue de um hospedeiro

vertebrado que contém formas tripomastigota sanguíneas, que ao atingirem o intestino do inseto se diferenciam em forma epimastigota (Figura 1).

Apesar de a transmissão vetorial ser predominante na América Latina (90%), há uma parcela de transmissão congênita (0,5-8%) e por transfusão de sangue (5-20%) (Dias, 2000), que também têm sido observadas em áreas não-endêmicas como nos Estados Unidos, Europa, Austrália e Japão (Pellegrino, 1949; Grant et al., 1989; Coura & Viñas, 2010).

Epidemiologicamente podem ser considerados três ciclos de transmissão, sendo o de maior importância o doméstico (que perpetua a infecção em humanos). No ciclo silvestre participam roedores, marsupiais e outros animais silvestres. No ciclo peridoméstico participam roedores, marsupiais, gatos e cães, servindo de ligação entre os ciclos doméstico e silvestre (Rey, 2008). Diversas abordagens tentaram classificar a estrutura das populações de *T. cruzi*, tentando definir o número de sub-grupos existentes, que, dependendo da técnica empregada, podem ser chamados de zimodema, schizodema, biodema, clones e linhagens. Atualmente estas diferentes populações são divididas em seis unidades de tipificação discreta (DTUs) distintas, de acordo com seu conteúdo genético, designados *T. cruzi* (TC) I a VI (revisto por Zingales et al., 2009). A distribuição geográfica destes grupos indica que as cepas TC II a VI são os principais agentes causais da doença de Chagas no sul da América do Sul. TC I estaria presente apenas no ciclo silvestre, apesar de alguns estudos demonstrarem que este grupo é o mais encontrado em infecções na Colômbia, Venezuela e América Central (Añez et al., 2004, Espinoza et al., 2010).

1.2. *Trypanosoma cruzi* - Aspectos biológicos

O *Trypanosoma cruzi* é um protozoário digenético, que pertence ao filo *Protozoa*, subfilo *Sarcomastigophora*, superclasse *Mastigophora*, classe *Zoomastigophorea*, ordem *Kinetoplastida*, subordem *Trypanosomatina*, família *Trypanosomatidae*, gênero *Trypanosoma* e subgênero *Schizotrypanum* (Hoare, 1966). Dentro da subordem *Trypanosomatina* estão também *Trypanosoma brucei* e *Leishmania spp*., agentes causadores da tripanossomíase africana e Leishmaniose, respectivamente. O *T. cruzi* tem como característica a presença de corpúsculo basal

nas três formas evolutivas (amastigota, epimastigota e tripomastigota), de onde se origina o flagelo. O flagelo de *T. cruzi* segue o mesmo padrão de microtúbulos de outros flagelos, porém o tamanho desta estrutura pode variar de acordo com a forma evolutiva do parasito. As formas amastigotas apresentam flagelo de aproximadamente 1 μm de comprimento, enquanto que em tripomastigotas e epimastigotas esta estrutura pode atingir 20 μm. Além disso, em epimastigotas e tripomastigotas, o flagelo está aderido lateralmente ao corpo celular por uma junção considerada similar a desmossomos (de Souza et al., 1978a).

O cinetoplasto, presente nos tripanossomatídeos, é encontrado em todos os membros da ordem Kinetoplastida. Esta estrutura faz parte da mitocôndria única, cujo DNA é correspondente a 20-25% do DNA total de epimastigotas (Benard et al., 1979). Em epimastigotas, o cinetoplasto encontra-se anterior ao núcleo e nas formas amastigota e tripomastigota, na região posterior ao núcleo (Figura 2). Os tripanossomatídeos apresentam também uma camada de microtúbulos associados fortemente à membrana plasmática, chamados microtúbulos subpeliculares, que estão associados à rigidez da membrana e ao tamanho destes parasitos (revisto em de Souza, 2002).

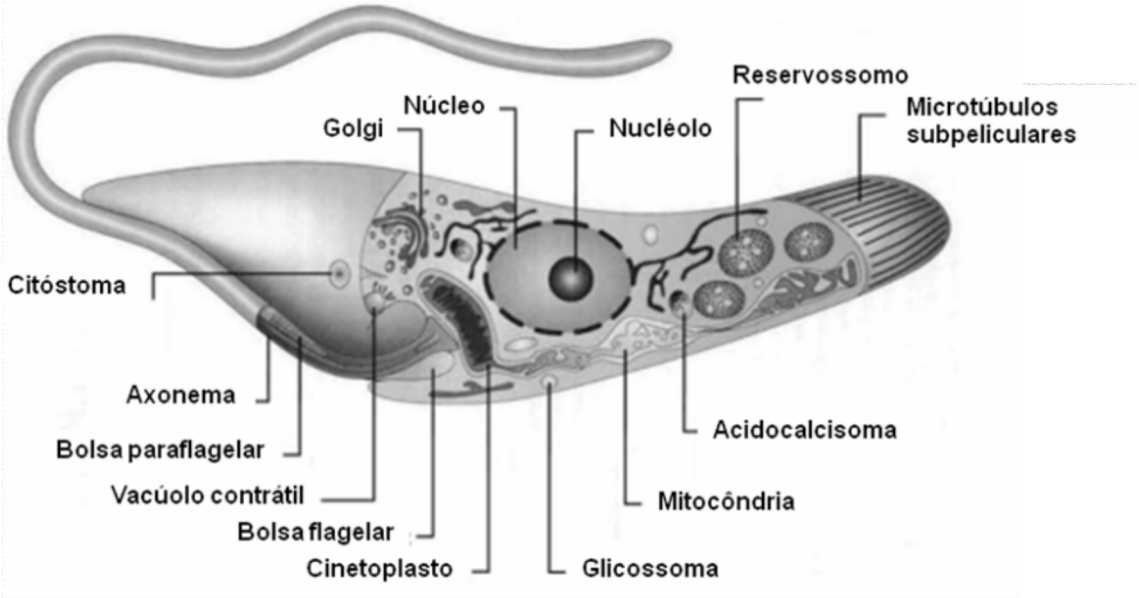


Figura 2: Representação esquemática da ultraestrutura de epimastigota de *Trypanosoma cruzi* e suas organelas, descritas por microscopia eletrônica de transmissão. Adaptado de Souza, 2008.

O *T. cruzi* apresenta outras organelas peculiares, tais como os acidocalcisomos, descritos como organelas eletrondensas de aproximadamente 200 nm, responsáveis pelo estoque intracelular de cálcio e outros íons (Docampo et al., 1995). Os acidocalcisomas estão presentes nas três formas de *T. cruzi e* são organelas acídicas, assim como os reservossomos, que são encontrados apenas em epimastigotas (Correa et al., 2002). Os reservossomos foram os primeiros compartimentos endocíticos descritos em epimastigotas de *T. cruzi* (de Souza et al., 1978b), caracterizados como grandes vesículas desprovidas de membranas internas e contendo inclusões lipídicas. Estas organelas estão localizadas na parte posterior do corpo do parasito, ocorrendo uma progressiva redução na quantidade destas estruturas durante o processo de metaciclogênese, não sendo encontradas em tripomastigotas (revisto em de Souza et al., 2002). Os reservosomos têm pH luminal igual a 6,0, semelhante a compartimentos tardios da via endocítica e foram descritos como sítios de acúmulo de proteínas endocitadas, como observado em estudos utilizando-se transferrina, albumina e peroxidase (Soares & de Souza, 1991). Apesar disso, não foi observada marcação positiva para proteínas de lisossomos nestas organelas (Cunha-e-Silva et al., 2002). Os reservosomos são responsáveis por acúmulo de proteínas, entre elas uma cisteína peptidase (cruzipaína), principal protease de *T. cruzi* (Soares, 1999).

As formas amastigota e epimastigota possuem o citóstoma, uma profunda invaginação da membrana plasmática, que gera uma depressão que pode chegar até a região próxima ao núcleo. O citóstoma está ligado ao flagelo e é associado à endocitose de fase fluida e mediada por receptores (Soares, 1999).

1.3. Fisiopatogenia

Após a inoculação das formas metacíclicas no hospedeiro vertebrado, inicia-se o processo de diferenciação para formas amastigotas no citoplasma das células, que sofrem diversos ciclos de divisão binária, e se diferenciam novamente para tripomastigota. Em seguida os parasitos rompem a célula hospedeira e podem atingir a circulação sanguínea e também órgãos distantes do local da infecção primária. Esta

fase inicial da doença é chamada de fase aguda, e é caracterizada pela infecção de diferentes tipos celulares, inclusive macrófagos, células musculares lisas e estriadas e fibroblastos. O diagnóstico clínico é obtido em menos de 10% dos casos devido à sintomatologia não específica (Punukollu et al., 2007; Marin-Neto et al., 2007), apesar de ser possível observar linfadenopatia, hepatoesplenomegalia, náusea, vômito, diarréia, anorexia, irritação de meninge e aumento nos níveis de enzimas cardíacas no sangue (revisto em Tanowitz et al., 1992). A primeira reação à infecção é uma inflamação mononuclear local, devido à ruptura das células parasitadas. Há uma resposta humoral composta de anticorpos IgM, proteínas do complemento e também migração de células mononucleares para o sítio de infecção (Andrade , 1999).

Esta fase dura de um a três meses e após este período há uma diminuição na produção de imunoglobulinas de classe IgM e aumento naquelas de classe IgG, além de uma drástica redução na parasitemia. Neste momento há a transição para fase crônica, na qual 20-50% dos pacientes podem permanecer em uma forma indeterminada da doença por 10 a 20 anos e 25-35% irão desenvolver a forma cardíaca (Dias, 2000). O diagnóstico fica limitado a métodos parasitológicos indiretos tais como

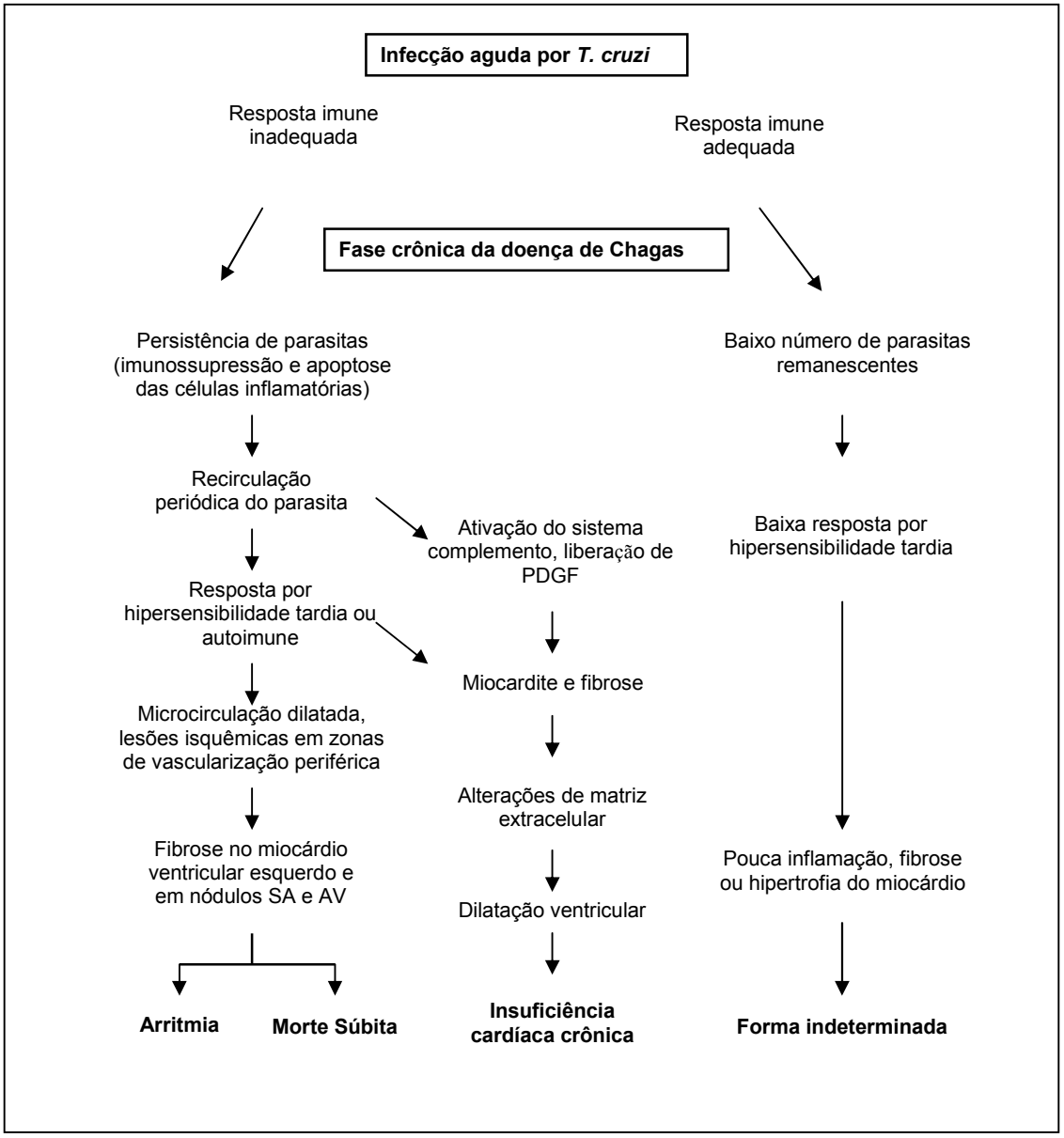


Figura 3: Esquema hipotético dos principais fatores envolvidos na cardiopatia chagásica crônica e suas possíveis interações que podem levar a diferentes graus de lesões cardíacas. Traduzido de Higuchi et al., 2003. SA: nódulo sino-atrial; AV: nódulo átrio-ventricular.

xenodiagnóstico, hemocultivo ou sorologia e o paciente em geral não apresenta sintomas graves durante o período indeterminado. Apesar de as formas digestivas (megaesôfago e megacólon) não serem incomuns, a mais importante manifestação é a cardiomiopatia (Higuchi et al., 2003), que será descrita a seguir.

Durante a fase aguda da doença, é observado parasitismo em quase todos os tecidos do hospedeiro. Apesar disso, em cerca de 90% dos casos há envolvimento ou comprometimento do tecido cardíaco, incluindo necrose miocitolítica focal e fibrose reparadora causadas pela destruição dos miócitos e por células inflamatórias, como macrófagos e linfócitos (Tanowitz et al., 1992). O alto tropismo de algumas cepas de *T. cruzi* por tecido muscular estriado (Andrade et al., 1974) resulta em acentuado parasitismo das células miocárdicas. A infecção de cardiomiócitos estimula a secreção de citocinas pró-inflamatórias (ex: IL-12, IFN-γ, TNF-α), quimiocinas (CCL3, CCL5) (Marino et al., 2004; Roffe et al., 2010) e óxido nítrico (Borges et al., 2009). Estas moléculas estimulam a migração e ativação de células do sistema mononuclear tais como macrófagos e linfócitos T $CD4^+$ e principalmente $CD8^+$ (revisto em Martin & Tarleton, 2004). A correta expressão destas moléculas pró-inflamatórias é crítica para o controle da infecção por *T. cruzi*, incluindo a distribuição, a composição de células mononucleares e o estado de ativação destes infiltrados inflamatórios (Paiva et al., 2009). Devido ao dano tecidual, a miocardite pode ocasionar taquicardia, insuficiência cardíaca congestiva e cardiomegalia. Em alguns pacientes pode ocorrer prolongamento do intervalo P-R (nódulo átrio-ventricular), alterações inespecíficas da onda T (repolarização do ventrículo) e baixa voltagem (Higuchi et al., 1987). Estes fatores são determinantes para a progressão da doença durante a fase crônica e a manifestação ou não da forma cardíaca da doença, a cardiomiopatia chagásica crônica (CCC) (Figura 3).

Baseadas em evidências reunidas através de modelos experimentais e estudos em humanos, existem atualmente quatro principais hipóteses para explicar os mecanismos de patogênese da CCC (revisto por Marin-Neto et al., 2007):

(a) Mecanismos Imunológicos: apesar da presença de antígenos e DNA de *T. cruzi* juntamente com a presença de linfócitos T $CD8^+$ ativados no coração durante a fase crônica, há escassez de parasitos detectáveis (Fonseca et al., 2005) promovendo dano tecidual. Alguns cientistas hipotetizaram que algumas proteínas de *T. cruzi*, incluindo a cruzipaína possuem homologia a proteínas cardíacas, tais como a cadeia

pesada de miosina, assim gerando uma resposta auto-imune humoral ou celular (revisto em Kierszenbaum, 2005). Esta hipótese afastou por muitas décadas o desenvolvimento de novas abordagens quimioterápicas específicas, inclusive para a fase crônica (revisto em Urbina, 2010). Além disso, não foi criado nenhum programa de vacinação para doença de Chagas, com a justificativa de que aumentando a resposta imune contra o *T. cruzi* poderia causar uma maior severidade da doença auto-imune na fase crônica. A maior evidência para a teoria da autoimunidade é a observação de que danos teciduais são observados mesmo quando da ausência de parasitos e a presença de auto-anticorpos no tecido cardíaco (revisto em Higuchi et al., 2003).

(b) Desenervação Parassimpática (Teoria Neurogênica): A observação de que a infecção experimental ou humana por *T. cruzi* induz uma redução na população de neurônios no tecido cardíaco, associada à inflamação perigangliona r e a uma reação autoimune antineural (Ribeiro dos Santos et al., 1979; Soares & Santos, 1999), gerou a hipótese de que CCC estaria principalmente relacionada a danos no sistema nervoso parassimpático. No entanto, esta hipótese perdeu força após a constatação de que a patogênese da doença de Chagas é muito complexa e encontrou alguns obstáculos conceituais, tais como o fato de que a CCC pode ser causada aparentemente por qualquer cepa de *T. cruzi*, inclusive por aquelas não-neurotrópicas (Miles et al., 1981). Mesmo assim a teoria neurogênica é relevante principalmente porque os danos ao sistema parassimpático podem ser um dos mecanismos que induzem a morte súbita nos pacientes chagásicos (revisto em Marin-Neto et al, 2007).

(c) Alterações na Microcirculação: Diversos trabalhos vêm demonstrando que a infecção por *T. cruzi*, e o consequente desenvolvimento da CCC podem estar relacionados a graves alterações na microcirculação do tecido cardíaco, incluindo aumento da viscosidade do sangue (Berra e cols., 2005), diminuição da velocidade de hemácias (Tanowitz et al., 1996) e aumento da agregação plaquetária (Tanowitz et al., 1990), durante infecção experimental pelo parasito. Este efeito foi revertido com o uso experimental de verapamil, que inibe a agregação plaquetária (Morris et al., 1989). Baseado em investigações clínicas (Hiss et al., 2009), seria possível hipotetizar que a hipoperfusão crônica no miocárdio pode contribuir como um mecanismo patogênico através da geração de regiões de micro-isquemias, com o desenvolvimento de miocitólise e fibrose reparadora no ventrículo esquerdo observadas na doença de

Chagas, independente do parasitismo. No entanto esta hipótese ainda necessita de comprovação clínica, tais como o estudo mostrando o efeito benéfico do tratamento em longo prazo de vasodilatadores ou anti-agregação plaquetária no curso clínico da CCC.

(d) Persistência do parasito: No início da década de 1990, alguns grupos descreveram que, apesar da drástica redução na parasitemia após a fase aguda, persiste o parasitismo por *T. cruzi* na fase crônica no tecido cardíaco, e que este parasitismo é acompanhado de uma reação inflamatória (Higuchi et al, 1993; Jones et al., 1993). Mais recentemente, com o desenvolvimento de métodos mais modernos de detecção como PCR quantitativo, tornou-se evidente que durante a fase crônica é possível detectar a presença de *T. cruzi* em sítios específicos do hospedeiro tais como tecido cardíaco e adiposo (Combs et al., 2005), resultando numa reação inflamatória multi-focal. Esta teoria está apoiada em várias evidências experimentais e clínicas, dentre elas (i) a presença de parasitos no local ou nas proximidades ao local de dano tecidual, (ii) o desenvolvimento de métodos moleculares de diagnóstico, com maior sensibilidade de detecção do parasito (Britto et al., 1995; Galvão et al., 2003) e (iii) as evidências de que a imunossupressão experimental (Taniwaki et al., 2005) ou em pacientes, como no caso de transplante de órgãos (Almeida et al., 1996) ou pacientes de AIDS (Rivera et al., 2004; Vaidian et al., 2004), com reduzido número de linfócitos T $CD4^+$ (Del Castillo et al., 1990), há reativação da infecção (Simões et al., 1995). Nestes casos, uma melhora na resposta imune resultaria numa menor gravidade da doença de Chagas crônica (Tarleton, 2001). Além disso, segundo Higuchi e colaboradores (2003) apenas a autoimunidade não seria suficiente para explicar o aspecto multifocal da miocardite e a localização de fibrose preferencialmente em certas regiões como as paredes apical e posterior esquerda durante a CCC. A fisiopatologia da CCC consiste principalmente da prevalência de miocardite, acompanhado de acúmulo de densas fibras de colágeno ao redor de fibras ou grupos de fibras cardíacas, que em geral estão hipertrofiadas (Higuchi et al., 1999).

Este fenômeno pode ocorrer em resposta a um aumento na demanda por trabalho cardíaco causado por vários estresses patológicos (incluindo, possivelmente, a miocitólise observada na infecção pelo *T. cruzi*). O coração se adapta através da hipertrofia compensatória dos miócitos em doenças cardiovasculares, tais como hipertensão, doença vascular e infarto do miocárdio, e é um fator de risco independente

para morbidade cardíaca e mortalidade. Um estímulo hipertrófico induz aumento no tamanho celular, com ausência de divisão celular através de sinalização de cálcio e ativação de PKC, MAPK e PKB/Akt (Dorn & Force, 2005) e é acompanhado por aumento na síntese de proteínas com reprogramação da expressão gênica (Takeo et al., 2000). A hipertrofia cardíaca é induzida por uma variedade de fatores, tais como peptídeos vasoativos, fatores de crescimento, citocinas e hormônios (revisto em Jeong et al., 2009).

Na doença de Chagas, além da lise dos cardiomiócitos induzida pelo parasitismo e também pela ação de células inflamatórias, há um processo de fibrose reparadora que interfere na propagação do impulso elétrico cardíaco, gerando uma insuficiência cardíaca progressiva (Andrade, 1999). Por este motivo, há hipertrofia das fibras cardíacas num mecanismo que compensa a deficiência causada pela perda destas fibras e mantém a dinâmica circulatória.

O desenvolvimento da fibrose pode ser favorecido pela produção de citocinas e fatores de crescimento secretados por macrófagos e linfócitos, tais como IFN-γ, IL-4, IL-6 e TNF-α (Reis et al., 1997), apesar de estudos *in vitro* apontarem para a contribuição dos cardiomiócitos na produção de citocinas e quimiocinas durante infecção pelo *T. cruzi* (Machado et al., 2000; Waghabi et al., 2005). A exacerbação da expressão de proteínas de matriz extracelular (ex: fibronectina e colágeno) ocorreria como consequência da redução nos níveis cardíacos de MMP9 (Soares et al., 2004) e de aumento na expressão de TGF-β e de TIMP-1 (Araujo-Jorge et al., 2002; Soares et al., 2010). O esquema na Figura 3 resume os principais fatores envolvidos na CCC e as interações que podem levar às diferentes lesões cardíacas. Essas lesões podem causar alterações diversas no funcionamento do coração como taquicardia (taquiarritmia) ventricular sustentada, que pode incluir palpitações e dispnéia (Leite et al., 2001); alterações na sístole e diástole do ventrículo esquerdo (Nunes et al., 2006); fibrilação atrial; bloqueio átrio-ventricular e morte súbita (Valente et al., 2006).

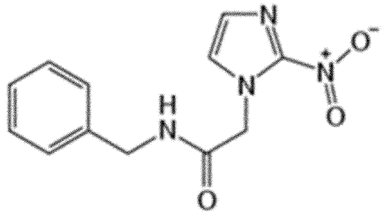
1.4. Tratamento

Do fim da década de 60 até a década de 80 o Nifutimox (Lampit®, Bayer; 3-metil-4-[5'-nitrofurfurilideneamina] tetrahidro-4H-1,4-tiazina-1,1-dióxido) (Figura 4) era

utilizado como quimioterápico para o tratamento anti-*T. cruzi*. Devido aos efeitos colaterais causados aos pacientes a droga foi descontinuada no Brasil, Argentina, Chile e Uruguai. Atualmente, o tratamento para doença de Chagas é feito principalmente com o composto Benzonidazol (Rochagan® no Brasil ou Radanil® na Argentina, Roche; N-benzil-2-nitroimidazol acetamida) (Figura 4). Esta droga atua por ligações covalentes ou outras interações de intermediários de nitroredução com componentes do parasito ou se ligando a DNA, lipídeos e proteínas do parasito (revisto em Coura & de Castro, 2002). Benzonidazol (Bz) tem um índice de eficácia média de 60% para casos de infecção aguda e infecções recentes (Silva et al., 1974). Não se sabe ao certo por que estes compostos nitro-heterocíclicos têm eficácia tão baixa, principalmente durante a fase crônica, mas acredita-se que isto ocorra devido às propriedades farmacocinéticas desfavoráveis e sua baixa penetração tecidual (revisto em Urbina, 2010). No entanto, alguns estudos recentes mostraram que o uso de Bz tanto na infecção crônica experimental (Garcia et al., 2005) e humana (Viotti et al., 2006) reduziu o parasitismo, miocardite, alterações na condução cardíaca, extrassístole ventricular e sorologia. Estes resultados promissores estimularam, por exemplo, a criação de um amplo estudo para avaliar a eficácia do uso de Bz em pacientes portadores de doença de Chagas crônica cardíaca, chamado BENEFIT (*Benzonidazole Evaluation for Interrupting Trypanosomiasis*) (Marin-Neto et al., 2009), mas até o momento não há nenhum estudo que demonstre total cura parasitológica com uso deste composto durante a fase crônica.

Outro aspecto importante a respeito do tratamento é que existe uma importante variabilidade biológica entre as cepas de *T. cruzi* em relação à virulência, patogenicidade, tropismo tissular, estresse oxidativo e resistência à quimioterapia (Sanchez et al., 1990; Veloso et al., 2001; Mielniczki-Pereira et al., 2007). A cepa Colombiana, por exemplo, é destacada por sua resistência a Bz (Filardi & Brener, 1987). Por esta razão, o Programa Integrado de Doença de Chagas (PIDC), iniciativa da Fundação Oswaldo Cruz, elaborou um protocolo para validação de novos compostos como possíveis candidatos para tratamento de doença de Chagas, que inclui o teste *in vitro* e *in vivo* da ação destes compostos contra cepa Y e Colombiana (Romanha et al., 2010).

Benzonidazole



Nifurtimox

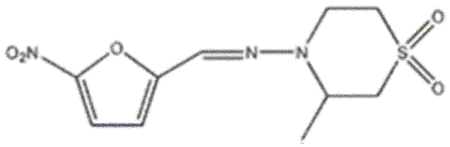


Figura 4: Estrutura química de Benzonidazol e nifurtimox

As novas classes de compostos que vêm sendo testados são direcionadas para inibir vias metabólicas exclusivas dos tripanosomatídeos. Dentre essas moléculas, destacam-se: (**a**) cisteína peptidase (cruzipaína), principal enzima proteolítica de *T. cruzi*. O composto K-777 inibe a proliferação de amastigotas e epimastigotas (Engel et al., 1998), porém sem induzir cura parasitológica; (**b**) síntese e metabolismo de tripanotiona, uma via metabólica exclusiva de tripanossomatídeos, importante para o combate a radicais livres (Urbina & Docampo, 2003). Alguns compostos testados como a tioridazina e cloripramina induziram redução da mortalidade de camundongos, mas não promoveram a cura parasitológica nem apresentaram ação seletiva contra *T. cruzi* (revisto em Wilkison & Kelly, 2009); (**c**) metabolismo de pirofosfato, responsável pela resposta a estresse, homoregulação e energia dos tripanossomatídeos. Os bisfofonatos foram testados contra *T. cruzi in vitro* e *in vivo* (Garzoni et al., 2004a,b, Bouzahzah et al., 2005) e mostraram efeito anti-proliferativo de formas amastigotas e epimastigotas porém sem causar cura parasitológica; (**d**) biossíntese de ergosterol, principal fonte de esteróis de fungos e tripanossomatídeos foi validada quimicamente como alvo para quimioterapia (de Souza & Rodrigues, 2009). Alguns compostos como cetoconazol, itraconazol e terbinafina, utilizados como anti-fúngicos, tem relevante atividade tripanocida em modelos murinos, mas não apresentaram resultados similares em

humanos (revisto em Urbina, 2002). Um novo composto, derivado dos triazóis, Posaconazol (SCH 56592), induz cura parasitológica em modelos animais e também em pacientes crônicos, causando profundas alterações ultraestruturais nas três formas evolutivas de *T. cruzi* (Lazardi et al., 1990), inclusive em cepas resistentes a Bz e mais eficaz em prevenir dano cardíaco do que esta droga padrão (Olivieri et al., 2010).

Apesar de promissores, estes compostos ainda não estão disponíveis ou aprovados para o tratamento etiológico da doença de Chagas. Dessa forma, o tratamento para CCC é o mesmo que para insuficiência cardíaca congestiva, que em geral inclui β-bloqueadores, diuréticos, inibidores da angiotensina e do receptor para angiotensina e antiarrítmicos como a amiodarona. À medida que a doença progride, as opções terapêuticas ficam ainda mais restritas e o transplante cardíaco surge como alternativa para as terapias padrão. Devido ao número limitado de doadores e o risco de re-agudização em pacientes que fazem uso de imunossupressores, alguns estudos vêm desenvolvendo modelos de terapia celular direcionado para doença de Chagas (revisto em Campos de Carvalho et al., 2009). Esta abordagem se baseia em estudos pioneiros utilizando transplante de células-tronco em modelos de infarto do miocárdio (Orlic et al., 2001). A injeção sistêmica de células mononucleares de medula óssea de camundongos chagásicos crônicos resultou em aumento de apoptose em células inflamatórias, redução de área fibrótica, consequente da redução dos níveis de MMP9 (Soares et al., 2004) e reversão da dilatação do ventrículo direito (Goldenberg et al., 2008).

