

Ministério da Saúde
Fundação Oswaldo Cruz
Centro de Pesquisas René Rachou
Programa de Pós-graduação em Ciências da Saúde

“Glicoconjugados (GIPLs e LPGs) de *Leishmania braziliensis* e *L. infantum*:
Modulação do Sistema Imune Inato e variações na estrutura de carboidratos.”

por

Rafael Ramiro de Assis

Belo Horizonte
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**Tese apresentada com vistas à
obtenção do Título de Doutor em
Ciências na área de concentração
Biologia Celular e Molecular**

Orientação: Dr. Rodrigo Pedro Pinto Soares

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Este trabalho é dedicado à minha Família, amigos e à Izabela

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Lista de Abreviaturas e símbolos

°C - Graus Célsius

AP-1 – Proteína ativadora 1

BH46 – *Leishmania infantum* (MHOM/BR/70/BH46)

CD – “cluster” de diferenciação

CPqRR – Centro de Pesquisas René Rachou

EDTA – Ácido etilenodiaminotetracético

ERK – Cinases reguladas por sinal extracelular

ESOAK – Água/etanol/etil éter/piridina/NH₄OH; 15:15:5:1:0,017

Gal – Galactose

GalNAc – N-acetil galactosamina

GIPLs – Glicoinositolfosfolípides

Glc – Glicose

GlcN – Glicosamina

GPI – Glicosilfosfatidilinositol

Hex – Hexose

IFN- γ – Interferon gama

IL – Interleucina

iNOS – Óxido nítrico sintase induzível

JNK – c-Jun amino-terminal cinases (c-Jun N-terminal kinases)

LC – Leishmaniose cutânea (LC)

Linfócitos Th1 – Linfócitos T auxiliares do tipo 1

Linfócitos Th2 – Linfócitos T auxiliares do tipo 2

LCM – Leishmaniose cutâneo-mucosa

LPG – Lipofosfoglicano

LPS – Lipopolissacarídeo

LV – Leishmaniose visceral

Man – Manose

MAPKs – Proteínas cinases ativadas por mitógenos

MyD88 – gene primário de diferenciação mielóide 88 (Myeloid differentiation primary response gene 88)

NaF – Fluoreto de sódio

NF- κ B – Fator nuclear kappa B

NK – células citotóxicas naturais (Natural Killer)

NO – Óxido nítrico

PAMPs – Padrão molecular associado à patógeno

PG – Fosfoglicano

PI – Fosfatidilinositol

PP75 – Leishmania infantum (MHOM/BR/74/PP75)

PPGs – Proteofosfoglicanos

PRR – Receptores de reconhecimento de padrões (Pattern recognition receptors)

PSPs ou gp63 – Proteases da superfície de promastigotas

RPMI – Roswell Park Memorial Institute (meio de cultura)

sAP – Fosfatases ácida secretadas.

SDS PAGE – Eletroforese em gel SDS-poliacrilamida

STAT – Moléculas de transdução de sinal e ativadores de transcrição

TLR – Receptores do tipo Toll (Toll-like receptor)

TNF- α – Fator de necrose tumoral alfa

TRAF6 – Receptor de TNF associado ao fator 6 (*TNF receptor associated factor 6*)

WHO/OMS – Organização Mundial de Saúde (*World Health Organization*)

Resumo

Os glicoconjugados de *Leishmania* tem sido extensivamente estudados, mas ainda pouco se sabe sobre o quanto polimorfismos intra e interespecíficos contribuem com o desenvolvimento das diferentes imunopatologias das leishmanioses. Por este motivo, duas espécies de importância epidemiológica foram examinadas, *L. braziliensis* e *L. infantum*, agentes causadores das leishmanioses cutânea e visceral, respectivamente. O LPG de *L. braziliensis* não possui cadeias laterais enquanto o LPG de *L. infantum* carrega em sua estrutura oligômeros de até três β -glicosos como cadeias laterais. Por outro lado a estrutura dos GIPLs destas espécies era desconhecida e foi objeto de estudo deste trabalho.

A análise estrutural dos GIPLs mostrou que *L. infantum* possui GIPLs pequenos e ricos em manose, sugerindo predominância de GIPLs do tipo I e híbridos enquanto *L. braziliensis* apresenta GIPLs grandes e ricos em galactose, sugestivo do tipo II. Para analisar o papel destas moléculas na interação com o hospedeiro, macrófagos peritoneais murinos foram tratados com LPG ou GIPLs e a produção de nitrito, citocinas, bem como a ativação de MAPKs foram avaliados.

De forma geral, macrófagos estimulados com LPG de *L. braziliensis*, demonstraram uma produção maior de $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 e NO do que os estimulados com LPG de *L. infantum*, adicionalmente, células tratadas com LPG de ambas as espécies mostraram uma resposta pro inflamatória mais proeminente. Além disto, os GIPLs mostraram a capacidade de inibir a produção de IL-12 e NO em macrófagos estimulados com $\text{IFN-}\gamma$ e LPS. Finalmente, os glicoconjugados destas duas espécies resultaram em uma cinética diferencial na ativação de MAPKs. O LPG de *L. braziliensis* mostrou uma ativação transiente enquanto o de *L. infantum* uma ativação gradual. Os GIPLs de ambas espécies falharam em ativar MAPKs.

Abstract

Leishmania LPG has been extensively studied but little is known about in what extent interspecies variations contribute to the different immunopathologies of leishmaniasis. Similarly, for GIPLs, a relatively neglected molecule, little is known about its polymorphisms among Leishmania species and its role during infection. To address this issue, two epidemiologically important South American species of Leishmania were examined, *L. braziliensis* and *L. infantum*, causative agents of cutaneous and visceral leishmaniasis, respectively. The LPG from these two species differ in structure, being *L. braziliensis* LPG devoid of side chains while *L. infantum* LPG carries one to three β -glucoses on its side chains. In the other hand the structure of the GIPLs from these two species is still unknown and was addressed in this present work. A structural analysis of the GIPLs showed that *L. infantum* has small, mannose rich GIPLs, suggestive of type I and Hybrid GIPLs while *L. braziliensis* has larger and galactose rich GIPLs, suggestive of Type II GIPLs. To address the role of these molecules upon macrophage invasion, mouse peritoneal macrophages were treated with either LPG or GIPLs and Nitrite and cytokine production, as well as MAPKs activation were evaluated.

Overall, macrophages stimulated with *L. braziliensis* LPG, had a higher TNF- α , IL-1 β , IL-6 and NO production than those stimulated with that of *L. infantum*, also, IFN- γ primed macrophages stimulated with LPG, had a higher production of NO and TNF- α than GIPLs stimulated cells. Additionally, GIPLs showed the capacity of inhibit IL-12 and NO production in IFN- γ and LPS stimulated macrophages. Furthermore, the glycoconjugates from the two species resulted in differential kinetics of signaling via MAPK activation. *L. infantum* LPG exhibited a gradual activation profile, whereas *L. braziliensis* LPG showed a sharp but transient activation, while GIPLs failed to activate MAPKs.

1. Introdução

Leishmania infantum (sin. *L. chagasi*); e *L. braziliensis* são os agentes causadores de leishmaniose visceral (LV) e leishmaniose tegumentar (LT), respectivamente.

Uma parte fundamental de seu ciclo de vida envolve a modulação da resposta imune do hospedeiro vertebrado, em especial a ativação das células hospedeiras, primariamente os macrófagos.

Por este motivo, muitos estudos tem focado na interação parasito/hospedeiro com um foco especial nas moléculas que fazem parte desta interface. Para sobreviver e se multiplicar no ambiente hostil encontrado no tubo digestivo do vetor e nos tecidos do hospedeiro vertebrado, o parasito conta com uma diversa gama de moléculas dentre as quais, os glicoconjugados assumem um papel fundamental.

As moléculas associadas à GPIs estão amplamente expressas na superfície dos eucariotos e procariotos. Além de âncoras lipídicas, os lipídios associados a GPIs ainda desempenham um papel no transporte de sinais através da membrana, transporte intracelular de proteínas e atuam como padrões moleculares associados a patógenos (PAMPS).

Por este motivo, este trabalho tem como foco principal o estudo do papel desempenhado por dois importantes glicoconjugados de superfície de *Leishmania*, os GIPLs e o LPG. Neste trabalho foram avaliados aspectos da estrutura e composição bioquímica destas moléculas bem como sua participação nos eventos fundamentais da ativação dos macrófagos como sinalização celular e produção de mediadores inflamatórios.

2. Objetivos

2.1. Objetivo Geral

Avaliar os aspectos da imunologia inata na interação entre GIPLs e LPGs de *L. braziliensis* e *L. infantum* e macrófagos murinos levando em conta as variações de estrutura bioquímica dos GIPLs.

2.2. Objetivos específicos

1. Avaliar a inibição da produção de NO por macrófagos estimulados por GIPLs e LPG de *L. braziliensis* e *L. infantum*;
2. Avaliar a produção de citocinas (IL1- β , IL-2, IL-4, IL-10, IL-12p40, IFN- γ e TNF- α) por macrófagos murinos estimulados com GIPLs e LPGs das duas espécies;
3. Avaliar a ativação de MAPKs em macrófagos murinos estimulados com GIPLs e LPG das duas espécies;
4. Determinar os tamanhos relativos e composição da porção glicídica dos GIPLs das duas espécies;

3. Revisão da literatura

3.1. As Leishmanioses como problema de saúde pública

As leishmanioses compõem um grupo de doenças negligenciadas causadas por protozoários tripanosomatídeos do gênero *Leishmania* (Kinetoplastida: Triponosomatidae). Nas Américas, elas são amplamente distribuídas desde o sul dos Estados Unidos até o norte da Argentina (Grimaldi & Tesh, 1993). As manifestações clínicas das leishmanioses podem variar de pequenas úlceras cutâneas de progressão geralmente benigna que se curam espontaneamente até formas mais graves como a leishmaniose visceral, que é letal se não tratada (Herwaldt, 1999). Na América Latina, especialmente no Brasil, *Leishmania braziliensis* (Subgênero *Viannia*) e *Leishmania infantum* (syn. *Leishmania chagasi* do subgênero *Leishmania*) estão entre as espécies de maior importância epidemiológica como os agentes causadores da Leishmaniose tegumentar (LT) e visceral (LV), respectivamente (Herwaldt, 1999; Reithinger *et al.*, 2007).

No Brasil, a LT causada por *L. braziliensis* é bastante comum e de ampla distribuição no Brasil com ocorrência em todas as regiões do país (Ashford, 2000; Kaye & Scott, 2011). Classicamente, as manifestações clínicas da leishmaniose tegumentar causada por *L. braziliensis* são classificadas como leishmaniose cutânea (LC) e leishmaniose cutâneo-mucosa. A LC, forma mais comum, pode se apresentar como uma infecção inaparente na qual o paciente possui reatividade positiva ao teste de Intradermorreação de Montenegro (IDRM) sem histórico de LT e sem lesão ou cicatriz característica da LC. Além da forma inaparente, o paciente ainda pode apresentar um quadro de leishmaniose linfonodal no qual o paciente apresenta um quadro de linfadenopatia localizada também na ausência de lesão tegumentar. A LC típica apresenta-se como uma lesão uniforme e indolor, normalmente iniciada como uma pápula avermelhada no

sítio da infecção que geralmente evolui para uma úlcera com bordas bem demarcadas e elevadas com fundo avermelhado e granulações grosseiras (Ashford, 2000; Herwaldt, 1999).

