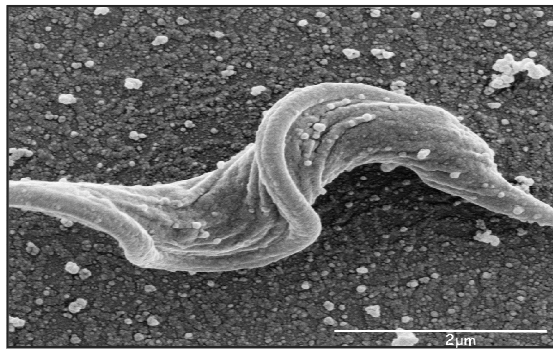**UNIVERSIDADE FEDERAL DE MINAS GERAIS**

Instituto de Ciências Biológicas

Departamento de Parasitologia

Programa de Pós-Graduação em Parasitologia

Aspectos da glicobiologia de Tripanosomatídeos:

as vesículas extracelulares de *Trypanosoma cruzi* e o lipofosfoglicano (LPG) de *Leishmania amazonensis* na interação parasito-hospedeiro.

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"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos."

Albert Einstein

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RESUMO I – 1ª publicação

Os parasitos tripanosomatídeos são responsáveis por graves doenças no homem. Durante seu ciclo de vida são submetidos a condições extremamente adversas. Moléculas ancoradas por glicosilfosfatidilinositol (GPI) são os principais antígenos expressados na membrana plasmática dos tripanosomatideos e auxiliam no processo de infecção. Dentre essas, as GPI-mucinas são glicoconjugados importantes em *Trypanosoma cruzi* e desempenham um papel crucial na sobrevivência dos parasitos. Este parasito é capaz de liberar vesículas extracelulares (EVs) enriquecidas por GPI-mucinas contendo resíduos terminais de α-galactosil (α-Gal) e TS/gp85. Contudo, o comportamento de células do sistema imune no curso da infecção da doença de Chagas após a estimulação com EVs de *T. cruzi* de cepas de diferentes DTUs ainda é desconhecido. Assim, o este trabalho avaliou o impacto destas vesículas das cepas Colombiana (Tc I), YuYu (Tc I), Y (Tc II) e CL-14 (Tc VI) no perfil de ativação da resposta imune inata e crônica. As EVs foram purificadas e exibiram diferenças intraespecíficas na quantidade de proteína e de epítopo α-Gal. Durante a resposta imune inata, vesículas das cepas YuYu e CL-14 induziram altos níveis de citocinas pró-inflamatórias (TNF-α e IL-6) e óxido nítrico via TLR2. Não foi observada diferença na ativação de MAPKs (ERK1/2, p38 e JNK) após estimulação com as vesículas. A produção de citocinas no sobrenadante de cultura de células esplênicas derivadas de camundongos crônicos infectados com os quatro tipos de cepas mostrou perfil pro-inflamatório após estimulação com vesículas de Colombiana e Y, independente do tipo de cepa usada na infecção *in vivo*. Para testar a importância funcional desta modulação, foi avaliada a expressão de citocinas intracelulares após exposição *in vitro* apenas nas cepas Colombiana e YuYu. As vesículas foram capazes de induzir produção de citocinas. Foi observada alta frequência de IL-10 em linfócitos T CD4+ e CD8+, além de um perfil misto de produção de TNF-α e IL-10 em células B. Finalmente, células dendríticas produziram TNF-α após estimulação com as vesículas. Em conclusão, variações intra-específicas nas EVs de *T. cruzi* são importantes para a ativação do sistema imune inato e modular os eventos imunopatológicos nas fases aguda e crônica da Doença de Chagas.

RESUMO II – 2ª. publicação

Vários trabalhos têm elucidado o papel do LPG em diferentes espécies de *Leishmania*. Neste trabalho foram utilizadas duas cepas de referência de *L. amazonensis* anteriormente isoladas do vetor (cepa PH8) e do hospedeiro vertebrado (cepa Josefa). Análises preliminares dos LPGs destas cepas indicaram que a cepa PH8 apresentou uma e duas cadeias laterais de β-glicose, enquanto que da cepa Josefa possui duas a três cadeias de β-galactose (Nogueira et al. *em preparação*). Este trabalho caracterizou o papel imunomodulatório destes LPGs durante interação *in vitro* com macrófagos murinos, células CHO e infecção *in vivo* do vetor *Lutzomyia migonei*. Os experimentos com macrófagos murinos demonstraram um papel pró-inflamatório dos LPGs, na produção de NO e citocinas (TNF-α e IL-6). Esta estimulação foi preferencialmente via TLR4. Ambos LPGs foram capazes de ativar MAPKs (p38 e JNK) e IκBα, sem ativação posterior de NF-κB em células CHO. Não houve diferença na infecciosidade para o flebotomíneo e ambas as cepas foram capazes de alcançar altas densidades após a excreção do bolo alimentar no terceiro dia após alimentação sanguínea. O polimorfismo intraespecífico em *L. amazonensis* não ocasiona diferença no perfil de ativação do sistema imune e na infectividade do vetor. Em conclusão, os LPGs de *L. amazonensis* apresentam um perfil pró-inflamatório e ao mesmo tempo imunossupressor para o hospedeiro vertebrado e seus polimorfismos não afetam a interação com o vetor.

ABSTRACT I

Tripanosomatid parasites are the causative agents of a wide spectrum of clinical manifestations. The parasites have an outstanding ability to avoid destruction in the hostile environments during their life cycles. Their main strategies include the expression of major glycoconjugates attached to the membrane by glycosylphosphatidylinositol (GPI) anchors. The GPI-mucins are important glycoconjugate of *Trypanosoma cruzi*. Those molecules play an important role on the protozoan infection and survival. Trypomastigote forms of *T. cruzi* shed extracellular vesicles (EVs) enriched with trans-sialidase (TS)/gp85 glycoproteins and other α-galactosyl (α-Gal)-containing glycoconjugates, like mucins. However, the role of EVs from different *T. cruzi* strains and some aspects of the immune system stimulation remain unknown. Here, vesicles from pathogenic (Colombiana (Tc I), YuYu (Tc I), Y (Tc II)) and non-pathogenic (CL-14 (Tc IV)) *T. cruzi* strains were purified and exhibited differences in their protein and α-galactosyl contents. Later, those polymorphisms were evaluated in the modulation of immune responses (innate and in the chronic phase) in C57BL/6 mice. EVs from YuYu and CL-14 strains induced higher levels of proinflammatory cytokines (TNF-α and IL-6) and nitric oxide by macrophages via TLR2. In general, no differences were observed in MAPKs activation (p38, JNK and ERK 1/2) after EV stimulation. In splenic cells derived from chronically infected mice, a different modulation pattern was observed, where Colombiana and Y strain EVs were more pro-inflammatory. This modulation was independent of the strain used in the mice infection. To test the functional importance of this modulation, the expression of intracellular cytokines after in vitro exposure was evaluated using EVs from YuYu and Colombiana strains. Both EVs induced cytokine production with the appearance of IL-10 in the chronically infected mice. A high frequency of IL-10 in CD4+ and CD8+ T lymphocytes was observed. A mixed profile of cytokine induction was observed in B cells with the production of TNF-α and IL-10. Finally, dendritic cells produced TNF-α after stimulation with EVs. Intraspecific variation in *T. cruzi* EVs are very important to immune system activation and it is responsible to modulate the immunopatologycal events in acute and cronic Chagas disease.

ABSTRACT II

Many studies have detected intra and interspecies polymorphisms in the *Leishmania* LPGs. However, the immunomodulatory properties and the degree of intraspecies polymorphism in *Leishmania amazonensis* LPG are not known. Here, the immunomodulatory activity of LPGs from *L. amazonensis* was evaluated in two strains from Brazil. One strain (PH8) was originally isolated from the sand fly and the other (Josefa) was isolated from a human case. Preliminary analysis in both strains exhibited structural polymorphism in the LPG, with the former possessing 1 and 2 β-glucose and the latter 1 to 3 β-galactose as side chains (Nogueira et al. *in preparation*). The ability of purified LPGs from both strains was investigated during *in vitro* interaction with peritoneal murine macrophages and CHO cells and *in vivo* infection with *Lutzomyia migonei*. In peritoneal murine macrophages, the LPGs from both strains activated NO and citokines (TNF-a and IL-6) via TLR4. Both LPGs equally activate MAPKs and the NF-κB inhibitor p-IκBα, but were not able to translocate NF-κB. *In vivo* experiments with sand flies showed that both stains were able to sustain infection in *L. migonei*. Also those polymorphisms did not affect infectivity to the sand fly. Intraspecific polymorphisms in the LPGs from *L. amazonensis* they did not result in different activation profiles of the innate immune system and the sand fly infectivity. Finally, both *L. amazonensis* LPGs are proinflamatory and immunosuppressive in vertebrate host and those polymorphisms did not affect the interaction with sand fly.

Lista de Abreviaturas e Siglas

DTUs – *Discreet Typing Units*-DTUs, Tc I-VI

TS – trans-sialidase

GIPLs – glicoinositolfosfolípides

LPG – lipofosfoglicano (l*ipophosphoglycan*)

GPI – glicosilfostatidilinositol

EVs – vesículas extracelulares (*extracellular vesicles*)

MASP – proteína de superfície associada a mucina (*mucin-associated surface protein*)

TLR – receptor do tipo Toll (*Toll Like Receptor*)

MAPKs – proteíno-quinases ativadas por mitógenos (*Mitogen Activated Protein Kinases*)

CHO – células de ovário (*chinese hamster ovary*)

IFN-γ – interferon gama

TNF-α – fator de necrose tumoral alfa

IL – interleucina

TGF-β – fator de transformação do crescimento (*Transforming growth factor beta*)

PBS – tampão fosfato-salina (*phosphate buffered saline*)

PMA – *Phorbol-12-miristato-13-acetato*

EDTA – ácido etilenodiamino tetra-acético (*ethylenediamine tetraacetic acid*)

CD – cluster de diferenciação (*cluster of differentiation* – exemplo CD4)

PAMPs – padrões moleculares associados ao patógenos (*Pathogen-associated molecular patterns*)

SUMÁRIO

INTRODUÇÃO

Parasitos dos gêneros *Leishmania* e *Trypanosoma* (família Trypanosomatidae) são protozoários unicelulares com flagelos simples capazes de causar doenças no homem com considerável morbidade e mortalidade. A Leishmaniose e a Doença de Chagas são consideradas um grave problema de saúde pública mundial afetando mais de 27 milhões de pessoas no mundo todo (WHO, 2010). Os parasitos do gênero *Leishmania* e *Trypanosoma* são heteroxênicos e durante seu ciclo de vida alternam entre hospedeiros invertebrado e vertebrado, onde são submetidos a condições extremamente adversas. Para conseguir sobreviver e completar seu ciclo, esses parasitos sofrem fortes adaptações fisiológicas e bioquímicas, relacionadas ao meio ambiente específico ou em antecipação à próxima etapa do ciclo.

O estudo da glicobiologia desses microrganismos aumenta a compreensão do envolvimento de glicoconjugados nos diversos processos imunopatológicos da interação parasito-hospedeiro. Neste trabalho, serão abordadas estratégias utilizadas por *Trypanosoma cruzi* e *Leishmania amazonensis* que auxiliam na sua sobrevivência e sucesso no estabelecimento da infecção nos hospedeiros vertebrado e invertebrado. De acordo com as publicações, este trabalho foi dividido em duas partes: I) O papel de vesículas extracelulares de *T. cruzi* na resposta imune do hospedeiro vertebrado (Nogueira et al. 2015); II) Papel do Lipofosfoglicano (LPG) de *L. amazonensis* na interação com hospedeiros vertebrado e invertebrado (Nogueira et al. 2016). Estes são projetos multidisciplinares que contaram com a colaboração de vários pesquisadores do Brasil e exterior.

PARTE I) O papel de vesículas extracelulares de *T. cruzi* no processo de interação com hospedeiro vertebrado

1 – A Doença de Chagas e as vesículas de *T. cruzi*

A doença de Chagas é causada pelo protozoário parasito *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida: Tripanosomatidae). Esse parasito é transmitido ao homem pelas fezes de insetos reduviídeos da subfamília Triatominae, sendo que na América Latina, o *Triatoma infestans* e o *Rhodnius prolixus* são os vetores mais importantes. As formas de transmissão mais importantes incluem: vetorial, transfusional e congênita (Dias 2007). Entretanto, recentemente têm sido relatados vários casos de transmissão por via oral (Coura & Borges-Pereira 2010) WHO, 2010). Estima-se que cerca de 11 milhões de pessoas estejam infectadas, distribuídas principalmente em 21 países da América Latina, com incidência anual de 40 mil novos casos por ano. Mesmo considerando os recentes avanços na eliminação da transmissão vetorial e transfusional da Doença de Chagas na América Latina, ainda há cerca de 5-8 milhões de chagásicos residindo nesta área e cerca de seis mil mortes por ano (Dias 2009; Rassi et al. 2010; Silveira & Pimenta Jr 2011). Com os movimentos migratórios, a doença tem se expandido a outros países da América do Norte, Europa, Ásia e África onde a mesma não é endêmica (Basile et al. 2011; Bern et al. 2011), sendo neste caso a transmissão transfusional mais importante. A significativa carga global da doença de Chagas junto com a limitação do tratamento disponível indica a necessidade iminente de desenvolver novas metodologias alternativas de controle da doença.

Durante a história natural da Doença de Chagas, os indivíduos infectados desenvolvem uma fase aguda curta, caracterizada pelo alto parasitismo no sangue e tecidos, muitas vezes com sintomas inespecíficos que podem ou não evoluir para sinais e sintomas graves. O sucesso da terapia é obtido em aproximadamente 70% dos casos, quando diagnosticados rapidamente e principalmente em faixas etárias mais jovens (de Andrade et al. 1996; Rassi et al. 2010). A maioria dos indivíduos evolui para fase crônica após dois a quatro meses de infecção, acompanhada por níveis de parasitemia

subpatentes e elevação de anticorpos específicos da classe IgG, que perduram por toda vida. Aproximadamente 60 a 70% dos pacientes crônicos desenvolvem a forma indeterminada da doença de Chagas e não apresentam sintomas clínicos associados à infecção. Outros indivíduos podem desenvolver formas sintomáticas da doença, que afeta tecido cardíaco e/ou digestivo (Rassi et al. 2000; Rocha et al. 2007). O envolvimento cardíaco é a manifestação mais séria e mais frequente da fase crônica.

Os mecanismos específicos envolvidos com o estabelecimento/manutenção das diferentes formas clínicas da doença de Chagas são complexos. Acredita-se que a heterogeneidade das manifestações clínicas sejam consequências de múltiplos fatores ligados ao *T. cruzi* (cepa, virulência, antigenicidade, tropismo e tamanho do inóculo) e ao hospedeiro (idade, sexo, raça e perfil da resposta imune) (Dias et al. 2015). Análises de biologia molecular de diferentes isolados de *T. cruzi* demonstram que populações de parasito são extremamente polimórficas (Buscaglia & Di Noia 2003). Atualmente, o táxon de *T. cruzi* é classificado em seis subgrupos de "unidades discretas de tipagem" (*Discret Typing Units*-DTUs, Tc I-VI) (Zingales et al. 2009). Esta classificação foi proposta com a finalidade de uniformização das diferentes classificações anteriores e reúne populações mais relacionadas entre si do que qualquer outra amostra. Foram identificáveis por meio de marcadores genéticos, moleculares ou imunológicos em amostras de *T. cruzi* isoladas de diferentes hospedeiros, áreas geográficas e associadas à complexidade ecoepidemiológica (Miles et al. 2009; Andrade et al. 2010). Porém, o desenvolvimento de um consenso para as diferentes cepas de *T. cruzi* ainda não forneceu bases moleculares para as diferentes manifestações clínicas da doença de Chagas.

O subgrupo TcI é amplamente disperso nas Américas. No Brasil TcI está relacionado ao ciclo silvestre, mas nos outros países como Argentina e Colômbia os ciclos silvestre e doméstico podem se sobrepor, além de ser associada a cardiopatias graves (Ramírez et al. 2010; Zingales et al. 2012). O subgrupo TcII é predominante entre os pacientes chagásicos nas regiões sul e central da América do Sul, onde a doença de Chagas é considerada mais grave, associada a manifestações cardíacas, megaesôfago e megacólon (Miles et al. 2009). O grupo híbrido TcVI aparece nas regiões sul e centro da América do Sul, no Gran Chaco, porém o conhecimento do seu ciclo está incompleto. O tropismo tecidual diferencial entre DTUs tem sido demonstrado para as

linhagens TcI, II e VI (Vago et al. 2000; Franco et al. 2003) (Fig. 1). Além disso, padrões diferenciais de parasitemia e respostas imunes específicas contra determinadas DTUs têm sido reportados (Andrade et al. 2010; Poveda et al. 2014).

Figura 1: Distribuição geográfica aproximada de três subgrupos de *T. cruzi* na transmissão com ciclo silvestre e doméstico (Adaptado de Zingales et al., 2009).

Independentes da forma clínica existem fortes evidências da participação do sistema imune na patogênese da doença de Chagas (Tarleton 2007). Durante o ciclo de infecção no hospedeiro vertebrado, as formas tripomastigotas invadem diferentes tipos de células, diferenciam-se em amastigotas no citoplasma celular e multiplicam-se por sucessivas divisões binárias. Ao final, as amastigotas voltam a se diferenciar em tripomastigotas que rompem a membrana da célula hospedeira, atingem a matriz extracelular e a corrente sanguínea, disseminando a infecção para os diferentes órgãos e sistemas (Dias 2000). O *T. cruzi* possui uma variedade de moléculas e estratégias elaboradas para invadir células hospedeiras e escapar de mecanismos de defesa imune do hospedeiro (Almeida & Gazzinelli 2001; Andrade & Andrews 2005; Soares et al. 2012).

A superfície das tripomastigotas é composta por glicoconjugados, principalmente da superfamília *trans*-sialidase/gp85 (TS), glicoinositolfosfolípides (GIPLs) e mucinas (tGPI-muc) associados à membrana por âncoras de glicosilfostatidilinositol (GPI) (Schenkman et al. 1994; Almeida & Gazzinelli 2001; Oliveira et al. 2004; Soares et al. 2012). Essas moléculas desenvolvem importante papel na invasão do parasito e controle da resposta imune. Além de proteínas de superfícies, estudos têm demonstrado que o parasito libera antígenos em meio de cultura de forma solúvel ou com fragmentos de

membrana plasmática. Estes últimos são denominadas de exossomos ou simplesmente vesículas extracelulares (EVs) (Torrecilhas et al. 2012; Marcilla et al. 2014).

A liberação de vesículas já foi descrita em vários protozoários, como *Leishmania*, *T. cruzi*, *Trypanosoma brucei*, *Plasmodium*, *Trichomonas vaginalis*, *Toxoplasma gondii* e *Eimeria* e helmintos (revisado por (Campos et al. 2015; Szempruch et al. 2016) (Marcilla et al. 2014). A produção dessas vesículas representa um mecanismo adicional de comunicação entre células sem contato direto e atua sobre o microambiente, em que o parasito apresenta sistematicamente seus antígenos exercendo efeitos a curta e longa distância no hospedeiro (Montaner et al. 2014). O *T. cruzi* produz EVs espontaneamente além de induzir a liberação das mesmas em células infectadas com presença de antígeno próprio (Gonçalves et al. 1991; Trocoli Torrecilhas et al. 2009; Cestari et al. 2012; Torrecilhas et al. 2012). Análises bioquímicas e proteômicas preliminares destas estruturas em *T. cruzi* revelam a presença de proteases (cruzipaína) e glicoconjugados enriquecidos com epítopos de α-galactose, encontrados preferencialmente nas GPI-mucinas (Torrecilhas et al. 2012). Também são encontradas várias glicoproteínas de superfície, como Tc-85, MASP e TS, além de pequenos RNAs (tRNA) (Garcia-Silva et al. 2014; Campos et al. 2015). Essas moléculas estão envolvidas no processo de adesão, invasão celular e modulação da resposta imune do hospedeiro (Almeida & Gazzinelli 2001; Soares et al. 2012; Torrecilhas et al. 2012). A liberação de vesículas com esses antígenos pode representar um mecanismo adicional utilizado pelo parasito para obter sucesso durante a infecção.

O papel de vesículas na patologia da doença de Chagas foi sugerido por Torrecilhas *et al*., (2009) em camundongos que foram previamente desafiados com EVs. Posteriormente, estes animais foram infectados com *T. cruzi* (cepa Y) e tiveram alta taxa de mortalidade e intensa reação inflamatória, com aumento do número de ninhos de amastigota no tecido cardíaco. Recentemente, foi descrito que vesículas contêm fosfatases ácida e alcalina que aumentam a capacidade do parasito em aderir e infectar novas células hospedeiras atuando diferencialmente para cepas Y e CL (Neves et al. 2014). Contudo, são necessárias mais pesquisas para descrição do papel de EVs de diferentes cepas de *T. cruzi* na ativação do sistema imune inato e durante a fase crônica no hospedeiro.

Vários estudos têm descrito a atividade biológica de moléculas de membrana do *T. cruzi* isoladamente e na sua interação com o hospedeiro vertebrado. As GPI-mucinas têm capacidade de induzir forte resposta pró-inflamatória em macrófagos por ativação de receptor do tipo Toll-2 (TLR2) dependente de MyD88 e promove sinalização de MAPKs, que são elementos chave na produção de citocinas. Durante esse processo ocorre aumento da produção de IL-12 e TNF-α, que ativa as células para eliminar o parasito (Campos et al. 2001; Ropert & Gazzinelli 2004). As mucinas são capazes de induzir resposta celular e humoral levando a produção de anticorpos líticos contra resíduos de α-galactosil (Almeida et al. 1994; Galili 2005). Outros TLRs (TLR9 e TLR4) podem estar envolvidos na resposta imune durante estágios iniciais da infecção. A participação de TLR4 ainda não está bem definida. Alguns estudos sugerem o envolvimento de TLR4 no reconhecimento de GIPLs de *T. cruzi* na resistência à infecção (Oliveira et al. 2004). Adicionalmente, motivos de CpG não metilados de DNA estimulam a produção de citocinas de maneira dependente de TLR9 em células apresentadoras de antígenos (Bafica et al. 2006; Bartholomeu et al. 2008).

As citocinas desempenham papel importante na modulação da resposta imune e estão envolvidas tanto na resistência à infecção quanto nos mecanismos relacionados à evolução da doença de Chagas. Nos eventos iniciais da infecção, o parasito induz intensa resposta inflamatória com produção de citocinas, tais como IL-1, IL-6, IL-12, IL-18, IL-27, TNF-α e IFN-γ, e de reativos de oxigênio e nitrogênio por meio da ativação de células "*natural killer*" (NK) e macrófagos/monócitos contribuindo para efetiva diminuição e/ou eliminação do parasito e também para posterior ativação da resposta imune adaptativa (Brener & Gazzinelli 1997; Gazzinelli et al. 2004). Após o processo de endocitose e apresentação de antígenos, ocorre ativação dos macrófagos, células dendríticas e céluas B promovendo futura produção de anticorpos (Teixeira et al. 2002; Golgher and Gazzinelli 2004; Savino et al. 2007). O óxido nítrico (NO) pode, diretamente ou indiretamente, modular a efeito da maquinaria leucocitária através de diversos mecanismos. Esse processo envolve efeitos microbicidas, pela produção de radicais livres tóxicos, assim como regulação e aumento da resposta inflamatória durante este tipo de infecção (Silva et al. 1995). De fato, a intensa reação inflamatória em resposta ao parasito pode destruí-lo mas pode também gerar danos teciduais, com consequente desenvolvimento crônico de imunipatologias.

A expressão desbalanceada de citocinas inflamatórias, como por exemplo, TNF-α e IFN-γ, está relacionada com o desenvolvimento de patologia, tanto na forma clínica cardíaca quanto na forma digestiva (Dutra et al. 2009). Além disso, IL-10 e TGF-β desenvolvem um papel crítico na regulação da resposta imune ao *T. cruzi* (Hunter et al. 1996). A citocina IL-10, produzida principalmente por células T, é capaz de inibir a ativação de macrófagos, bloqueando tanto a liberação de NO quanto a diferenciação de células Th1, que controla a intensa reação inflamatória gerada na fase aguda. Também foi observado que monócitos derivados de pacientes com quadro indeterminado apresentaram características moduladoras, com alta expressão de IL-10, enquanto monócitos derivados de pacientes com cardiopatia produzem intensa resposta inflamatória devido à alta expressão de TNF-α (Souza et al. 2004), sugerindo uma participação crítica dessas células na infecção. Em outro estudo, utilizando-se camundongos *knockout* para IL-10, foi observado que, após infecção com *T. cruzi*, estes animais apresentaram menor parasitemia, parasitismo tecidual, maior produção de IFN-γ e óxido nítrico por células do baço, quando comparados a camundongos selvagens. Além disso, o aumento da parasitemia após neutralização de IFN-γ, TNF-α ou IL-12 demonstra a importância da produção endógena dessas citocinas no controle do parasitismo pelas respostas imunológicas inatas e adaptativas (Abrahamsohn & Coffman 1996).

Interessantemente, exossomos de *Leishmania* são capazes de modular a produção de citocinas em células do sistema imune. Essas vesículas promovem aumento de IL-10 e inibição de TNF-α e INF-γ em células esplênicas (Silverman et al. 2010) e parecem regular a expressão gênica de IFN-γ e IL-12 em macrófagos murinos (Hassani et al. 2014) sugerindo seu potencial papel na exacerbação da infecção pelo parasito.

Estudos em animais têm demonstrado que a resistência ou susceptibilidade à infecção pelo *T. cruzi* é, pelo menos em parte, determinada nos estágios iniciais da infecção, antes do desenvolvimento de resposta imune adquirida (Hölscher et al. 2000). Além disso, o efeito combinatório das diferentes vias de sinalização desencadeadas pelo *T. cruzi*, em células teciduais e da imunidade inata, pode ter uma importante consequência em diferentes aspectos da infecção, tais como parasitismo, tropismo e a patogênese da doença de Chagas (Vitelli-Avelar et al. 2007).

Durante a fase crônica da infecção chagásica, as duas subpopulações de linfócitos T (CD4+ e CD8+) apresentam importância central quanto à capacidade de controlar a infecção pelo *T. cruzi*. Células T desses pacientes, de todas as formas clínicas, apresentam características de ativação, sendo capazes de produzir citocinas em resposta a antígenos do parasito (Dutra et al. 1994; Dutra et al. 2009). É sabido que a falta de células T CD4+ ou CD8+ resulta em uma infecção descontrolada com consequente morte em modelos experimentais. Roffê *et al*., (2012) confirmaram a participação de IL-10 produzidas por células T CD4+ e CD8+ como fator de proteção contra miocardite aguda fatal no modelo murino, contudo parece haver outras fontes desta citocina. Recentemente foi evidenciada a influência de vesículas de *T. cruzi* sobre a população de linfócitos T, em que foram capazes de aumentar a infiltração de células T CD4+ e macrófagos em tecido cardíaco de camundongos infectados com o parasito, podendo assim influenciar na patologia da doença de Chagas (Trocoli Torrecilhas et al. 2009). Embora vários trabalhos utilizando modelos experimentais tenham definido a importância das subpopulações de linfócitos T CD4 e CD8, bem como de células NK, macrófagos e células B na infecção pelo *T. cruzi* o comportamento dessas células na produção de citocinas frente a estímulos liberados pelo parasito ainda não é totalmente esclarecido (Silva et al. 1995; Brener & Gazzinelli 1997; Sathler-Avelar et al. 2003; Souza et al. 2004; Savino et al. 2007; Souza et al. 2007; Tarleton 2007; Sathler-Avelar et al. 2012; Fares et al. 2013).

Variações nas moléculas do *T. cruzi* podem ter implicações na antigenicidade, patogênese e virulência entre diferentes cepas. Soares *et al*., (2012) demonstraram que variação estrutural de açúcares α-galactosil-*O*-ligados em GPI-mucinas das cepas Colombiana, Y e CL (Tcs I, II e VI, respectivamente) foram capazes de induzir diferencialmente a produção de IL-12 e NO em macrófagos murinos. Adicionalmente, houve modulação da invasão de *T. cruzi* em células LLC-MK2 quando pré-incubadas com GPI-mucinas, em que taxa de infecção foi maior para cepas CL e Y quando comparadas à cepa Colombiana (Soares et al. 2012). Como esse glicoconjugado é o principal componente das membranas de *T. cruzi* (60-80%) espera-se que o mesmo também esteja presente nas vesículas. Entretanto, uma importante lacuna no conhecimento da glicobiologia das vesículas é se parasitos pertencentes a diferentes cepas/DTUs podem estimular diferencialmente a resposta imune no hospedeiro.

