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Impacto da galectina-3 no curso da infecção experimental por *Trypanosoma cruzi*

Uberlândia,
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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural Aplicadas da Universidade Federal de Uberlândia como requisito parcial à obtenção do título de mestre em Biologia Celular

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RESUMO

A galectina-3 (Gal-3) é uma proteína que pertence à família lectina, apresenta afinidade a carboidratos contendo β-galactosídeos, e pode estar localizada no núcleo, citoplasma, associada à membrana ou secretada. Esta proteína está envolvida em muitos processos imunoregulatórios, tais como adesão de linfócitos T/CD, respostas inflamatórias, migração de células para focos inflamatórios e proliferação celular. Foi visto que a infecção por *Trypanosoma cruzi*, agente etiológico da doença de Chagas, aumenta a expressão de Gal-3. Assim, o objetivo do trabalho foi explorar as atividades biológicas da Gal-3 nas fases aguda e crônica da infecção experimental por *T. cruzi.* . Camundongos C57 / BL6 *Wild-Type* (WT) e galectina-3 *knockout* (Gal-3 KO) foram infectados por via intraperitoneal e foram avaliados quanto a parasitemia, recrutamento de células inflamatórias na cavidade peritoneal, produção de citocinas do baço e coração e fibrose cardíaca. Os dados aqui apresentados demonstraram que a falta de Gal-3 aumentou a parasitemia e reduziu o recrutamento de leucócitos. Em amostras de coração, verificamos um aumento da secreção de TNF-α e IFN-γ em animais WT, enquanto que em animais Gal-3 KO foi detectado o aumento da produção de IL-1β e IL-4 durante a fase aguda. Este cenário pode ter sido responsável pela maior taxa de infecção durante a infecção aguda observada em camundongos deficientes em Gal-3. Observamos que na fase crônica da infecção o tecido cardíaco de camundongos WT apresentou uma resposta imune com perfil Th2, importante para o controle do dano tecidual, com níveis basais de TNF-α e IFN-γ, diminuição na concentração de IL-1β e aumento de IL-4. O aumento de IL-4 é importante para diminuir danos cardíacos e não foi observado em animais Gal-3 KO. Na fase crônica, observou-se um aumento de recrutamento de mastócitos em animais Gal-3 KO e maior fibrose do órgão. Portanto, a Gal-3 apresentou funções quimiotáticas e imunorreguladoras que são necessários para controlar a fase aguda da infecção e diminuir o comprometimento cardíaco na fase crônica.

Palavras-chave: Galectina-3, *T. cruzi*, resposta imune

ABSTRACT

Galectin-3 (Gal-3) is a protein of the lectin-family, has affinity for β-galactose-containing carbohydrates, and can be localized in nucleus, cytoplasm, membrane associated or secreted. This protein is involved in many immunoregulatory processes, such as DC/T lymphocyte adhesion, inflammatory responses and cell migration toward inflammatory foci and cell proliferation. It was also seen that the *T. cruzi* infection, that is the etiological agent of Chagas' disease, increases the expression of Gal-3. Thus, in this paper we aim to explore the biological activities of galectin-3 in acute and chronic *T. cruzi* experimental infection. Mice C57/BL6 Wild-Type (WT) and galectin-3 knockout (Gal-3KO) were infected intraperitoneally and was evaluate parasitaemia, recruitment of inflammatory cells in the peritoneal cavity, production of cytokines in spleen and heart and cardiac fibrosis. The data presented here demonstrate that the lack of Galectin-3 enhanced the parasitaemia and reduced the recruitment of leukocytes. In heart samples, we observed an increased secretion of TNF-α and IFN-γ in WT while in galectin-3 knockout mice we detected increased production of IL-1β and IL-4 during the acute phase. This scenario may have accounted to the higher infection rate during acute infection observed in knockout mice. We observed that in the chronic phase of infection the heart tissue of WT mice showed an immune response to Th2 profile, important to control tissue damage, with basal levels of IFN-γ and TNF-α, decrease in the concentration of IL-1β and increased IL-4. The increase in IL-4 is important for reducing heart damage and was not observed in animals Gal-3 KO. In chronic phase we observed an increased recruitment of mastocyte in Gal-3 KO animals and larger fibrosis of the heart. Therefore, the Gal-3 showed chemotactic and immunoregulatory functions that are needed to control the acute phase of infection and decreased chronic heart damage.

Keywords: Galectin-3, *T. cruzi*, immune response

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AE	Amastigotas Extracelulares
CCC	Cardiopatia Chagásica Crônica
CEUA	Comitê de Ética em Pesquisa no Uso de Animal (CEUA)
CDR	Domínio de Reconhecimento a Carboidratos
D-MEM	*Dulbecco's Modified Eagle's Medium* (D-MEM)
Gal-3	Galectina-3
G3CS	Estruturas Contendo Gal-3 (G3CSs).
KD	*Knockdown*
KO	*Knockout*
LAMP-2	Proteína de Membrana Associada a Lisossomos 2
IFN-γ	Interferon-γ
IL-12	Interleucina-12
iNOS	Óxido Nítrico Sintase induzível
NK	*Natural Killer*
NO	Óxido Nítrico
OMS	Organização Mundial de Saúde
PAMP	Padrões Moleculares Associados A Patógenos
PBS	Tampão Fosfato Salino
TLR	Receptores Toll-like
TNF	Fator de Necrose Tumoral
WT	*Wild-Type*

SUMÁRIO

1.0 INTRODUÇÃO

1.1 *Trypanosoma cruzi*

Trypanosoma cruzi é um protozoário flagelado e agente etiológico da doença de Chagas. De acordo com a Organização Mundial da Saúde cerca de 7 a 8 milhões de pessoas no mundo estão infectadas pelo parasito, principalmente na América Latina (WHO, 2014). No Brasil, é estimado de 2 a 3 milhões de pessoas infectadas e 6.000 mortes anuais pela doença (MARTINS-MELO *et al*., 2014).

O protozoário apresenta ciclo de vida heteroxênico, alternando entre hospedeiro vertebrado (mamíferos de várias espécies) e invertebrado (vetor). Sua classificação o inclui na Ordem Kinetoplastida e Família Trypanosomatidae, apresentando três estágios evolutivos: epimastigota, tripomastigota e amastigota (BRENER, 1973; DE SOUZA, 1984). Os vetores são insetos hemípteros da Família *Reduvidae*, sendo os principais gêneros: *Triatoma*, *Rhodnius* e *Panstrongylus* (BARRETO, 1979).

O ciclo inicia quando o vetor ingere formas tripomastigotas de um hospedeiro portador de *T. cruzi* durante o repasto sanguíneo. Os tripomastigotas passam para o intestino médio do inseto e multiplicam-se como epimastigotas, que após algumas semanas migram para o intestino posterior, onde se diferenciam em tripomastigota metacíclicos. Ao fazer um novo repasto sanguíneo esses vetores defecam em um vertebrado mamífero, e juntamente com as fezes e urina, os parasitas são depositados na pele ou mucosas. Quando os parasitas são depositados na mucosa a penetração ocorre diretamente, no entanto quando são depositados sobre a pele é necessário uma lesão tecidual para que ocorra a penetração, como por exemplo, o ato de coçar o local da picada. Dessa forma, tripomastigotas metacíclicos podem invadir várias células e se diferenciar em amastigotas, que após 20 a 30 horas começam a se multiplicar. Após a multiplicação os amastigotas se diferenciam em tripomastigotas e saem da célula hospedeira para o espaço intercelular ou circulação sanguínea. Esses tripomastigotas, chamados tripomastigotas sanguíneos, são capazes de invadir macrófagos e outras células de vários tecidos e órgãos do hospedeiro, ou serem ingeridos pelo vetor durante o repasto sanguíneo (VIANNA, 1911).

Além da transmissão vetorial a doença de Chagas pode ser transmitida por meio de transfusão sanguínea, transmissão congênita, acidentes laboratoriais, transplante de órgãos e por via oral (DIAS, 1992; LATTES; LASALA, 2014). No Brasil, vários relatos recentes de surtos de contaminação oral pela ingestão de açaí in natura e/ou caldo de cana-de-açúcar tem

sido notificados às autoridades sanitárias do país, que alertam para uma vigilância e controle maior na manipulação e comercialização destes produtos (STEINDEL *et al.*, 2008. MARTINS-MELO, 2014).

Os agentes terapêuticos disponíveis (Benznidazol e o Nifurtimox) são eficazes apenas na fase aguda da doença e apresentam alta toxicidade (VIOTTI *et. al,* 1994; LAURIA-PIRES *et. al,* 2000; CANÇADO, 2002, ROJO *et al.*, 2014). Há décadas não são produzidos novos fármacos que apresentam maior eficácia e menores efeitos colaterais do que os medicamentos utilizados no tratamento da doença de Chagas (SANTOS *et al.*, 2004, MEIJA *et al.*, 2012; HALL; WILKINSON, 2012). Dessa forma, pesquisas que relacionam o agente causador da doença de Chagas e possíveis proteínas envolvidas nessa doença são essenciais para melhora do entendimento da doença.

1.2 Doença de Chagas

1.2.1 Fase aguda

A fase aguda da doença de Chagas compreende os fenômenos clínicos dos primeiros meses de infecção e delimita-se pela demonstração de parasito no sangue. Assim, ocorre uma parasitemia que pode durar de 40 a 60 dias, com sintomas moderados e atípicos, podendo ser confundido com os de outras infecções, por isso a doença de Chagas com grande freqüência não é reconhecida neste estágio (LARANJA, 1953; WHO, 2002; BASTOS *et al.*, 2010).

Nesta fase, as formas amastigotas podem ser encontradas no interior de fibras cardíacas, células musculares lisas e esqueléticas, células endoteliais, gliais, macrófagos e fibroblastos; podendo causar insuficiência cardíaca ou meningoencefalite (TORRES, 1917; JUNQUEIRA *et al.*, 2010).

Alguns sinais e sintomas podem aparecer, como: febre diária, dor de cabeça, gânglios linfáticos aumentados, palidez, dor muscular, dificuldade em respirar, inchaço e dor abdominal ou torácica (WHO, 2014). O exame físico frequentemente mostra linfadenopatia generalizada, com linfonodos palpáveis em regiões cervicais, axilares e inguinais; e hepatoesplenomegalia moderada. Podendo ser observadas, ainda, manifestações de comprometimento cardíaco e de meningoencefalite (CHAGAS, 1936; RASSI; MARIN-NETO, 2010, WHO, 2014).

A fase aguda apresenta uma taxa de letalidade baixa (2 a 10%), observada

principalmente em crianças e indivíduos imunodeprimidos que adquirem a infecção por transfusão ou transplante de órgãos. As principais causas de óbito são a miocardite acompanhada de insuficiência cardíaca refratária e meningoencefalite (CHAGAS, 1916; DIAS, 1992; TEIXEIRA *et al.*, 2006).

1.2.2 Fase crônica

Após a fase aguda da Doença de Chagas o paciente entra, na fase crônica que é caracterizada por parasitemia ausente ou baixa, mesmo que o paciente apresente sorologia positiva para a doença em exames de rotina. Essa fase apresenta a seguintes formas clínicas: indeterminada ou assintomática e sintomática (cardíaca e digestiva).

Entende-se por fase indeterminada da doença de Chagas a presença de infecção chagásica na ausência de manifestações clínicas, eletrocardiográficas ou radiológicas de acometimento cardíaco ou digestivo (Reunião de pesquisa aplicada em doença de Chagas, 1985).

A fase indeterminada é a fase mais comum da doença de Chagas e é de grande interesse médico-social em que os pacientes apesar de serem assintomáticos podem evoluir para forma determinada e por isso necessitam de um acompanhamento médico. Nessa fase o parasito está presente, mas os indivíduos infectados apresentam focos de amastigotas raros e esparsos e a reação inflamatória focal é pouco intensa. Cerca de 40% dos infectados irão manifestar alguma das formas crônicas da tripanossomíase após um período de 10 a 20 anos (MACEDO, 1980; RASSI, 2010; WHO, 2014).

Até 30% dos pacientes infectados sofrem de distúrbios cardíacos e até 10% sofrem de distúrbios digestivos (tipicamente com megacólon e/ou megaesôfago), alterações neurológicas ou ambas. Nos anos posteriores, a infecção pode levar à morte súbita ou insuficiência cardíaca causada pela destruição progressiva do músculo cardíaco (WHO, 2014).

1.3 Resposta imunológica contra *T. cruzi*

A resposta imune inata e adaptativa desempenha um importante papel no controle da infecção por *T. cruzi*, com o envolvimento de vários tipos celulares incluindo células *Natural Killer* (NK), linfócitos T CD4+, T CD8+, macrófagos bem como de anticorpos produzidos por linfócitos B (BRENER; GAZZINELLI, 1997; DOS REIS, 1997; GOLGHER; GAZZINELLI, 2004).

Os receptores Toll-like (TLR) estão presentes em todas as células do sistema imume inato e são capazes de reconhecer padrões moleculares associados a patógenos (PAMP) de *T. cruzi*, com isso há o recrutamento de proteínas intracelulares, com posterior ativação de fatores de transcrição e expressão de genes envolvidos na resposta inflamatória, como o Fator de Necrose Tumoral (TNF), Interferon-γ (IFN-γ) e Interleucina-12 (IL-12), controlando a replicação do parasito (JANEWAY, 1998; ABBAS; LICHTMAN, 2002; CAMPOS; GAZZINELLI, 2004; GAZZINELLI; DENKERS, 2006).

A IL-12 é produzida principalmente por macrófagos, sendo capaz de atuar em células NK da resposta imune inata e em linfócitos T da resposta adaptativa, induzindo-as a produzirem IFN-γ e TNF-α (MICALLEF *et al.*,1996, WATFORD *et al.*, 2003). As citocinas IFN-γ e TNF-α podem induzir a enzima óxido nítrico sintase induzível (iNOS) a sintetizar óxido nítrico, composto tóxico que pode causar a eliminação de patógenos intracelulares ou a inibição da replicação (GAZZINELLI, 1992, PAVANELLI; SILVA, 2010; ABEL *et al.*, 2014).

IFN-γ é uma citocina pró-inflamatória cuja atuação na ativação de macrófagos mostra-se essencial para que estas células sejam capazes de eliminar patógenos intracelulares. Nossos resultados em modelos murinos mostraram que a cepa G é altamente suscetível à IFN-γ logo no início da infecção e que tal citocina tem significativa importância na resposta a *T. cruzi*, sendo que animais IFN- *knockout*, são mais susceptíveis a infecção, e apresentam maior índice de parasitemia e mortalidade (RODRIGUES *et al.*, 2012). A atividade anti-parasitaria de IFN-γ resulta de uma combinação de efeitos, os quais incluem a produção de óxido nítrico (NO) (GAZZINELLI, 1992, de MELO *et al.*, 2012), indução de MHC de classe II, polarização de células T para um perfil Th1, troca isotípica de imunoglobulinas, induzindo predomínio de IgG2a, e produção de quimiocinas (GAZZINELLI, 1992; CHESSLER *et al.*, 2009).

TNF-α possui função similar a de IFN-γ, potencializando a ativação de macrófagos em sua atividade microbicida (BEUTLER; CERAMI, 1989; ALIBERTI *et al.*, 2001; PEREIRA *et*

al., 2014). Em contrapartida, o perfil de citocinas IL-10, IL-4 e TGF-β estão associadas com a susceptibilidade ao parasito (RODRIGUES *et al.*, 2000; HIYAMA *et al.*, 2001, PEREIRA *et al.*, 2014).

Portanto, estudos envolvendo a regulação da resposta imune e as citocinas secretadas durante a infecção são importantes para compreender o processo de controle parasitário e a ação protetiva e deletéria dessas citocinas para o hospedeiro.

1.4 Galectina-3

Lectinas são proteínas que se ligam a carboidratos e podem reconhecer glicoproteínas, glicolipídeos e outras moléculas como proteínas e lipídeos (LIS; SHARON, 1998). As galectinas são pertencentes à família das lectinas, possuem afinidade por ß-galactosídeos e podem ser encontrada na matriz extracelular, superfície celular e no meio intracelular (citoplasma e núcleo) (MENON; HUGHES, 1999; HOUZELSTEIN *et al.*, 2004; LEFFLER *et al.*, 2004; FUNASAKA; RAZ; NANGIA-MAKKER, 2014). Dentre elas, a mais estudada é a galectina-3 (Gal-3) que possui peso molecular de 31 kDa e em solução encontra-se como um monômero de 2 domínios funcionais: o N-terminal e o C-terminal que contem o domínio de reconhecimento a carboidratos (CRD) (BARONDES *et al*., 1994; LIU; RABINOVICH, 2005).

A Gal-3 está envolvida em vários eventos biológicos como adesão celular, regulação do ciclo celular, apoptose, sinalização intracelular e resposta imunológica (DUMIC; DABELIC; FLÖGEL, 2006; SALOMONSSON *et al*., 2010). A função extracelular dessa proteína é dependente da propriedade lectina do domínio C-terminal, podendo ser inibida por ligantes sacarídicos específicos como a lactose (SANO *et al.*, 2000).

Estudos mostraram que ocorre interação entre a Gal-3 e proteínas de superfície de tripomastigotas de *T. cruzi*, e esse acontecimento pode ser inibido pela lactose. Além disso, foi demonstrado que a expressão de Gal-3 é requerida para adesão de tripomastigotas às células, e que com a ligação da Gal-3 exógeno à superfície do parasito e também à superfície de células musculares há um aumento das taxas de adesão. Foi visto também que a infecção por *T. cruzi* aumenta a expressão de Gal-3 e de seus ligantes (MOODY; OCHIENG; VILLALTA, 2000; KLESHCHENKO *et al*., 2004; VRAY *et al*., 2004).

Nossos experimentos anteriores demonstraram que a Gal-3 desempenha um papel no tráfego intracelular de amastigotas extracelulares (AE) em macrófagos peritoneais, foi visto

recrutamento de Gal-3 e actina nos tempos inicias correspondentes aos sítios de entrada do parasito. Esse recrutamento se prolongou até o tempo de 6 horas onde houve uma polarização da Gal-3 em uma das extremidades do parasito formando estruturas contendo Gal-3 (galectin-3-containing structures - G3CSs). Observamos que a formação de G3CS era concomitante com o desaparecimento da Proteína de Membrana Associada a Lisossomos 2 (LAMP-2) e, portanto, essa estrutura pode ser considerada um marcador de lise de vacúolo parasitóforo de *T. cruzi* (MACHADO *et al.*, 2014). A formação de G3CS não foi observada em vacúolos contendo esferas fluorescentes, confirmando sua presença apenas em eventos de lise de vacúolo (REIGNAULT *et al.*, 2014).

Trabalhos relacionados com carcinoma gástrico sugerem que elevadas taxas de Gal-3 promovem o aumento da motilidade celular e metástase (BALDUS *et al.*, 2000; NANGIA-MAKKER; BALAN, RAZ, 2012; FORTUNA-COSTA *et al.*, 2014). Isso ocorre supostamente por meio da regulação exercida pela Gal-3 em uma proteína chamada fascina-1 (SEOK *et al.*, 2010) . A fascina-1 é uma proteína que se liga à actina, se localiza ao longo dos filopódios e induz protuberâncias da membrana, aumentando assim a motilidade da célula (KUREISHY; SAPOUNTZI; PRAG, 2002; KIM *et al.*, 2010). Esses trabalhos são evidencias que a Gal-3 está relacionada ao citoesqueleto de actina e que estudos com essa proteína são essenciais para a compreensão da sua função.

Em experimentos anteriores observamos um aumento da multiplicação de AE de *T. cruzi* da cepa G em células BeWo Gal-3 *knockdown* (KD) e macrófagos peritoneais Gal-3 *knockout* (KO) quando comparadas com as respectivas células tipo selvagem. Ou seja, as células que possuíam a expressão de Gal-3 comprometida apresentaram um aumento da multiplicação de AE, sugerindo que essa proteína desempenha um importante papel no controle da multiplicação parasitária ao longo da infecção. A partir desses dados, este trabalho busca explicar o envolvimento da Gal-3 na infecção por *T. cruzi*.

Dessa forma, a ausência da Gal-3 se mostra suficiente para propiciar um ambiente favorável à multiplicação intracelular de amastigotas. Este resultado sugere a possibilidade da participação da Gal-3 na polimerização do citoesqueleto de actina durante a infecção por *T. cruzi*, e dessa forma, essa proteína possui um papel importante no controle da replicação do parasito e, por conseguinte, na propagação da doença. Acreditamos que após a fase aguda da doença de Chagas, fatores (citocinas, quimiocinas ou outras moléculas) liberados pelo hospedeiro induzam uma maior polimerização de actina na célula infectada para silenciar a infecção. Uma outra hipótese, que não exclui a primeira, seria a de que o parasito por meio de controle de expressão gênica possa evitar a resposta do hospedeiro induzindo uma maior

expressão de proteínas que atuam diretamente na polimerização da actina.

Trabalhos relatam que a Gal-3 tem um papel pró-inflamatório e fibrogênico (JIANG *et al.*, 2012; LI; LI; GAO, 2014). Baseado nesses achados, investigamos a progressão da infecção por *T. cruzi* em animais galectina-3 *knockout* (Gal-3 KO) em comparação com animais *Wild-Type* (WT) e analisamos os níveis de parasitemia e fibrose cardíaca. A hipótese de que a Gal-3 pode estar relacionada com a fibrose e inflamação em vários órgãos, mostra que a aquisição destes conhecimentos é de grande importância, dentre outras razões, pela possibilidade de adoção de medidas profiláticas e terapêuticas.