Também causada por *L. braziliensis*, a leishmaniose cutânea-mucosa (LCM), se caracteriza por lesões desfigurantes da mucosa oral, nasal e da faringe. A LCM clássica é secundária à LC que, por um mecanismo ainda pouco conhecido, se caracteriza por uma disseminação linfática ou sanguínea dos parasitas para a mucosa naso-orofaríngea e se manifesta após cura clínica das lesões cutâneas iniciais. Na maioria dos casos, a lesão se torna evidente alguns anos após a resolução da lesão cutânea original e o paciente apresenta uma positividade bastante elevada para a IDRM mas de diagnóstico parasitológico muito difícil devido à escassez de parasitos no local da lesão (Desjeux, 2004). O sintoma inicial da LCM é uma irritação branda na ponta do nariz ou outra região afetada. Em decorrência de uma reação imunológica hiperativa, inicia-se uma erosão progressiva da mucosa, e da cartilagem associada, que resulta em lesões desfigurantes. A LCM é considerada uma das formas mais graves da doença pela grande morbidade e estigma social causados pelas lesões (Ashford, 2000; Herwaldt, 1999).

Além da LC e LCM, a LV também possui ampla distribuição no Brasil e sua incidência tem aumentado inclusive nas grandes cidades, incluindo Belo Horizonte (Gontijo, 2004). A LV caracteriza-se por um amplo espectro clínico, que pode variar desde as manifestações clínicas oligossintomáticas até manifestações mais graves, que, se não tratadas, levam ao óbito. Seus principais sintomas incluem: febre branda intermitente, anemia, linfadenopatia, hepatoesplenomegalia, perda de peso, caquexia progressiva e hemorragias associadas a um quadro de plaquetopenia (Ashford, 2000).

3.2. Ciclo Biológico

Os protozoários do gênero *Leishmania* pertencem à ordem Kinetoplastida, família Trypanosomatidae. São parasitos heteroxênicos, necessitando de hospedeiros vertebrado e invertebrado nos quais alternam entre a forma amastigota, intracelular e imóvel no hospedeiro vertebrado e a forma promastigota, extracelular e móvel que habita o interior do tubo digestivo do inseto vetor.

Ao realizar o repasto sanguíneo em um hospedeiro infectado, as formas amastigotas são ingeridas e no interior do tubo digestivo do inseto se diferenciam nas formas promastigotas. As formas promastigotas, nos vetores, sofrem por uma série de modificações passando pelas formas promastigotas procíclicas, altamente replicativas, até se diferenciarem nas formas promastigotas metacíclicas, estas últimas são as formas infectantes que serão inoculadas em um hospedeiro vertebrado durante o próximo repasto sanguíneo (Lawyer *et al.*, 1990) (figura 1).

Ao serem inoculadas no hospedeiro vertebrado durante o repasto sanguíneo do inseto vetor, as promastigotas são fagocitadas e, dentro da célula hospedeira, rapidamente se diferenciam em amastigotas (Handman, 1999), estas são capazes de infectar novos fagócitos e manter a infecção no hospedeiro vertebrado. O ciclo biológico se perpetua quando estas formas amastigotas são ingeridas pelo inseto vetor durante o repasto sanguíneo. No interior do vetor as formas amastigotas se diferenciarão nas formas promastigotas. (Handman, 1999; Kaye & Scott, 2011; Sacks & Kamhawi, 2001).

Os vetores responsáveis pela transmissão de *Leishmania* são representados no Novo Mundo por dípteros do gênero *Lutzomyia*, enquanto que no Velho Mundo pelo gênero

Phlebotomus (Família: *Psychodidae*, Subfamília: *Phlebotominae*) (Young & Duncan, 1994). Apenas as fêmeas são hematófagas e sua distribuição é fator limitante para a disseminação da doença (Ashford, 2000; Singh *et al.*, 2006). No Brasil, a espécie de flebotomíneo apontada como vetor da *L. infantum* é *Lutzomyia longipalpis* enquanto *Lutzomyia whitmani* e *Lutzomyia intermedia* são apontados como principais vetores de *L. braziliensis* (Grimaldi & Tesh, 1993; Rangel & Lainson, 2003; Soares *et al.*, 2010; Soares & Turco, 2003).

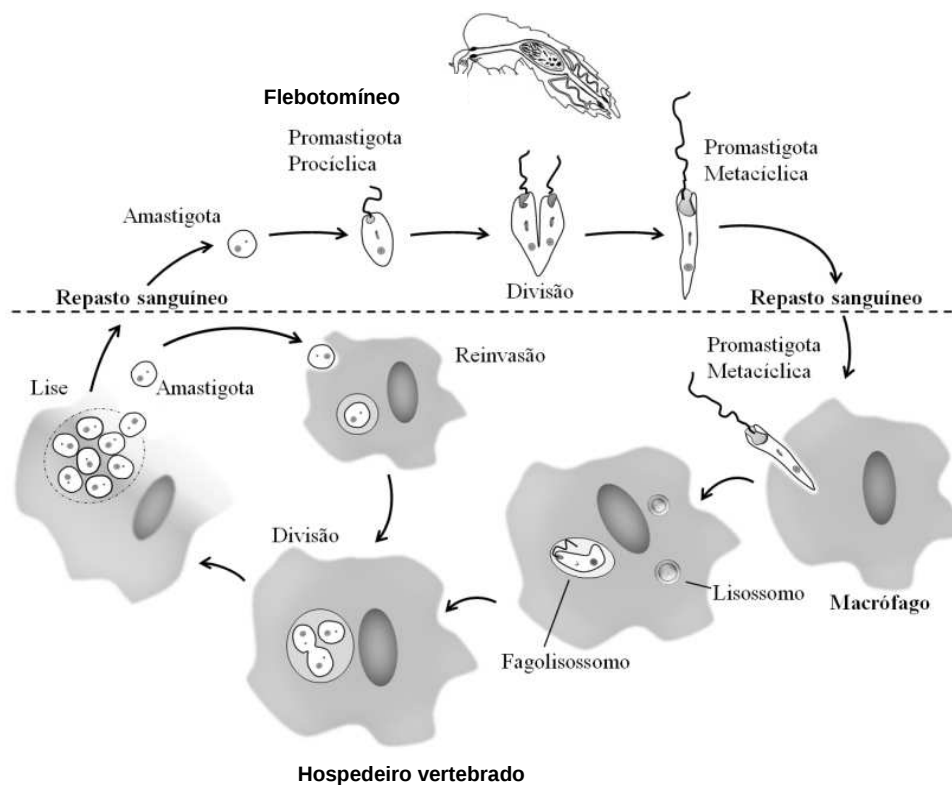


Figura 1. Representação esquemática do ciclo biológico de *Leishmania*: Representação esquemática do ciclo de vida de *Leishmania*. (adaptado de Assis *et al.*, 2012b).

Apesar de possuir um ciclo evolutivo relativamente simples quando comparado com outros tripanosomatídeos, os parasitos do gênero *Leishmania* encontram diversas barreiras biológicas à infecção em ambos os hospedeiros. Dentre estas barreiras pode-se citar no vetor: a matriz peritrófica; o ataque de enzimas digestivas, além da necessidade de adesão ao epitélio do intestino para evitar sua eliminação durante a excreção do bolo alimentar (Sacks & Kamhawi, 2001). Entretanto, é no hospedeiro vertebrado que o parasito encontra as barreiras mais complexas onde diversos receptores e moléculas estão envolvidos no reconhecimento (Peters *et al.*, 1995), fagocitose (Mosser & Edelson, 1985), modulação da atividade celular, resistência ao efeito lítico do sistema do complemento (Brittingham & Mosser, 1996) e redes extracelulares de neutrófilos (Guimaraes-Costa *et al.*, 2009) além do efeito tóxico de radicais intermediários reativos de oxigênio (ROI) e nitrogênio (RNI), dentre outras (Feng *et al.*, 1999; Gazzinelli *et al.*, 2004; Liew *et al.*, 1990; Murray, 1982).

3.3. Os glicoconjugados de *Leishmania*

Para sobreviver e se multiplicar no ambiente hostil encontrado no tubo digestivo do vetor e nos tecidos do hospedeiro vertebrado, o parasito conta com uma diversa gama de moléculas dentre as quais, os glicoconjugados assumem um papel fundamental (Turco, 2003).

O termo “glicoconjugados” se refere a qualquer molécula que possua algum motivo de carboidrato ligado covalentemente a outra classe de moléculas como lipídeos e proteínas. Neste trabalho daremos foco para os glicoconjugados de *Leishmania* ancorados à membrana por meio de âncoras de glicosilfosfatidilinositol (GPI). Lipídeos associados a GPI são tipicamente considerados como âncoras de outras moléculas de superfície como glicoproteínas e glicolipídeos (McConville & Ferguson, 1993; McConville & Menon, 2000) e estão presentes na

maioria, se não em todos, os eucariotos. Entretanto, em *Leishmania*, estas estruturas estão presentes não somente associadas à membrana (como o lipofosfoglicano (LPG) e glicoproteínas como a gp63) com a função de ancoragem, mas também estão presentes como uma família de glicolípides com distintas funções como os glicoinositol-fosfolípides (GIPLs) (Figura 2) (McConville & Ferguson, 1993).

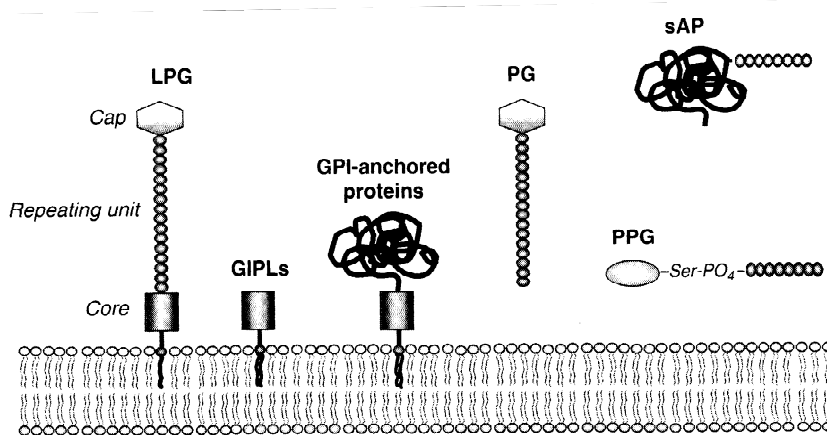


Figura 1. Representação esquemática dos glicoconjugados de superfície de *Leishmania* spp.

As pequenas estruturas ovais representam as unidades repetitivas Gal-Man-PO₄. Nos PPGs (proteofosfoglicanos) e sAPs (fosfatases ácidas secretadas), as unidades repetitivas estão ligadas ao polipeptídeo *via* Man-PO₄-serina. GIPLs, glicoinositol-fosfolípides; LPG, lipofosfoglicano; PG, fosfoglicano (extraído de Turco, 2003).