2. Interação *T. cruzi*-célula hospedeira.

Dado o envolvimento do tecido cardíaco durante a patogênese da doença de Chagas, nosso laboratório desenvolveu um modelo de cultivo de células cardíacas em monocamadas adaptado do método da gota pendente (Meyer & Oliveira, 1948). Neste modelo, as células são isoladas por método de dissociação enzimática e sedimentadas em substrato de gelatina e apresentam estruturas e funcionalidade similares às características dos cardiomiócitos *in vivo* (Meirelles et al., 1986).

Resultados do nosso grupo demonstraram que as células em cultura passam por um processo miogênico, que inclui fusão celular (Meirelles et al., 1986) e aumento

no mRNA para α-actina cardíaca (Pereira et al., 2000). As análises ultraestruturais demonstraram que as células apresentavam estruturas contráteis (miofibrilas), sistema sarcotubular, junções celulares, mitocôndrias próximas à miofibrilas (Meirelles et al., 1986; Barbosa & Meirelles, 1995) e estruturas endocíticas (Soeiro et al., 2002). Como muitos outros microorganismos intracelulares obrigatórios, o *T. cruzi* desenvolveu mecanismos que permitem a invasão de praticamente qualquer tipo celular, sejam células fagocíticas profissionais ou não (de Meirelles et al., 1980; Barbosa & Meirelles, 1995; Nagajyothi et al., 2008). Esses mecanismos envolvem o reconhecimento de moléculas na superfície da célula hospedeira e do parasito, que resultam na incorporação das mesmas.

O estudo da interação *T. cruzi*-célula hospedeira ajudou a esclarecer aspectos do reconhecimento e invasão da célula hospedeira pelo *T. cruzi* (Meirelles et al., 1986; Barbosa & Meirelles, 1992; Araujo-Jorge et al., 1992; Barbosa et al., 1993; Carvalho et al., 1999); e para testar quimioterápicos anti-*T. cruzi* (de Castro & Meirelles, 1987; de Castro et al., 1992; Garzoni et al., 2004a; Silva et al., 2006).

Cardiomiócitos embrionários de camundongos infectados pelo *T. cruzi*, sofreram alterações eletrofisiológicas (Aprigliano et al., 1993) e estruturais, tais como quebra de filamentos de citoesqueleto (Pereira et al., 1993, 2000), incluindo miofibrilas (Taniwaki et al., 2006; Melo et al., 2006) e perda de adesão focal (Melo et al., 2004). O modelo de cultivo *in vitro* de células cardíacas também revelou o possível papel destas no remodelamento cardíaco observado durante o curso da doença de Chagas. Estes estudos revelaram que a infecção de cardiomiócitos, células endoteliais e fibroblastos (Costales et al., 2009) induz a produção de moléculas efetoras microbicidas como o óxido nítrico (NO), citocinas e quimiocinas tais como CCL-5/RANTES, TNF, IL-1β (Machado et al., 2000) e TGF-β (Waghabi et al.. 2005), bem como um aumento na taxa de proliferação celular (Hassan et al., 2006; Nagajyothi et al., 2006). .

Recentemente desenvolvemos um novo modelo de cultivo de células cardíacas tri-dimensional para estudar a infecção por *T. cruzi* (Garzoni et al., 2008). Neste modelo as células são cultivadas em frascos de cultura nos quais não é possível a adesão ao substrato, formando agregados multicelulares, que expressam proteínas características do tecido cardíaco como tropomiosina sarcomérica, conexina43, fibronectina e VEGF (Garzoni et al., 2009). Quando infectados por *T. cruzi* (cepa Y), estes microtecidos

sofreram hipertrofia e fibrose, com aumento da imunorreatividade para laminina, colágeno tipo IV e fibronectina (Garzoni et al., 2008). Este é um modelo promissor para estudar migração de células inflamatórias e mecanismos envolvidos na gênese de fibrose e hipertrofia cardíacos durante a infecção.

Os diferentes modelos de cultivo celular existentes atualmente são ferramentas altamente necessárias e confiáveis para a elucidação dos mais variados aspectos envolvidos no desenvolvimento da doença de Chagas no hospedeiro vertebrado e sua importância no contexto da infecção *in vivo*. Abaixo descrevemos alguns destes aspectos com maior detalhamento.

O íon cálcio é um importante segundo-mensageiro em células eucarióticas e diversos trabalhos já descreveram os efeitos da infecção pelo *T. cruzi* nos níveis e na dinâmica do cálcio na célula hospedeira. Um estudo pioneiro de infecção de células HeLa pelo *T. cruzi* demonstrou que houve um aumento nos níveis de cálcio intracelular, e que este aumento está relacionado ao processo de invasão da célula (Osuna et al., 1990).

Este influxo de Ca^{+2} intracelular parece ser induzido por fatores solúveis secretado pelas formas tripomastigotas, criando um cenário favorável para a entrada do parasito na célula hospedeira: um aumento no cálcio citoplasmático libera mais cálcio estocado no retículo endoplasmático via IP3 e induz a despolimerização de filamentos de actina, facilitando a migração de lisossomos necessária para a invasão da célula pelo parasito, (Tardieux et al., 1992, 1994; Rodriguez, et al., 1995). Utilizando lisados protéicos de tripomastigotas, Barr e colaboradores (1996) verificaram um aumento nos níveis de cálcio em cardiomiócitos obtidos de cães, induzido por uma enzima processadora do agonista de cálcio, mais tarde chamada de oligopeptidase B (Caler et al., 1998). Quando ativado, este agonista se liga ao seu receptor na célula hospedeira, ativando fosfolipase C e gerando inositol 1,4,5-trifosfato (IP3). O IP3 se ligaria ao seu receptor na membrana do retículo endoplasmático promovendo um aumento nos níveis de cálcio intracelular (Caler et al., 1998).

Por outro lado, em cardiomiócitos murinos, apenas a adesão da forma tripomastigota à membrana do miócito induziu aumentos sustentados no cálcio intracelular, também essencial para a invasão da célula hospedeira (Garzoni et al., 2003). Além de ter um papel na invasão do parasito, os níveis citoplasmáticos de cálcio

parecem ter relevância para a multiplicação das formas amastigotas, conforme demonstrado num modelo *in vitro* de infecção de mioblastos (Schettino et al., 1995). Nesta etapa do ciclo intracelular do *T. cruzi*, nosso grupo observou uma drástica redução nas variações cíclicas de Ca^{+2} intracelular em cardiomiócitos altamente parasitados (L.R.G., dados não publicados), concomitantemente com uma diminuição significativa da carga de superfície dos cardiomiócitos (Soeiro et al., 1995), provavelmente graças a uma enzima presente na membrana plasmática das formas tripomastigotas chamada transialidase, que remove o ácido siálico da superfície da célula hospedeira (Schekman et al., 1994)

Estudos prévios demonstraram que alterações na permeabilidade celular ao cálcio foram induzidas após a remoção de ácido siálico de cardiomiócitos com tratamento com neuraminidase (Frank et al., 1977). Subsequentemente, foi demonstrado que este tratamento altera as correntes de cálcio tipo T- e tipo L- e leva a alterações eletrofisiológicas na célula (Yee et al., 1991). No músculo cardíaco, os *sparks* de cálcio são induzidos pela entrada de cálcio dependente de potencial de membrana através de canais de cálcio ativados por voltagem presentes na membrana plasmática em domínios de túbulos T e cavéolas (Furstenau et al., 2000; Lohn et al., 2000).

2.1. Estrutura e função das cavéolas no tecido cardíaco.

As cavéolas foram identificadas em 1953 através de análises por microscopia eletrônica de células endoteliais de capilar de rato (Palade, 1953) e possui esse nome devido a seu formato, que se assemelha a pequenas “cavernas”. Cavéolas são compartimentos móveis de 50-100 nm, envolvidos em eventos de endocitose, que possuem forma característica de vaso, estando presentes na membrana plasmática. Além da morfologia clássica, invaginada, as cavéolas podem apresentar variadas formas na célula incluindo vesículas intracelulares, que podem se fundir e formar túbulos e estruturas similares e “cachos de uva”, com tamanhos superiores a 100 nm (revisto por Smart et al., 1999).

Uma importante característica das cavéolas é a presença de caveolina, principal componente protéico destas estruturas, juntamente com colesterol e

esfingolipídeos, que interagem e se oligomerizam, dando o formato característico das cavéolas (Chang et al., 1992). Estas podem estar tanto abertas para comunicação direta com o espaço extracelular como fechadas para processar moléculas ou estocá-las (Suzuki & Sugi, 1989), em um processo conhecido como "potocitose": as cavéolas possuem grandes quantidades de receptores específicos (por exemplo: receptores para ácido fólico ou HDL) e a ligação destas moléculas a seus receptores promove uma incorporação independente de um processo endocítico (Anderson et al., 1992). As cavéolas podem também ser um sítio de entrada de cálcio, similar aos túbulos T das células musculares, uma vez que altas concentrações deste íon estão presentes nesses sítios. A localização destas estruturas na periferia celular pode também controlar a distribuição espacial de cálcio, regulando sua entrada e saída da célula. As cavéolas são plataformas de sinalização, por compartimentalizar e concentrar moléculas sinalizadoras como subunidades de proteína G e óxido nítrico sintase endotelial (eNOS). As caveolinas inibem também a ativação e a sinalização de muitas proteínas, incluindo c-Src, H-Ras e as quinases ativadas por mitógenos (MAPK) e eNOS (revisto por Williams & Lisanti, 2005; Tourkina et al., 2008).

Caveolina é a proteína formadora das cavéolas e pode também funcionar como molécula sinalizadora a partir de proteínas ligadas a âncoras de GPI até uma quinase residente (Sargiacomo et al., 1993; Chang et al., 1994). A família de genes de caveolina consiste de três membros (revisto em Krajewska & Maslowska, 2004):

- **caveolina-1:** presente em humanos no cromossomo 7, possui 3 exons que codificam duas isoformas (Cav-1α e Cav-1β), sendo a classe mais abundante, expressa constitutivamente em diferentes tipos celulares tais como células epiteliais e endoteliais, adipócitos, fibroblastos e células musculares lisas. Cav-1α apresenta 24 KDa e 178 resíduos e é traduzida de um RNAm diferente de Cav-1β, que apresenta 21 KDa e 147 resíduos. A caveolina-1 é uma proteína transmembrana e sua estrutura parece um grampo de cabelo, com as porções N- e C- terminais voltadas para a face citoplasmática e uma região hidrofóbica inserida na bicamada lipídica. A porção N-terminal é a região mais variável entre as isoformas de caveolina e é responsável por permitir a interação de Cav-1 com outras proteínas através de um domínio de oligomerização, composto por 40 aminoácidos. O domínio transmembrana desta proteína está envolvido

principalmente na formação de oligômeros de caveolina-1 e -2 de até 350KDa (Das et al., 1999);

- **caveolina-2:** é comumente co-expressa com Cav-1 apresentando 38% de identidade e 58% de similaridade com a mesma (Scherer et al., 1997). Caveolina-2 apresenta três isoformas: Cav-2α (162 aminoácidos), Cav-2β (149 aminoácidos) e Cav-2γ (menor e menos abundante). Cav-2 é encontrada apenas como monômeros ou homodímeros, sendo incapaz de gerar grandes oligômeros sem a presença de Cav-1 (revisto por Krajewska & Maslowska, 2004). Apesar de ser aparentemente uma proteína acessória para Cav-1, Cav-2 possui um papel seletivo para a fisiologia dos mamíferos, uma vez que animais deficientes nesta proteína apresentam disfunções pulmonares (Razani et al., 2002);
- **caveolina-3:** pode ser chamada também de M-caveolina, por ser encontrada praticamente apenas em células diferenciadas de músculo liso, cardíaco e esquelético (Song et al., 1996), enquanto que outros trabalhos também descreveram a expressão de Cav-3 por astrócitos e condrócitos (Schwab et al., 1999). O gene CAV3 está presente no cromossomo 3 humano e traduz uma proteína de 151 aminoácidos com massa molecular de 23 KDa e 85% de similaridade com Cav-1 (Tang et al., 1996). Assim como Cav-1, Cav-3 forma homo-oligômeros de 14-16 monômeros que são capazes de formar cavéolas independentemente da co-expressão de Cav-1 ou -2.

A expressão de Cav-3 em células musculares está restrita a células completamente diferenciadas tais como miotubos multinucleares ou cardiomiócitos *in vitro*, os quais apresentam Cav-3 em grandes quantidades na membrana plasmática, sendo indetectável em mioblastos indiferenciados (Volonte et al., 2003). Além disso, Cav-3 está localizada nos túbulos-T da membrana de células musculares maduras ou durante o desenvolvimento do músculo esquelético em camundongos (Parton et al., 1997), sugerindo um papel na formação dos túbulos-T durante o desenvolvimento muscular.

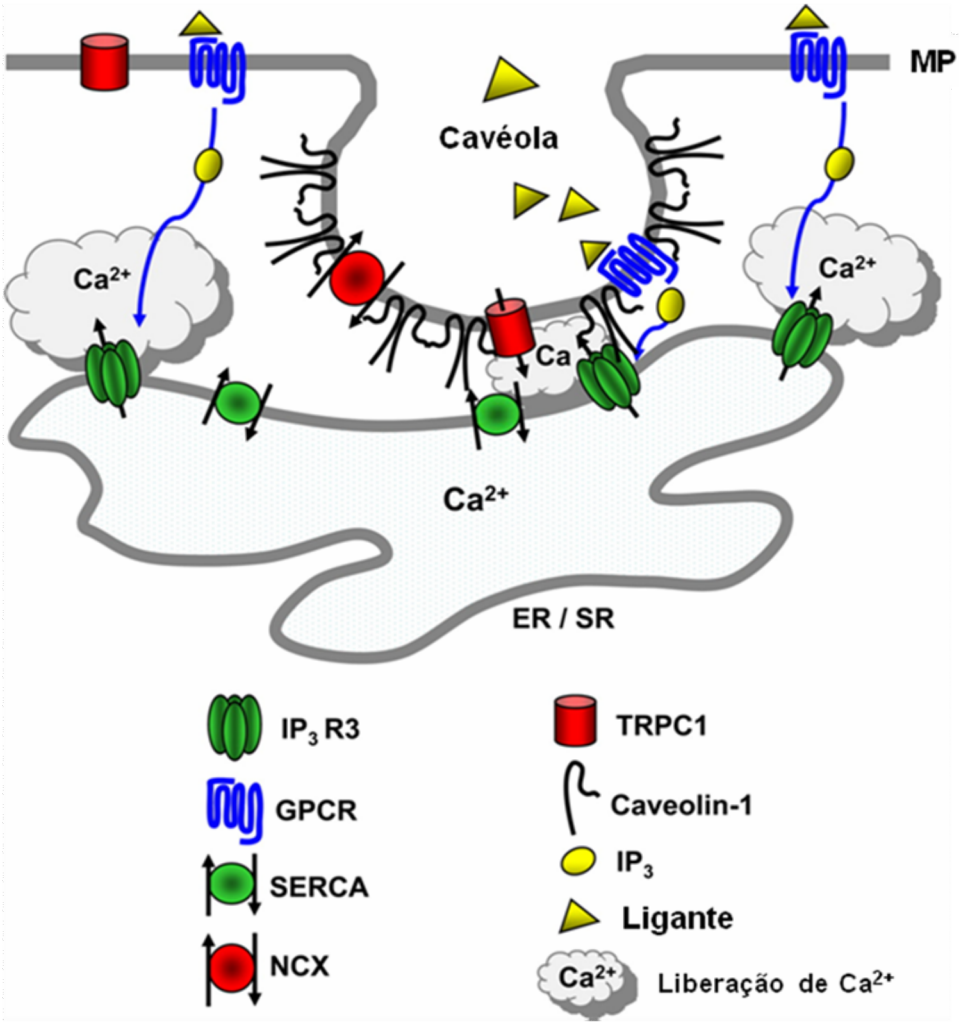


Figura 5: Visão esquemática da interação entre caveolina-1 e outras proteínas, incluindo canais de cálcio e receptor de IP3 para gerar um influxo de cálcio citoplasmático. IP3: inositol trifosfato; IP_3R3: receptor de IP3; SERCA: Ca^{2+} ATPase de retículo endoplasmático; NCX: Na^{+}/Ca^{2+} ATPase; GPCR: receptor ligado a proteína G; TRPC1: canal de Ca^{2+} acionado por voltagem; ER/SR: retículo endoplasmático ou retículo sarcoplasmático (em células musculares); MP: Membrana Plasmática. Traduzido de Hardin & Vallejo, 2008.

Essa idéia é reforçada pela verificação de que camundongos *knock-out* (KO) para Cav-3 (-/-) apresentam anormalidades nos túbulos-T (Galbiati et al., 2001). De maneira similar, camundongos KO duplo (dKO) para Cav-1/3 são viáveis (Park et al., 2002), porém apresentam uma progressiva cardiomiopatia dilatada. Este trabalho demonstrou que, apesar de haver diminuição no número de cavéolas no coração de

camundongos Cav-1 e -3 KO, nos animais dKO Cav-1/-3 não foi possível detectar a presença de cavéolas no sarcolema (Park et al., 2002).

Além dos danos à formação de túbulos T em cardiomiócitos pela ausência de caveolinas, as cavéolas são abundantes em canais iônicos e transportadores tais como o trocador Na^+/Ca^{2+}, canais de cálcio ativados por voltagem tipo L (Cav1.2), canais de K^+ e de $Na^{+,}$ criando complexos de sinalização celular importantes para a fisiologia da célula (Figura 5) (Balijepalli et al., 2006; Hardin & Vallejo, 2008). Dada a variedade de diferentes canais iônicos localizados nas cavéolas, é provável que estas contribuam para a gênese de arritmias. Tanto as arritmias congênitas quanto as adquiridas, como observado em pacientes com insuficiência cardíaca, podem envolver alterações nas funções das cavéolas (revisto por Balijepalli & Kamp, 2009). Estes danos congênitos podem incluir mutações no gene CAV3, gerando a Síndrome do QT longo tipo 9 (Vatta et al., 2006) e a Síndrome da Morte Súbita Infantil (Cronk et al., 2007).

A importância da correta expressão de caveolinas para a fisiologia cardiovascular fica ainda mais evidenciada com os modelos animais KO para caveolina-1, -3 e -1/-3, uma vez que estes animais apresentaram severo espessamento da parede do ventrículo esquerdo, fibrose intersticial, miocitólise e ativação da via de p42/44 MAPK, também chamada ERK (quinase regulada por sinal extracelular) (Woodman et al., 2002). Esta quinase apresenta um papel importante como efetora de resposta hipertrófica cardíaca e proliferação celular no sistema cardiovascular (Gillepsie-Brown et al., 1995). Dados obtidos por experimentos *in vitro* também indicam um papel das Cav-1 e -3 como reguladores negativos da sinalização por ERK 1/2 (Engelman et al., 1998).

Durante a fase aguda da doença de Chagas em modelo murino, Nagajyothi e colaboradores (2006) descreveram uma redução nos níveis de Cav-1, -2 e -3 e aumento na forma fosforilada de ERK nos corações de animais infectados por *T. cruzi*. Este efeito também foi observado na infecção *in vitro* de adipócitos, nos quais houve redução da expressão de Cav-1 e aumento da expressão de citocinas, quimiocinas e ativação de p38 MAPK e ERK (Nagajyothi et al., 2008). Estes dados apontam para um papel importante desempenhado pelas caveolinas cardíacas durante a infecção por *T. cruzi*.

2.2. Efeito da infecção sobre junções comunicantes e eletrofisiologia

Devido às alterações elétricas no tecido cardíaco durante a doença de Chagas e à importância da correta expressão das conexinas para manutenção da homeostase do coração, alguns trabalhos tentaram esclarecer que efeito a infecção de cardiomiócitos por *T. cruzi* geraria nas junções comunicantes.

O acoplamento entre duas células é feito através de junções comunicantes (*gap junctions*), que são canais intercelulares, onde ocorre livre troca de íons e pequenas moléculas. No tecido cardíaco, por exemplo, a presença dessas junções permite a formação de um sincício entre os cardiomiócitos, auxiliando na propagação da corrente elétrica e, assim, a manutenção da sincronia da contração do tecido.

2.2.1. Estrutura e funcionamento das junções comunicantes

A unidade funcional de uma junção comunicante é o conexon, um hemi-canal formado por seis subunidades protéicas. Para ocorrer o acoplamento elétrco entre duas células é preciso haver interação entre seus conexons para formação de canais. Apesar de haver um espaço (*gap*) entre as duas membranas plasmáticas, os dois conexons interagem e se ligam no espaço extracelular para formar um canal intercelular bimembranar fortemente selado (Unger et al., 1999). Cada conexon é formado por seis unidades da proteína transmembrana, não-glicosilada, conexina (Cx) (Figura 6). Estas proteínas fazem parte de uma família de genes que, em humanos, possui aproximadamente 20 isoformas, nomeadas de acordo com o peso molecular predito pelo produto de tradução do RNAm (ex: Cx43 tem 43KDa). As conexinas são divididas em dois grupos principais: tipo α (Cx33, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50, Cx57) e tipo β (Cx26, Cx30, Cx31, Cx32) (Urban et al., 1999). As conexinas possuem uma porção N-terminal citoplasmática, quatro domínios trans-membrana, que formam duas alças extracelulares e uma volta (*loop*) citoplasmática.

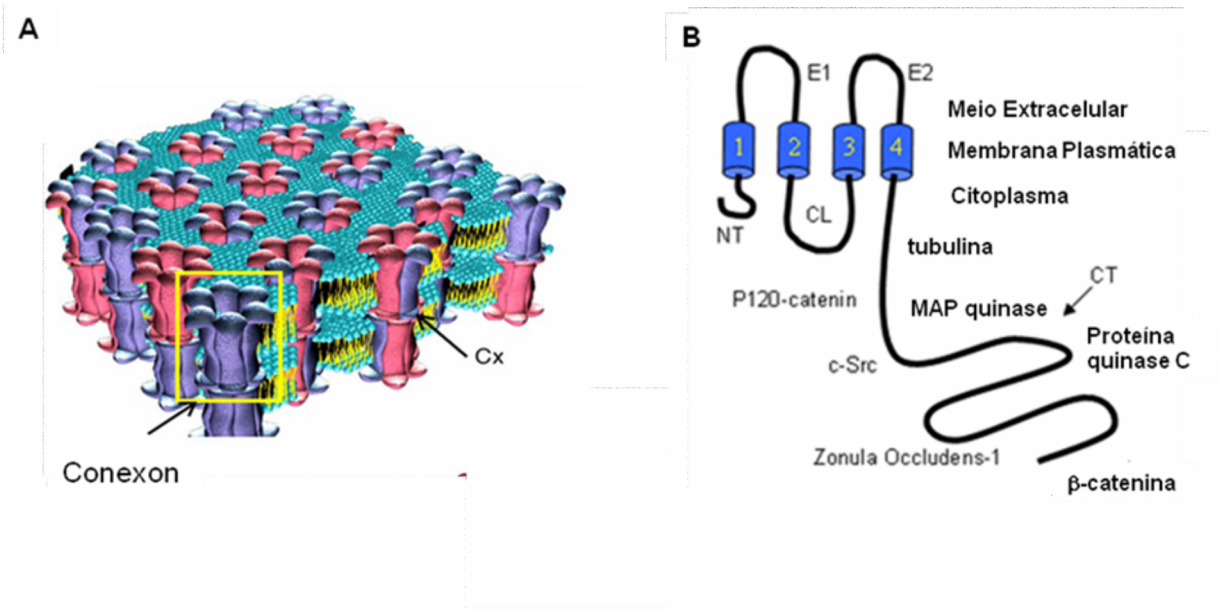


Figura 6: Representação esquemática da estrutura e composição das junções comunicantes em A, evidenciando o conexon formado pelo hexâmero de conexinas. A estrutura da conexina está representada no esquema em B, mostrando as duas alças extracelulares, o *loop* intracelular e as porções C- e N- terminal, citoplasmáticas. Na cauda C-terminal estão apontados os sítios de interação de Cx com outras proteínas. Adaptado de Cottrel & Burt, 2005.

Para a contração do coração, o impulso elétrico é iniciado por células auto-excitatórias no nódulo sinoatrial e é transferido com alta velocidade até o nódulo átrio-ventricular, onde a condução é atrasada. Em seguida, os impulsos elétricos se propagam rapidamente através do sistema His-Purkinje aos miócitos ventriculares que contraem simultaneamente (Kreuzberg et al., 2006). A ativação elétrica do miocárdio requer a transferência intercelular de corrente, que é feita pelas junções comunicantes, já que apenas nestas junções é possível haver a transferência da corrente de despolarização. A Cx43 é a principal forma encontrada no tecido cardíaco, além de Cx40 e Cx45, localizadas nos discos intercalares, regiões de contato entre dois cardiomiócitos onde são também encontradas junções aderentes e desmossomos. As células endoteliais presentes no coração também expressam Cx37 além de Cx40 e

Cx43 (Kanno & Saffitz, 2001). As conexinas estão distribuídas pelo coração de forma que no ventrículo a proteína majoritária é a Cx43; no átrio e sistema de condução (fibras de Purkinje), a Cx40 (alta condutância) e no sistema de condução atrioventricular, a Cx45 (baixa condutância).

Durante uma injúria, o tecido cardíaco sofre remodelamento de Cx43 que em alguns casos pode ter direta relação com o dano elétrico resultante. O remodelamento de Cx43 pode ser dividido em duas modalidades: distribuição de placas de Cx43 e alterações na expressão de Cx43 (proteína ou mRNA). Em zonas do miocárdio próximas à cicatriz do infarto, há perda da ordem normal de distribuição de Cx43, cuja imunomarcação passa a ser localizada nas laterais das fibras cardíacas. Da mesma forma, em casos de hipertrofia cardíaca e morte súbita, é possivel observar lateralização, desorganização e internalização de Cx43. Baixos níveis de expressão de proteína Cx43 ou mRNA para Cx43 foram detectados em pacientes com insuficiência cardíaca terminal, cardiomiopatia dilatada idiopática e doença cardíaca isquêmica (Severs et al., 2004**;** Gourdie et al., 2006). No entanto, apenas danos que causem uma redução de mais de 50% na quantidade de junções comunicantes no coração são capazes de gerar efeitos na velocidade de condução (Jongsma & Wilders, 2000).

2.2.2. Impacto da infecção por *T. cruzi* na expressão de conexina43

A infecção de cardiomiócitos obtidos de neonatos de rato pela cepa Tulahuen de *T. cruzi* (TC I) por 48 h resultou na perda de comunicação celular das células parasitadas como revelado por microinjecção de corante (*dye transfer*). Além disso, as células infectadas apresentaram redução na imunomarcação para Cx43 e redução na automaticidade da contração. Curiosamente, as formas amastigotas intracelulares também apresentaram forte marcação quando o anticorpo anti-Cx43 (181A) foi utilizado (de Carvalho et al., 1992). Este trabalho indicou que as alterações na distribuição e funcionalidade de Cx43 estão relacionadas com a presença do parasito na célula, independente de fatores solúveis envolvidos na infecção das culturas. Além disso, astrócitos e células da meninge (pia-máter e aracnóide) quando infectados *in vitro* e *in vivo* por *T. cruzi* (Tulahuen) e *Toxoplasma gondii*, também apresentaram redução no acoplamento celular e na marcação para Cx43 e Cx26. No entanto, culturas infectadas

após 72h não apresentaram variação nos níveis de conexina ou nos níveis de fosforilação de Cx43 (Campos de Carvalho et al., 1998). Os autores argumentaram que estes resultados conflitantes poderiam ser explicados por uma possível alteração no transporte de Cx43 para a membrana plasmática ou porque a região de Cx43 que o anticorpo reconhece estaria mascarada devido a alterações conformacionais da proteína. Curiosamente o modelo de infecção experimental de cardiomiócitos de feto de camundongo Swiss Webster pela cepa Y de *T. cruzi* (TC II) resultou no aumento da contratilidade dos miócitos (Aprigliano et al., 1993) e em alterações nos níveis de Cx43 *in vitro* e *in vivo* (Adesse et al., 2008). Outra observação importante foi a de que o uso de soro de pacientes chagásicos crônicos em culturas de miócitos murinos, resultou em uma redução no acoplamento celular (Costa et al., 2000). Este resultado pode indicar um papel de fatores solúveis presentes na cardiopatia chagásica na regulação da expressão de conexina durante a infecção *in vivo*. Os níveis séricos de TGF-β são aumentados durante a doença de Chagas e estão diretamente relacionados com a geração de resposta fibrótica no tecido cardíaco (revisto em Araújo-Jorge et al., 2008). TGF-β parece estar diretamente relacionado à redução de Cx43 observada na infecção *in vitro* de cardiomiócitos de camundongo e humana por *T. cruzi* (Waghabi et al., 2009).

JUSTIFICATIVA

A doença de Chagas afeta aproximadamente 11 milhões de pessoas na América Latina e em 30% dos casos crônicos há comprometimento da função cardíaca com hipertrofia das fibras cardíacas e fibrose, gerando arritmias, bloqueio átrio-ventricular e morte súbita.

Sabe-se que os vários isolados de *T. cruzi* existentes apresentam diferenças quanto à infectividade, tropismo tissular, resistência à quimioterapia e ao estresse oxidativo. Outras diferenças também são observadas quando comparadas diferentes combinações de cepa de *T. cruzi*-hospedeiro, como demonstrado em estudos que s investigaram o impacto da infecção na miogênese (Rowin et al., 1983; Meirelles et al., 1986); eletrofisiologia e expressão de conexinas (Campos de Carvalho et al., 1998; Adesse et al., 2008) e homeostase de cálcio intracelular (Barr et al., 1992; Garzoni et al., 2003).