2 – JUSTIFICATIVA

O sucesso do *T. cruzi* na manutenção do seu ciclo de vida está relacionado à sua habilidade em causar infecção permanente, empregando estratégias elaboradas para escapar dos mecanismos de defesa do hospedeiro vertebrado. A participação de componentes imunológicos na instalação, desenvolvimento ou manutenção da patogênese chagásica constitui, ainda, um ponto de grande importância a ser investigado.

Formas tripomastigotas de *T. cruzi* são capazes de liberar continuamente fragmentos de membrana plasmática que são compostas por moléculas importantes para o estabelecimento da infecção do patógeno. Vários trabalhos têm discutido a importância de vesículas secretadas em diferentes doenças parasitárias, porém muitas questões importantes ainda precisam ser respondidas. Por exemplo, como estas estruturas afetam a resposta imune inata e adquirida durante a interação parasito-hospedeiro. Em especial, as implicações da variabilidade genética de cepas de *T. cruzi* de diferentes DTUs. Neste trabalho abordaremos as cepas Colombiana (TcI), YuYu (TcI), Y (TcII) e CL-14 (TcVI).

Dessa forma, pretendemos avaliar o papel de receptores de membrana e o perfil de produção de citocinas em células do sistema imune do hospedeiro na interação com vesículas do *T. cruzi* em diferentes cepas. Entender os mecanismos envolvidos no controle da resposta celular frente à estimulação por diferentes vesículas de *T. cruzi* fornecerá uma importante informação a respeito da possível estratégia do parasito no controle da resposta imune e permanência da infecção no hospedeiro.

3 – OBJETIVOS

3.1 - Objetivo Geral

Avaliar o papel de vesículas de diferentes cepas de *T. cruzi* na interação com células de hospedeiro vertebrado.

3.2 - Objetivos Específicos

• Avaliar a ativação de macrófagos murinos e o padrão de reconhecimento dos receptores do tipo Toll (TLR2 e TLR4) no reconhecimento de vesículas de *T. cruzi* das cepas Y, Colombiana, CL-14 e YuYu;

• Avaliar a ativação de MAPKs (ERK 1/2, p38 e JNK) por macrófagos J774.A1 após exposição às vesículas de *T. cruzi*;

• Avaliar o perfil de produção de citocinas por células esplênicas de camundongos em fase crônica da doença após estimulação com vesículas de *T. cruzi*;

• Avaliar o perfil de citocinas intracelulares (IL-10, IFN-γ e TNF-α) produzidas por células esplênicas de camundongos em fase crônica da doença após estimulação com vesículas de *T. cruzi*.

4 – ARTIGO

Nogueira PM, Campos JH, Silveira ACO, Martins-Filho OA, Bela SR, Campos MA, Pessoa NL, Soares RP, Torrecilhas AC (2015).** Vesicles from different *Trypanosoma cruzi* strains trigger differential innate and chronic immune responses. ***Journal of Extracelular Vesicles***. doi.org/10.3402/jev.v4.28734

ORIGINAL RESEARCH ARTICLE

Vesicles from different *Trypanosoma cruzi* strains trigger differential innate and chronic immune responses

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Trypomastigote forms of *Trypanosoma cruzi*, the causative agent of Chagas Disease, shed extracellular vesicles (EVs) enriched with glycoproteins of the gp85/*trans*-sialidase (TS) superfamily and other α-galactosyl (α-Gal)-containing glycoconjugates, such as mucins. Here, purified vesicles from *T. cruzi* strains (Y, Colombiana, CL-14 and YuYu) were quantified according to size, intensity and concentration. Qualitative analysis revealed differences in their protein and α-galactosyl contents. Later, those polymorphisms were evaluated in the modulation of immune responses (innate and in the chronic phase) in C57BL/6 mice. EVs isolated from YuYu and CL-14 strains induced in macrophages higher levels of proinflammatory cytokines (TNF-α and IL-6) and nitric oxide via TLR2. In general, no differences were observed in MAPKs activation (p38, JNK and ERK 1/2) after EVs stimulation. In splenic cells derived from chronically infected mice, a different modulation pattern was observed, where Colombiana (followed by Y strain) EVs were more proinflammatory. This modulation was independent of the *T. cruzi* strain used in the mice infection. To test the functional importance of this modulation, the expression of intracellular cytokines after in vitro exposure was evaluated using EVs from YuYu and Colombiana strains. Both EVs induced cytokine production with the appearance of IL-10 in the chronically infected mice. A high frequency of IL-10 in CD4+ and CD8+ T lymphocytes was observed. A mixed profile of cytokine induction was observed in B cells with the production of TNF-α and IL-10. Finally, dendritic cells produced TNF-α after stimulation with EVs. Polymorphisms in the vesicles surface may be determinant in the immunopathologic events not only in the early steps of infection but also in the chronic phase.

Keywords: *Trypanosoma cruzi*; extracellular vesicles; innate and chronic immunity; TLR2; α-galactosyl

*T*rypanosoma cruzi is the causative agent of Chagas disease, one of the most important neglected infectious diseases in Latin America. Recent studies estimate that approximately 11 million people are infected, and about 100 million are at risk (1). This protozoan parasite is transmitted by insect vectors, orally, blood transfusion, organ transplantation and congenitally. During the life cycle, the parasites must face extremely adverse conditions both in the vertebrate and invertebrate hosts (2).

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In this context, *T. cruzi* employs a highly elaborated array of molecules and strategies to invade a wide range of host cells (3–6) and to escape from host immune responses (7–11). Invasion and immune resistance are vital processes required for survival, proliferation and establishment of *T. cruzi* infection. A number of parasite surface molecules have been implicated in host cell invasion and/or immunomodulation (12–16). Those include gp85/TS and mucin superfamilies of developmentally regulated glycosylphosphatidylinositol (GPI)-linked glycoproteins (14,17).

In addition to surface proteins, secreted factors also play an important role in parasite virulence. Some of those studies indicate that surface antigens can be released in soluble and/or membrane-bound forms (18–20). Secretion of virulence factors via extracellular vesicles (EVs) is well described in pathogens including protozoa, helminths, bacteria, fungi and virus (21–23). For example, Gram-negative bacteria secrete outer-membrane vesicles (OMVs) that are important for vaccination and delivery of a variety of virulence factors (23).

Recent secretome analyses of the trypanosomatids *Trypanosoma brucei* and *Leishmania donovani* have shown that a large proportion, if not most, of the secreted proteins are released as membrane-bound vesicles (24–27). Consistent with those observations, trypomastigote forms of *T. cruzi* also secrete proteins in EVs that are enriched with gp85/TS and mucins, crucial molecules for the host–parasite interaction (18). A distinguished feature of those EVs from Y strain is their ability to exacerbate parasite load and modulation of inflammation of the heart (19).

Studies comparing glycoconjugates from different *T. cruzi* strains and discrete typing units (DTUs) are not common (16,28). Purified GPI mucins isolated from Colombiana, Y and CL (DTUs I, II and VI, respectively) had the ability to differentially activate nitric oxide (NO) and cytokine production via TLR2 and modulate parasite invasion (8,16). However, those aspects remain unknown in EVs from different *T. cruzi* strains. In this work, we evaluated their role in the innate immune compartment and in the chronic phase.

Materials and methods
All experiments described in this session are summarized in the workflow (Fig. 1).

Ethics statement
All animals were handled in strict accordance with animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CEUA) of Federal University of São Paulo (UNIFESP), Diadema, São Paulo, Brazil (protocol no. 3598). Knock-out mice handling protocol was approved by the National Commission on Biosafety (CTNBio) (protocol no. 01200.006193/2001-16).

Cell lines and culture
Tissue culture-derived trypomastigote forms from Colombiana (DTU I), YuYu (DTU I), Y (DTU II) and CL-14 (DTU VI) strains of *T. cruzi* were obtained after infection of green monkey (*Rhesus*) kidney LLC-MK$_2$ epithelial cells (ATCC, Manassas, VA) (29). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS; Invitrogen, Carlsbad, CA) at 37°C and 5% CO$_2$. Murine macrophages (J774.1) were obtained from ATCC. Murine C57BL/6 macrophages were obtained after peritoneal washing. Both macrophage types were cultured in Roswell Park Memorial Institute (RPMI) supplemented with 10% FCS (37°C, 5% CO$_2$). Parasites and mammalian cells were regularly tested for the absence of *Mycoplasma* contamination (30).

Isolation, microscopy and characterization of EVs
Trypomastigotes from different *T. cruzi* strains (Y, Colombiana, CL-14 and YuYu) were obtained from the supernatant of LLC-MK$_2$ cells. Recovered parasites were washed 5 times in PBS (1,000g, 15 min) incubated for 2 h in RPMI with 5% glucose (37°C, 5% CO$_2$) for EVs release. Parasites were fixed and subjected to scanning electron microscopy as described (31). Trypomastigotes were removed by centrifugation (15 min, 1,000*g*), and EV-containing supernatants were filtered through 0.45-μm sterile cartridges (Fig. 1). For gel-exclusion chromatography, the filtered supernatant (1 mL) was concentrated 4 times and diluted to 100 mM ammonium acetate, pH 6.5. Then, it was loaded onto a Sepharose CL-4B column (1 × 40 cm, GE Healthcare, Piscataway, NJ) pre-equilibrated with 100 mM ammonium acetate, pH 6.5. The column was eluted with the same buffer at a flow rate of 0.2 mL/min. Fractions of 1 mL were collected, and then screened by chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) with chagasic anti-α-Gal antibodies, as previously described (19), and by a polyclonal antibody antiparasite membrane 460 (1:1,000), a kind gift from Dr Sérgio Schenkman (18,19). To identify the differential expression of α-galactosyl-containing glycoconjugates in the EVs, the fractions were pooled, concentrated in a speed-vac and its reactivity against anti-α-Gal antibodies (1:500) was tested by ELISA. Antirabbit or antihuman IgG-peroxidase conjugate (1:2,000) was used as secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was developed using TMB substrate reagent set (BD Biosciences, San Diego, CA). Protein concentrations were determined using the Micro BCA protein assay kit (Thermo Scientific, Waltham, MA). To test for LPS contamination, CHO reporter cell lines TLR2 −/− TLR4 −/− (which do not express TLR2 or TLR4) and TLR4+ (expressing TLR4) were used as described elsewhere (8).
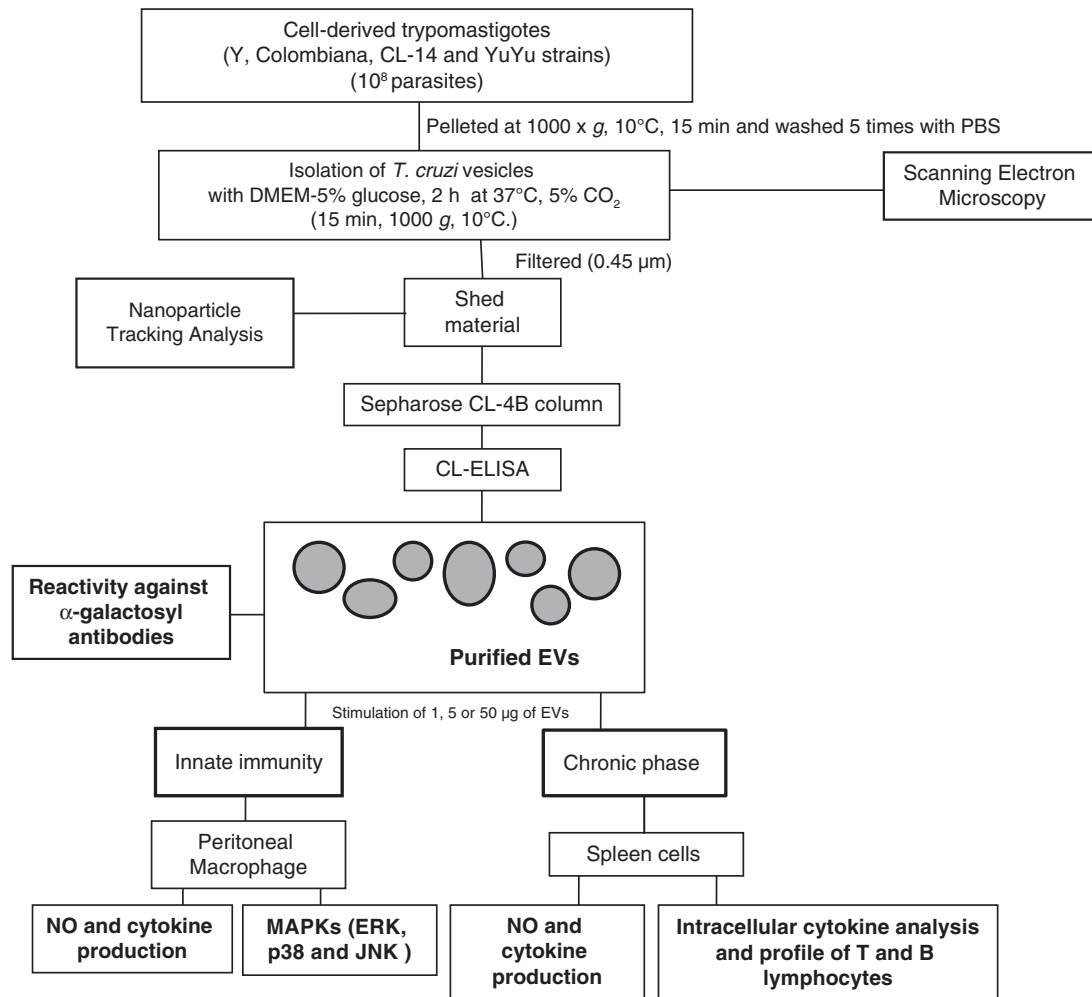
Fig. 1. Procedures employed for the production, fractionation and characterization of *T. cruzi* EVs from different strains.

Nanoparticle tracking analysis

Size, distribution and concentration of isolated vesicles were measured in a Nanosight NS300 instrument (Malvern Instruments Ltd, Malvern, UK) equipped with a 405-nm laser and coupled to a CCD camera (the laser emitting a 60-mW beam at 405-nm wavelength), and data were analysed using the nanoparticle tracking analysis (NTA) software (version 2.3 build 0017). The detection threshold was set to 10. Blur, Min track Length and Min Expected Particle Size were set to auto. To perform the measurements, samples were diluted 100 times with PBS. Readings were taken in triplicates during 30 s at 20 frames per second, at camera level set to 14 and manual monitoring of temperature (19°C).

Purification of murine peritoneal macrophages

For the innate immune experiments, thioglycollate-elicited macrophages were extracted from wild-type C57BL/6 and its respective knockouts (TLR2 $-/-$ and TLR4 $-/-$) by peritoneal washing with ice-cold serum-free RPMI and enriched by plastic adherence for 1h (37°C, 5% CO_2).

Cells (3×10^5 cells/well) were washed and cultured in RPMI, 2 mM glutamine, 50 U/mL of penicillin and 50 μg/mL streptomycin supplemented with 10% FBS in 96-well culture plates (37°C, 5% CO_2). Cells were primed with interferon-γ (IFN-γ) (25 IU/mL) for 18 h prior to incubation with vesicles (1, 5 and 50 μg/mL) from Colombiana, YuYu, Y or CL-14 strains. A positive control included live parasites from Y strain (MOI 10:1). Control glycoconjugates included lipophosphoglycan (LPG) from *Leishmania braziliensis* (10 μg/mL) and lipopolysaccharide from *Escherichia coli* (LPS; 100 ng/mL) (32). Negative controls included medium and medium-containing IFN-γ (25 IU/mL). Cells were incubated for 48 h and the supernatants collected for cytokine (IL-1β, IL-6, IL-10, IL-12/IL-23p40, IL-12p70 and TNF-α) and NO measurements. Results are representative of 2 experiments performed in duplicate.

Preparation of cell lysates and MAPKs

In order to evaluate the signalling events prior to EVs stimulation, J774.1 macrophages were placed on 24-well

tissue culture plates (3×10^6/well) for 18 h. The cells were washed with warm RPMI and incubated with vesicles isolated from Colombiana, YuYu, Y or CL-14 (5 μg/mL) strains. Four time points (5, 15, 30 and 45 min) were assayed. Controls with medium (negative control) or LPS (100 ng/mL, positive control only for 45 min incubation) were included. After each time point, cells were washed with ice-cold PBS and lysed in RIPA lysis buffer (Sigma) in the presence of protease inhibitor cocktail (Thermo Fisher Scientific). Cells were harvested with a plastic scraper and centrifuged at 13,000g (4°C, 10 min). Supernatants were transferred to new tubes and stored at -20°C. Cell lysates were resolved by SDS–PAGE, transferred to a nitrocellulose membrane and blocked (5% non-fat milk in TBS–0.1% Tween 20) for 1 h. Primary Abs (1:1,000) [anti-dually phosphorylated ERK (Sigma), anti-dually phosphorylated p38 (Santa Cruz Biotechnology, Dallas, TX) and JNK (Sigma)] were added. Total p38 primary antibody (Sigma) was used as the normalizer. All mAbs were incubated for 16 h at 4°C. Membranes were washed (3×10 min) with TBS–0.1% Tween 20 and incubated 1 h with antimouse IgG conjugated with peroxidase (1:10,000). The reaction was visualized using luminol (33).

Splenocyte cultures derived from chronic infections

For the chronic assays, the 4 strains of *T. cruzi* (Y, Colombiana, CL or YuYu) were intraperitoneally injected (50 parasites/animal, 15 per group) in C57BL/6 mice. Parasitaemia was detected daily up to 15 days to confirm infection. Only the animals whose infection was parasitologically diagnosed were selected. Those mice were maintained for 6 months ($\sim$180 days). After this period, the animals were anaesthetized and the spleens removed (34). Spleen cells (1×10^6/well) from mice infected with each *T. cruzi* strain were incubated for 48 h with EVs (5 μg/mL) isolated from the 4 strains. Supernatants were collected for NO and cytokine assays. Total *T. cruzi* antigen (10 μg/mL) and culture medium were added as positive and negative controls, respectively. Results are representative of 2 experiments in duplicate.

Cytokines and nitrite measurements

For CBA multiplex cytokine detection, supernatants were collected and cytokines were determined using the BD CBA Mouse Cytokine assay kits according to the manufacturer's specifications (BD Biosciences). IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12/IL-23p40, IL-17, IFN-γ and TNF-α were measured. Flow cytometry measurements were performed on a FACSCalibur flow cytometer (BD Biosciences). Cell-Quest™ software package provided by the manufacturer was used for data acquisition, and the FlowJO software 7.6.4 (Tree Star, Inc., Ashland, OR) was used for data analysis. A total of 2,400 events were acquired for each preparation. Nitrite concentrations were determined by Griess reaction (Griess Reagent System, 2009).

Immunostaining for cell surface markers and intracellular cytokines

To identify the sources of the cytokines, splenocytes (1×10^6 cells) were cultured with RPMI supplemented with 10% FCS and incubated with vesicles (5 μg/mL) for 24 h (37°C, 5% CO_2). Three positive controls were performed to evaluate the sample viability. Lymphocytes were treated with phorbol 12-myristate 13-acetate (25 ng/mL) (Sigma) and ionomycin (1 μg/mL) (Sigma), whereas monocytes and DCs were treated with LPS (100 ng/mL). The viability of these controls was confirmed by the high levels of cytokine production (data not shown). Afterward, all cultures were incubated in the presence of Brefeldin (10 μg/mL) (4 h, 37°C, 5% CO_2) and treated with 100 μL of 2 mM EDTA in PBS for 15 min to block the reaction (35).

After incubation, spleen cells were washed once (800g, 5 min at 4°C) with FACS buffer prepared in PBS (0.15 M) supplemented with 0.5% of bovine serum albumin and 0.1% sodium azide. Cells were resuspended in 100 μL of FACS buffer and immunostained with antimouse monoclonal antibodies including: F4/80 (clone BM8/PE-Cy5), CD11b (clone M1/70/ PE-Cy7), CD11c (clone N418/ FITC) and CD19 (clone 1D3/PE-Cy7) from eBioscience (San Diego, CA); and MHCII (clone M5/114.15.2/ APC), CD3 (clone 145-2C11/PerCP), CD4 (clone GK1.5/Alexa fluor 700) and CD8 (clone 53–6.7/Alexa fluor 647) from BioLegend (San Diego, CA) in the dark (30 min, 4°C). The lysing/fixation occurred in the presence of 150 μL FACS brand Lysing solution (BD Biosciences) for 10 min. Cells were washed once (800 g, 10 min, 4°C) with FACS buffer. Membrane-stained cells were permeabilized by incubation for 15 min with 200 μL of FACS permeabilization buffer (FACS buffer supplemented with 0.5% saponin) and washed again (800g, 10 min at 4°C) with FACS buffer. Finally, cells were incubated in the dark (30 min) at room temperature, in the presence of 25 μL (1:50) of PE-labelled antimouse cytokines (IL-10, IFN-γ and TNF-α) (eBioscience). The material was fixed with 200 μL of FACS FIX Solution (10 g/L of paraformaldehyde, 10.2 g/L of sodium cacodilate and 6.63 g/L of sodium chloride, pH 7.2) (Sigma) and stored at 4°C prior to flow cytometry acquisition and analysis.

Flow cytometry acquisition and analysis

Data were collected using FACSFortessa (BD Biosciences – Immunocytometry Systems) with Diva software (BD Biosciences) and analysed by FlowJo software (Tree Star). Flow cytometry acquisitions of 100,000 and 200,000 immunostained cells/samples were performed for lymphocytes and monocytes, respectively. Distinct gating strategies were used to analyse the cytokine-expressing leucocyte subpopulations from innate and adaptive immunity, including monocytes, DCs, T and B lymphocytes. The singlets (single cells) were selected excluding the doublets (FSC-A/FSC-H), and the cells were gated by forward and side scatter to
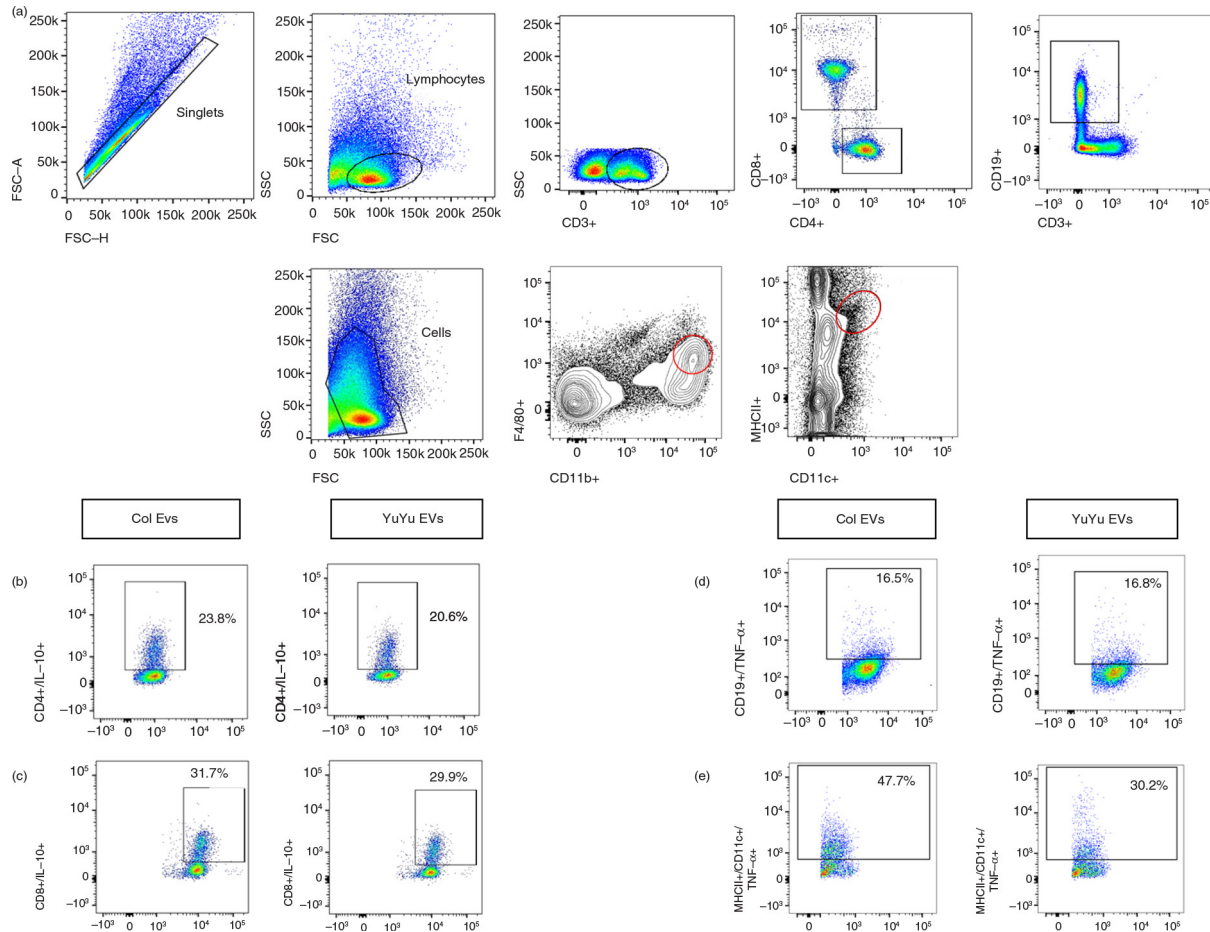
Fig. 2. Flow cytometry strategy for intracellular cytokine production by spleen cells from infected mice. (a) Dot plots of CD4, CD8, CD19, F4/80/CD11b and MHCII/CD11c expression. The representative dot plots illustrating the frequency (%) of cytokines from spleen cells are shown for Colombiana and YuYu strains. (b) CD4+IL-10+ cells. (c) CD8+IL-10+. (d) CD19+TNF-α+. (e) MHC/CD11c+TNF-α+.

separate lymphocytes from monocytes (Fig. 2a). Following the initial gate selection, the frequencies of cytokine-positive cells were quantified by statistics applied on T lymphocytes (CD3+/CD4+ or CD3+/CD8+), B lymphocytes (CD19+), DCs (CD11c+/MHC-II+) and monocytes (CD11b+/F4/80+) vs. anticytokine-PE dot plots (Fig. 2b–e).

Statistical analysis

For nitrite and cytokine measurements, the Shapiro–Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution. The p-value ($p > 0.05$) showed that data did not deviate from Gaussian distribution. For this reason, Student's t-test and ANOVA were performed to test equality of population medians among groups and independent samples. The comparative analysis between control and stimulated culture was performed by Mann-Whitney pair test. Data were analysed using GraphPad Prism 5.0 software (Graph Prism, San Diego, CA) and $p < 0.05$ was considered significant.

Results

Purification and α-galactosylation in T. cruzi-derived EVs

Trypomastigotes from *T. cruzi* constantly shed EVs into the culture medium [18–20] throughout the whole body, as shown for Y strain (Fig. 3a–d). EVs were collected from the supernatant of 1×10^8 trypomastigotes from Y, Colombiana, CL-14 and YuYu strains after incubation of the parasites for 2 h at 37°C in culture medium. NTA analysis showed a heterogeneous profile for the different strains (Fig. 4a–f). The average size was very similar for all strains (Fig. 4e), whereas the particles' concentrations were lower for strains Y, Colombiana and CL-14 (Fig. 4f), in contrast with YuYu strain. Size distribution according to the D10, D50 and D90 did not vary significantly among the 4 strains analysed (Supplementary Fig. 1).