2.0 JUSTIFICATIVA

De acordo com a Organização Mundial de Saúde (OMS) há uma estimativa que em 2014 existam cerca de 7 a 8 milhões de pessoas no mundo infectadas por *T. cruzi*, principalmente na América Latina (WHO, 2014).

No Brasil foram relatados diversos surtos de doença de Chagas por via oral, o primeiro foi em 1968 em uma escola agrícola do município de Estrela (RS) onde 17 alunos apresentaram um quadro agudo da doença de Chagas e seis morreram. No período de 1965-2009 foi relatado de 7 a 8 surtos com 112 casos agudos da doença. Em contraste, em um período de 10 anos (2000-2010) foram reportados mais de 1000 casos agudos em 138 surtos, principalmente na Amazônia do Brasil, sendo 71% desses casos ocorreram devido à ingestão de alimentos contaminados (SHIKANAI-YASUDA; CARVALHO, 2012).

O tratamento convencional contra o *T. cruzi* com Benznidazol (N-benzil-2-nitro-imidazol-1-acetamida) e nifurtimox não apresentam atividade significativa na fase crônica da doença de Chagas (VIOTTI *et. al*, 1994; LAURIA-PIRES *et. al*, 2000; CANÇADO, 2002). Além disso, os metabólitos (eletrofílicos) formados por meio do mecanismo de ação dessas drogas possuem baixa especificidade de ação pelas vias bioquímicas do parasita, o que contribui para os efeitos citotóxicos observados no tratamento dos pacientes (CASTRO, MECA, BARTEL, 2006).

Experimentos *in vitro* anteriores mostraram que a Gal-3 é uma proteína essencial para o controle da multiplicação do *T. cruzi*. Desta forma, estudos relacionados com a atividade da Gal-3 são importantes para melhor compreensão da infecção por *T. cruzi*. A melhor compreensão de moléculas envolvidas na interação parasito-hospedeiro pode futuramente contribuir na identificação de novos alvos terapêuticos para minimizar os efeitos da doença de Chagas. Neste projeto, tentamos esclarecer o papel da proteína Gal-3 no controle do parasito, resposta imune e fibrose frente à infecção por *T. cruzi*.

3.0 OBJETIVOS

3.1 Objetivo Geral

1. Estudar o envolvimento da proteína Gal-3 na infecção por *T. cruzi in vivo*.

3.2 Objetivos específicos

1. Avaliar a parasitemia e o número de parasitos livres na cavidade peritoneal em animais WT e Gal-3 KO infectados.

2. Analisar o recrutamento de leucócitos totais após a infecção intraperitoneal em animais WT e Gal-3 KO.

3. Comparar a área do baço e coração de animais WT e Gal-3 KO infectados.

4. Comparar o perfil de citocinas pró e anti-inflamatórias dos animais WT e Gal-3 KO nas fases aguda e crônica da infecção por *T. cruzi.*

5. Analisar a presença de mastócitos e a fibrose do coração de camundongos WT e Gal-3 KO infectados.

4.0 MATERIAL E MÉTODOS

4.1 Aspectos Éticos e Legais

Este estudo faz parte do projeto intitulado: "Estudos de moléculas envolvidas na invasão e tráfego intracelular das formas infectivas de *Trypanosoma cruzi in vitro* e *in vivo*. Emprego da P21 na indução de proteção contra a doença de Chagas.", aprovado pelo Comitê de Ética na Utilização de Animais da Universidade Federal de Uberlândia (CEUA/UFU) (Protocolo número 059/08).

Os procedimentos experimentais foram realizados no Laboratório de Tripanosomatídeos (Bloco 6T sala 07) do Instituto de Ciências Biomédicas da UFU (ICBIM – UFU).

4.2 Animais

Camundongos C57BL/6 Gal-3 KO foram gentilmente cedidos pela Prof. Maria Cristina Roque Antunes Barreira, da Universidade de São Paulo. Camundongos C57BL/6 selvagens (WT) e C57BL/6 Gal-3 KO foram mantidos em condições padrões de 12 horas de luz-12 horas na ausência de luz, em temperatura de 25 ± 2 °C, com comida e água "*ad libitum"*. Os animais utilizados nos experimentos tinham de 6 a 8 semanas. Toda manutenção, manipulação e eutanásia foram realizadas de acordo com o Comitê de Ética em Pesquisa no Uso de Animal (CEUA), UFU.

4.3 Células Vero

Células Vero foram utilizadas para manutenção do parasito. As células foram cultivadas em meio *Dulbecco's Modified Eagle's Medium* (D-MEM) (Gibco BRL, Gaithersburg, MD) contendo L-glutamine and D-glucose (4500 mg/l), bicarbonato de sódio (2000 mg/L), e suplementado com tampão HEPES (2380 mg/L), piruvato de sódio, soro fetal bovino (10%) e complexo de três antibióticos: penicilina (60 mg/L), gentamicina (40 mg/L) e estreptomicina (10 mg/L). As garrafas de cultura foram mantidas em estufa de CO_2 (5% de CO_2) a 37ºC.

4.4 Parasito

Neste estudo utilizamos tripomastigotas de *T. cruzi* da cepa CL mantidos em células Vero e meio D-MEM suplementado com 2,5% de soro fetal bovino.

4.5 Leucócitos da cavidade peritoneal

Para análise do recrutamento de leucócitos e do número de parasitos viáveis foi injetado na cavidade peritoneal de camundongos C57BL/6 WT e Gal-3 KO 10^5 tripomastigotas de *T. cruzi* da cepa CL. Após 72 horas os animais foram eutanasiados por deslocamento cervical e uma lavagem peritoneal foi realizada com 5 mL de meio DMEM 10% gelado. Foram utilizados 4 animais de cada grupo.

Os leucócitos peritoneais e os parasitos livres na cavidade peritoneal foram contados em câmara de Neubauer.

4.6 Infecção de WT e Gal-3 KO para análise *in vivo*

C57BL/6 WT e Gal-3KO de seis a oito semanas de idade, foram infectados com tripomastigotas de *T. cruzi* da cepa CL via intra-peritoneal (10^5 parasitos por camundongo), usando seringa de 1 mL. Um grupo de camundongos não infectados foi utilizado como controle negativo. Foram utilizados 6 animais de cada grupo.

Os animais foram sacrificados 15 dias (fase aguda) e 90 dias (fase crônica) pós-infecção e foram removidos cirurgicamente o baço e o coração. Imagens dos órgãos com escalas foram capturadas por meio de câmera de 13 megapixels do aparelho SONY Xperia ZQ e analisadas no programa Image J. Posteriormente, uma quantidade padronizada de cada órgão foi colocada em 1 mL de inibidor de protease diluído em tampão fosfato salino (PBS) para dosagem de citocinas por meio de ELISA e outra parte foi utilizada para análises histológicas.

4.7 Parasitemia

Após 7, 15, 30, 60 e 90 dias de infecção foi coletado sangue da cauda dos camundongos para análise da parasitemia (quantidade de parasitas no sangue) dos grupos WT e Gal-3 KO. 5 µL de sangue foi adicionado em uma lâmina e foi contado o número total de parasitos para posterior comparação e avaliação da importância da proteína Gal-3 na infecção por *T. cruzi*.

4.8 Área dos órgãos

Para avaliação da área do baço e coração, dos animais infectados e não infectados, imagens foram capturadas na presença de uma escala (régua graduada em centímetros) e analisadas no software Image J.

4.9 Dosagem de Citocinas

Foi feito a dosagem de citocinas do baço e coração de animais WT e Gal-3 KO infectados e não infectados por *T. cruzi.* Uma quantidade padronizada dos órgãos foram mantidos em 1 mL de inibidor de protease para evitar a degradação dessas proteínas e as amostras foram sonicadas. Todos os procedimentos e reagentes para dosagem de IFN-γ, TNF-α, IL-1β e IL-4 foram provenientes do kit BD Biosciences®.

Primariamente, a placa de ELISA de 96 poços foi sensibilizada *overnight* com o anticorpo de captura da citocina em questão, diluído em tampão específico. A seguir, a placa foi lavada e bloqueada com PBS suplementado com soro fetal bovino (10%) por 1 hora. Subsequentemente, as amostras foram adicionadas nos poços e para obter-se a curva padrão utilizou-se 50 µL das citocinas recombinantes em concentrações decrescentes. Finalmente, foi adicionado o anticorpo de detecção e a enzima a cada poço. Posteriormente adicionou-se substrato para revelação do resultado. A leitura foi feita em absorbância de 450 nm, e foi comparada a dosagem de citocinas nos órgãos dos animais WT e Gal-3 KO para verificarmos o papel dessa proteína na imunomodulação.

4.10 Análise histológica

O coração foi processado para análises histológicas. Os órgãos foram fixados em formaldeído (10% em PBS), posteriormente desidratados em álcoois de crescentes concentrações, diafanizados em xilol e finalmente incluídos em parafina. Foram feitos cortes de 5 µm de espessura e colocados em lâminas de vidro. Subsequentemente as lâminas foram processadas para coloração.

4.10 Coloração de azul de toluidina

Para análise do número de mastócitos recrutados nos diferentes grupos, os cortes foram corados com azul de toluidina.

Primeiramente, os cortes foram desparafinados com 3 banhos de xilol de 15 minutos cada e passaram pelo processo de hidratação com concentrações decrescentes de álcool (100, 100, 100, 95, 85, 70 % por um minuto), 10 minutos de água corrente e 5 minutos de água destilada. Os cortes foram então colocados em tampão fosfato citrato pH 3.0 por 5 minutos e corados com azul de toluidina 0.5% por 3 minutos. O excesso do corante foi retirado mergulhando as lâminas no tampão.

Posteriormente, foi feito a diafanização com concentrações crescentes de álcool (95% e três banhos de 100%) por 2 segundos e três banhos em xilol por 10 minutos cada. A montagem da lâmina foi feita com Entellan®.

O *software* Image J foi utilizado para mensuração da área do corte histológico a partir de imagens capturadas no microscópio de luz com câmera acoplada Leica DM500. Os dados foram analisados por meio da proporção entre o número total de mastócitos e a área do corte histológico (número total de mastócitos/ cm^2).

4.11 Coloração de picrosirius

Para análise da participação da Gal-3 na fibrose após a infecção por *T. cruzi* foram realizadas análises histológicas dos corações corados com picrossirius.

Os cortes foram submetidos a desparafinação em três banhos sucessivos de um minuto

com xilol, hidratados em soluções de concentrações decrescentes de etanol (100%, 90%, 70%) e água por um minuto; e então corados, com solução de picrossirirus durante 50 minutos e lavados em água destilada. A seguir, os cortes foram colocados em solução de hematoxilina por quatro segundos, lavados em água corrente por cinco minutos e água destilada por um minuto e corados durante um minuto com eosina aquosa. Posteriormente, foram desidratados em soluções crescentes de etanol (70%, 90% e dois banhos de absoluto) por um minuto. A diafanização foi feita em três banhos de xilol de um minuto cada. A montagem da lâmina foi feita com Entellan®.

A análise da quantidade de fibras colágenas foi feita por meio do *software* Image J a partir de imagens de 30 campos de cada corte histológico utilizando o microscópio de luz com câmera acoplada Leica DM500 com a objetiva de 40X .

4.12 Normas de Biossegurança

Todos os procedimentos envolvendo manuseio dos parasitas, de animais, bem como a utilização de equipamentos foram realizados de acordo com as normas de biossegurança descritas por Mineo (2005).

4.13 Análise Estatística

A análise estatística dos dados foi feita com o programa GraphPad Prism, versão 6.01. Os dados foram expressos como média ± desvio padrão. A comparação de resultados entre os grupos foi analisada pelo teste *t* ou ANOVA. Os resultados foram considerados significantes quando $p<0,05$.

5.0 RESULTADOS E DISCUSSÃO

5.1 Avaliação da atuação da Gal-3 no controle do *T. cruzi* e recrutamento de leucócitos após a infecção

Com o intuito de observar o papel da proteína Gal-3 na infecção por *T. cruzi* foram feitos experimentos *in vivo* com animais WT e Gal-3 KO. Os animais *knockout* apresentaram uma maior parasitemia (Figura 1A) quando comparados com os animais WT nos tempos de 7, 15 e 30 dias após a infecção, com significância nos tempos de 15 e 30 dias.

Na Figura 1A é possível observar que os dois grupos apresentaram pico de parasitemia no tempo de 15 dias e decréscimo no tempo de 30 dias, entretanto nos animais Gal-3 KO a quantidade de parasitos no sangue foi significativamente maior. 60 e 90 dias após a infecção houve ausência de parasitemia nos animais dos dois grupos. Ou seja, a falta da proteína Gal-3 retardou o controle parasitário e comprometeu a diminuição do número de parasitos no sangue nos primeiros dias de infecção. Dessa forma, a proteína pró-inflamatória Gal-3 se mostra importante no controle inicial da infecção experimental por *T. cruzi*.

Para determinar se a Gal-3 pode estar envolvida na regulação da migração de leucócitos para a cavidade peritoneal e no controle da infecção por *T. cruzi*, foi feita infecção intraperitoneal por 72 horas em animais WT e Gal-3 KO. É possível perceber que o grupo Gal-3 KO infectado apresentou um maior número de parasitos livres no lavado peritoneal e um menor número de leucócitos totais quando comparado com o grupo WT infectado. Confirmando que a Gal-3 é uma proteína importante para o controle da multiplicação parasitária e aumenta significativamente o recrutamento de células do sistema imune.

Dessa forma, os dados *in vivo* estão de acordo com nossos estudos anteriores *in vitro* que mostraram maior multiplicação de *T. cruzi* em células deficientes em Gal-3 (da SILVA, 2012).

Estudos com *Toxoplasma gondii* também evidenciam que animais Gal-3 KO apresentam uma maior carga parasitária no cérebro e pulmões, menor infiltrado de neutrófilos e macrófagos na cavidade peritoneal e menor taxa de sobrevivência (BERNARDES *et al.*, 2006). Além disso, animais Gal-3 KO infectados com *Paracoccidioides brasiliensis* também apresentaram uma maior susceptibilidade à infecção quando comparados com os animais WT (RUAS *et al.*, 2009).

Considerando esses relatos e os resultados do presente estudo, é notável que a Gal-3 pode exibir diversos papéis em diferentes modelos de infecção e se mostra importante tanto

no controle da infecção por *T. gondii* e *P. brasiliensis* quanto no controle da fase aguda da infecção por *T. cruzi*.

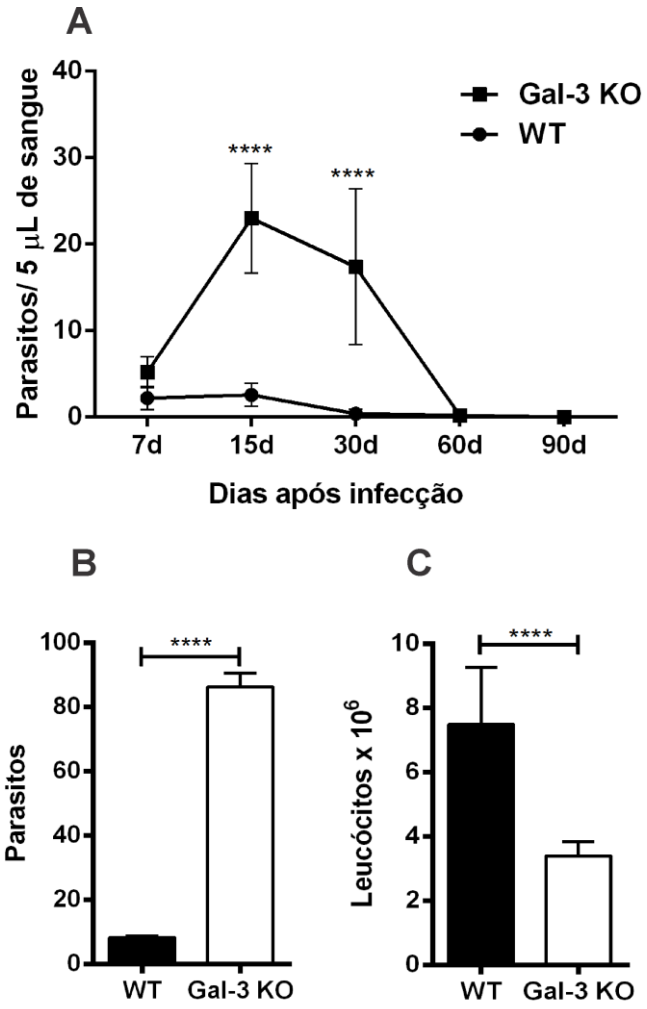

Figura 1. Parasitemia, número de parasitos livres na cavidade peritoneal e recrutamento de leucócitos em camundongos C57BL/6 WT e Gal-3 KO infectados com tripomastigotas de *T. cruzi* da cepa CL. O número de parasitos em 5 µl de sangue foi contado nos tempos de 7, 15, 30, 60 e 90 dias pós-infecção. Animais Gal-3 KO apresentaram maiores parasitemias nos tempos de 15 dias ($p<0,0001$) e 30 dias ($p<0,0001$) quando comparados com os animais WT (A). Quantificação de parasitos livres na cavidade peritoneal (B) e do recrutramento de leucócitos totais (C). Após a infecção com tripomastigotas da cepa CL por 3 dias, os animais do grupo Gal-3 KO apresentaram um maior numero de parasitos livres na cavidade peritoneal ($p<0,0001$) (B) e menor recrutamento de leucócitos totais ($p<0,0001$) (C).

5.2 Relação entre a área do baço e coração de animais WT e Gal-3 KO e a infecção por *T. crzui*

O software Image J foi utilizado para análise da área dos órgãos, e foi possível observar que os animais infectados apresentaram baços maiores em relação aos animais não infectados (Figura 2 A e C) nos dois grupos. Esse resultado pode ser explicado pelo fato de que o baço é o principal local de respostas imunológicas provenientes do sangue, sendo composto por linfócitos, macrófagos, células dendríticas e plasmócitos.

A infecção por *T. cruzi* pode levar a alta reatividade do baço, expansão dos folículos linfoides e proliferação de linfócitos durante a fase aguda da doença (PEREIRA, 1999). Alguns autores já relataram alterações sistêmicas da resposta imune durante a fase inicial da infecção, levando a esplenomegalia e linfadenopatia, com ativação policlonal de linfócitos T e B (MINOPRIO, 2001; BRENER; GAZINELLI, 1997). É possível observar que após a esplenomegalia da fase aguda, houve uma diminuição da área do baço durante a fase crônica. Entretanto, os animais infectados, mesmo na fase crônica, apresentaram áreas do baço maiores que o controle (Figura 2C). Essa resposta pode estar relacionada à presença de pequenas quantidades de *T. cruzi* e seus antígenos nos tecidos na fase crônica.

Trabalhos relatam aumento da expressão de Gal-3 em linfócitos T ativados (JOO *et al.*, 2001). Em nossos experimentos foi visto que tanto camundongos WT quanto Gal-3 KO infectados apresentaram esplenomegalia, o que sugere que a Gal-3 não é responsável pelo processo de esplenomegalia observada nestes animais. Ainda não se sabe se a esplenomegalia em animais Gal-3 KO infectados é resultado da proliferação de linfócitos T e se a deficiência em Gal-3 pode levar ao comprometimento da ativação de linfócitos. Dessa forma, a causa do aumento do baço em animais Gal-3 KO ainda precisa ser investigada.

Foram vistas diferenças na infecção por *T. cruzi* na ausência e presença de Gal-3 e apesar do tropismo cardíaco da cepa CL, não foi observado alterações na área do coração dos diferentes grupos (Figura 2B e D).