As moléculas associadas à GPIs estão amplamente expressas na superfície dos eucariotos e procariotos. (Jones, 2005; McConville & Ferguson, 1993; McConville & Menon, 2000). Além de âncoras lipídicas, os lipídios associados a GPIs ainda desempenham um papel no transporte de sinais através da membrana, transporte intracelular de proteínas (Englund, 1993; Medof *et al.*, 1996) e atuam como padrões moleculares associados a patógenos (PAMPS) (Takeda *et al.*, 2003).

3.4. Os glicoinositolfosfolípides (GIPLs) de *Leishmania*

Os GIPLs de *Leishmania* são pouco estudados apesar de presentes em todas as fases de vida do parasito. Os GIPLs estão presentes na superfície celular e revestindo estruturas intracelulares de *Leishmania* onde representam o glicolípide mais abundante (McConville & Ferguson, 1993). Apesar de estruturalmente análogos às âncoras GPI, os GIPLs apresentam diversas modificações bioquímicas durante sua síntese, como adição de cadeias laterais e intensas substituições em sua âncora lipídica além de estarem expressos em grandes quantidades (McConville & Ferguson, 1993), por isso representam produtos metabólicos distintos ao invés de simples precursores de âncoras (Lillico *et al.*, 2003; Zawadzki *et al.*, 1998).

Estruturalmente, os GIPLs, apresentam um grande polimorfismo, entretanto possuem uma estrutura básica conservada de Man α 1-4GlcN ligada à porção lipídica, normalmente composta por alquil-acil-glicerol ou lisoglicerolliso-alquil-glicerol (liso-PI), por um resíduo de fosfatidilinositol (PI) (McConville *et al.*, 1990). Esta estrutura básica esta presente em todos os GIPLs de *Leishmania* descritos até o momento e representa uma entidade metabólica final expressa na membrana bem como um precursor para a síntese de outros GIPLs sendo a estrutura mais básica entre todas (Figura 3).

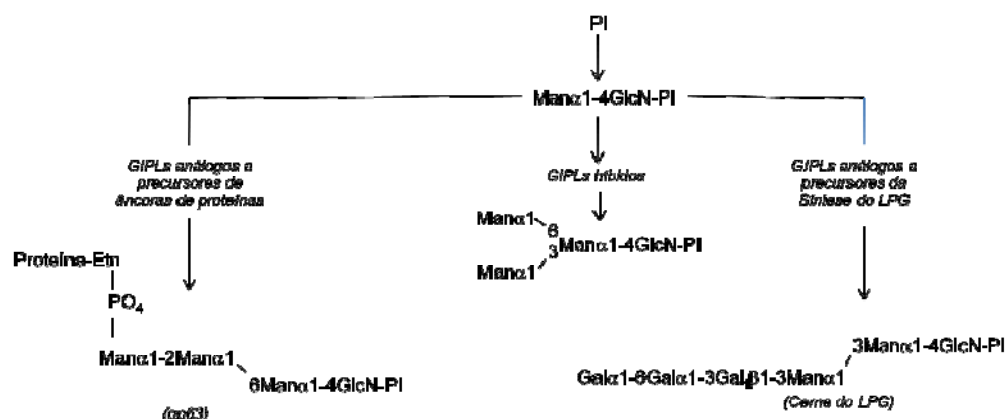


Figura 3. Esquema básico dos GPIs. A estrutura $\text{Man}\alpha 1\text{-4GlcN}$, além de entidade metabólica final, atua como precursor de outras estruturas de GPIs bem como âncoras lipídicas de moléculas de superfície associadas à GPI (McConville et al., 1993).

A partir desta estrutura básica, dependendo da espécie ou linhagem analisados, os GPIs apresentam um intenso polimorfismo relacionado tanto com as substituições dos ácidos graxos na porção lipídica e de açúcares na parte glicídica e são comumente classificados dentro de três grupos (Figura 4). Os GPIs do tipo I, em sua maioria apresentando um resíduo de manose como açúcar mais distal, é identificado pela adição ao sexto carbono da manose proximal de um resíduo de manose (**$\text{Man}\alpha 1\text{-6Man}\alpha 1\text{-4GlcN-PI}$**). Os GPIs mais comuns deste grupo são representados por M2 e M3 cujas estruturas são $\text{Man}\alpha 1\text{-6Man}\alpha 1\text{-4GlcN-PI}$ e $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4GlcN-PI}$, respectivamente. Esta família é estruturalmente análoga às âncoras GPI de proteínas de superfície e seu ácido graxo predominante é o ácido esteárico (C18:0) (McConville & Blackwell, 1991). Os GPIs do tipo I são a forma predominante em *Leishmania donovani*, *Leishmania tropica* e *Leishmania aethiopica* (McConville & Blackwell, 1991; Schneider et al., 1994).

Os GIPLs do tipo II possuem como característica a adição ao terceiro carbono da manose proximal de um resíduo de manose (**Man α 1-3Man α 1-4GlcN-PI**). Estes possuem uma composição lipídica mais heterogênea que varia entre combinações de ácido esteárico (C18:0), ácido behênico (C22:0), ácido lignocérico (C24:0) e ácido hexacosanóico (C26:0). Os GIPLs do tipo II variam entre os pequenos iM2(Man α 1-3Man α 1-4GlcN-PI) até mais longos como o GIPL A (Gal β 1-3Gal α 1-6Gal α 1-6Gal β 1-3Man α 1-3Man α 1-4GlcN-PI) e são estruturalmente análogos à âncora GPI do LPG. Estes GIPLs são comumente encontrados em *L. major*, *Leishmania mexicana* (McConville & Ferguson, 1993) e *Leishmania panamensis*. É importante salientar que em *L. panamensis* é observada uma composição lipídica incomum entre os GIPLs de *Leishmania* sendo em sua maioria composta por diacilglicerol ao invés de alquil-acil-glicerol ou liso alquilglicerol (Zawadzki *et al.*, 1998).

O terceiro grupo, o dos GIPLs híbridos, compartilha características estruturais com os dois primeiros tipos e possuem adição de uma manose em ambos os terceiro e sexto carbonos da manose proximal (Man α 1-3(Man α 1-6)Man α 1-4GlcN-PI) encontrados em *L. mexicana* e *L. donovani* (McConville & Ferguson, 1993) (Figura 4).

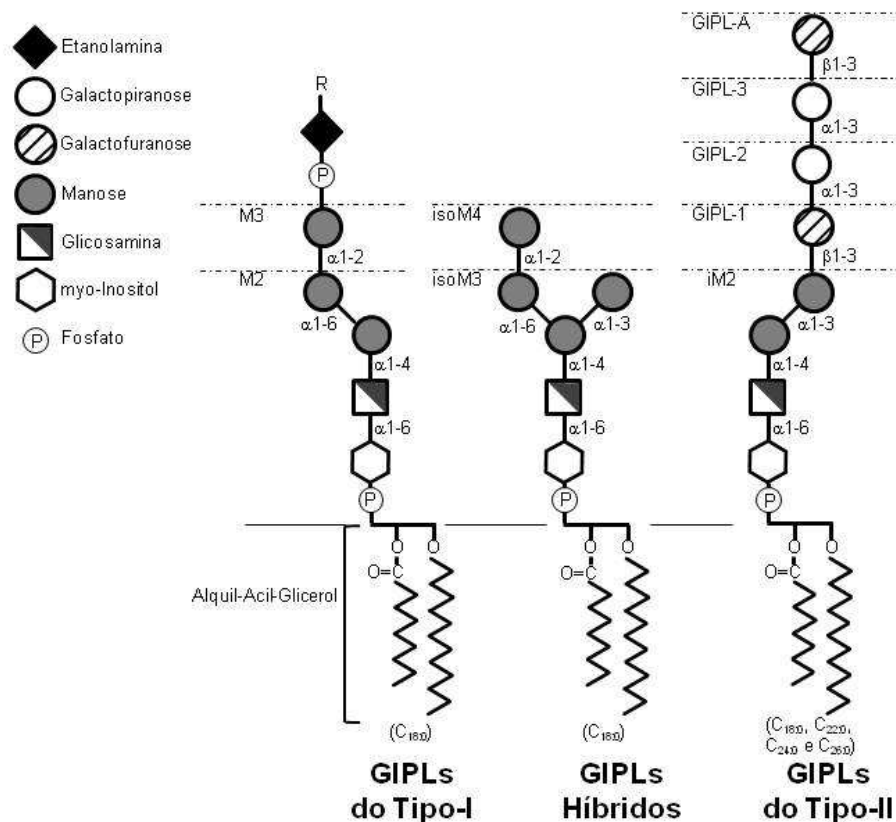


Figura 4. Tipos de GIPLs. Principais estruturas de GIPLs descritos em *Leishmania*. O ácido graxo predominante nos GIPLs do tipo-I e híbridos é C18:0, Os ácidos graxos predominantes nos GIPLs do tipo-II são C18:0, C22:0 C24:0 e C26:0. É possível a adição de um radical "R" na manose mais distal dos GIPLs do tipo-I, este radical é em geral uma proteína de superfície (ex: a metaloprotease gp63) ligada ao GIPL via um resíduo de etanolamina-fosfato (McConville & Ferguson, 1993; Ralton & McConville, 1998).

As estruturas dos GIPLs de *L. braziliensis* e *L. infantum* ainda são desconhecidas e são foco deste trabalho.

3.5. Os lipofosfoglicanos (LPGs) de *Leishmania*

Ao contrário dos GIPLs, os LPGs são os glicoconjugados de superfície de *Leishmania* mais bem estudados. Estão amplamente expressos nas formas promastigotas aonde formam um denso glicocálice que recobre toda a superfície do parasito (Turco & Descoteaux, 1992). Entretanto estão ausentes nas formas amastigotas (McConville & Blackwell, 1991).

Bioquimicamente, os LPGs possuem uma estrutura geral bem conservada, composta por quatro domínios: (i) uma âncora lipídica conservada representada por 1-*O*-alquil-2-*liso*-fosfatidilinositol (PI) ligado a (ii) uma porção central composta por um heptassacarídeo também conservado representado por Gal(a1-6)Gal(a1-3)Gal β (b1-3)[Glc1-PO₄]Man(a1-3)Man(a1-4)-GlcN(a1-); (iii) uma região de repetições de dissacarídeos fosforilados Gal(b1-4)Man(a1-)-PO₄ que podem possuir substituições de um ou mais açúcares dando origem a um grande polimorfismo intra e interespecífico e finalmente, (iv), um oligossacarídeo neutro terminal (Descoteaux & Turco, 1999) (Figura 5).