A correta expressão de conexinas e caveolinas é essencial para correta sincronia e sinalização celular para contração do tecido cardíaco. Nosso grupo descreveu recentemente que a infecção por *T. cruzi* induz redução na expressão de Cx43 em cardiomiócitos infectados *in vitro* e no coração de camundongos infectados durante a fase aguda (Adesse et al., 2008). Cx43 forma canais intercelulares e depende de diversas interações proteína-proteína para manter o acoplamento célula-célula (revisto em Herve et al., 2004). A família das caveolinas tem papel importante na regulação de Cx43, uma vez que Cx43 interage diretamente com Cav-1 e -2 em queratinócitos humanos (Langlois et al., 2008) e com av-3 em células musculares cardíacas (Liu et al., 2010; Adesse et al., resultados não publicados).

As cavéolas são estruturas formadas por caveolina e estão presentes em domínios da membrana plasmática que participam da regulação de cálcio intracelular em cardiomiócitos (Rothberg et al., 1992). No coração de camundongos infectados por *T. cruzi* foi descrita uma redução no RNAm de Cav-1, -2 e -3 (Nagajyothi et al., 2006). Esta redução pode ter relevância para geração de um fenótipo hipertrófico na doença de Chagas, como observado em camundongos KO para Cav-1, Cav-3 e duplo KO Cav-

1/-3 (Woodman et al., 2002), que apresentaram inclusive ativação da via de MAPK/ERK.

Neste trabalho nos propusemos a esclarecer alguns aspectos relacionados à doença de Chagas. Primeiramente, comparamos a infecção de células musculares por quatro cepas de *T. cruzi* distintas, com intuito de identificar grupos de genes comumente afetados nos quatro modelos, visando a busca de biomarcadores. Em seguida investigamos o efeito da infecção de cardiomiócitos murinos na expressão de Cav-3 e a conseqüente ativação de 1/2 ERK (quinase regulada por sinal extracelular), além de transpor estas observações para um modelo *in vivo* de infecção experimental murina, nas fases aguda e crônica. Por último, testamos a capacidade tripanocida de amiodarona em culturas de cardiomiócito. Este composto é um conhecido antiarrítmico, que também possui a capacidade de inibir a síntese de esteróis de fungos e protozoários como *T. cruzi* e *Leishmania mexicana.* Neste estudo avaliamos também o potencial de recuperação das células hospedeiras após o tratamento, validando este composto como potencial candidato para o tratamento etiológico da doença de Chagas.

OBJETIVOS

Objetivo geral: Utilizar modelos experimentais de infecção *in vitro* e *in vivo* por *Trypanosoma cruzi* para estudar mecanismos envolvidos na patogênese da doença de Chagas.

Objetivos específicos:

1) Comparar as assinaturas transcriptômicas induzidas por diferentes cepas de *T. cruzi* em uma linhagem de célula muscular esquelética de rato;

2) Avaliar o efeito da infecção *in vitro* e *in vivo* pelo *T. cruzi* na expressão de Cav-3 cardíaca e a possível ativação da via de uma quinase ativada por mitógeno (MAPK/ERK);

3) Testar a ação tripanocida do composto amiodarona em culturas de cardiomiócito infectadas e verificar a possível recuperação da fisiologia das células através da análise do citoesqueleto de actina, expressão de Cx43 e eletrocontratilidade espontânea.

RESULTADOS

Os resultados gerados nesta tese foram divididos em três artigos e um capítulo de livro. No **Trabalho #1** investigamos as assinaturas transcriptômicas que quatro cepas de *Trypanosoma cruzi* induzem em uma mesma linhagem de célula mioblástica esquelética (L_6E_9). Para este fim, utilizamos a técnica de microarranjos de cDNA ("microarray"), que permite analisar simultaneamente 27.000 transcritos de forma imparcial nas diferentes condições experimentais. Neste trabalho nós apresentamos as categorias de genes que foram alteradas após a infecção com as cepas Y, CL, Brazil e Tulahuen e apontamos alguns genes como possíveis candidatos a biomarcadores da doença.

O impacto da infecção por *T. cruzi* na expressão de Cav-3 e sua relevância na patogênese da de algumas doenças infecciosas foi abordado no **Trabalho #2** e em um capítulo de livro (**Trabalho #3**). Estas contribuições não só mostram as alterações na expressão de Cav-3, mas também a concomitante ativação da via de sinalização da quinase regulada por sinal extracelular (ERK 1/2).

Na última parte desta tese (**Trabalho #4**), aprofundamos nosso estudo sobre o impacto da infecção por *T. cruzi* na expressão de Cx43 cardíaca. Nossos dados prévios mostraram que a infecção de cardiomiócitos murinos por *T. cruzi* (cepa Y) reduziu significativamente a expressão desta proteína. Neste trabalho, nós testamos o potencial tripanocida do composto amiodarona em culturas infectadas e verificamos recuperação da célula hospedeira, incluindo a expressão de Cx43 e F-actina após erradicação do parasitismo.

Trabalho #1:

Daniel Adesse, Dumitru A. Iacobas, Sanda Iacobas, Luciana R. Garzoni, Maria de Nazareth Meirelles, Herbert B. Tanowitz and David C. Spray.

"Transcriptomic Signatures of Alterations in a Myoblast Cell Line Infected with Four Distinct Strains of *Trypanosoma cruzi*"

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Transcriptomic Signatures of Alterations in a Myoblast Cell Line Infected with Four Distinct Strains of *Trypanosoma cruzi*

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Abstract. We examined the extent to which different *Trypanosoma cruzi* strains induce transcriptomic changes in cultured L_6E_9 myoblasts 72 hours after infection with Brazil (TC I), Y (TC II), CL (TC II), and Tulahuen (TC II) strains. Expression of 6,289 distinct, fully annotated unigenes was quantified with 27,000 rat oligonucleotide arrays in each of the four replicas of all control and infected RNA samples. Considering changes greater than 1.5-fold and P values < 0.05, the Tulahuen strain was the most disruptive to host transcriptome (17% significantly altered genes), whereas the Y strain altered only 6% of the genes. The significantly altered genes in the infected cells were largely different among the strains, and only 21 genes were similarly changed by all four strains. However, myoblasts infected with different strains showed proportional overall gene-expression alterations. These results indicate that infection with different parasite strains modulates similar but not identical pathways in the host cells.

INTRODUCTION

Chagas disease, caused by infection with the flagellate protozoan parasite *Trypanosoma cruzi*, is a widespread disease in Latin America affecting millions of people.[1] Infective trypomastigotes invade peripheral cells and transform into multiplicative amastigote forms. The initial (acute) phase of the disease is characterized by intense tissue parasitism involving the heart, skeletal and smooth muscle cells, liver, fat, and brain that is accompanied by intense focal inflammation and necrosis.[2] Some patients can evolve to a chronic phase of the disease that can include cardiac and/or digestive forms. The severity of the chronic phase may be related to the efficiency of the host immune response in resolving the infection during the acute phase,[3] but this has never been proven. Moreover, there are several reports of differences of tropism of *T. cruzi* to host tissue, which is also associated with the pathogenesis of chronic Chagas disease.[4,5]

Differences in the pathogenesis of the disease among patients may vary according to differences in both hosts and parasite strain.[6] Among differences in *T. cruzi* strains are their resistance to chemotherapy, oxidative stress, and infectivity in the mouse.[7–9] Although previous *in vivo* and *in vitro* microarray analyses using cultured cells[10,11] and hearts of mice[12–14] infected with *T. cruzi* showed that this infection results in profound alterations in the host cells, the degree to which these results are applicable to all *T. cruzi* strains found in infected individuals has not been explored previously.

Since host immune response, tissue parasitism, and parasite strains may be important factors in the pathogenesis of chronic Chagas disease, we have used gene-array analysis to compare the alterations in host cells caused by four different stocks of *T. cruzi*. The present study characterizes the transcriptomic changes in cultured rat myoblasts that result from infection with each strain and highlights common genes that were similarly or differentially modulated by each strain. Analysis reveals host cell changes that might lead to an understanding of previously observed differences in pathogenesis *in vivo*.

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METHODS

Cells and parasites. The L_6E_9 rat myoblast cell line was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin at 37°C with 5% CO_2 atmosphere.[15] Cells were dissociated with trypsin/ethylenediaminetetracetic acid (EDTA) solution (Gibco), and 10^6 cells were plated in 100-mm^2 cell-culture dishes. After 24 hours of plating, cells were washed with Phosphate Buffered Saline (PBS) containing Ca^{2+} and Mg^{2+} (Gibco) and infected with 2×10^6 trypomastigote forms of *T. cruzi* in DMEM. Parasites of the Y, CL Brener,[16] Tulahuen, and Brazil strains were obtained from supernatants of infected L_6E_9 cultures. Forty-eight hours post-infection, cells were washed twice with Ca^{2+}/Mg^{2+} PBS to remove free trypomastigotes in the supernatant, and they were re-fed with fresh supplemented DMEM. Total RNA was harvested at 72 hours post-infection using guanidinium thiocyanate-phenol-chloroform extraction (TRIZOL) reagent (Invitrogen, Carlsbad, CA), following the protocol indicated by the manufacturer, when at least 25% of the cultured cells were infected, presented only intracellular amastigotes, and had no release of trypomastigotes, which would lead to re-infection of culture.

***T. cruzi* genotyping.** The different isolates of *T. cruzi* used in this work were identified using the method described by Fernandes and others[17] according to their phylogenetic lineage. Briefly, genomic DNA from 5×10^8 epimastigote forms of the Y, CL, Brazil, and Tulahuen strains was extracted using the DNeasy kit (Qiagen, Hilden, Germany). Multiplex polymerase chain reaction (PCR) was performed using 150 ng of DNA, and the primers were designed to recognize the mini-exon gene of the parasites using a pool of five nucleotides: three were derived from a hypervariable region of the *T. cruzi* mini-exon repeat (*T. cruzi* 1 [TC1], 5′ ACA CTT TCT GTG GCG CTG ATC G; TC 2, 5′ TTG CTC GCA CAC TCG GCT GCA T; TC 3, 5′ CCG CGW ACA ACC CCT MAT AAA AAT G) and an oligonucleotide from a specific region of the *Trypanosoma rangelii* non-transcribed spacer (TR; 5′ CCT ATT GTG ATC CCC ATC TTC G). A common downstream oligonucleotide corresponds to sequences present in the most conserved region of the mini-exon gene (ME;

5′ TAC CAA TAT AGT ACA GAA ACT G). PCR reaction was performed using the Multiplex PCR kit (Qiagen) with the initial denaturing cycle of 95°C (15 seconds) followed by 30 cycles of 94°C (30 seconds, denaturing), 60°C (30 seconds, annealing), and 72°C (30 seconds, extension); a final extension cycle of 72°C lasts for 10 minutes and is followed by a soak cycle (4°C) using a PTC-100 Thermocycler (M.J. Research Inc., Massachusetts, (USA)). PCR fragments were loaded into a 2% agarose/ Tris base, boric acid, EDTA (TBE) gel with 0.008% ethidium bromide, and images were acquired using Kodak 1D Scientific Imaging Systems.

Light microscopy. Cells (6×10^4) were plated into glass cover slips in 24-well plates. After 24 hours, 10^6 trypomastigotes were added to cultures in fresh supplemented DMEM, and infection was followed as described above. After 72 hours of infection, cells were washed three times and fixed with glutaraldehyde for Giemsa staining.[18] Cover slips were digitally photographed using a Zeiss Axioplan microscope.

Microarray analysis. We used the protocol optimized in our laboratory[19] according to the standards of the Microarray Gene Expression Data Society. Briefly, 20 µg Trizol extracted total RNA from each culture dish was reverse transcribed in the presence of fluorescent Alexa Fluor 555-aha-dUTP (green fluorescent emission) or Alexa 647-aha-dUTP (red emission; Invitrogen) to label cDNAs. Differently labeled RNA samples from biological replicas of control (uninfected cells cultured for the same duration) or infected with one strain at a time were co-hybridized ("multiple yellow" strategy[20]) overnight at 50°C with rat 27k oligonucleotide arrays printed by Duke University (full technical information available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18175). All spots affected by local corruption, with saturated pixels, or with foreground fluorescence less than twice the background fluorescence (where noise may obscure the quantity) were removed from the analysis. The background subtracted signals were normalized iteratively,[19] alternating red/green, interblock, lowess and scale intraslide and interslide normalization until the fluctuation of the ratio between the spot median and the corresponding block median of valid spots became less than 5% between successive iteration steps. Normalized expression levels were organized into redundancy groups (each group composed of all spots probing the same gene) and were represented by the weighted average of the values of individual spots. The abundance of host cell transcripts was considered as significantly altered after infection if the absolute fold-change was greater than 1.5-fold and the *P* value of the heteroscedastic *t* test (two-sample, unequal variance), applied to the means of the background-subtracted normalized fluorescence values in the four biological replicas of the compared transcriptomes, was greater than 0.05. This composite criterion to identify the significantly altered gene expression minimizes the number of false hits without eliminating too many true hits. The 50% change cut-off (1.5-fold) was selected to be significantly larger than the overall less than 10% interslide technical noise determined for the bacterial controls.

Gene categories. GenMapp and MappFinder programs (www.genmapp.org; Gladstone Institute, University of California,

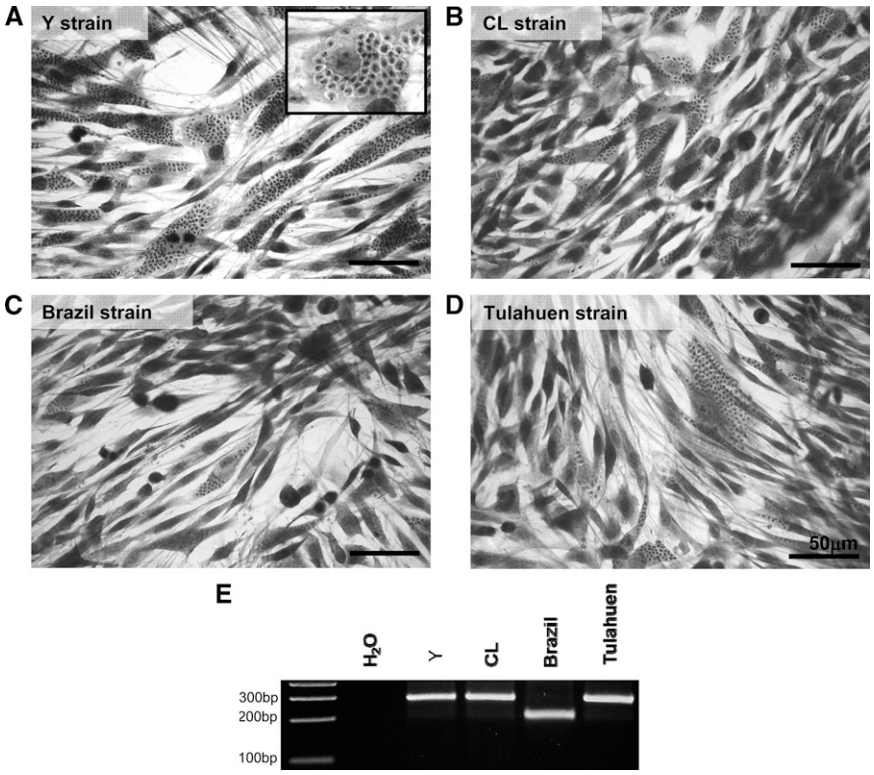

FIGURE 1. Characterization of L_6E_9 cell infection by four *T. cruzi* strains. Giemsa staining of L_6E_9 cells after 72 hours of infection with (**A**) Y, (**B**) CL, (**C**) Brazil, and (**D**) Tulahuen strains of *T. cruzi* was performed. All strains studied successfully infected the myoblasts, and at 72 hours post infection (hpi), it was possible to observe amastigote forms in the host cell's cytoplasm as shown in details in **A** inset. (Bars = 50 µm.) To confirm genetic background of the parasite stocks used for infection, genomic DNA from epimastigote forms was isolated and amplified with Multiplex PCR with primers derived from a hypervariable region of the *T. cruzi* mini exon. (**E**) The Y, CL, and Tulahuen strains had a PCR product of 250 bp, indicating that they belong to the *T. cruzi* (TC) II family, and the Brazil strain had a 200-bp product, indicating that it belongs to TC I family.

San Francisco, CA) were used to determine whether or not altered gene expressions differed significantly from chance for the overlapping functional and structural Gene Ontology (GO) categories.

RESULTS

Multiplex PCR was performed to confirm correspondence of the strains of *T. cruzi* used in this work to the phylogenetic classification as described in the literature. We verified that the Y, CL, and Tulahuen parasites displayed a 250-bp PCR product, indicating that they belong to the *T. cruzi* II (TC II) group. The Brazil strain, whose effects in host cells and animals have been characterized previously by our group,[15,21] belongs to the TC I group, because it displays a 200-bp PCR product (Figure 1E).

Data complying with the Minimum Information about Microarray Experiments (MIAME) were deposited in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18175) as series GSE18175. In this experiment, we quantified 6,289 distinct, well-annotated unigenes in all samples.

The impact of infection with each of the four strains of *T. cruzi* on the transcriptome changes of the same immortalized cell line was strikingly different (Figure 2). Thus, as shown in Table 5, there were only two (0.03%) genes significantly decreased, and 19 (0.3%) increased by all four *T. cruzi* strains. However, 4,340 (69%) genes were not significantly altered by any of the strains. The Venn diagrams in Figure 2 illustrate this observation by showing the number of genes equally increased (Figure 2B) or decreased (Figure 2C) by two, three, or all four strains of *T. cruzi*.

Analysis of functional classes of genes modulated by the infection with different *T. cruzi* strains was performed with GenMapp software, and it revealed a very small number of gene functional categories with an abundance that was similarly affected by each pair of parasite strains (Table 7). Below, we describe in more detail the differences that were observed.

Y strain. We observed that the Y strain altered expression of 426 (6%) of the 6,996 quantified host cell genes. Among these genes, 150 were decreased, and 276 were increased after 72 hours of infection. When we identified individual genes whose expression was altered exclusively by the Y strain, we found that 87 genes were increased, such as α-1-integrin (1.76-fold), intercellular adhesion molecule (1.79-fold), presenilin 2 (3.81-fold), transforming growth-factor beta regulated gene (1.73-fold), and vascular cell-adhesion molecule 1 (1.91-fold). Sixty-five genes were decreased, such as cardiac Ca^{2+} ATPase (–1.6-fold), cadherin (–2.88-fold), and Cyp26b1 (cytochrome P450, family 26, subfamily b, polypeptide 1; –2.83-fold) (Table 1). The Y strain affected the expression of 11 gene categories including calcium and metal binding, regulation of transcription, and complexes of protein (Table 7).

CL strain. When the rat myoblasts were infected with the CL strain, we observed that 763 of 7,133 (11%) genes were significantly altered (53 decreased and 710 increased). The CL strain uniquely modulated the expression of 517 genes (494 were increased and 23 decreased), which corresponded to alterations in 14 gene categories including protein, intracellular protein, proton and ion transport, and ubiquitin cycle (Table 7). Some of the genes that the CL strain modulated were adenylate cyclase 2 (1.56-fold), α-spectrin (1.7-fold), annexin A1 (1.7-fold), caspase 7 (1.76-fold), epsin 2 (1.97), glutamate receptor (3.38), matrix metallopeptidase (MMP) 3 (2.51-fold), and syntaxin (–1.56-fold) (Table 2).

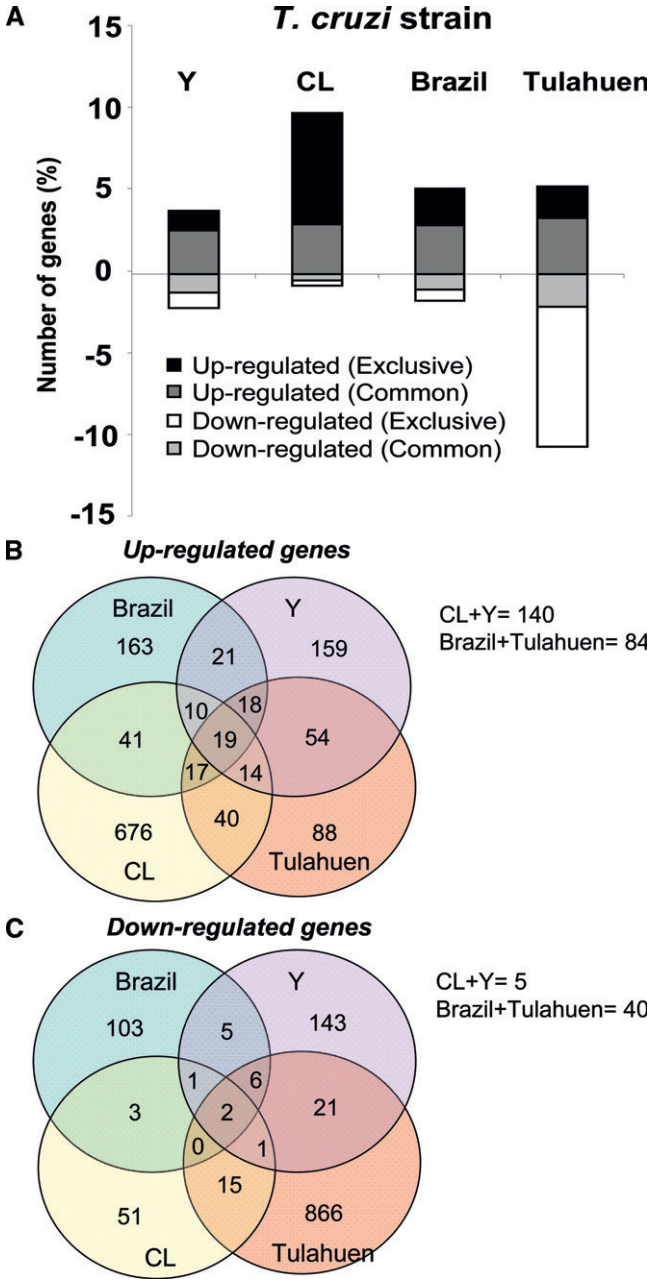

FIGURE 2. Profiles of transcriptomic changes caused by each strain of *T. cruzi* in host cells. The percentage of genes modulated in L_6E_9 cells by each *T. cruzi* strain is shown in **A**. Genes with fold-change value ≥ 1.5 and *P* value ≤ 0.05 were quantified and plotted in this histogram, in which bars represent a percentage of the valid genes. Bars in white and black represent genes whose expression was decreased or increased exclusively by each strain, respectively. Bars in gray and light gray represent genes that were increased and decreased, respectively, and shared this modulation by at least two strains. The Venn diagrams represent the number of genes significantly (fold change ≥ 1.5 and *P* value < 0.05) (**B**) increased and (**C**) decreased in common by all four strains, by three strains, by pairs of strains, and by individual strains of the parasite. The number of genes equally altered by CL + Y and Brazil + Tulahuen strains is indicated below the diagrams. Note the low number of genes altered in the same direction by all strains studied (19 increased and 2 decreased).

TABLE 1
Y Strain

Gene name	Gene symbol	Fold change
Alanyl-tRNA synthetase	Aars	1.85
Actin, gamma, cytoplasmic 1	Actg1	1.55
Chloride intracellular channel 2	Clic2	1.55
Cyclin-dependent kinase inhibitor 2B (p15; inhibits CDK4)	Cdkn2b	1.65
Glycogen synthase kinase 3 beta	Gsk3b	1.52
Integrin alpha 1	Itga1	1.76
Intercellular adhesion molecule 1	Icam1	1.79
Lectin, galactose binding, soluble 3	Lgals3	1.52
Presenilin 2	Psen2	3.814
Protein C receptor, endothelial	Procr	2.04
Pyruvate kinase, muscle	Pkm2	1.70
Syndecan -protein	Sdcbp	1.854
Transforming growth-factor beta-regulated gene 1	Tbrg1	1.73
Vascular cell-adhesion molecule 1	Vcam1	1.91
X-linked myotubular myopathy gene 1	Mtm1	1.53
Actin, alpha 1, skeletal muscle	Acta1	–4.84
Acyl-CoA synthetase long-chain family member 3	Acsl3	–2.63
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Atp2a2	–1.60
Cadherin 15	Cdh15	–2.88
Calsenilin, presenilin-binding protein, EF hand transcription factor	Csen	–1.62
Cytochrome P450, family 26, subfamily b, polypeptide 1	Cyp26b1	–2.83
Guanine monphosphate synthetase	Gmps	–1.94
Myosin light chain, phosphorylatable, fast skeletal muscle	Mylpf	–1.68
Rho family GTPase 2	Rnd2	–1.62

Brazil strain. The Brazil strain induced significant alteration of 7.31% genes of L_6E_9 by more than 1.5-fold. Among these 494 modulated genes, 117 were decreased, and 377 were increased. Some interesting genes that had their transcription altered by infection with the Brazil strain were cardiomyopathy associated gene (3.37-fold), MMP 1B (2.44-fold), mitogen-activated protein kinase 9 (2.12-fold), fibroblast growth factor (1.94-fold), desmin (1.63-fold), and tropomyosin 4 (–2.45-fold) (Table 3). Ten functional categories of genes were highly modulated in L_6E_9 by Brazil strain, including metallopeptidase

TABLE 2
CL strain

Gene name	Gene symbol	Fold change
Adenylate cyclase 2	Adcy3	1.56
Alpha-spectrin 2	Spna2	1.70
Angiopoietin-like 2	AF159049	1.56
Annexin A1	Anxa1	1.56
Casein kinase 1, gamma 1	Csnk1g1	1.95
Caspase 7	Casp7	1.76
Chemokine-like factor	Cklf	1.76
Chondroitin sulfate proteoglycan 6	Cspg6	1.64
Desmuslin	Dmn	1.60
Epsin 2	Epn2	1.97
Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	Grin2b	3.38
Integrin alpha 5	Itga5	1.84
Interleukin 1 receptor, type I	Il1r1	2.27
Matrix metallopeptidase 10	Mmp10	2.40
Matrix metallopeptidase 3	Mmp3	2.52
Phosphatidylethanolamine N-methyltransferase	Pemt	2.03
Phospholipase D1	Pld1	1.58
Plasminogen activator, urokinase receptor	Plaur	1.76
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	Psmd2	1.99
Chaperonin subunit 4 (delta)	Cct4	–1.57
Syntaxin 8	Stx8	–1.56
T-cell immunomodulatory protein	Cda08	–2.61

TABLE 3
Brazil strain

Gene name	Gene symbol	Fold change
H2A histone family, member Y	H2afy	3.70
Cardiomyopathy associated 3	Cmya3	3.37
Protein kinase C, gamma	Prkcc	2.70
Myocyte enhancer factor 2D	Mef2d	2.43
Mitogen-activated protein kinase 9	Mapk9	2.12
Fibroblast growth factor 21	Fgf21	1.95
ATPase, H+/K+ exchanging, beta polypeptide	Atp4b	1.90
Phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	Pik3c2g	1.78
Paladin	Pald	1.67
Desmin	Des	1.63
Phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3	Pik3r3	–1.53
Coronin, actin-binding protein, 1B	Coro1b	–1.91
Synaptotagmin XI	Syt11	–1.91
Proteasome (prosome, macropain) 28 subunit, beta	Psme2	–2.35
Keratin 25D	Krt25d	–2.38
Tropomyosin 4	Tpm4	–2.46

TABLE 4
Tulahuen strain

Gene name	Gene symbol	Fold change
Activin A receptor type II-like 1	Acvrl1	7.60
Cadherin 3, type 1, P-cadherin (placental)	Cdh3	4.65
Cardiac ankyrin repeat kinase	Cark	2.54
CDK5 regulatory subunit-associated protein 1	Cdk5rap1	2.40
Chymotrypsinogen B	Ctrb	2.64
Glucose 6 phosphatase, catalytic, 3	G6pc3	1.65
Inositol 1,4,5-trisphosphate 3-kinase C	Itpkc	2.37
Interleukin 11	Il11	1.68
Kinesin family member C1	Kifc1	2.04
Myotrophin	Mtpn	1.51
ATPase, Ca++ transporting, plasma membrane 1	Atp2b1	–2.76
Bcl2 modifying factor	Bmf	–2.93
Calcium channel, voltage-dependent, beta 3 subunit	Cacnb3	–1.88
Calreticulin	Calr	–2.04
Caspase 9	Casp9	–1.75
Cytochrome c oxidase, subunit Va	Cox5a	–1.62
Cytokine-induced apoptosis inhibitor 1	Ciapin1	–1.67
Dynein cytoplasmic 1 heavy chain 1	Dync1h1	–1.86
Ectonucleoside triphosphate diphosphohydrolase 1	Entpd1	–2.90
Farnesyltransferase, CAAX box, alpha	Fnta	–1.66
Hypoxia inducible factor 1, alpha subunit	Hif1a	–1.52
Inositol 1,4,5-triphosphate receptor 3	Itpr3	–3.70
Janus kinase 3	Jak3	–1.64
Junctional adhesion molecule 3	Jam3	–2.25
Laminin, beta 2	Lamb2	–2.21
Matrix metallopeptidase 11	Mmp11	–3.46
Muscle, skeletal, receptor tyrosine kinase	Musk	–2.73
Phospholipase D2	Pld2	–2.88
Protein kinase C, nu	Prkcn	–2.57
Synaptojanin 2	Synj2	–2.35
Troponin T2, cardiac	Tnnt2	–2.89
Tumor protein p53	Tp53	–1.92

TABLE 5
Similar results

Gene name	Gene symbol	Fold change (strains)			
		Brazil	CL	Tulahuen	Y
C1q and tumor necrosis factor-related protein 1	C1qTNF1	–1.90	–1.80	–2.70	–1.80
Matrix metallopeptidase 14	MMP14	–2.00	–1.90	–2.00	–1.70
Cardiotrophin-like cytokine factor 1	Clcf1	2.20	2.30	1.80	2.30
Cut-like 1 (Drosophila)	Cutl1	7.72	2.58	5.36	1.59
DNA-damage inducible transcript 3	Ddit3	9.30	3.08	2.41	2.64
Excision repair cross-complementing rodent repair deficiency, complementation group 3	Ercc3	2.19	2.67	1.59	2.36
G protein-coupled receptor, family C, group 5, member A	Gprc5a	1.94	2.53	1.87	1.99
GrpE-like 1, mitochondrial	Grpel1	1.60	1.95	1.66	1.91
Pericentriolar material 1	Pcm1	2.70	4.25	2.23	3.70
Proprotein convertase subtilisin/kexin type 7	Pcsk7	6.93	2.87	2.38	2.15
Serologically defined colon cancer antigen 3	Sdccag3	3.36	2.71	2.67	2.50
Solute carrier family 1 (glutamate/neutral amino acid transporter)	Slc1a4	1.59	2.50	1.82	1.68
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	Ywhaq	2.84	2.85	1.77	2.56

activity, small GTPase-mediated signal transduction, and ubiquitin cycle (Table 7).