EVs released into the medium were then purified by gel-exclusion chromatography [18–19] for further experiments (Fig. 1). Polyclonal antibodies raised against total
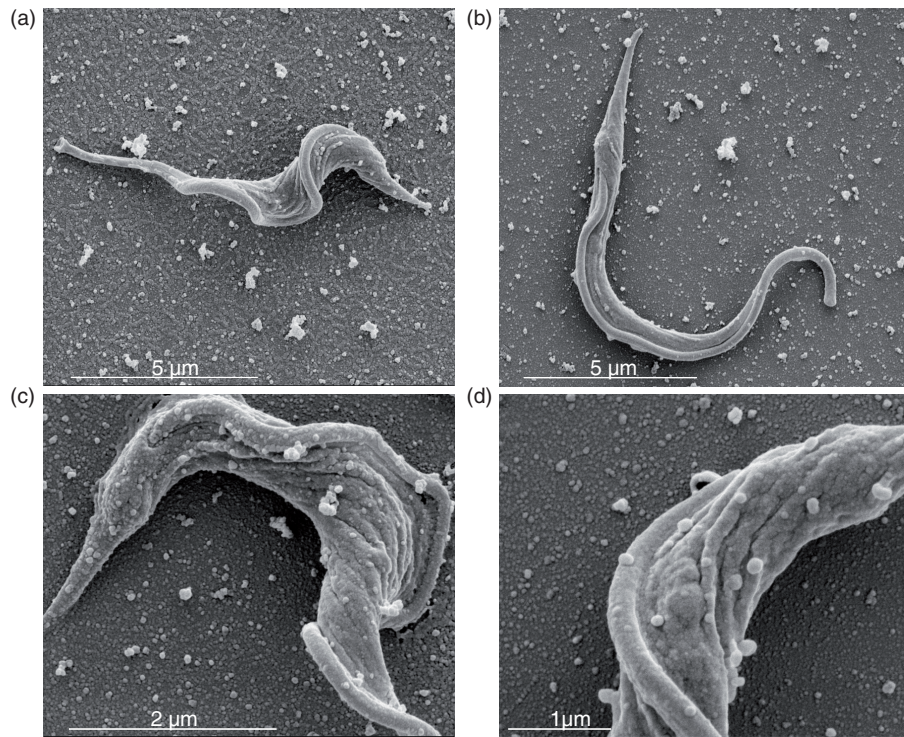
Fig. 3. *Trypanosoma cruzi* (Y strain) trypomastigotes spontaneously shed vesicles from their entire membrane surface. Scanning electron microscopy (SEM) of parasite membrane shedding after incubation in culture medium (a–d, bars: 1–5 μm). Magnification: (a) 27,383 ×, (b) 25,242 ×, (c) 60,470 × and (d) 92,084 ×.
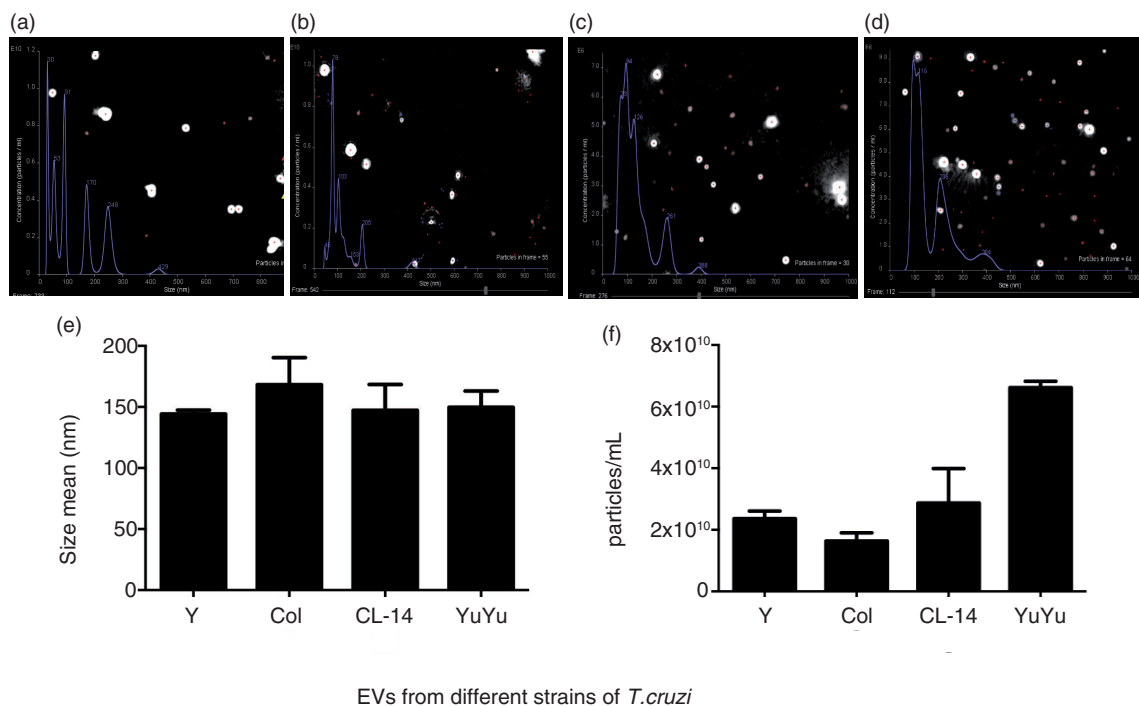

EVs from different strains of *T.cruzi*

Fig. 4. Nanoparticle tracking analysis of the EVs isolated from different strains of *T. cruzi*. Graphic demonstration of size distribution and concentration for Y (a), Colombiana (b), CL-14 (c) and YuYu (d) strains. The average size (nm) (e) and concentration (particles/mL) (f) of the vesicles for all strains are represented. Data are representative of 3 independent experiments.

trypomastigote membrane (antibody named 460) were employed for the selection of positive fractions by ELISA. Those antigen-containing fractions were pooled and the protein content was estimated. Lower protein content was detected in EVs derived from Y, Colombiana and CL-14 strains. EVs from the YuYu strain exhibited the highest protein level of liberated proteins (Fig. 5a), in contrast to EVs from Colombiana strain. Analysis of α-galatosyl-containing glycoconjugates expression in EVs has shown that those obtained from Y, CL-14 and YuYu strains were richer in α-galactosyl residues (Fig. 5b). EVs derived from the Y strain showed the lowest protein and the highest carbohydrate contents. Altogether, those results suggest that EVs from different strains have protein and α-galactosyl contents that are not directly correlated with each other.

Trypanosoma cruzi *EVs differentially activate TLR2*

Since EVs from different strains of *T. cruzi* are polymorphic when expression of α-galactosyl residues and protein content are considered, their ability to induce NO and cytokines in murine macrophages was investigated. A pilot experiment was designed to test a panel of pro- and anti-inflammatory cytokines (IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12/IL-23p40, IL-17, IFN-γ and TNF-α). No production of the cytokines IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, IL-17 and IL-12/IL-23p40 was detected when macrophages were primed with EVs from each of the strains (data not shown). For this reason, only the remaining cytokines were used in the next experiments. In comparison to EVs from YuYu and CL-14, no significant levels of cytokines were detected after incubation with EVs from Y and Colombiana strains in all concentrations tested, especially for TNF-α and NO (Figs. 6 and 7). A similar profile was observed for IL-6

(Supplementary Fig. 2). Consistent with those observations, no activation of the CHO cells transfected with CD25 reporter coupled to NF-κB was detected in TLR2-TLR4- and TLR4+ subsets confirming that the EVs preparations were free of LPS (Supplementary Fig. 3). Since the results were similar when 1 or 5 μg/mL was employed, the concentration 5 μg/mL was chosen for the next experiments.

EVs from T. cruzi *strains activate MAPKs in J774.1 macrophages*

To better characterize the signalling events after EVs exposure, J774.1 macrophages were incubated with vesicles (5 μg/mL) from each of the strains. MAPK activation was assessed as a function of time and analysed by densitometry. Although all EVs activated ERK 1/2, JNK and p38, no visible differences were detected among the strains (Fig. 8a–c).

T. cruzi *EVs differentially activate chronic-infected mice splenocytes*

Previous studies demonstrated that EVs are very important for heart tissues invasion and pathology during infection (19). The impact of *T. cruzi* EVs isolated from different strains during the chronic phase was assessed in splenocyte cells after 180 days post-infection of the animals with *T. cruzi*. No production of IL-2, IL-4 and IL-5 was detected (data not shown). Regardless of the type of strain employed for mice infection, all strain-derived vesicles were able to induce expression of TNF-α, IFN-γ, IL-6 and IL-10 in all groups. Similar data were observed for NO (Supplementary Fig. 4). Interestingly, Colombiana and Y EVs were more pro-inflammatory than those from YuYu and CL-14 strains ($p > 0.05$; Figs. 9 and 10).
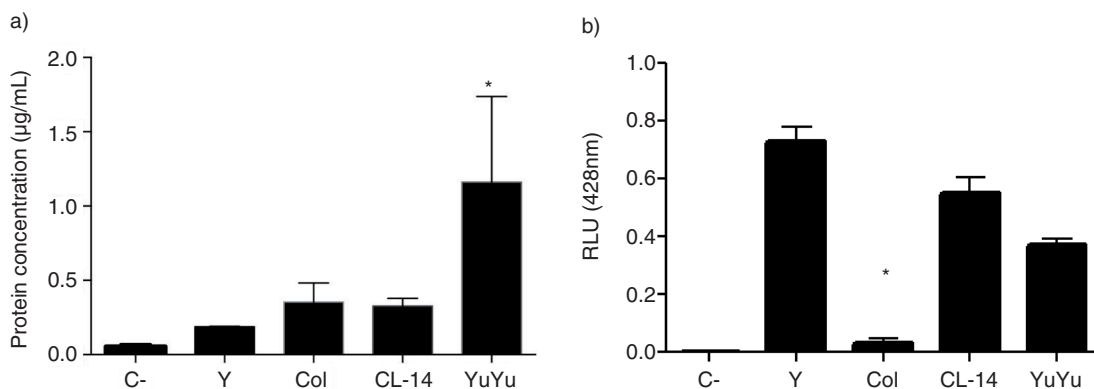

Fig. 5. Protein and terminal α-galactosyl residues measurement in EVs isolated from 4 *T. cruzi* strains. (a) Protein concentration in EV-pooled (b) EVs reactivity with anti-α-Gal antibodies (1:500) determined by ELISA. Negative control corresponds to the medium with 5% glucose without EVs. Bars express the mean value ± SD of 4 separate studies (*$p < 0.05$).
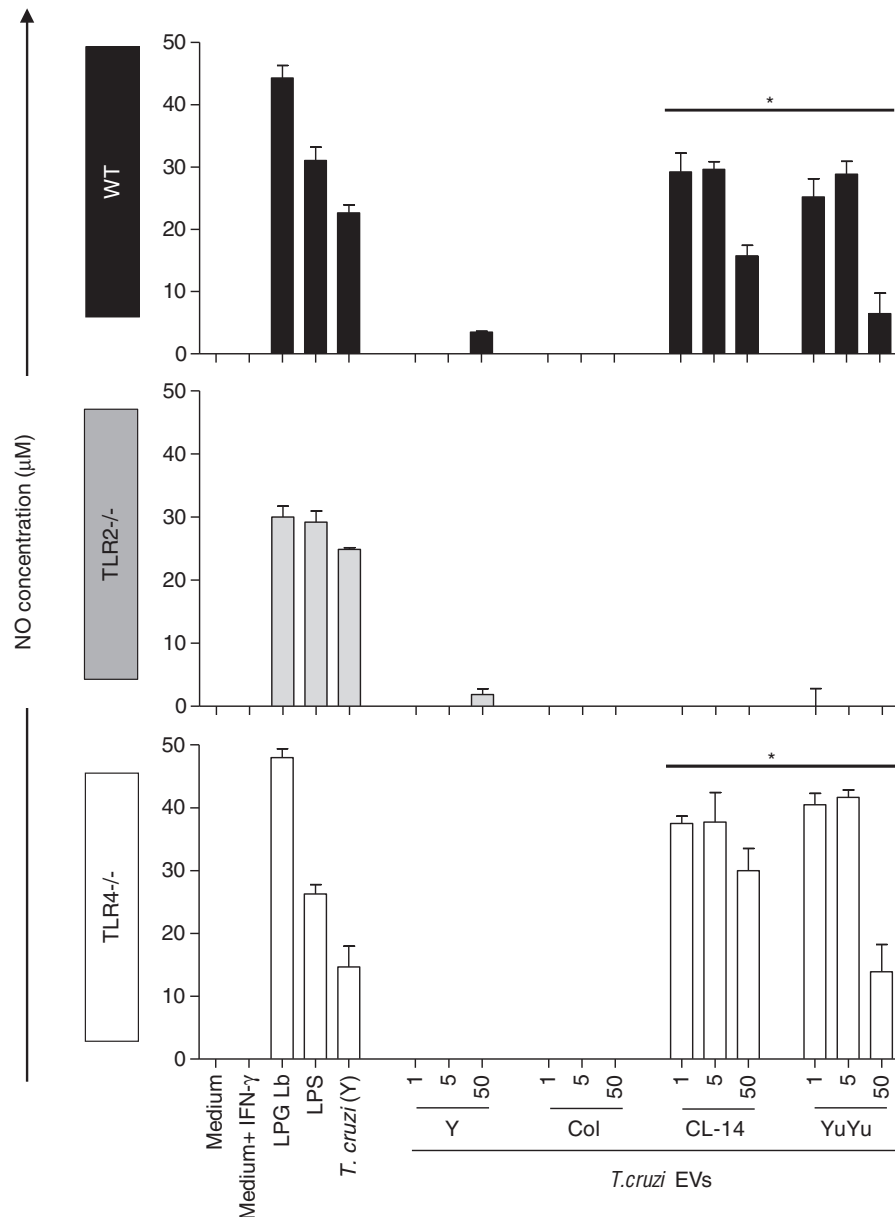
Fig. 6. Nitric oxide (NO) production by murine macrophages stimulated by EVs from CL-14 and YuYu strains is dependent on TLR2. Murine macrophages (C57BL/6, TLR2 − / − and TLR4 − / −) were stimulated with different concentrations of *T. cruzi* EVs (1, 5 and 50 μg). Cells were pre-incubated with IFN-γ (100 U/mL) for 18 h prior to addition of EVs or LPS, LPG and live parasites MOI 10:1 (positive controls). Negative controls included medium and medium + INF-γ. LPG Lb, *L. braziliensis* LPG; LPS, lipopolysaccharide (LPS) from *E. coli*; *T. cruzi* (Y), live parasites of *T. cruzi* (Y strain). Bars express the mean value ± SD of 2 separate studies (*p < 0.05).

EVs from Colombiana and YuYu strains induce similar levels of intracellular cytokines in leucocyte subpopulations

The previous cytokine analyses did not show major differences after EVs stimulation in mice infected with each of the 4 strains. For this reason, only 2 strains (Colombiana and YuYu) were chosen for intracellular cytokine detection in spleen cells (monocytes, DCs, T and B lymphocytes). In general, no quantitative cytokine differences were detected between splenocytes from YuYu and Colombiana-infected mice (Figs. 11 and 12). In CD4+ and CD8+ T lymphocytes, a higher frequency of IL-10+ than INF-γ+ or TNF-α+ cells (Fig. 11) was observed. A mixed profile of cytokine induction was observed in B cells with the production of TNF-α and IL-10 (Fig. 12a–c). Dendritic cells also produced TNF-α after stimulation with EVs from both strains (Fig. 11d). No relevant production of those cytokines was detected in monocytes (data not shown).
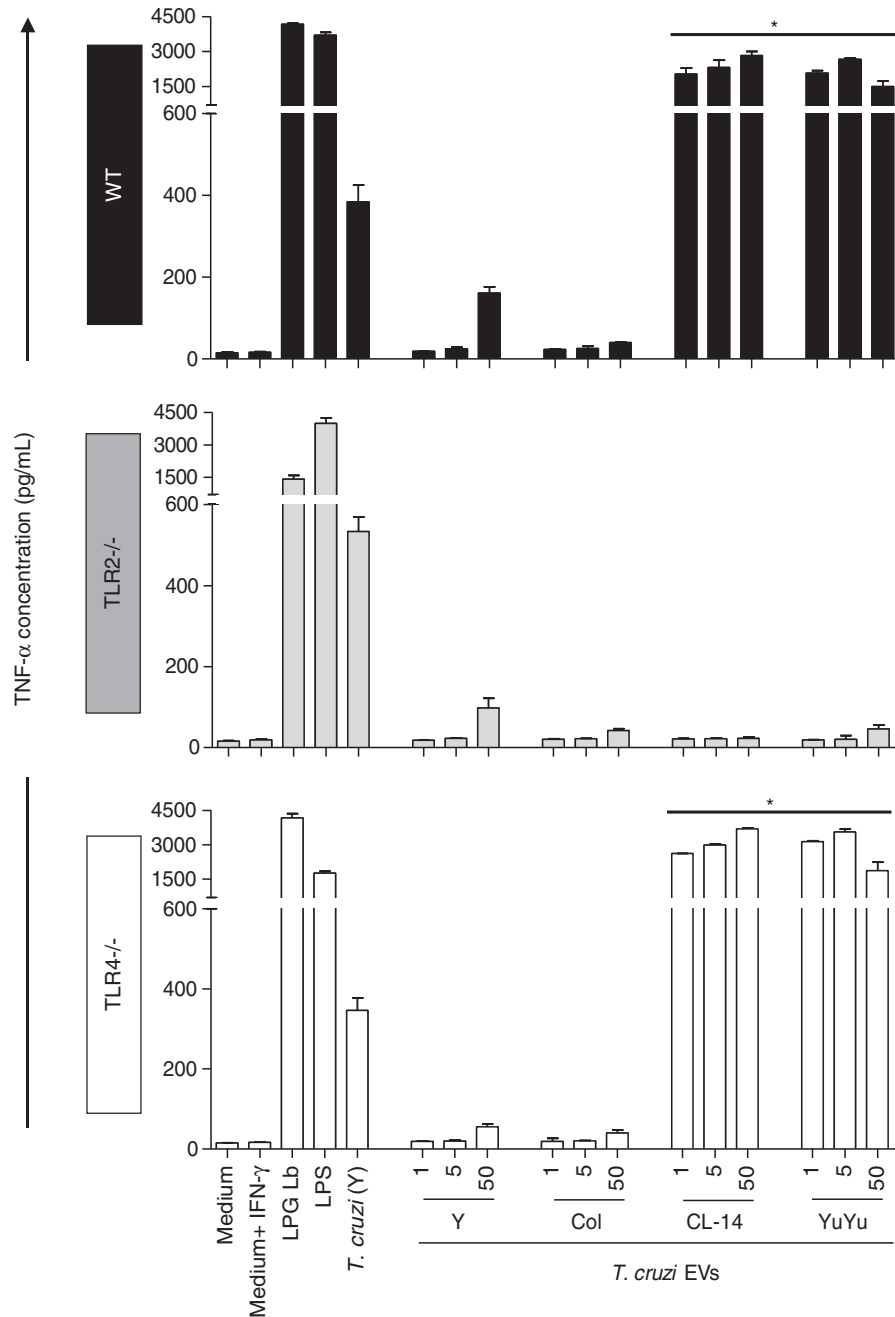
Fig. 7. TNF-α production by murine macrophages stimulated by EVs from CL-14 and YuYu strains is dependent on TLR2. Murine macrophages (C57BL/6, TLR2 −/− and TLR4 −/−) were stimulated with different concentrations of *T. cruzi* EVs (1, 5 and 50 μg). Cells were pre-incubated with IFN-γ (100 U/mL) for 18 h prior to addition of the EVs, and controls (LPS, LPG and live parasites MOI 10:1) (positive controls). Negative controls included medium and medium + INF-γ. IFN-γ, gamma-interferon; LPG Lb, *L. braziliensis* LPG; LPS, lipopolysaccharide from *E. coli*; *T. cruzi* (Y), live parasites of *T. cruzi* (Y strain). Bars express the mean value ± SD of 2 separate studies (*p < 0.05).

Discussion

T. cruzi infection induces important changes in the host's cellular immune response not only in the innate compartment but also in the adaptive immunity context. Parasite and host-related factors may affect the onset of clinical forms. In the host, T cells especially are critical for pathology modulation (36). Many studies have evaluated the cytokine production in different clinical forms (cardiac/intestinal and or indeterminate) providing insights into the understanding of the immunopathological events in Chagas disease (9). Consistent with those observations, a recent report has associated clinical forms
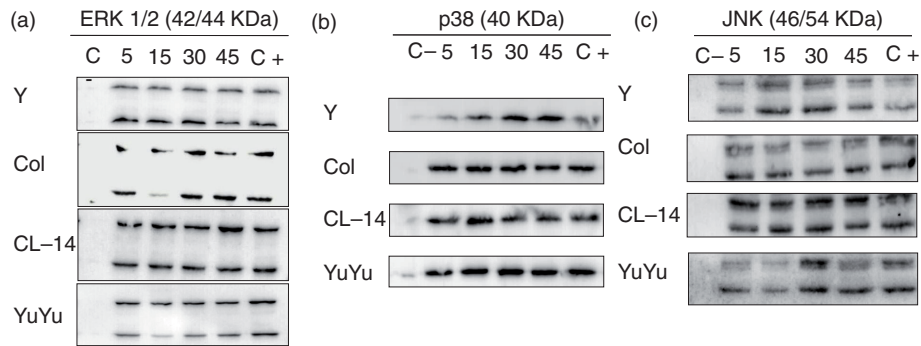
Fig. 8. Trypanosoma cruzi EVs equally activate MAPKs (ERK 1/2, p38 and JNK) from J774.1 macrophages. Cells were stimulated with EVs (5 µg/mL) at different time points (5, 15, 30 and 45 min). Dually phosphorylated MAPKs were detected by western blot: (a) ERK 1/2, (b) p38 and (c) JNK. C-, negative control (medium); C+, positive control (LPS from *E. coli*) (100 ng/mL).

with different DTUs. Here, similar to the tGPIs (16), a clear association between DTU and the parameters studied was not demonstrated, suggesting that those variations are probably strain-specific. For example, strains YuYu and Colombiana (DTU I) exhibited a different pro-inflammatory profile in the previous experiments.

EVs have been isolated and characterized in a number of pathogens including viruses, bacteria, fungi and parasites, and were described as having a pivotal role during the modulation of the host immune system. Those vesicles exhibit a complex array of molecules and may be considered a packet of pathogen-associated molecular patterns (PAMPs) reviewed in (22,23). In this sense, trypomastigote forms of *T. cruzi* continuously shed vesicles that are enriched with gp85/TS, mucin-associated surface proteins and other α-Gal-containing glycoconjugates,
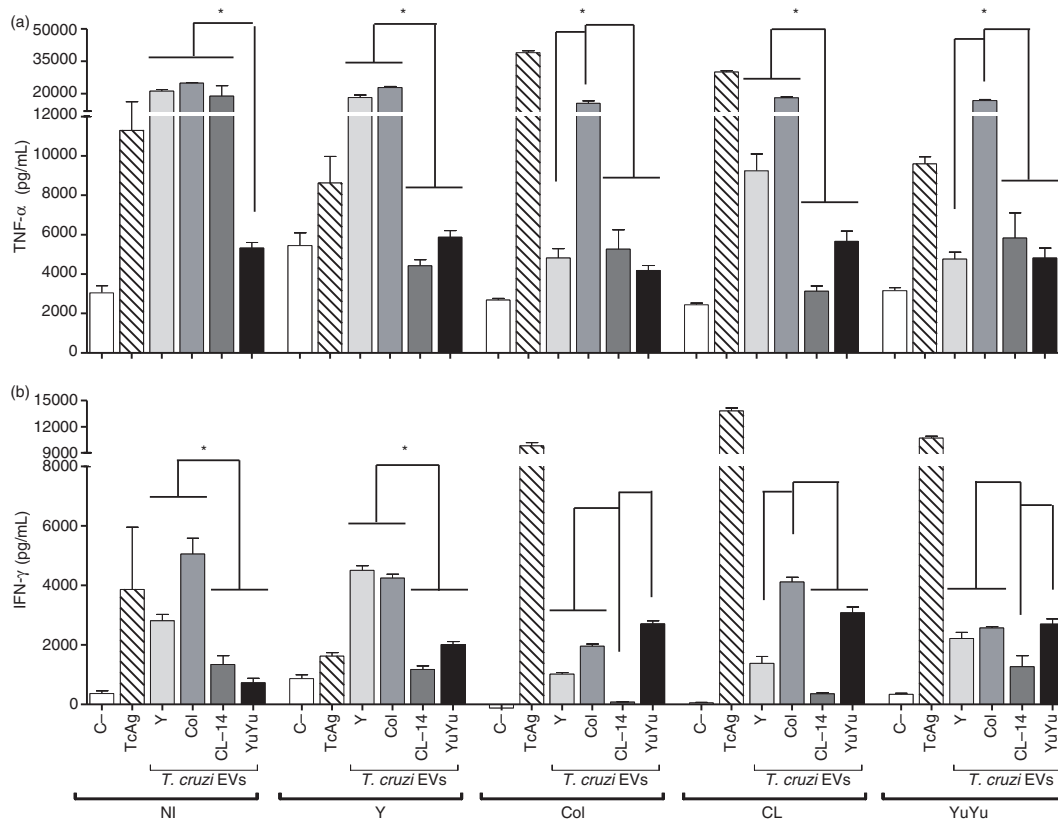

Fig. 9. Trypanosoma cruzi EVs from Colombiana and Y strains display a higher pro-inflammatory activity in splenocytes derived from chronically infected mice. Cells were incubated with EVs (5 µg/mL) or total *T. cruzi* antigen (10 µg/mL) (positive control). Spleen cells from non-infected mice with and without the addition of a new stimulus were also used as controls. TNF-α (a) and IFN-γ (b) concentrations (pg/mL) were determined by CBA. Medium, negative control; TcAg, soluble trypomastigote antigen of *T. cruzi*; Bars express the mean value ±SD of 2 separate studies (*$p < 0.05$).
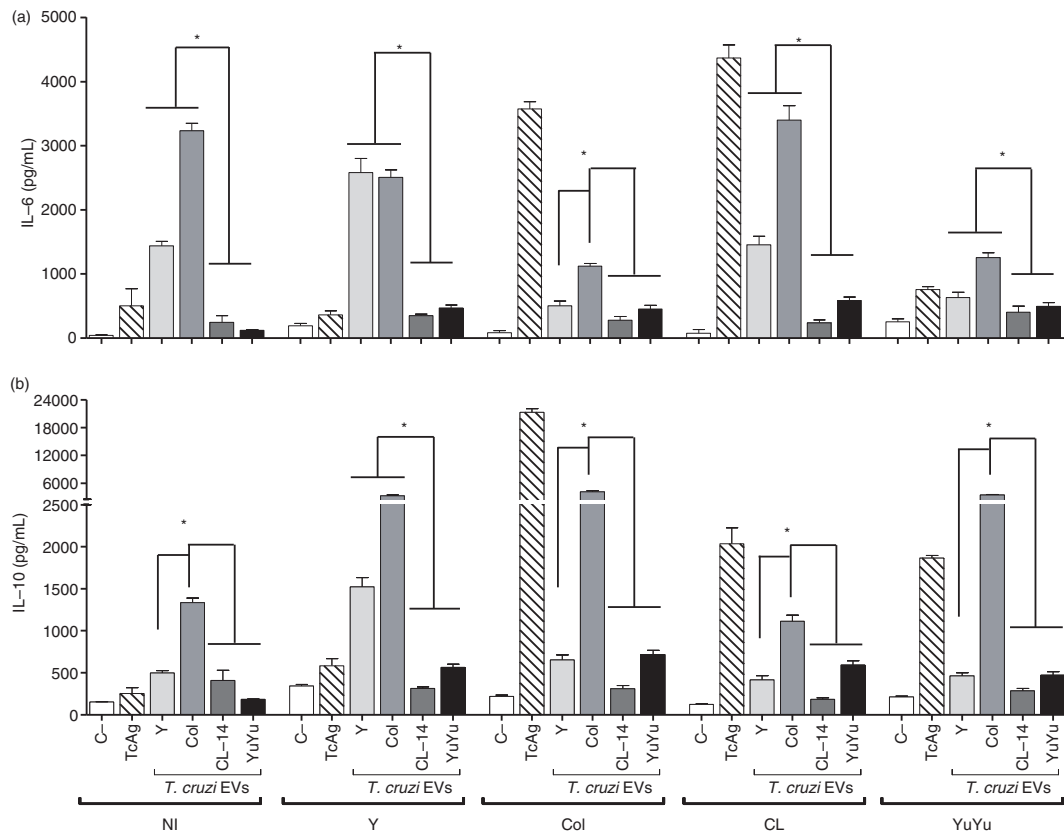
Fig. 10. *Trypanosoma cruzi* EVs from Colombiana and Y strains display differential immunomodulatory activity in splenocytes derived from chronically infected mice. Cells were incubated with EVs (5 μg/mL) or total *T. cruzi* antigen (10 μg/mL) (positive control). Spleen cells from non-infected mice with and without the addition of a new stimulus were also used as controls. IL-6 (a) and IL-10 (b) concentrations (pg/mL) were determined by CBA. Medium, negative control; TcAg, soluble trypomastigote antigen of *T. cruzi*; bars express the mean value $\pm$ SD of 2 separate studies (*$p < 0.05$).

such as mucins (16,37). BALB/c mice injected with *T. cruzi* EVs and challenged with tripomastigote forms developed severe heart pathology with an intense inflammatory reaction, an increased number of intracellular amastigotes and mortality (19). These results indicate that molecules present in vesicles are important during the immunopathological events in Chagas disease. However, no information about the role of EVs from different *T. cruzi* strains in those processes is available. In this paper, purified EVS, free of LPS from 4 strains belonging to DTUs I (Colombiana and Yu-Yu), II (Y) and VI (CL-14) (28), were chosen to evaluate their role in the innate immune compartment and in spleen cells from chronically infected mice.