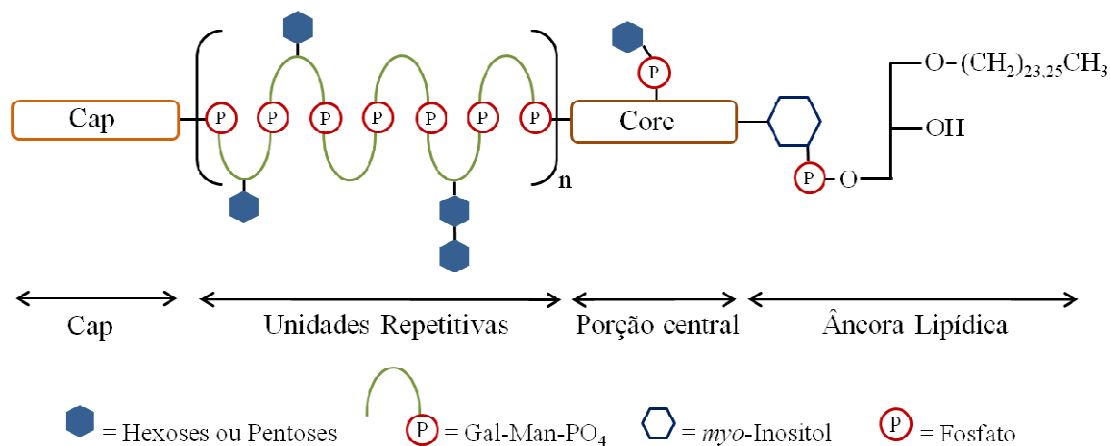


Figura 5. Representação esquemática do LPG. Bioquimicamente, possui quatro domínios: (i) uma âncora lipídica de 1-O-alkil-2-liso-fosfatidilinositol (PI) ligado à; (ii) porção central fosfatidilinositol representada por Gal(α 1-6)Gal(6)Gal(α 1-3)Gal(β 1-3)[Glc α 1 PO₄]Man(α 1-3)Man(3)Man(α 1-4)-GlcN(α 1-); (iii) uma região de repetições de dissacarídeos fosforilados Gal(β 1-4)Man(Gal(4)Man(α 1-)-PO₄ que podem possuir substituições de um ou mais açúcares e (iv), um oligossacarídeo neutro terminal polimórfico (adaptado de Assis et al., 2012b).

Variações qualitativas e quantitativas nas unidades repetitivas ocorrem dependendo da espécie, linhagem e fase no ciclo de vida do parasito. Durante o processo de metaciclogênese, as unidades repetitivas dobram em número passando de aproximadamente 15 para aproximadamente 30 unidades (Barron & Turco, 2006; Guha-Niyogi *et al.*, 2001; Ilg *et al.*, 1992; Sacks *et al.*, 1995; Soares *et al.*, 2002). Em *L. braziliensis* O LPG das formas promastigotas procíclicas não possuem substituições, sendo estruturalmente semelhante ao LPG de *L. donovani* (Sudão) (Sacks *et al.*, 1995). Durante a metaciclogênese, em *L. braziliensis*, ocorre a adição de mono ou dissacarídeos de β -glicoses no carbono 3 da galactose (Soares *et al.*, 2005). O LPG de *L. infantum* de diversas linhagens já foi descrito e pode variar de estruturas sem cadeias laterais

como no LPG de *L. braziliensis* na forma promastigota procíclica (LPGs do tipo 1) (Coelho-Finamore *et al.*, 2011), até LPGs com adição de resíduos de uma a duas β - glicoses (LPGs do tipo II) (Coelho-Finamore *et al.*, 2011; Soares *et al.*, 2002) e LPGs mais complexos formando cadeias laterais de três ou mais glicoses (LPGs do tipo III) (Coelho-Finamore *et al.*, 2011). Nas formas promastigotas metacíclicas, apenas o LPG da linhagem PP75 (MHOM/BR/1974/PP75) foi estudado até o momento e a regulação da adição de glicoses à cadeia lateral segue um padrão contrário ao de *L. braziliensis* ocorrendo a remoção destas glicoses, enquanto que em *L. braziliensis* ocorre a adição (Soares *et al.*, 2005; Soares *et al.*, 2002). Por ocasionarem patologias distintas e por apresentarem LPGs com estruturas diferentes, este trabalho focou em *L. braziliensis* (MHOM/BR/1975/2903) (LPG não substituído) e *L. infantum* (MHOM/BR/1970/BH46) cujo LPG possui até 3 glicoses na cadeia lateral (Coelho-Finamore *et al.*, 2011; Soares *et al.*, 2002) (Figura 6).

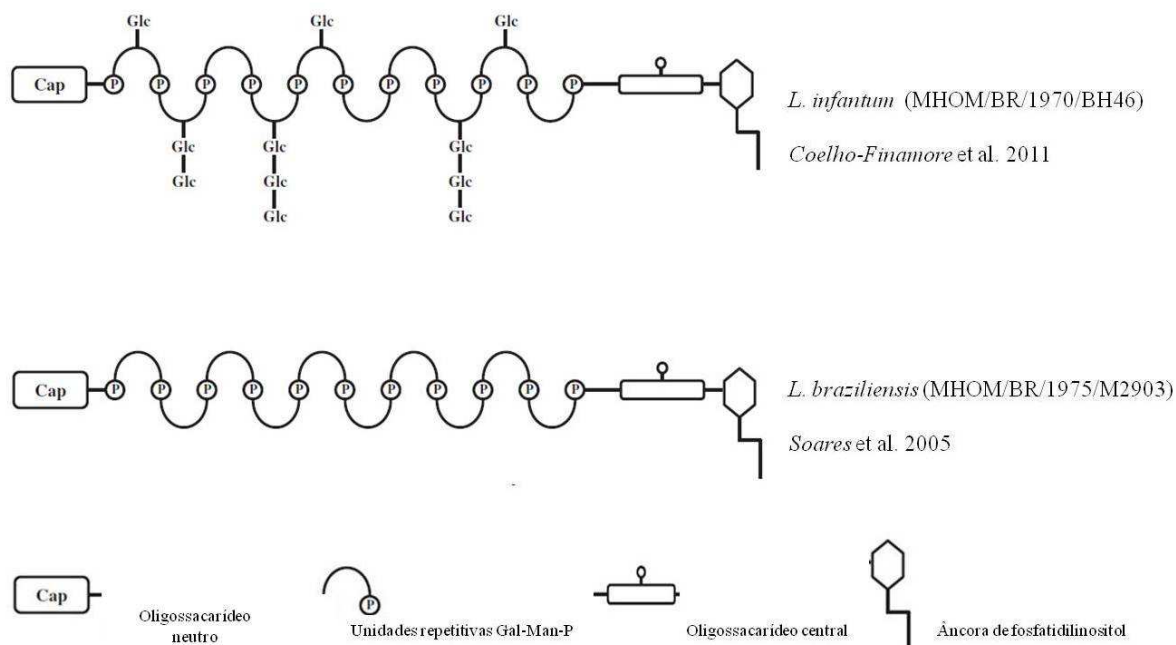


Figura 6. Diferenças estruturais do LPG das formas promastigotas procíclicas de *L. braziliensis* e *L. infantum*. O polimorfismo presente nas unidades repetitivas dos LPGs de *L. braziliensis* e *L. infantum* está na adição de resíduos de glicose ao resíduo de manose, formando cadeias laterais. Enquanto que na forma promastigota procíclica de *L. infantum* ocorre a substituição com oligômeros de um a três resíduos de glicose, no LPG das formas promastigotas procíclicas de *L. braziliensis* não ocorre esta substituição (Soares *et al.*, 2002; 2005).

3.6. Aspectos imunológicos das leishmanioses

Em um âmbito geral, a modulação do sistema imune por *Leishmania* tem sido estudada a fim de se compreender os mecanismos de proteção ou susceptibilidade à infecção. Por muito tempo acreditou-se que a leishmaniose se iniciava pela direta infecção de macrófagos após a inoculação dos parasitos na pele pelo vetor. Parte disso se deve pela dificuldade de se estudar os eventos iniciais da infecção.

Neste sentido, modelos experimentais têm proporcionado um importante avanço na compreensão dos eventos imunológicos que marcam o curso da infecção. Dentre estes modelos, pode se destacar o estabelecimento de um importante paradigma que envolve células T auxiliaadoras (Células T CD4+).

Após ativação, as células T CD4+ não estimuladas (Th0) podem se diferenciar em distintas subpopulações com papéis bastante específicos na regulação da resposta imune. Por exemplo, as células T CD4+ do tipo 1 (Th1) secretam IFN- γ , que estimula uma resposta celular caracterizada pela ativação dos mecanismos microbicidas dos macrófagos e está diretamente relacionada a um fenótipo protetor contra a LT e LV (Revisado por Reiner & Locksley, 1995).

No modelo clássico de infecção por *L. major*, este fenótipo chamado tipo 1 está associado a linhagens de camundongo resistentes a infecção como C57BL/6 que é dependente diretamente da produção de IL-12 por células apresentadoras de antígenos (APC), em um microambiente pro inflamatório, onde diversas citocinas como IL-1 α , IL-18, IL-23 e IL-27 e TNF- α também estão presentes (Revisado por Alexander & Bryson, 2005; Bogdan & Rollinghoff, 1998).

Por outro lado, a diferenciação das células T CD4+ no tipo 2 (Th2), característica da imunidade contra helmintos e nos processos alérgicos (Kool *et al.*, 2012), leva a produção de outras citocinas, como IL-4, IL-5 e IL-13. Este fenótipo das células T CD4+ está associado à estimulação de uma resposta humoral à infecção por *Leishmania* e a sua predominância está diretamente associada a um fenótipo de susceptibilidade à infecção, fenômeno observado em camundongos BALB/c (Rogers *et al.*, 2002).

Além dos já bem descritos e estudados fenótipos Th1 e Th2, a diferenciação dos linfócitos TCD4+ no subtipo Th17 têm demonstrado um papel importante mas ainda pouco compreendido na LCM. Estudos tem demonstrado que a produção de IL-17 pode ser detectada

nas lesões ativas e está relacionada com seu agravamento. Este fenômeno parece estar relacionado com a capacidade da IL-17 de estimular um grande influxo de neutrófilos para o sítio da lesão e, mesmo num cenário de escassez de parasitos, estimular a ativação destas células com produção de proteinases e liberação de proteínas celulares (Boaventura *et al.*, 2010). Este fenômeno também pode ser observado pelo agravamento de lesões causadas pela IL-17 em camundongos susceptíveis infectados com *L. major* (Lopez Kostka *et al.*, 2009).

Assim como descrito, o modelo clássico de diferenciação das células TCD4⁺ como marcador da polarização da resposta imune se mostra restrito quando se leva em conta o leque de modificações que podem ocorrer e pela ocorrência de perfis mistos (WHO 2010) e não se aplica completamente a outras espécies de *Leishmania*, principalmente nas Américas. Em infecções por *L. braziliensis*, observa-se que a polarização Th1/Th2 não é bem definida e provavelmente não é um fator determinante na infecção de camundongos BALB/c. Quando infectados por esta espécie, estes animais controlam a multiplicação dos parasitas que desaparecem após 42 dias. Este fenótipo de resistência em resposta à infecção por *L. braziliensis* não se dá devido a uma resposta Th1 eficiente, e sim por uma falha ao montar uma resposta Th2, uma vez que os níveis de IL-4 são de 10-15 vezes menores se comparados à infecção por *L. major* (DeKrey *et al.*, 1998). Por outro lado, em camundongos BALB/c infectados por *L. infantum* tendem a apresentar uma maior produção de IL-10 por células T CD4⁺ DC25⁻ *foxp3*⁻ nos sítios de infecção (principalmente baço e linfonodos) além do acúmulo de células T reg (células T CD4⁺ CD25⁺ *foxp3*⁺) nestes sítios cuja produção de TGF- β promove imunossupressão que previne o início precoce dos sintomas imunopatológicos (Rodrigues *et al.*, 2009). Além disso, a presença de células T reg também parece reduzir a imunopatologia de lesões cutâneo-mucosas causadas por *L. amazonensis* (Ji *et al.*, 2005).