Tulahuen strain. The Tulahuen strain of *T. cruzi* induced the highest percentage of altered gene expression in the myoblasts (17.35%) with 761 decreased genes and 383 increased genes, of which, 617 and 139, respectively, were uniquely observed in Tulahuen-infected dishes. Additionally, infection with this strain significantly modulated the expression of 18 gene categories, including cholesterol biosynthesis, immune response, lipid metabolism, and receptor activity (Table 7). Some relevant examples of host cell genes significantly modulated during infection were p-cadherin (4.64-fold), cardiac ankyrin repeat kinase (2.54-fold), chymotrypsinogen B (2.64-fold), H2A histone family member Z (3.18-fold), interleukin 11 (1.68-fold), myotrophin (1.51-fold), caspase 9 (–1.75-fold), cytochrome c oxidase, subunit Va (–1.61-fold), heavy and light chain dynein (–1.86- and –1.81-fold, respectively), farnesyltransferase (–1.65-fold), hypoxia-inducible factor 1α subunit (–1.51-fold), janus kinase 3 (–1.64-fold), matrix metallopeptidase 11 (–3.46-fold), and cardiac troponin T2 (–2.89-fold) (Table 4).

Genes modulated equally by all *T. cruzi* strains as possible disease biomarkers. We observed that 13 (0.18%) host cell genes had the same pattern of significant modulation by all four strains of *T. cruzi* studied. These genes, thus, may represent disease biomarkers that could be useful in detecting the disease independent of the parasite strain. Only two (0.027%) were decreased, MMP-14 (–1.9-fold) and C1q and tumor necrosis factor-related protein 1 (–2.0-fold), and 11 (0.15%) increased, such as solute carrier family 1 (glutamate/neutral amino acid transporter; 1.9-fold), G protein-coupled receptor (2.1-fold), cardiotrophin-like cytokine factor 1 (2.13-fold), pericentriolar material 1 (3.22-fold), and DNA damage-inducible transcript 3 (4.37-fold). Table 5 shows the list of these thirteen genes and their modulation by each strain of the parasite. GenMapp analysis revealed that only nine gene categories were equally expressed by some pairs of strains such as receptor activity (Brazil and Tulahuen) and ubiquitin cycle (Brazil and CL) (Table 7).

Oppositely modulated genes. Surprisingly, our arrays revealed that some transcripts increased by one specific strain were decreased by another strain. Table 6 contains all the 24 genes that behaved in this manner. Some genes of interest were cytochrome P450, family 2, subfamily d, polypeptide 22 (3-fold by Brazil strain and –2.9-fold by Y strain), neuropathy target esterase-like 1 (2.1-fold by Y strain and –1.8-fold by CL strain), platelet-derived growth-factor receptor, β polypeptide (1.7-fold by CL strain and –1.6-fold by Tulahuen strain), protein kinase D2 (1.5-fold by Brazil strain and –1.6-fold by Y strain), and RNA polymerase 1-1 (1.6-fold by CL strain and –1.6-fold by Tulahuen strain).

TABLE 6
Opposite results

Gene name	Gene symbol	Fold change (strains)			
		Brazil	CL	Tulahuen	Y
ADP-ribosylation factor guanine nucleotide-exchange factor 2	Arfgef2	–	1.56	–1.54	–
CDC-like kinase 2	LOC365842	–	1.52	–1.64	–
Cytochrome P450, family 2, subfamily d, polypeptide 22	Cyp2d22	2.97	–	–	–2.92
NAD synthetase 1	Nadsyn1	–	1.56	–1.63	–
Neuropathy target esterase-like 1	Ntel1	–	–1.78	–	2.11
Platelet-derived growth-factor receptor, beta polypeptide	Pdgfrb	–	1.68	–1.64	–
Pleckstrin homology, Sec7, and coiled-coil domains 1	Pscd1	–	1.53	–1.53	–
Preoptic regulatory factor-2	PORF-2	–	1.62	–1.56	–
Protein kinase D2	Prkd2	1.50	–	–	–1.62
Regenerating islet-derived 1	Reg1	1.67	–2.31	–	–
RNA polymerase 1-1	Rpo1-1	–	1.56	–1.56	–
Son of sevenless homolog 1	Sos1	–1.53	1.82	–	–
Sulfotransferase family 1A, phenol-preferring, member 1	Sult1a1	–	–	1.90	–1. 53
Thymoma viral proto-oncogene 2	Akt2	–	1.54	–1.64	–
Transducer of ErbB-2.1	Tob1	–	–	1.58	–1.72

TABLE 7
Gene categories

Gene Ontology (GO) ID	GO Name	No. changed	No. measured	No. in GO	Percent changed	*P* value
	Y strain					
5634	Nucleus	22	557	1,643	3.95000	0.004
45449	Regulation of transcription	12	264	884	4.55000	0.010
6355	Regulation of transcription, DNA-dependent	11	237	790	4.64000	0.012
5509	Calcium-ion binding	8	149	538	5.37000	0.013
6350	Transcription	12	278	920	4.32000	0.014
46872	Metal-ion binding	20	593	1,913	3.37000	0.049
5743	Mitochondrial inner membrane	4	22	63	18.18000	0.006
19866	Organelle inner membrane	4	29	79	13.79000	0.026
16757	Transferase activity, transferring glycosyl groups	5	48	144	10.42000	0.042
43234	Protein complex	9	396	1,280	2.27000	0.052
16853	Isomerase activity	4	34	74	11.77000	0.052
	CL strain					
5829	Cytosol	4	68	121	5.88000	0.002
5622	Intracellular	18	1,331	3,766	1.35000	0.004
15031	Protein transport	5	154	348	3.25000	0.011
6512	Ubiquitin cycle	3	102	281	2.94000	0.049
3824	Catalytic activity	8	1,611	5,073	0.50000	0.054
6886	Intracellular protein transport	3	103	237	2.91000	0.054
16757	Transferase activity, transferring glycosyl groups	10	48	144	20.83000	0.011
15992	Proton transport	6	24	64	25.00000	0.023
4197	Cysteine-type endopeptidase activity	7	34	91	20.59000	0.032
6811	Ion transport	18	118	555	15.25000	0.038
3924	Gtpase activity	5	22	45	22.73000	0.040
16491	Oxidoreductase activity	25	185	539	13.51000	0.045
6812	Cation transport	14	86	375	16.28000	0.046
	Brazil strain					
8237	Metallopeptidase activity	3	38	133	7.89000	0.020
4984	Olfactory receptor activity	9	24	1,034	37.50000	0.000
1584	Rhodopsin-like receptor activity	9	46	1,391	19.57000	0.001
4872	Receptor activity	20	195	2,013	10.26000	0.002
7186	G-protein–coupled receptor protein-signaling pathway	11	82	1,581	13.41000	0.005
43234	Protein complex	12	396	1,280	3.03000	0.022
7264	Small gtpase mediated signal transduction	8	66	179	12.12000	0.027
5615	Extracellular space	4	23	136	17.39000	0.029
6512	Ubiquitin cycle	1	102	281	0.98000	0.046
5525	GTP binding	10	99	237	10.10000	0.050
	Tulahuen strain					
6457	Protein folding	16	68	138	23.53000	0.001
5488	Binding	219	1,786	5,708	12.26000	0.010
3743	Translation initiation factor activity	7	25	37	28.00000	0.019
6695	Cholesterol biosynthesis	4	11	15	36.36000	0.021
6955	Immune response	10	46	258	21.73913	0.033
6629	Lipid metabolism	20	120	304	16.67000	0.053
4984	Olfactory receptor activity	7	24	1,034	29.17000	0.000
1584	Rhodopsin-like receptor activity	9	46	1,391	19.57000	0.001
16020	Membrane	63	740	3,798	8.51000	0.003
19866	Organelle inner membrane	7	29	79	24.14000	0.003
5743	Mitochondrial inner membrane	5	22	63	22.73000	0.012
4872	Receptor activity	20	195	2,013	10.26000	0.014
16021	Integral to membrane	41	494	2,888	8.30000	0.018
5856	Cytoskeleton	13	114	337	11.40000	0.021
7186	G-protein–coupled receptor protein-signaling pathway	10	82	1,581	12.20000	0.029
9058	Biosynthesis	9	284	923	3.17000	0.036
16874	Ligase activity	2	124	329	1.61000	0.046
5739	Mitochondrion	15	149	290	10.07000	0.048

Correlations between infections with four strains. Our studies identifying individual genes that were significantly altered by the four strains of *T. cruzi* revealed a surprising diversity with only a few genes similarly changed by infection with all strains. However, because this analysis selects only individuals, it does not compare subtle global changes throughout the transcriptome. To compare global trancriptomic alteration patterns in L_6E_9 cells infected by the four strains used in the current investigation, we compared the entire gene-array datasets obtained from the infection with each strain against each of the other strains. With Origin software (OriginLab, Northampton, MA), we plotted results of these pairs as $\log_2$ values of their expression ratios. In all six of the comparisons of expression changes induced by infection with separate

T. cruzi strains (Figure 3), the regression coefficients (r^2 values) for these linear relations were highly significant, and *P* values in all cases were less than 0.0001. This finding indicates that although infection with each of the parasite strains leads to only partially overlapping alterations in the genes that are most affected, there is an overall similarity in the pattern of gene-expression alterations resulting from infection with all the strains.

DISCUSSION

Chagas disease represents a spectrum of pathogenesis, varying both in its severity and the organ systems afflicted. Although various host factors, such as competence to launch immune response, may account in part for differences in the pathogenesis of the disease, parasite strain is also an important variable,[6] resulting in differences in viability, infectivity, and tissue tropism.[7–9] The present study was undertaken to evaluate the extent to which global gene-expression alteration was similarly altered after *in vitro* infection of a myoblast cell line with four distinct *T. cruzi* strains.

The microarray analyses described in this study were performed on the myoblast cell line L_6E_9 during infection with four reference strains of *T. cruzi*, each with well-characterized rates of *in vivo* and *in vitro* infectivity, resistance to chemotherapy, and pathogenesis *in vivo*. We used the Y and CL strains as representatives of the TC II group of *T. cruzi*, known to be found in central and eastern Brazil, which is commonly associated with the "mega" syndromes (cardiomegaly, megacolon, and megaesophagous).[22] The Tulahuen strain was chosen to represent the TC I group, however, the Multiplex PCR performed with genomic DNA of parasites of this strain revealed that it was actually a strain belonging to the TC II family. The Brazil strain was selected, because

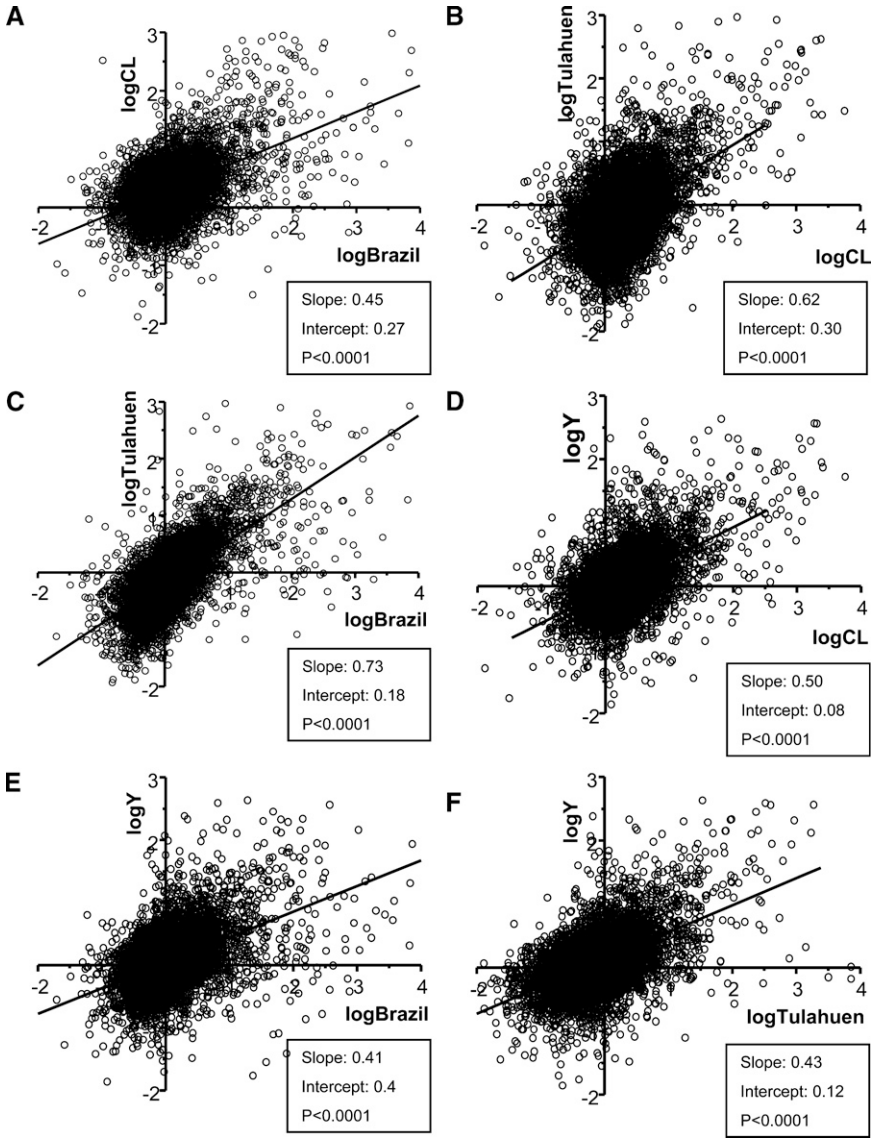

FIGURE 3. Correlation of datasets obtained by microarray analyses of the infection of L_6E_9 cell line with Y, CL, Brazil, and Tulahuen strains of *T. cruzi*. The datasets of each strain were plotted against each other in pairs of parasite strains. The histograms show how the transcriptomic changes induced by all strains have a highly significant correlation (all have *P* values < 0.0001). R^2 values were (**A**) 0.5, (**B**) 0.51, (**C**) 0.67, (**D**) 0.52, (**E**) 0.47, and (**F**) 0.53.

it has been shown to cause a dilated cardiomyopathy associated with a reduction in fractional shortening and myocardial wall thinning in mice.[13] Genotyping revealed that the Brazil strain belongs to the TC I group, the predominant group in Venezuela and central Brazil, which is usually associated with electrocardiographic (ECG) abnormalities.[22]

We have compared the datasets obtained from the microarray analyses in two ways, resulting in different but complementary conclusions regarding the pathogenesis of *T. cruzi* infection *in vitro* and perhaps, applicable to *in vivo* infection as well. First, through identification of the genes that had altered expression after infection, we identified a large number of gene expression changes in the infected cells, but only a very small number of these were common to infection with each parasite strain. We concluded from this analysis that each strain induces a particular fingerprint of pathology. One such fingerprint may include genes encoding cell-junction proteins, which was evidenced by our finding that both Tulahuen and Y strains down-regulated several cellular junction genes such as junction plakoglobin, junctional adhesion molecule 3, and adipocyte-specific adhesion molecule as well as cadherin 15 (Y strain). *T. cruzi* infection was also shown to alter adhesion molecules of the host such as connexin43[23] and cadherin-catenin.[11,24] These findings highlight an evolving concept that many types of cardiomyopathy target expression or involve mutations in molecular components of the intercalated disk (see reference 25 for review and reference 26 for changes in sepsis). Thus, as pointed out in a recent review,[27] cardiomyopathies, including chronic chagasic cardiomyopathy, may be considered to be junctionopathies.

An additional conclusion from the small number of genes found to be commonly regulated in infections with all strains (e.g., cut-like 1, DNA-damage inducible transcript 3, proprotein convertase subtilisin/kexin type 7) may provide a subset of biomarkers that could be potentially useful for diagnosis of acute *T. cruzi* infection and possibly, also reliably determine both indeterminate phase and chronic disease.

The second type of analysis that we performed on the gene-expression datasets was to compare overall transcriptomic changes in myoblasts infected with each *T. cruzi* strain through regression analysis of relative expression levels of each gene. For this, we compared each of the six pair-wise combinations of parasite strains, in each case finding a highly significant positive slope. This analysis emphasizes the concept that although Chagas disease shows a spectrum of manifestations, it does indeed represent a syndrome of common phenotypic alterations. The shallow slopes of the regression lines indicate that the changes induced by the parasite strains, although similar, are on average very low in amplitude; this emphasizes again the potential utility of the few commonly altered biomarkers.

The significant alteration in few overlapping genes among myoblasts infected with all strains poses a formidable challenge to the development of disease biomarkers that can be used to detect disease endemic areas where endogenous infective strains differ, although the few genes that we have detected with strong and significant expression changes offer such a possibility. Nevertheless, the overall transcriptomic signature of the disease that is revealed in the strong correlations between subtle expression alteration of all genes may provide a source whereby additional biomarkers may be discovered as common principal components of the acute response.

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Trabalho #2

Daniel Adesse, Michael P. Lisanti, David C. Spray, Fabiana S. Machado, Maria de Nazareth Meirelles, Herbert B. Tanowitz and Luciana Ribeiro Garzoni

Trypanosoma cruzi infection results in the reduced expression of caveolin-3 in the heart

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Trypanosoma cruzi infection results in the reduced expression of caveolin-3 in the heart

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Key words: *Trypanosoma cruzi*, cardiac myocytes, caveolin-3, caveolae, ERK

Caveolae are motile, membrane-bound compartments that contain a number of molecules that participate in cell signaling. Caveolins are protein markers of caveolae and function in a variety of biological processes. Caveolin-3 (Cav-3) is expressed in muscle cells and Cav-3 null mice display a cardiomyopathic phenotype. Ultrastructural cytochemistry, confocal microscopy and immunoblotting revealed a reduction in Cav-3 expression and an activation of ERK (extracellular-signal-regulated kinase) 48 hours after *Trypanosoma cruzi* infection of cultured cardiac myocytes. CD-1 mice infected with the Brazil strain of *T. cruzi* displayed reduced expression of Cav-3 and activation of ERK 66 days post infection (dpi). By 180 dpi there was a normalization of these values. These data suggest that the reduction in Cav-3 expression and the activation of ERK during the early phase of infection may contribute to the pathogenesis of chagasic cardiomyopathy.

Introduction

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*,[1] and results in heart disease in endemic areas of Latin America.[2] Acute infection is usually associated with intense myocardial inflammation characterized by an upregulation of cytokines and chemokines.[3,4] The ensuing cardiovascular remodeling may result in cardiomyopathy. The dilated cardiomyopathy is associated with congestive heart failure, arrhythmias, conduction abnormalities and thrombo-embolic events.[2,5] The mouse model of *T. cruzi* infection has been intensely studied because it recapitulates many of the pathological, functional and immunological features of the human disease. In addition, primary cultures of rodent neonatal cardiac myocytes have been utilized as a tool to investigate the effect of *T. cruzi* on the heart.[6-13] For example, *T. cruzi* alters cardiac myocyte Ca^{2+} homeostasis.[6-13] and gap junctional communication.[14,15] These infection-associated changes may contribute to the arrhythmias observed in this infection.

Caveolae are motile, membrane-bound compartments containing molecules that participate in cell signaling such as enzymes that generate messengers from substrates in the environment, substrates that are enzymatically converted into messengers and high-affinity binding sites that concentrate chemical signals. Caveolins are protein components of caveolae that are important in modulating a variety of biological functions including the modifications of signaling systems.[18] Caveolae and caveolins play critical roles in calcium homeostasis in the heart[16] and are disrupted by *T. cruzi* infection.[17]

Caveolae are signaling platforms that compartmentalize and concentrate signaling molecules such as G-protein subunits and endothelial nitric oxide synthase. Caveolins inhibit the downstream activation and signaling of many proteins, including c-Src, H-Ras, mitogen-activated protein (MAP) kinases, and eNOS.[19,20] Caveolin-1 (Cav-1) is expressed ubiquitously, although at different levels in different tissues; Cav-2 is tightly co-expressed with Cav-1, whereas Cav-3 is expressed predominantly in striated muscle cells.[21] Importantly, Cav-1 and Cav-3 null mice display a cardiomyopathy, with left ventricle wall thickening, fibrosis and p42/44 MAPK (ERK 1/2) activation.[22]

The activation of ERK1/2 plays an important role in the pathogenesis of cardiac hypertrophic responses and cellular proliferation in the cardiovascular system.[23] In vitro data support a role for Cav-1 and Cav-3 as negative regulators of ERK signaling.[24] During acute infection with the Tulahuen strain of *T. cruzi* Nagajyothi et al.[17] described reduced levels of Cav-1, -2 and -3 and increased ERK phosphorylation in hearts from infected mice. *T. cruzi* infection of mice and of cultured endothelial and smooth muscle cells resulted in activation of AP-1 which participates in cardiovascular remodeling through ERK 1/2 phosphorylation.[25,26]

We now report that the infection of cultured cardiac myocytes with *T. cruzi* resulted in a reduction of Cav-3 expression

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and activation of ERK 1/2. This was also observed in infected mice. These observations strongly suggest that infection with *T. cruzi* results in the reduced expression of Cav-3 and the activation of ERK. The cardiomyopathic phenotype of Cav-3 null mice suggests that a reduction in Cav-3 expression contributes to the cardiomyopathy of chagasic cardiomyopathy. ERK likely plays an important role in cardiac remodeling and arrhythmogenesis that accompany chagasic cardiomyopathy.

Results

Caveolae and caveolin-3 (Cav-3) expression are reduced as a result of *T. cruzi* infection. Ultrastructural analysis revealed that uninfected cardiac myocytes displayed features of differentiated myocytes such as myofibrils near mitochondria, sarcoplasmic reticulum, T-tubules and cell-cell junctions (**Fig. 1A–C**). Use of the lanthanum (La) as an electron opaque tracer for divalent ion (Ca^{2+}) binding sites revealed numerous caveolae on the surface of cultured cardiac myocytes and subsurface vesicles containing the tracer (**Fig. 1B and C**). After 24 hours of infection, trypomastigotes were identified by the presence of basket shaped kinetoplasts and subpellicular microtubules (**Fig. 1D**). We observed intracellular amastigotes in the cytoplasm of host cells presenting typical structures such as reservosomes and acidocalcisomes at 48 h (**Fig. 1E**). Beginning at 24 hr post infection, there was both reduced surface staining with La and a smaller number of caveolae in infected cells (**Fig. 1D**). These differences persisted at longer time-points, with surface La labeling virtually absent at 72 hours post infection (**Fig. 1E and F**).

To quantitate alterations in caveolae and Cav-3 abundance and distribution, we performed immunostaining and immunoblotting during the course of infection. Cultured cardiac myocytes displayed specific reactivity to anti-Cav-3 antibody, since no staining was detected in endothelial cells or fibroblasts (not shown). Cav-3 expression was abundant at and beneath the surface membrane of cardiac myocytes cultured for 96 hours (**Fig. 2**). However, 72 hours post infection, highly infected cells showed a striking reduction in Cav-3 immunoreactivity (**Fig. 2B**). Immunoblot analyses of the myocytes were consistent with both La cytochemistry and Cav-3 immunostaining. We observed that infected cardiac myocytes displayed Cav-3 levels similar to controls at 24 hours post infection and that there was a significant reduction at 48 (74%) and 72 (51%) hours post infection ($p < 0,05$, ANOVA) (**Fig. 2C and D**).

***T. cruzi* in vivo infection and caveolin-3 (Cav-3) expression.** We further analyzed the changes in Cav-3 in infected mice 66 and 180 days post infection (dpi). Immunoblot analysis of the heart tissue at these time points revealed that at 66 dpi there was a 46% decrease in Cav-3 levels, which were fully restored at 180 dpi (**Fig. 3**). Immunohistochemistry of the heart tissue at 66 dpi revealed that non-infected animals displayed abundant Cav-3 amongst cardiac myocytes, with T-tubule patterns (**Fig. 3** and arrows). This pattern was altered in infected hearts, where we observed areas of tissue damage (**Fig. 3B**) and areas in which cardiac myocytes displayed mostly intracellular staining.

***T. cruzi* and ERK phosphorylation.** Since an inverse relationship has been reported between the activation of ERK and the reduction in Cav-3 expression[24] we examined the impact of infection on ERK activation in cardiac myocytes at various time-points post infection, from one to 72 hours. The protein lysates were subjected to immunoblot analysis and probed with specific antibodies. We verified that at all time points studied, the infection did not alter total ERK expression (**Fig. 4A and B**). However, there was a progressive activation of ERK, as evidenced by increased phosphorylation in the cardiac myocytes; starting at 2 hours post infection (40%) and a 3-fold increase 72 hours post infection (**Fig. 4A and B**). We next analyzed ERK expression activation in hearts of infected mice at 66 and 180 dpi. While total ERK expression remained unaltered, phosphorylated forms of ERK at 66 dpi (when Cav-3 levels were decreased) had a 2.3-fold increase when compared to age-matched controls. At 180 dpi we observed that phosphoERK expression was 68% decreased in infected heart tissue (**Fig. 4C and D**).

Discussion

The effects of *T. cruzi* infection and its progression to heart disease is an ongoing interest by our groups and of others, since chagasic cardiomyopathy is the main cause of heart disease in Latin America. The parasite alters many of the host cell processes such as intracellular calcium homeostasis,[9,27] cell-cell communication[14,15] and cytoskeleton stability.[28,29] Alterations caused to host cells in the heart during acute infection where tissue parasitism is high and is associated with an intense inflammatory response and myonecrosis, may determine the development of the chronic disease.