A clear polymorphism was detected early in the EVs. Vesicles from Y, CL-14 and YuYu strains were richer in α-Galactosyl epitopes (Galα1-3Galβ1-(3)4GlcNAc-R) (38). Those glycoconjugates are extremely immunogenic resulting in the production of high levels of lytic anti-α-Gal antibodies in chagasic patients and are important for infection control (39–41). A distinguished feature was observed for Colombiana strain, which is extremely virulent

and often leads to mortality in mice (42). Since EVs from that strain express much less α-Gal epitopes, one can hypothesize that this fact would make Colombiana strain less susceptible to the action of lytic antibodies. Consistent with those observations, purified tGPI-mucins from Y, CL and Colombiana strains were also differentially recognized by anti-α-Gal antibodies from human patients (16). Those data indicate the presence of not only variable amounts of terminal α-Gal residues but also other changes in the structure of this epitope. No substantial differences in protein content were observed for the Y, CL-14 and Colombiana EVs. However, EVS isolated from YuYu strain have the highest protein amount. To further characterize and understand the role of EVs, proteomic analyses are under way to determine EVs composition from *T. cruzi*, as performed for other pathogens, such as *Plasmodium yoelii* (43), *Leishmania mexicana* (44) and the trematodes *Echinostoma caproni* and *Fasciola hepatica* (45). To evaluate the implications of such polymorphisms in antigenicity, pathogenesis and virulence among different strains, purified EVs from *T. cruzi*, were incubated with host cells.
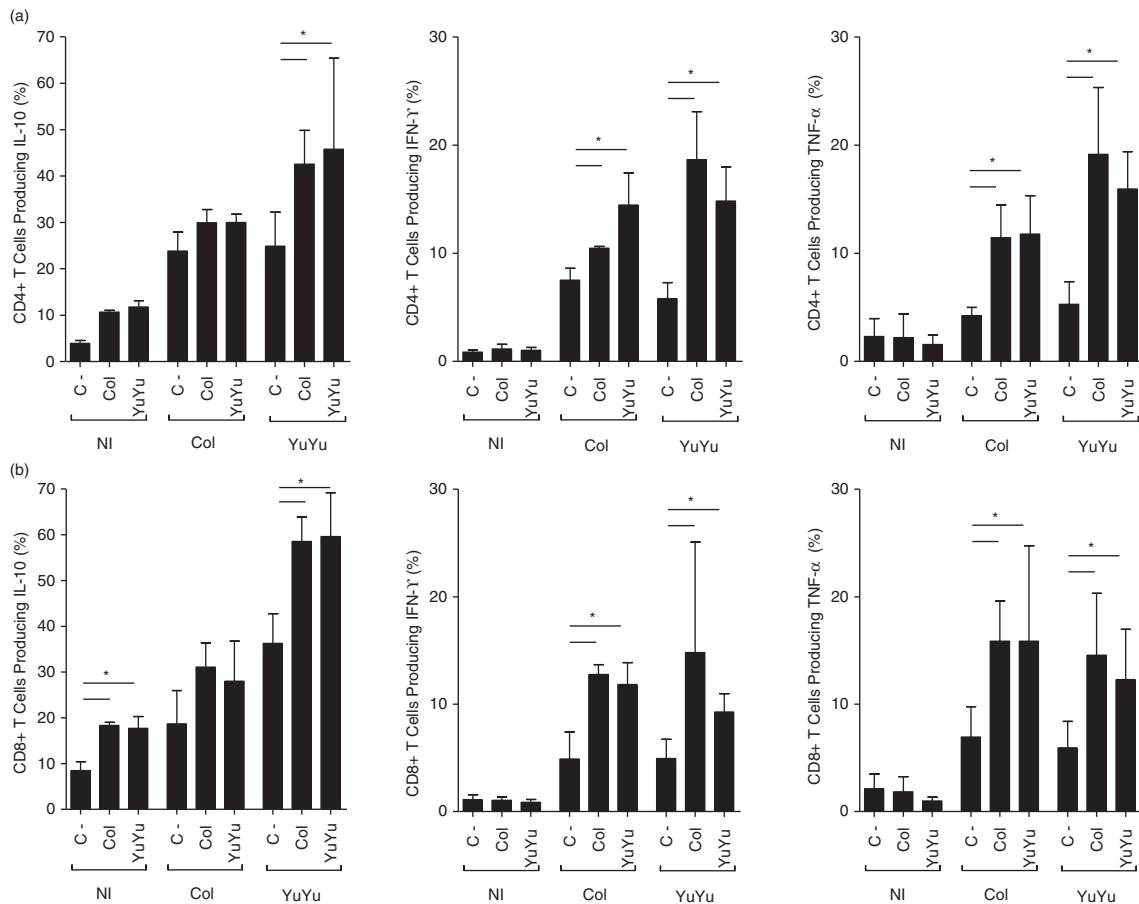
Fig. 11. Intracellular cytokine production by T lymphocytes is similar after stimulation with EVs from Colombiana or YuYu strains. (a) CD4+ production of intracytoplasmic cytokines (IL-10 with IFN-γ and TNF-α). (b) CD8+ production of intracytoplasmic cytokines (IL-10 with IFN-γ and TNF-α). Immunophenotypic staining was performed as described in the Material and Methods section. Bars express the mean value±SD of 2 separate studies (*p < 0.05).
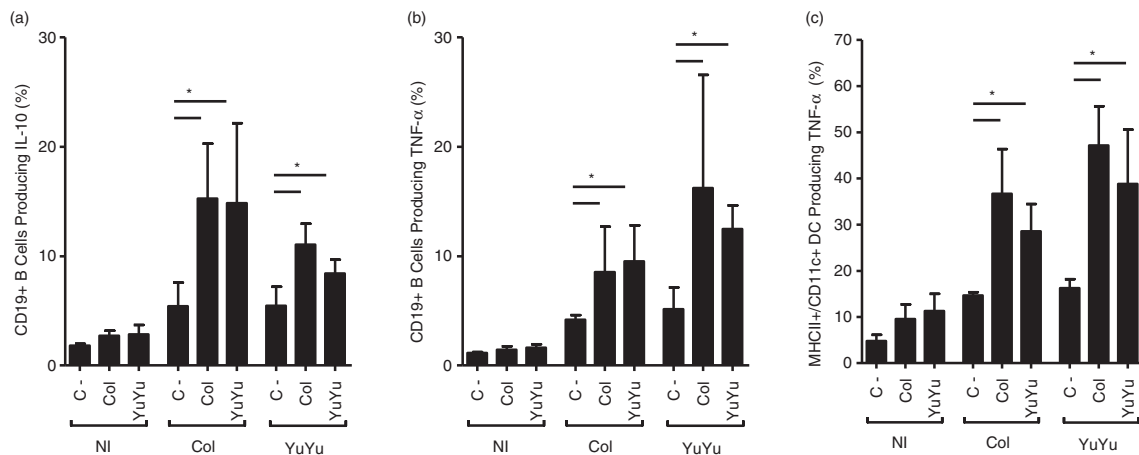

Fig. 12. Intracellular cytokine production by B and DC is similar after stimulation with EVs from Colombiana or YuYu strains. (a) CD19+IL-10+ production. (b) CD19+TNF-α+production. (c) MHCII/CD11c+TNF-α+ production. Immunophenotypic staining was performed as described in the Material and Methods section. Bars express the mean value±SD of 2 separate studies (*p < 0.05).

Extensive studies have reported on the biological activities of tGPI-mucins, which corresponds to 60–80% of the parasite *T. cruzi* surface molecules (37,46) and may be one of the main components of EVs. Those are potent agonists of TLR1/TLR2 and TLR2/TLR6 (47). Interestingly, tGPIs from Colombiana, Y and CL strains differentially induced NO and proinflammatory cytokines (IL-12 and TNF-α) in murine macrophages (16,37).

EVs from all strains induced different levels of NO, IL-6 and TNF-α in macrophages. An interesting aspect of this activation is that although they have the expression of many molecules, EVs were very specific for TLR2, particularly in the case of CL-14 and YuYu strains. This ability was lower for Y and Colombiana strain, showing that differential stimulation by EVs varies in the innate immune compartment. This result may be due to the content of other components, such as GIPL, a TLR4 agonist (48), did not seem to be important in this activation. On the other hand, membrane molecules like cruzipain could also be involved. Those were able to decrease NO production by down-regulating the iNOS expression in macrophages (49).

Those data are in accordance with previous in vivo results showing that EVs from the Y strain induced a local reduction of iNOS expression resulting in higher tissue parasitism (19). Recently, it was demonstrated that strains belonging to different DTUs vary in the ability to bind to human galectins, suggesting the existence of glycoconjugate polymorphisms in *T. cruzi* (13,14,50). In other tripanosomatids, such as *L. donovani*, a more immunomodulatory profile was observed by promoting IL-10 production and consequent inhibition of TNF-α by the parasite EVs (27).

The MAPKs transduce a variety of extracellular stimuli through a cascade of protein phosphorylations like ERK1/2, JNK and p38, leading to the activation of transcription factors (51). Previous studies have correlated that tGPI mucins from Y strain were able to trigger phosphorylation of ERK-1/2 and p38 in murine macrophages resulting in the production of IL-12 and TNF-α (52,53). Since tGPI mucins are major glycoconjugates on the membrane surface and may be also found in EVs, the next step was to evaluate their ability to activate MAPKs. All EVs were equally able to induce MAPKs (ERK 1/2, p38 and JNK) signalling in J774.1 macrophages during the very initial steps of innate immune response. However, this did not result in NO and cytokine production for the EVs from Y and Colombiana strains, reinforcing that inhibition of MAPKs is likely to occur downstream in the pathway. This is in accordance with the fact that EVs are internalized by the cells (20), thus modulating the further intracellular events such as NO and cytokine production after 48 h. This may suggest 2 possible mechanisms: (a) EVs can activate MAPKs via TLR2 and/or via internalization (CL-14 and YuYu strains) and (b) EVs can

be internalized and activate MAPKs (Colombiana and Y strains). Glycoconjugates (LPGs and GIPLs) from other trypanosomatids including *Leishmania braziliensis*, *Leishmania infantum* and different strains of *Leishmania enriettii* were also able to differentially activate MAPKs, thus reinforcing that intra and interspecies variation are important in the early events of immune response (32,33,54). In summary, those data indicate that regardless of the type of *T. cruzi* EVs, they were all sensed early by the cells from the innate immune compartment. Therefore, a differential expression of NO and pro-inflammatory cytokines were observed later on.

The transition from the acute to the chronic phase is a well-known mechanism in the natural history of Chagas Disease. It is characterized by the establishment of an effective immune response and a decrease in blood parasitaemia (55). The initial events in the immune compartment are crucial for the development of a successfully acquired immune response (56). Based on these observations, our next step was to evaluate the role of EVs from all *T. cruzi* strains in splenocytes from chronically infected C57BL/6 mice. Mice were infected with a low number of parasites from all strains and those that survived the acute phase were kept for 6 months (∼180 days) prior to splenocyte recovery. Since CL-14 strain is not infective to mice (57), its parental isolate (CL) has been employed, resulting in detectable parasitaemia. An interesting aspect was observed in these experiments, in which the strains that did not activate the innate immune compartment (Y and Colombiana) were the most pro-inflammatory in the chronic phase. Regardless of the type of strain used for in vivo infection, Colombiana and Y strains EVs exhibited a more pro-inflammatory role than those from YuYu and CL-14 strains. Similar to macrophages, splenocytes also produced NO, TNF-α and IL-6 in addition to IFN-γ. As expected, the appearance of IL-10 in detectable levels occurred in those cells confirming its role modulating the inflammatory events in the chronic phase (58–60). Depending on the strain circulating in the host and considering the fact that blood tripomastigotes may shed vesicles during the acute and chronic phase, those data indicate that EVs from *T. cruzi* may be crucial for the immunopathological mechanisms occurring in the tissues and spleen. Altogether, differential expression of antigenic molecules in all EVs was able to activate either the cells from innate compartment or the splenocytes from infected and non-infected mice. This stimulation was independent on the strain used for the mice infection reinforcing the role of EVs as antigenic stimuli.

Interestingly, for the intracellular cytokine production, they exhibited a similar profile, especially in their ability to activate spleen cells from infected mice with them. Those responses were in general higher than those seen for spleen-derived cells from mice infected with the Y and CL strains. CD4+ T cells and monocyte/macrophages

act as key orchestrators of the cellular response during chronic Chagas disease. These cells produce pro- and anti-inflammatory cytokines after stimulation with total crude parasite extract of *T. cruzi* (60,61). Analysis of the inflammatory infiltrates in infected mice primed with vesicles from Y strain also revealed a dominance of CD4+ and CD8+ T lymphocytes and macrophages (19). Our data demonstrated that, regardless the strain, in vitro stimulation with EVs increased the frequency of IL-10+ CD4+ and CD8+ T cells in mice infected with YuYu and Colombiana strains. This is consistent with the previous data showing that IL-10 was detected in splenocytes derived from mice in the chronic phase. IL-10 is a very important cytokine involved in the immunomodulatory mechanisms during chronic Chagas disease (62). The ability to produce IL-10 in chronic phase may be important to modulate the fine balance between inflammatory and anti-inflammatory cytokines avoiding tissue damage (58).

The specific role of B cells in Chagas disease is still unclear. In general, these cells can contribute to immunity in multiple ways including producing antibodies, antigen presentation or cytokine secretion (41,63,64). A mixed profile of cytokine induction was observed after EVs stimulation with the production of IL-10 and TNF-α by CD19+ B cells, key cytokines often involved in the immunomodulatory processes triggered by pathogens. Additionally, B-cell activation may account for some of the observed increases in cytokine levels post-infection with *T. cruzi* (65). Recently, it was observed that an increase in the expression of regulatory B cells in patients from Chagas disease is associated with higher levels of IL-10 (66). These observations reinforce the role of B cells to produce a variety of cytokines, and there is growing interest in the potential of B cells to modulate immune responses by regulating their profiles of effector cytokine secretion (67). In summary, B and T cells from chronically infected mice were readily able to trigger cytokine production after EV exposure. However, their role in antigen presentation remains unclear for EVs.

Finally, monocytes and DCs are innate immune cells that are very important in the early events of immune responses against *T. cruzi* (68,69). Here, their role in the chronic phase was also determined. No production of IL-10 was detected after stimulation with EVs by either monocytes and or DC, suggesting that those cells are not related to its production in the later phases of the disease. On the other hand, DCs were still an important source of TNF-α. However, in *L. donovani*, treatment with vesicles prior to challenge with parasites exacerbated infection and also promoted IL-10 production in the spleen by DCs (27). Those experiments suggest that during the chronic phase, the EVs from *T. cruzi* seem to be more pro-inflammatory for DCs than those from *L. donovani*, which were immunosuppressive.

Conclusions

We have demonstrated that EVs from *T. cruzi* strains were able to differentially modulate the early and later events of immune responses. Although a clear association with DTUs was not observed, those EVs were very potent agonists of TLR2, especially for CL-14 and YuYu strains. On the other hand, EVs from Y and Colombiana strains were weak activators of the innate immune compartment but were very pro-inflammatory in chronic phase-derived splenocytes. An important aspect of EVs is their ability to modulate the events in the chronic phase due to the production of IL-10 by T and B cells. Additional studies are still needed to understand better the impact of EVs on the immune system of patients and correlate with distinct clinical forms of Chagas disease. Understanding the mechanisms involved in the immune responses to infection by parasites is important to prevent and control the immunopathological events during Chagas disease.

Authors' contributions

Conceived and designed the experiments: PMN, OAM-F, ACOS, SRB, RPS, MAC and ACTT. Performed the experiments: PMN, SRB, KR, JHC, ACOS, NLP, RPS and ACTT. Analysed the data: PMN, ACOS, OAM-F, MAC, NLP, MJMA, RPS and ACTT. Wrote the paper: PMN, MJMA, WC, RPS and ACTT.

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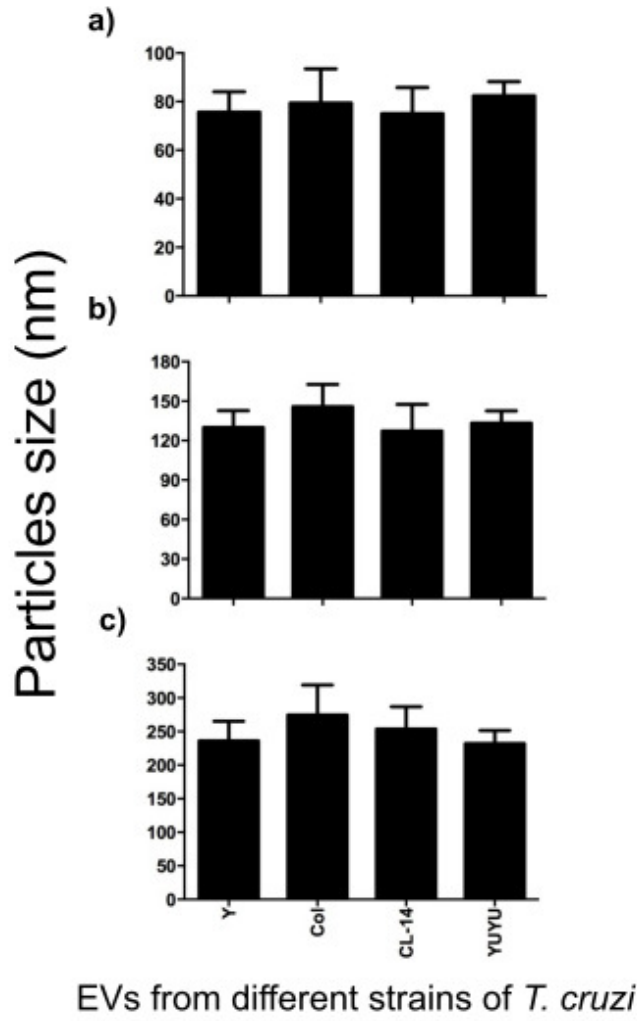
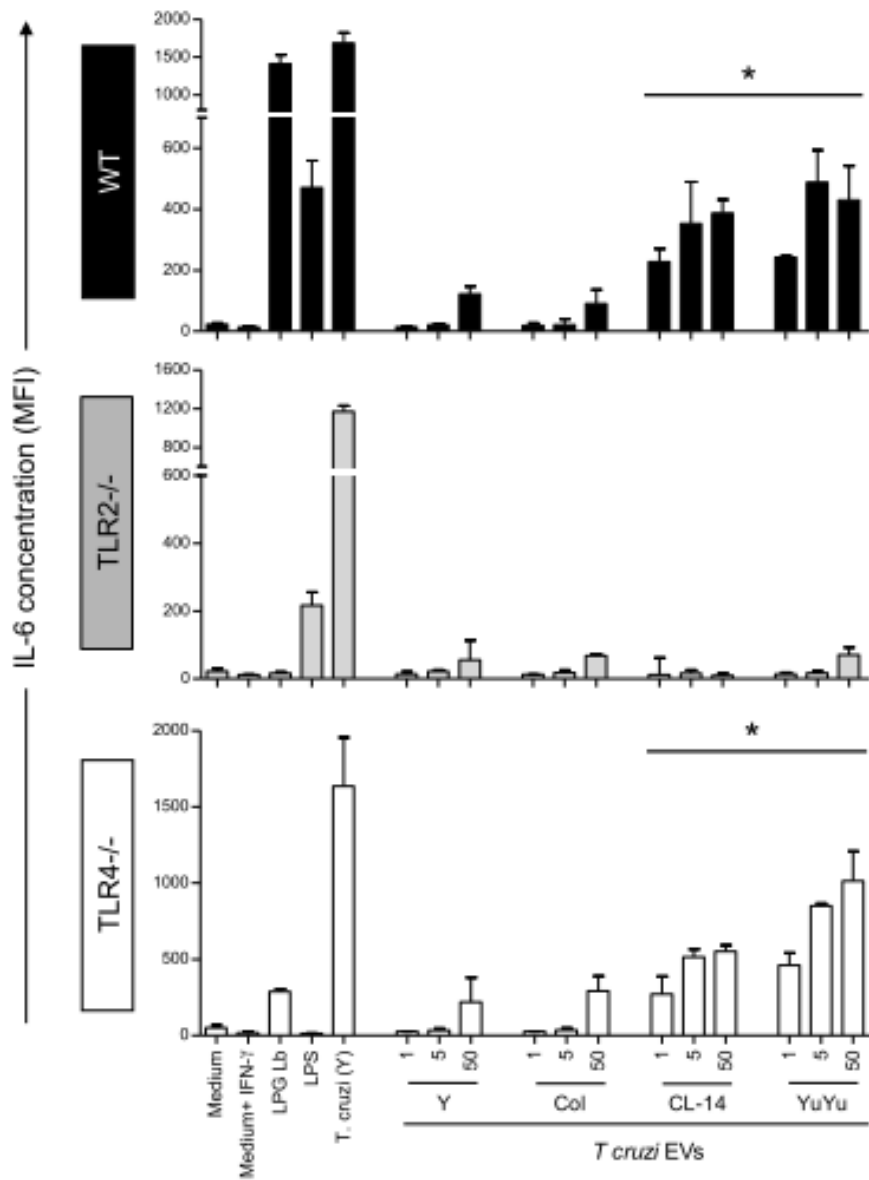**Fig. sup 1:** The average size (nm) of the vesicles for all strains is represented. a) D10 b) D50 and c) D90.

Fig. sup 2: IL-6 stimulation is dependent on TLR2 for strains CL-14 and YuYu. Murine macrophages (C57BL/6, TLR2-/- and TLR4-/-) were stimulated with different concentrations of *T. cruzi* EVs (1, 5 and 50 µg). Cells were pre-incubated with IFN-γ (100 U/ml) for 18 h prior to addition of the EVs, and controls (LPS, LPG and live parasites MOI 10:1) (positive controls). Negative controls included medium and medium + INF-γ. LPG Lb, *Leishmania braziliensis* LPG; LPS, lipopolysaccharide from *Escherichia coli*; T. cruzi (Y), live parasites *T. cruzi* (Y strain). Bars express the mean value ± SD of 2 separate studies. *p <0.05.
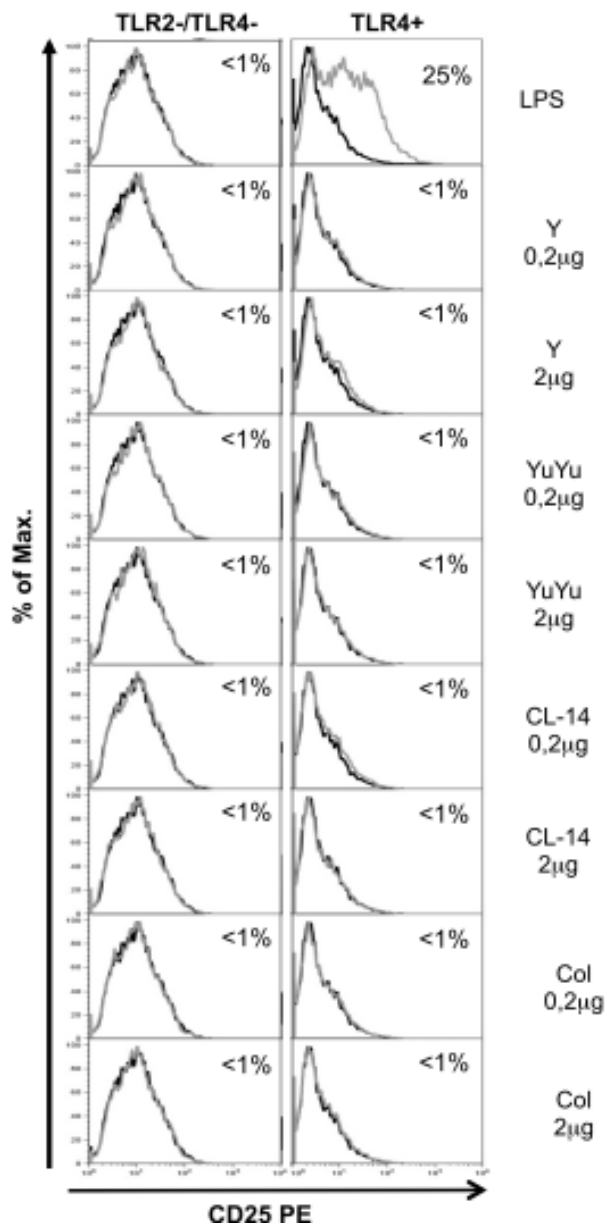
Fig. sup 3: EVs purified of *T. cruzi* do not induce translocation of NF-κB through TLR4 indicating is free of LPS contamination. CHO cells expressing TLR4 (TLR4+) or neither (TLR2-/TLR4-) were either untreated (black line) or treated (grey line) with EVs from all strains of *T. cruzi*. Control: LPS (TLR4 control). CD25 expression was measured by flow cytometry 18 h after stimulation. Results shown as percentage of CD25 expression on stimulated cells minus percentage of CD25 expression on non-stimulated cells.
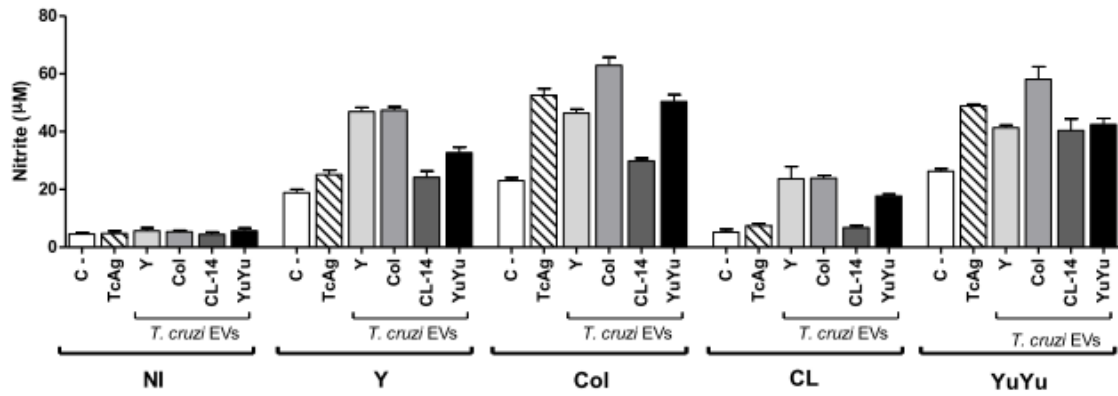
Fig. sup 4: EVs from *T. cruzi* strains Colombiana and Y display a differential immunomodulatory activity in splenocytes derived from chronically infected mice. Cells were incubated with EVs (5 µg/mL) or total *T. cruzi* antigen (10 µg/mL) (positive control). Spleen cells from non-infected mice with and without the addition of a new stimulus were also used as controls. NO concentration (µM) were determined by Griess Protocol. Medium, negative control; TcAg, soluble trypomastigote antigen of *T. cruzi*.

5 – DISCUSSÃO

O controle do parasito na doença de Chagas requer ativação adequada de diversos mecanismos imunes efetores, tanto da resposta imune inata quanto adaptativa, decisiva para este processo. A produção de determinadas citocinas e espécies reativas de oxigênio tem um importante papel na imunopatogênese da doença de Chagas. A infecção pelo *T. cruzi* induz importantes mudanças na ativação celular do hospedeiro para garantir a sua sobrevivência e acredita-se que fatores relacionados à variabilidade genética do parasito (DTUs) possam determinar a forma clínica da doença, seja pela geração uma resposta imune específica ou por tropismo tecidual (Vago et al. 2000; Poveda et al. 2014). Dado que a variabilidade genética pode influenciar na expressão de proteínas, é possível que a expressão diferencial de moléculas antigênicas pertencentes a diferentes subgrupos influencie na resposta imune do hospedeiro. Porém neste trabalho, não foi possível observar uma associação entre vesículas produzidas por *T. cruzi* de DTUs e os parâmetros avaliados, sugerindo que variações na ativação do sistema imune são cepas-específicas.