Por este motivo, diversos trabalhos têm focado na modulação da ativação das células hospedeiras como produção de citocinas/quimiocinas e de sua capacidade de produção de radicais ativos de nitrogênio e oxigênio. Por representarem o primeiro contato com o hospedeiro e formarem uma interface de interação bastante complexa, as moléculas de superfície do parasito assumem um papel central. Neste contexto se destacam os glicoconjugados de superfície. Esta diversa classe de moléculas atua nas diversas fases do ciclo de vida do parasita como fatores de virulência multifuncionais (Descoteaux & Turco, 1999; Schlein *et al.*, 1990; Soares *et al.*, 2004; Soares *et al.*, 2002).

3.7. A resposta imune inata e as leishmanioses

Como descrito anteriormente, a habilidade do hospedeiro de controlar a infecção e resolver a doença requer a capacidade de montagem de uma forte resposta imune celular, em especial com produção de citocinas do tipo I, capaz de ativar os macrófagos hospedeiros para eliminar as formas amastigotas intracelulares (Cummings *et al.*, 2010). Este tipo de resposta depende de diversos fatores sendo a capacidade de reconhecer, com certa especificidade, o protozoário invasor e regular de uma resposta direcionada.

O reconhecimento do parasito, assim como de qualquer outro microrganismo, parasita ou não, depende da capacidade do sistema imune do hospedeiro de reconhecer padrões moleculares que estão normalmente associados a patógenos, os chamados PAMPs. Estes PAMPs são reconhecidos por receptores especializados chamados “receptores de reconhecimento de padrões” ou PRRs (do inglês *pattern recognition receptors*).

Os PRRs podem ser de diversos tipos e podem estar associados à membrana como os receptores do tipo toll (TLRs) bem como podem estar localizados no citoplasma como os receptores do tipo NOD (NLR) dos quais se destacam duas famílias principais: Os NODs (NOD1 e NOD2) e as NALPs (por volta de 14 proteínas em humanos)(Harton *et al.*, 2002; Inohara & Nunez, 2003). Neste trabalho o foco será dado para os receptores do tipo toll (figura 7).

Estruturalmente, os TLR são proteínas transmembrana que contém dois domínios principais: um extracelular caracterizado por uma região rica em leucinas (LRR) responsável pelo reconhecimento dos motivos moleculares associados aos microrganismos invasores e um domínio intracelular em comum ao receptor de IL-1 chamado de domínio Toll/IL-1, ou domínio TIR responsável pela transdução do sinal para as diferentes vias intracelulares (Akira, 2003; Akira & Takeda, 2004).

O primeiro TLR humano identificado foi o TLR4 (Medzhitov & Janeway, 1997) e seu principal agonista identificado como sendo o lipopolissacarídeo (LPS), componente abundante da superfície de bactérias gram-negativas (Poltorak *et al.*, 1998). Subsequentemente outros TLRs e seus ligantes foram sendo identificados. Pela capacidade marcante de formar heterodímeros com o TLR1 e TLR6, o TLR2 é capaz de reconhecer uma largo espectro de compostos microbianos como lipoproteínas (Aliprantis *et al.*, 1999), peptidoglicanos (Schwandner *et al.*, 1999; Takeuchi *et al.*, 1999) bem como diversos lipídeos (Revisado por Akira & Takeda, 2004) e em especial GIPLs de *T. cruzi* (Coelho *et al.*, 2002) e GIPLs e LPG de *Leishmania* (Becker *et al.*, 2003; de Veer *et al.*, 2003). Um lista de agonistas conhecidos dos TLRs pode ser encontrada em Akira & Takeda (2004) e Carpenter & O'Neill (2007).

De forma geral, o reconhecimento dos diversos PAMPs pelos TLRs leva à produção de citocinas pro inflamatórias e a subsequente resposta imune. Após reconhecimento do ligante à

porção LRR, a região TIR do receptor sofre mudanças conformacionais necessárias para o recrutamento das proteínas que irão transferir este sinal de ativação para as diversas vias de sinalização intracelular. Estas proteínas são comumente chamadas de proteínas adaptadoras e incluem o fator de diferenciação mieloide 88 (MyD88), adaptador do tipo MyD88 (MAL), proteína adaptadora contendo o domínio TIR (TIRAP), proteína adaptadora contendo o domínio TIR induzido por IFN- α (TRIF), molécula adaptadora relacionada a TRIF (TRAM) e proteína adaptadora contendo SAM e ARM (SARM) (Revisado por Carpenter & O'Neill, 2007).

A via dependente de MyD88 é a mais estudada e está envolvida na ativação do fator nuclear κB (NF- κB), um importante fator de transição para expressão de citocinas e mediadores inflamatórios, por todos os TLRs descritos exceto pelo TLR3. As principais vias ativadas por TLRs são a via de NF- κB através de κB cinase (IKK), proteínas cinase ativadas por mitógenos (MAPKs) e a via de fosfatidilinositol 3-cinase (PI3K/Akt) (Akira & Takeda, 2004). De forma geral, a ligação de algum agonista à porção LRR do TLR leva a uma mudança conformacional na porção TIR do receptor, esta mudança leva à interação com a proteína adaptadora MyD88, que também possui um motivo TIR, ativando IRAK-1/4 que por sua vez fosforila TRAF6. A fosforilação de TRAF6 culmina com a degradação de κB e consequente liberação e translocação de NF- κB para o núcleo (Janssens & Beyaert, 2003; Suzuki *et al.*, 2002b; Suzuki *et al.*, 2002).

Apesar da maioria dos TLRs serem dependentes de MyD88, os TLR3 e TLR4 são capazes de ativar vias de sinalização independentes desta molécula adaptadora como a ativação de IRF3 em vias controladas por TRIF (TLR3) e TRIF/TRAM (TLR4) levando a expressão de genes controlados por IRF3 como a produção de interferons do tipo 1 (Krishnan *et al.*, 2007). Um resumo das vias dependentes e independentes de MyD88 esta representado na Figura 7.

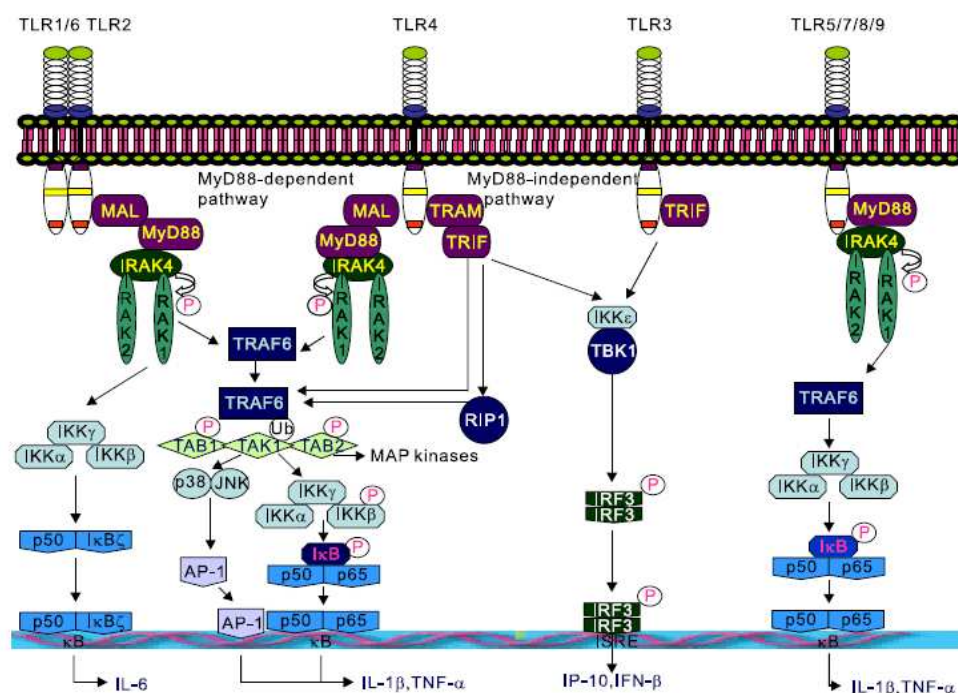


Figura 7. Vias de sinalização dos TLRs. A ligação de PAMPs aos TLRs leva a ativação de moléculas adaptadoras como MyD88 e TRIF/TRAM que levam a ativação de genes por meio de diferentes vias como MAPKs, IRF-3 e NF-κB. (figura adaptada de Krishnan *et al.*, 2007).

O primeiro trabalho a estudar as vias dependentes de MyD88 e a infecção por *Leishmania* foi realizado por Hawn em 2002 (Hawn *et al.*, 2002). Neste trabalho, avaliando a infecção por *L. major*, foi demonstrado que macrófagos de camundongos deficientes em MyD88 expressam níveis reduzidos de IL-1 α . Em 2003, o TLR2 foi identificado como receptor de LPG (Becker *et al.*, 2003; de Veer *et al.*, 2003). Posteriormente foi demonstrado que camundongos deficientes em TLR4 possuem uma menor resistência à infecção por *L. major* (Kropf *et al.*, 2004). Em *L. donovani*, foi demonstrado que por meio da modulação de MAPKs, o parasito é capaz de reduzir a expressão de TLR2, e consequentemente reduzir a produção de IL-12 acompanhado de uma elevação da produção de IL-10 (Chandra & Naik, 2008). Porém, estes mecanismos, para espécies do Novo Mundo, são ainda pouco conhecidos. Trabalhos recentes com *L. braziliensis* demonstram que apesar de MyD88 ser importante para o controle da infecção, no reconhecimento *in vitro* e *in vivo* do parasito por células dendríticas, o TLR2 possui um papel modulatório, reduzindo a produção de IL-12 (Vargas-Inchaustegui *et al.*, 2009).

3.8. O papel biológico dos Glicoconjugados de *Leishmania*

A interação entre *Leishmania* e hospedeiro é complexa e assume diversas formas dependendo das espécies envolvidas e fatores intrínsecos como status nutricional e imunológico do hospedeiro, bem como linhagens do parasito envolvidas, mas não se sabe o quanto, variações nas moléculas de superfície do parasito, influenciam na resposta imune subsequente. Entretanto, estudos mostram que, principalmente glicoconjugados de superfície, assumem o papel de fatores de virulência multifuncional e são indispensáveis para o estabelecimento de uma infecção bem sucedida.

O LPG tem sido associado com diversos processos durante a infecção incluindo resistência ao efeito lítico do sistema do complemento, bem como reconhecimento, fagocitose por macrófagos e proteção contra o ambiente ácido dos vacúolos parasitóforos (Bogdan & Rollinghoff, 1999; Wilhelm *et al.*, 2001); inibição da maturação fagossomal (Dermine *et al.*, 2000), bem como intervenção na integridade de microdomínios de membrana em fagossomos (Dermine *et al.*, 2005), modulação da produção de óxido nítrico e IL-12 (Brittingham & Mosser, 1996; Piedrafita *et al.*, 1999; Proudfoot *et al.*, 1996), inibição de proteína cinase C (Piedrafita *et al.*, 1999), modulação de MAPKs (Feng *et al.*, 1999; Prive & Descoteaux, 2000), indução de redes extracelulares de neutrófilos (NETs) (Guimaraes-Costa *et al.*, 2009), indução da proteína cinase R (PKR) (de Carvalho Vivarini *et al.*, 2011) e indução da heme-oxigenase I (Luz *et al.*, 2012).