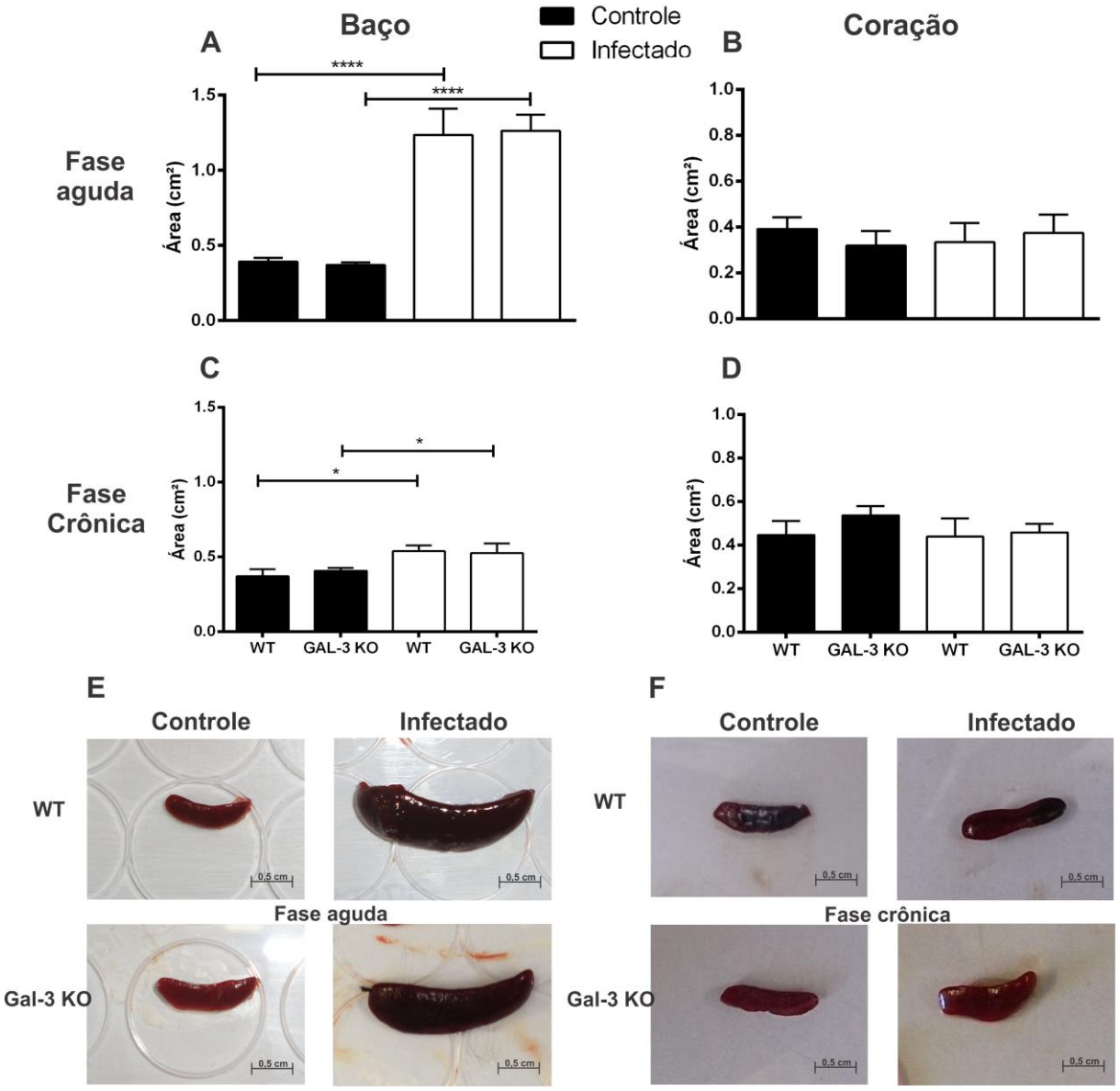

Figura 2- Análise da área do baço e coração de animais WT e Gal-3 KO infectados ou não com tripomastigotas da cepa CL. A área do baço (A e C) e coração (B e D) nas fases: aguda (A e B -15 dias) e crônica (C e D- 30 dias) foi mensurada utilizando o software ImageJ. Animais infectados (WT e Gal-3 KO) apresentaram uma área maior do baço durante fase aguda ($p<0,0001$) (A e E) e durante a fase crônica ($p<0,05$) (C e F). Não houve diferença estatística na área do coração nos diferentes grupos.

5.3 Análise de citocinas na fase aguda da infecção por *T. cruzi*

Nossos resultados anteriores mostraram que a deficiência em Gal-3 favorece a multiplicação do parasita. Neste sentido foram avaliados IL-1β, TNF-α, IFN-γ e IL-4 em amostras de baço e coração de animais WT e Gal-3 KO, durante a fase aguda e crônica da infecção por *T. cruzi* da cepa CL.

A dosagem de citocinas por meio da técnica ELISA mostrou que os animais WT infectados durante a fase aguda apresentaram um aumento de IL-1β no baço (Figura 3A), aumento de TNF-α no coração (Figura 3D) e aumento de IFN-γ no coração e baço em relação ao controle não infectado (Figura 3 E e F), mas em animais Gal-3 KO isso não foi observado.

Observou-se também um aumento da citocina IL-1β no coração de animais Gal-3 KO infectados por *T. cruzi* (Figura 3B) na fase aguda. O sistema imunológico possui uma complexidade que envolve a participação de muitos componentes estruturais, celulares e moleculares. Assim, o aumento de IL-1β na ausência de Gal-3 pode ser explicado como forma de compensação da deficiência de outras citocinas pró-inflamatórias, como IFN-γ e TNF-α. Entretanto, esse aumento não foi suficiente para o controle da infecção, visto que animais Gal-3 KO apresentaram uma parasitemia relativamente alta.

A figura 3 demonstra que não houve alteração na concentração de IL-4 no baço e coração de animais WT infectados quando comparados com animais WT não infectados (G e H). Camundongos Gal-3 KO apresentaram um aumento na concentração de IL-4 no coração após a infecção, indicando que a deficiência na proteína Gal-3 ocasiona a diminuição de citocinas do perfil Th1 e aumento de citocinas do perfil Th2 na fase aguda.

Segundo alguns autores, existe uma relação entre o tipo de citocinas produzidas após a infecção por *T. cruzi* e o padrão de resistência ou susceptibilidade à doença de Chagas. Destacando que citocinas do perfil Th1, tais como IFN-γ, TNF-α e IL-12 promoveria resistência ao parasito, enquanto citocinas do perfil Th2, tais como IL-10, IL-4 e TGF-β promoveriam a susceptibilidade (SAVINO *et al.*, 2007).

Os dados de quantificação de citocinas e parasitemia, que revelam a importância da Gal-3 na polarização da resposta imune para o perfil Th1 com secreção de TNF-α, IFN-γ e diminuição de IL-4, estão em concordância com outros trabalhos que relatam que a ativação precoce do sistema imune, com estimulação da síntese de IL-12, TNF-α e IFN-γ, ativam as células NK e favorecem a diferenciação dos linfócitos T no fenótipo Th1, levando ao controle da replicação do parasito na fase aguda da doença e maior resistência do hospedeiro ao *T. cruzi* (VESPA *et al.*, 1994; ALIBERTI *et al.* 1996; HOLSCHER *et al.*, 1998).

É conhecido que a resposta imunológica do hospedeiro é extremamente importante na determinação dos resultados da doença de Chagas (DUTRA; GOLLOB, 2008), exercendo papel importante nos seus efeitos patológicos (BILATE; CUNHA-NETO, 2008). Tendo em vista que a doença de Chagas é uma doença infecciosa com lesões de caráter inflamatório, o entendimento da resposta imune e do papel de proteínas do hospedeiro frente a uma infecção é essencial para se entender a biologia básica da relação parasito-hospedeiro e os efeitos patológicos causados.

Assim, os dados indicam fortemente que a Gal-3 desempenha um papel importante como mediador pró-inflamatório na infecção por *T. cruzi* e está relacionada com o controle parasitário (JEON *et al*., 2010).

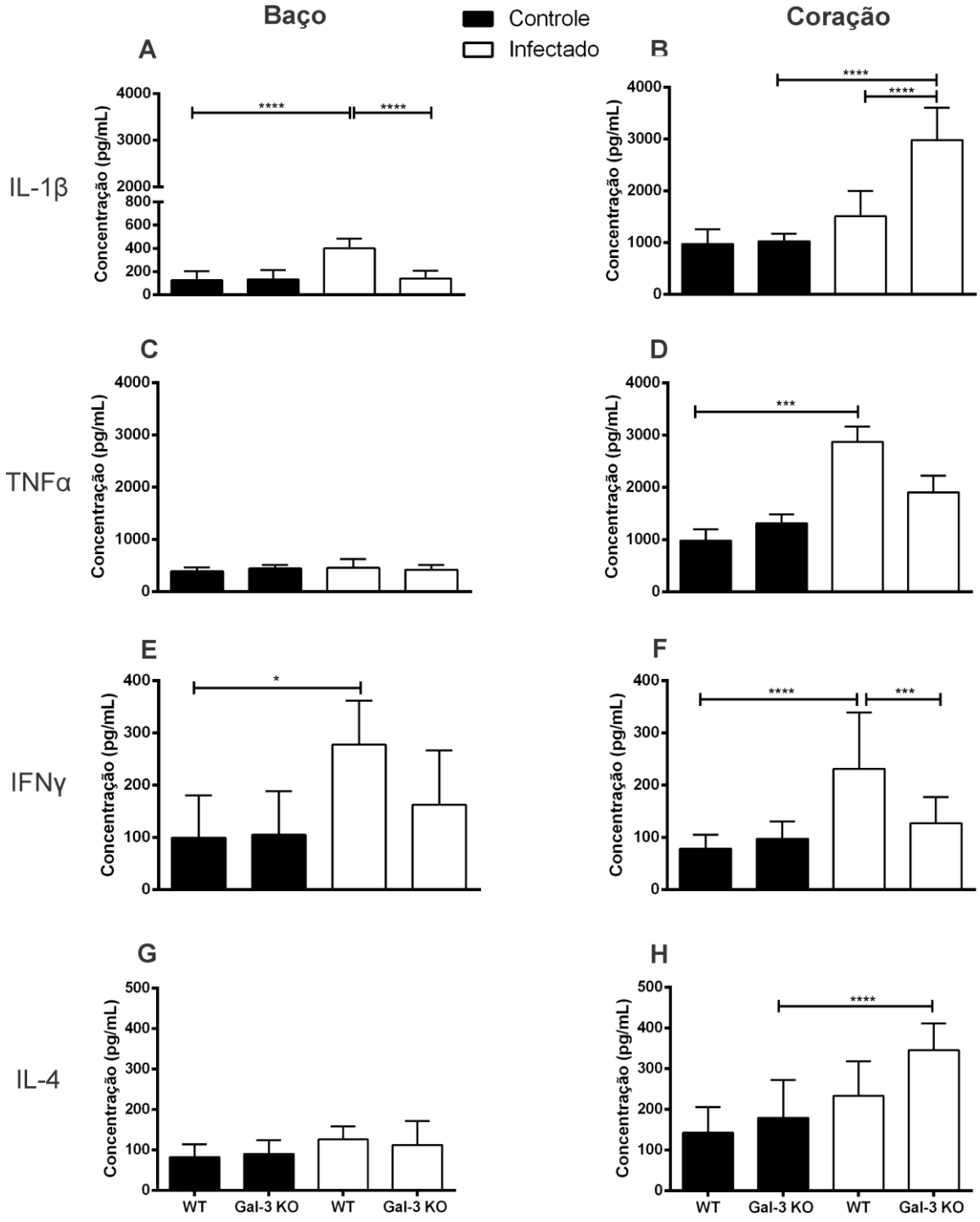

Figura 3. Dosagem das citocinas IL-1β, TNF-α, IFN-γ e IL-4 do baço e coração de animais infectados por *T. cruzi* da cepa CL por 15 dias para avaliação da fase aguda. Houve aumento significativo na concentração de IL-1β no baço (A) de animais WT infectados ($p<0,0001$) e no coração (B) de animais Gal-3 KO infectados ($p<0,0001$) quando comparados com seus respectivos controles não infectados. A citocina TNF-α apresentou em maiores concentrações no coração (D) de animais WT infectados ($p< 0,01$) em relação aos animais não infectados. Houve maior produção de IFN-γ no baço (E) ($p<0,05$) e coração (F) ($p<0,0001$) de animais WT infectados em relação ao controle não infetado. A concentração de IL-4 foi maior no coração (H) de animais Gal-3 KO infectados ($p<0,001$) quando comparados com Gal-3 KO não infectados.

5.4 Análise de citocinas na fase crônica da infecção por *T. cruzi*

O papel da Gal-3 nas respostas imunitárias e inflamatórias Th1 / Th2 parece variar de acordo com os modelos experimentais utilizadas e nas diferentes fases da infecção (ZUBERI *et al.*, 2004). Para investigar ainda mais o seu papel, neste contexto, foi analisado camundongos Gal-3 KO no que diz respeito à sua resposta à infecção por *T. cruzi* na fase crônica.

Analisando a Figura 4 é possível perceber que na fase crônica não houve alteração na produção das citocinas TNF-α (C e D) e IFN-γ (E e F) no baço e coração dos grupos WT e Gal-3 KO infectados ou não. No baço não observamos alterações na concentração das citocinas IL-1β (Figura 4A) e IL-4 (Figura 4G) nos diferentes grupos. Já no coração houve uma diminuição da citocina IL-1β (Figur 4B) tanto nos animais WT quanto nos animais Gal-3 KO infectados quando comparados com seus respectivos controles não infectados. Os animais WT apresentaram uma maior produção de IL-4 no coração após a infecção, o que não foi observado em animais Gal-3 KO (Figura 4H).

Estudos mostraram que a Cardiopatia Chagásica Crônica (CCC) está relacionada com a presença de um infiltrado inflamatório e ausência ou redução da expressão de TGF-β em células T regulatórias e de IL-4 e IL-13 em células Th2, sugerindo que a intensa resposta inflamatória Th1 no miocárdio de pacientes com essa forma da doença ocorre na ausência de mecanismos regulatórios (CUNHA-NETO *et al*., 2009; ARAUJO *et al*., 2007; GOMES *et al*., 2003; ABEL *et al*., 2001).

Há evidencias de que a forte resposta inflamatória no tecido cardíaco e secreção exacerbada de citocinas do perfil Th1 durante a fase crônica, em que há uma quantidade reduzida de parasitos, não são benéficas para o hospedeiro, podendo levar a resposta auto-imune e lesões teciduais (HYLAND; ENGMAN, 2006; TARLETON, 2003; ENGMAN; LEON, 2002).

Dessa forma, percebemos que no tecido cardíaco os animais WT infectados apresentaram uma resposta similar aos pacientes com a forma indeterminada da doença, direcionada para o perfil Th2 com níveis basais de IFN-γ e TNF-α, diminuição de IL-1β e aumento de IL-4. Já no coração de animais Gal-3 KO infectados não houve o aumento da citocina IL-4. A resposta observada nos animais WT pode ser considerada mais adequada no sentido de controlar o dano tecidual e diminuir o comprometimento funcional do órgão.

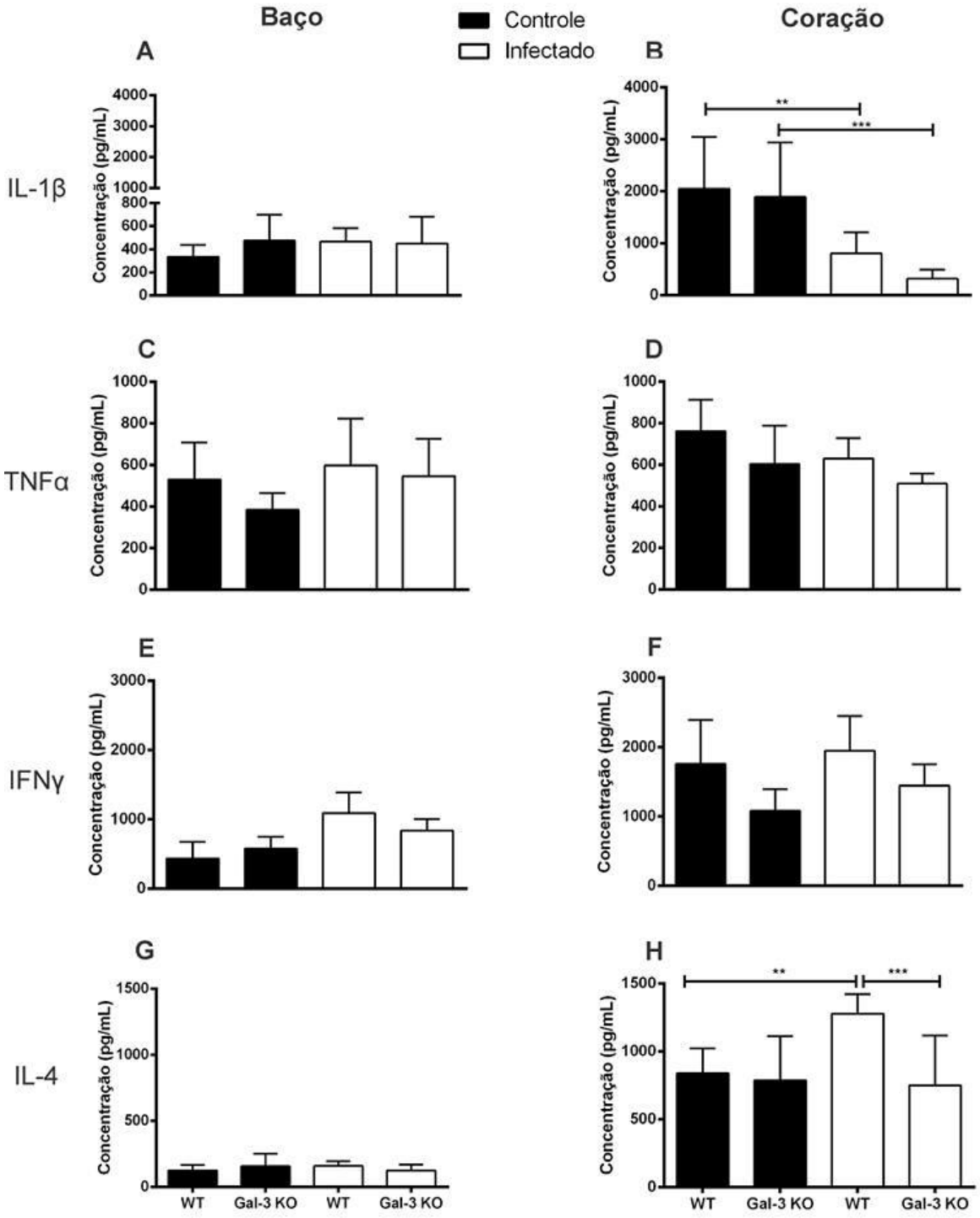

Figura 4. Dosagem das citocinas IL-1β, TNF-α, IFN-γ e IL-4 do baço e coração de animais infectados por *T. cruzi* da cepa CL por 90 dias para avaliação da fase crônica. Houve diminuição significativa na concentração de IL-1β no coração (B) de animais WT infectados ($p<0,01$) e Gal-3 KO infectados ($p<0,001$) quando comparados com seus respectivos controles. Não houve diferença significativa na concentração TNF-α (C e D) IFN-γ nos diferentes grupos. A citocina IL-4 apresentou em maiores concentrações no coração (H) de animais WT infectados ($p< 0,01$) quando comparado com seu controle.

5.5 Presença de mastócitos e fibrose no coração de animais WT e Gal-3 KO infectados por *T. cruzi*

Mastócitos são células residentes no tecido conjuntivo capazes de secretar numerosos mediadores inflamatórios em resposta à agressão do tecido e podem participar de processos fibróticos (BERTON *et al.*, 2000; WYNN, 2011) e imunológicos (DVORAK *et al.*, 1983; ABRAHAM; ST JOHN, 2010).

Na figura 5 é possível perceber que após a infecção por *T. cruzi* na fase aguda há um aumento no recrutamento de mastócitos no coração de animais WT e Gal-3 KO (A). Já na fase crônica há uma diminuição da quantidade de mastócitos encontrados no tecido cardíaco, entretanto, nos animais Gal-3 KO infectados o número de mastócitos continua maior quando comparado com o controle não infectado (B).

A ativação de receptores de mastócitos pode levar a distintas vias de sinalização e síntese de quimiocinas e citocinas que contribuem para a inflamação. Essa célula apresenta função na imunidade natural, na defesa contra parasitos, imunomodulação do sistema imune, no reparo tecidual e angiogênese (METCALFE *et al.*, 1997; GILFILLAN; BEAVEN, 2011).

Foi relatado que mastócitos se acumulam nas bordas de lesões e contribuem para a inflamação local pelo recrutamento de outros tipos celulares, como neutrófilos, e pelo aumento da permeabilidade vascular por meio da secreção de histamina, mediadores lipídicos e VEGF (DVORAK, 2005; WELLER *et al.*, 2006; TAKATO *et al.*, 2011).

T. cruzi pode ser o responsável direto ou indireto de alterações moleculares e/ou morfológicas em diferentes tecidos e órgãos. Esse parasito pode induzir no tecido dos vertebrados uma resposta inflamatória, lesões celulares e fibrose (MACEDO; PENA, 1998).

Durante o ciclo evolutivo o ninho de parasitas rompe a célula, e dessa forma, há a liberação no interstício de restos de células hospedeiras e parasitos, induzindo uma resposta inflamatória. Essa resposta inflamatória inicialmente é focal e relacionada com o parasitismo, e de acordo com a evolução da infecção pode confluir e ocasionar inflamação com aspecto difuso (BRENER; GAZZINELLI, 1997; KIERSZENBAUM, 2005).

Nota-se que tanto na fase aguda quanto na fase crônica há uma maior área de fibrose nos animais Gal-3 KO infectados (Figura 5 C e D). A presença elevada de fibras colágenas em camundongos Gal-3 KO infectados pode ser explicada pela maior multiplicação parasitária nesses animais, levando a um maior número de parasitos viáveis para invasão, multiplicação e lise celular. O grande número de células lisadas pode induzir uma resposta inflamatória intensa e acarretar na substituição de células funcionais por colágeno,

caracterizando a fibrose no órgão.

A fibrose é uma das alterações que ocorre de forma lenta e progressiva na Doença de Chagas. Já foi visto que em condições experimentais, observa-se fibrose no coração já nos primeiros dias de infecção e pode ser a causa da progressiva perda da atividade contrátil do miocárdio nos chagásicos crônicos. Geralmente, essa fibrose ocorre por substituição de miocélulas por áreas irregulares de neoformação colágena, e o conjunto de fibroses focais ao longo dos anos pode evoluir para um quadro grave (SWYNGHEDAUW, 2006).

Mastócitos podem liberar triptase e trombina que potencializam a diferenciação de fibrocitos humanos e iniciam a formação de fibras colágenas no tecido. Portanto, essa célula pode estar relacionada com fibrose em tecidos (WHITE; GALVIS-CARVAJAL; GOMER, 2015).

Na figura 5 podemos observar que na fase aguda nos grupos WT e Gal-3 KO o número de mastócitos encontrados no tecido cardíaco foi similar, entretanto, a fibrose nos animais Gal-3 KO foi maior. Na fase crônica os animais Gal-3 KO apresentaram um maior nível de fibrose e um maior recrutamento de mastócitos. Estudos relacionados com cardiopatia chagásica crônica verificaram que a mastocitose está relacionada topograficamente com a miocardite e que o aumento de mastócitos no tecido cardíaco pode ser um dos fatores agravantes da fibrose na doença de Chagas (ALMEIDA *et al.*, 1975; PINHEIRO *et al.*, 2003).