Vesículas extracelulares (Evs) têm sido isoladas e caracterizadas em diversos patógenos que incluem vírus, bactérias, fungos e parasitos, com importante papel na patogênese e modulação da resposta imune do hospedeiro. Essas vesículas possuem um complexo arranjo de moléculas e podem ser consideradas um pacote de PAMPs (revisado por Marcilla et al. 2014; Campos et al. 2015; Szempruch et al. 2016). Formas tripomastigotas de *T. cruzi* liberam continuamente vesículas que são enriquecidas com proteínas de superfície como tGPI-mucinas, gp85/TS, MASP e outros glicoconjugados (Torrecilhas et al. 2012; Ribeiro et al., *em preparação*). A aplicação de vesículas em camundongos BALB/c e posterior desafio com formas tripomastigotas de *T. cruzi* (cepa Y) resultaram no desenvolvimento de patologia cardíaca grave com intensa reação inflamatória (Trocoli Torrecilhas et al. 2009), indicando que essas as vesículas foram importantes durante os eventos imunopatológicos na doença de Chagas. Contudo, devido à vasta variabilidade genética, tornaram-se necessários estudos sobre o papel de vesículas de diferentes cepas de *T. cruzi* na interação com células do sistema imune. Neste trabalho, vesículas extracelulares de quatro cepas de *T. cruzi* pertencentes aos subgrupos TcI (Colombiana e YuYu), TcII (Y) e TcVI (CL-14) foram avaliadas durante

o processo de interação com células do sistema imune inato e células esplênicas de camundongos cronicamente infectados.

Foram observadas diferenças importantes entre as vesículas produzidas por *T. cruzi*. Vesículas das cepas Y, CL-14 e YuYu apresentaram maiores quantidades de epítopos α-galactosil (Galα1-3Galβ1-(3)4GlcNAc-R) expressos em glicoconjugados de GPI-muc. Esses glicoconjugados são extremamente imunogênicos resultando na produção de altos níveis de anticorpos líticos anti-α-Gal em pacientes chagásicos e são importantes para o controle da infecção (Gazzinelli et al. 1991; Almeida et al. 1994). Já para a cepa Colombiana, que é extremamente virulenta e patogênica no modelo murino (Talvani et al. 2000), apresentou menor reatividade ao anticorpo α-Gal. Este fato faria da cepa Colombiana menos susceptível à ação de anticorpos líticos. Consistente com essas observações, já é descrito o polimorfismo do glicoconjugado tGPI-muc para as cepas Y, CL e Colombiana (Soares et al. 2012), indicando não só a presença de variações na quantidade de α-Gal terminal mas também na quantidade de moléculas expressas na superfície das vesículas. A completa análise proteômica dos antígenos de superfície presentes em vesículas de diferentes cepas de *T. cruzi* está em andamento pelo nosso grupo (Ribeiro *et al.*, *em preparação*) podendo estabelecer potenciais marcadores para cada população de vesículas extracelulares. A fim de avaliar as implicações do polimorfismo na patogênese e virulência durante o processo de interação com células do hospedeiro, as vesículas extracelulares de diferentes cepas de *T. cruzi* foram purificadas e incubadas com macrófagos murinos.

Extensivos estudos reportam atividade biológica de tGPI-muc, que correspondem a 60-80% do total de moléculas de superfície em *T. cruzi* (Ferguson 1999) e pode ser um dos principais componentes das vesículas. Esse glicoconjugado é potente agonista de TLR1/2 e TLR2/6 (Gazzinelli & Denkers 2006; Gravina et al. 2013) e capaz de induzir NO e citocinas pró-inflamatórias de maneira cepa-específica em macrófagos murinos (Campos et al. 2001; Soares et al. 2012). Interessante aspecto da ativação dos macrófagos neste estudo é que, embora as vesículas derivadas do parasito sejam enriquecidas com diversas moléculas imunogênicas, a produção de NO e citocinas foi especificamente pela ativação de TLR2 para cepas CL-14 e YuYu. Essa habilidade foi menor ou nula para cepas Y e Colombiana, evidenciando que a estimulação diferencial por vesículas varia no compartimento imune inato.

Provavelmente a presença de outros componentes, como GIPLs que são agonistas de TLR4 (Oliveira et al. 2004), parece não ser importante nesta ativação. Por outro lado, moléculas de membrana como cruzipaína podem estar envolvidas, uma vez que são capazes de diminuir a expressão de iNOS em macrófagos (Giordanengo et al. 2002).

Estudos prévios *in vivo* demonstraram que vesículas da cepa Y induziram redução da expressão de iNOS resultando em alto parasitismo tecidual (Trocoli Torrecilhas et al. 2009). Recentemente foi demonstrado que cepas pertencentes a diferentes DTUs variam na ligação a galectinas humanas purificadas, sugerindo a existência de polimorfismos em glicoconjugados de *T. cruzi* (Pineda et al. 2015), que resulta em uma complexa rede de interações com hospedeiro. Em outros tripanosomatídeos, como *L. donovani*, foi observado um perfil imunomodulatório com produção de IL-10 e inibição de TNF-α pelas vesículas desses parasitos promovendo progressão da doença (Silverman et al. 2010). Esse fenótipo foi específico na presença da proteína HSP100, indicando que o conteúdo proteico das vesículas influencia diretamente no perfil modulator do sistema imune.

As MAP Quinases (MAPKs) convertem vários estímulos extracelulares em uma ampla gama de resposta celular, via cascata de fosforilação de proteínas como ERK, JNK e p38, que resultam na ativação de fatores de transcrição (Arthur & Ley 2013). Já foi demonstrado que as tGPI-muc da cepa Y são capazes de fosforilar ERK-1/2 e p38 em macrófagos murinos, e consequente aumento da produção de IL-12 e TNF-α (Ropert et al. 2001; Ropert & Gazzinelli 2004). No nosso estudo todas as vesículas foram capazes de induzir fosforilação de MAPKs (ERK1/2, p38 e JNK) em macrófagos J774.1. Contudo esta ativação não resultou na produção de citocinas e NO para as vesículas das cepas Y e Colombiana, indicando a ocorrência de inibição de outras MAPKs ou da translocação do fator de transcrição para o núcleo. De fato, as vesículas podem ser internalizadas pelas células (Torrecilhas et al. 2012), e então modular os eventos intracelulares por ação de proteases ou RNAs (Garcia-Silva et al. 2014). Glicoconjugados (LPGs e GIPLs) de outros tripanosomatídeos que incluem *Leishmania braziliensis*, *Leishmania infantum,* diferentes cepas de *Leishmania enriettii* e *Leishmania amazonensis* também foram capazes de ativar diferencialmente MAPKs, em que variações intra e interespecíficas foram importantes nos eventos iniciais da resposta imune (Assis et al. 2012; Ibraim et al. 2013; Paranaíba et al. 2015; Nogueira et

al. 2016). Sumariamente, nossos dados indicam que independente do tipo de linhagem da qual as vesículas de *T. cruzi* pertencem, todas foram percebidas pela célula do compartimento imune inato. Em contrapartida, ocorreu produção diferencial de NO e citocinas pró-inflamatórias de maneira cepa-específica.

A transição de fase aguda para fase crônica é caracterizada pelo estabelecimento de uma resposta imune efetiva e controle da parasitemia, mas baixos níveis de parasitismo ainda são detectáveis em certos tecidos (Rassi et al. 2010). A grande variabilidade dos parasitos pode influenciar na evolução da doença e refletir na interação diferencial das células do sistema imune. Baseado nessas observações, nosso próximo passo foi avaliar o potencial imunomodulador das vesículas de *T. cruzi* em esplenócitos de camundongos em fase crônica da doença de Chagas. Um interessante aspecto foi observado, em que vesículas de cepas que não ativaram o compartimento imune inato foram mais pró-inflamatórias na fase crônica. Independente da cepa usada na infecção *in vivo*, vesículas das cepas Y e Colombiana exibiram perfil mais estimulador que as cepas CL-14 e YuYu. Como esperado, foi detectada presença da citocina IL-10, confirmando o papel modulador nos eventos inflamatórios da fase crônica (Souza et al. 2004; Vitelli-Avelar et al. 2008; Sathler-Avelar et al. 2009). Dependendo da cepa que circula no hospedeiro e considerando o fato que tripomastigotas podem liberar vesículas continuamente, estes resultados indicam que vesículas de *T. cruzi* podem ser cruciais para o mecanismo imunopatológico que ocorre nos órgãos. Até agora, o papel de diferencial de vesículas de uma mesma espécie resultando em determinado fenótipo ainda não havia sido explorado. A estimulação das células foi cepa-específica independente da cepa utilizada na infecção *in vivo*, reforçando o papel das vesículas como estímulo antigênico. Futuros estudos poderão determinar a utilização dessas vesículas de *T. cruzi* como ferramenta terapêutica para tratar inflamação crônica na doença de Chagas.

O subgrupo TcI é prevalente no norte da América do Sul e América Central, e engloba as cepas Colombiana e YuYu. Interessante observar que as vesículas dessas cepas tiveram desempenho antagônico, por isso analisamos o comportamento das vesículas das cepas Colombiana e YuYu na produção de citocinas em células do compartimento imune separadamente. Em geral não houve diferença na detecção de citocinas entre células de camundongos infectados com as duas cepas. Nos linfócitos T

CD4+ e CD8+ foi observada produção maior de IL-10 que IFN-γ e TNF-α. A habilidade de produzir IL-10 na fase crônica é importante para modular o balanço entre citocinas pró e anti-inflamatórias evitando dano tecidual (Souza et al. 2004). Foi observado um perfil misto de indução de citocinas em células B. Recentemente, foi identificada uma nova subpopulação de células B com características reguladoras, capazes de suprimir a progressão e/ou melhorar a recuperação de inflamações mediadas pela imunidade adquirida, principalmente pela produção de IL-10 e TGF-β (Fares et al. 2013). Adicionalmente, monócitos e células dendríticas parecem não ser responsáveis pela alta produção de IL-10 após estimulação com vesículas das cepas Colombiana e YuYu. As células dendríticas foram importante fonte de TNF-α, sugerindo que durante a fase crônica, vesículas de *T. cruzi* parecem ser pró-inflamatórias. Diferente do que ocorre para vesículas de *L. donovani*, que promove produção de IL-10 em células dendríticas do baço, as quais são consideradas imunossupressoras (Silverman et al. 2010).

As vesículas de diferentes cepas de *T. cruzi* foram capazes de modular diferencialmente os eventos imunes em macrófagos e células na fase crônica. Embora uma clara associação entre DTUs não tenha sido observada, vesículas das cepas CL-14 e YuYu foram potentes agonistas de TLR2. Por outro lado, vesículas das cepas Colombiana e Y foram ativadoras de citocinas pró-inflamatórias em células esplênicas de camundongos em fase crônica. Um importante aspecto das vesículas é a sua capacidade de modular produção de citocinas na fase crônica com produção de IL-10 por células T e B. Estudos adicionais para entender melhor o impacto de vesículas no sistema imune de pacientes *in vivo* e correlacionar com diferentes formas clínicas da doença de Chagas ainda serão realizados pelo grupo. Este trabalho proveu valiosa ferramenta para entender diferentes processos de ativação por vesículas de uma mesma espécie de parasito e seus efeitos na ativação celular.
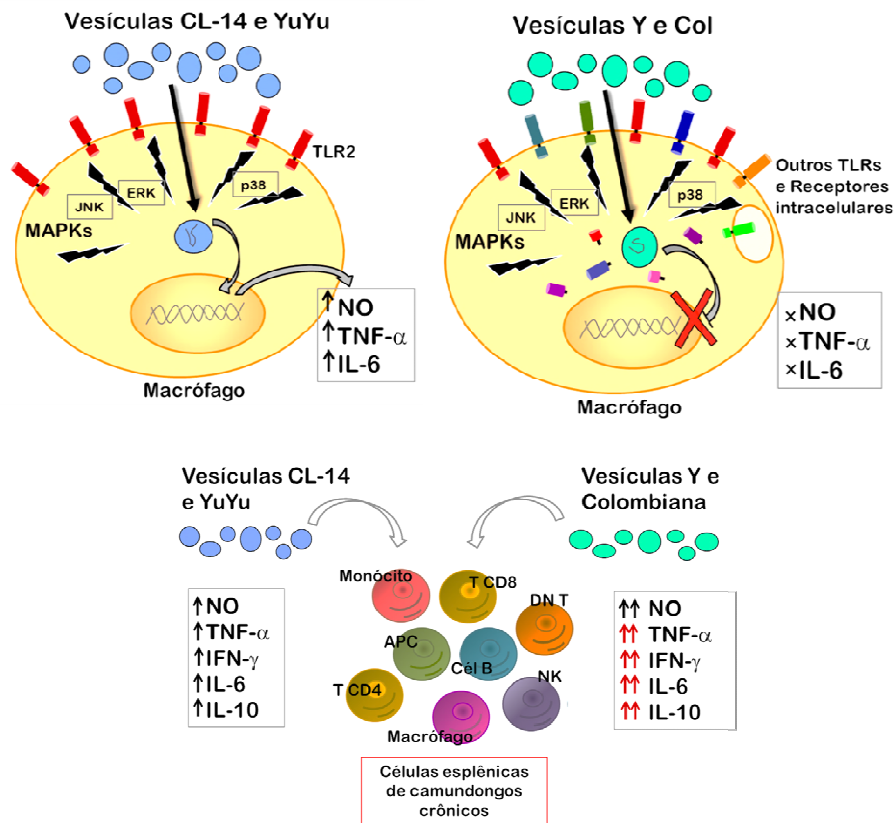
Figura 2: Esquema representativo da modulação da resposta imune após estímulo com EVs de diferentes cepas de *T. cruzi*. Em um primeiro contato, as vesículas de todas as cepas ativam fosforilação de MAPKs, seja por TLR2 (CL-14 e YuYu) ou por outros receptores (Y e Colombiana). Porém, após internalização, as vesículas atuam de maneira diferenciada, em que vesículas de CL-14 e YuYu são capazes de ativar a produção de NO e citocinas (TNF-a e IL-6). Essas citocinas favorecem o estabelecimento de um ambiente inflamatório, que ativa o macrófago. Por outro lado, vesículas das cepas Y e Colombiana bloqueiam a produção de citocinas por mecanismos ainda desconhecidos, com consequente inativação do macrófago. Durante a fase crônica ocorre inversão do papel inflamatório e ativação de citocinas. Ao contrário do que foi observado anteriormente, vesículas das cepas CL-14 e YuYu ativam menos a produção de citocinas em células esplênicas que vesículas das cepas Y e Colombiana. Não foi possível observar associação entre DTUs e ativação resposta imune

Parte II) Papel do Lipofosfoglicano (LPG) de *L. amazonensis* na interação com hospedeiros vertebrado e invertebrado

1 – Leishmanioses e seus glicoconjugados

As Leishmanioses são doenças negligenciadas que possuem ampla distribuição mundial e estima-se que 350 milhões de pessoas vivam em áreas de risco tanto no Novo quanto no Velho Mundo. As dificuldades no controle de reservatórios e vetores, a ineficácia dos tratamentos devido a cepas resistentes e a inexistência de vacinas humanas são alguns dos fatores que contribuem para sua expansão. A transmissão do parasito para o hospedeiro vertebrado é realizada por flebotomíneos do gênero *Phlebotomus* no Velho Mundo e do gênero *Lutzomyia* no Novo Mundo (Desjeux 2004). No homem, as leishmanioses são causadas por aproximadamente 21 espécies de *Leishmania*, sendo responsáveis por um amplo espectro de manifestações clínicas incluindo as formas cutânea (LC), muco-cutânea (LMC) e visceral (LV).

A leishmaniose cutânea (LC) é a forma mais comum da doença caracterizada por lesões ulcerosas na pele, indolores, únicas ou múltiplas que podem curar-se espontaneamente. É causada por *Leishmania guyanensis*, *Leishmania panamensis*, *Leishmania peruviana*, *Leishmania mexicana, L. amazonensis* e comumente associada à *L. braziliensis* no Novo Mundo (Herwaldt 1999). Uma variante da LC, a leishmaniose cutânea difusa (LCD) é freqüentemente causada por *L. amazonensis*, *Leishmania aethiopica* e *L. mexicana* (Reithinger et al. 2007). A LCD é caracterizada por infiltração difusa na pele, com grande número de nódulos, que geralmente não ulceram e se alastram por todo o corpo (Silveira et al. 2004). Nestes casos não ocorre cura espontânea.

Um aspecto importante que difere *L. amazonensis* das outras espécies, é que ela capaz de causar uma anergia imunológica no homem, responsável pela ausência de imunidade celular que resulta em não reatividade de resposta ao teste de Montenegro e é frequentemente refratária ao tratamento (Silveira et al. 2004). Tem sido demonstrado que essa espécie de *Leishmania* pode causar uma forma intermediária da doença, denominada leishmaniose cutânea disseminada *borderline* (LCDB), na qual a disseminação da *L. amazonensis* é observada alguns meses após o surgimento da lesão

cutânea primária, resultando em um limitado número de lesões secundárias com características semelhantes à inicial (Silveira et al. 2005). Contudo, a razão pela qual essa mesma espécie provoca amplo espectro clínico é desconhecida.

A infecção humana por *L. amazonensis* é menos comum que as outras espécies que causam LC, principalmente devido à restrição do habitat do seu vetor *Lutzomyia flaviscutellata*, apesar de apresentar grande potencial patogênico (Silveira et al. 2004). Esse flebotomíneo é pouco antropofílico e tem atividade essencialmente noturna. Porém, tem ocorrido um substancial aumento do número de novos casos, devido ao desmatamento, como doença ocupacional, e proximidade do contato do vetor com o homem (Silveira et al. 1991; Silveira et al. 2005). Além disso, a espécie de *Lutzomyia migonei* também é capaz de transmitir a infecção do parasito para o vertebrado (Nieves & Pimenta 2000).

A *Leishmania amazonensis* é um dos parasitos mais estudados devido a sua facilidade de cultivo e alta infectividade para o modelo animal. Muitos trabalhos abordam o uso desta espécie como modelo para testes de drogas e de compostos derivados de plantas (Aguiar et al. 2010; García et al. 2010; Pinheiro et al. 2011; Rocha et al. 2013). Essa espécie é endêmica em muitos países da América do Sul e Central, incluindo o Brasil (Grimaldi et al. 1989).

Durante seu ciclo de vida, os parasitos possuem duas formas de desenvolvimento que se alternam entre os hospedeiros vertebrado e invertebrado: uma forma promastigota extracelular alongada, flagelada e móvel no intestino do hospedeiro invertebrado e uma forma amastigota ovóide, imóvel e sem flagelo aparente que se desenvolve no interior de fagócitos mononucleados do hospedeiro vertebrado (Kaye & Scott 2011). A infecção do hospedeiro mamífero é iniciada durante o repasto sanguíneo pela fêmea infectada, que inocula as formas promastigotas metacíclicas na pele. Estas deverão escapar dos mecanismos de defesa do hospedeiro (Sacks & Kamhawi 2001; De Assis et al. 2012). Elas podem ser fagocitadas pelos macrófagos diretamente ou, após a infecção de neutrófilos que são rapidamente recrutados para o sítio de inoculação (Peters et al. 2008). Dentro dos vacúolos parasitóforos dos macrófagos as promastigotas diferenciam-se em formas amastigotas. Após o processo de divisão binária e ruptura dessas células, as amastigotas liberadas podem ser fagocitadas por outros macrófagos ou serem ingeridas pelo inseto vetor. Durante um novo repasto sanguíneo juntamente

com o bolo alimentar, estas formas diferenciam-se em promastigotas procíclicas, que são as formas de divisão. Essas sofrem várias mudanças em sua morfologia, sendo capazes de resistir à ação das enzimas digestivas do intestino médio e de aderirem ao epitélio intestinal. No final desse processo, elas diferenciam-se em promastigotas metacíclicas infectantes, que se desprendem do epitélio e migram para a porção anterior do intestino onde poderão ser novamente transmitidas ao hospedeiro vertebrado. O processo de diferenciação de promastigotas procíclicas em metacíclicas é chamado metaciclogênese (De Assis et al. 2012).

No seu ciclo biológico, os parasitos do gênero *Leishmania* encontram condições extremamente adversas em ambos hospedeiros. Vários estudos têm identificado diversas moléculas estágio e espécie-específicas importantes durante o processo de interação parasito-hospedeiro. Neste contexto, destacam-se os glicoconjugados de membrana. Essas moléculas estão presentes na superfície do parasito e podem ser ligadas à membrana por âncoras de glicosilfosfatidilinositol (GPI), que incluem os lipofosfoglicanos (LPGs), os glicoinositolfosfolípides (GIPLs) e a glicoproteína 63 (gp63). Eles podem também ser secretados sob a forma de proteofosfoglicanos (PPGs), fosfoglicanos (PGs) e fosfatases ácidas (*s*APs) (Turco & Sacks, 2003) (Fig. 2).
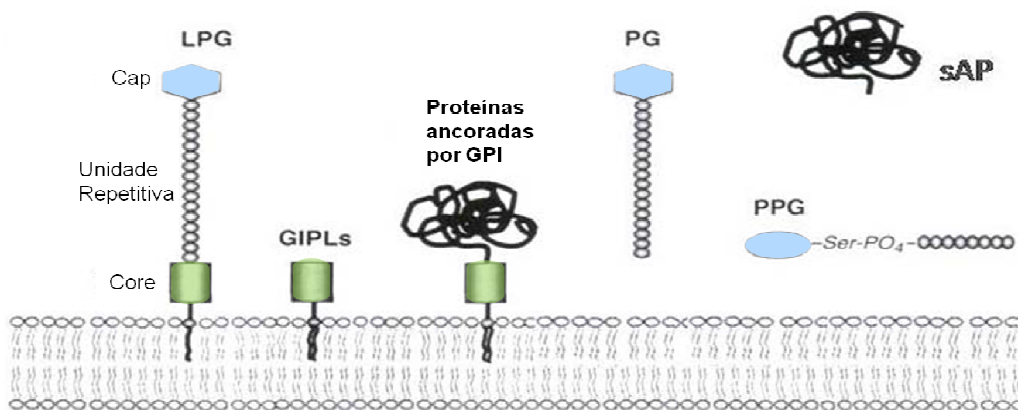

Figura 3: Representação esquemática dos glicoconjugados de *Leishmania.* Bioquimicamente, a âncora de GPI é composta por uma porção glicídica contendo 6-7 oligossacarídeos ligados a uma porção lipídica contendo 1-*O*-alquil-2-*liso*fosfatidilinositol. Legenda: LPG – Lipofosfoglicano; GIPLs – Glicoinositolfosfolípides; GPI – glicosilfosfatidilinositol; PG – Fosfoglicano; PPG – Proteofosfoglicano; sAP – Fosfatase ácida secretada. (adaptado de Turco, 2003).

O LPG é o glicoconjugado majoritário presente na superfície de promastigotas de todas as espécies conhecidas de *Leishmania*, localizado em todo o parasito inclusive no flagelo (Descoteaux & Turco 1999). É considerado um fator de virulência multifuncional, tendo importância na interação com os hospedeiros vertebrado e invertebrado. Sua estrutura básica consiste em quatro domínios: (I), uma âncora lipídica contendo glicosilfosfatidilinositol; (II), uma porção central composta por um heptassacarídeo Gal(α1,6)Gal(α1,3)Gal$_f$(β1,3)[Glc(α1)-PO$_4$]Man(α1,3)Man(α1,4)-GlcN(α1); (III), uma região de unidades repetitivas fosforiladas Gal(β1,4)Man(α1)-PO$_4$ e (IV), um oligossacarídeo neutro terminal "*cap*" (Turco & Descoteaux 1992) (Fig. 3).
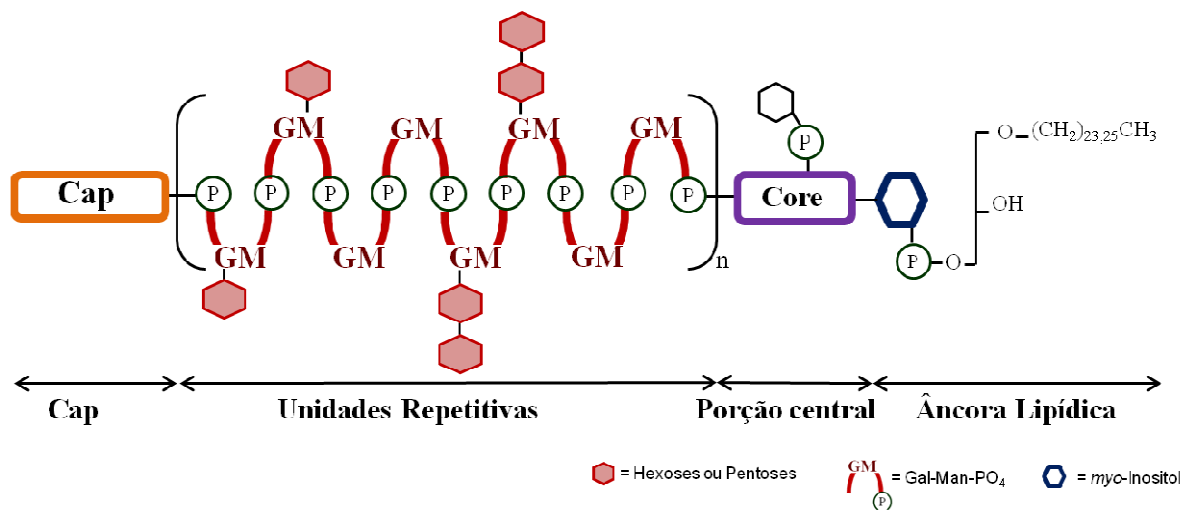

Figura 4: Desenho esquemático da estrutura do LPG (Adaptado de Assis *et al.*, 2012).

Análises estruturais do LPG revelam ampla conservação da âncora lipídica e do heptassacarídeo da porção central. No entanto, observa-se variabilidade na quantidade, composição e seqüência de açúcares ligados às unidades repetitivas e ao cap. Além destes polimorfismos interespecíficos, variações estágio-específicas também ocorrem durante a metaciclogênese. Isto é devido ao aumento do número de unidades repetitivas que dobram em quantidade nas formas metacíclicas (McConville et al. 1992; Sacks et al. 1995; Mahoney et al. 1999; Barron & Turco 2006). Diferentes substituições de açúcares podem ocorrer nas unidades repetitivas tanto no C3 da galactose quanto no C2 da manose incluindo glicoses, galactoses ou arabinoses (De Assis et al. 2012).

A caracterização bioquímica estrutural de LPGs tem sido objeto de muitos estudos desde a década de 1990. O primeiro LPG caracterizado foi o de *Leishmania major* (FV1), o qual apresenta de um a quatro resíduos de β-galactose eventualmente substituídos por α-arabinose após a metaciclogêne (McConville et al. 1992; McConville & Homans 1992). Ao mesmo tempo, o LPG das formas procíclicas de *L. mexicana* foi descrito contendo um resíduo de β-glicose nas unidades repetitivas (Ilg et al. 1992). Ao contrário de *L. major*, a estrutura do LPG da forma metacíclica não foi descrita para esta espécie. Em seguida o LPG de *Leishmania donovani* (Sudão) foi caracterizado não possuindo substituições nas cadeias laterais (Sacks et al. 1995), enquanto que o da cepa indiana possuía uma ou duas β-glicoses em suas unidades repetitivas (Mahoney et al. 1999). Dentre os LPGs descritos, o de *Leishmania tropica* (LRC-L36) mostrou-se o mais complexo com pelo menos 19 combinações diferentes de açúcares contendo glicose, galactose e arabinose (McConville et al. 1995; Soares et al. 2004). Outro LPG com estrutura incomum é o de *L. aethiopica*, onde suas substituições ocorrem geralmente no carbono 2 (C2) da manose por α-manose (McConville et al. 1995). Todos estes trabalhos, com exceção de *L. mexicana*, estudaram preferencialmente espécies do Velho Mundo. No entanto, espécies de *Leishmania* do Novo Mundo começaram a ter seus LPGs caracterizados na última década.