A diversidade e importância do LPG, bem como dos GPIs tem sido demonstradas no contexto da resposta imune celular. De fato, *L. major* deficiente em LPG tem sua infectividade e capacidade de sobreviver no interior de macrófagos reduzida, enquanto que em *L. mexicana* o desenvolvimento de mutantes deficientes em LPG é normal (Ilg, 2000; Ilg *et al.*, 2001; Turco *et al.*, 2001). A importância do polimorfismo do LPG na infecção também pode ser observada em *L. donovani* (Prive & Descoteaux, 2000), onde o LPG de *L. donovani* da linhagem indiana foi capaz de ativar simultaneamente, mas com cinética diferente, as MAPKs ERK, JNK e p38. Esta linhagem de *Leishmania* possui um LPG com cadeias laterais de 1 a 2 β -Glicoses (Mahoney & Turco, 1999). Por outro lado, o LPG da linhagem 1S2D, de origem sudanesa, de *L. donovani*, não possui cadeias laterais. Nesta linhagem o LPG está implicado na ativação de MAPKs uma vez que promastigotas deficientes em LPG não ativam MAPKs em macrófagos (Prive & Descoteaux, 2000). Adicionalmente, o LPG de *L. donovani* da linhagem indiana (sem cadeias

laterais) não induz a formação de NETs por neutrófilos, bem como aumenta a resistência do parasito à sua ação microbicida (Gabriel *et al.*, 2010) enquanto o LPG de *L. amazonensis*, em cuja estrutura ocorre a adição de uma a 2 β - glicoses (dados não publicados), induz a formação de NETs de forma dose dependente (Guimaraes-Costa *et al.*, 2009).

Por outro lado, apesar da estrutura bioquímica dos GIPLs de diversas espécies de *Leishmania* já ter sido descrita, seu papel na biologia e infecção tem sido ainda negligenciado. Em parte, isto se deve à dificuldade em se isolar os efeitos específicos dos GIPL, já que os mesmos compartilham vias metabólicas e estrutura bioquímica com outros glicoconjugados ancorados a GPI.

Os primeiros estudos descreveram a participação dos GIPLs de *L. major* na resposta humoral (McConville & Bacic, 1989), desencadeando a produção de altos títulos de anticorpos anti- α -Gal em humanos. Resultados semelhantes foram observados por outro grupo em *L. major*, *L. donovani*, *L. mexicana* e *L. braziliensis* (Avila *et al.*, 1991). A primeira evidência de que os GIPLs de *Leishmania* desempenhariam um papel na imunidade inata foi demonstrada durante a inibição da produção de NO por macrófagos murinos por estas moléculas (Proudfoot *et al.*, 1995).

Em parte, a dificuldade de se estudar o papel dos GIPLs se deve ao fato de que os GIPLs compartilham vias metabólicas com outras moléculas semelhantes e a ausência de mutantes específicos. Entretanto, a geração de mutantes deficientes parcial ou totalmente de GIPLs sugeriu uma participação na infecção por *L. amazonensis* (Mensa-Wilmot *et al.*, 1999) e *L. mexicana* (Garami *et al.*, 2001; Ralton *et al.*, 2003).

Após o reconhecimento dos receptores na membrana, os GIPLs são capazes de desencadear diferentes respostas intracelulares. Foi demonstrado que os GIPLs de *L. mexicana*

são capazes de ativar vias de sinalização através de proteínas tirosina-quinase (PTKs) (Tachado *et al.*, 1997). PTKs modulam vários processos como diferenciação celular, crescimento, metabolismo e apoptose (Geer *et al.* 1994). Elas representam uma família de cinases cuja ativação desencadeia uma série de fenômenos celulares entre os quais pode-se citar, a ativação da proteína Ras (Margolis & Skolnik, 1994). Esta é capaz de se ligar às isoformas de Raf (MAP3K) como A-Raf, B-Raf e C-Raf, ou seja, levando a ativação de vias de MAP quinases (Avruch *et al.*, 2001) que modulam várias funções intracelulares. Entretanto, esta ativação de PTKs parece não levar à ativação de PKC nem à produção de NO (Tachado *et al.*, 1997).

Em sintonia com estes achados, o LPG também é capaz de interagir de forma intrincada com os mecanismos de sinalização celular. Em *L. donovani* e *L. mexicana*, o LPG é capaz de inibir *in vitro* a ativação de PKC α , o que resulta em inibição da despolimerização dos filamentos de F-actina e fusão do fagossomo com lisossomos (Holm *et al.*, 2003; Holm *et al.*, 2001) bem como a explosão respiratória em camundongos Balb/c de fenótipo susceptível à infecção (Delgado-Dominguez *et al.*, 2010). Variações na estrutura do LPG parecem também ser importantes na interação com as células hospedeiras. De forma geral, a capacidade de modular a produção de NO, em sinergia com IFN- γ , parece estar diretamente ligado com a complexidade das cadeias laterais das unidades repetitivas, uma vez que LPGs mais complexos tendem a possuir uma atividade pro-inflamatória mais proeminente (Coelho-Finamore *et al.*, 2011; Gabriel *et al.*, 2010; Guimaraes-Costa *et al.*, 2009; Prive & Descoteaux, 2000; Proudfoot *et al.*, 1996).

Baseado no exposto, os GIPLs e LPGs de *Leishmania*, em conjunto com outras moléculas de superfície, desempenham um papel importante na infecção. Mas as informações sobre seu efeito isolado sobre a atividade de macrófagos ainda são escassas. Como parte de um amplo projeto em Glicobiologia, este projeto teve como objetivo investigar o papel destes dois

glicoconjugados de espécies de *Leishmania* do Novo Mundo. Neste trabalho, os GIPLs e LPGs de *L. braziliensis* e *L. infantum* apresentaram variações importantes em sua porção glicídica. Estas variações resultaram na inibição e modulação de diferentes mecanismos efetores em macrófagos murinos incluindo a produção NO, citocinas e a ativação de MAP quinases.

4. Métodos

Todos os animais utilizados neste trabalho foram manejados de acordo com boas práticas de manejo animal definidas pela Comissão Ética no Uso de Animais (CEUA) da Fundação Oswaldo Cruz (Fiocruz), Belo Horizonte, Minas Gerais. Protocolo P-0297-06. A utilização de camundongos *Knockout* foi aprovada pela Comissão Técnica Nacional de Biossegurança (CTNBio) Protocolo #01200.006193/2001-16.

4.1. Parasitos

As Linhagens de referência da Organização Mundial de Saúde de *L. braziliensis* (MHOM/BR/1975/M2903), *L. infantum* (MHOM/BR/1974/PP75), *L. infantum* (MHOM/BR/70/BH46), e *L. donovani* (MHOM/SD/00/1S-2D) foram utilizadas. Os parasitos foram cultivados em meio M199 suplementado com 10% de soro fetal bovino (SFB), 100 U/ml de penicilina, 50 mg/ml de estreptomicina, 12,5mM de glutamina, 0,1M de adenina, 0,0005% de hemina e 40mM de Hepes, pH7,4 a 26°C (Soares *et al.*, 2002).

4.2. Extração e purificação do LPG e dos GIPLs

As promastigotas de *Leishmania* foram cultivadas como descrito no item anterior até o início da fase estacionária quando foram centrifugadas e lavadas duas vezes com PBS. Ao sedimento foram adicionados 2,5ml de clorofórmio/metanol (3:2, v/v) e 0,5ml de cloreto de magnésio (MgCl₂) 4mM seguindo uma nova centrifugação a 2100g por 7min. O sedimento foi sonificado e o material centrifugado a 2100g por 7min. As fases aquosas, superior e inferior, foram descartadas e o procedimento anterior repetido uma vez. À fase sólida intermediária foram

adicionados 2,5ml de $MgCl_2$ 4mM e novamente sonicada e centrifugada a 2100g por 7min. O procedimento foi repetido uma vez desprezando-se o sobrenadante. Ao sedimento foram adicionados 3ml de clorofórmio/metanol/água (10:10:3, v/v) e 0,5ml de clorofórmio/metanol (1:1, v/v), este último adicionado apenas na primeira centrifugação. Este passo foi repetido por três vezes, com centrifugação a 2100g por 7min.

Para a extração do LPG, foram adicionados 2,5 mL de ESOAK (água/etanol/etil-éter/piridina/ NH_4OH ; 15:15:5:1:0,017 v/v) ao sedimento resultante da extração dos GIPLs. O sedimento foi então sonificado e centrifugado (7 min, 2100g) e o sobrenadante contendo o LPG recolhido. O procedimento foi repetido mais 3 vezes. Os sobrenadantes contendo os GIPLs foram combinados e secos por evaporação em banho sob fluxo de N_2 , o mesmo procedimento foi aplicado ao LPG.

O material seco contendo os GIPLs foi diluído em acetato de amônio a 0,1M com 5% de 1-propanol e aplicado em uma coluna de Octil-sepharose (80 ml) equilibrada na mesma solução. À coluna foi aplicado um gradiente contínuo de 1-propanol de 5 a 60% em acetato de amônio 0,1M. Frações de 3 ml foram coletadas e a presença dos GIPLs nas frações foi detectada aplicando-se 2 μ l de cada fração em uma placa de Cromatografia em camada delgada (CCD). Após aspersão da placa com uma mistura de orcinol:ácido sulfúrico (1:1) e aquecendo a placa a 100°C por 5 min, as frações contendo os GIPLs foram identificadas pelo aparecimento de uma coloração roxa (Orlandi & Turco, 1987).

O material seco contendo o LPG foi solubilizado em 1 mL da solução ácido acético 0,1 N/ NaCl 0,1 M, sonificado e aplicado em uma coluna contendo fenil Sepharose equilibrada com o mesmo solvente. A coluna foi então lavada de acordo com a seguinte sequência: 1 ml de ácido acético 0,1 N/NaCl 0,1 M seguido de 1 ml de ácido acético 0,1 N seguido de 1 ml de dH_2O .

Foram utilizados 4 ml de ESOAK para eluir o LPG. O material foi então seco por evaporação em sob fluxo de N₂ (Soares *et al.*, 2002).

Os GIPLs e LPGs secos foram solubilizados em água livre de endotoxinas e armazenados a -20°C. Para utilização nos experimentos de interação *in vitro*, os glicoconjugados foram diluídos em meio RPMI em várias concentrações dependendo do experimento.

4.3. Purificação de macrófagos peritoneais murinos e cultura celular

Os macrófagos peritoneais foram extraídos de camundongos BALB/c, C57BL/6, TLR2 -/- e TLR4 -/- (*knockouts* cujo *background* genético é C57BL/6) por lavagem peritoneal com meio RPMI gelado (4°C) sem soro 72h após a injeção peritoneal de 2 ml de tioglicolato de sódio (3% em água). As células foram diluídas em meio RPMI suplementado com 2mM de glutamina, 50 U/ml de penicilina e 50µg/ml de estreptomicina e plaqueadas a 3 X 10⁵ células/ml em placa de 96 poços. A suspensão foi mantida em RPMI por 30 minutos a 37°C/5% CO₂. As células não aderentes foram removidas após lavagem com RPMI. Os macrófagos enriquecidos foram incubados com IFN-γ (100 UI/ml) (Kolodziej *et al.*, 2008), promastigotas de *Leishmania* (10:1), GIPLs (1, 5, 10 e 25 µg/mL), LPG (1 a 10 µg/mL) ou lipopolissacarídeo (LPS) (100 ng/mL) em RPMI.