Dessa forma, percebemos que a deficiência de Gal-3 na fase aguda provoca uma resposta imune que promove uma maior suscetibilidade do parasito ao hospedeiro, maiores níveis de: multiplicação parasitária, parasitemia e número de parasitos viáveis na cavidade peritoneal e consequentemente, maior fibrose quando comparados com animais WT. Na fase crônica da infecção os animais Gal-3 KO apresentaram eventos que levam a um maior dano tecidual e maior comprometimento das funções do coração, como: diminuição da citocina IL-4, aumento de mastócitos e fibrose no tecido cardíaco (Figura 6).

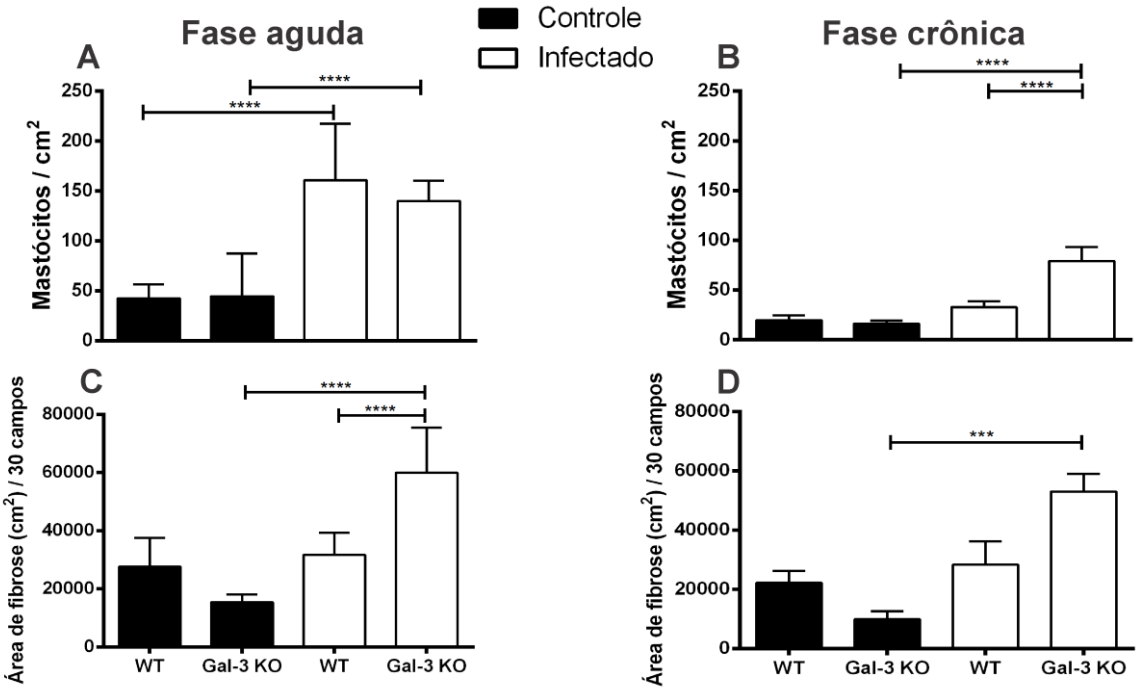

Figura 5. Análise histológica do número de mastócitos e fibrose presentes no tecido cardíaco durante as fases aguda e crônica da infecção por *T. cruzi*. Na fase aguda os animais WT e Gal-3 KO apresentaram maiores quantidades de mastócitos ($p<0,0001$) após a infecção quando comparados com seus respectivos controles não infectados (A). Na fase crônica houve uma diminuição do recrutamento de mastócitos em todos os grupos, mas os animais Gal-3 KO ainda apresentaram uma maior taxa de mastócitos ($p<0,0001$) (B). A área de fibrose foi mensurada em 30 campos do coração corado com picrossirius. Animais Gal-3 KO apresentaram maiores taxas de fibrose em ambas as fases da infecção (C e D).

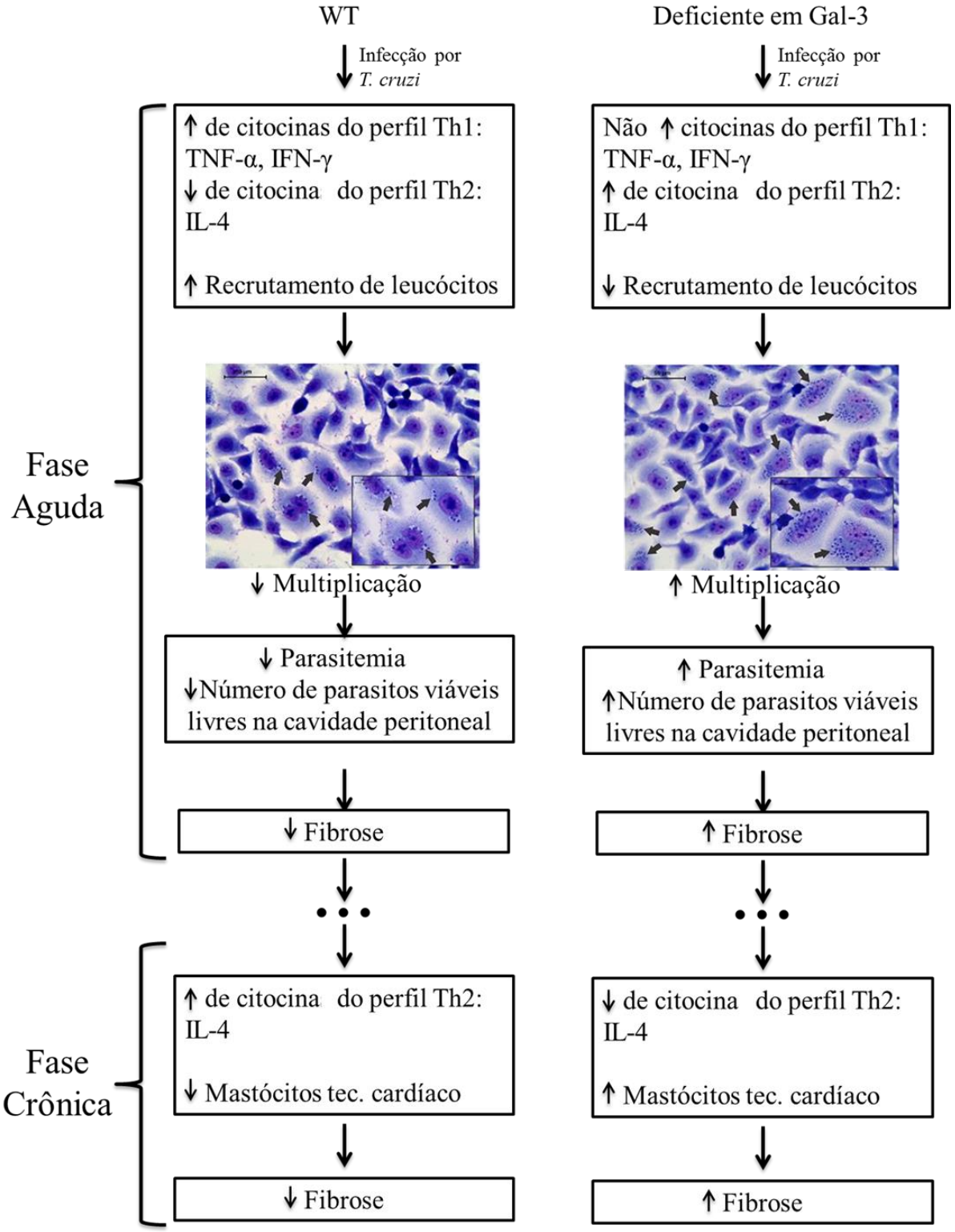

Figura 6. Participação da Gal-3 na infecção por *T. cruzi.* (A) Em animais WT a infecção por *T. cruzi* durante a fase inicial leva a um aumento da produção de citocinas TNF-α e IFN-γ do perfil Th1, diminuição de citocinas IL-4 do perfil Th2 e maior recrutamento de leucócitos para o local da infecção (cavidade peritoneal). Com isso, esses animais apresentam níveis menores de: multiplicação parasitária, parasitemia e número de parasitos viáveis na cavidade

peritoneal e, portanto, menor fibrose. Na fase crônica, em animais WT observa-se aumento de IL-4 e menor quantidade de mastócitos no coração. (B) Em animais Gal-3 KO a infecção durante a fase inicial apresenta níveis basais das citocinas TNF-α e IFN-γ, aumento de IL-4 e menor recrutamento de leucócitos para o local da infecção. Com isso, a deficiência de Gal-3 leva a maiores níveis de: multiplicação parasitária, parasitemia e número de parasitos viáveis na cavidade peritoneal, e consequentemente, maior fibrose. Na fase crônica da infecção em animais Gal-3 KO percebe-se diminuição da citocina IL-4, aumento de mastócitos e fibrose no tecido cardíaco, provavelmente esses animais terão maior comprometimento funcional do coração.

6.0 CONCLUSÃO

- A Gal-3 participa no controle da infecção por *T. cruzi* sendo que sua ausência leva a uma maior parasitemia e maior número de parasitos livres na cavidade peritoneal.

-Animais Gal-3 KO apresentam menor recrutamento de leucócitos totais na cavidade peritoneal, sugerindo um papel quimiotático da proteína.

-Gal-3 está envolvida na imunomodulação, sua presença garante a secreção de citocinas durante a fase aguda que ajudam no controle da infecção por *T. cruzi* e durante a fase crônica produção de citocinas que diminuem os danos teciduais.

-A deficiência de Gal-3 em camundongos leva a um maior recrutamento de mastócitos e fibrose do tecido cardíaco.

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APÊNDICE A- Artigo publicado na Glycobiology

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Recruitment of galectin-3 during cell invasion and intracellular trafficking of *Trypanosoma cruzi* extracellular amastigotes

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The invasion of host cells by the intracellular protozoan *Trypanosoma cruzi* requires interactions with host cell molecules, and the replication of the parasite requires escape from a parasitophorous vacuole into the host cell cytosol. Galectin-3, a member of β-galactosidase-binding lectin family, has numerous extracellular and intracellular functions. In this study, we investigated the role of galectin-3 during the invasion and intracellular trafficking of *T. cruzi* extracellular amastigotes (EAs). Endogenous galectin-3 from mouse peritoneal macrophages accumulated around the pathogen during cell invasion by EAs. In addition, galectin-3 accumulated around parasites after their escape from the parasitophorous vacuole. Thus, galectin-3 behaved as a novel marker of phagolysosome lysis during the infection of host cells by *T. cruzi*.

Keywords: galectin-3 / intracellular traffic / phagolysosome lysis / *Trypanosoma cruzi*

Introduction

Obligate intracellular parasites, such as *Trypanosoma cruzi*, must enter host cells to survive. A host cell provides a favorable environment for replication and protection from immune defenses through the subversion of cell signaling pathways (Sibley 2004). Both developmental forms of *T. cruzi* are capable of invading host cells: the classical infective trypomastigotes and the amastigotes derived from the extracellular differentiation of trypomastigotes or by premature release from infected cells. However, they use different invasion mechanisms that involve multiple interactions (Behbehani 1973; Pan 1978; Hudson et al. 1984; Ley et al. 1988; Mortara 1991; Burleigh 2005; Yoshida 2006). After entering the cell, *T. cruzi* is retained within a parasitophorous vacuole that rapidly fuses with host cell lysosomes (Tardieux et al. 1992; Woolsey et al. 2003). The parasite is able to resist the acidic conditions within this vacuole and escape into the cytoplasm, where it replicates (Nogueira and Cohn 1976; Ley et al. 1990). Studies focusing on the host cell molecules that participate in the invasion and intracellular trafficking of *T. cruzi* are important to identify possible therapeutic targets for the reduction of parasite survival. Previous studies in murine dendritic cells have shown that *T. cruzi* infection up-regulates the expression of galectin-3, both in vivo and in vitro, as well as several carbohydrate-binding sites (Acosta-Rodriguez et al. 2004; Vray et al. 2004).

Galectin-3 is a member of the galectin family, which is characterized by affinity for β-galactose-containing carbohydrates, and has pleiotropic biological functions. It localizes to both the nucleus and cytoplasm and is both secreted and membrane-associated (Dumic et al. 2006). Human galectin-3 binds specifically to *T. cruzi* trypomastigote surface proteins, enhancing trypanosome adhesion to the extracellular matrix component laminin (Moody et al. 2000). In addition, the expression of human galectin-3 by coronary artery smooth muscle cells is required for and mediates the adhesion of *T. cruzi* to these cells (Kleshchenko et al. 2004). Although the role of extracellular galectin-3 in the immune response to infection by *T. cruzi* trypomastigotes has been established, there have been no studies regarding the role of endogenous galectin-3 in cell invasion by and intracellular trafficking of *T. cruzi*. Invasion of host cells by *T. cruzi* extracellular amastigotes (EAs) seems to occur through a process similar to phagocytosis (Mortara 1991; Procopio et al. 1998), which may involve lipid rafts (Fernandes et al. 2007).

Galectin-3 is a major component of phagosomes in a murine macrophage cell line (Garin et al. 2001) and of exosomes derived from a dendritic cell line (Théry et al. 2001). Furthermore, galectin-3 interacts with two lysosomal–/late endosomal–lysosomal proteins, lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2, Dong and

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Hughes 1997; Sarafian et al. 1998). Galectin-3 localizes to a network of tubules and vesicles intersecting with the endocytic pathway (Paz et al. 2010), in membrane ruffles and lamellipodia, and is enriched in lipid raft domains of stimulated dendritic cells and macrophages (Hsu et al. 2009). Moreover, *galectin-3*$^{-/-}$ macrophages are defective in the phagocytosis of both immunoglobulinG (IgG)-opsonized erythrocytes and apoptotic cells (Sano et al. 2003). These features of galectin-3 may influence *T. cruzi* EA interactions with host cells and survival. Thus, we proposed to study the role of galectin-3 during invasion and intracellular trafficking of *T. cruzi* EAs.

In this study, we report that endogenous galectin-3 plays a role during intracellular trafficking of EAs. In murine peritoneal macrophages, galectin-3 was recruited to the EA entry site. In different cell types, galectin-3 accumulated around vacuole-free parasites. Thus, galectin-3 may be a novel marker of parasitophorous vacuole lysis during *T. cruzi* infection.

Results and discussion

To verify the participation of galectin-3 in host cell invasion and intracellular trafficking of *T. cruzi* EAs, we performed invasion kinetics assays using peritoneal macrophages from C57BL/6 mice, mouse embryonic fibroblasts (MEFs) or breast carcinoma cell line (SKBR) cells. The SKBR cells were transfected with either galectin-3-green fluorescent protein (GFP) or with galectin-3 mutated on its lectin domain. At early time points, galectin-3 accumulated at the site of parasite entry in peritoneal macrophages (Figure 1A–C). Galectin-3 was present, but not accumulated, in the cytoplasmic expansions associated with

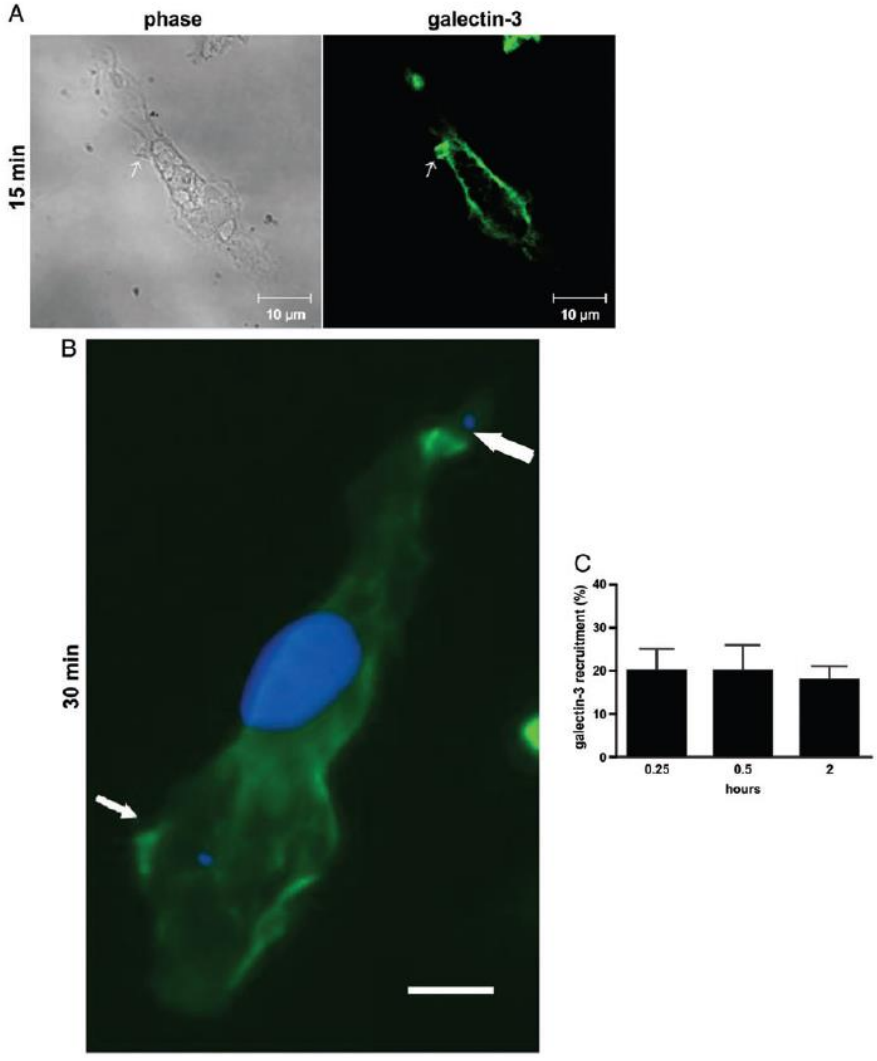

Fig. 1. Recruitment of galectin-3 during cell invasion by *T. cruzi* EAs. Localization of galectin-3 at initial time points of EA invasion in mouse peritoneal macrophages. Parasite location was confirmed by bright microscopy (**A**) and 4′,6-diamidino-2-phenylindole staining (**B**) (white arrows). (**C**) Percentage of recruitment of galectin-3 at the initial time points of EA invasion in murine peritoneal macrophages. Bars: 10 µm.

endocytosis of EAs in MEF and SKBR cell lines (data not shown). Sano et al. (2003) described the presence of galectin-3 in F-actin-enriched phagocytic cups in macrophages after the addition of opsonized erythrocytes. It has also been shown that galectin-3 localizes to the cytosolic side of lipid-enriched membrane microdomains (i.e. lipid rafts) through a mechanism independent of lectin–carbohydrate interactions and that *galectin-3*$^{-/-}$ cells contain ruffled structures with lower complexity as well as impaired motility in comparison with wild-type cells. Therefore, galectin-3 might regulate signal transduction and cell motility mediated by lipid rafts (Hsu et al. 2009). Our observation of the accumulation of galectin-3 at the site of parasite entry in macrophages and the presence of galectin-3 in the cytoplasmic expansions during the initial invasion may be explained by the fact that *T. cruzi* EAs mobilize the lipid raft pathway to enter host cells (Fernandes et al. 2007). Another study demonstrated that galectin-3 accumulated on the cytosolic face of vacuoles containing viable *Mycobacterium tuberculosis*, suggesting that the most likely source of the accumulated galectin-3 is either continued vesicular delivery to the phagosome via the sorting/recycling endocytic pathway or sequestration from the cytosol (Beatty et al. 2002). Thus, the galectin-3 in the vicinity of *T. cruzi* EA vacuoles may be interacting with the cytosolic side of membrane lipid rafts at the EA entry site.

Using immunofluorescence microscopy, Paz et al. (2010) observed that galectin-3 was recruited to lysed vacuoles, which they termed galectin-3-containing structures (G3CSs), following cell invasion by both Gram-positive and Gram-negative bacteria. They confirmed that the formation of G3CSs depended on the lectin characteristics of galectin-3 and verified that galectin-3 did not directly interact with the bacteria but instead interacted with galactose-containing glycoconjugates in the membrane of the lysed vacuole. Vacuole lysis is a poorly understood process due to the lack of markers associated with disrupted phagosomes. The investigators proposed that the detection of galectin-3 during late bacterial invasion, corresponding to bacterial escape from vacuoles, may serve as a marker for such events. This noteworthy finding led us to consider whether this same phenomenon could be observed during protozoan vacuole escape. Our results showed that galectin-3 accumulated around *T. cruzi* amastigotes that had lysed the phagolysosome along a kinetic curve (Figure 2A–E), and this accumulation was dependent on the lectin domain of galectin-3 (Figure 3A and C). These results were similar to the results of Paz et al. in regard to the detection of galectin-3 during bacterial trafficking. Hence, this feature may be considered a vacuole lysis marker for *T. cruzi*.