O primeiro LPG de uma cepa do Brasil foi o de *L. infantum* (cepa PP75). Similar a *L. mexicana*, o LPG de *L. infantum* também apresentou uma β-glicose nas unidades repetitivas (Soares et al. 2002). Nesta espécie, após a metaciclogênese, o açúcar desaparece e o número de unidades repetitivas aumenta de 19 para 34 nas formas procíclica e metacíclica, respectivamente (Soares et al. 2002; Barron & Turco 2006). Em *L. braziliensis* acontece o oposto do que ocorre em *L. infantum*. As formas procíclicas não possuem cadeias laterais e as metacíclicas possuem uma ou duas β-glicoses (Soares et al. 2005). Recentemente, nosso grupo observou que além de variabilidade interespecífica, polimorfismos intraespecíficos também ocorrem no LPG. Neste trabalho, as unidades repetitivas de 16 cepas de *L. infantum* foram caracterizadas, sendo classificadas em três grupos: (I), sem cadeias laterais (maioria das cepas); (II), com uma cadeia lateral de glicose (cepa PP75) e (III), com até três cadeias laterais de glicose (cepa BH46) (Coelho-Finamore et al. 2011) (Fig. 4). Já os LPGs de *Leishmania*

shawi e *Leishmania enriettii*, causadores de formas tegumentares, não apresentam cadeias laterais em seu LPG (Paranaíba et al. 2015; Passero et al. 2015).
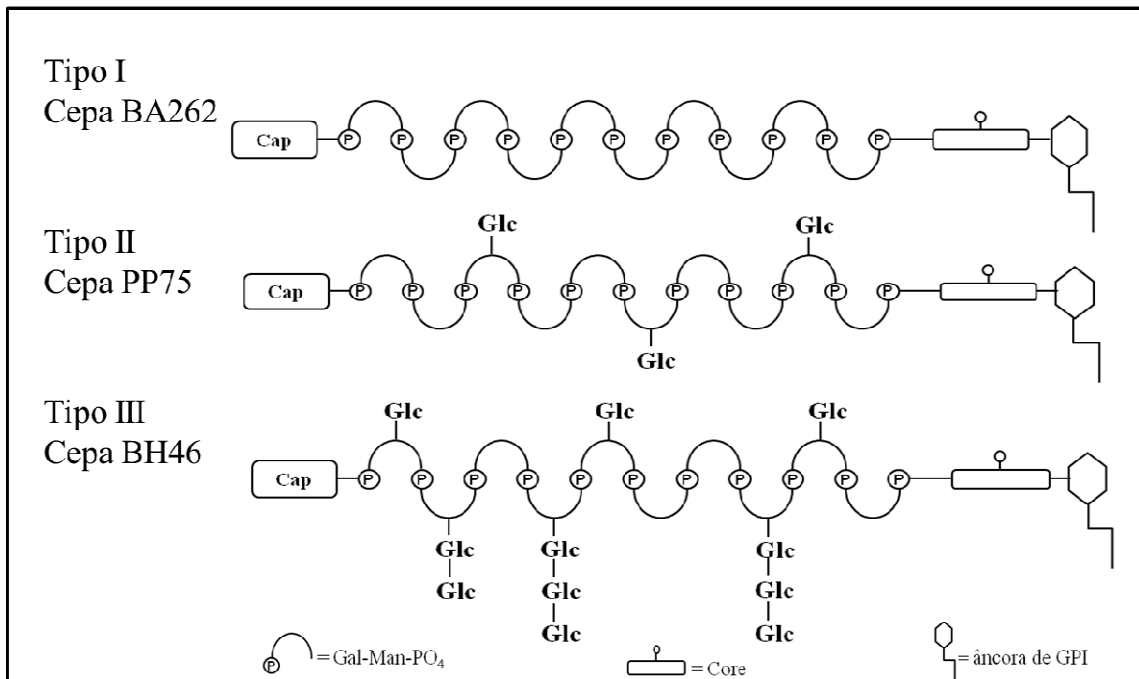

Figura 5: Diagrama esquemático da estrutura do LPG das formas procíclicas de *L. infantum*. Glc = glicose.

Até o momento já foram descritas 10 estruturas do LPG de espécies de *Leishmania* presentes no Novo e Velho Mundo (Tabela 1). Entretanto, não só a estrutura do LPG de *L. amazonensis*, mas também seus polimorfismos intraespecíficos ainda permaneciam desconhecidos.

Tabela 1: Estado da arte dos LPGs de *Leishmania* descritos.

Lipofosfoglicanos	Referências
Velho Mundo	
L. donovani	(McConville & Blackwell 1991) (Thomas et al. 1992) (Sacks et al. 1995)
L. major	(McConville & Homans 1992) (Moody et al. 1993)
L. tropica	(McConville et al. 1995) (Soares et al. 2004)
L. aethiopica	(McConville et al. 1995)
L. turanica	(Volf et al. 2014)
Novo Mundo	
L. mexicana	(Ilg et al. 1992)
L. infantum	(Soares et al. 2002) (Coelho-Finamore et al. 2011)
L. braziliensis	(Muskus et al. 1997) (Soares et al. 2005)
L. enriettii	(Paranaíba et al. 2015)
L. shawi	(Passero et al. 2015)

A caracterização envolvendo a variabilidade intra-específica de LPGs são escassos tanto em cepas do Novo quanto do Velho Mundo. Nossos resultados preliminares evidenciam a existência de dois tipos de LPG (I e II) para *L. amazonensis* (Fig. 5) (Nogueria et al. *em preparação*). É importante ressaltar que a cepa PH8 foi isolada de flebotomíneo, enquanto a cepa Josefa foi isolada de hospedeiro humano. Além de características genéticas, este achado poderia estar correlacionado também ao tipo de hospedeiro de onde as cepas foram isoladas.
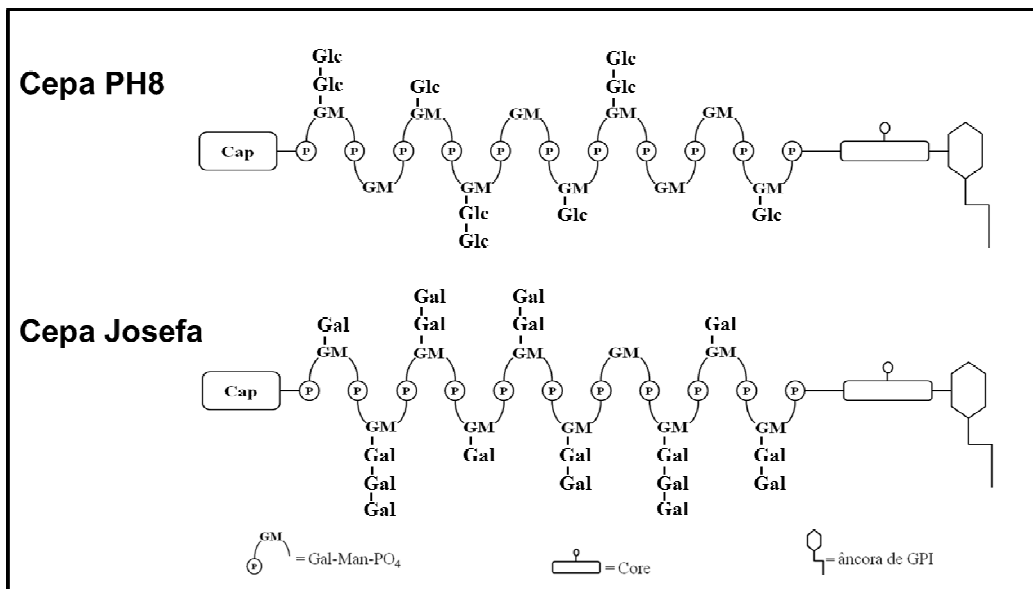
Figura 6: Representação esquemática do polimorfismo intraespecífico dos lipofosfoglicanos (LPGs) de formas procíclicas das cepas PH8 e Josefa de *L. amazonensis*. Glc = glicose; Gal = galactose (Nogueira et al., *em preparação*).

Estudos da interação *Leishmania*-hospedeiro são importantes para o entendimento dos processos envolvidos no desenvolvimento e transmissão do parasita. A compreensão das estruturas bioquímicas e funções do LPG podem ajudar a elucidar como esse patógeno sobrevive a estes processos. Esse glicoconjugado é considerado um fator de virulência multifuncional, com uma ampla variedade de funções que incluem: ligação do parasito ao epitélio intestinal (Kamhawi et al. 2004), resistência à ação lítica do sistema de complement (Brittingham & Mosser 1996), ligação e indução de fagocitose no macrófago (Descoteaux & Turco 1999), proteção contra dano proteolítico no vacúolo parasitóforo, atraso ou inibição da maturação fagossomal (Lodge et al. 2006), inibição da proteína quinase C (PKC) (Descoteaux et al. 1992), modulação da produção de óxido nítrico (NO) e de citocinas (Proudfoot et al. 1996; Piedrafita et al. 1999; Paranaíba et al. 2015) modulação de MAPKs (Feng et al. 1999; Ibraim et al. 2013; Rojas-Bernabé et al. 2014) e ativação de receptores do tipo toll (TLR) (Becker et al. 2003; de Veer et al. 2003; Ibraim et al. 2013; Paranaíba et al. 2015). Importantes trabalhos que foram recentemente publicados mostraram a importância do LPG de *L. amazonensis* na indução de proteína quinase R (PKR) (de Carvalho Vivarini et al. 2011), na indução de redes extracelulares de neutrófilos (NETs) (Guimarães-Costa et al.

2009) e de leucotrieno B4 (LTB4) (Tavares et al. 2014). Entretanto, apesar destas funções marcantes, a estrutura de seu LPG não era conhecida.

A importância do LPG para a viabilidade de *Leishmania* foi confirmada após estudos com *L. major* e *L. donovani* deficientes para sua produção. Foi observado que estes mutantes não foram capazes de manter a infecção em macrófagos, sendo que sua viabilidade foi restabelecida após a restauração da expressão do LPG (Butcher et al. 1996; Lodge & Descoteaux 2005). Porém, ao contrário destes, no caso de *L. mexicana*, o parasito mutante é capaz de sobreviver, indicando provavelmente o uso de outros mecanismos (Ilg 2000). Isso demonstra que a dependência de tipos específicos de glicolípides pode variar de acordo com a espécie de *Leishmania*, o que enfatiza a necessidade de se estudar a função do LPG em outras espécies, incluindo as do Brasil. Recentemente foi demonstrado que o LPG de *L. braziliensis* foi mais pró-inflamatório que o de *L. infantum* (Ibraim et al. 2013), indicando que polimorfismos podem ser importantes durante a imunopatologia da doença. Por outro lado, demonstramos que o LPG de *L. enriettii* de duas cepas (L88 e Cobaia) possuíam diferentes perfis pró-inflamatórios e capacidade de gerarem lesões (Paranaíba et al. 2015). Isto demonstra que mesmo entre cepas da mesma espécie pode haver diferentes graus de virulência.

O reconhecimento de PAMPs por receptores do tipo Toll (TLRs) é importante durante os eventos iniciais da resposta imune inata (Gazzinelli & Campos 2004). Estudos prévios indicam que o LPG de *L. major* é um importante agonista de TLR2 em macrófagos humanos e células NK, induzindo a produção de TNF-α e IFN-γ via proteína MyD88, sendo que a integridade da âncora lipídica é muito importante para ativação (Becker et al. 2003; de Veer et al. 2003). Em macrófagos murinos estimulados com LPG de *L. braziliensis* e *L. infantum*, houve participação tanto de TLR2 e TLR4. Adicionalmente, LPG de *L. enriettii* foi capaz de estimular a produção de NO, TNF-α, IL-12 e IL-6 preferencialmente via TLR2. De modo interessante, ao se utilizar células CHO transfectadas com TLRs, foi observado que o LPG de *L. braziliensis* é agonista de TLR2, enquanto o LPG de *L. infantum* não ativa este receptor (Ibraim et al. 2013). Do mesmo modo, apenas GIPLs de *L. enriettii* foram capazes de ativar essas células via TLR2 e TLR4 (Paranaíba et al. 2015). Esses dados evidenciam que a presença de polimorfismo interespecífico pode atuar de maneira diferenciada durante a interação parasito-hospedeiro. Um recente trabalho em colaboração com nosso grupo demonstrou

que o LPG de *L. amazonensis* foi capaz de controlar a síntese de PKR-dependente de RNA de fita dupla via TLR2 (de Carvalho Vivarini et al. 2011).

Ao contrário do que é observado nas infecções por *L. major*, em que há polarização da resposta imune em tipo Th1 (resistente à infecção) e tipo Th2 (susceptível à infecção), vários trabalhos indicam que a susceptibilidade a *L. amazonensis* por camundongos resistentes a *L. major* está relacionada mais ao desenvolvimento de uma resposta mista Th1/Th2, ou a uma ausência de resposta Th1 eficiente, do que à presença de resposta Th2 (Afonso & Scott 1993; Ji et al. 2002). Por outro lado, IL-10 parece ter um papel importante na modulação negativa da resposta Th1 após a infecção por *L. amazonensis* (Jones et al. 2002). Ademais, amastigotas de *L. amazonensis* são muito mais resistentes à morte mediada por NO do que amastigotas *de L. major*, visto que níveis muito maiores de NO são requeridos para sua eliminação (Gomes et al. 2003; Mukbel et al. 2007; Wanasen et al. 2008). Além disso, o parasita tem a capacidade de modular negativamente a produção de NO por mecanismos independentes da produção de IL-4, IL-10 ou TGF-β (Balestieri et al. 2002), porém outros fatores parecem estar envolvidos na limitação de respostas Th1 na fase crônica da infecção. A infecção com promastigotas *de L. amazonensis* ativa o complexo repressor de NF-κB em macrófagos, regulando negativamente a expressão de iNOS, o que favorece o estabelecimento e sucesso da infecção por *L. amazonensis* (Calegari-Silva et al. 2009). Portanto, em nosso trabalho utilizamos macrófagos murinos afim de elucidar qual tipo de TLR poderia estar envolvido na imunomodulação de citocinas e NO.

Durante a infecção do inseto vetor, a ligação do parasito ao epitélio intestinal por meio do LPG é um passo crucial para prevenir a perda da *Leishmania* no processo de excreção do bolo alimentar. O papel do LPG na interação com o vetor foi demonstrado com uso de parasitos mutantes deficientes em LPG (-/-) que não são capazes de se ligar e sustentar a infecção no intestino (Sacks et al. 2000). A significância das modificações estruturais durante a metaciclogênesis no LPG de *L. infantum* (cepa PP75) foi demonstrada na interação com vetor *Lutzomyia longipalpis in vitro*, em que o LPG é crucial para ligação da forma procíclica ao epitélio intestinal (Soares et al. 2002). Porém, o polimorfismo intraespecífico dos LPGs de *L. infantum* não alterou a densidade dos parasitos no vetor *in vivo* (Coelho-Finamore et al. 2011). Uma proteína de 65-kDa

foi identificada em *Phlebotomus papatasi* como receptor para LPG de *L. major*, que permite apenas o desenvolvimento dessa espécie (Dillon & Lane 1999). Porém apenas cinco anos depois foi identificado um ligante para o LPG desta espécie no intestino deste vetor (Kamhawi et al. 2000). Recentemente nosso grupo descreveu a presença de resíduos de β-galactose no LPG de *L. turanica*, o que explicaria a sua capacidade de manter infecção em *P. papatasi* (Chajbullinova et al. 2012; Volf et al. 2014). Este achado quebrou um paradigma importante o qual dizia que *L. major* só poderia ser transmitido por *P. papatasi* e *P. duboscqi*. A competência vetorial destes insetos para a transmissão de diferentes espécies de *Leishmania* parece ser controlada por polimorfismos interespecíficos na estrutura do LPG. O principal vetor de *L. amazonensis* é *Lutzomyia flaviscutellata,* amplamente distribuído na América do Sul (Carvalho et al. 2015). Mas já foi demonstrado que *Lutzomyia migonei* é capaz de albergar a infecção dessa espécie (Nieves & Pimenta 2000). Devido ao acesso a esta espécie, neste trabalho optamos por utilizar *L. mogonei* em colaboração com o Laboratório de Ecologia de Doenças Transmissíveis na Amazônia (EDTA), CPq Leônidas e Maria Deane - FIOCRUZ Manaus/AM.

Com base no que foi exposto este projeto teve como objetivo elucidar aspectos desconhecidos da interação do LPGs de duas cepas de *L. amazonensis* com células do hospedeiro vertebrado e invertebrado. Este estudo faz parte de um amplo projeto que visa conhecer a Glicobiologia das espécies de *Leishmania* do Novo Mundo.

2 – JUSTIFICATIVA

Estudos que auxiliem a compreensão dos mecanismos de interação de *Leishmania* com seus hospedeiros constituem uma ferramenta importante no entendimento da biologia destes parasitos. Desta forma, um grande número de estudos busca identificar marcadores de virulência e correlacioná-los a imunopatologia da doença. A resposta imune a *Leishmania* tem sido bastante estudada no modelo murino de infecção por *L. major*. No entanto, estes estudos não refletem o que acontece nas infecções por outras espécies de *Leishmania*.

Durante o processo de interação parasito-hospedeiro, o LPG desempenha um papel crucial na sobrevivência do parasito. Ele está envolvido em várias funções tanto no hospedeiro vertebrado quanto invertebrado. Estudos prévios de nosso grupo caracterizaram as estruturas dos LPGs de *L. infantum*, *L. braziliensis*, *L. enriettii* e *L. shawi* os quais apresentaram diferenças importantes na modulação de células do sistema imune do hospedeiro vertebrado. Alguns papéis biológicos já foram descritos para o LPG de *L. amazonensis*. Porém, devido ao desconhecimento de sua estrutura e polimorfismos, este trabalho teve como foco caracterizar preliminarmente o LPG em duas cepas desta espécie, bem como avaliar a ativação de macrófagos por estas moléculas e seu papel na interação com vetor *Lutzomyia migonei*.

3 – OBJETIVOS

3.1 - Objetivo Geral

Avaliar o papel do LPG de duas cepas de referência de *L. amazonensis* (PH8 e JOSEFA) na interação com células do hospedeiro vertebrado e com o vetor *L. migonei*.

3.2 - Objetivos Específicos

- Avaliar a produção de citocinas e NO em macrófagos murinos de diferentes linhagens após exposição aos LPGs de *L. amazonensis* das cepas PH8 e Josefa;

- Avaliar participação dos receptores do tipo Toll 2 e 4 (TLR2 e TLR4) e translocação de NF-κB após estimulação por LPGs das duas cepas;

- Avaliar a ativação de MAPKs (p38 e JNK) por macrófagos murinos após exposição aos LPGs das duas cepas;

- Avaliar capacidade de infecção das cepas de *L. amazonensis* ao intestino médio de *L. migonei*.

4 – ARTIGO

RESEARCH ARTICLE

Lipophosphoglycans from *Leishmania amazonensis* Strains Display Immunomodulatory Properties via TLR4 and Do Not Affect Sand Fly Infection

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Abstract

The immunomodulatory properties of lipophosphoglycans (LPG) from New World species of *Leishmania* have been assessed in *Leishmania infantum* and *Leishmania braziliensis*, the causative agents of visceral and cutaneous leishmaniasis, respectively. This glycoconjugate is highly polymorphic among species with variation in sugars that branch off the conserved $Gal(\beta1,4)Man(\alpha1)-PO_4$ backbone of repeat units. Here, the immunomodulatory activity of LPGs from *Leishmania amazonensis*, the causative agent of diffuse cutaneous leishmaniasis, was evaluated in two strains from Brazil. One strain (PH8) was originally isolated from the sand fly and the other (Josefa) was isolated from a human case. The ability of purified LPGs from both strains was investigated during *in vitro* interaction with peritoneal murine macrophages and CHO cells and *in vivo* infection with *Lutzomyia migonei*. In peritoneal murine macrophages, the LPGs from both strains activated TLR4. Both LPGs equally activate MAPKs and the NF-κB inhibitor p-IκBα, but were not able to translocate NF-κB. *In vivo* experiments with sand flies showed that both stains were able to sustain infection in *L. migonei*. A preliminary biochemical analysis indicates intraspecies variation in the LPG sugar moieties. However, they did not result in different activation profiles of the innate immune system. Also those polymorphisms did not affect infectivity to the sand fly.

Author Summary

Leishmania amazonensis, a member of the *Leishmania mexicana* complex, is the causative agent of localized cutaneous leishmaniasis (LCL) and anergic diffuse cutaneous leishmaniasis (ADCL) [1,2]. It is widely distributed throughout the Amazon basin, where it infects a wide range of terrestrial rodents and, less frequently, marsupials. Its main vector is *Lutzomyia flaviscutellata* (Diptera: Psychodidae) widely distributed in South America and a recent study has predicted its expansion towards South of Brazil [3]. Moreover, *Lutzomyia migonei* (França, 1920) can also harbor the infection of this species [4,5]. Although its transmission to man is very uncommon, *L. amazonensis* triggers an incurable and disseminated form of cutaneous leishmaniasis [2,6]. However, most of the mechanisms involved in *L. amazonensis* pathogenesis are still unknown, especially those related to surface molecules. Glycoconjugates have been extensively characterized as important for the establishment of infection as they protect the parasite from the early action of the host immune system and therefore acting as invasive/evasive strategies. Consequently, we here present the role of lipophosphoglycan (LPG) of *L. amazonensis* in the interaction with vertebrate and invertebrate hosts.

Introduction

The major cell surface glycoconjugate of *Leishmania* is the lipophosphoglycan (LPG), implicated in a wide range of functions, both in vertebrate and invertebrate hosts [7]. In the invertebrate host, LPG variations are important for *Leishmania* specificity to the sand fly [8], where attachment of the parasite to a midgut receptor is a crucial event [9]. In the vertebrate host, the main functions of this virulence factor during the earlier steps of infection include: protect the parasite from complement-mediated lysis, attachment and entry into macrophages [10], able to inhibit phagolysosomal fusion [11], modulation of nitric oxide (NO) production [12] and inhibition of protein kinase C (PKC) [13]. Interestingly, although *L. major* LPG mutants ($lpg1^-$) were highly susceptible to complement mediated lysis, they were able to invade macrophages reinforcing the role of other molecules and the host defenses during the interaction [11].

Many functions have been attributed to *L. amazonensis* LPG including induction of neutrophil extracellular traps (NETs) [14], induction of protein kinase R (PKR) [15], triggering and killing of the parasite via Leukotriene B4 (LTB4) [16]. Although *L. amazonensis* LPG is important in many steps of host infection, its role during the interaction with macrophages and sand flies remains unknown.

LPG structures have been described for several dermotropic and viscerotropic *Leishmania* [17–26]. LPGs have a conserved glycan core region of Gal(α1,6)Gal(α1,3)Gal$_f$(β1,3)[Glc(α1)-PO$_4$]Man(α1,3)Man(α1,4)-GlcN(α1) linked to a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. The salient feature of LPG is another conserved domain consisting of the Gal(β1,4)Man(α1)-PO$_4$ backbone of repeat units ($n = \sim15$–30). The distinguishing feature of LPGs that is responsible for the polymorphisms among *Leishmania* spp. is variable sugar composition and sequence of branching sugars attached to the repeat units and cap structure [27]. For example, the LPG of *Leishmania major* (Friedlin) has β-1,3 galactosyl side-chains, often terminated with arabinose, whereas the LPGs of *Leishmania donovani* (Mongi) and *L. infantum* (PP75 and BH46 strains) possess β-glucoses in their repeat units [17,20,24]. However, there is no available information on the degree of variability in the LPG structure for *L. amazonensis*.

The *L. major* LPG was identified as potent agonist of Toll-like receptor 2 (TLR2) in human natural killer (NK) cells and murine macrophages, triggering the production of TNF-α and

IFN-γ through MyD88 [28,29]. Recently, the LPGs of two New World species (*L. infantum* and *Leishmania braziliensis*) differentially activated TLR2. In this case, *L. braziliensis* LPG was more pro-inflammatory being able to induce the translocation of NF-κB to the nucleus [30].

As a part of a wider project on the glycobiology of New World species of *Leishmania*, we evaluated the role of *L. amazonensis* LPGs (PH8 and Josefa strains) during the interaction with host cells and the sand fly *L. migonei*. The present study might help to improve our understanding on the immune modulation mediated by glycoconjugates of *L. amazonensis*, the etiological agent of diffuse cutaneous leishmaniasis (DCL).

Materials and Methods

Ethics statement

The animals were kept in the Animal Facility of the Centro de Pesquisas René Rachou/FIO-CRUZ. All animals were handled in strict accordance with animal practice as defined by Internal Ethics Committee in Animal Experimentation (CEUA) of Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais (MG), Brazil (Protocol P-82/11-4). This protocol followed the guidelines of CONCEA/MCT, the maximum ethics committee of Brazil. Knockout mice handling protocol was approved by the National Commission of Biosafety (CTNBio) (protocol #01200.006193/2001-16).

Parasites, growth curves, and molecular typing

World Health Organization Reference strains of *L. amazonensis* (IFLA/BR/1967/PH8 and MHOM/BR/75/Josefa) were used. The PH8 strain was originally isolated from the sand fly *L. flaviscutellata* from Pará State, Brazil, and the Josefa strain was isolated from a human case from Bahia State, Brazil. Promastigotes were cultured in M199 medium supplemented with 10% fetal bovine serum (FBS), penicillin 100 units/mL, streptomycin 50 μg/mL, 12.5 mM glutamine, 0.1 M adenine, 0.0005% hemin, and 40 mM Hepes, pH 7.4 at 26°C until late log phase [21]. Parasites were seeded in triplicate (1 x 10^5 cells/mL), and growth curves of PH8 and Josefa strains were determined daily using a Neubauer improved haemocytometer until cells reached a stationary phase. Both strains exhibited a similar division profile reaching stationary phase after 7 days of culture. For this reason the 6th day was chosen for harvesting parasites for LPG extraction and molecular typing (S1A Fig).

For molecular typing, genomic DNA was extracted from log-phase *Leishmania* using the phenol/chloroform method (1:1) for amplification of the HSP70 fragment prior to digestion with HaeIII as previously described [31]. Positive controls included DNA from *L. braziliensis* (MHOM/BR/75/M2903), *L. infantum* (MHOM/BR/74/PP75), *Leishmania guyanensis* (MHOM/BR/75/M4147) and *L. amazonensis* (IFLA/BR/67/PH8). After PCR-RFLP both *L. amazonensis* strains were confirmed (S1B Fig).

Extraction and purification of LPG

For optimal LPG extraction, late log phase cells were harvested and washed twice with PBS prior to extraction of LPGs (Fig 1). The LPG extraction was performed as described elsewhere with solvent E (H_2O/ethanol/diethylether/pyridine/NH_4OH; 15:15:5:1:0.017) after a sequential organic solvent extraction [32]. For purification, the solvent E extract was dried under N_2 evaporation, resuspended in 2 mL of 0.1 M acetic acid/0.1 M NaCl, and applied onto a column with 2 mL of phenyl-Sepharose, equilibrated in the same buffer. The column was washed with 6 mL of 0.1 M acetic acid/0.1 M NaCl, then 1 mL of 0.1 M acetic acid and finally 1 mL of endotoxin free water. The LPGs were eluted with 4 mL of solvent E then dried under N_2 evaporation. LPG
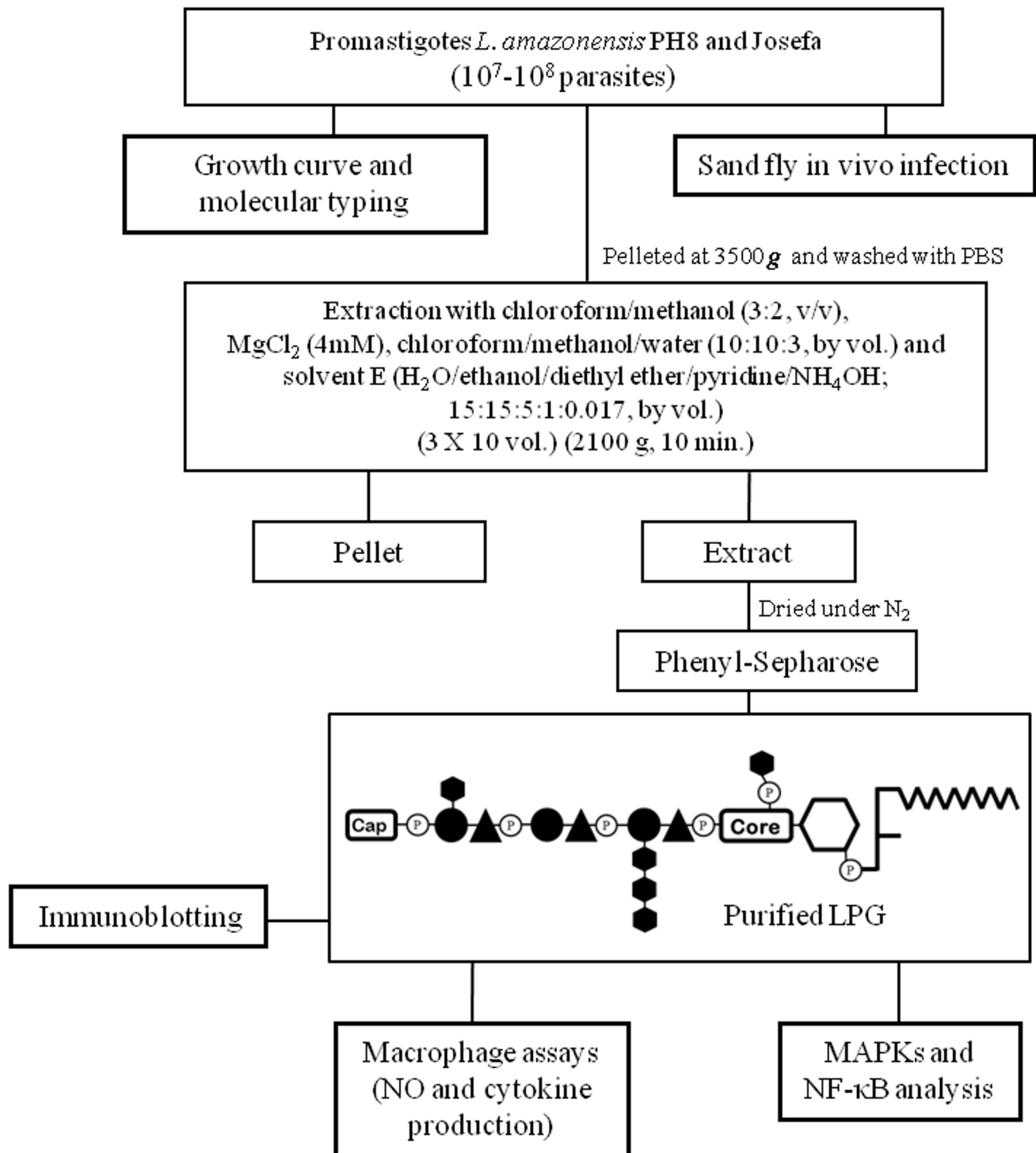
Fig 1. Procedures for extraction, purification, preliminary characterization of *L. amazonensis* LPG, interaction with vertebrate cells and *L. migonei*. Late log phase cells were harvested and washed with PBS. For studies with vector, *L. migonei* midguts were dissected on days 2 and 4 post feeding containing *L. amazonensis* from each strain. Parasite cell pellets were subject to extraction with organic solvents as described elsewhere. For purification, the solvent E extract was dried under N_2 evaporation and applied into a phenyl-Sepharose column. The purified LPG was used for biological and immunological assays.