In the present study, we have focused on caveolae, which are membrane-bound signaling complexes involved in Ca^{2+} homeostasis as well as other cell functions.[19] We evaluated the distribution of caveolae on infected cardiac myocytes by ultra-structural cytochemistry using lanthanum nitrate (La). La displaces Ca^{2+} from its binding sites on sarcolemma and can be used as a tracer for surface Ca^{2+} binding sites, abundantly found in caveolae.[30,31] The La staining permits the observation of caveolae distribution and intracellular vesicles, with positive La staining indicating sites of transport and storage of Ca^{2+}. In the current study we observed that *T. cruzi*-infected cardiac myocytes displayed a reduction in La staining on sarcolemma when compared with non infected cells after 48 hours of infection.[32] This is in agreement with previous results showing (a) that *T. cruzi* could alter cardiac myocyte endocytotic uptake of small molecules,[32] and (b) that caveolin (Cav)-1 null mice also display impaired endocytosis.[33] We confirmed the alterations in caveolae distribution during infection with *T. cruzi* by immunostaining and immunblot analysis demonstrating a significant reduction in the expression of Cav-3 48 and 72 hours post infection. Since caveolae are involved in Ca^{2+} regulation in cardiac myocytes and *T. cruzi* infection alters these structures, it is possible that caveolae disruption could be involved with the changes on Ca^{2+} homeostasis in cardiac myocytes. By depleting caveolae with cyclodextrin[34] a dose-dependent decrease in frequency, amplitude and spatial

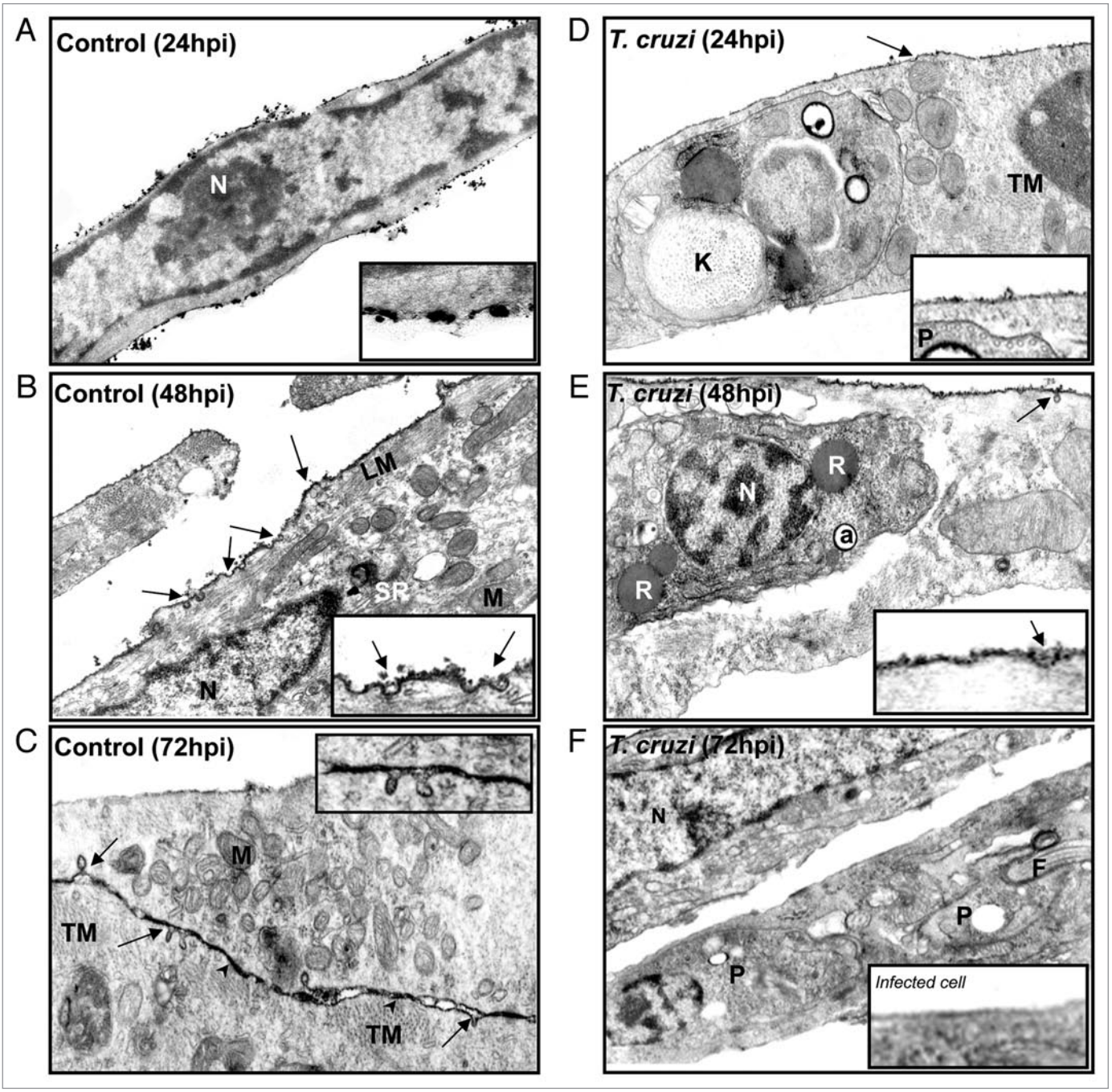

Figure 1. Ultrastructural cytochemistry. The tracer Lanthanum (La) Nitrate was used to reveal Ca^{2+} binding sites in cardiac myocytes. (A–C) Representative uninfected cardiac myocytes displayed longitudinal (LM) and transversal (TM) myofibrils, abundant mitochondria (M) and T-tubules (TT). Note in (C) the cell-cell junctions are depicted by arrowheads. La staining reveals intense electron dense staining in the plasma membrane with many caveolae (arrows), which can be observed in more detail in the inserts. (C) Some vesicles close to the membrane show staining with the tracer. (D) After 24 hours of infection a parasite is observed with subpellicular microtubules and a round kinetoplast (K). (E) A representative infected cardiac myocyte 48 hours post infection containing an intracellular amastigote with a reservosome (R) and an acidocalcisome (a). (N = Nucleus). (F) There was a reduction in La staining and alterations in caveolae abundance 72 hours after infection. Trypomastigotes (P) are observed in the cell cytoplasm and a decrease in surface staining by La. Original magnification: (A and F) = 26,000X; (B, C and E) = 41,000X; (D) = 47,350X.

extent of Ca^{2+} sparks was observed in smooth muscle cells and in cardiac myocytes: These observations suggest that alterations in the molecular assembly and ultra-structure of caveolae may lead to pathophysiological changes in Ca^{2+} signaling.[16] Through disruption of Ca^{2+} homeostasis, alteration in caveolae may contribute to the occurrence of arrhythmias observed in chagasic heart disease.

One major mechanism by which expression of caveolins is downregulated is activation of the mitogen-activated protein kinase family (MAPK), including the p42/44 MAPK, also

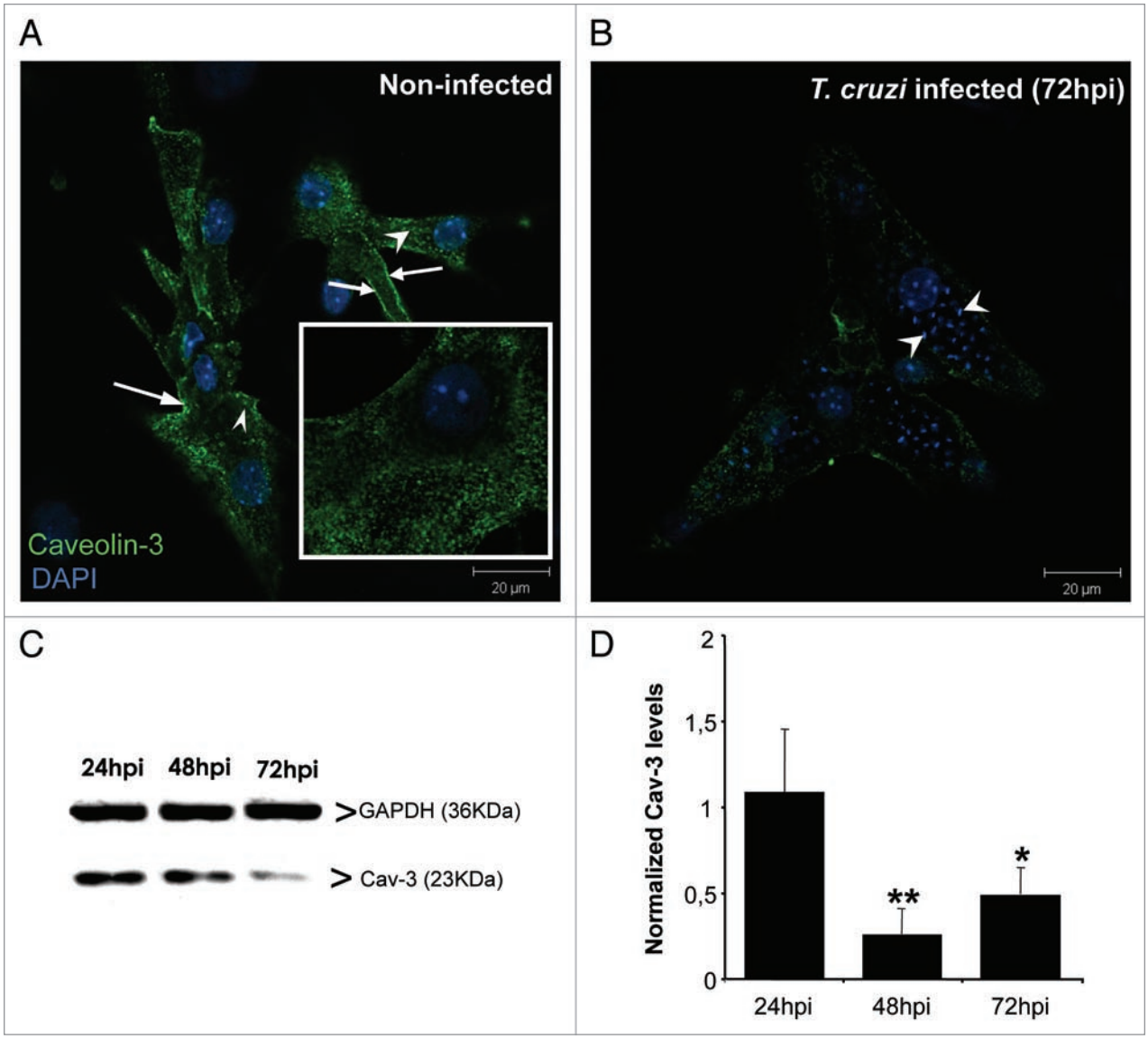

Figure 2. Caveolin-3 (Cav-3) expression is decreased after *T. cruzi* infection. Cultured cardiac myocytes were infected for 3 days. (A) Representative confocal Laser Scanning analysis of Cav-3 distribution in uninfected cardiac myocytes fixed and stained after 96 hours in culture. Cells presented abundant caveosomes (arrowheads) and peripheral staining (white arrows). The inset depicts higher magnification of the Cav-3 staining. (B) After 72 hours of infection, Cav-3 signal was reduced among highly parasitized cardiac myocytes, present mostly at cell periphery and with reduced presence of caveosomes. (C) Representative immunoblot of Cav-3 (23 KDa) expression in infected and uninfected cardiac myocytes. Anti-GAPDH (36 KDa) antibody was used as a loading control. (D) Bar graph demonstrating quantification of Cav-3 expression as normalized by GAPDH. There was a significant decrease of Cav-3 expression 48 (74%) and 72 (51%) hours post infection (n = 4) $^{**}p < 0.01$; $^{*}p < 0.05$, ANOVA. Bars = 20 µm.

known as extracellular signal regulated kinase (ERK 1/2).[19,35] The infected myocytes displayed activation of this protein kinase starting at 2 hours post infection as determined by immunoblot analysis using phospho-specific antibodies, without any significant alteration in total protein expression. This observation is consistent with our previous demonstration of ERK activation in cultured endothelial and smooth muscle cells after infection with the Tulahuen strain of *T. cruzi*.[25]

Since ERK phosphorylation is involved in cardiac remodeling, we studied Cav-3 and phospho-ERK expression in heart lysates of infected mice 66 days and 180 days post infection. Cav-3 levels were drastically reduced at 66 dpi as observed by immunohistochemistry and immunoblot; and a 3-fold increase in ERK phosphorylation was observed. This in accordance with previous studies that shown reduced Cav-1, Cav-2 and Cav-3 during acute murine *T. cruzi* infection followed by ERK phosphorylation.[3,17] Cav-1 null mice display hyperactivation of ERK in the heart and these mice also display cardiac hypertrophy with normal substrate utilization and expression of genes involved in energy metabolism.[36-38] In addition, Cav-3 null mice display increased expression of phosphorylated ERK and a cardiomyopathic phenotype.[22]

The observations in the current report demonstrate that *T. cruzi* infection alters Cav-3 expression in cardiac myocytes.

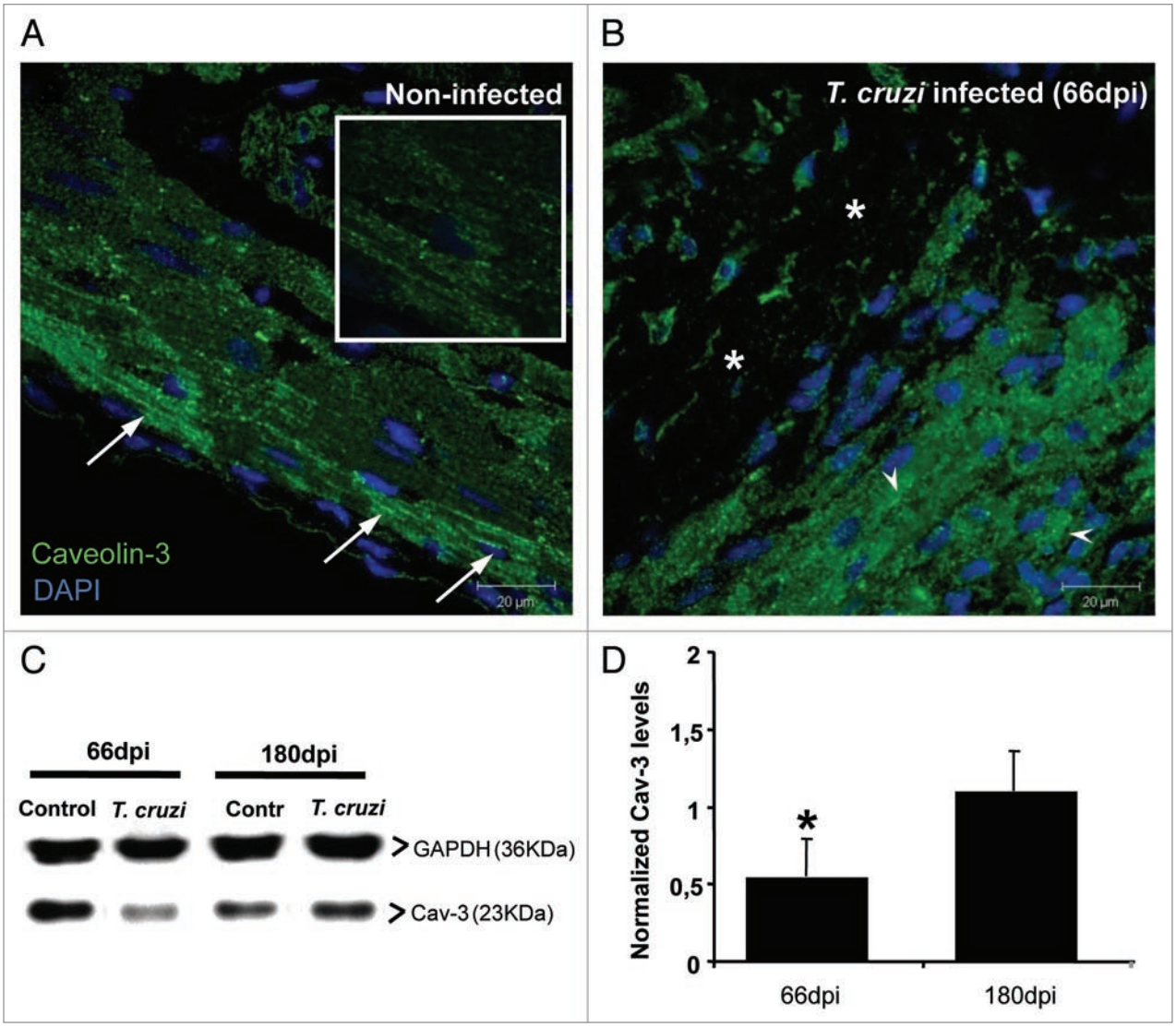

Figure 3. Caveolin-3 (Cav-3) in infected hearts. Hearts obtained from infected (Brazil strain) and uninfected CD-1 mice were embedded in paraffin and processed for immunohistochemistry. Sections were stained with anti-Cav-3 antibody (green) and DAPI (blue) and observed by confocal microscopy. (A) Representative uninfected cardiac tissue displayed intense Cav-3 staining of cardiac myocytes, especially in pericardial area with T-tubule patterns (arrows). The inset shows higher magnification of the Cav-3 staining. (B) This staining pattern was altered in the hearts of infected mice at 66 days post infection (dpi) where areas of tissue damage were evident (*). In other areas Cav-3 staining was observed mainly in the cytoplasm of cardiac myocytes (arrowheads). Bars = 20 µm. (C) Representative immunoblot lot analysis of Cav-3 expression in cardiac tissue. Hearts of mice 66 and 180 dpi were harvested and protein lysates probed with anti-Cav-3 antibody. GAPDH (36 KDa) was used as a loading control. At 66 dpi, infection induced 46% reduction in Cav-3 expression, which was restored to control levels at 180 dpi (n = 4 for each group). (*$p < 0.05$, ANOVA).

The ERK 1/2 activation indicates that Cav-3 downregulation is an important contributor in the cardiac injury observed during chagasic cardiomyopathy, including the hypertrophy, inflammation, fibrosis and arrhythmias, as observed in Cav-1, Cav-3 and the double knockout mice.[22,36,37] These observations also suggest that the caveolins may provide a novel target for potential therapeutic agents.

Material and Methods

Parasites and mice. The Brazil strain of *T. cruzi* was maintained in C3H/Hej (Jackson Laboratories, Bar Harbor, ME) mice. For mouse experiments 6–8 week old male CD-1 mice were injected with 5 x 10^4 trypomastigotes of the Brazil strain and parasitemia was evaluated by counting in a hemocytometer. All experiments were approved by the Institutional Animal Care Committee of the Albert Einstein College of Medicine. For in vitro experiments the Y strain was utilized. These trypomastigotes were maintained in cultured cardiac myocytes as previously described until used.[39]

CD-1 mice infected with the Brazil strain had a mortality of 50% by 45 days post infection (dpi). The parasitemia peaked at 30 to 35 dpi at 7.5 x 10^5 trypomastigotes/ml and then waned. There was no mortality after 45 dpi. Hearts were collected at 66

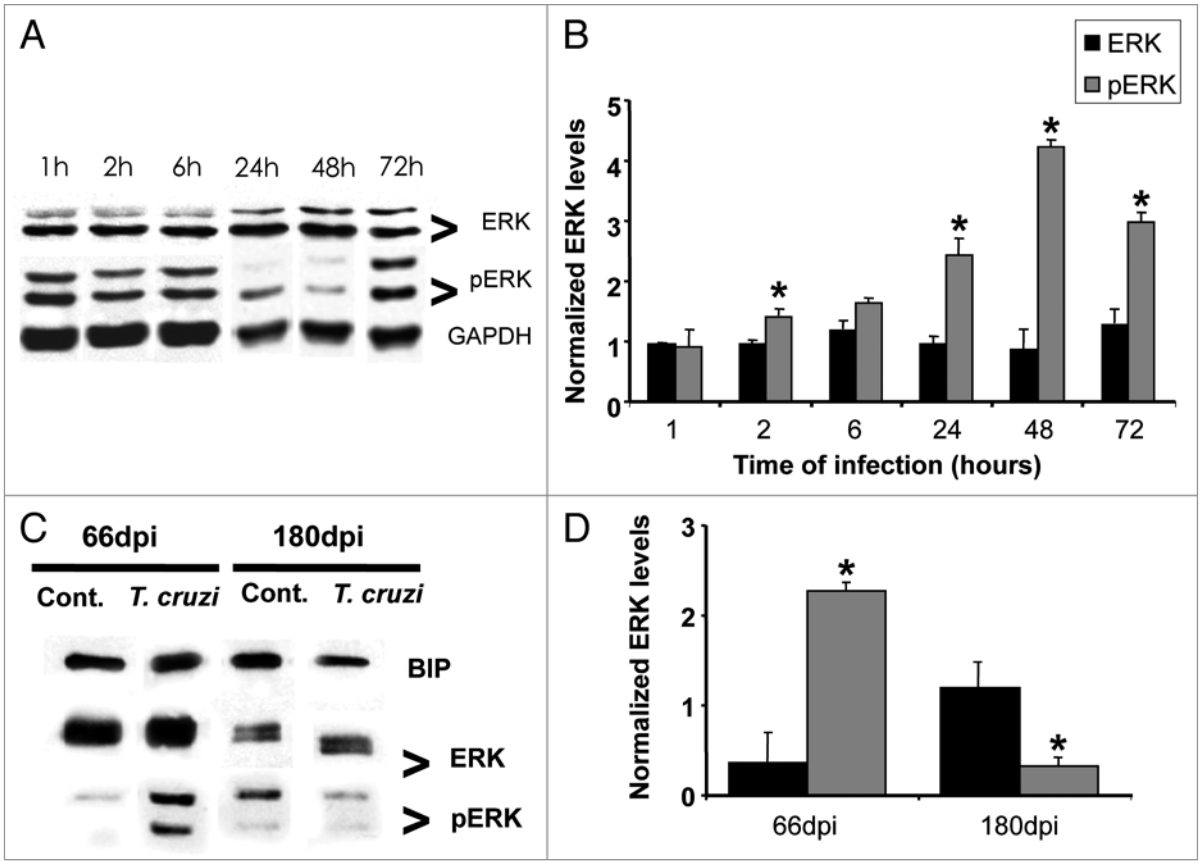

Figure 4. *Trypanosoma cruzi* infection induces activation of ERK 1/2 in cultured cardiac myocytes and in the myocardium of infected mice. (A) Cultured cardiac myocytes were infected with *T. cruzi* and blots were probed against anti-total ERK and pERK. There was no change in total ERK over a 72 hours infection. However, a progressive increase in pERK (activation) over the same period of time was observed. GAPDH (36 KDa) was used as loading control in myocyte samples (n = 3 for each group). (C and D) Hearts obtained from Brazil strain infected mice (66 and 180 days post infection, dpi) were subjected to SDS-PAGE analysis and probed with anti-phosphoERK antibody. *T. cruzi* infection induced a 2.3-fold increase in pERK (activation) at 66 dpi and a 68% decrease at 180 dpi as compared with age-matched uninfected controls. BIP (78 KDa) was used as loading control for heart tissue samples (n = 4 for each group) (*$p < 0.05$, ANOVA).

dpi and 180 dpi. Samples were either processed for immunohistochemistry or quick frozen in liquid nitrogen, crushed using a mortar and pestle (Humboldt, Schiller Park, IL), resuspended in lysis buffer and sonicated for immunoblotting.

Reagents and antibodies. Trypsin was obtained from Difco Laboratories (Detroit, Michigan), Type II collagenase was obtained from Worthington Biochemical Corporation (Lakewood, NJ). Fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, $CaCl_2$, Dulbecco's Modified Eagle's Medium (DMEM), RPMI, glutaraldehyde, sodium cacodylate, osmium tetroxide,acetoneand bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Lanthanum nitrate was obtained from Merk KGaA, (Darmstadt, Germany) and Epon 812 resin from Polysciences Inc., (Warrington, PA). Anti-caveolin-3 antibody was obtained from Affinity Bioreagents (Golden, CO), polyclonal goat anti-rabbit Alexa Fluor 488 antibody was obtained from Invitrogen (Carlsbad, CA), BCA Protein Assay Reagent (bicinchoninic acid) and 4'-6-Diamidino-2-phenylindole (DAPI) from Thermo Scientific (Rockford, IL). Rabbit polyclonal anti-phosphorylated ERK and total ERK were obtained from Cell Signaling (Beverly, MA) and the Protease Inhibitor Cocktail was obtained from Roche Molecular Biochemicals (Indianapolis, IN.) Goat anti-rabbit IgG and goat anti-mouse IgG HRP-labeled antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-BIP antibodies were obtained from Affinity Bioreagents (Rockford, IL).

Experiments with cardiac myocytes. Hearts were obtained from 18 day old embryos of mice and disassociated by mechanical and enzymatic dissociation methods using 0.05% trypsin and 0.01% collagenase in phosphate buffered saline (PBS, pH 7.2) at 37°C, following methods previously described.[5] Briefly, ventricular heart muscle cells were plated on 0.02% gelatin-coated glass cover slips maintained in 24-well plates for immunostaining assays or on plastic dishes for electron microscopy and immunoblotting. The cells were maintained at 37°C in 5% CO_2 atmosphere in DMEM supplemented with 5% FBS, 1 mM $CaCl_2$, 1 mM L-glutamine, 2% chick embryo extract, 1,000 U/mL penicillin and streptomycin 50 $\mu g/ml^{-1}$ (complete Eagle medium). Cardiac myocytes were infected with the Y strain trypomastigotes at a multiplicity of infection of 10:1.

Ultra-structural cytochemistry. Uninfected and *T. cruzi*-infected cardiac myocytes cultured in plastic culture dishes were

washed with PBS, fixed with 2.5% glutaraldehyde for 1 hour at 4°C, followed by washes first with Na-cacodylate buffer and then collidine buffer. Cells were post-fixed with 1% osmium tetroxide, 1.3% lanthanum nitrate in collidine buffer for 2 hours at 4°C. Cells were then dehydrated in an ascending series of acetone (30–100%) and embedded in Epon 812 resin. Ultra-thin sections were picked up with 300-mesh copper grids, stained with uranyl acetate and lead citrate and examined using a Zeiss EM 10C transmission electron microscope.

Immunofluorescence. Cells were washed with PBS (pH 7.2) at 72 hours post infection and fixed in 4% paraformaldehyde for 5 minutes at 20°C. After the washes cells were permeabilized with 0.05% Triton and non-specific staining was blocked with 4% BSA. Anti-Cav-3 antibody was incubated overnight 4°C, after which cells were washed and incubated with secondary polyclonal goat anti-rabbit Alexa Fluor 488 antibody for 1 hour at 37°C. DNA was stained with DAPI. For immunohistochemistry of heart tissue sections, samples were embedded in paraffin, sectioned with a microtome and placed on slides. Paraffin was removed incubating slides at 60°C, then with xylene and descending ethanol series. After washes with PBS, samples were blocked with BSA and incubated with anti-caveolin-3 antibody for 72 hours at 4°C. Secondary anti-rabbit Alexa Fluor 488 antibody was incubated for 2 hours at 20°C. Auto-fluorescence of the heart tissue was removed by incubating samples with 0.01% Evans blue solution and DNA was stained with DAPI. All slides were mounted with DABCO and images were acquired with a Zeiss 510 Meta Laser Scanning Confocal Microscope.

Immunoblotting. At desired times, cells were washed three times with PBS and scraped with 300 µl of lysis buffer (2 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM $NaHCO_3$, 10% Roche Protease Inhibitor Cocktail at 1, 2, 6, 24, 48 and 72 hours post infection). Hearts from infected CD1 mice and age-matched control mice were frozen in liquid nitrogen, resuspended in lysis buffer and sonicated. Samples were frozen at -80°C until used, the lysates were sonicated and protein concentration was measured using the BCA Protein Assay Reagent. Five to 10 µg of protein was loaded and resolved in 12% SDS-polyacrylamide gels. After resolving, proteins were transferred to nitrocellulose membranes (Whatman) and incubated with rabbit polyclonal anti-caveolin-3 antibody or rabbit polyclonal anti-phosphorylated ERK or total ERK diluted in TBST with 5% skim milk overnight at 4°C. For loading controls mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 36 KDa) monoclonal antibody and anti-BIP antibody were used. Membranes were washed with TBST and incubated with secondary goat anti-rabbit IgG and secondary goat anti-mouse IgG HRP-labeled antibody for 1 h at 25°C, followed by incubation with chemoluminescent kit ECL.

Statistical analysis. Statistical analyses were performed using the ANOVA test, with the level of significance set at $p < 0.05$. The data represent average (±standard error of the mean) and a minimum of three independent experiments.

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Trabalho #3

Fabiana S. Machado, Nilda E. Rodriguez, **Daniel Adesse**, Luciana R. Garzoni, Lisia Esper, Michael P. Lisanti, Robert D. Burk, Chris Albanese, Koenraad Van Doorslaer, Louis M. Weiss, Fnu Nagajyothi, Joshua D. Nosanchuk, Mary E. Wilson and Herbert B. Tanowitz

"Recent developments in the interactions between caveolin and pathogens"

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CHAPTER

RECENT DEVELOPMENTS IN THE INTERACTIONS BETWEEN CAVEOLIN AND PATHOGENS

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Abstract: The role of caveolin and caveolae in the pathogenesis of infection has only recently been appreciated. In this chapter, we have highlighted some important new data on the role of caveolin in infections due to bacteria, viruses and fungi but with particular emphasis on the protozoan parasites *Leishmania* spp., *Trypanosoma cruzi* and *Toxoplasma gondii*. This is a continuing area of research and the final chapter has not been written on this topic.

Caveolins and Caveolae: Roles in Signaling and Disease Mechanisms,
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INTRODUCTION

The first steps in the initiation of an infection are the attachment and entry of a pathogen into a host cell. It has long been assumed that an understanding of these initial events may result in new methods for control and treatment of infections. A role for caveolae and caveolin proteins in these processes has only recently been investigated. In this chapter, it is not our intent to review all of microbiology and describe how each and every microorganism interacts with caveolae and caveolin proteins, but rather to focus our attention on some recent important new developments. Pathogens enter mammalian cells to escape the immune system of the host and/or as part of their requirement to maintain a replicative cycle. In the past several years, this topic has been reviewed by others.[1-5] These articles highlighted the role of caveolae and caveolin proteins, such as caveolin-1 (Cav-1) and lipid rafts in the entry of diverse pathogens into the host cell. As discussed in detail in other chapters in this book, Cav-1 is a critical structural protein in the formation of the flask shaped caveolae lining the plasma membrane. In addition, the role of caveolins in the pathogenesis of infection may be related to an effect on components of the immune system such as lymphocytes and macrophages.[6,7]

VIRUSES

Caveolae are enriched in cholesterol and glycolipids, such as the glycosphingolipid GM1, glycosylphosphatidylinositol-anchored proteins and caveolin. Caveolae–mediated viral entry into human cell lines has been described and viruses that enter cells via caveolae apparently act as signaling ligands, triggering signal transduction events and actin rearrangement in the host cell, resulting in pathogen uptake (Table 1).[8,9]

The mechanisms by which a virus gains entry via caveolae are still not completely understood. The SV40 virus may bind to the MHC Class I complex, which recruits Cav-1 to the site of viral attachment from preformed caveolae and the associated lipid rafts are then formed around the virus. Alternatively, the SV40 virus bound to MHC may be associated with preformed caveolae. Regardless of the precise mechanism, SV40 containing caveolae pinch off from the plasma membrane and are transported to the endoplasmic reticulum via a caveosome.[8] Polyoma virus, Echovirus, Respiratory Syncitial Virus (RSV) and the filoviruses (Ebola and Marburg viruses) are additional examples of viruses that are associated with Cav-1. The effects of lipid raft disrupting agents on Ebola infection indicate that membrane lipid rafts are important in the entry of filoviruses.[1]

The HIV-1 receptors are associated with lipid rafts in T-cells and the disruption of the integrity of these lipid rafts likely inhibits HIV infection. The blood brain barrier (BBB) integrity is maintained by tight junctions,[10,11] and an intact BBB is crucial for preventing the trafficking of HIV into the brain. Early in the course of HIV infection, virus crosses the BBB via HIV-1 infected monocytes in macrophages and microglial cells in the brain. During the subsequent inflammatory response, leukocytes enter into the central nervous system through breaches in the BBB. Weiss et al [12] demonstrated that HIV-1-Tat protein is a powerful pro-inflammatory agent that causes transendothelial cell migration of monocytes. Recently, Zhong et al[13] demonstrated that Tat-mediated activation of Ras signaling is regulated by Cav-1 in brain endothelial cells and that

Table 1. Summary of papillomavirus entry pathways

HPV Type	Pathway Identified	Methods Used	Ref.
BPV1	clathrin mediated entry, shuttling from endosomes to caveosomes	biochemical inhibitors, co-localization studies, caveolinl shRNA, dominant negative cavl, 293 cells, pseudovirions	27
BPV1/HPV16	clathrin mediated entry	biochemical inhibitors, co-localization studies, C127 cells, virions and VLPs	19
HPV16	clathrin/caveolae independent, lipid raft independent, dynamin independent, tetraspanins involved	siRNA KO of clathrin, cavl, dynamin and tetraspanins, biochemical inhibitors, caveolin –/– cells, dominant negative inhibitors 293TT and HELA cells, pseudovirions	23
HPV16	clathrin mediated entry, shuttling from endosomes to caveosomes	biochemical inhibitors, co-localization studies, caveolinl shRNA, HaCaT cells, pseudovirions	26
HPV16/HPV31	clathrin mediated entry (HPV16), caveolar uptake (HPV31)	biochemical inhibitors, dominant negative inhibitors, HaCaT cells, pseudovirions	22
HPV16/HPV31	clathrin mediated entry	biochemical inhibitors, 293TT and COS7 cells, pseudovirions	20
HPV31	Caveolar mediated uptake, Rab5 mediated shuttling to endosome	biochemical inhibitors, co-localization studies, dominant negative Rab5, HaCaT cells, pseudovirions	28
HPV16/31 and 58	clathrin mediated entry (HPV16 and 58) caveolar uptake (HPV31)	biochemical inhibitors co-localization studies, COS7 cells, VLPs and pseudovirions	18
HPV33	non-caveolar uptake	biochemical inhibitors, COS7 and HELA cells, pseudovirions	21

inhibition of Cav-1 and Ras signaling attenuates Tat-induced disruption of the tight junction proteins.