Lysosomal membrane proteins, such as LAMP-1 and LAMP-2, have been used to indicate *T. cruzi* vacuole lysis (Ley

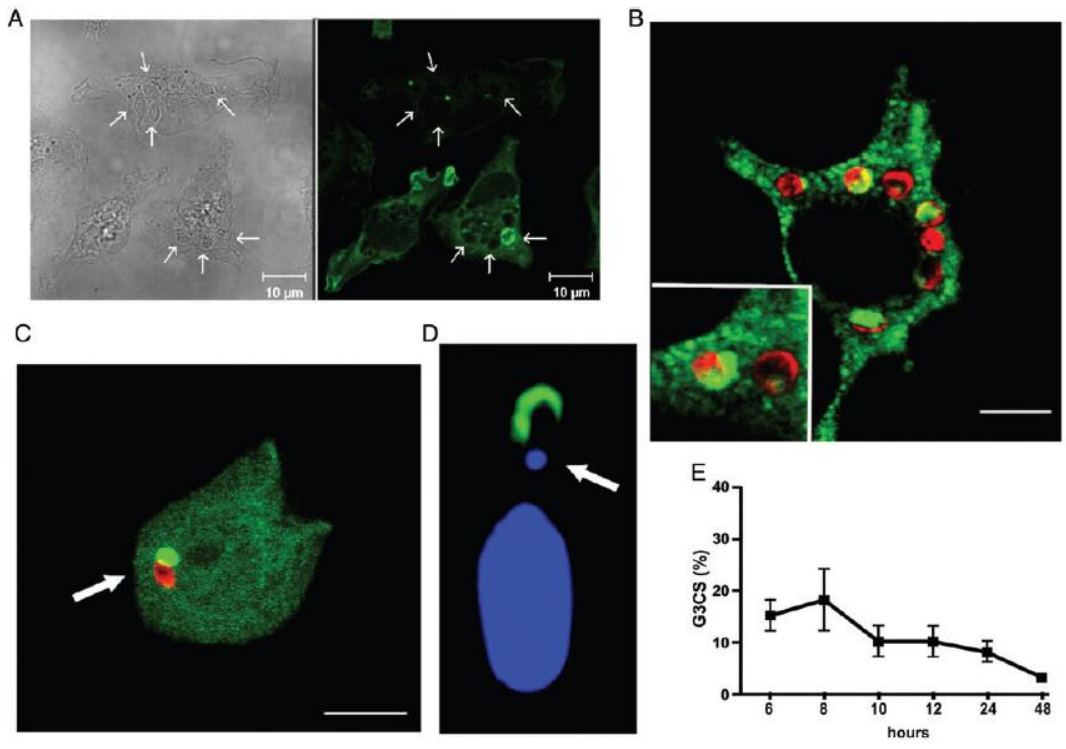

Fig. 2. Formation of G3CSs in different cell types infected with *T. cruzi*. G3CSs were observed around *T. cruzi* EAs in murine peritoneal macrophages (**A**) MEF (**B**) and green fluorescent protein-galectin-3 wild-type transfected breast carcinoma cell line (SKBR) cell (**C**) (illustration images were captured at 6 h post-infection). A brightness and contrast-treated image showed intense fluorescence depicting G3CS formation around the parasite in a murine peritoneal macrophage at 6 h post-infection (**D**). Percentage of recruitment of galectin-3 at the final time points of EA invasion in murine peritoneal macrophages (6–48 h post-infection) (**E**). Bars: 10 µm.

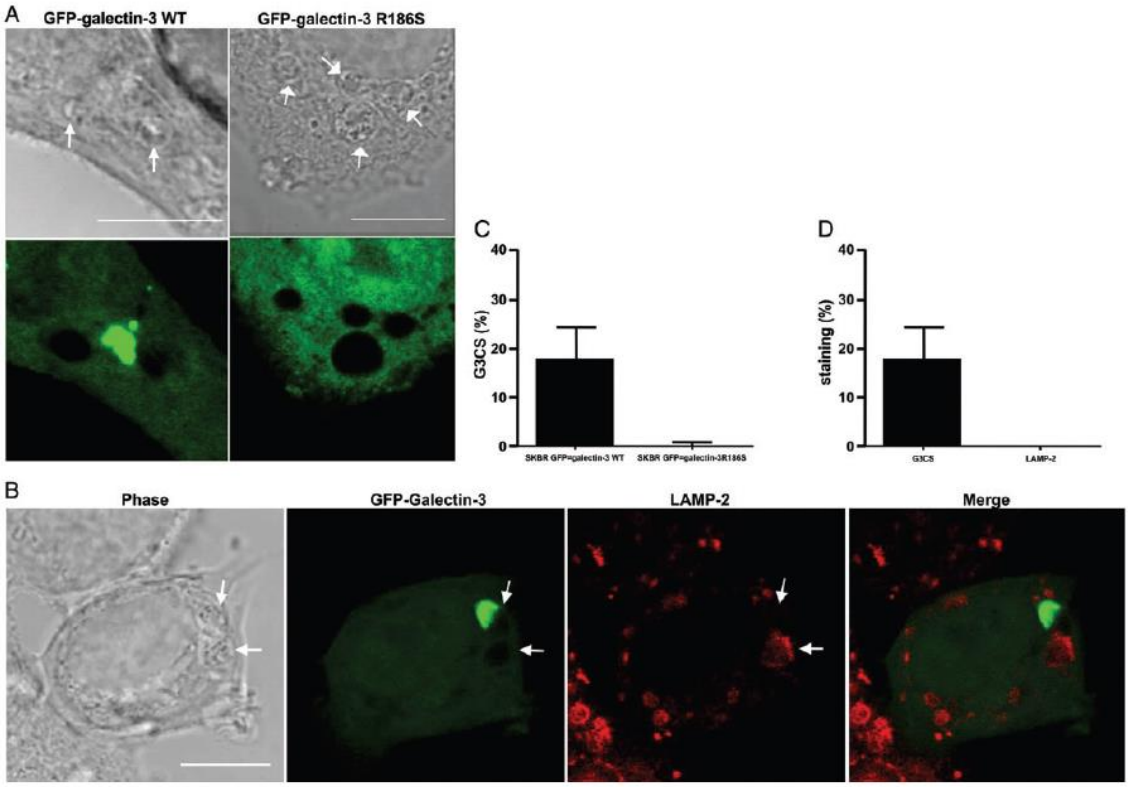

Fig. 3. Formation of G3CSs is related to vacuole lysis by *T. cruzi* EAs and depends on the galectin-3 lectin domain. G3CS was not observed in SKBR transfected with galectin-3 mutated on the lectin domain (**A** and **C**). Moreover, G3CS was only observed around LAMP-2 negative parasites (**B** and **D**). White arrows indicate the presence of parasites. Bars: 10 µm. (Images and quantification were performed at 6 h post-infection)

et al. 1990; Tardieux et al. 1992). Negative staining for LAMP proteins indicates that the parasite is already free in the cytosol of the host cell. We investigated whether galectin-3 accumulated around amastigotes at the same time that LAMP-2 protein disappeared. We observed that galectin-3 accumulated around the parasites only at the time when LAMP-2 was not detected (Figure 3B and D). Assuming that the lysis of *T. cruzi* phagolysosomes is not synchronous, this result suggests that the formation of G3CSs occurred around the portion of parasites that had recently escaped from the vacuole at each time point. This hypothesis raised intriguing questions regarding the lifespan of G3CSs, the mechanism of formation of G3CSs, the role of G3CSs during the *T. cruzi* life cycle and infection and the clearance of G3CSs from the cytosol. The questions will be addressed in future studies.

Our results showed for the first time the localization of expression of galectin-3 during cell invasion by *T. cruzi* and intracellular trafficking of the parasite. Galectin-3 was recruited to the site of EA entry in macrophages and G3CSs were formed during the lysis of parasitophorous vacuoles in different cell types.

Material and methods

Cell culture

Peritoneal macrophages from C57BL/6 mice and MEFs were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% sodium pyruvate, 0.1 mg/mL streptomycin, 100 U/mL penicillin and 0.04 mg/mL gentamycin at 37°C in a humidified 5% CO_2 atmosphere. SKBR3 cells were cultured under the same conditions, except that the culture medium for GFP-galectin-3-transfected cells was supplemented with G418 (1 µg/mL, Gibco, USA) to select stably transfected cells. Transfection of SKBR3 cells was performed as previously described (Paz et al. 2010). SKBR cells were generously provided by Philippe Sansonetti, PhD (Institut Pasteur, France).

Parasites

Trypanosoma cruzi EAs from G strain (Yoshida 1983) were obtained by differentiation of tissue culture trypomastigotes in liver infusion tryptose medium supplemented with 5% FCS (pH 5.8) for 16 h as described previously (Ley et al. 1988; Mortara 1991).

Infection experiments

For intracellular trafficking analysis, cells were incubated with *T. cruzi* EAs from G strain and maintained at 4°C for 10 min to synchronize invasion. The parasites were then allowed to invade for 15 min, cells were gently washed three times with phosphate buffered saline (PBS), and the culture medium was replaced. At 15 min, 30 min, 1, 3, 6, 8, 10, 12, 24 and 48 h

after invasion, the cells were fixed with 4% formaldehyde (Merck, Germany) for 1 h at room temperature. Following fixation, the cells were washed in PBS and soaked in PGN (PBS containing 0.25% gelatin and 0.05 M NaN_3) until indirect immunofluorescence reactions were performed.

Immunofluorescence analysis

To analyze the kinetics of LAMP-2 vacuole acquisition in wild-type and galectin-$3^{-/-}$ MEF cells, the cells were incubated with chagasic human serum (diluted 1:500 in PGN) to label parasites outside of the cells. After 15 min, the coverslips were washed three times with PBS and incubated for 1 h with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-human IgG (1:100 in PGN). After washing three times in PBS, the cells were incubated with rat anti-human-LAMP-2 (diluted 1:1 in PGN-0.1% saponin for cell permeabilization) for 1 h, washed again, and incubated with Alexa Fluor® 488-conjugated anti-rat IgG (diluted 1:100) for 1 h. Cell and parasite nuclei were labeled with 5 µM 4′,6-diamidino-2-phenylindole (DAPI) (4,6-diamidino-2-phenylindole dilactate, Invitrogen, USA). Using this method, the percentage of LAMP-2 positive internalized parasites was determined at different times.

To analyze the kinetics of invasion using GFP-galectin-3-transfected SKBR3 cells, parasites were stained with chagasic human serum (diluted 1:500 in PGN-0.1% saponin for cell permeabilization). After 15 min the coverslips were washed three times with PBS and incubated for 1 h with TRITC-conjugated anti-human IgG (1:100 in PGN). In the case of MEF cells, the parasites were labeled under the same conditions and then cells were incubated with rat anti-galectin-3 (1:1 in PGN-0.1% saponin) for 1 h. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated anti-rat IgG (1:100 in PGN-0.1% saponin) for 1 h. In both experiments, nuclei were stained with DAPI. The coverslips were mounted on glass slides, and the samples were analyzed by confocal fluorescence microscopy (Zeiss, LSM 510 Meta, Germany) using an inverted microscope (Zeiss Axiovert 200 M). Digital images were captured using a 63× oil immersion objective and were processed with ImageJ software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij). Alternatively, images were captured using Digital inverted microscope EVOS® FL.

Statistical analysis

VassarStats (©Richard Lowry 1998–2006) software was used to determine statistically significant differences by the ANOVA test. A P-value of <0.05 was considered to be significant. Data are presented as the means ± SE.

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Conflict of interest

None declared.

Abbreviations

EA, extracellular amastigote; FCS, fetal calf serum; G3CS, galectin-3-containing structure; LAMP, lysosome-associated membrane protein; MEF, mouse embryonic fibroblast; PGN, PBS containing 0.25% gelatin and 0.05 M NaN_3.

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APÊNDICE B- Documentação de encaminhamento do artigo para Scientific Reports

Enc: Manuscript SREP-14-11560A peer review

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Trypanosoma cruzi P21: a potential novel target for chagasic cardiomyopathy therapy

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Abstract

Chagas disease, which is caused by the parasite *Trypanosoma cruzi,* is an important cause of cardiomyopathy in Latin America. It is estimated that 10%–30% of all infected individuals will acquire chronic chagasic cardiomyopathy (CCC). The etiology of CCC is multifactorial and involves parasite genotype, host genetic polymorphisms, immune response, signaling pathways and autoimmune progression. Herein we verified the impact of the recombinant form of P21 (rP21), a secreted *T. cruzi* protein involved in host cell invasion, on progression of chronic inflammatory processes in a polyester-induced inflammation model using chemotaxis, angiogenesis and actin polymerization assays. Results indicate that rP21 can recruit immune cells and decrease blood vessels compared to controls *in vitro* and *in vivo*. Furthermore, rP21 induced actin polymerization. *T. cruzi* P21 could be a potential target for development of P21 antagonist compounds to treat symptomatic CCC.

Chagas disease, which is caused by the parasite *Trypanosoma cruzi,* is an important cause of cardiomyopathy in Latin America. It is estimated that 10%–30% of all infected individuals will acquire chronic chagasic cardiomyopathy (CCC). This represents anywhere between 1.6 to 5.4 million CCC patients in Latin America[1]. CCC has been shown to cause social and economic burdens in endemic areas because of increased health care costs[2]. An estimated 20.000 deaths occur annually in endemic countries due to complications of CCC[1]. The prognosis for chagasic patients is rather bleak. In fact, CCC has been reported to be the main prognostic mortality factor among patients with heart failure of various etiologies[1,2].

The etiology of CCC is multifactorial and involves parasite genotype[3,4], host genetic polymorphisms[5-9], immune response[10-13], signaling pathways[14] and autoimmunity[15,16]. An intriguing question remains unanswered: could trypanosome-derived components play an active role in CCC onset rather than serving as passive targets for the host immune response? In this sense, trapped intracellular parasites may continue to secrete proteins that can enter the extracellular space after plasma membrane wounding and/or lysis and influence disease progression. Herein we verified the impact of the recombinant form of P21 (rP21), a secreted *T. cruzi* protein involved in host cell invasion[17-19], on progression of chronic inflammatory processes in a polyester-induced inflammation model.

Results and Discussion

Our previous studies using the recombinant form of *T. cruzi* P21 showed that this secreted protein is involved in host cell invasion[17]. In addition, rP21 enhanced macrophage phagocytosis and actin polymerization by binding to the CXCR4 receptor[18].

The ability of rP21 to bind to CXCR4 indicated possible chemotaxis activity. We observed that rP21 attracted neutrophils *in vitro* (Figure 1A). To assess whether rP21 has chemoattractive activity *in vivo*, we injected it intraperitoneally into mice and observed similar cell recruitment over time (Figure 1B). Phenotypic cell identification showed that the infiltrated was composed mainly of neutrophils at earlier time points, followed by lymphocytes and macrophages at later time points (Figure 1C). Similar results were obtained after injection of 4% thioglycolate (data not shown). rP21 attracted peritoneal murine macrophages in a trans-well system through a polycarbonate

membrane over a thin layer of extracellular matrix. Detection of migrating cells was performed via crystal violet staining (Figure 1D, E).

The literature has shown that the inflammatory process that characterizes CCC is accentuated during the acute phase of the disease. Although it may be clinically silent after the acute phase, inflammation is continuously present in patients with indeterminate and chronic phases[20,21]. Moreover, trypanosome antigens and/or its genomic material are found in inflammatory foci[22-26]. These observations provide a basis to propose a role for P21 regarding the onset and maintenance of chagasic heart inflammation.

We observed high N-acetylglucosaminidase levels in rP21-treated polyester sponge implants (Figure 2A, B), indicating that rP21 not only recruits, but also activates macrophages. A high rP21 dose impacted myeloperoxidase expression (Figure 2A, B). This observation corroborated our previous results on the ability of rP21 to recruit immune cells.

In vitro and *in vivo* studies have shown the presence of mast cells associated with cardiac *T. cruzi* infection[27-29]. In the present study, we observed rP21-triggered mast cells in sponge implants (Figure 2C), suggesting a potential role of *T. cruzi* P21 in promoting recruitment of mast cells to damaged cardiac tissue and upregulating the inflammatory process.

We observed a significant decrease in hemoglobin content (Figure 3A), suggesting a potential anti-angiogenic role of rP21. To confirm this hypothesis, we measured the number of blood vessels on histological preparations. Sponge implants treated with different rP21 concentrations showed a decreased number of blood vessels compared to the control (Figure 3B). We also treated a murine endothelial cell line derived from thymus hemangioma (tEnd) with similar rP21 concentrations and observed inhibition of blood vessel formation *in vitro* (Figure 3 C-F). This activity was not due to cell adhesion impairment to the matrix gel or cell toxicity (Figure 3 G, H).

Functional and structural microvascular abnormalities occur in CCC[30,31]. Consequently, vasospasms, decreased blood flow, focal ischemia, platelet thrombi, increased platelet aggregation, and elevated levels of thromboxane A-2 and endothelin-1 are frequently observed[32-35]. As such, P21 could impact microvascular ischemia, which is believed to amplify chronic inflammatory aggression toward myocardial tissue[36].

We previously observed that rP21 induced actin polymerization by binding to the CXCR4 receptor[18]. Herein we evaluated whether rP21 directly induced actin polymerization using a cell free biochemical assay (Figure 3I). The ability of rP21 to induce actin polymerization suggests that the native protein may induce polymerization in infected cells to restrict intracellular parasites from migrating. In this sense, native P21 may contribute to the establishment of chronic infection without a parasitological cure.

rP21 pretreatment with polymixin B showed similar activity (data not shown), as previously observed[18].

CCC treatment regimens include β-blockers, diuretics, angiotensin-enzyme inhibitors, angiotensin receptor blockers and amiodarone[1]. While parasitological treatment is still a matter of debate[37,38], researchers have studied host defense components such as NADPH oxidase[39] and transforming growth factor beta signaling[40] and approaches based on treatment with granulocyte colony-stimulating factor have been described[41]. All of these therapeutic schemes have shown promising results.

We propose a novel line of investigation that targets components of parasitic origin that could have an impact on CCC symptoms. *T. cruzi* P21 may be a potential target for developing antagonist compounds to treat symptomatic CCC.

Experimental procedures

Animals and ethics

Six- to eight-week-old male C57BL/6 and BALB/c mice were maintained under standard conditions on a 12-h light-dark cycle in a temperature controlled setting (25 °C) with food and water *ad libitum*. Maintenance and care of animals complied with the guidelines of the Laboratory Animal Ethics Committee from the Universidade Federal de Uberlândia. Animal euthanasia was performed based on international welfare grounds according to the American Veterinary Medical Association Guidelines on Euthanasia. This study was approved by the ethics committee for animal research at Universidade Federal de Uberlândia.

Cell culture

Murine endothelial cell lines derived from thymus hemangioma (tEnd)[47] were cultivated in RPMI 1640 medium supplemented with 10% bovine fetal serum, 2 mM l-

glutamine, 2 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin and incubated at 37 ºC and 5% CO_2.

Cell viability

The viability of cultured tEnd cells treated with rP21 was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cells were seeded at 1.5×10^4 cells/well in 96-well microplates. After adhesion, cells were treated with different concentrations of rP21 or culture medium (control group) for 24 h at 37 ºC and 5% CO_2. After 24 h, cells were incubated with 5 mg/mL MTT for 3 h at 37 ºC. Formazan crystals resulting from MTT reduction were dissolved by addition of 100 µL of PBS containing 10% SDS and 0.01 M HCl (18 h, 37 ºC and 5% CO_2). Absorbance (570 nm) was read on a multiwell scanning spectrophotometer (Thermo Scientific).

rP21 purification

rP21 (GenBank: EU004210.1) was purified as previously described[17,19]. The quality of purification was demonstrated by SDS-PAGE.

***In vitro* and *in vivo* chemotaxis assays**

The *in vitro* chemotaxis assay for peritoneal macrophages was performed in a trans-well system through a polycarbonate membrane with a defined pore size of 8 µm (Chemicon Cell Invasion Assay Kit, EMD Millipore Corporation, Darmstadt, Alemanha). Peritoneal macrophages from BALB/c mice were seeded at 10^6 in the top of the insert in serum-free media, while medium with FBS as a positive control, 50 µg/mL of rP21 or serum-free media as a negative control was added in the bottom well. Cells were incubated for 72 hours. Migratory cells were stained, photographed and counted.

For neutrophil chemotaxis, a Boyden chamber assay was used, consisting of upper and lower chambers separated by a polycarbonate membrane with an 8-µm pore. Neutrophils were extracted from C57BL/6 bone marrow using ficoll to separate them from other cells. Some of the cells were treated with pertussis toxin (G-protein coupled receptor inhibitor) for 1 hour. Treated and non-treated cells were placed in the upper chamber. In the lower chamber, only media, rP21 or macrophage inflammatory protein (MIP-2), a chemotaxis positive control, were added. After 1 hour, the membrane was removed, fixed and colored with panothic dyes. Cells that migrated were counted using an optical microscope.

In vivo, C57BL/6 mice received two different intraperitoneal treatments: PBS and 40 µg/mL rP21. Animals were euthanized at intervals of 6 hours, 24 hours and 72 hours. Peritoneal lavage was performed using 4 mL PBS, with 2 mL collected. Total leukocyte recruitment was quantified in a Neubauer chamber. Fifty microliters of each sample was placed on a microscope slide, dried and fixed in pure methanol (Synth A1085.01.BJ) for 5 minutes and then subjected to Giemsa stain (Sigma) at a concentration of 1:20 diluted in water for 15 minutes. Analysis was performed by optical microscopy wherein 100 cells were counted and differentiated between lymphocytes, monocytes and polymorphonuclear cells.

Contents from washing the peritoneal cavities of the mice were centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 1 mL of 4% formaldehyde. After fixation for 2 hours, samples were centrifuged at 2500 rpm for 5 minutes and resuspended in PBS for subsequent flow cytometry (BD FacsCanto II) to evaluate the size and granularity of cells and to separate populations of monocytes, polymorphonuclear cells and lymphocytes.

Polyester chronic inflammation induction and rP21 treatment scheme

A polyester sponge disc with a 1.2 cm diameter (Vitafoam Ltd.) was stored in 70% ethanol v/v prior to deployment for at least 24 hours and subsequently boiled in distilled water for 30 minutes[43,44].