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concentrations were determined as described elsewhere [33]. Prior to use on *in vitro* cells cultures, LPGs were diluted in RPMI. All solutions were prepared in sterile, LPS-free distilled water (Sanobiol, Campinas, Brazil). All extractions and purifications procedures are depicted in Fig 1.

Immunoblotting and preliminary characterization of LPGs

Purified LPGs (5 μg) were subjected to dot-blot, blocked (1 h) in 5% milk in PBS and probed for 1 h with monoclonal antibody (mAb) CA7AE (1:1000), that recognizes the unsubstituted Gal(β1,4)Man repeat units [34]; mAb LT22 (1:1000) that recognizes β-glucose side chains and WIC 79.3 (1:1000) that recognizes β-galactose side chains [21,35]. After three washes in PBS (5 min), the membrane was incubated for 1 h with anti-mouse IgG conjugated with peroxidase (1:5,000) and the reaction was visualized using luminol.

Purification of murine peritoneal macrophages and cell culture

Thioglycollate-elicited macrophages were extracted from C57BL/6 and C57BL/6 knockouts TLR2 (-/-) and TLR4 (-/-) by peritoneal washing with ice cold RPMI and enriched by plastic adherence (1 h, 37°C, 5% CO_2). Cells (3 x 10^5 cells/well) were washed with fresh RPMI then culture in RPMI, 2 mM glutamine, 50 U/mL of penicillin and 50 μg/mL streptomycin supplemented with 10% FBS in 96-well culture plates (37°C, 5% CO_2). Cells were primed with interferon-gamma (IFN-γ) (3 IU/mL) for 18 h prior to incubation with LPGs from both strains (10 μg/mL), live stationary *Leishmania* parasites (MOI 10:1) and lipopolysaccharide (LPS: 100 ng/mL) [30,36].

Cytokine and nitrite measurements

For CBA multiplex cytokine detection, cells were plated, primed as describe above and incubated with LPGs and live stationary promastigotes (MOI 10:1) for 48 h. LPS was added as a positive control and medium as negative control. Supernatants were collected and IL-1β, IL-6, IL-10, IL-12p40 and TNF-α were determined using BD CBA Mouse Cytokine assay kits according to the manufacturer's specifications (BD Biosciences, CA, USA). Flow cytometry measurements were performed on a FACSCalibur flow cytometry (BD Bioscience, Mountain View, CA, USA). Cell-QuestTM software package provided by the manufacturer was used for data acquisition and the FlowJo software 7.6.4 (Tree Star Inc., Ashland, OR, USA) was used for data analysis. A total 1,500 events were acquired for each preparation. Results are representative of six experiments in duplicate. Nitrite concentrations were determinate by Griess reaction (Griess Reagent System, 2009).

MAPKs and NF-κB translocation assay

For MAPKs, peritoneal murine macrophages were obtained as described above. They were applied on 24 wells tissue culture plates (10^6 cells/well) for 18 h prior to assay. The cells were washed with warm RPMI and incubated with LPG from both species for different times (5, 15, 30, 45 and 60 min) or with medium (negative control) or *E. coli* extracts (100 ng/mL, only 45 minutes) as positive control. p-p38, p-JNK, p-IκBα and total p38 were assayed as previously described [25]. p-IκBα antibody was provided by Dr. L. P. de Sousa. NF-κB translocation using CHO reporter lines (a kind gift by M. A. Campos) was determined as described elsewhere [30]. CHO reporter cells were plated (1 x 10^5 cells/well) in 24-well tissue culture dishes and the LPG (0.02 and 0.2 μg/mL) from both strains was added in a total volume of 0.25 mL medium/well.

The cells were examined by flow cytometry (BD Biosciences, CA, USA) and the analyses were performed using CellQuestTM software.

Sand fly *in vivo* infection

Lutzomyia migonei (Baturite strain) sand flies were kept under laboratory conditions and were fed on 30% sucrose solution for 3–4 days prior to experiments. The insects were artificially fed using a chick skin membrane in a glass-feeder device. The chick skin membrane was provided by the Animal Facility of Centro de Pesquisas René Rachou/FIOCRUZ under the Protocol LW 30/10. Heparinized mouse blood (drawn intracardially from Balb/C), with penicillin (100 U/mL) and streptomycin (100 μg/mL) (37°C) containing 2 x 10^7/mL logarithmic phase promastigotes (PH8 and Josefa strains) offered for 5 h under dark conditions [5]. Blood engorged flies were separated and maintained at 26°C with 30% sucrose. Engorged sand flies had their midguts dissected on days 2 and 4 post feeding. The midguts were homogenized in 30 μl of PBS and the number of viable promastigotes determined by counting under a Neubauer improved haemocytometer [24].

Statistical analyses

For nitrite, cytokine measurements and *in vivo* sand fly experiments, the Shapiro Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution [37]. For the non-parametric distribution, it was performed the Mann-Whitney test. Data were analyzed using GraphPad Prism 5.0 software (Graph Prism Inc., San Diego, Ca). $P < 0.05$ was considered significant.

Results

The LPGs from *L. amazonensis* strains display intraspecific polymorphism

The purified LPGs from *L. amazonensis* PH8 and Josefa strains were differentially recognized by the mAbs CA7AE and LT22 (S2 Fig). LPG from PH8 strain was recognized by CA7AE and LT22 as well as the positive control represented by *L. infantum* (BH46). However, a different recognition profile was observed for the Josefa strain since its LPG was weakly recognized by LT22 but not by CA7AE, indicating the presence of side-chains branching-off the repeat units. Because CA7AE recognizes Gal(β1,4)Man unsubstituted repeat units in LPG [34], these results indicate that at least some of the repeat units are indeed unsubstituted in the LPG of PH8 strain. On the other hand, the presence of side-chains suggestive of glucoses, due to LT22 reactivity, was detected in the LPGs of PH8 and Josefa strains. However, LT22 also recognized the galactose-branched repeat units of *L. major* (strains FV1 and LV39) indicating cross-reactivity of the antibodies, thus suggesting the presence of either glucose or galactose as side chains (S2 Fig). These data suggested an intraspecific polymorphism in the LPGs of *L. amazonensis* strains.

LPGs from *L. amazonensis* strains equally activate NO and cytokine production via TLR4

We investigated whether LPGs purified from different strains could have an impact on the parasite's interaction with host cells, the ability to elicit NO and cytokine production by murine macrophages. LPGs from both strains were incubated with murine peritoneal macrophages from C57BL/6 and respective knockouts for TLR2 (-/-) and TLR4 (-/-). We did not detect any production of the cytokines IL-1β, IL-10 and IL-12 (S3A–S3C Fig). Both LPGs and respective

parasites were able to activate through TLR4, resulting in NO, TNF-α and IL-6 production (Fig 2A–2C) (P < 0.05). As expected, LPS (positive control) activated TLR4 in the TLR2 (-/-) (Fig 2A–2C).

LPGs from *L. amazonensis* equally activate MAPKs and the NF-κB inhibitor p-IκBα via TLR4

No difference in MAPKs phosphorylation (p38 and JNK) and p-IκBα was observed after incubation with LPGs from both strains. In peritoneal murine macrophages this activation was mainly via TLR4 (Fig 3A and 3B). We also evaluated if the LPGs from these strains were able to translocate NF-κB in CHO cells. No activation of NF-κB was detected in those cells (Fig 4).

Leishmania amazonensis strains equally infected the sand fly *L. migonei*

In vivo midgut infections of the sand flies were determined on days 2 and 4 post feeding, in order to evaluate the number of parasites after the blood meal digestion, as well as, after its excretion on day 3, where non-attached parasites are lost. Although a higher parasite density was detected for PH8 strain on day 2 (P < 0.05), no statistical differences in the parasite densities from both *L. amazonensis* strains were observed on day 4, and both strains were able to colonize *L. migonei* midgut (P > 0.05, Fig 5).

Discussion

Leishmania amazonensis, etiologic agent of the cutaneous and anergic diffuse leishmaniasis, is characterized by disseminated non-ulcerative skin lesions and constantly proportion of negative delayed hypersensitivity skin-test (DTH), resulting in a high resistance of this disease to any type of chemotherapy [1,38,39]. In the Old and New World, parasite glycoconjugates have being implicated in a variety of events during parasite-host interactions [40,41]. More recently, the role of LPG and GIPLs in the *L. braziliensis* and *L. infantum* was determined, suggesting that two distinct LPGs were able to differentially modulate macrophage functions [30,41]. Regarding *L. mexicana* complex, from where *L. amazonensis* is a member, a recently study has demonstrated the inflammatory role of LPG [42]. This glycoconjugate naturally exposed to the host immune system could contribute to the maintenance of infection by interfering with the assembly immune response, like modulation of cytokine production and non-activation of effectors cells. In the present work, we investigated whether LPGs from two *L. amazonensis* strains would account for differences in the interaction with macrophages and *L. migonei*.

LPG polymorphisms are common in the composition of branching sugars attached to the conserved repeat units of its backbone. While in the Old World species, a wide spectrum of sugar composition and structure is commonly observed, in New World species only glucose residues in the side chains of *Leishmania* were documented to date [17,21,23,24,43]. Our preliminary characterization of the repeat units using specific antibodies suggested the existence of intraspecies polymorphism in *L. amazonensis* LPGs with differences in the side-chains and in the level of glycosylation. The LPG of PH8 strain strongly reacted with CA7AE, that recognizes the basic backbone of the repeat units is Gal(β)Man-PO$_4$ [21,34]. However, Josefa LPG did not reacted with this antibody, thus suggesting the existence of sugars as side-chains in the repeat units. This feature is commonly found in the LPG of *L. major* reference strain FV1, which does not react with CA7AE [17]. In order to evaluate the quality of the sugars branching-off the repeat units, LT22 and WIC.79.3 antibodies were used to detect the presence of glucose and galactose, respectively [21,35]. Based on *L. major* LPGs used as controls, they were either recognized by those antibodies, suggesting cross-reactivity. Moreover, those data
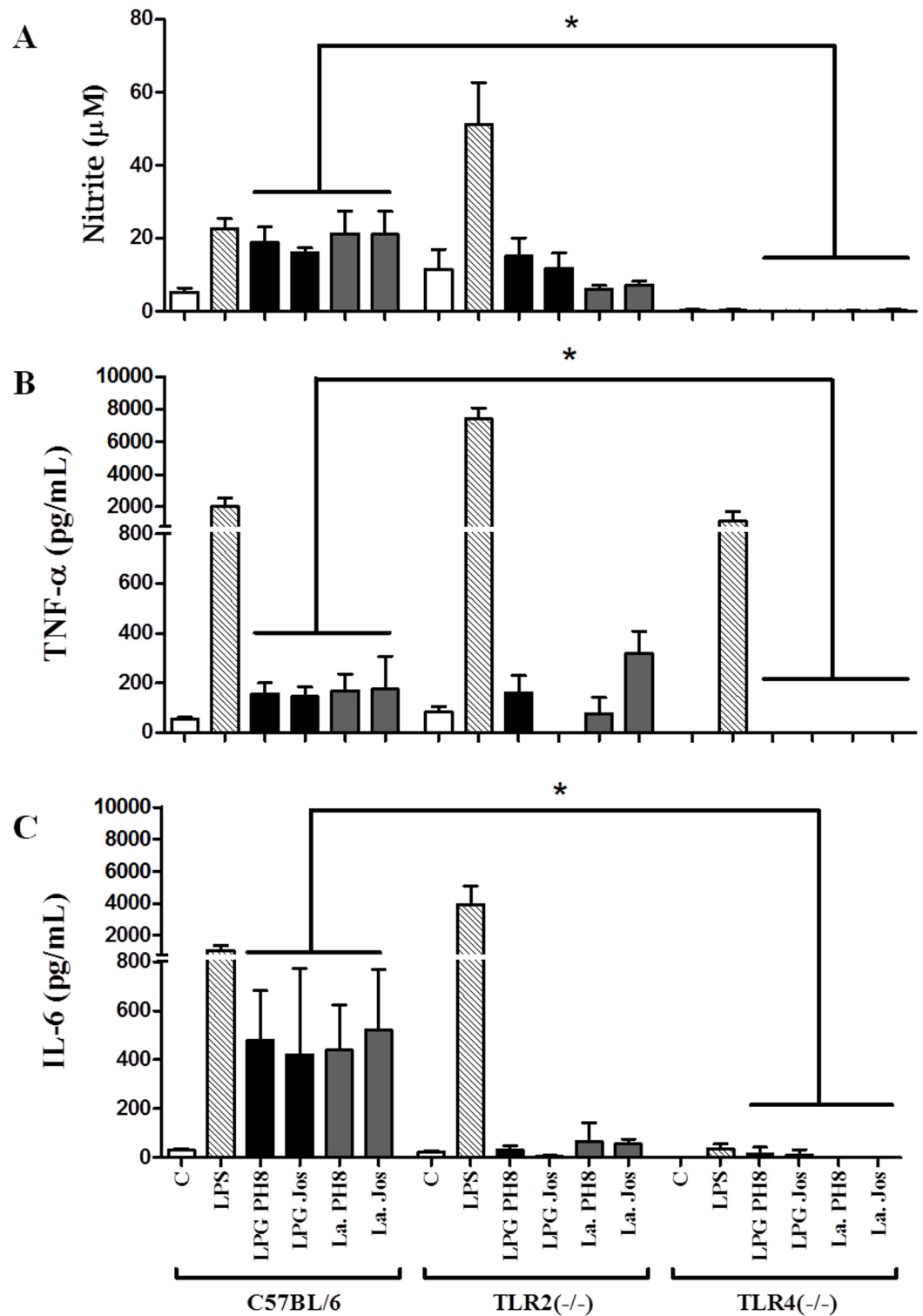
Fig 2. Nitrite (A) and cytokines TNF-α (B) and IL-6 (C) production by IFN-γ primed macrophages stimulated with LPG and live parasites. Cells were pre-incubated with IFN-γ (3 IU/mL) for the 18 h then 10 µg/mL of LPG, and supernatants used for cytokine and nitrite measurements were collected 48 h latter. Fresh medium alone was used as negative control cells and LPS (100 ng/mL) as a positive control. Nitrite concentration was measured by Griess reaction and cytokine concentrations were determined by flow cytometry. C = negative control; LPG PH8 = *L. amazonensis* LPG PH8 strain; LPG Jos = *L. amazonensis* LPG Josefa strain; La PH8 = *L. amazonensis* PH8 live

reinforced the presence of either glucoses or galactoses as side-chains in *L. amazonensis* LPGs. A fully detailed biochemical analysis must await the results of further investigations.

Understanding variations and the LPG structures are crucial for the comprehension of the mechanisms of how parasites survive under extremely adverse conditions. Although the role of LPG in the interaction with the vertebrate host immune system has been studied, it is still unclear how its polymorphism affects the parasite survival. *L. amazonensis* LPG induces release of NETs and LTB4 production by neutrophils, thus contributing to diminish parasite burden in the *Leishmania* inoculation site [14,16]. Additionally, *L. mexicana* LPG induce TNF-α and IL-10 in monocytes, modulates IL-12 production and diminishes NF-κB nuclear translocation
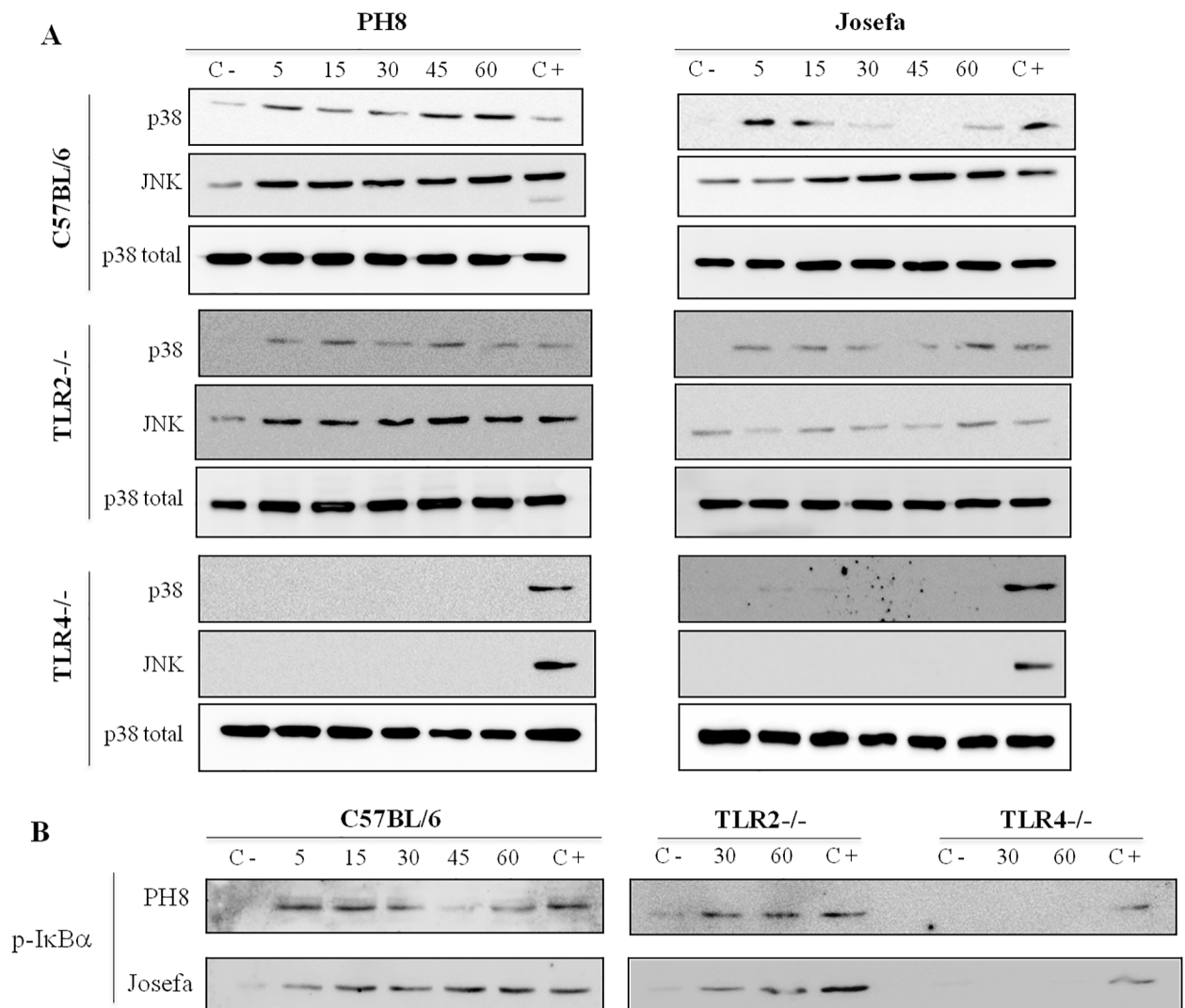

Fig 3. Activation of p38/JNK (A) and p-IκBα (B) in peritoneal murine macrophages (C57BL/6, TLR2 -/- and TLR4 -/-) by *L. amazonensis* LPGs (PH8 and Josefa). Macrophages were stimulated for 5, 15, 30, 45 and 60 min with 10 µg/mL of LPG from *L. amazonensis* PH8 and Josefa strains. Dually phosphorylated MAPKs (p38 and JNK) and p-IκBα were detected by Western blot analysis. C- = negative control; C+ = *E. coli* extract, positive control (100 ng/mL, 45 min). Total p38 content was used as the normalizing protein.

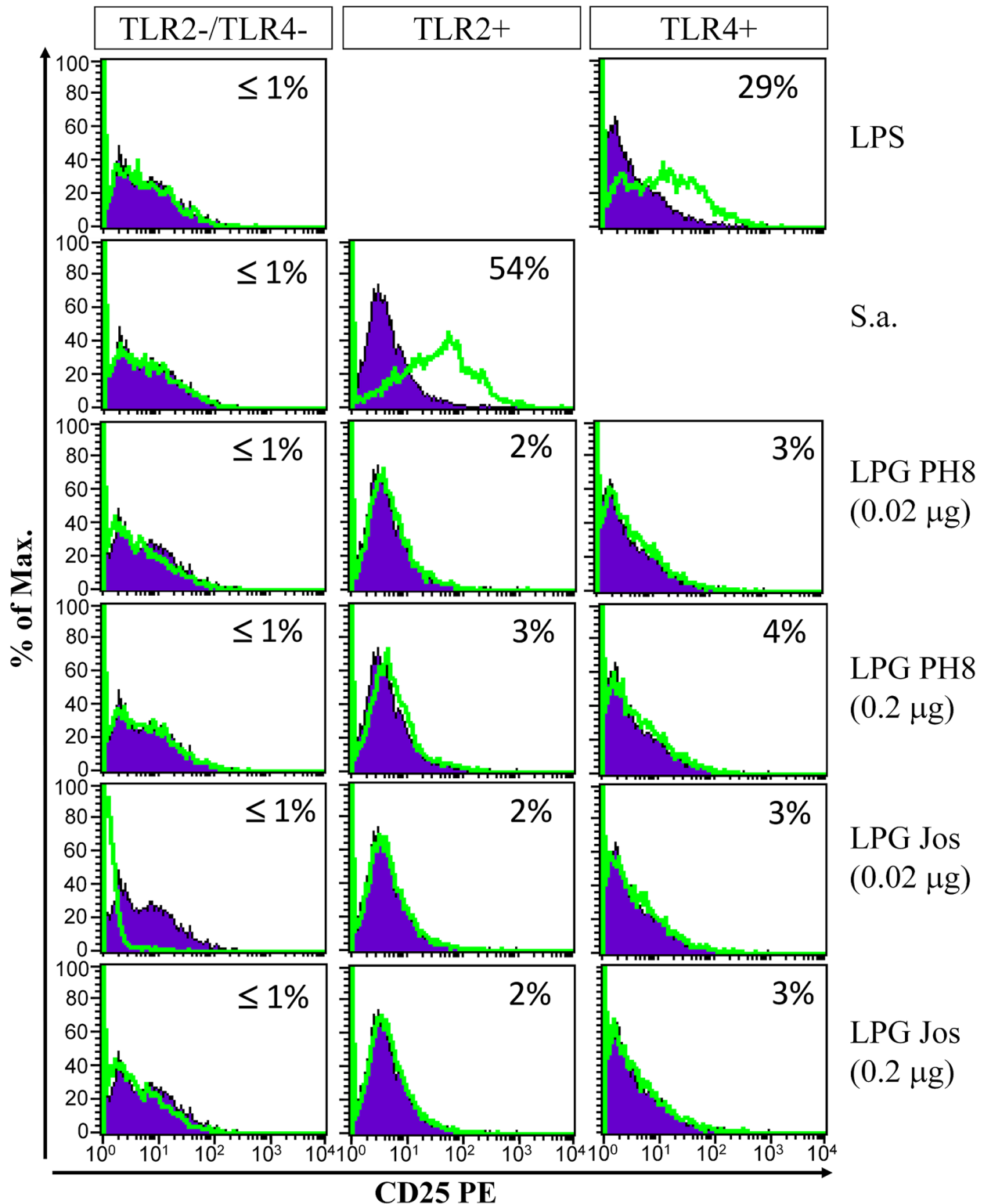doi:10.1371/journal.pntd.0004848.g003

Fig 4. LPGs purified of *L. amazonensis* do not induce translocation of NF-κB through TLRs. CHO cells expressing TLR2 (TLR2 +), TLR4 (TLR4+), or neither (TLR2-/TLR4-) were either untreated (purple line) or treated (green line) with LPGs from both strains of *L. amazonensis*. Legend: PH8 and Josefa LPGs (0.2 and 0.02 μg), Controls: LPS (TLR4 control) and *S. aureus* (S.a.) (TLR2 control). CD25 expression was measured by flow cytometry 18 h after stimulation. Results shown as percentage of CD25 expression on stimulated cells minus percentage of CD25 expression on non-stimulated cells.

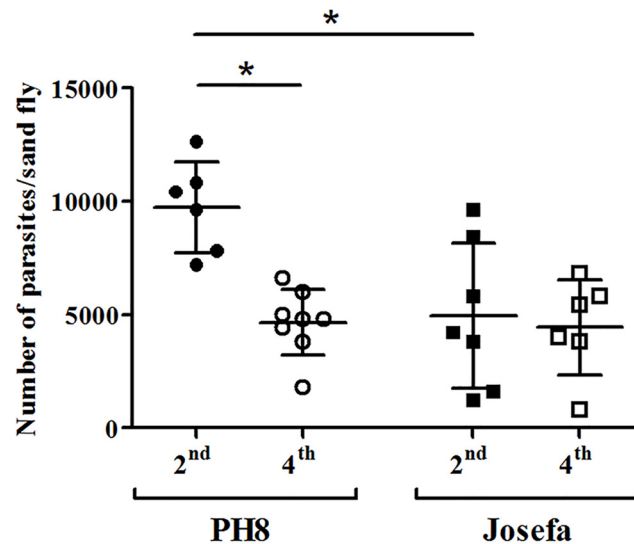doi:10.1371/journal.pntd.0004848.g004

Fig 5. Development of *L. amazonensis* (PH8 and Josefa strains) in *Lutzomyia migonei*. Sand flies were infected with promastigotes (2×10^7 parasites/mL) of PH8 and Josefa strains. Day 2 (2nd) parasites counted before blood excretion; Day 4 (4th) parasites remaining after blood excretion. Results are representative of two experiments and * = $P < 0.05$ was considered significant.

[44]. Here we show that LPGs from both *L. amazonensis* strains stimulates NO and cytokine production (TNF-α and IL-6) by peritoneal murine macrophages via TLR4. A similar cytokine production was also observed for other species such as *L. braziliensis* LPG, another important dermotropic species. However, this activation was primarily via TLR2 [30]. The NO production by macrophages play a central role in determining intracellular killing of *Leishmania* [45] and the intact structure of LPG appears to be important for this activation [12,29]. In many models, NO synthesis is dependent on a combination of IFN-γ and TNF-α via TLR-dependent mechanisms as an important leishmanicidal effector complex to macrophages [46]. In conclusion, the preliminary variations in the sugar motifs of LPG, did not result in any difference in macrophage activation/signaling thus suggesting the role of conserved motifs such as the lipid anchor [29].

Previous studies have demonstrated that different macrophage receptors mediate the uptake and phagocytosis of *Leishmania*. The early recognition of pathogens by cells capable of synthesizing cytokines is crucial for the adequate control of intracellular pathogens. Gene knockout studies in mice have suggested that TLR signaling is essential for the immune response against *Leishmania* parasites. Moreover, *Leishmania* LPGs and GIPLs are agonists of TLR2 and TLR4 [28–30,41,42]. Glycoconjugates can modulate the host immune response and their activity seems to be structure dependent. The *L. braziliensis* LPG exerts a pro-inflammatory interaction with TLR2, inducing the production of NO and cytokines (IL-1β, TNF-α and IL-6). On the other hand, the *L. infantum* LPG was shown to be immunosuppressive and did not induce NO, cytokines and NF-κB translocation [30]. Our results indicate that LPG from both *L. amazonensis* strains induce the production of NO and cytokines in IFN-γ-primed macrophages via TLR4. However in other members of the *L. mexicana* complex, *L. mexicana* LPG activates either TLR2 or TLR4 leading to ERK and p38 MAPK phosphorylation and production of cytokines in human macrophages [42].