4.4. Dosagem de citocinas e nitrito

Para a dosagem de citocinas pela metodologia multiplex CBA (BD), as células foram plaqueadas como descrito acima por 1 h. RPMI foi então adicionado com a adição de IFN-γ (3 UI/ml) para células primadas (Hu X *et al.*, 2002) ou sem a adição de IFN-γ (para células não primadas) e incubadas por 18 h (37°C, 5% CO₂). Após este período, as células foram lavadas

com meio RPMI sem soro e foi adicionado meio fresco, sem IFN- γ suplementado com 2 mM de glutamina, 50 U/ml de penicilina e 50 mg/ml de estreptomicina. GIPLs (25 μ g/ml), LPG (10 μ g/mL) ou LPS (100 ng/ml) foram adicionados e incubados por 48h. Sobrenadantes foram coletados e armazenados a -70°C até o uso. A concentração de citocinas (IL1- β , IL-2, IL-4, IL-5, IL-10, IL-12p40, IFN- γ e TNF- α) nos sobrenadantes foi determinada usando o Kit *CBA Mouse Cytokine assay kits* (BD® Biosciences, CA, EUA) de acordo com as recomendações do fabricante. As medições por citometria de fluxo foram realizadas em citômetro de fluxo FACS Calibur (Becton Dickinson, Mountain View, CA, EUA). A aquisição dos dados foi realizada utilizando o programa *Cell-Quest™ software package* fornecido pelo fabricante. A análise dos dados foi feita no programa *FlowJo software 7.6.4* (Tree Star Inc., Ashland, OR, USA). Foi realizada uma aquisição de 300 eventos para cada citocina testada em cada preparação. Os resultados representam a média de 2 experimentos em duplicata. A concentração de nitrito foi dosada nos mesmos sobrenadantes pela reação de Griess (Drapier *et al.*, 1988).

Para os experimentos de inibição utilizando GIPLs, as suspensões celulares extraídas dos camundongos como descrito anteriormente, foram incubadas enriquecidas por aderência em plástico e incubadas por 18h sem a adição de IFN- γ . GIPLs foram adicionados e incubados por 15 a 30 minutos a 37°C quando então foram adicionadas 3 U/ml de IFN- γ . Os sobrenadantes coletados após 24 h para dosagem de nitrito, TNF- α e IL-12. Quando usados, LPS e IFN- γ foram adicionados 15 min antes da adição dos GIPLs. Os sobrenadantes foram coletados e as concentrações de nitrito determinadas pela reação de Griess (Drapier *et al.*, 1988). As concentrações de TNF- α e IL-12 foram determinadas por ELISA (BD). Para os experimentos de inibição utilizando LPG, as suspensões celulares extraídas dos camundongos como descrito anteriormente, foram incubadas enriquecidas por aderência em plástico e primadas por 6h com 3

U/ml de IFN- γ . Em seguida foi adicionado o LPG (10 μ g/ml) e as células incubadas por 18h. Após este período LPS (100 ng/mL) foi adicionado ao meio e as células incubadas por mais 24h a 37°C. Os sobrenadantes foram coletados e as concentrações de nitrito determinadas pela reação de Griess (Drapier *et al.*, 1988).

4.5. Tratamento com fosfolipase C PI específica (PI-PLC)

Com o intuito de avaliar a necessidade da estrutura intacta para a sua atividade. Os GIPLs purificados foram diluídos em 150 ml de tampão CHAPS (298mg HEPES, 47mg EDTA e 50mg CHAPS em 50ml de água livre de endotoxinas). Foram então adicionadas 2 U de PI-PLC (Sigma) e o material foi incubado em banho-maria a 37°C por 16h. Macrófagos peritoneais foram então extraídos e plaqueados como descrito anteriormente, e incubados com GIPLs intactos ou GIPLs tratados com PI-PLC por 15 a 30 min a 37°C. Foram então adicionadas 3 U/mL de IFN- γ e a concentração de nitrito dosada nos sobrenadantes após 24h pela reação de Griess.

4.6. Preparação dos lisados celulares e *imunoblot*

Macrófagos (3×10^6 células por poço) estimulados por GIPLs (25 μ g/ml) ou LPGs (10 μ g/ml) foram lavados em PBS gelado (4°C) e rompidos com tampão de lise (Tris-HCl 20mM pH7,5, 1% Triton X-100, Ortovanadato de sódio 1mM, fenilmetilsulfonil fluoreto (PMSF) a 1mM, Fluoreto de Sódio a 50 mM, NaCl a 150 mM, EDTA a 5 mM, Glicerol a 10% (v/v), ditioneitol (DTT) a 0,5 mM e um coquetel de inibidores de protease (Sigma®).

Em seguida as células foram raspadas e centrifugadas a 13.000 x g (4°C, 10 min), e os sobrenadantes transferidos para tubos novos e mantidos a -20°C até o uso. Os lisados celulares

foram resolvidos por SDS-PAGE (100V), transferidos para uma membrana de nitrocelulose e a membrana bloqueada por 1h (Caseína a 5% em TBS-Tween 20 0,1%). As membranas foram incubadas com os anticorpos primários (específicos para as formas fosforiladas de ERK ou p38/SAPK) na diluição de 1:1000 por 16 h a 4°C. As membranas foram lavadas três vezes por 10 minutos com TBS-Tween 20 0,1% e incubadas por 1h com os anticorpos secundários anti-IgG conjugados com peroxidase (1:10.000) e as reações visualizadas usando Luminol. No caso do LPG também foi testada a MAPK JNK.

Caracterização bioquímica preliminar dos GIPLs

4.7. Desaminação pelo ácido nitroso

Para determinação de sua estrutura bioquímica, os GIPLs purificados foram delipidados por desaminação pelo ácido nitroso (300 µl de acetato de sódio a 0,5 M pH 4,0 e 300 µl de NaNO₂ a 0.5 M) por 16 h a 37 °C (Soares *et al.*, 2002). As amostras foram secas em *speed-vac* e diluídas em 500 µl de ácido acético 0,1N em HCl 0,1M e aplicadas em uma coluna de fenil-sepharose (1 ml). A porção glicana foi eluída usando-se a mesma solução (3 ml). Em seguida a coluna foi lavada com 2 ml de água e as porções lipídicas e GIPLs não delipidados foram eluídos com Solvente E (H₂O:etanol:dietil eter:piridina:NH₄OH 15:15:5:1:0.017 v/v) (Orlandi & Turco, 1987).

4.8. Filtração em gel

Para retirar o sal da preparação, colunas Sephadex G-25 (1 cm X 5 cm) foram equilibradas com 10ml de água. As amostras (oligossacarídeos desaminados) foram aplicadas na

coluna e eluídas com 5 ml de água. Foram coletadas frações de 0,5ml e a presença de sal foi avaliada adicionando uma gota de Nitrato de prata à uma alíquota de cada fração. As frações dessalinizadas foram combinadas e secas em centrífuga evaporadora (Soares *et al.*, 2002).

4.9. Hidrólise ácida forte

Para estudo da composição de monossacarídeos, as porções glicídicas dos GIPLs, obtidas por desaminação, foram submetidas a hidrólise ácida forte (ácido trifluoroacético a 2N, 3h a 100°C) e secas em *speed-vac*. Para remover o ácido, foram adicionados ao material seco 500µl de tolueno, o material foi homogeneizado em vórtex e evaporado sob N₂. O processo foi repetido 1 vez. As amostras foram então dissolvidas em 500µl de água e o sal foi removido por cromatografia de troca iônica (Soares *et al.* 2002).

4.10. Cromatografia de troca iônica

Para remoção do sal dos monossacarídeos neutros, as amostras dissolvidas em 500 µl de água foram aplicadas em uma coluna contendo a resina AG1-X8 sobre a resina AG50W-X12 (cerca de 500 µl de cada). As amostras foram eluídas com 5 ml de água e secas em centrífuga evaporadora (Coelho-Finamore *et al.*, 2011).

4.11. Cromatografia em camada delgada (CCD)

Os GIPLs foram separados por cromatografia em camada delgada (CCD) em placas de Sílica Gel 60 (Merck). Os GIPLs intactos foram cromatografados utilizando-se o solvente 1-butanol:metanol:água (4:4:3 v/v) por 20 h (ou até atingir o topo da placa). Os GIPLs delipidados foram cromatografados em clorofórmio:metanol: hidróxido de amônio 13M: acetato de amônio

1M:água (180:140:9:9:23 v/v) por 20 h (ou até atingir o topo da placa). As amostras foram visualizadas como descrito no item 4.5 (Schneider *et al.*, 1993; Orlandi & Turco, 1987).

4.12. Eletroforese de carboidratos marcados por fluoróforos (FACE)

Para análise de oligossacarídeos, as amostras foram marcadas com ANTS 0,05M (8-aminonaftaleno-1,3,6-trissulfato) em ácido acético 5% e cianoborohidreto de sódio 1M em Tetrahidrofurano (THF) (37°C por 16h). Para análise dos monossacarídeos, as amostras foram marcadas com AMAC 0,1M (2-aminoacridona) em ácido acético 5% e cianoborohidreto de sódio 1M em THF.

A separação dos oligossacarídeos foi realizada por eletroforese em gel de resolução (19% acrilamida-1% bis-acrilamida em Tris-HCl 0,18M, pH 8,9) sob um gel de concentração (5% acrilamida-1,25% bis-acrilamida em Tris-HCl 0,125M, pH 6,8). O tampão de corrida, foi composto por Tris-base 0,025M, Glicina 0,192M, pH 8,3.

Para os monossacarídeos a eletroforese foi realizada como descrito acima, entretanto, o gel de resolução utilizado continha: 19% acrilamida-1% bis-acrilamida em Tris-HCl 0,5M, ácido bórico 0,5M, pH 7,0 e o gel de concentração continha: 5% acrilamida-1,25% bis-acrilamida em Tris-HCl 0,5M, ácido bórico 0,5M, pH 6,8. O tampão de corrida também foi modificado para glicina 0,1M, Tris-base 0,12M e ácido bórico 0,1M, pH 8,3.

Os açúcares submetidos a FACE foram visualizados sob Luz UV. Um padrão de oligoglicoses (G1 a G7) foi usado para se estimar o tamanho dos oligossacarídeos e um padrão contendo 0,5 µg/ml de monossacarídeos D-galactose, D-glicose e D-manose (Sigma) foi usado para a determinação da composição dos açúcares, respectivamente (Coelho-Finamore *et al.*, 2011; Soares *et al.*, 2004).

4.13. HPLC

Monossacarídeos dessalinizados foram separados utilizando o sistema DX-500 HPLC (Dionex Corp). Foi utilizado o detector eletroquímico ED40 para detecção dos picos. As amostras foram separadas utilizando a coluna CarboPac PA10 (4 mm X 250 mm) na presença de NaOH 18mM (fluxo contínuo de 1ml/min a 2000 psi). D-galactose, D-glucose and D-mannose (100 µg/mL) foram usados como padrão.