To facilitate spongy matrix insertion, C57BL/6 mice were divided into groups of 10 animals for biochemical analysis and 5 animals for histological analysis, treated with and without rP21. The animals were anesthetized intraperitoneally with ketamine-xylazine (Syntec) (60 mg-4 mg/kg) and subjected to trichotomy and antisepsis in the dorsal region with alcohol 70% v/v. An approximately 10 mm dorsal midline incision was made and dilatation was performed in the subcutaneous cranial direction. The sponge disc was introduced by midline incision in the interscapular region. Incisions were closed with a Donati suture using nylon 3.0. After recovery from anesthesia, animals were placed in individual cages with food and water ad libitum[43]. Treatments were injected into the sponge every 72 hours and analyzed during the chronic (9 days) phase of inflammation.

Hemoglobin content

Determination of the intra-implant hemoglobin content was done using Drabkin's reagent, which was developed in 1932 and adapted as an desvascularization index[45,46].

Implants were removed and masses determined 9 days post-implantation. Samples that showed macroscopic bleeding or infection were excluded from analysis. Each implant was homogenized (Tekmar TR-10, Ohio, USA) in 2 mL of a hemoglobin-specific chromogen reagent (Drabkin kit, Labtest) and added to 2 mL Eppendorf tubes. Samples were centrifuged at 4ºC for 30 minutes at 12,000 rpm and homogenates were filtered through 0.22 µm filters (Millipore). Spectrophotometric analysis was performed on an ELISA reader at a wavelength of 540 nm using 96-well plates. The hemoglobin concentration of each sample was calculated from a known standard curve (diagnostic analyses) and the results were expressed as hemoglobin concentration (micrograms) per milligram wet weight of the implant.

Cellular infiltration by myeloperoxidase activity (MPO)

After determining the hemoglobin content, the supernatant was discarded and the pellet was resuspended in 2 mL sodium phosphate buffer (pH 5.4). Samples were homogenized by vortexing for 30 seconds, and 300 µL was transferred to Eppendorf tubes and supplemented with 600 µL of 0.5% w/v HTAB (hexadecyltrimethylammonium bromide, Sigma) diluted in phosphate buffer (pH 5.4). After further vortex homogenization, the samples were frozen for subsequent dosing. After freezing, samples were thawed and centrifuged at 10,000 g for 10 minutes at 4 °C and the supernatant was used in an enzymatic assay.

The enzymatic assay was performed in 1.5 ml Eppendorf tubes, with reagents added in the following order: 100 µL of 0.003% hydrogen peroxide and 100 µL TMB (3,3',5,5'-tetramethylbenzidine, Sigma) at 4 mM diluted in DMSO (dimethyl sulfoxide, Merck). A total of 200 µL of the sample supernatant was added for 1 minute. The reaction was stopped by adding 100 µL of 4M H2SO4 (sulfuric acid, Merck). Then 200 µL was added to the 96-well plate and spectrophotometric analysis was performed at a wavelength of 450 nm. Results are expressed as the MPO index (absorbance/mg wet weight of the implant).

Cellular infiltration activity of N-acetylglucosaminidase (NAG)

After using the sponge for hemoglobin assessment, the supernatant was discarded and the pellet was resuspended in 2.0 mL 0.9% saline with 0.1% Triton X-100 (Promega). Samples were homogenized by vortexing and centrifuged at 3000 rpm for 10 minutes at 4 °C. The supernatant was used in the assay.

A total of 100 µL of the samples was added to a 96-well plate in duplicate. An additional 100 µL of substrate (p-nitrophenyl-N-acetyl-beta-D-glucosaminide, Sigma) diluted in citrate buffer/phosphate pH 4.5 was added to the samples and incubated at 37 °C for 30 minutes. Last, 100 µL of 0.2M glycine buffer pH 10.6 was added. Absorbance was measured using a spectrophotometry ELISA reader at a wavelength of 400 nm. NAG activity of the implant was calculated from a standard curve of p-nitrophenol evaluated simultaneously. P-nitrophenol is the product of the reaction between the chromogen p-nitrophenol-N-acetyl-glucosamine and beta-D-N-acetylglucosaminidase (NAG). Results are expressed as nmol mL-1/mg wet weight of the implant.

Histological analyses

Sponges removed only for histological processing were fixed in Metacarn buffer (60% methanol, 30% chloroform, 10% acetic acid) for 3 hours in the refrigerator. Sponges were then prepared for inclusion in ethyl alcohol PA for 30 minutes with 4 washes in xylene, followed by an additional 30 minutes incubation and three washes. Sponges were embedded in paraffin I, II and III for 90 minutes each. The blocks were processed for hematoxylin and eosin (HE) and toluidine blue staining. Images were obtained in the Histology Laboratory at the Federal University of Uberlândiawith a Leica DM500 microscope coupled to Las Ez camera and software.

Angiogenesis assay

The influence of rP21 on endothelial cell tube formation was evaluated by Matrigel tube formation assays. Experiments were performed as described[47]. tEnds cells (2×10^5 cells/well) were preincubated with 10, 40 and 80 µg/mL of rP21 or culture medium (control group) for 30 min at room temperature and plated on 24-well plates previously coated (60 min at 37 °C) with 250 µL of 5.25 mg/mL Matrigel (BD Bioscience) in a total volume of 200 µL culture medium supplemented with bFGF (30 ng/mL). After 18 h, cells were photographed under a microscope at ×20 magnification.

Polymerization of actin cytoskeleton assay

An actin polymerization biochem kit (muscle actin) (Cytoskeleton, Inc.) was used to verify the effect of rP21 on polymerization of actin monomers. The kit contains actin monomers and the fluorophore pyrene. Pyrene becomes fluorescent if it binds to polymerized actin.

Statistical analysis

Significant differences were determined by one-way ANOVA performed according to the VassarStats program (Richard Lowry 1998–2006) available at http://faculty.vassar.edu/lowry/VassarStats.html, or using the GraphPad Prism program, version 5.01 for Student's t-test analysis. Differences were considered significant when $p<0.05$. All experiments were performed 4 to 8 times in triplicate.

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Author contribution statement

AAS, BCB, FCM, TLT, SCT: performed the biological experiments, acquired, analyzed and interpreted the data and drafted the manuscript.

AAR, AFON, SRD, DSL: performed experiments.

VMRA, FAA, TCT, MJBS, CVS: designed the biological experiments, drafted the manuscript, critically revised the manuscript and contributed to its intellectual content.

Additional information

Competing financial interests: The authors declare no competing financial interests.

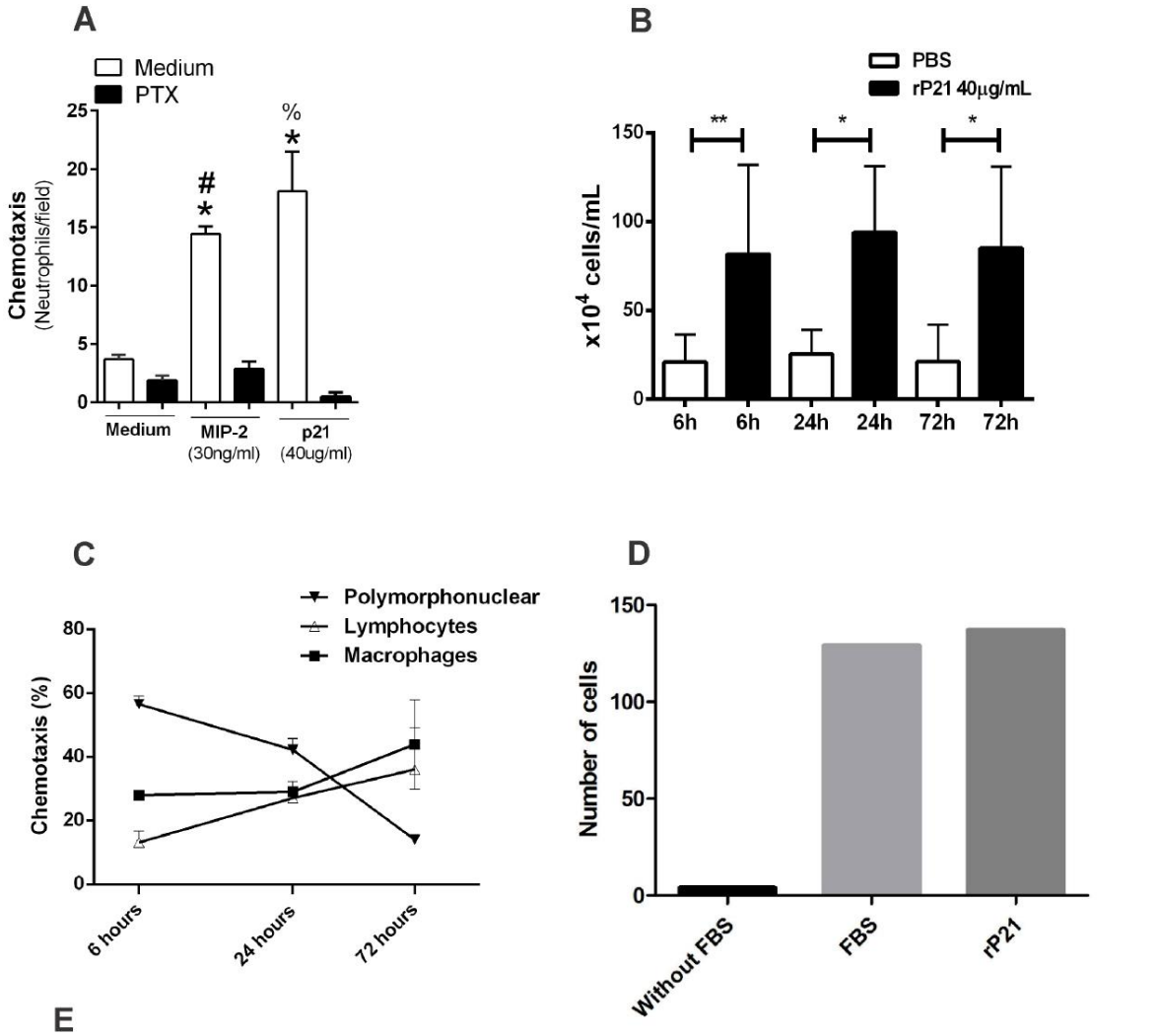
Figure legends

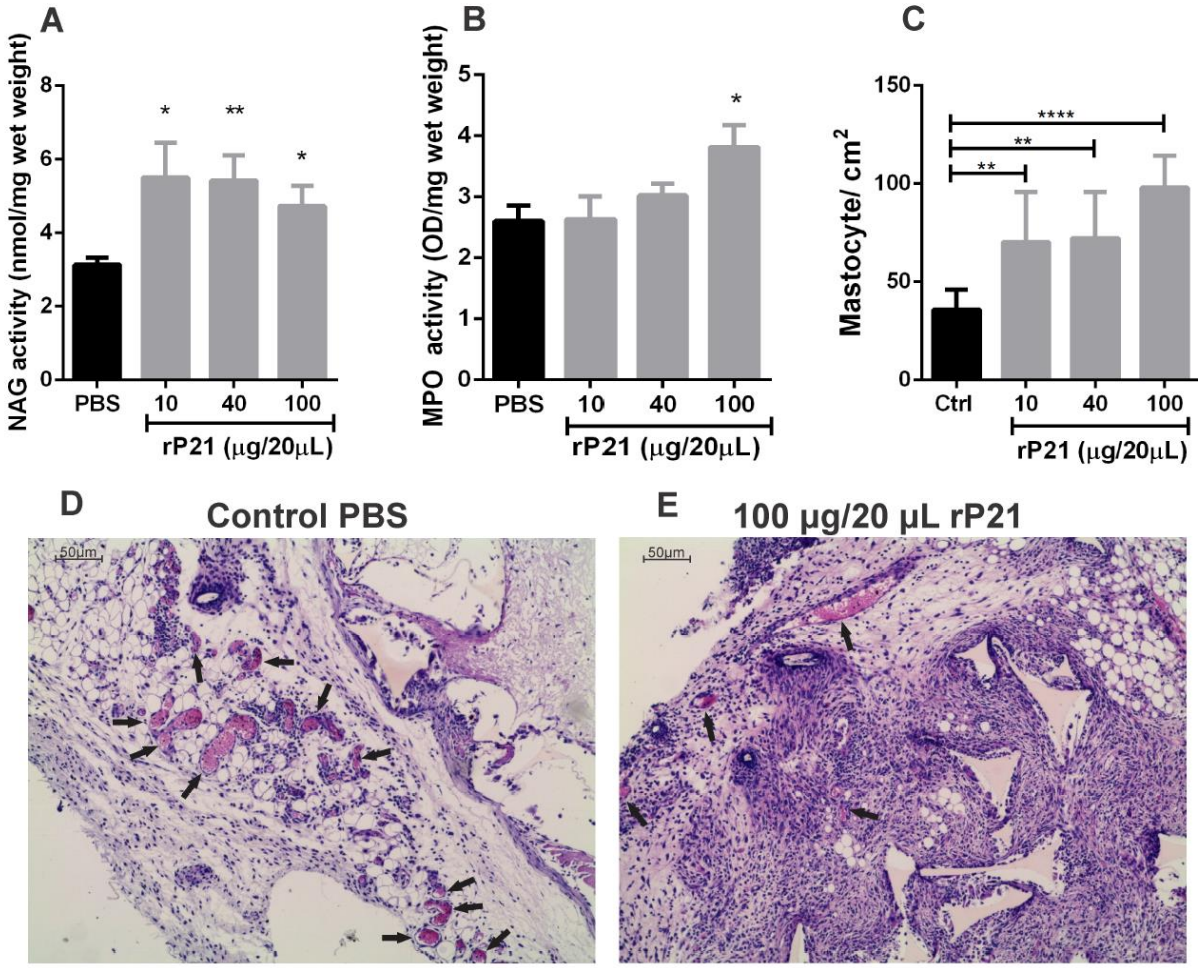
Fig. 1: rP21 and chemotaxis. A. Chemotaxis of neutrophils *in vitro* was determined using the Boyden chamber assay. Cells were placed in the upper chamber and medium, rP21 and the positive control MIP-2 were placed in the lower chamber. B. To evaluate chemotaxis *in vivo*, 40 µg/mL rP21 was injected into the peritoneal cavity of C57BL/6 mice. Results show the capacity of the protein to recruit neutrophils at different exposure times. C. Flow cytometry demonstrated a prevalence of neutrophils at early time points, while infiltrated lymphocytes and macrophages were predominant at later time points, suggesting an exchange of the population recruited. D and E. In a trans-well

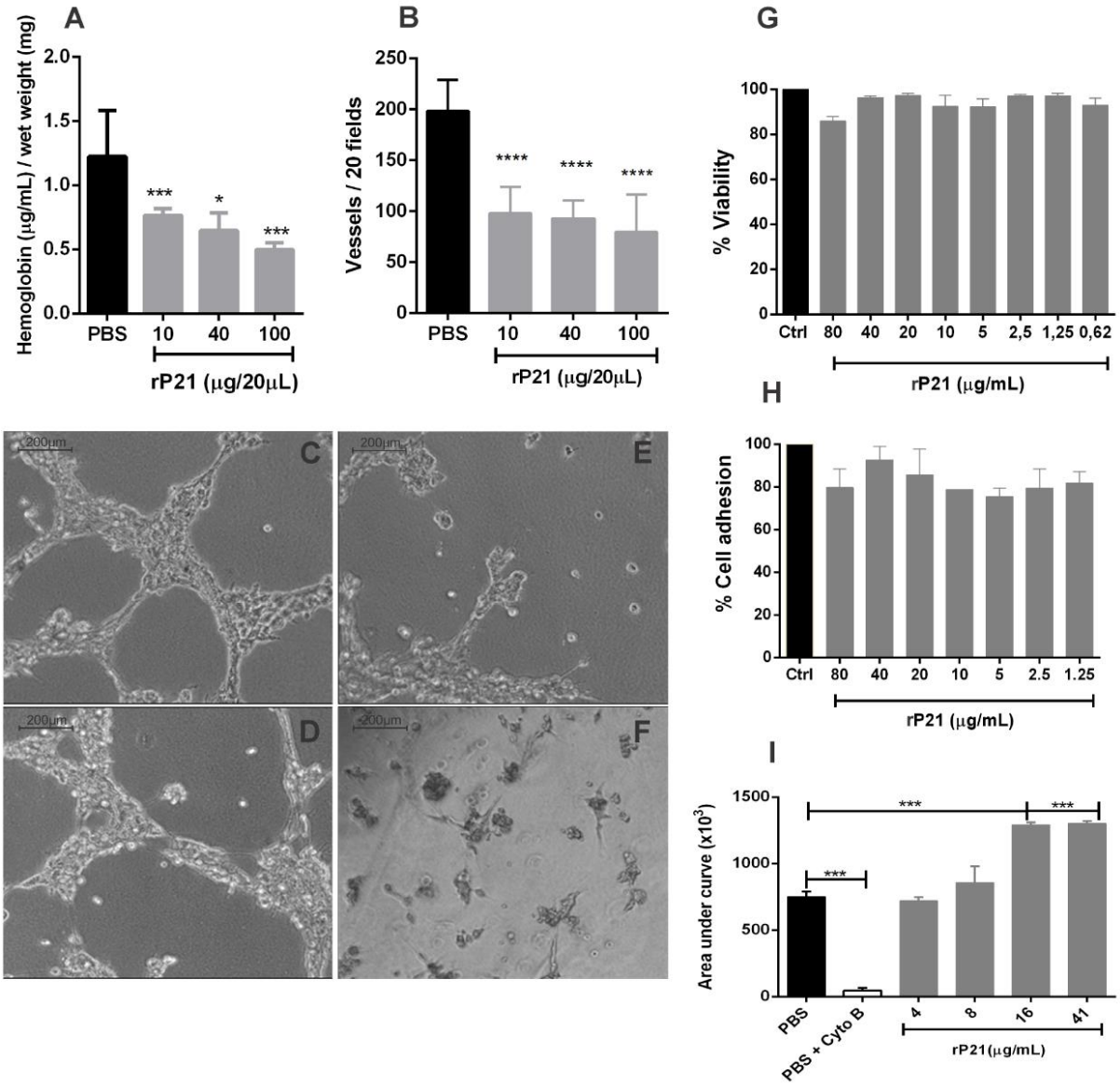
system through a polycarbonate membrane, the protein showed chemoattractive properties in murine peritoneal macrophages. Medium with FBS served as a positive control. Arrows indicate the presence of recruited macrophages (*p<0.05; **p<0.001; ***P<0.0001).

Fig. 2: rP21 recruited immune cells, as determined by treating polyester sponge discs with different concentrations of rP21. A and B. High levels of N-acetylglucosaminidase and myeloperoxidase after treatment indicate that rP21 not only recruits but also activates macrophages and neutrophils. C. Mast cells were also recruited. D and E. Histological images show that the sponges treated with rP21 had a lower number of blood vessels (arrows). (*p<0.05; **p<0.001; ***P<0.0001).

Fig. 3: rP21 inhibited angiogenesis. A. Hemoglobin concentrations (g/mL) per mg wet weight of sponge were obtained. There was a significant reduction in hemoglobin content with increasing rP21 concentrations, suggesting an anti-angiogenic effect. B. There was decrease in blood vessels present in the groups treated with rP21. C. tEnd cells preincubated with medium were used as controls. D. tEnd cells treated with 10 µg/mL, E. 40 µg/mL, and F. 80 µg/mL of rP21. After 18 h, cells were observed under the microscope at x20 magnification and photographed. G. Despite inhibiting vessel formation, the protein was not cytotoxic. H. Also, it did not affect cell adhesion. I. rP21 can polymerize the actin cytoskeleton in a dose-dependent manner. PBS with cytochalasin B (cyto B - 1 µg/mL) showed that fluorescence was not observed when actin polymerization was inhibited (*p<0.05; **p<0.001; ***P<0.0001).


A
Medium
PTX
Chemotaxis
(Neutrophils/field)
25
20
15
10
5
0
#
*
%
*
Medium
MIP-2
(30ng/ml)
p21
(40ug/ml)
B
PBS
rP21 40µg/mL
x10^4 cells/mL
150
100
50
0
**
*
*
6h
6h
24h
24h
72h
72h
C
Polymorphonuclear
Lymphocytes
Macrophages
Chemotaxis (%)
80
60
40
20
0
6 hours
24 hours
72 hours
D
Number of cells
150
100
50
0
Without FBS
FBS
rP21
E
Control without FBS
Control with FBS
rP21
100 µm
100 µm
100 µm


A
NAG activity (nmol/mg wet weight)
PBS
10
40
100
rP21 (μg/20μL)
B
MPO activity (OD/mg wet weight)
PBS
10
40
100
rP21 (μg/20μL)
C
Mastocyte/ cm2
Ctrl
10
40
100
rP21 (μg/20μL)
D
Control PBS
50μm
E
100 μg/20 μL rP21
50μm


A
Hemoglobin (µg/mL) / wet weight (mg)
2.0
1.5
1.0
0.5
0.0
***
*
***
PBS
10
40
100
rP21 (µg/20µL)
B
Vessels / 20 fields
250
200
150
100
50
0
****
****
****
PBS
10
40
100
rP21 (µg/20µL)
G
% Viability
100
80
60
40
20
0
Ctrl
80
40
20
10
5
2,5
1,25
0,62
rP21 (µg/mL)
H
% Cell adhesion
100
80
60
40
20
0
Ctrl
80
40
20
10
5
2.5
1.25
rP21 (µg/mL)
C
D
E
F
200µm
I
Area under curve (x10³)
1500
1000
500
0
***
***
***
PBS
PBS + Cyto B
4
8
16
41
rP21(µg/mL)

APÊNDICE C – Artigo enviado para revista

Galectin-3 expression is crucial for *Trypanosoma cruzi* control during the acute infection leading to lower cardiac tissue damage and fibrosis.