Thus, although it has been shown that LPG of *Leishmania* activates TLRs and that the engagement of these receptors is important for the infection, the complete intracellular

processes that are involved in this activation remain unknown. Here we bring some light into the effects of LPG on MAPK and NF-κB signaling, a kinase and transcription factor known for their crucial role in immune defense against pathogens [44,47–49]. According to previous reports, infection by *L. amazonensis* altered phosphorylation of ERK1/2 in response to LPS in murine macrophages [50] and also activates a transcriptional repressor of the NF-κB [48,51]. Consistent with those observations, here LPGs from both *L. amazonensis* strains also activated p-IκBα, a NF-κB translocation inhibitor, via TLR4. Since no further NF-κB translocation was detected in the CHO cells, a possible mechanism that has been suggested favors its inhibition by p50/p50 NF-κB homodimer [55]. Moreover, *L. donovani* and *L. major* infection caused inactivation of ERK1/2 and p38, respectively, which was accompanied by the inhibition of transcription factors also modulation of cytokine production [52,53]. In contrast to GIPLs (with fail to activate MAPKs) [41], our data show that LPG from both *L. amazonensis* strains is equally activating MAPKs (p38 and JNK) and p-IκBα in peritoneal murine macrophages via TLR4 (Fig 3). On the other hand, these LPGs do not activate the NF-κB translocation. These and our results strongly suggest that *Leishmania* species have distinct mechanism of modulating the signaling pathways during immunopathological events.

The role of LPG during the interaction with the invertebrate host is a very controversial subject and it has been extensively investigated using *in vitro* and *in vivo* models [8,21,24,54,55]. Although the *in vitro* system has limitations [56], this model provided important evidence for parasite attachment in the sand fly midgut using many restricted and specific vector as classified elsewhere [57,58]. For example, successful binding to the midgut was reported using the Old World pairs *L. major/Phlebotomus papatasi* [8,54], *L. major/Phlebotomus duboscqi* [59] and *L. tropica/Phlebotomus sergenti* [60]. Perhaps, due its similarity to *L. major* LPG, who also possesses terminal β-galactosyl residues, *L. turanica* LPG may also be important for development in *P. papatasi* [61,62]. Moreover, the role of LPG has been questioned in permissive vectors such as *Lutzomyia longipalpis* and *Phlebotomus perniciosus*, where LPG mutants of *L. mexicana* and *L. major* were able to sustain infection in those vectors [63]. Recently, an alternative mechanism was suggested that flagellar protein FLAG1/SMP1 has been also implicated as an attachment binding candidate for specific and restricted vectors. In this work, a competitive binding assays using an antibody against FLAG1/SMP1 inhibited interaction using the pair *L. major* and *P. papatasi*. However, no effect was observed for permissive *L. longipalpis* [64].

The significance of LPG modifications was investigated during *in vivo* interaction of *L. amazonensis* with *L. migonei*. Although *L. amazonensis* is naturally transmitted by *L. flaviscutellata*, the absence of a colony led us to use an alternative sand fly, which had been previously shown to successfully harbor this parasite and *L. braziliensis* [5]. Since this species, although suspected, is not yet considered a natural proven vector of *L. amazonensis*, a high parasite doses was artificially offered to the sand flies. In spite of a loss after the 3rd day, parasite multiplication inside the alimentary tract of the *L. migonei* was successful for both *L. amazonensis* strains. To survive, the parasites need avoid a number of barriers including the lethal effects of digestive enzymes in the early blood-fed midgut and the excretion with the digested blood meal [5,7,65,66]. The strong correlation between the excretion of blood meal and the sudden loss of promastigotes suggests that the inability of *Leishmania* strains to persist in an inappropriate sand fly is related to their failure to remain anchored to the gut wall via specific attachment sites [22,67]. Nevertheless, *L. migonei* was able to sustain infection with both of the *L. amazonensis* strains tested, regardless of the type of LPG. It seems likely that *L. migonei* together with *L. longipalpis* might be considered a permissive vector as previously suggested [57,58,68]. However, the fully development of those two *L. amazonensis* strains should be further investigated.

Some studies have determined that polymorphisms in the phosphoglycan domains of LPG might be crucial for *Leishmania* promastigotes to attach to the midgut and to maintain vector

infection after blood meal excretion [9]. Additional support is based on the altered behavior of LPG deficient *L. donovani* and *L. major* mutant promastigotes (lpg-) who showed diminished capacity to maintain infection within the sand fly midgut [54,69]. Furthermore, it was recently presented the occurrence of intraspecies polymorphism in *L. infantum* LPG. Also, the biological role of the three LPG types (I, II and III) was studied during the interaction with the vector *L. longipalpis* [24]. Consistent with our results, all strains could successfully sustain infection in this vector, indicating that LPG polymorphisms did not affect this process. In spite of having a strong evidence for the existence of a midgut receptor for LPG, there is no current information in *L. migonei*. Indeed, the only known receptor was described for *L. major*, a galectin receptor found in the midgur of *P. papatasi* binding to LPG β-galactose residues [9,70]. The existence of midgut glycoproteins bearing terminal N-acetylgalactosamine in sand fly was also suggested as a putative parasite ligand [71].

Here we describe for the first time the immunomodulary properties of two LPGs isolated from different hosts. Those LPGs were equally able to trigger NO and cytokine (TNF-α and IL-6) production via TLR4. The preliminary differences in carbohydrate structure did not seem to affect the interaction of these strains with macrophages and the sand fly vector.

Supporting Information

S1 Fig. Growth curves of *L. amazonensis*. (A) *L. amazonensis* (PH8 and Josefa strains) were grown in M199 medium and counts determined daily (initial concentration of 1×10^5/mL). (B) Restriction fragment length polymorphisms of 120 bp kDNA amplicons from *Leishmania* obtained with restriction enzyme Hae III and analyzed on silver-stained 10% polyacrylamide gel. MM: 50 bp molecular size marker; lanes: Lb–*L. braziliensis* (MHOM/BR/75/M2903), Li–*L. infantum* (MHOM/BR/74/PP75); La–*L. amazonensis* reference (IFLA/BR/67/PH8), PH8 –*L. amazonensis* PH8 (IFLA/BR/67/PH8) and Jos–*L. amazonensis* Josefa (MHOM/BR/75/Josefa). (TIF)

S2 Fig. Dot-blots of *Leishmania* LPGs using different mAb antibodies. Purified LPGs from *L. amazonensis* strains (PH8 and Josefa), *L. infantum* (BH46 strain) and *L. major* strains (FV1 and LV39) were probed with the mAbs CA7AE (1:1000), LT22 (1:1000) and WIC 79.3 (1:1000). Peroxidase-conjugated anti-mouse IgG (1:5000) was used as secondary antibody. The reaction was developed with luminol. (TIF)

S3 Fig. Cytokine production by IFN-γ primed macrophages stimulated with LPG and live parasites. Cells were pre-incubated with IFN-γ (3 IU/mL) for the 18 h then 10 μg/mL of LPG, and supernatants used for cytokine IL-10 (A), IL-1β (B) and IL-12 (C) measurements were collected 48 h latter. Fresh medium alone was used as negative control cells and LPS (100 ng/mL) as a positive control. Cytokine concentrations were determined by flow cytometry. C = negative control; LPG PH8 = *L. amazonensis* LPG PH8 strain; LPG Jos = *L. amazonensis* LPG Josefa strain; La PH8 = *L. amazonensis* PH8 live promastigotes and La Jos = *L. amazonensis* Josefa live promastigotes. Results represent the mean ± SD of 3 experiments in duplicate, * = P< 0.05 was considered significant. (TIF)

Author Contributions

Analyzed the data: RPS PMN RRA ACT NFS MNM.

Contributed reagents/materials/analysis tools: RPS SJT MNM MAC ACT EN NFS FAP CMRV.

Wrote the paper: RPS PMN RRA MNM SJT.

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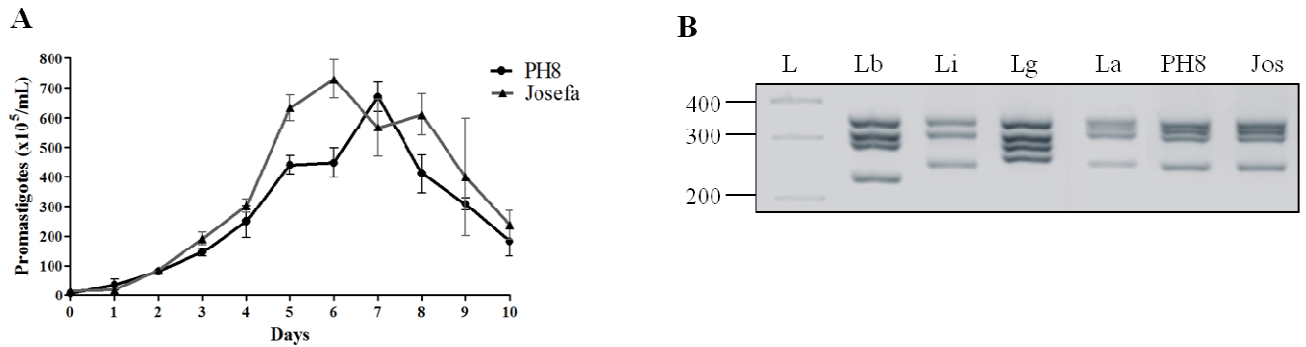
Fig. S1. Growth curves of *L. amazonensis*. (A) *L. amazonensis* (PH8 and Josefa strains) were grown in M199 medium and counts determined daily (initial concentration of 1×10^5/mL). (B) Restriction fragment length polymorphisms of 120 bp k 0 DNA amplicons from *Leishmania* obtained with restriction enzyme Hae III and analysed on silver-stained 10% polyacrylamide gel. Lanes: Lb – *L. braziliensis* (MHOM/BR/75/M2903), Li – *L. infantum* (MHOM/BR/74/PP75); La – *L. amazonensis* reference (IFLA/BR/67/PH8), PH8 – *L. amazonensis* PH8 (IFLA/BR/67/PH8) and Jos – *L. amazonensis* Josefa (MHOM/BR/75/Josefa).

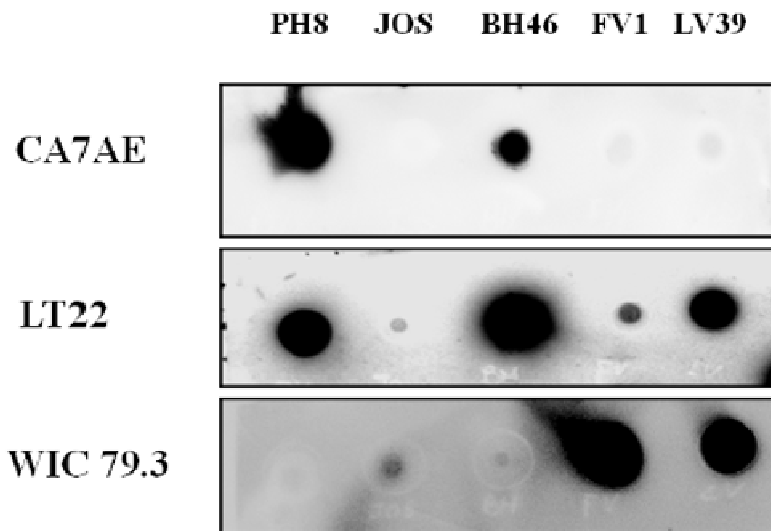**Fig. S2.** Dot-blots of *Leishmania* LPGs using different mAb antibodies. Purified LPGs from *L. amazonensis* strains (PH8 and Josefa), *L. infantum* (BH46 strain) and *L. major* strains (FV1 and LV39) were probed with the mAbs CA7AE (1:1000), LT22 (1:1000) and WIC 79.3 (1:1000). Peroxidase-conjugated anti-mouse IgG (1:5000) was used as secondary antibody. The reaction was developed with luminol.

Fig. S3. Cytokine production by IFN-γ primed macrophages stimulated with LPG and live parasites. Cells were pre-incubated with IFN-γ (3 IU/mL) for the 18 h then 10 µg/mL of LPG, and supernatants used for cytokine and nitrite measurements were collected 48 h latter. Fresh medium alone was used as negative control cells and LPS (100 ng/mL) as a positive control. Nitrite concentration was measured by Griess reaction and cytokine concentrations were determined by flow cytometry. C = negative control; LPG PH8 = *L. amazonensis* LPG PH8 strain; LPG Jos = *L. amazonensis* LPG Josefa strain; La PH8 = *L. amazonensis* PH8 live promastigotes and La Jos = *L. amazonensis* Josefa live promastigotes. Results represent the mean ± SD of 3 experiments in duplicate.

5 – DISCUSSÃO

Leishmania amazonensis, membro do complexo *L. mexicana*, é agente causador da LC e LCD no Brasil. Este parasito é muito estudado principalmente por seu aspecto único na resposta imune em comparação com as outras espécies de *Leishmania*. É responsável por provocar lesões não ulceradas que se disseminam pelo corpo, com hipersensibilidade retardada e frequentemente resistente a terapia convencional (Herwaldt 1999; Silveira et al. 2005). Em *Leishmanias* tanto do Velho quanto Novo Mundo, os glicoconjugados de membrana possuem importante papel em vários eventos durante o processo de interação parasito-hospedeiro (Sacks & Kamhawi 2001; De Assis et al. 2012). Recentemente foi elucidado o papel dos LPGs e GIPLs de *L. braziliensis* e *L. infantum,* que foram capazes de modular diferencialmente a ativação de macrófagos (Assis et al. 2012; Ibraim et al. 2013). Adicionalmente, LPG de *L. mexicana* possui papel inflamatório, contribuindo para manutenção da infecção por interferir na montagem da resposta imune, na modulação da produção de citocinas e de células efetoras (Rojas-Bernabé et al. 2014). No presente trabalho, foi investigado o papel do LPG de duas cepas de *L. amazonensis* no processo de interação com células do hospedeiro vertebrado para avaliar seu potencial imunomodulador diferencial nos eventos iniciais da infecção, e com *L. migonei*.

Análises bioquímicas e estruturais revelam amplo polimorfismo na composição de açúcares que se ramificam das unidades repetitivas do LPG. As cadeias laterais do LPG em espécies de *Leishmania* do Velho Mundo possuem diversas composições, com resíduos de galactose, glicose e arabinose, diferente do que ocorre com espécies do Novo Mundo, que foram documentados apenas resíduos de glicose (Ilg et al. 1992; McConville et al. 1992; Soares et al. 2002; Soares et al. 2005; Paranaíba et al. 2015). Em caracterização preliminar utilizamos de técnicas apuradas como western blot, eletroforese de carboidratos e eletroforese capilar e foi possível evidenciar a existência de polimorfismo intra-específico no LPG das duas cepas de *L. amazonensis* (Nogueira et al. *em preparação*). O LPG da cepa PH8 foi fortemente reconhecido pelo anticorpo CA7AE, que reconhece unidades repetitivas de Gal(β)Man-PO$_4$, que são comuns a todos LPGs. Contudo o LPG da cepa Josefa não foi reconhecido, sugerindo a existência de açúcares que se ramificam e poderiam impedir a ligação desse anticorpo, como

ocorre para o LPG de *L. major* (cepa FV1), qual não reage ao anticorpo CA7AE (McConville et al. 1992). Para avaliar os possíveis açúcares que compõem as unidades repetitivas de *L. amazonensis*, foram utilizados anticorpos LT22 e WIC.79.3 que detectam a presença de glicose e galactose, respectivamente (Kelleher et al. 1994; Soares et al. 2002). Adicionalmente, realizamos tratamento enzimático com β-glicosidase e β-galactosidase nas amostras identificadas por eletroforese capilar, que confirmou a presença de uma a duas cadeias laterais de glicose para cepa PH8 e de uma a três cadeias laterais de galactoses para cepa Josefa.

Compreender as variações e a estrutura dos LPGs é fundamental para entender os mecanismos de sobrevivência do parasito em condições extremamente adversas. Embora vários estudos já tenham elucidado a importância do LPG na interação com sistema imune do hospedeiro vertebrado, ainda não está claro como o polimorfismo afeta a sobrevivência do parasito. O LPG de *L. amazonensis* induz NETs e produção de LTB4 em neutrófilos, contribuindo para diminuição da carga parasitária no sítio de inoculação da *Leishmania* (Guimarães-Costa et al. 2009; Tavares et al. 2014). Também é possível observar que o LPG de *L. mexicana* induz citocinas TNF-α e IL-10, modula a produção de IL-12 e diminui a translocação de NF-κB para o núcleo em monócitos, contribuindo para sobrevivência do parasito e no resultado da infecção (Argueta-Donohué et al. 2008). No presente trabalho, o LPG de ambas cepas de *L. amazonensis* estimularam produção de NO e citocinas (TNF-α e IL-6) em macrófagos peritoneais estimulados com IFN-γ. Foi observada produção similar de citocinas para outras espécies nesses mesmos parâmetros, como *L. braziliensis*, outra importante espécie de LC no Brasil. Contudo essa ativação foi preferencialmente via TLR2 (Ibraim et al. 2013). A produção de NO por macrófagos é determinante na morte de parasitos (Holzmuller et al. 2006) e a estrutura intacta do LPG parece ser muito importante para essa ativação (Proudfoot et al. 1996; de Veer et al. 2003). Em muitos modelos experimentais, a síntese de NO é dependente da combinação de IFN-γ e TNF-α por mecanismo dependente de TLR para efetiva ativação dos macrófagos e morte dos parasitos (Mosser and Edwards 2008). Aqui, nós evidenciamos que variações nos açúcares dos LPGs de *L. amazonensis* não resultam em ativação/sinalização diferencial dos macrófagos, sugerindo um papel ativador de motivos conservados da âncora lipídica (de Veer et al. 2003).

Estudos anteriores demonstraram que diferentes receptores nos macrófagos são responsáveis por reconhecer e fagocitar a *Leishmania*. O rápido reconhecimento dos patógenos pelas células e mediado por TLR para sintetizar citocinas e NO é crucial para controle adequado dos parasitos intracelulares. Porém os glicoconjugados podem modular a resposta imune dessas células e sua atividade parece ser dependente da integridade de sua estrutura. O LPG de *L. braziliensis* exerce uma interação pró-inflamatória com ativação de TLR2, induzindo produção de NO e citocinas (IL-1β, TNF-α e IL-6). Por outro lado, LPG de *L. infantum* parece ser imunossupresivo, sem indução de NO, citocinas e translocação de NF-κB (Ibraim et al. 2013). Nossos resultados indicam que LPGs de ambas cepas de *L. amazonensis* induziram igualmente produção de NO e citocinas em macrófagos murinos via TLR4, independente da sua estrutura bioquímica. Já o LPG de *L. mexicana* ativou TLR2 ou TLR4 em macrófagos humanos e induziram diretamente a fosforilação de MAPKs (ERK e p38) e produção de citocinas (Rojas-Bernabé et al. 2014).

Então, embora tenha sido evidenciado que LPG de *L. amazonensis* ativa TLR após estímulo com IFN-γ e que sua ligação a este receptor é importante para combater a infecção, o processo intracelular que é envolvido na modulação do parasito para sua sobrevivência permanecia desconhecido. Aqui nos avaliamos o efeito do LPG na sinalização de MAPKs e NF-κB em macrófagos de camundongos C57BL.6 selvagem e respectivos *knockouts* (TLR2-/- e TLR4-/-), elementos cruciais na defesa contra patógenos (Yang et al. 2007; Argueta-Donohué et al. 2008). De acordo com resultados anteriores, a infecção com parasito vivo de *L. amazonensis* altera a fosforilação de ERK1/2 em resposta ao LPS em macrófagos murinos (Martiny et al. 1999) e ativa a porção repressora do fator de transcrição p50/p50 de NF-κB (Calegari-Silva et al. 2009; Calegari-silva et al. 2015). A infecção por *L. donovani* e *L. major* causa inativação de ERk1/2 e p38, respectivamente, acompanhadas pela inibição do fator de transcrição e modulação da produção de citocinas (Srivastava et al. 2012; Sarkar et al. 2013). Em contraste aos GIPLs que falham em ativar MAPKs (Assis et al. 2012), nossos dados demonstram que LPG de *L. amazonensis* ativam igualmente a fosforilação de p38 e JNK em macrófagos murinos não primados via TLR4. Adicionalmente, esses LPGs também ativam IκBα, um inibidor de NF-κB. Porém não foi observada translocação de NF-κB em células CHO extremamente sensíveis, sugerindo que ocorra posterior

inibição do fator de transcrição pelo homodímero p50/p50 (Calegari-silva et al. 2015). Essa diferença pode ser encontrada, pois espécies de *Leishmania* possuem distintos mecanismos de modular as vias de sinalização durante os eventos imunopatológicos. Embora tenhamos observados que os LPGs são pró-inflamatórios, a não ativação/inibição posterior da translocação de NF-kB sugere um papel também imunossupressor no hospedeiro vertebrado, o que explicaria em parte a anergia provocada por esta espécie.

O papel do LPG durante a interação com hospedeiro invertebrado é muito controverso e tem sido investigado usando modelos *in vitro* e *in vivo* (Pimenta et al. 1992; Pimenta et al. 1994; Soares et al. 2002; Rocha et al. 2010; Coelho-Finamore et al. 2011). Embora o sistema *in vitro* tenha limitações (Wilson et al. 2010), este modelo provê evidências importantes da ligação do parasito ao intestino do flebotomínio usando vetores restritos e específicos (Kamhawi 2006; Volf & Myskova 2007). Como por exemplo, foi possível identificar com sucesso a ligação dos pares do Velho Mundo *Phlebotomus papatasi/L. major* (Pimenta et al. 1992; Pimenta et al. 1994), *Phlebotomus duboscqi/L. major* (Svárovská et al. 2010) e *Phlebotomus sergenti/L. tropica* (Kamhawi et al. 2000). Acredita-se que devido à similaridade ao LPG de *L. major*, que possui resíduos de β-galactose, o LPG de *L. turanica* seja importante para seu desenvolvimento em *P. papatasi* (Chajbullinova et al. 2012; Volf et al. 2014). Por outro lado, o papel do LPG é questionado em vetores permissivos, como *Lutzomyia longipalpis* e *Phlebotomus perniciosus*, que foram capazes de sustentar a infecção mesmo após a utilização de *L. mexicana* e *L. major* mutantes para o LPG (Jecna et al. 2013). Recentemente, um mecanismo alternativo foi sugerido, em que a proteína flagelar FLAG1/SMP1 seria responsável pela ligação ao vetor específico ou restrito. Neste trabalho, foi realizado um ensaio de competitividade de ligação usando anticorpo contra FLAG/SMP1 e o par *L. major* e *P. papatasi*. Contudo, não foi observado efeito dessa proteína no vetor permissivo *L. longipalpis* (Di-Blasi et al. 2015).

A significância das modificações no LPG das duas cepas de *L. amazonensis* foi investigada durante interação in vivo com *L. migonei*. Embora *L. amazonensis* seja naturalmente transmitida por *L. flaviscutellata*, a ausência de uma colônia para experimentação nos levou a utilizar este vetor, o qual foi previamente demonstrado sua capacidade de albergar esta espécie e também *L. braziliensis* (Nieves & Pimenta 2000).

Como *L. migonei* ainda não é considerado vetor natural de *L. amazonensis*, uma grande quantidade de parasitos foi oferecida durante alimentação sanguínea. Apesar da perda de parasitos ocorrida após excreção do bolo sanguíneo no terceiro dia, as duas cepas de *L. amazonensis* conseguiram sobreviver e se multiplicar no trato digestivo de *L. migonei* com sucesso. Para sobreviver, os parasitos precisam enfrentar várias barreiras que incluem efeito letal de enzimas digestivas e excreção após digestão do bolo sanguíneo (Sacks 2001; Kamhawi 2006; Dostálová & Volf 2012). A forte correlação entre excreção sanguínea e perda de promastigotas sugere que a inabilidade de algumas cepas de *Leishmania* em persistir em determinado vetor está relacionado à sua falha em permanecer ancorada a parede intestinal por uma ligação específica (Soares & Turco 2003; Soares et al. 2004). Contudo, *L. migonei* foi capaz de sustentar a infecção de ambas as cepas utilizadas neste estudo, independente do tipo de LPG que elas apresentam. Parece que *L. migonei* pode ser considerado um vetor permissivo, como sugerido para *L. longipalpis* (Kamhawi 2006; Volf & Myskova 2007; Guimarães et al. 2016). Contudo, o completo desenvolvimento dessas cepas por todo trato intestinal no vetor deverá ser melhor investigado investigado.

Adicionalmente, o papel biológico de três tipos de LPG (I, II e III) em *L. infantum* foi estudado durante interação com *L. longipalpes* (Coelho-Finamore et al. 2011). Consistente com nossos resultados, todas cepas foram capazes de sobreviver com sucesso neste vetor, indicando que o polimorfismo no LPG não afeta esse processo. Apesar da forte evidência da existência de um receptor intestinal para o LPG, ainda não há informações para *L. migonei*. Na verdade, o receptor de galectina conhecido foi encontrado no intestino de *P. papatasi* capaz de ligar a resíduos de β-galactose no LPG de *L. major* (Kamhawi et al. 2004). A existência de glicoproteína N-acetilgalactosamina terminal nos intestinos dos vetores também foi sugerida como possível ligante (Myskova et al. 2007).

Neste trabalho foi descrito pela primeira vez o papel imunomodulatório de dois LPGs isolados de diferentes hospedeiros. Esses LPGs foram capazes de ativar igualmente a produção de NO e citocinas em macrófagos via TLR4, após estimulação com IFN-γ. Assim, as diferenças na estrutura e composição dos carboidratos desse glicoconjugado parece não afetar a interação dessas cepas com macrófagos e com vetor.
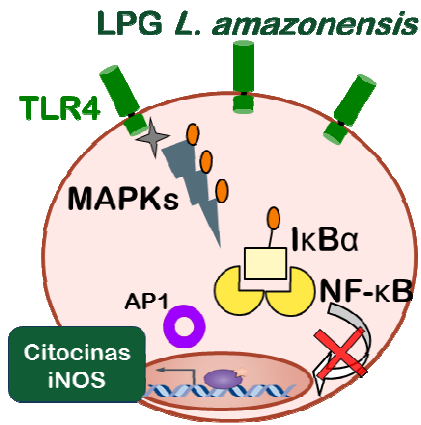
Figura 7: Esquema representando ativação de macrófagos após estímulo com LPGs de *L. amazonensis*. Os LPGs das cepas PH8 e Josefa são reconhecidos por TLR4 e induzem uma cascata de sinalização e fosforilação de MAPKs (JNK e p38). Adicionalmente, os LPGs fosforilam IκBα, proteína inibitória que se desliga do NF-κB, permitindo sua translocação para o núcleo. Porém, observamos uma inibição desse processo, provavelmente por ativação da porção repressora p50/p50 de NF-κB. Produção de citocinas pode ser por outras vias – AP1, IRF5, 3.

CONCLUSÕES

A partir desses estudos podemos concluir que:

- Vesículas das cepas Y, Colombiana, CL-14 e YuYu de *T. cruzi* possuem diferenças na quantidade de proteína e expressão de carboidratos de superfície.

- As vesículas de CL-14 e YuYu foram capazes de induzir NO e citocinas (TNF-α e IL-6) via TLR2 em macrófagos murinos.

- Não foi observada diferença na ativação de MAPKs (ERK1/2, p38 e JNK) após estimulação com as vesículas.

- Na avaliação da resposta imune em camundongos crônicos, as vesículas das cepas Colombiana e Y induziram maiores níveis de citocinas que as cepas CL-14 e YuYu.

- Não houve diferença na produção de citocinas nos compartimentos celulares dos camundongos crônicos após estimulação com vesículas das cepas Colombiana e YuYu.

- As vesículas das cepas Colombiana e YuYu foram capazes de estimular produção de citocinas nos linfócitos T CD4+ e CD8+, nas células B e nas células dendríticas.

- Os LPGs das cepas PH8 e Josefa de *L. amazonensis* apresentaram polimorfismo em sua estrutura, com cadeias laterais de β-glicose e de β-galactose, respectivamente.

- Os LPGs possuem papel inflamatório capazes de ativar produção de NO e citocinas (TNF-α e IL-6), via TLR4.

- Ambos LPGs foram capazes de ativar MAPKs (p38 e JNK) e IκB, porém não ativaram translocação de NF-κB.

- Não houve diferença na infecção do parasito em *L. migonei* para as duas cepas.

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