Papillomaviruses (PVs) infect the mucosal and cutaneous stratified squamous epithelia. These infections are associated with both benign and malignant neoplasias and the human papillomaviruses (HPVs) cause virtually all cases of cervical cancer.[14] The 8 kb, circular viral genome is encapsulated by a complex of L1 (major) and L2 (minor) structural proteins.[15] Upon binding to heparan sulfate proteoglycans, the PV capsid undergoes a series of conformational changes resulting in the N-terminus of L2 becoming sensitive to cleavage by furin.[16] A recent study suggested that these conformational changes may occur on the extracellular matrix prior to transfer to the cell.[17] Earlier work employed chemical inhibitors and/or microscopic localization to suggest that HPV16, HPV33, HPV58 and Bovine PV1 (BPV1) use a clathrin-dependent pathway, whereas similar studies suggest the use of a caveolae-dependent pathway for HPV31.[18-22] However, more recent work suggests that HPV31 may also use a clathrin-dependent pathway.[20] Other authors have suggested that PVs can enter cells independent of either pathway.[23]

The lack of in vitro culture methods for the production of infectious virus has limited the study of PV entry. Most studies have used viral-like particles (VLPs) produced in insect cells or pseudovirions in which the L1/L2 capsid carries a reporter gene. It is possible that the use of these laboratory-made particles and nontarget cell lines could explain the observed differences between entry mechanisms. For example, it has been demonstrated that HPV16 enters dendritic cells and Langerhans cells via distinct pathways.[24]

The observations that a simian virus, JC virus (a polyoma virus), enters cells via clathrin-mediated endocytosis before being shuttled to caveolae-derived vesicles[25] led to the identification of a similar pathway for HPV16 and BPV1.[26,27] Based on recent studies, HPV16 virions first colocalize with markers of early endosomes; then, beginning at around 20 minutes post-entry, increasing amounts of the virons colocalize with Cav-1. Four hours post-entry the HPV16 virions are present in the endoplasmic reticulum. These observations strongly suggest that HPV16 and BPV1 enter the host cell via a clathrin-dependent pathway, after which they get shuttled from endosomes to caveolae. Conversely, HPV31 enters cells through the caveolar pathway followed by Rab5-dependent shuttling towards endosomes.[28] Since Rab5 controls the transport from endosomes to caveosomes and vice-versa, it is tempting to speculate that both HPV16 and HPV31 could shuttle back and forth between the caveolar and endosomal pathways following entry. This hypothesis is supported by the ultrastructural observation that both HPV16 and HPV31 end up in similar looking vesicles.[18] However, studies in which cells have been co-infected with HPV16 and HPV31 have not shown colocalization of both viral types.[23] It is possible that the differences in entry half-time (4 hours for HPV16 vs 14 hours for HPV31) could explain these observations. Thus, it appears that HPV31 has evolved to (predominantly) enter cells through a uniquely different pathway from the other tested PV types. This is surprising since HPV16 and HPV31 are evolutionary more closely related to each other than HPV16 is to HPV58 or BPV1 (HPV16 and HPV31 share approximately 83% amino acid similarity (PAM250 matrix) across the L1 structural protein). One hypothesis is that these two highly related viruses evolved to use different entry mechanisms to avoid competition. However, the much higher prevalence of HPV16 may suggest otherwise. One key observation is that it appears that the HPV16-E5 protein up-regulates Cav-1 at the plasma-membrane of

cervical cells. Taken together, these findings suggest that caveolin proteins might play a role in PV entry, but it will be necessary to sort out discrepant results and inter-type differences in order to adequately assess their role.

BACTERIA

There is limited information regarding the role of caveolins in bacterial infection. However, early studies suggested that caveolae may be an important alternative pathway for endocytosis of bacteria.[1,2,4,5] Chemical agents have been used to study the function of caveolae including nystatin, filipin and methyl-β-cyclodextrin (MβCD). These agents disrupt the cholesterol enriched lipid rafts. Many pathogens require lipid rafts for entry and colocalize with markers of caveolae to invade host cells. However, only a small number of bacterial pathogens have actually been shown to require caveolin expression for host cell entry.

Escherichia coli is an important cause of human infection including those of the urinary and gastrointestinal tracts. Studies involving *E. coli* invasion into mast and bladder epithelial cells have revealed that caveolae-dependent endocytosis is a mechanism for bacteria to invade both phagocytic and nonphagocytic cells. Importantly, intracellular bacteria can colocalize with Cav-1 and compounds that cause disruption of caveolae by removing membrane cholesterol inhibited bacterial invasion. *E. coli* uptake and invasion is dependent on the organization of lipid rafts and Cav-1 expression.[29] Furthermore, *Campylobacter jejuni,* is an important cause of diarrhea world-wide whose invasion of intestinal epithelial cells is dependent on Cav-1 expression.[30]

In an intraperitoneal model of sepsis using lipopolysaccharide injection, Cav-1 null mice were observed to be resistant to lung injury and their mortality was reduced due to a reduction in inflammation.[31] However, in a Cav-1 null mouse model of *Salmonella typhimirium*, an important cause of human diarrhea and systemic illness, Medina et al[32] found that higher levels of proinflammatory cytokines, chemokines and nitric oxide accompanied increased bacterial burden in the spleen. Surprisingly, no differences in *S. typhimurium* invasion of macrophages between Cav-1 null or wild type macrophages were observed.

Infections with *Pseudomonas aeruginosa* are observed most commonly in hospitalized immunocompromised individuals, resulting in urinary tract infections, pneumonia and sepsis. This is especially true of those individuals on mechanical ventilation and those suffering from severe burns. Also, over 80% of individuals with the genetic disorder Cystic fibrosis (CF) have pulmonary infection with *P. aeruginosa*.[33] Individuals with CF have a defect in the transmembrane conductance regulator (CFTR).[34,35] In normal individuals, infection with this bacteria stimulates the formation of lipid rafts that contain the CFTR thus allowing the organism to invade the respiratory epithelium and initiate inflammatory and apoptotic processes leading to shedding of bacteria-containing epithelial cells.[35-37] However, in CF patients these responses are absent and *P. aeruginosa* becomes established as a chronic infection.[38] Mechanistically, knockdown of either Cav-1, which also eliminates Cav-2, or Cav-2 alone reduces the uptake of *P. aeruginosa* into rat bronchial epithelial cells.[39] Importantly, Bajmoczi et al[40] demonstrated that following entry into host cells, *P. aeruginosa* colocalizes with Cav-1 and CFTR. More recently, increased mortality, bacterial burden and inflammation was demonstrated in Cav-1 KO compared with wild type mice, which correlated with a decreased ability of

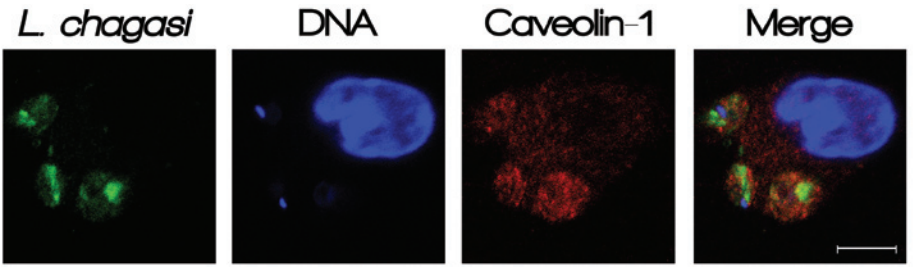

Figure 1. Intracellular *L. i. chagasi* colocalizes with caveolin-1. Bone marrow macrophages were infected with carboxy-fluorescein diacetate succinimidyl (CFSE) labeled *L. i. chagasi* promastigotes (green). Macrophage and parasite DNA was stained with TO-PRO-3 (blue). Confocal microscopy was used to assess colocalization of markers at serial time points. Caveolin-1 (red) clustered at the entry site of *L. i. chagasi* promastigotes and remained associated with parasites for 24 to 48 hours after infection. Shown is a picture taken at 24 hours of infection. Scale bar: 5 μm. A color version of this image is available at www.landesbioscience.com/curie.

Cav-1-deficient neutrophils to phagocytose *P. aeruginosa*. Additionally, the colonization of *P. aeruginosa* was more efficient in Cav-1 KO mice. Taken together, these preclinical observations strongly suggest that Cav-1 contributes to innate immunity to *P. aeruginosa* infection, which may have clinical implications for human CF patients.[41]

PROTOZOAN PARASITES

Leishmania

Depending on the species, infection with members of the genus *Leishmania* may result in cutaneous, mucocutaneous, or visceral leishmaniasis. These diseases are found wide-spread in tropical and sub-tropical areas of the world and are major causes of morbidity and mortality. Additionally, leishmaniasis is an opportunistic infection in patients with HIV/AIDS.[42]

Leishmania spp. have a life cycle consisting of two stages, the promastigote and the amastigote. The extracellular promastigote develops in the gut of the sand fly vector until it becomes a fully virulent metacyclic promastigote. Metacyclogenesis is a process during which surface molecules associated with virulence, such as lipophosphoglycan (LPG) and MSP (called GP63), are modulated in their expression and/or posttranslational modifications.[43-45] Attainment of full promastigote virulence coincides with the feeding cycle of the insect vector.[43-45] During a blood meal, the sand fly inoculates the parasite in the skin whereupon it is phagocytosed first by neutrophils and then by macrophages, the ultimate host cell.[46] Inside the macrophage, parasites transform from promastigotes to amastigotes over two to five days. Thereafter, amastigotes are the only form found in the mammalian host. Amastigote replication leads to the release of amastigotes from infected macrophages; amastigotes in turn are taken up by non-infected macrophages, thus spreading the infection.[47,48]

Leishmania enter macrophage phagosomes that ultimately fuse with lysosomes. The survival of *Leishmania* spp. in this hostile intracellular environment has been primarily attributed to the capacity of amastigotes to withstand the phagolysosomal compartment and

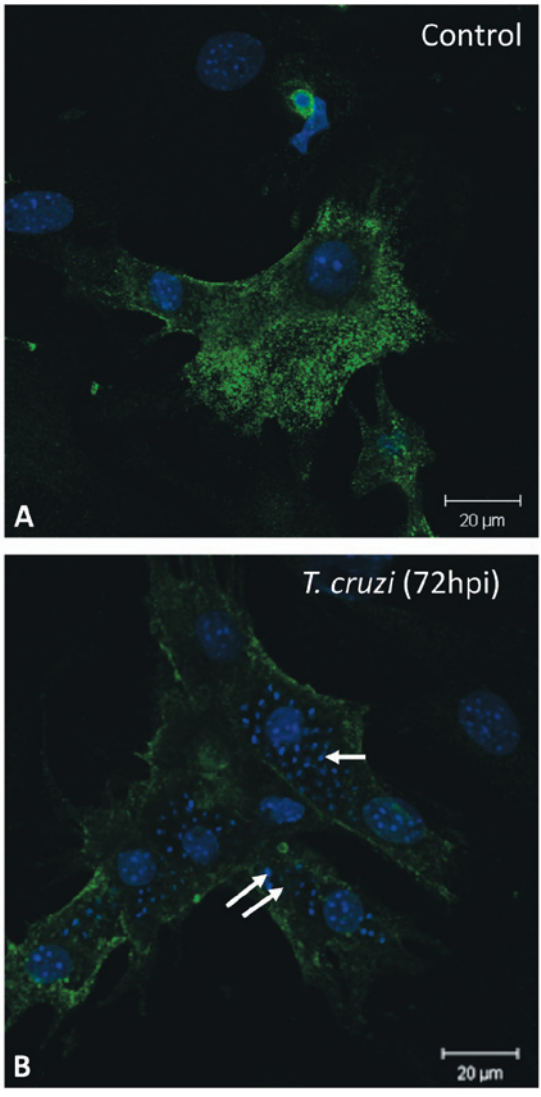

Figure 2. Caveolin-3 (Cav-3) expression is diminished after *Trypanosoma cruzi* infection: Cardiac myocytes were isolated from mouse embryos and infected with trypomastigotes. Confocal microscopy showed that uninfected cultures displayed abundant Cav-3 immunoreactivity (A), including peripheral staining. Cav-3 signal was reduced among highly parasitized myocytes (B) and was present predominantly at the cell periphery. DNA staining by DAPI permitted visualization of host cell nucleus and the amastigote kinetoplastid DNA. Bars = 20 mm. Reproduced with permission from: Adesse D et al. Cell Cycle 2010; 9:1639-164.81

to the ability of the parasite to down-modulate macrophage activation.[49-54] Accumulating evidence demonstrates that different external stimuli induce distinctive types of macrophage activation with divergent pro and/or anti-inflammatory profiles.[55-57] *L. infantum chagasi* is a cause of visceral leishmaniasis. Infection of BALB/c mouse macrophages with *L.i chagasi*

promastigotes initiates a pattern of gene expression that is neither classically activated nor alternatively activated, but which demonstrates a novel type of macrophage activation characterized by an anti-inflammatory profile and an increase in the caveolae-related molecules dynamin-2, Cav-1 and Cav-3.[58] This increase in caveolae components upon *L. i. chagasi* infection suggests the presence of a feedback mechanism that may be triggered by the depletion of surface caveolae upon parasite uptake.

Employing confocal microscopy, virulent *L. i. chagasi* promastigotes were found to colocalize with the caveolae markers GM1 and Cav-1 both during entry and up to 24 hours after murine macrophage infection (Fig. 1). Entry of promastigotes via Cav-1 correlated with a delay in lysosome fusion of approximately 24 to 48 hours; a time coinciding with their promastigote-to-amastigote conversion.[48,59] Co-localization of promastigotes with Cav-1 also correlated with increased parasite survival. In contrast, serum opsonization of attenuated (avirulent) *L. i. chagasi* promastigotes precluded parasites to enter macrophages through lipid rafts/caveolae. Unlike virulent *L. i. chagasi,* avirulent parasites entered compartments that fused early with lysosomes usually within the initial three hours of infection and failed to survive in macrophages.

Because caveolae are enriched in cholesterol, transient treatment of macrophages with the cholesterol chelating agent MβCD was used to investigate the role of lipid rafts/caveolae in Leishmania infection. MβCD treatment does not affect macrophage viability if the cell membrane cholesterol is extracted while preserving the intracellular cholesterol pools.[59-61] Under these conditions, pretreatment with MβCD significantly impaired parasite entry and inhibited replication for up to 72 hours after phagocytosis, even though surface cholesterol was restored by 4 hours after treatment. Furthermore, macrophage pretreatment with MβCD accelerated the rate of lysosome fusion and led to rapid intracellular killing. Side by side comparisons showed that transient disruption of lipid rafts/caveolae caused virulent metacyclic *L. i. chagasi* to enter macrophages through a phagocytic pathway, leading to early lysosome fusion and intracellular death, resembling the entry of attenuated parasites.[59]

Surface LPG and MSP on metacyclic promastigotes are able to bind and inactivate the serum protein C3b to C3bi, facilitating parasite uptake through the macrophage receptor CR3, which localizes in caveolae.[62-65] A comparison of the receptors used by avirulent log phase versus virulent metacyclic *L. i. chagasi* in human macrophages demonstrated that metacyclic parasites preferentially enter through CR3 but not the mannose receptor and that the metacyclic parasites colocalize with Cav-1 during the initial hour of infection. In contrast, log-phase parasites ligated both the mannose receptor and CR3 and failed to colocalize with Cav-1. The ability of metacyclic, but not log phase promastigotes to associate with CR3 and Cav-1 also correlated with a slower kinetics of lysosome fusion and increased parasite survival.[66]

The amastigote form of the parasite expresses low levels of MSP and no LPG; the latter of which inhibits lysosome fusion with promastigotes. Amastigotes can survive in the phagolysosomes and it has been suggested that the unique nutritional requirements of the amastigote can only be met in this degradative compartment.[67-69] As such, amastigotes might not benefit from entering macrophages through a cholesterol-rich/caveolae uptake mechanism that delays lysosome fusion. Indeed, side by side experiments with metacyclic promastigotes and hamster-derived amastigotes of *L. i. chagasi* supported this hypothesis. Transient depletion of cholesterol from BALB/c mouse macrophages did not affect the entry of amastigotes or their kinetics of lysosome fusion (submitted manuscript, Rodriguez, Gaur, Allen and Wilson), suggesting that the entry and survival of

amastigotes is independent of cholesterol-rich microdomains including caveolae. Taken together, these results suggest that virulent, but not attenuated or log phase promastigotes, are able to exploit a caveolae-mediated pathway to facilitate their entry and intracellular survival by a mechanism that includes delayed lysosome fusion until their conversion into amastigotes and the establishment of infection.

Trypanosoma cruzi

Trypanosoma cruzi causes Chagas disease. It is an important cause of acute myocarditis, chronic cardiomyopathy and gastrointestinal disorders in endemic areas.[70] Chagas disease is found in endemic areas of Mexico, Central and South America. In recent years, there has been an increased recognition of Chagas disease among immigrants from endemic areas into North America and Europe. Chagas disease is also an opportunistic infection in patients with HIV/AIDS.[71-73]

The parasite has a complex life cycle. During a blood meal from an infected mammalian host, the insect vector ingests blood-form trypomastigotes, which undergo transformations and after 3 to 4 weeks, infective, nondividing metacyclic trypomastigotes are present in the hindgut of the vector and are deposited with the feces of the vector during subsequent blood meals. Transmission to the new host occurs when the parasite-laden feces contaminate oral or nasal mucous membranes, the conjunctivas, or other vulnerable surfaces. Other modes of transmission include blood transfusion, organ donation, congenital, breast milk, ingestion of contaminated food or drink and laboratory accident.

When the trypomastigotes enter a host cell they transform into amastigotes where they multiply by binary fission and again transform to trypomastigotes. Trypomastigotes are released as the host cell ruptures and disseminate through the lymphatics and the bloodstream to find new cells to invade. The precise mechanism(s) by which the parasite enters the host cell is not entirely understood, however several receptors have been implicated in this process. Although any nucleated mammalian cell can be parasitized, those of the cardiovascular system, including cardiac myocytes, cardiac fibroblasts, endothelial cells and vascular smooth muscle cells, as well as cells of the reticuloendothelial, nervous and muscle systems and adipose tissue, appear to be favored.

T. cruzi infection is characterized by an intense inflammatory reaction accompanied by an upregulation of cytokines and chemokines.[73,74] Pathological examination of the cardiovascular system in both human samples and experimental acute chagasic myocarditis reveals inflammation, myonecrosis, vasculitis and numerous parasite pseudocysts. In chronic chagasic cardiomyopathy, inflammation, fibrosis, myocytolysis and vasculitis persist but there are few parasites in the infected tissues. In many chronically infected individuals there is a dilated cardiomyopathy. In order to understand the mechanisms involved in progression of disease and the development of the resulting cardiomyopathy, many groups have focused their research either on the in vitro infection of cardiac cells with *T. cruzi* or through the use of murine models of infection.

Caveolin is a negative regulator of extracellular signal-regulated kinases (ERK) and cyclin D1.[75] Thus, a reduction in the expression of Cav-1 and Cav-3 generally results in the upregulation of ERK activity and an induction of *cyclin D1* expression, which contribute to cardiac myocyte hypertrophy and ultimately cardiomyopathy. Interestingly, Cav-1 and Cav-3 null mice as well as the Cav-1/Cav-3 double null mice display a cardiomyopathic phenotype associated with cardiac myocyte hypertrophy and interstitial fibrosis.[76-78] Thus,

it was of great interest that during the acute phase of *T. cruzi* infection a reduction in the expression of Cav-1, Cav-2 and Cav-3 was observed. This was accompanied by activation of ERK, activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and increased cyclin D1 expression.[79,80] The change in Cav-1 expression in infected mice was the result in part of infection of the cardiac fibroblasts since cardiac myocytes do not express Cav-1. At 60 days post-infection, which is considered the sub-acute/chronic phase, there was a reduction in Cav-3 expression which normalized by day 180 post-infection,[81] though the increase in the expression of ERK persisted. Thus, it would appear that the initial reduction in Cav-3 expression may trigger an increase in ERK, leading to cardiac myocyte hypertrophy.

Chagas disease is also a vasculopathy and in carotid arteries obtained from *T. cruzi* infected mice there was a reduction in the expression of Cav-1 and Cav-3 and activation of ERK, cyclin D1 and endothelin-1(ET-1).[82] These findings may in part explain the vasoconstriction observed as a result of this infection. *T. cruzi* infection also results in a reduction of Cav-1 and Cav-2 expression and an increase in activated ERK, cyclin D1 and ET-1.[82,83] In addition, infection of cardiac myocytes results in a reduction of Cav-3 expression and a concomitant increase in activated ERK (Fig. 2).[81] Taken together *T. cruzi* infection results in cardiomyopathy and a vasculopathy in which caveolin, ERK, cyclin D1 and ET-1 contribute to cardiovascular remodeling and the pathogenesis of chagasic heart disease.

Employing the highly virulent Tulahuen strain of *T. cruzi* infection, Medina et al[84] infected both Cav-1 wild type and Cav-1 null mice and while the resulting mortality was 100% in both genetic backgrounds, death was slightly delayed in the wild type mice. The parasitemia in the Cav-1 null mice was significantly reduced compared with wild type mice. In both groups there were numerous pseudocysts, myonecrosis and marked inflammation. Interestingly, infection of cultured cardiac fibroblasts obtained from Cav-1 null and wild type mice revealed no differences in infectivity. Determination of serum levels of several inflammatory mediators revealed a significant reduction in IFN-γ, TNF-α and components of the nitric oxide pathway in infected Cav-1 null mice, while infection of wild type mice resulted in an increase in these inflammatory mediators. The defective production of chemokines and cytokines observed in vivo is, in part, attributed to Cav-1 null macrophages. These results suggest that Cav-1 may play an important role in the normal development of immune responses. Recently, Barrias et al demonstrated that infection of mouse peritoneal macrophages with the Y strain of *T. cruzi* was impaired when cells were treated with MβCD.[85] The contributions of caveolin in Chagas disease are not entirely understood and investigators are continuing to explore these interactions.

Toxoplasma gondii

Toxoplasma gondii is a ubiquitous Apicomplexan obligate intracellular protozoan parasite of mammals and birds. It has long been recognized as being an important cause of congenital infection with chorioretinitis and central nervous system manifestations and has also emerged as an opportunistic pathogen in immune compromised hosts where it primarily causes encephalitis.[86-88] Although overwhelming disseminated toxoplasmosis has been reported, the predilection of this parasite for the central nervous system causing necrotizing encephalitis and the eye causing chorioretinitis constitutes its major threat to patients. The development of these diseases is a consequence of the transition of the resting or latent bradyzoite stage to the active rapidly replicating form, the tachyzoite

stage.[86-87,89-93] It is likely that in chronic toxoplasmosis tissue cysts (bradyzoites) regularly transform to tachyzoites and that these active forms are removed or sequestered by the immune system, while some invade host cells differentiating to new tissue cysts.[86,89,90,93]

T. gondii replicates within a parasitophorous vacuole, isolated from host vesicular traffic.[94] *T. gondii* are capable of actively invading host cells[95,96] and these invasion processes require parasite motility, orientation toward the host cell and sequential discharge of three secretory organelles termed micronemes, rhoptries and dense granules.[97,98] Host cell cholesterol is required for entry and intracellular replication of this and other pathogens.[99-102] Central roles for cholesterol are suggested at the attachment, penetration and intracellular multiplication stages. Depletion of membrane cholesterol leads to a loss of invaginated caveolae.[103] The predominant sterol in *T. gondii* membranes is cholesterol and it has been unequivocally demonstrated that this parasite is auxotrophic for this major lipid.[101] *T. gondii* actively intercepts low-density lipoprotein (LDL)-derived cholesterol that has transited through host lysosomes by a yet undefined mechanism. Cholesterol trafficking from mammalian lysosomes to intravacuolar *T. gondii* requires functional host Niemann–Pick type cholesterol proteins (NPC), which are known to mediate cholesterol egress across the endosomal (mainly NPC1) and lysosomal (mainly NPC2) membranes,[104] and trafficking is independent of pathways involving the host Golgi and endoplasmic reticulum.[101] The uncoupling between LDL uptake and cholesterol biosynthesis occurring during *T. gondii* infection[101] leads to the assumption that these pathways are dramatically perturbed in infected cells.

Caveolae/caveolins have been proposed to function in a number of cholesterol-trafficking steps. These steps include selective cholesterol uptake from HDL via the scavenger receptor SR-B1 located in caveolae (cholesterol influx) and the delivery of newly synthesized cholesterol from the endoplasmic reticulum membrane caveolae, where it is delivered to HDL (cholesterol efflux).[105] However, studies have shown that high levels of host caveolae vesicles are not needed for trafficking of LDL-derived lysosomal cholesterol to Toxoplasma in mammalian cells.[101] It has been suggested that a probable scenario during *T. gondii* infection is that following LDL receptor-mediated endocytosis, cholesterol is liberated from LDL cholesteryl ester in early hydrolytic compartments containing the enzyme acid lipase. The cholesterol then effluxes from the NPC-containing late endosome/lysosome before trafficking to the parasitophorous vacuole. The post-endolysosomal movement of cholesterol to the parasitophorous vacuole is blocked by inhibitors of vesicular transport but does not require vesicle fusion or host endolysosome fusion with the parasitophorous vacuole. Transit to the parasitophorous vacuole is direct, rather than across host organelles, e.g., endoplasmic reticulum, Golgi[101,106] or through the host plasma membranes.[107] However, the parasitophorous vacuole is accessible to sterol acceptors in the medium by an unknown mechanism. Neither the host sterol carriers SCP-2 nor caveolins are involved and the process is independent of vimentin intermediate filaments. Host cholesterol is delivered to the parasitophorous vacuole via lipid extractor-or transporter-like proteins on the parasitophorous vacuole membrane, then trafficked within the vacuolar space, perhaps in association with the tubulo-vesicular network (TVN) secreted by *T. gondii* before internalization into the parasite interior via parasite plasma membrane proteins and storage as cholesteryl esters in lipid bodies.

Rhoptries are elongated club-shaped organelles containing a densely packed granular material in their basal bulbous portion and are related to secretory lysosomes or exosomes.[108] *T. gondii* rhoptries are formed via the endocytic pathway[109] and contribute to the formation

of the *Toxoplasma* and *Plasmodium* parasitophorous vacuole membranes (PVMs) by releasing their contents from the anterior end of the parasite during invasion.[98,110,111] *Toxoplasma* rhoptries also contain lipids, including large amounts of cholesterol and phosphatidylcholine.[112,113] In these organelles, the cholesterol/phospholipid molar ratio (1.5/1) is too high for lipid bilayer stability, suggesting that some rhoptry cholesterol molecules may be organized in a crystalline array inside the organelle. Although it is plausible that rhoptry cholesterol is incorporated into the PVM during invasion and that rhoptry discharge can effectively compensate for the absence of caveolae in host cells, this possibility requires testing.

The biogenesis of the PVM surrounding *Toxoplasma* is only partially understood. Capacitance measurements in patch-clamped host cells indicate that at least 80% of the membrane in the nascent vacuolar membrane is host cell derived.[114] This is consistent with observations showing that fluorescent tracers inserted into the host plasma membrane before infection were incorporated into the nascent PVM of *Toxoplasma*[110,115,116] as well as into the *Plasmodium* PVM.[117] Additionally, multiple lines of evidence suggest that rhoptry contents contribute directly to formation of the vacuolar membrane.[109,110,118-120] These apparently conflicting observations can be resolved by postulating a two-step process of invasion. Initial discharge of the rhoptry contents directly into the host cytoplasm leads to coalescence of multivesicular structures, which then fuse with the nascent vacuole that is derived primarily from the host cell plasma membrane.[121] Whether and how cholesterol contributes to this unusual process of cell invasion by *T. gondii* is not clear. Studies have evaluated the ability of cholesterol-depleted parasites to invade normal cells, of cholesterol-depleted cells to be invaded by untreated parasites, as well as the ability of normal parasites to invade caveolin-minus cells. The results demonstrate that *T. gondii* is dependent upon host plasma membrane cholesterol to trigger organelle discharge. Neither rhoptry-derived cholesterol nor caveolae microdomains in the host cell plasma membrane are required to complete invasion. These results identify a heretofore unexpected mechanism by which cholesterol regulates microbial entry into mammalian cells.