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Abstract

Galectin-3 is a member of the lectin family of proteins, characterized by its affinity for β-galactose-containing carbohydrates; it can be found in both the nucleus and the cytoplasm and can be either membrane-associated or secreted. This lectin is involved in many immunoregulatory processes. Here, we explore the biological activities of galectin-3 during acute and chronic *Trypanosoma cruzi* infection. Our results demonstrate that a lack of galectin-3 enhances the replication of intracellular parasites *in vitro*, results in increased parasitaemia *in vivo*, and reduces the recruitment of leukocytes. Moreover, we observed decreased secretion of pro-inflammatory cytokines in the spleen and heart in infected galectin-3 knockout mice. In addition, we observed higher mast cell recruitment and fibrosis in heart tissue from knockout and infected mice. In conclusion, galectin-3 expression plays a pivotal role in controlling acute *T. cruzi* infection, thus preventing consequent heart damage and fibrosis.

Keywords: Galectin-3, *T. cruzi*, heart, fibrosis, leukocytes

Introduction

Obligate intracellular parasites, such as *Trypanosoma cruzi*, must enter host cells to survive. The host cell provides a favorable environment for replication and protection from immune defenses through the subversion of cell signaling pathways (Sibley 2004). After entering the cell, *T. cruzi* is retained within a parasitophorous vacuole that rapidly fuses with host cell lysosomes (Tardieux et al. 1992; Woolsey et al. 2003). The parasite is able to resist the acidic conditions within this vacuole and escape into the cytoplasm, where it replicates (Nogueira and Cohn 1976; Ley et al. 1990). We have verified that galectin-3 transiently accumulates around vacuole-free parasites. Thus, this observation indicates that galectin-3 is a marker of *T. cruzi* phagosome lysis (Machado et al., 2014).

Moreover, authors have observed that *T. cruzi* infection upregulates the expression of galectin-3, both *in vivo* and *in vitro* (Acosta-Rodriguez et al. 2004; Vray et al. 2004). Human galectin-3 binds specifically to *T. cruzi* trypomastigote surface proteins, enhancing trypanosome adhesion to the extracellular matrix component laminin (Moody et al. 2000). In addition, the expression of human galectin-3 by coronary artery smooth muscle cells is required for and mediates the adhesion of *T. cruzi* to these cells (Kleshchenko et al. 2004).

In order to gather novel evidence of the role of galectin-3 during *T. cruzi* infection, we investigated parasite invasion *in vitro* and parasite replication in macrophages from wild-type and *galectin-3*$^{-/-}$ mice. In addition, we examined the impact of galectin-3 expression during *in vivo* acute and chronic infection.

Results and Discussion

Galectin-3, a multifunctional 35-kDa protein, is the only known member of the chimera-type group of galectins and is composed of a non-lectin domain connected to a typical carbohydrate-recognition domain (Cooper, 2002; Gabius, 1997; Kasai and Hirabayashi, 1996). Galectin-3 is involved in many immunoregulatory processes such as dendritic cell/T lymphocyte adhesion (Swarte et al., 1998), adhesion of cells to matrix glycoproteins (Kaltner and Stierstorfer, 1998), cellular proliferation, and the inflammatory response and cell migration toward inflammatory foci (Rabinovich et al., 2002). Thus, galectin-3 has been characterized as a chemoattractant (Sano and Liu, 2001). Galectin-3 participates in myelin phagocytosis in Schwann cells (Reichert et al., 1994) and contributes to the phagocytosis of microorganisms and apoptotic cells by macrophages.

While galectin-3 plays an important role in the initial control of *Leishmania* infection (Sato et al., 2014) and functions as an alarmin by augmenting the inflammatory response during sepsis development during pulmonary *F. novicida* infection (Mishra et al., 2013), it seems not to have a significant impact on the control of gut bacterial pathogens. In addition, researchers have demonstrated an association between galectin-3 expression and an unfavorable host response in leprosy (Chung et al., 2013).

Lack of galectin-3 expression does not alter T. cruzi *phagocytosis by murine peritoneal macrophages but enhances parasite intracellular replication.*

Researchers have observed that *galectin-3*$^{-/-}$ macrophages are defective in the phagocytosis of both IgG-opsonized erythrocytes and apoptotic cells during the initial time points of interaction. However, after one hour of macrophage and particle interaction, galectin-3-deficient phagocytes exhibited wild-type phagocytosis ability. In addition, galectin-3- deficient peritoneal macrophages displayed attenuated phagocytic clearance of apoptotic thymocytes *in vivo* (Sano et al. 2003). In agreement with this study, we verified that after two hours of interaction between macrophages and trypomastigotes, both wild type and galectin-3 knockout macrophages displayed a similar phagocytosis index (Figure 1C). Similar results were observed in human BeWo galactin-3 knockdown cells (Figure 1A). We previously observed the accumulation of galectin-3 at the site at which *T. cruzi* enters macrophages (Machado et al., 2014).

One intriguing observation in our *in vitro* studies was that at later time points, *T. cruzi* showed a higher proliferation index in galectin-3 knockout macrophages (Figure 1 D) and BeWo galactin-3 knockdown cells (Figure 1B). It is worth mentioning that galectin-3 overexpression promotes actin redistribution and cellular adhesion to the extracellular matrix (Matarrese et al., 2000). Also, galactin-3 overexpression was found to stimulate focal adhesion kinase, increase F-actin turnover, and enhance integrin activation and recruitment to elongated fibrillar adhesions (Granovsky et al., 2000; Lagana et al., 2006). Taken together, these data suggest that galectin-3 plays important role in actin cytoskeleton dynamics during *T. cruzi* infection. In this sense, galectin-3 expression and actin polymerization may contribute to impair parasite intracellular multiplication, leading to the chronic phase without detectable blood stream parasitemia. The mechanism behind this activity and a possible link to the actin polymerization machinery remains to be elucidated.

Galectin-3 knockout mice infected with T. cruzi *show delayed clearance of intra-peritoneal parasites and higher parasitemia than wild type animals.*

In order to check the ability of galectin-3 knockout mice to clear parasites inoculated intraperioneally, we collected peritoneal content three days after infection and counted the number of viable parasites (Figure 2B) and the number of total leukocytes. In accordance with Sano and colleagues, we also noted a delayed clearance of parasites (Figure 2B) and a lower recruitment of leukocytes into the peritoneal cavity (Figure 2C) as compared to wild-type animals. A reasonable explanation for this defect has yet to be described; however, we can envisage several different scenarios and explanations. First, this phenotype may be the result of an *in vivo* defect of phagocytosis in galectin-3 knockout mice which was not observed in our *in vitro* assays. On the other hand, it could also be a result of a defect in macrophage homing, due to low actin polymerization and membrane dynamics resulting in the retention of macrophages in the peritoneum and in the release of intracellular parasites due to a higher proliferation rate. In addition, we cannot discard the possibility of a defective innate immune response as an important player in pathogen clearance.

Corroborating our *in vitro* results, galectin-3 knockout mice displayed higher levels of parasitemia than wild-type animals (Figure 2A). This phenotype may be a result of the higher proliferation rate of the parasite within galectin-3 knockout cells and/or by an inappropriate host immune response by galectin-3 knockout mice during the acute phase of the infection. It is important to note that on by 90 days post-inoculation, both animal groups had completely controlled parasitemia.

Expression of cytokines in spleen and heart from galectin-3 knockout and wild type animals.

Our results have shown that galectin-3 knockout mice favor parasite replication. Thus, we evaluated IL-1β, TNF-α, IFN-γ, and IL-4 expression levels in spleen and heart samples from infected wild-type and galectin-3 knockout mice during both the acute and chronic phases of *T. cruzi* infection. Studies in galectin-3–deficient mice have strongly indicated that galectin-3 plays an important role as a pro-inflammatory mediator in inflammatory responses associated with pathological conditions (Jeon et al., 2010). For example, galectin-3–deficient mice displayed defective neutrophil recruitment and decreased macrophage survival (Colnot et al., 1998). It has also been reported that galectin-3–deficient mice exhibit reduced NF-kB responses and decreased cytokine production in several cell types (Chen et al., 2006; Hsu et al., 2000). Galectin-3 was found to be an inhibitory regulator of T-cell activation that functions intracellularly by promoting TCR down-regulation (Chen et al., 2009). Accordingly, we observed that IL-1β (Figure 3A) and IFN-γ (Figure 3E) were upregulated only in spleen from wild-type mice during the acute infection. In heart samples, we observed increased secretion of TNF-α (Figure 3C) and IFN-γ (Figure 3E) in wild-type animals, while in galectin-3 knockout mice we detected increased production of IL-1β (Figure 3B) and IL-4 (Figure 3H). This scenario may account for the higher infection rate during acute infection observed in knockout mice. We have previously described the importance IFN-γ produced by natural killer cells in controlling infection by low virulence *T. cruzi* strains (Rodrigues et al., 2012), thus revealing the important role played by this cytokine in parasite clearance. Taken together, these data suggest that IFN-γ production early in infection is crucial for the control of parasitemia and that the primary source of this cytokine during acute infection is natural killer cells. Therefore, galectin-3 may

174 have a stimulatory effect on natural killer cells which is in contrast to the inhibitory
175 effect of galactin-3 on T-cells observed by Chen and colleagues.

176 During the chronic phase of infection, we observed that there was no variation in
177 the concentration of IL-1β, TNF- α, IFN- γ, or IL-4 in spleen regardless of galactin-3
178 expression status, even after infection (Figure 3 A, C, E, G). We also observed that the
179 heart tissue of infected wild-type mice showed a similar response to patients with the
180 indeterminate form of the disease, i.e. a Th2- skewed response with basal levels of
181 TNF-α (Figure 3 D) and IFN-γ (Figure 3 F), decreased levels of IL-1β (Figure 3 B), and
182 increased levels of IL-4 (Figure 3H). This response observed in wild-type animals can
183 be considered an appropriate response that controls tissue damage and decreases the
184 functional impairment of the organ. However, in galactin-3 knockout mice, heart tissue
185 to did not exhibit increased levels of IL-4 (Figure 3H).

186 *In vitro* and *in vivo* studies have shown the presence of mast cells is associated
187 with cardiac *T. cruzi* infection (Almeida et al., 1975; Meuser-Batista et al., 2008). Here,
188 we observed increased recruitment of mast cells in wild-type and galectin-3 knockout
189 animals during the acute phase of infection (Figure 5A). During the chronic phase, only
190 galactin-3 knockout animals showed high levels of mast cells in the heart (Figure 5B).
191 This observation indicates that galectin-3 plays a different role in mast cell recruitment
192 than it does for the recruitment of leukocytes. The higher presence of mast cells in
193 cardiac tissue of galectin-3 knockout mice may account for the observed increased
194 levels of tissue damage and fibrosis. Thus, we analyzed the content of collagen in
195 histological specimens and verified that during both the acute and the chronic phase,
196 there are larger areas of fibrosis in infected galectin-3 knockout animals (Figure 5 C and
197 D). The high presence of collagen fibers in knockout animals can be explained by
198 increased parasite replication in these animals, leading to an increased number of viable

parasites for invasion, proliferation, and cell lysis. The large number of lysed cells can induce a strong inflammatory response and can result in the deposition of collagen on functional cells. This process is termed organ fibrosis.

In conclusion, galectin-3 expression during acute *T. cruzi* infection is important for optimal control of the infection; the absence of galactin-3 expression leads to increased levels of parasitemia and free parasites in the peritoneal cavity. Galectin-3 knockout animals have lower total leukocyte recruitment into the peritoneal cavity, suggesting that galactin-3 may act directly or indirectly as a chemotactic protein; conversely, increased recruitment of mast cells and fibrosis of the cardiac tissue was also observed.

Galectin-3 expression may contribute to the secretion of pro-inflammatory cytokines that control *T. cruzi* infection during the acute phase and result in a consequential decrease in tissue damage during the chronic phase. Our main results are summarized in Figure 7.

Material and Methods

Animals

C57BL/6 WT and Gal-3KO mice were maintained in standard conditions: a 12 hours light/dark cycle at 25 ± 2°C with food and water "ad libitum". The animals used in the experiments were 6 to 8 weeks of age. Animal protocols were approved by the Animal Care and Use Committee of the Federal University of Uberlândia.

Parasite and cells

223 *T. cruzi* was maintained in Vero cells cultured in DMEM (Sigma Aldrich) supplemented
224 with 2.5% fetal bovine serum. Human choriocarcinoma BeWo cells were treated with
225 lentiviral particles containing shRNA against galectin-3 (Santa Cruz Biotechnology);
226 WT and galectin-3 knockodow BeWo cell lines were generously provided by Prof. Dr.
227 Eloísa Amália Vieira Ferro, Universidade Federal de Uberlândia. BeWo cells were
228 cultured in DMEM (Sigma) supplemented with 10% FBS.

229 **Leukocytes from the peritoneal cavity**

230 To obtain peritoneal leukocytes in uninfected mice, 3% thioglycollate medium was
231 injected into the peritoneal cavity of C57BL/6 wild-type or galectin-3 knockout mice.
232 In the infected group, 10^5 trypomastigotes of *T. cruzi* CL strain were injected into the
233 peritoneal cavity. After 72 hours, the animals were euthanized by cervical dislocation
234 and peritoneal lavage was performed with 5 ml of ice cold 10% DMEM.
235 The peritoneal leukocytes and the free parasites in peritoneal cavity were counted in a
236 Neubauer chamber.

237

238 **Animal infection**

239 C57BL/6 wild-type and galectin-$3^{-/-}$ mice six to eight weeks of age were infected with
240 trypomastigotes of the CL strain of *T. cruzi* via intraperitoneal injection (10^5 parasites
241 per mouse). A group of uninfected mice was used as negative control.
242 The animals were euthanized 15 days (acute phase) or 90 days (chronic phase) post-
243 infection; next, the spleen and heart were removed. Thereafter, part of each organ was
244 placed in protease inhibitors for the measurement of cytokines by ELISA and the
245 remaining portion was used for histological analysis.

246

247 **Parasitemia**

248 At 7, 15, 30, 60 and 90 days post-infection, blood samples were obtained from each
249 mouse via tail vein in order to analyze the level of parasitemia (amount of parasites in
250 the blood) in wild-type and galectin-3 knockout mice. Approximately, 5 µl of blood was
251 added to a slide and the total number of parasites was counted for subsequent
252 comparison and evaluation of the importance of the galectin-3 protein in *T. cruzi*
253 infection.
254
255 **Cytokine Expression**
256 The level of cytokines in tissues from wild-type and galectin-3 knockout control mice
257 and mice infected with *T. cruzi* was performed by macerating spleen and heart tissue,
258 then analyzing cytokine levels in the supernatant using ELISA . The organs were kept in
259 1 ml of protease inhibitor (Sigma Aldrich) solution to prevent degradation of these
260 proteins. The levels of IFN-γ, TNF-α, IL-1β, and IL-4 were measured using the ELISA
261 kit from BD Biosciences ®.
262
263 **Histological analysis**
264 The spleen and heart were processed for histological analysis. The organs were fixed in
265 formaldehyde (10% in PBS), then dehydrated in increasing concentrations of alcohol,
266 diaphanized in xylene, and finally embedded in paraffin. Using a microtome, the organs
267 were sliced into 5 mm thick sections and placed on glass slides; subsequently the slides
268 were processed for staining.
269
270 **Toluidine blue staining**
271 For analysis of the number of mast cells recruited in the different groups, sections were
272 stained with toluidine blue.

First, sections were deparaffinized with xylene and passed through a hydration process using decreasing concentrations of alcohol and water. The sections were then placed in phosphate citrate buffer pH 3.0 for 5 min and stained with 0.5% toluidine blue for 3 minutes. The excess dye was removed by dipping the slides in the buffer.

Next, further clarification was achieved using increasing concentrations of alcohol and xylene. The slides were mounted with Entellan®.

Data were analyzed using the ratio of the total number of mast cells per area of histological section (total number of mast cells/cm^2).

Picrosirius staining

For analysis of galectin-3 participation in the histological fibrosis of organs infected by *T. cruzi,* a picrosirius staining method was utilized for the visualization of collagen.

Sections were subjected to successive baths with xylene, hydrated in solutions of decreasing concentrations of ethanol and water, and then stained with picrossirirus solution for 50 minutes and washed in distilled water. Next, the sections were placed in hematoxylin solution for four minutes, washed in water, and stained for one minute with aqueous eosin. Subsequently, they were dehydrated in ethanol solutions and diaphanized with xylene. The slides were mounted with Entellan®.

Quantification of collagen fibers was performed using Image J software; images of 30 fields of each sample were taken with a light microscope with a camera attached.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.01. Results were considered significant when $p < 0.05$.

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299

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444 **Figure Legends:**

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446 **Figure 1:** *Galectin-3 knock down did not effect parasite cell invasion but increased*
447 *parasite replication at later time points*. BeWo cells (A, B) and mouse peritoneal
448 macrophages (C, D) were plated and submitted to invasion by trypomastigotes of the *T.*
449 *cruzi* CL strain. At two hours after invasion and additional later time points, coverslips
450 were washed, fixed with Bouin, and stained with Giemsa. The number of parasites was
451 counted using a bright light microscope. Representative images of parasite invasion and
452 replication are also shown (E, F).

453

454 **Figure 2:** *Lack of galectin-3 leads to higher systemic parasitemia, delayed parasite*
455 *clearance, and low leukocyte recruitment to the peritoneal cavity*. Mice were inoculated
456 intraperitoneally with trypomastigotes of the *T. cruzi* CL strain. At different time points
457 post-inoculation, parasitemia was observed in tail peripheral blood samples (A). The
458 ability of animals from both groups in clear peritoneal parasites and to recruit
459 leukocytes to peritoneal cavity was measured (B, C).

460

461 **Figure 3:** *Spleen area was higher in wild type and galectin-3 knockout animals during*
462 *the acute phase of* T. cruzi *infection*. Spleen (A, C) and heart (B, D) from infected and
463 uninfected wild-type and galectin-3 knockout mice during acute (A, B) and chronic
464 phases of infection (C, D) were removed and images were taken. Organ area was
465 measured using ImageJ software.

466

Figure 4: *Pro-inflammatory cytokine expression was down-regulated in spleen and heart tissue of infected galectin-3 knockout mice during the acute phase of infection.* IL-1β (A, B), TNFα (C, D), IFNγ (E, F), and IL-4 (G, H) from the supernatant of macerated spleen and heart was measured using ELISA.

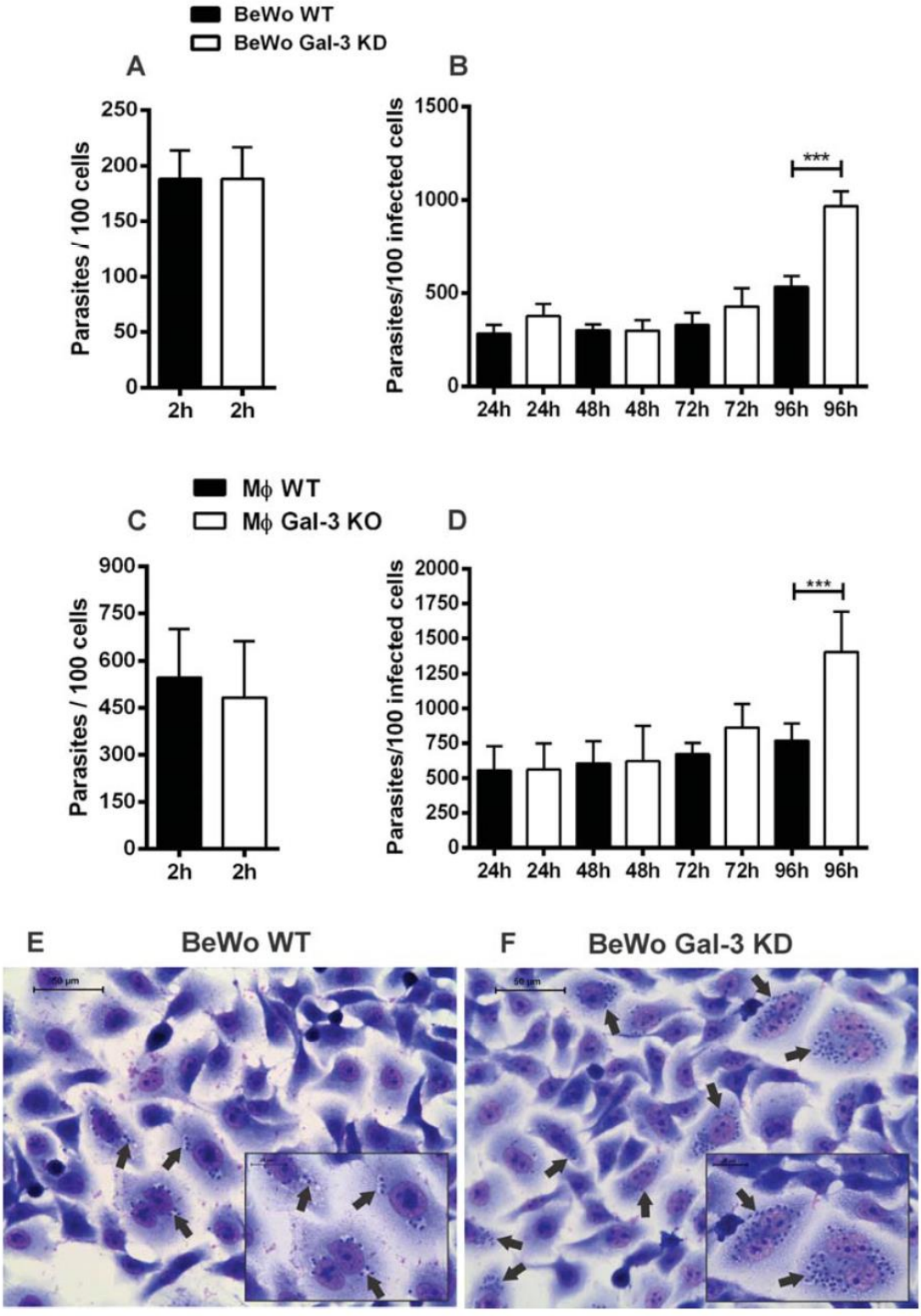
Figure 5: *A similar pro-inflammatory cytokine expression profile was observed in spleen and heart tissue of both wild-type and galectin-3 knockout infected animals during the chronic phase of infection.* IL-1β (A, B), TNFα (C, D), IFNγ (E, F) and IL-4 (G, H) were measured by ELISA from the supernatant of macerated spleen and heart.

Figure 6: *Lack of galectin-3 leads to recruitment of mast cells and higher levels fibrosis in heart samples*. Mast cell recruitment to heart tissue in infected galectin-3 knockout mice was increased during chronic infection (A, B). High levels of fibrosis were observed in heart histological samples from these animals during both the acute and chronic phase of infection (C, D).

Figure 7: *Impact of galectin-3 expression over* T. cruzi *infection progression*. WT animals infected with *T. cruzi* during the acute infection showed an increased production of TNF-α and IFN-γ. Decreased secretion of IL-4 and increased recruitment of leukocytes into the site of infection (peritoneal cavity). Hence, these animals showed lower levels of parasitemia and viable parasites in the peritoneal cavity. During the chronic phase, WT animals increased IL-4 production and showed lower amounts of mast cells in the heart tissues that appeared with low fibrosis. Gal-3 KO animals, on the other hand, showed basal levels of TNF-α and IFN-γ; increased IL-4 and reduced recruitment of leukocytes to the site of the infection. Therefore, Gal-3 deficiency leads

to higher levels to parasite multiplication and also higher levels of viable parasites in the peritoneal cavity. Heart tissues of these animals displayed higher fibrosis area.

517 Figure 1:



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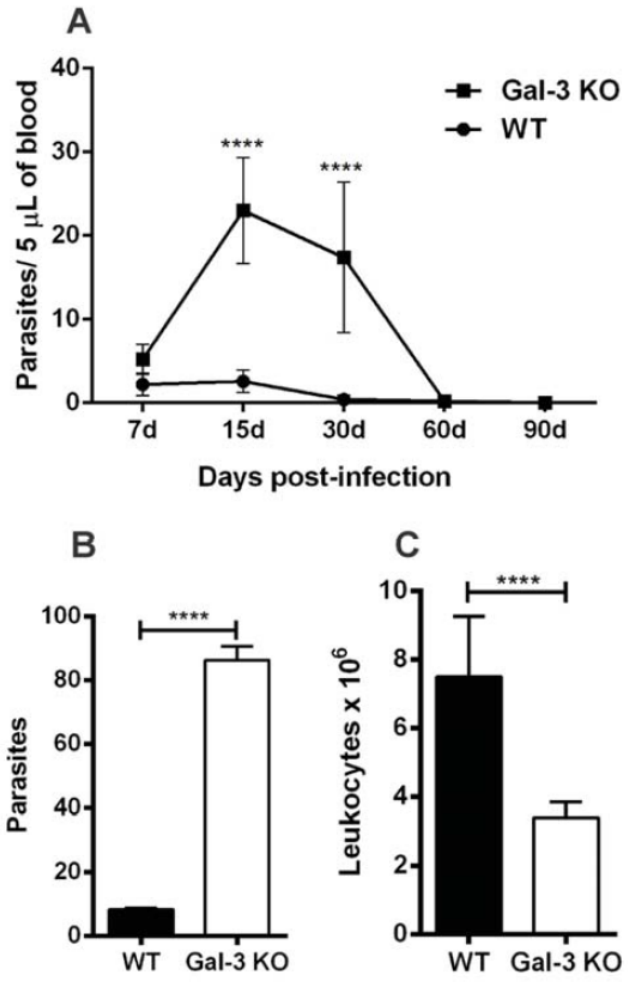

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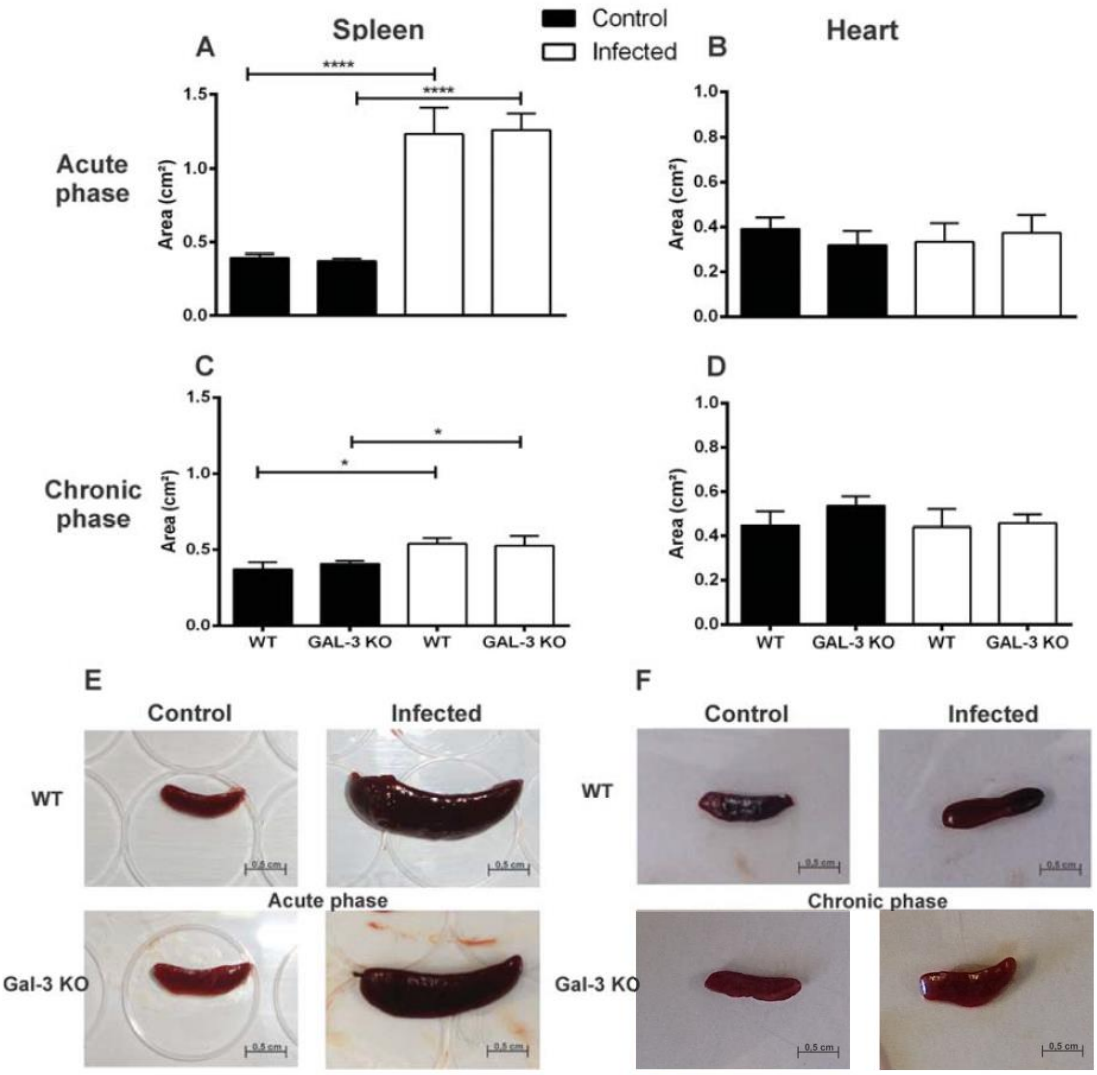

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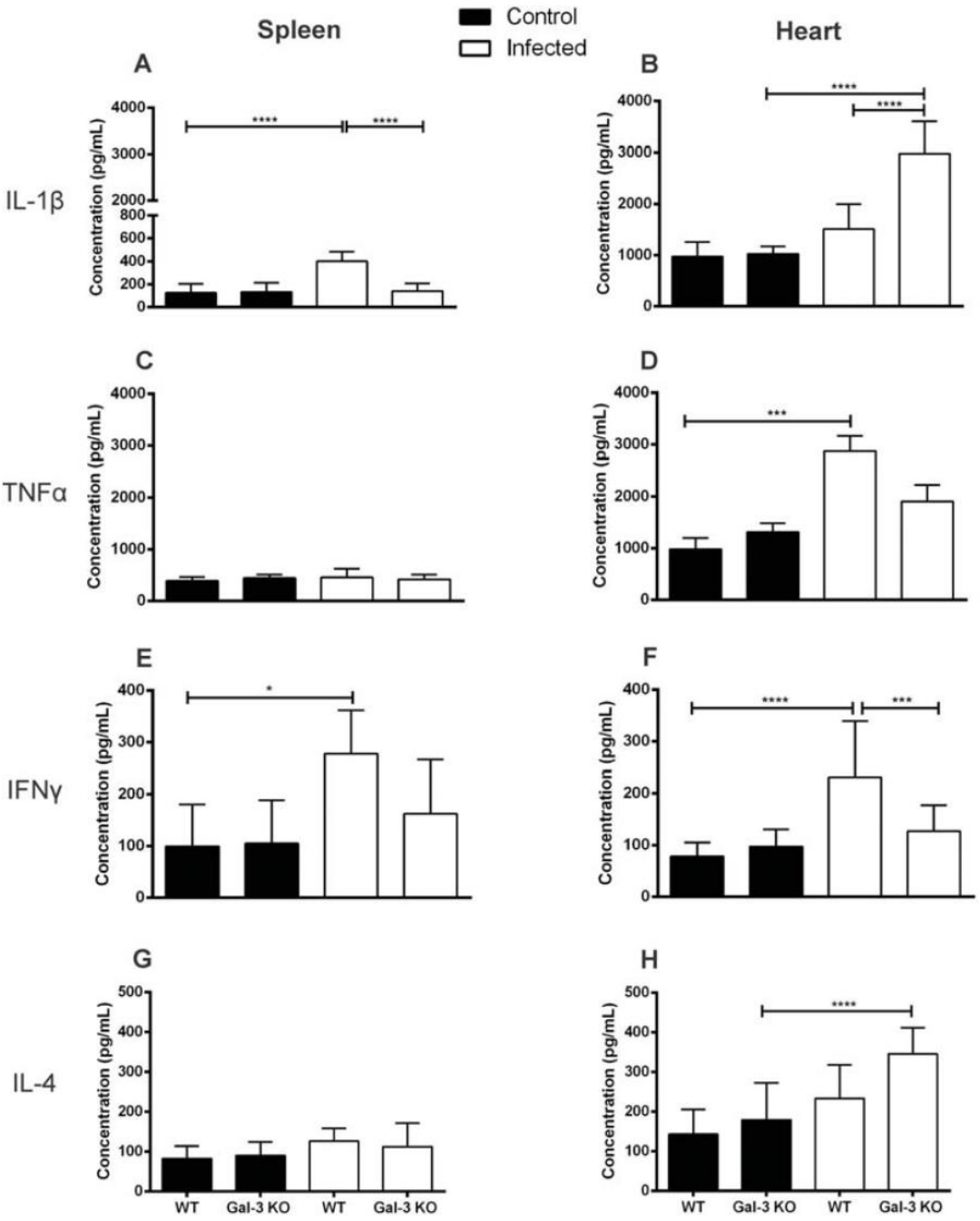

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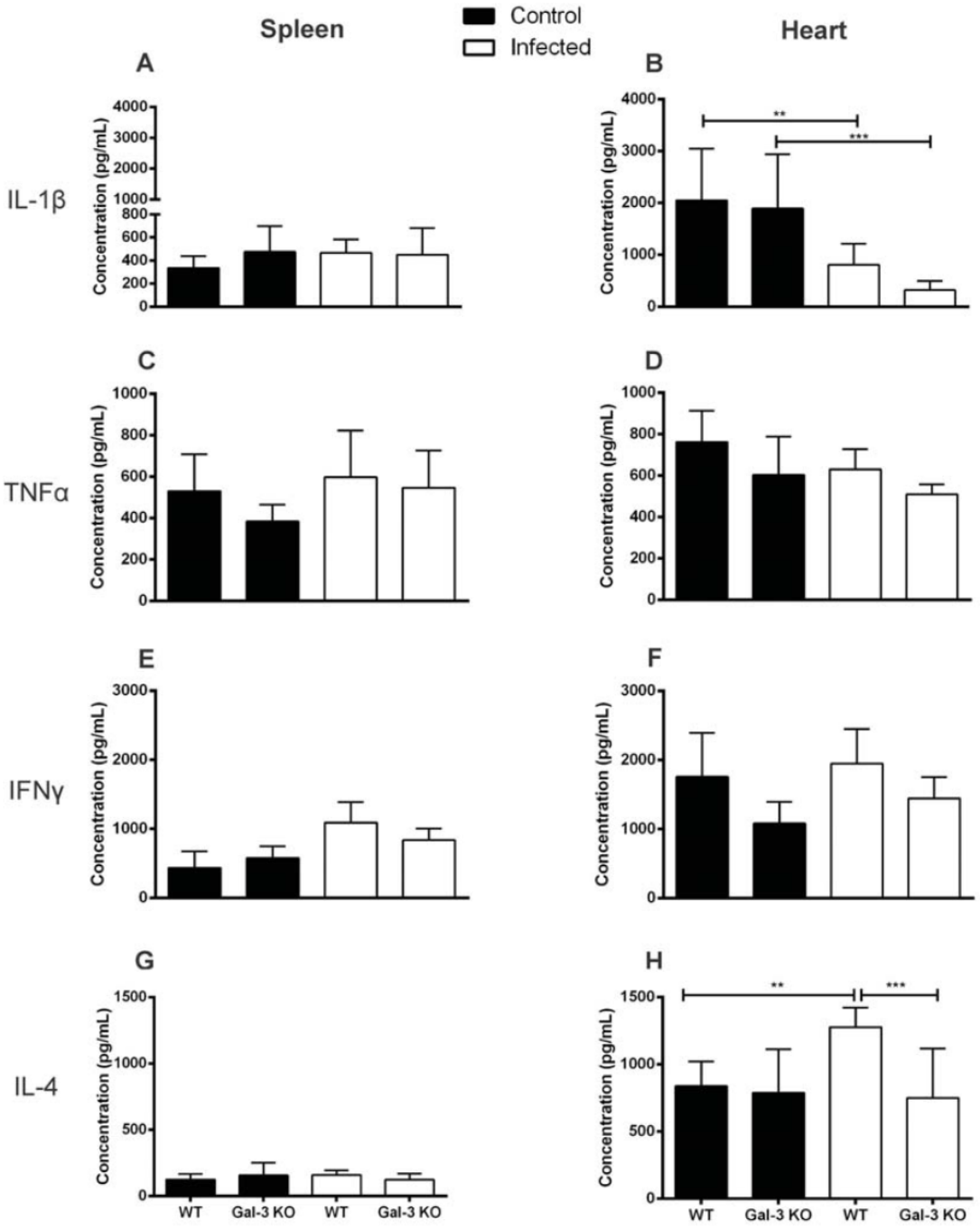

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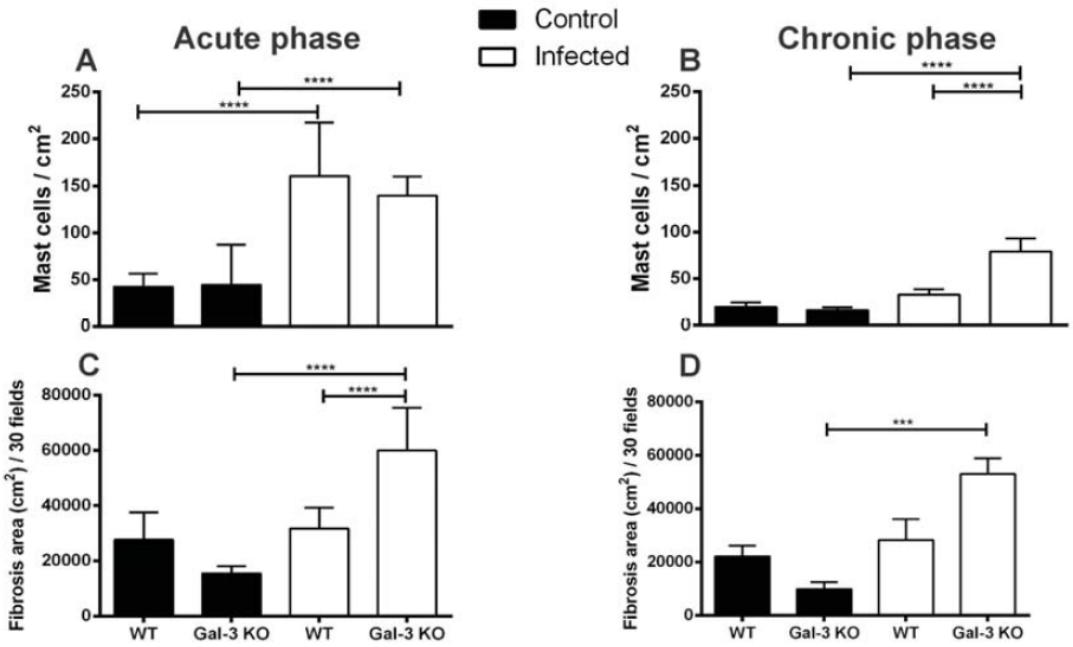

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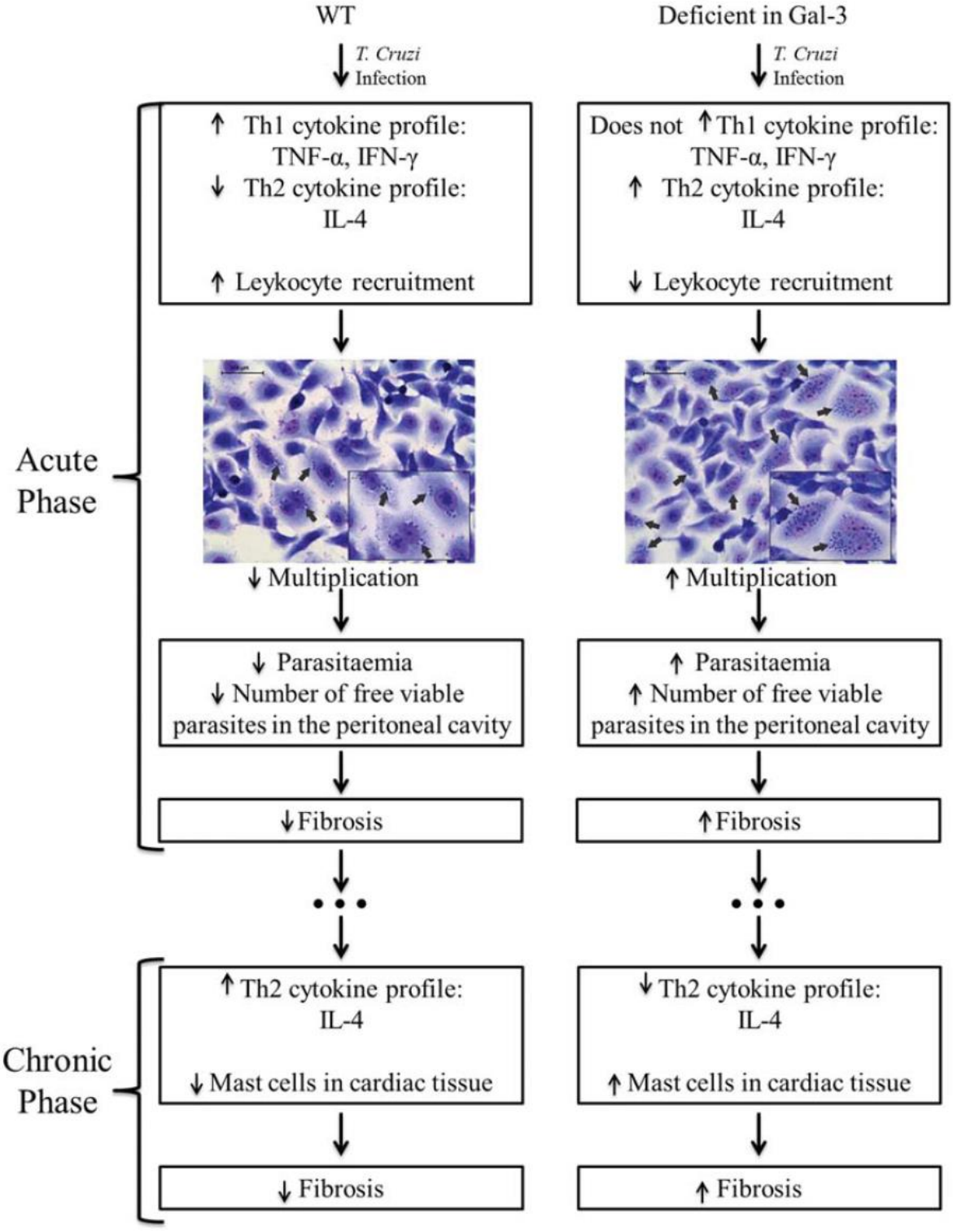

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