Lisanti and colleagues[122,123] discovered that purified caveolae microdomains contained an abundance of signaling molecules, such as Src-like tyrosine kinases and heterotrimeric G proteins and proposed that Cav-1 and caveolae may serve as docking points for numerous cell surface receptors, which, when activated by ligand binding, are recruited to caveolae.[122,123] It has been demonstrated that GTPase activity of G protein-subunits could be suppressed by a peptide derived from the NH2 terminus of Cav-1 termed the caveolin scaffolding domain, demonstrating the interdependence of these proteins for functional activity.[124] Finally, studies have shown that interaction of the *T. gondii*-derived molecule cyclophillin-18 (C-18) with a chemokine receptor (CCR5) on dendritic cells is critical for IL-12 (a pro-inflammatory mediator) induction in vivo and the subsequent control of parasite growth.[125] Since CCR5 is associated with G-protein-subunits, it is interesting to consider the possible participation of caveolin in the signaling pathways and transcriptional events downstream of CCR5 triggered by *T. gondii*.

FUNGI

Although significant efforts have led to an appreciation that sterol-rich membrane domains significantly impact the function of fungal membranes,[126] there is limited information regarding the role of lipid rafts and caveolae in the pathogenesis of fungal disease in mammalian hosts. The most in depth analysis of a fungal interaction with cellular membrane rafts comes from a study on *Paracoccidioides brasiliensis,* a dimorphic fungus endemic in the environment in Central and South America.[125] This fungus causes a spectrum of human disease ranging from mild localized infection to disseminated deep lesions, characterized by granulomatous inflammation. *P. brasiliensis* is frequently acquired via inhalation. Using epithelial alveolar cells, Maza et al[127] demonstrated that host cell lipid rafts are critical for engaging *P. brasiliensis* cells. They demonstrated that ganglioside GM1 appeared to be recruited to the point of adhesion of *P. brasiliensis* to this host cell. Furthermore, pretreatment of the alveolar cells with either MβCD to deplete host cell cholesterol, or nystatin, to bind host cholesterol, significantly reduced fungal adhesion.

Pneumocystis species are frequent pulmonary pathogens in hosts with compromised cellular immune responses, especially individuals with AIDS and the fungus can intimately engage type I alveolar epithelial cells.[128] Although *Pneumocystis* associates with cells, the fungus is an extracellular pathogen. Nevertheless, the fungus interdigitates with the alveolar cells and activates caveolae of the host cells, resulting in the close proximity of plasmalamellar vesicles in the areas of contact. Presumably, this allows the fungus to parasitize the host cells for nutrients.

Beta-glucans are major components of many fungal cell walls including that of *Candida albicans*, which is the most prevalent cause of systemic mycoses. Beta-glucans from *C. albicans* can interact with very long fatty acid-containing lactosylceramide lipid rafts on the plasma membrane of human neutrophils, inducing migration of the neutrophil toward the fungus[129,130] and, presumably, enhancing phagocytosis of the yeast.

Histoplasma capsulatum, a dimorphic fungus endemic to the Mississippi and Ohio River Valleys of the USA and in regions within Central and South America, causes pulmonary and disseminated infections, especially in individuals with compromised immunity. Calcium binding protein (CBP) is a well known virulence factor for the fungus that recently was shown to have structural homology with mammalian saposin B.[131] Hence, it has been proposed that CBP may interact with host glycolipids, including those present in caveolae. This finding underscores the probable broad and yet undiscovered import of caveolae in interactions with pathogenic fungi. Future work will continue to elucidate the functional importance of caveolae interactions with fungi during adhesion, internalization and internal processing.

CONCLUSION

A growing list of pathogens, including viruses, bacteria and their associated toxins, fungi and even prions, can interact with caveolae membrane domains.[2] The intracellular trafficking of these agents via caveolae differs dramatically from the usual route of ligands internalized by clathrin-mediated endocytosis. The use of caveolae for cellular entry allows the pathogen to avoid classical endosome-lysosome trafficking and, consequently, avoid degradative compartments within the cell.

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Trabalho #4

Amiodarone inhibits *Trypanosoma cruzi* infection and promotes cardiac cell recovery with gap junction and cytoskeleton reassembly in vitro

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Amiodarone Inhibits *Trypanosoma cruzi* Infection and Promotes Cardiac Cell Recovery with Gap Junction and Cytoskeleton Reassembly *In Vitro*▽

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We present the results of the first detailed study of the antiproliferative and ultrastructural effects of amiodarone on *Trypanosoma cruzi*, the causative agent of Chagas' disease. Moreover, we report the effects of this compound on the recovery of F-actin fibrils, connexin43, and contractility in *T. cruzi*-infected cardiac myocytes. Amiodarone is the most prescribed class III antiarrhythmic agent and is frequently used for the symptomatic treatment of Chagas' disease patients with cardiac compromise. In addition, recent studies identified its antifungal and antiprotozoal activities, which take place through Ca^{2+} homeostasis disruption and ergosterol biosynthesis blockage. We tested different concentrations of amiodarone (2.5 to 10 μM) on infected primary cultures of heart muscle cells and observed a dose- and time-dependent effect on growth of the clinically relevant intracellular amastigote form of *T. cruzi*. Ultrastructural analyses revealed that amiodarone had a profound effect on intracellular amastigotes, including mitochondrial swelling and disorganization of reservosomes and the kinetoplast and a blockade of amastigote-trypomastigote differentiation. Amiodarone showed no toxic effects on host cells, which recovered their F-actin fibrillar organization, connexin43 distribution, and spontaneous contractility concomitant with the drug-induced eradication of the intracellular parasites. Amiodarone is, therefore, a promising compound for the development of new drugs against *T. cruzi*.

Fn2 Chagas' disease is the largest parasitic disease burden and a major cause of heart disease and heart-related deaths in Latin America, where it affects approximately 16 to 18 million people AQ: B (25, 38). The disease is caused by the protozoan parasite *Trypanosoma cruzi*, which possesses a life cycle involving a mammalian host and an insect vector (31).

Chemotherapy against *T. cruzi* is limited to two compounds, namely, benznidazole (a 2-nitroimidazole) and nifurtimox (a 5-nitrofuran), which are mostly active in acute- and early chronic-phase patients but are of limited efficacy in the prevalent chronic stage (8, 34). Moreover, *T. cruzi* exhibits considerable biological variability, indicating possible variations in virulence, pathogenicity, oxidative stress, and drug resistance (23, 27, 36), which may pose an important challenge in the search of safer and more effective chemotherapeutic agents for the specific treatment of Chagas' disease.

Studies in the last 2 decades have permitted the identification of several new drug targets for this parasite. Among the most promising are (i) the essential cathepsin L-like protease cruzipain, (ii) the unique Kinetoplastida enzymes trypanothione reductase and trypanothione synthase, and (iii) the inhibitors of *de novo* sterol biosynthesis pathways, such as imidazole and triazole derivatives (5, 34, 35). There is also strong evidence that bisphosphonates can accumulate in the parasite's acidocalcisomes and interfere with the activity of enzymes involved in isoprenoid biosynthesis, such as farnesyl diphosphate synthase (13). Using *T. cruzi*-infected cardiomyocytes, we previously demonstrated that the bisphosphonate risedronate has a potent and selective *in vitro* effect against this parasite, resulting in recovery of the cardiac cells after the treatment (16). This compound also exhibited marked *in vivo* antiparasitic activity in a murine model of acute Chagas' disease (18). Another promising approach is the recent discovery of the anti-*T. cruzi* activity of the antiarrhythmic drug amiodarone, which is frequently prescribed for the symptomatic treatment of Chagas' disease patients (4). In the heart, the effects of this compound include inhibition of Na^+ channels, L-type Ca^{2+} channels, K^+ channels, and the Na^+/Ca^{2+} exchanger, leading to its characteristic antiarrhythmic action. It was found that the *in vitro* and *in vivo* activity against *T. cruzi* was mediated by disruption of the parasite's Ca^{2+} homeostasis and a blockade of ergosterol biosynthesis at the level of oxidosqualene cyclase (4).

Although the antiparasitic activity of amiodarone has been demonstrated previously, there is a lack of data regarding the effect of this compound on the ultrastructure of *T. cruzi* and its host cells and the recovery of these cells after the antiparasitic treatment. Primary cultures of murine cardiac myocytes have been the method of choice to demonstrate alterations in the host cell induced by this parasite. In these studies, many as-

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pects of this relationship were clarified, such as alterations in intracellular calcium dynamics (2, 17), changes in the cell cytoskeleton (24, 30), and cell-cell junction (1, 12). Gap junction channels are critical to maintaining cardiac homeostasis by allowing the free flow of ions and metabolites between cardiac myocytes, which contributes to the synchronized contraction of and signal exchange throughout the tissue. Gap junctions are composed of the connexin family of transmembrane proteins that assemble as end-to-end alignments of hexameric connexon subunits, thereby forming intercellular conduits for molecules of up to 1 kDa. Connexin43 (Cx43) is the most abundant gap junction protein in ventricular myocytes, being localized at intercalated disks in normal myocardium (15).

In the present study, we demonstrate the effects of amiodarone on the proliferation and ultrastructure of intracellular amastigote forms of *T. cruzi* growing in cardiomyocytes and the recovery of the host cells. We evaluated the recovery of spontaneous contractility of cardiac myocytes and the distribution of F-actin and Cx43 after treatment.

MATERIALS AND METHODS

Parasites. The Y (MHOM/BR/1950/Y) strain of *T. cruzi* was used in this work. Trypomastigote forms of *T. cruzi* were obtained from the supernatant of infected heart muscle cells grown in Dulbecco's modified Eagle medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS; Cultilab, São Paulo, Brazil), 1 mM $CaCl_2$, 1 mM L-glutamine, 2% chicken embryo extract, 1,000 U/ml penicillin, and 50 μg/ml streptomycin. After 96 h of infection, the parasites were collected, centrifuged, and resuspended in DMEM.

Cardiac cell cultures. Hearts of 18-day-old Swiss Webster mouse embryos were submitted to mechanical and enzymatic dissociation as previously described (22). Briefly, cells were harvested using 0.05% trypsin and 0.01% collagenase in phosphate-buffered saline (PBS) at 37°C. Ventricular heart muscle cells (HMCs) were plated on 0.02% gelatin-coated plastic flasks, on glass coverslips, in 24-well plates, or in petri dishes. Cells were maintained at 37°C in a 5% CO_2 atmosphere in DMEM for 72 h before the experiments. Use and handling of the animals were approved by the Ethics Committee for the Use of Laboratory Animals, FIOCRUZ (CEUA), protocol P70/09.2.

Infection of cultures and treatments. Heart muscle cells were plated in 24-well plates at a density of 1.5×10^5 cells/well in glass coverslips and infected with culture-derived trypomastigotes (20:1, parasites/host cells) in a final volume of 300 μl DMEM. After 2 h, the cultures were washed with PBS to remove nonadherent parasites and maintained in DMEM. Treatment with 2.5 to 10 μM amiodarone was performed by using the following two protocols: (i) addition immediately after the interaction step and (ii) addition at 48 h after infection. The total volume in each well was 500 μl. At specific times, coverslips (in triplicate) were collected, fixed with Bouin's fixative solution (Electron Microscopy Sciences, Hatfield, PA), and stained in Giemsa solution (Merck, Darmstadt, Germany). The percentage of infection was quantified by randomly counting at least 300 cells. In addition, supernatants were collected, and released parasites were counted in a hemocytometer.

Ultrastructural studies. Cells were plated in 35-mm plastic petri dishes and, at chosen times, were fixed (60 min/4°C) with 2.5% glutaraldehyde (Sigma-Aldrich), 2.5 mM $CaCl_2$, and 0.1 M Na-cacodylate buffer (pH 7.2), followed by postfixation for 1 h in cacodylate buffer solution containing 1% OsO_4, 0.8% potassium ferricyanide, and 2.5 mM $CaCl_2$. Samples were dehydrated in acetone and then embedded in Poly/Bed 812 resin (Electron Microscopy Sciences). Thin sections (Ultracut UCT; Leica, Vienna, Austria) were stained with uranyl acetate and lead citrate and were examined by transmission electron microscopy using a Zeiss EM10C microscope.

Immunofluorescence. Cells were washed with PBS and fixed with 4% paraformaldehyde for 5 min at 20°C at the desired time points. After being washed in PBS, cells were permeabilized with 0.5% Triton X-100, and nonspecific staining was blocked with 4% bovine serum albumin (BSA). Primary anti-Cx43 antibody (Sigma-Aldrich, St. Louis, MO) was incubated overnight at 4°C, after which cells were washed and incubated with secondary polyclonal goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, Eugene, OR) for 1 h at 37°C. F-actin filaments were stained with Alexa Fluor 594 phalloidin (Invitrogen) for 30 min at 37°C, and DNA was stained with DAPI (4′,6-diamidino-2-phenylindole). Images were acquired with an Olympus laser scanning confocal microscope.

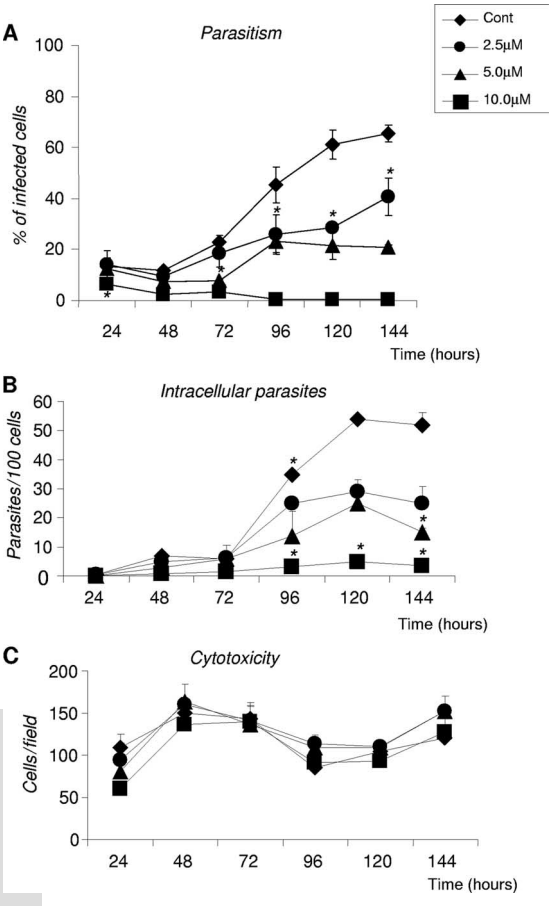

FIG. 1. Time and concentration dependence of the effects of amiodarone on infection of HMCs by *T. cruzi* (protocol i). Amiodarone (2.5 to 10 μM) was added to the cultures after 2 h of infection. (A, B) We observed significant effects of the drug on the percentage of infected cells (A) and the number of intracellular parasites (B), which were followed as a function of time. (C) The concentrations of the drug used in these studies were not toxic to the cardiac myocytes. Asterisks indicate statistical differences in relation to control cultures. The graphs show the means and standard deviations from triplicates of one representative experiment of four independent experiments. Cont, control.

Statistical analysis. Mean value comparisons were performed by using analysis of variance (ANOVA). *P* values below 0.05 were considered significant. IC_{50}s (50% inhibitory concentrations) were calculated from dose-response curves by using nonlinear regression analysis with the GraFit software package.

Materials. Amiodarone was purchased from Sigma-Aldrich. Stock solutions were prepared in phosphate-buffered saline (PBS) (pH adjusted to 7.4) and sterilized by using a 0.2-μM filter (Millipore, Billerica, MA). Trypsin was purchased from Sigma Chemical Co. (St. Louis, MO), and collagenase was purchased from Worthington Laboratories (Lakewood, NJ). All other reagents were analytical grade.

RESULTS

Effect of amiodarone on intracellular *T. cruzi* amastigotes and host cells. Using two different treatment protocols, amiodarone caused a strong inhibition of the infection of HMCs (Fig. 1 to 3). When the drug was added immediately after infection (protocol i) (Fig. 1), dose- and time-dependent re- F1-3

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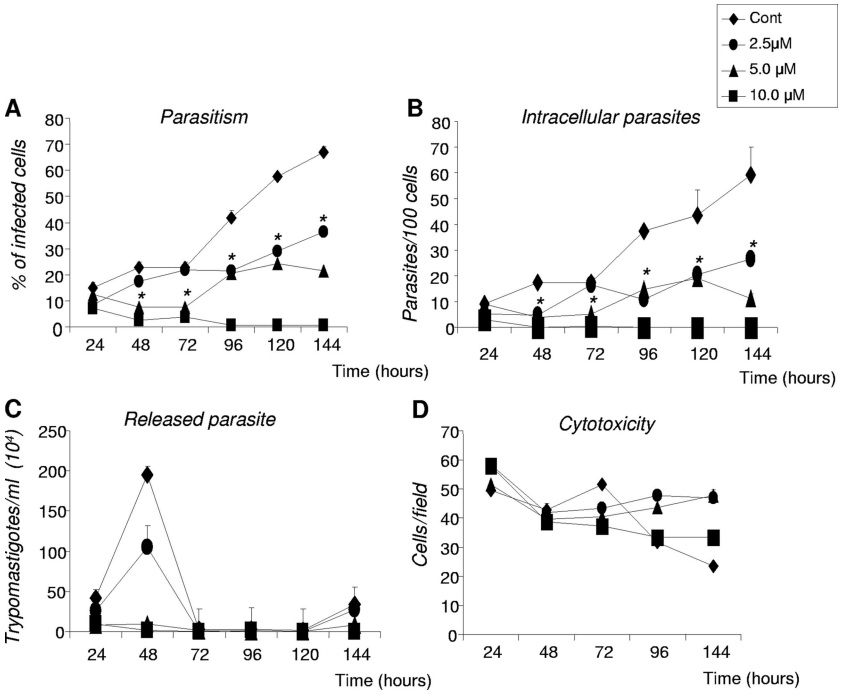

FIG. 2. Time and concentration dependence of the effects of amiodarone on infection of HMCs by *T. cruzi* (protocol ii). (A to C) Amiodarone (5 to 10 μM) was added to the cultures after 48 h of infection, and the percentage of infected cells (A), number of intracellular parasites per host cell (B), and number of released trypomastigotes in the supernatant (C) were followed as a function of time. (D) We did not observe significant cytotoxic effects of amiodarone with the concentrations used throughout the experiments. Asterisks indicate statistical differences in relation to control cultures. The graphs show the means and standard deviations from triplicates of one representative experiment of four independent experiments.

ductions in the percentages of infected cells were observed. The inhibition was statistically significant after 24 h of treatment with 10 μM amiodarone ($P < 0.05$), 48 h with 5 μM ($P < 0.01$), and 96 h with 2.5 μM ($P < 0.01$) (Fig. 1A). IC_{50}s with protocol i were 5.85 ± 1.4 μM at 96 h and 3.14 ± 1.2 μM at 120 h. Amiodarone had also an inhibitory effect on the number of intracellular amastigotes (Fig. 1B), as revealed by light microscopy (Fig. 3), significant for 10 μM amiodarone at 24 h of treatment ($P < 0.05$). Addition of amiodarone to cultures at 48 h after infection (protocol ii) also led to a highly significant reduction in the percentage of infected cells (Fig. 2). After 48 h of treatment (96 h of infection), 5 μM amiodarone already reduced significantly ($P < 0.05$) the parasitism of cultures, which was also observed at 96 h treatment with 2.5 μM amiodarone (Fig. 2A). In this protocol, IC_{50}s were 4.47 ± 0.3 μM and 2.24 ± 0.24 μM at 96 and 120 h, respectively. This inhibitory effect was also observed for the number of intracellular amastigotes in the HMCs (Fig. 2B). Moreover, amiodarone inhibited the release of trypomastigotes from infected cells in this model after completion of the intracellular cycle of the parasite. In control (untreated)-infected HMC cultures, the first two peaks of trypomastigote release to the supernatant occurred at 96 h and 192 h postinfection (Fig. 2C). Treatment with 5 and 10 μM amiodarone, starting 48 h postinfection, drastically inhibited such release after 48 h of treatment (96 h postinfection), while at 96 and 144 h, no trypomastigotes were detected in the supernatant (Fig. 2C). Amiodarone at the concentrations used in the experiments (up to 10 μM) had no apparent cytotoxic effects on the myocytes, as shown in Fig. 1C and 2D.

Light microscopy observations showed that treatment with amiodarone induced drastic morphological alterations on intracellular amastigotes (Fig. 3B, C, and E, insets). We confirmed this observation through transmission electron microscopy of infected HMCs and released parasites after 144 h of treatment of infected HMCs with 5 μM amiodarone. Infected untreated HMCs displayed abundant intracellular amastigotes in the cells' cytoplasm, with the expected kinetoplast morphology (bar shaped). A drastic loss of cytoplasmatic content and the formation of membrane inclusions inside the amastigotes were visualized in treated cultures; these cells also exhibited mitochondrial swelling, disorganization of reservosomes and the kinetoplast, probably associated with the disruption of Ca^{2+} homeostasis (4), and a blockade of amastigote-trypomastigote differentiation. Untreated amastigotes spontaneously released from their host cells also displayed their characteristic morphology (Fig. 4C). There was a marked damage in amastigotes released to the medium from amiodarone-treated (5 μM) cultures, such as kinetoplast alteration, and in the Golgi apparatus (Fig. 4D). F4 AQ: C

Cell physiology recovery after treatment with amiodarone. In order to assess the recovery of host cell ultrastructure and physiology after the treatment of infected cultures with amiodarone, we evaluated gap junction protein Cx43 (detected by immunofluorescence) and actin filaments (stained with phalloidin). *T. cruzi* infection is known to disrupt gap junctional

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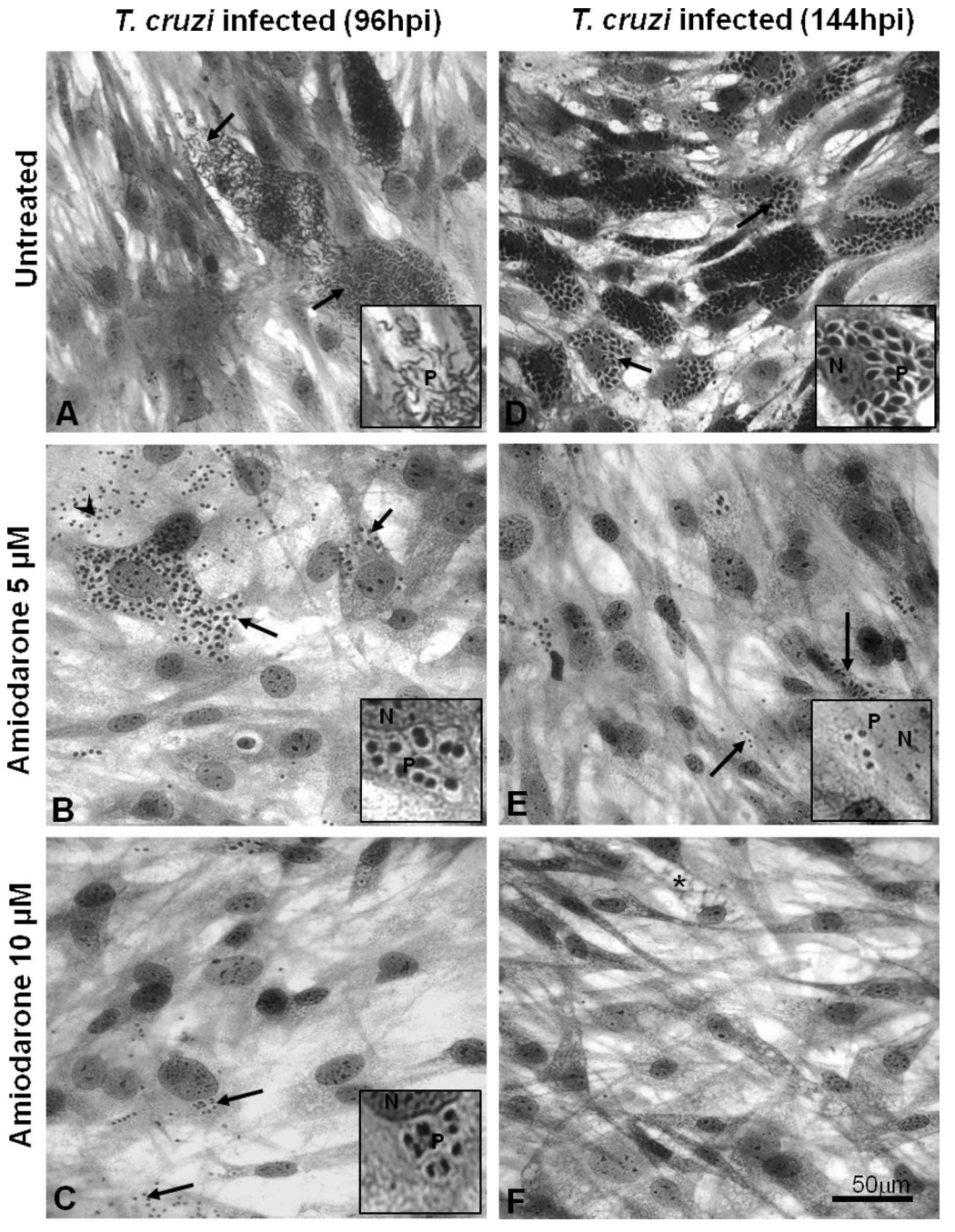

FIG. 3. Effect of amiodarone on the intracellular cycle of *T. cruzi*. Primary cardiac myocytes were obtained and infected with the Y strain of *T. cruzi*. After 2 h of infection, cultures were treated with 5 or 10 μM amiodarone. At 96 h postinfection (hpi), untreated cultures (A) displayed cells with trypomastigote forms of the parasite already evading host cells. Cultures treated with 5 μM (B) and 10 μM (C) of amiodarone displayed intracellular parasites with severe morphological alterations (arrows). (C) Interestingly, an amiodarone concentration of 10 μM induced a drastic reduction of parasitism. After 144 h of infection (corresponding to 142 h of treatment), we observed that in nontreated cultures (D), 70 to 80% of cardiomyocytes were infected. Treatment with 5 μM amiodarone (E) drastically reduced the number of parasites, whereas the use of 10 μM amiodarone (F) almost eliminated the parasitism of the cultures. Insets show details of intracellular parasites in higher magnification. P, parasites; N, nucleus.

F5 communication through Cx43 protein reduction (1). Figure 5 shows that infected untreated HMCs (Fig. 5D to F) totally lost Cx43 immunoreactivity at 192 h postinfection compared to that of control uninfected cells (Fig. 5A to C), which displayed well-formed gap junction plaques (Fig. 5C) and abundant myofibrils, as revealed by phalloidin staining. After treatment with 5 μM amiodarone for 144 h, infection was nearly abolished, as revealed by DAPI staining. In these cultures, Cx43 levels were comparable to those of age-matched uninfected cultures (Fig. 5I), and phalloidin staining revealed the presence of both po-

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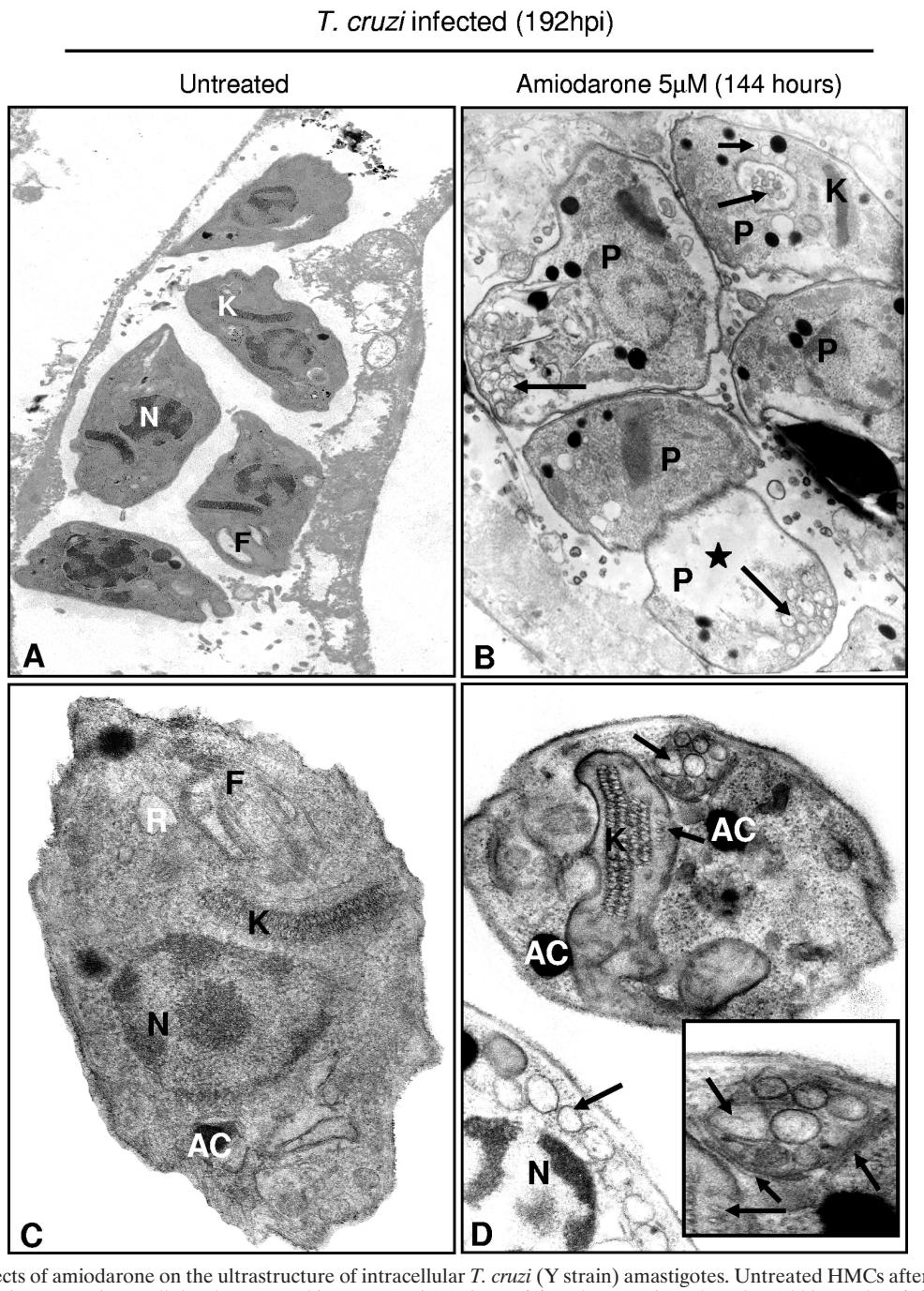

FIG. 4. (A) Effects of amiodarone on the ultrastructure of intracellular *T. cruzi* (Y strain) amastigotes. Untreated HMCs after 192 h of infection with *T. cruzi*, displaying severe intracellular damage and intact amastigote forms (P) and presenting a bar-shaped kinetoplast (K), nucleus (N), and flagellum (F). (B) Infected cultures treated with 5 μM amiodarone for 144 h showed parasites with membrane blebs (arrows), loss of intracellular material (stars), and kinetoplast (K) alterations. (C) Released parasites from untreated cultures displayed acidocalcisomes (AC) and a bar-shaped kinetoplast (K), reservosome (R), and flagellum (F), as expected. (D) Parasites obtained from cultures treated with 5 μM amiodarone (144 h) showed important alterations in the kinetoplast and Golgi apparatus (arrow).

lygonal and filamentous structures, indicative of cytoskeleton reassembly (Fig. 5H).

We also assessed the impact of *T. cruzi* infection on cardiac myocyte spontaneous electrocontractility, and the results are shown in Fig. 5J. The parasites' proliferation was associated with a progressive reduction in the cardiomyocytes' contraction, which followed the disorganization of the contractile apparatus as well as the disappearance of the gap junctions of these cells. After 48, 96, and 144 hours of infection (protocol i), untreated controls displayed a 28, 31, and 45% reduction in spontaneous contractility ($P < 0.05$), respectively, whereas treatment with amiodarone (5 μM) restored the number of spontaneous beatings to levels indistinguishable from those of uninfected cultures at 96 ($P < 0.05$) and 144 ($P < 0.01$) hours

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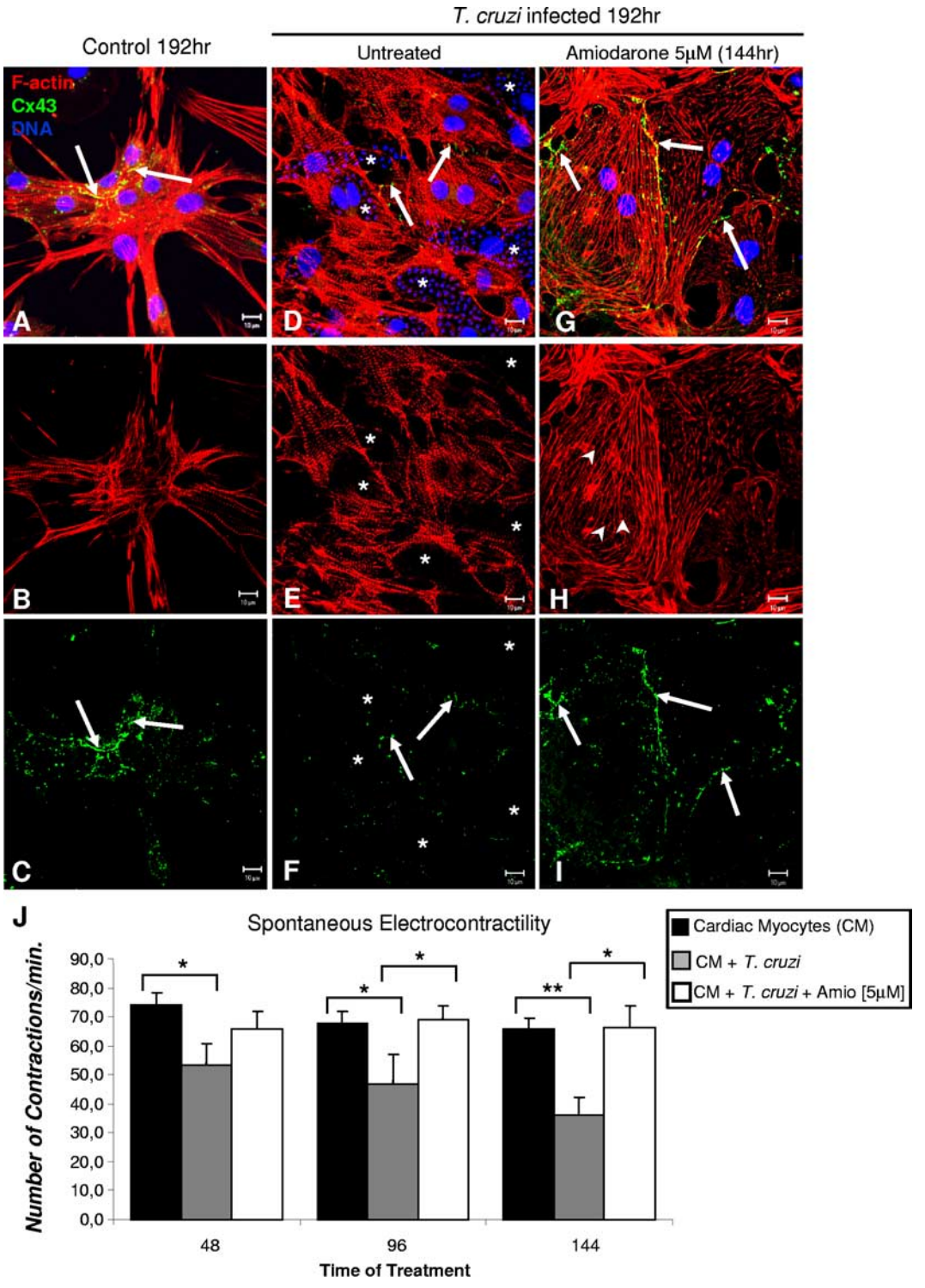

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FIG. 5. Amiodarone induced the recovery of host cell homeostasis. After eradicating the infection, we observed the recovery of morphological and functional aspects of cardiac myocytes cultures, as assessed by immunofluorescence and measuring of the spontaneous electrocontractility of the cells. (A to C) Uninfected cultured myocytes displayed abundant connexin43 immunoreactivity (C) as well as striated patterns of F-actin staining (B). (D to F) Highly infected cultures, at 144 h of infection, presented destruction of the F-actin cytoskeleton (E) and loss of Cx43 plaques (F). (G to I) Treatment with 5 μM amiodarone for 142 h decreased parasitism and induced recovery of host cells, as evidenced by F-actin and Cx43 recovery, which included striations (H) and the presence of gap junction plaques (I). (J) The spontaneous electrocontractility of the myocytes culture was evaluated by counting the number of contractions per 10 s. We observed that *T. cruzi*-infected cultures had a progressive decrease in electrocontractility, whereas treatment with 5 and 10 μM amiodarone restored cultures to normal levels of electrocontractility. *, $P < 0.05$; **, $P < 0.01$ (ANOVA).

of treatment. We observed no significant effect on contractility when the same dose of amiodarone was added to uninfected cultures (not shown).

DISCUSSION

Human Chagas' disease results from infection by *T. cruzi*, and tissue damage arises from both direct parasite action and the inflammatory process that ensues (7). Cytolysis and fibrosis are key components associated with Chagas' disease's pathological manifestations, along with a sustained and diffuse inflammation of the affected organs (31). The growing perception that chronic-phase manifestations are associated with the persistence of the parasite in the mammalian tissues (32) and that the two available drugs (benznidazole and nifurtimox) have important limitations, particularly in the chronic phase (6), has stimulated the search for new trypanocidal compounds. Amiodarone is frequently used as an antiarrhythmic in chronic-phase Chagas' disease patients with cardiac compromise (21, 26), and it also has antifungal (9, 10) and antiprotozoal (4, 28) activities, which were recently described. It was shown that the drug disrupts the parasite's Ca^{2+} homeostasis and also blocks ergosterol biosynthesis, resulting in excellent parasiticidal activity with low cytotoxicity (4). The results obtained in this work clearly confirm that amiodarone has potent and selective activity against *T. cruzi*, with no significant effects on their preferred host cells, cardiomyocytes, and at doses that do not induce the well-known antiarrhythmic action of the drug on these cells. This selective action was confirmed by ultrastructural analysis of amiodarone-treated cultures (Fig. 4), which revealed massive alterations of the parasites, allowing at the same time the full structural and functional recovery of the cardiomyocytes (see below). The ultrastructural effects of amiodarone on intracellular amastigotes were similar to those previously described for other sterol biosynthesis inhibitors, such as risedronate (16), ketoconazole, and terbinafine (19, 37).

We also investigated whether amiodarone treatment would induce host-cell recovery, concomitant with the elimination of the intracellular parasites' burden. We have previously shown that infection by *T. cruzi* induces a cytoskeletal disruption in HMCs due to myofibrillar breakdown (24) and that treatment with risedronate and posaconazole allowed reassembly of cytoskeleton elements (16, 29). The present results show that amiodarone allowed the reorganization of the actin-containing myofibrils to their normal state, with the occurrence of characteristic polygonal arrangement. Lin and coworkers (20) have described this polygonal configuration in dissociated cardiac myocytes as an indicator of myofibrillar reassembly. Moreover, it is known that the infection of cultured cardiac myocytes with *T. cruzi* decreases gap junction communication and Cx43 expression (1, 11). These observations were extended to acutely infected mice, which displayed reduced cardiac Cx43 expression (1). Our results demonstrate that Cx43 distribution among cardiac myocytes was fully restored after treatment of infected HMCs with amiodarone, and treated cultures displayed gap junction plaques comparable to those of uninfected controls. Cx43 has a turnover of approximately 1.5 h *in vitro* (3), and in *T. cruzi*-infected myocytes, Cx43 mRNA levels were largely unaffected (D. Adesse, unpublished data). This may provide evidence that the infection affects Cx43 translation and/or its subsequent trafficking to the plasma membrane, possibly due to microtubular damage (24). After the amiodarone-induced disappearance of the intracellular parasites, the host cells were capable of synthesizing new Cx43 molecules and made their successful delivery to the plasma membrane. More importantly, HMCs restored their spontaneous contractility after treatment with 5 μM amiodarone to levels comparable to those in control cultures. This observation is of interest since it permits us to speculate that during *in vivo* infection, *T. cruzi* disturbs synchronous contractility, which can be reverted with the use of amiodarone alone or in combination with another inhibitor of the parasite's sterol biosynthesis, such as posaconazole, which results in synergistic effects (4).

To summarize, our results showed that amiodarone has a selective antiproliferative effect on *T. cruzi* in an *in vitro* model of infection of cardiac cells. Treatment induced ultrastructural damage to intracellular amastigotes but promoted full structural and functional recovery of the host cells. This compound should be considered, beyond its known antiarrhythmic activity, as an important antiparasitic agent and a lead for the development of new specific treatments of this neglected disease.

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DISCUSSÃO

Um dos mais importantes desafios para a compreensão da fisiopatologia da doença de Chagas está no estudo dos mecanismos envolvidos na interação entre parasito e hospedeiro. Nesta tese, utilizamos modelos *in vitro* e *in vivo*, incluindo culturas primárias de cardiomiócito e a infecção experimental de camundongos por *T. cruzi* visando elucidar alguns destes aspectos.

Um importante fator consiste na diversidade de populações de *T. cruzi* que diferem em propriedades bioquímicas, que influenciam sua infectividade (Yoshida, 2006), virulência (Espinoza et al., 2010) e patogênese (Revollo et al., 1998). Diversas abordagens foram utilizadas para caracterizar a estrutura das populações de *T. cruzi*, com objetivo de definir subgrupos de relevância biológica. Estes subgrupos receberam diferentes designações, que incluem zimodema, esquizodema, biodema, linhagens e, mais recentemente, unidades de tipificação distintas (DTUs) (revisto em Zingales et al., 2009).

As diferentes combinações entre cepas de *T. cruzi* e hospedeiros com *backgrounds* diversos, gera um amplo espectro de manifestações da doença humana e experimental (Miles et al., 1981). Durante a fase aguda da doença, estas diferenças podem gerar um amplo espectro de sintomas e manifestações no hospedeiro, que podem determinar o curso da fase crônica, e o possível desenvolvimento da doença cardíaca crônica (Higuchi et al., 2003). Por esta razão os estudos *in vitro* da interação parasito-célula hospedeira são de extrema relevância, e já geraram uma vasta quantidade de informações a respeito do reconhecimento e invasão da célula hospedeira (revisto em Alves & Mortara, 2009); da instalação do ciclo intracelular do parasito e fatores relacionados à replicação das formas amastigotas (Schettino et al., 1995) e das alterações sofridas pela célula.

No entanto verificamos que alguns estudos apresentaram resultados conflitantes a depender do modelo experimental utilizado. Em nosso modelo de interação, cardiomiócitos de camundongos infectados pela cepa Y de *T. cruzi* apresentaram aumento na contratilidade espontânea, resposta a neurotransmissores diminuída e não sofreram alterações na miogênese (Meirelles et al., 1986; Aprigliano et al., 1993). Células musculares de ratos infectados pela cepa Tulahuen de *T. cruzi* tiveram resposta

à norepinefrina aumentada (Bergdolt et al., 1996) e quando aqueles infectados pela cepa Brazil apresentaram inibição de miogênese (Rowin et al., 1983).

Estas diferenças nos motivaram a avaliar até que ponto as alterações na expressão gênica global seriam similares após a infecção da linhagem L_6E_9 com quatro diferentes cepas de *T. cruzi*. Para isso utilizamos a técnica de microarranjos (*microarrays*) de oligonucleotídeos, que permite o estudo rápido e imparcial de alterações na expressão de um grande número de genes, no nível do RNAm.

Alguns trabalhos utilizaram a tecnologia de *microarrays* para investigar o impacto da infecção por *T. cruzi* no hospedeiro, tanto *in vitro* (Vaena-de-Avalos et al., 2002; Shigihara et al., 2008; Imai et al., 2005) como *in vivo* (Mukherjee et al., 2003; Mukherjee et al., 2008; Garg et al., 2003; Soares et al., 2010). No entanto, não podemos afirmar que os resultados demonstrados por estes autores são aplicáveis a outras combinações parasito-hospedeiro. Um estudo mais recente comparou, por *microarrays,* a resposta inicial à infecção intradérmica por três diferentes cepas de *T. cruzi* (Y, Brazil e G), verificando que o parasita induz uma resposta dependente de IFN-γ tipo I (Chessler et al., 2009).

Nós utilizamos a linhagem de mioblastos de rato L_6E_9, já que o músculo esquelético é também um alvo primário da infecção aguda. Esta linhagem apresenta crescimento rápido em cultura e foi muito utilizada em estudos de infecção por *T. cruzi* (Rowin et al., 1983; Morris et al., 1987; Rodriguez et al., 1996). Além disso, o uso de cultura de células evita a interferência de variáveis externas presentes na infecção *in vivo* como migração de células inflamatórias, fatores solúveis presentes no soro e presença de outros tipos celulares como fibroblastos e células endoteliais.

As análises de *microarray* descritas no **Trabalho #1** foram feitas utilizando células L_6E_9 após 72 horas de infecção com quatro cepas referência de *T. cruzi*, todas bem caracterizadas quanto à infectividade *in vitro* e *in vivo*, resistência à quimioterapia e patogênese. Nós realizamos a classificação filogenética segundo Fernandes e colaboradores (2001) e verificamos que as cepas Y, CL e Tulahuen são representativas do grupo *T. cruzi* II (TCII), encontrado em regiões do Centro e no Leste do Brasil e estão em geral associadas com as “mega” síndromes (cardiomegalia, megacólon e megaesôfago). A cepa Brazil foi selecionada por ser utilizada há muitos anos em modelos *in vivo*, gerando cardiomiopatia dilatada severa, associada com redução na

fração de ejeção do ventrículo esquerdo e afinamento da parede do miocárdio em camundongos (Mukherjee et al., 2008). A genotipagem revelou que esta cepa pertence ao grupo TCI, predominante na Venezuela e no centro do Brasil e está geralmente associada a importantes alterações eletrocardiográficas (Miles et al., 1981).

Nós analisamos os resultados obtidos destes *microarrays* de duas maneiras diferentes, porém complementares com relação ao impacto da infecção por *T. cruzi* nas células hospedeiras *in vitro*. Quando identificamos os genes dos mioblastos que sofreram alterações em sua expressão após a infecção, verificamos que uma pequena quantidade destes foi significativamente alterada pelas quatro cepas (>50%, $p<0,05$). No entanto, apenas um pequeno número de genes (21) foi comum às quatro cepas utilizadas, permitindo que concluíssemos que cada cepa de *T. cruzi* possui uma assinatura distinta na patologia.

Um gene comumente alterado foi o *cardiotrophin-like cytokine factor 1*, que pertence à família da interleucina-6 (IL-6), que inclui IL-11, LIF, oncostatina M, fator neurotrófico ciliar e cardiotrofina-1 (CT-1). Estas citocinas estão envolvidas em processos inflamatórios e imunológicos, assim como hematopoiese, regeneração hepática e neuronal, desenvolvimento embrionário e fisiologia cardiovascular. Através da ativação de genes-alvo envolvidos nos processos de diferenciação, sobrevivência, apoptose e proliferação, as citocinas tipo IL-6 tem um papel na homeostase celular e tissular. A proteína STAT3 (transdutor de sinal e ativador de fator de transcrição-3) é um mediador crítico para a sobrevivência dos cardiomiócitos (Sano et al., 2000) e parece ser essencial na indução de hipertrofia cardíaca através da glicoproteína gp130 (Kunisada et al., 1998, 2000). Todas as citocinas tipo IL-6 potencialmente ativam STAT3 e, em menor escala, STAT1, através de uma subunidade de gp130 em comum. A infecção de ratos Lewis por *T. cruzi* (cepa Sylvio X10/7) induziu um aumento transitório da expressão de cardiotrofina-1 e gp130 durante a fase aguda da infecção experimental (Chandrasekar et al., 1998). Estes resultados sugerem que a ativação desta via de sinalização possa ter um efeito cardioprotetor, uma vez que a superexpressão destas proteínas em culturas de cardiomiócitos promoveu aumento na sobrevida e hipertrofia destas células (Sheng et al., 1997). Apesar de haver aumento da expressão de gp130 no coração dos animais infectados (Chandrasekar et al.,1998) e da infecção de cardiomiócitos de ratos induzir hipertrofia destas células (Petersen &

Burleigh, 2003), ainda não foi elucidada a participação da via ativada por cardiotrofina durante a infecção por *T. cruzi*. No entanto, a redução na expressão deste transcrito observada em nossos experimentos sugere que a ativação de cardiotrofina-1 descrita por Chandrasekar e colaboradores (1998) pode ser proveniente de macrófagos ativados (Harris et al., 2006), presentes em infiltrados inflamatórios.

Outra observação interessante foi a de que diversos genes que codificam proteínas juncionais foram afetados pela infecção por *T. cruzi*, incluindo *plakoglobin*, *junctional adhesion molecule 3*, e *adipocyte-specific adhesion molecule*, bem como *cadherin 15* (pela cepa Y). Nosso grupo já demonstrou que a infecção de cardiomiócitos por *T. cruzi* altera a expressão de moléculas de adesão como Cx43 (Adesse et al., 2008) e caderina/catenina (de Melo et al., 2008). Como demonstrado num modelo murino de sepse (Celes et al., 2007), alguns tipos de cardiomiopatias apresentam alterações ou mutações em genes relacionados a proteínas do disco intercalar, sugerindo que estas doenças, possivelmente incluindo a CCC, são “doenças juncionais” (revisto por Spray & Tanowitz, 2007).

O segundo tipo de análise que realizamos em nossos estudos de *microarray* foi uma comparação global entre os transcriptomas dos mioblastos infectados com cada cepa, considerando inclusive as pequenas alterações (ou seja, menores que 1,5 vezes). Nestas análises, comparamos os dados obtidos com cada cepa com aqueles obtidos com as outras três cepas, gerando seis combinações altamente correlacionadas ($p<0,001$). Esta abordagem enfatiza o conceito de que apesar da doença de Chagas apresentar um amplo espectro de manifestações *in vivo*, na realidade representa uma síndrome de alterações fenotípicas em comum, como revelado por nossos resultados. Os genes comumente alterados pelas quatro cepas estudadas podem servir como biomarcadores de doença aguda em áreas endêmicas, uma vez que tais cepas são usadas como referência para diversos estudos (Gruson & Borodovitz, 2010).

Para compreender melhor o impacto da infecção por *T. cruzi* na fisiologia da célula hospedeira, estudamos a expressão de Cav-3 em cardiomiócitos infectados *in vitro* e no coração de camundongos durante infecção *in vivo*. Este estudo foi motivado por observações prévias de nosso laboratório, que mostraram alterações na homeostase de cálcio de cardiomiócitos durante infecção *in vitro* (Garzoni et al., 2003), que incluía a redução de sítios de cálcio de superfície e cavéolas das células

hospedeiras. Nossos resultados (**Trabalho #2**) mostraram que a infecção reduziu os níveis de Cav-3 nos cardiomiócitos após 48 h de infecção pela cepa Y e no coração dos camundongos após 66 dias de infecção pela cepa Brazil. Estes resultados corroboram observações prévias que demonstraram redução de caveolinas durante a infecção *in vitro* de adipócitos e no coração de camundongos infectados (Nagajyothi et al., 2006, 2008). A redução nos níveis de Cav-3 cardíaca pode estar relacionada a alterações na homeostase de cálcio intracelular e, consequentemente, à geração de arritmias no tecido cardíaco durante a infecção (Sargiacomo et al., 1993). Diversos patógenos, incluindo vírus, bactérias, fungos e protozoários utilizam caveolina para invasão da célula hospedeira ou causam profundos danos à composição das cavéolas, como discutido no **Trabalho #3**. O tratamento de macrófagos peritoneais (Barrias et a., 2007) ou células HeLa (Fernandes et al., 2007) com metil-β-ciclodextrina, que elimina o colesterol da membrana plasmática, inibiu a invasão de formas tripomastigotas, sugerindo que as cavéolas participem do processo de invasão da célula hospedeira (Barrias et al., 2007).

Além disso, a correta expressão de caveolinas inibe a ativação da via de MAP quinases incluindo a ativação (fosforilação) de ERK 1/2, como demonstrado em modelos KO de Cav-3 e Cav-1/-3 (Engelman et al., 1998, Park et al., 2002; Woodman et al., 2002). Verificamos no **Trabalho#2** que a redução na expressão de Cav-3 induzida pela infecção foi concomitante com a ativação de ERK nas culturas de cardiomiócito a partir da segunda hora de infecção *in vitro* e também na infecção *in vivo*. A ativação de ERK pode estar relacionada com o remodelamento cardíaco observado durante a doença de Chagas e à hipertrofia compensatória dos cardiomiócitos (Lorenz et al., 2009), através da redução de Cav-3.

Para avaliar a recuperação da célula hospedeira após a infecção por *T. cruzi*, testamos diferentes concentrações de amiodarona em culturas primárias de cardiomiócito (Trabalho #4). Utilizando dois protocolos de tratamento, observamos que este composto teve um efeito anti-proliferativo dose-dependente nas formas amastigotas intracelulares (cepa Y), sem efeitos citotóxicos significativos. Este composto já é utilizado como antiarrítmico em pacientes com CCC e recentemente foi demonstrado seu efeito na homeostase de cálcio intracelular de tripanossomatídeos e

na inibição da biossíntese de ergosterol de fungos e protozoários (Benaim et al., 2006; Serrano-Martin et al., 2009a, 2009b).

Utilizando microscopia eletrônica de transmissão observamos que o tratamento com amiodarona induziu importantes alterações morfológicas em amastigotas intracelulares e também nos parasitos liberados pelos cardiomiócitos após 192 h de infecção. Estas alterações incluíram perda de material citoplasmático, danos ao cinetoplasto e a formação de estruturas membranares intracelulares, induzidas possivelmente pela inibição da síntese de esteróis do parasito. Estas alterações foram similares a observações prévias que utilizaram outras classes de inibidores desta via tais como risedronato (Garzoni et al., 2004a), cetoconazol e terbinafina (Lazardi et al., 1990; Vivas et al., 1996).

Nós investigamos se após o tratamento as culturas infectadas seriam capazes de recuperar sua fisiologia, uma vez que a infecção de cardiomiócitos por *T. cruzi* causa a quebra do citoesqueleto da célula hospedeira, incluindo actina, tubulina, desmina, miosina e α-actinina (Pereira et al., 1993; Taniwaki et al., 2006; Melo et al, 2006), alterando a capacidade contrátil das células. Através de microscopia confocal de varredura a laser observamos que nas culturas não infectadas os miócitos exibiam abundantes miofibrilas e que, após 192 h de infecção, as células infectadas e não tratadas apresentaram total ausência de marcação para F-actina. O tratamento com amiodarona por 144 h permitiu o restabelecimento do citoesqueleto de actina nas células cardíacas. A marcação por faloidina demonstrou que as células apresentaram estruturas poligonais típicas de recuperação das miofibrilas (Lin et al., 1989), corroborando resultados prévios que demonstraram que o tratamento com os compostos risedronato (Garzoni et al., 2004a) e pozaconazol (Silva et al., 2006) também induziram recuperação do citoesqueleto de actina e tubulina em cardiomiócitos.

Em seguida avaliamos o efeito do tratamento na imunolocalização de Cx43 nestas culturas. Nós descrevemos previamente que a infecção de cardiomiócitos pela cepa Y de *T. cruzi* induz uma redução de 61% na expressão de Cx43 (Adesse et al., 2008), que pode estar relacionada à redução no acoplamento célula-célula causada pela infecção (de Carvalho et al., 1992). Após o tratamento com amiodarona verificamos a total recuperação da expressão desta proteína, em regiões de contato

célula-célula, como revelado por microscopia confocal. A meia-vida da Cx43 *in vitro* é de aproximadamente 1,5 horas, desde sua síntese no retículo sarcoplasmático, processamento e oligomerização, transporte até o sarcolema e degradação (Beardslee et al., 1998). Seu transporte até a membrana plasmática e estabilização depende de uma interação direta com tubulina (Giepmans et al., 2001), através de uma proteína associada chamada EB1 (Shaw et al., 2007). Esta interação é fundamental para a correta expressão da Cx43 no coração e, em casos de dano ao miocárdio, o transporte desta proteína para o sarcolema é reduzida (Smyth et al., 2010), afetando o acoplamento celular.

Apesar de sua ação anti-arrítmica, através da inibição de canais de K^+, a adição de amiodarona por 10 min não alterou a resistência juncional de cardiomócitos, mesmo na dose de 100 μM (Daleau et al., 1998). No entanto, o tratamento de camundongos com amiodarona por seis meses induziu uma importante redução na expressão de RNAm para Cx43 (Le Bouter et al., 2004). Estas observações indicam que a recuperação de Cx43 após o tratamento das culturas infectadas não está relacionada a uma ação direta de amiodarona sobre esta proteína e sim ao seu efeito tripanocida. É provável que os danos ao citoesqueleto causados por *T. cruzi* sejam responsáveis pela redução nos níveis de Cx43 e que, após o tratamento com amiodarona, haja reconstituição do citoesqueleto e, conseqüentemente, das junções comunicantes.

A expressão de Cx43 cardíaca também está diretamente relacionada à capacidade contrátil dos cardiomiócitos, como demonstrado por diferentes modelos de dano cardíaco (Haefliger & Meda, 2000, Lin et al., 2005). Nós verificamos uma redução significativa na contratilidade espontânea das culturas de cardiomiócito após 96 h de infecção, que foi revertida com o tratamento com amiodarona. Estes resultados estão de acordo com observações prévias demonstrando que a infecção *in vivo* por *T. cruzi* induz diminuição na contração do miocárdio já na fase aguda, consequência também da ação de citocinas pró-inflamatórias (Roman-Campos et al., 2009) ou ainda dos receptores β-adrenérgicos (Enders et al., 2004) no remodelamento mecânico do coração.

Em resumo, nossos resultados demonstraram que a amiodarona é um composto promissor para o tratamento da doença de Chagas, devido ao seu efeito

seletivo tripanocida e por promovera recuperação da fisiologia das células cardíacas, além de sua já conhecida ação antiarrítmica.

CONCLUSÕES

1) A infecção por diferentes cepas de *T. cruzi* gera assinaturas transcriptômicas únicas na célula hospedeira;

2) Os genes alterados da mesma maneira pelas quatro cepas (ex: *DNA-damage inducible transcript 3* e s*erologically defined colon cancer antigen 3*) são possíveis candidatos a biomarcadores para fase aguda da doença de Chagas;

3) A infecção por *T. cruzi* altera diversas vias envolvidas em adesão/comunicação celular (incluindo *junction plakoglobin* e *junctional adhesion molecule 3)*, que no tecido cardíaco podem contribuir para geração de arritmias;

4) Tanto no modelo de infecção *in vitro* quando no tecido cardíaco de camundongos infectados, há redução na expressão de Cav-3 induzida por *T. cruzi,* que pode contribuir com as alterações na homeostase de cálcio intracelular em cardiomiócitos;

5) A redução de caveolina-3 pela infecção foi acompanhada da ativação da via da MAP quinase ERK, tanto *in vitro* quanto *in vivo* e pode estar envolvida em eventos de hipertrofia cardíaca;

6) O composto antiarrítmico amiodarona apresenta atividade seletiva anti-*T. cruzi,* sem causar danos citotóxicos aos cardiomiócitos;

7) Após erradicar o parasitismo, amiodarona promove o restabelecimento da fisiologia de cardiomiócitos, com recuperação de citoesqueleto de actina, Cx43 e da contratilidade espontânea.

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