4.14. Análise estatística

Para a médias de citocinas e nitrito, foi realizado o teste de Shapiro-Wilk para análise da hipótese nula da distribuição Gaussiana (Shapiro & Wilk, 1950; 65) foi considerado o valor $P > 0,05$ para rejeição da hipótese de que as amostras variavam da distribuição Gaussiana. Neste cenário, foi realizado o teste “t de Student” e teste de variância ANOVA para análise das médias entre amostras independentes e população, respectivamente. Dados foram analisadas utilizando o programa GraphPad Prism 5.0 (Graph Prism Inc., San Diego, CA) e o valor de $P < 0,05$ foi considerado significativo.

5. Resultados / Artigos publicados

5.1. Artigos publicados

5.1.1. Primeiro artigo:

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Glycoinositolphospholipids from *Leishmania braziliensis* and *L. infantum*: Modulation of Innate Immune System and Variations in Carbohydrate Structure

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Abstract

The essential role of the lipophosphoglycan (LPG) of *Leishmania* in innate immune response has been extensively reported. However, information about the role of the LPG-related glycoinositolphospholipids (GIPLs) is limited, especially with respect to the New World species of *Leishmania*. GIPLs are low molecular weight molecules covering the parasite surface and are similar to LPG in sharing a common lipid backbone and a glycan motif containing up to 7 sugars. Critical aspects of their structure and functions are still obscure in the interaction with the vertebrate host. In this study, we evaluated the role of those molecules in two medically important South American species *Leishmania infantum* and *L. braziliensis*, causative agents of visceral (VL) and cutaneous Leishmaniasis (CL), respectively. GIPLs derived from both species did not induce NO or TNF- α production by non-primed murine macrophages. Additionally, primed macrophages from mice (BALB/c, C57BL/6, TLR22/2 and TLR42/2) exposed to GIPLs from both species, with exception to TNF- α , did not produce any of the cytokines analyzed (IL-1-b, IL-2, IL-4, IL-5, IL-10, IL-12p40, IFN- γ) or p38 activation. GIPLs induced the production of TNF- α and NO by C57BL/6 mice, primarily via TLR4. Pre incubation of macrophages with GIPLs reduced significantly the amount of NO and IL-12 in the presence of IFN- γ or lipopolysaccharide (LPS), which was more pronounced with *L. braziliensis* GIPLs. This inhibition was reversed after PI-specific phospholipase C treatment. A structural analysis of the GIPLs showed that *L. infantum* has manose rich GIPLs, suggestive of type I and Hybrid GIPLs while *L. braziliensis* has galactose rich GIPLs, suggestive of Type II GIPLs. In conclusion, there are major differences in the structure and composition of GIPLs from *L. braziliensis* and *L. infantum*. Also, GIPLs are important inhibitory molecules during the interaction with macrophages.

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Introduction

In the Americas, Leishmaniasis are widely distributed from the southern United States to northern parts of Argentina [1]. In Latin America, especially in Brazil, *Leishmania braziliensis* and *Leishmania infantum* are the causative agents of cutaneous (CL) and visceral leishmaniasis (VL), respectively. The severity of the disease may range from self-healing cutaneous ulcers to potentially lethal visceral form [2].

During the life cycle, *Leishmania* parasites have to survive to extreme adverse conditions in both vertebrate and invertebrate hosts [3]. In the vertebrate host, inoculation of metacyclic *Leishmania* promastigotes by the sand fly is followed by neutrophil phagocytosis prior to intracellular differentiation into amastigotes [4]. At the early steps of infection, innate cellular microbicidal mechanisms may include the production of reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI) and cytokines (IL-12, TNF- α and IFN- γ) [5,6]. This is crucial for Th1 polarization and subsequent parasite control in the mouse model.

Failure in this process can lead to higher parasite burden and increase severity of disease [7].

To avoid destruction, intracellular parasites must interfere with the cytotoxic signaling system of the host. In vivo and in vitro studies have demonstrated the importance of nitric oxide (NO) production in response to several stimuli such as bacterial lipopolysaccharide (LPS), IFN- γ and TNF- α [8]. It is known that *Leishmania*-infected macrophages fail to activate MAPKs, become less responsive to cytokine stimulation (IL-12 and IFN- γ) [9,10,11] and express lower amounts of iNOS and IL-12 [12,13], impairing T CD4⁺ cell differentiation to a TH1 phenotype.

The molecular mechanisms involved in the immune system modulation by *Leishmania* have been the focus of many studies. GPI-anchored molecules are closely associated with cell signaling and can act as agonists and second messengers in response to cytokines and other stimuli [9,14,15,16]. The most studied *Leishmania* glycoconjugate is lipophosphoglycan (LPG), whose functions include: attachment and entry into macrophages [17], modulation of NO production [18], inhibition of protein kinase C

Author Summary

Leishmania infantum (syn. *L. chagasi*) and *L. braziliensis* are the causative agents of VL and CL, respectively, in the New World. A vital part of the parasite's life cycle involves the circumvention of the host immune system and the infection of macrophages. This work focused on an important class of surface glycoconjugates, the glycoinositolphospholipids (GPIs), and their role in the interaction with murine macrophages. GPIs are expressed on every stage of the parasite life cycle and are the most abundant molecules on its surface. Here we show that these molecules modulate many macrophage functions such as cytokine production, release of nitric oxide and differentially activate MAPK. Although the GPIs of both New World species are capable of modulating the same mechanisms, they do so to different degrees requiring an examination of their glycan composition. We show that *L. infantum* synthesizes mannose rich GPIs whereas *L. braziliensis* express galactose rich GPIs.

(PKC) dependent cell activation [19,20], retardation of phagosome maturation [21], disruption of NADPH oxidase assembly at the phagosome membrane [22], induction of neutrophil extracellular traps (NETs) [23], induction of protein kinase R (PKR) [24], and attachment to the sand fly vector midgut [25]. In *Leishmania*, Toll-like receptor 2 (TLR2) is the main receptor for both LPG and glycoinositolphospholipids (GPIs), the latter as a less potent agonist [26,27]. Besides TLR2, *in vivo* studies have also demonstrated the importance of TLR4 and TLR9 during *Leishmania* infection [28,29,30].

Little is known about the functions of GPIs in *Leishmania* biology, although they are present as the major component of the parasite surface in numbers greater than LPG [31]. The basic GPI structure is a Mana1-4GlcN linked to an alkyl-acylglycerol through a phosphatidylinositol (PI) residue. Polymorphism in this family of molecules relies on the variety of fatty acid substitutions in the lipid anchor and monosaccharide substitutions in the glycan core moiety, leading to their classification into three groups (Figure 1): Type-I GPIs are characterized by having an α 1,6-mannose residue linked to the Mana1-4GlcN motif. This group is represented by M2 and M3 GPIs which structures are Mana1-6Mana1-4GlcN-PI and Mana1-2 Mana1-6Mana1-4GlcN-PI. Type I GPIs are closely related to GPI anchors of proteins with a very homogeneous lipid composition, predominantly C_{18:0}, C_{22:0}, C_{24:0} and C_{26:0} fatty acids. They can be found in Old World *L. major* [33,34] and New World *L. mexicana* [35,36] and *L. panamensis* [36]. Type II GPIs are characterized by having an α 1,3-mannose residue linked to the Mana1-4GlcN motif, similarly to the glycan core of LPG. Structurally, they can range from small iM2 GPI, Mana1-3Mana1-4GlcN-PI, to longer structures like GPI-A, Gal β 1-3Gala1-3Gal β 1-3Mana1-3Mana1-4GlcN-PI and GPI-3, Gala1-6Gala1-3Gal β 1-3Mana1-3Mana1-4GlcN-PI. The third group is the Hybrid-type GPIs, sharing common features to both Type-I and II with mannose residues located on both C-3 and C-6 positions of the Mana1-4GlcN motif (isoM3 and isoM4). There may be also other substitutions like phosphate sugars and ethanolamine residues [35,37]. Early studies have shown that GPIs from *L. major* were highly antigenic, being recognized by sera from chronic CL patients [38]. Recent findings have demonstrated that *L. braziliensis* GPIs are components of complex

membrane microdomains and that these structures were crucial for parasite infectivity and survival [39]. However, little is known about the role of GPIs in the innate immune compartment, especially in *L. braziliensis* and *L. infantum*.

This work is part of a wider study on the glycobiology of New World species of *Leishmania*. In previous studies, we reported on the LPGs of *L. braziliensis* and *L. infantum* [40,41] and showed that the differences in LPG structures were relevant in the parasite biology. In this study, we expanded those findings and show the GPI structures of the two New World *Leishmanias* also differentially modulate the innate immune system in mouse peritoneal macrophages.

Materials and Methods

All animals were handled in strict accordance with good animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CEUA) of Fundação Oswaldo Cruz (FIO-CRUZ), Belo Horizonte (BH), Minas Gerais (MG), Brazil (Protocol P-0297-06). Knock-out mice handling protocol was approved by the National Commission of Biosafety (CTNBio) (protocol #01200.006193/2001-16).

Parasites

World Health Reference strains of *L. braziliensis* (MHOM/BR/1975/M2903), *L. infantum* (MHOM/BR/1974/PP75) and *L. donovani* (MHOM/SD/00/1S-2D) were used. Promastigotes were cultured in M199 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin 100 units/ml, streptomycin 50 mg/ml, 12.5 mM glutamine, 0.1 M adenine, 0.0005% hemin, and 40 mM Hepes, pH 7.4 at 26°C [40].

Extraction and purification of GPIs

Cells were harvested and washed in PBS twice prior to GPIs extraction with methanol:chloroform:water (10:10:3). This material was dried under nitrogen stream, resuspended on 0.1 M ammonium acetate buffer containing 5% 1-propanol and loaded onto an octyl-sepharose column (80 ml) equilibrated in the same buffer. The column was subjected to a gradient of 1-propanol in 0.1 M ammonium acetate buffer (5–60%). Three mL fractions were collected and the presence of GPIs in the fractions was detected by staining aliquots of the fractions on a TLC plate with orcinol:sulfuric acid (100°C, 5 min) [34]. GPIs containing fractions were pooled, dried and resuspended in endotoxin-free water (Sanobiol, São Paulo, Brazil). GPIs concentrations determined as described elsewhere [42]. Prior to use on *in vitro* macrophage cultures, GPIs were diluted in fresh RPMI.

Purification of murine peritoneal macrophages and cell culture

Thioglycollate-elicited peritoneal macrophages were removed from BALB/c, C57BL/6 and respective TLR22/2 and TLR42/2 knockouts by peritoneal washing with RPMI and enriched by plastic adherence for 18 h. Cells (3–6 $\times 10^5$ cells/well) were cultured in RPMI, 2 mM glutamine, 50 U/ml of penicillin and 50 mg/mL streptomycin in 96-well culture plates (37°C/5% CO₂). They were incubated with gamma interferon (IFN- γ) (100 IU/mL) [43], live stationary *Leishmania* parasites (10:1), GPIs (1, 5, 10 and 25 mg/mL) and lipopolysaccharide (LPS) (100 ng/mL).

Cytokine and nitrite measurements

For CBA multiplex cytokine detection, cells were plated as described above for 1 h before washing with RPMI without

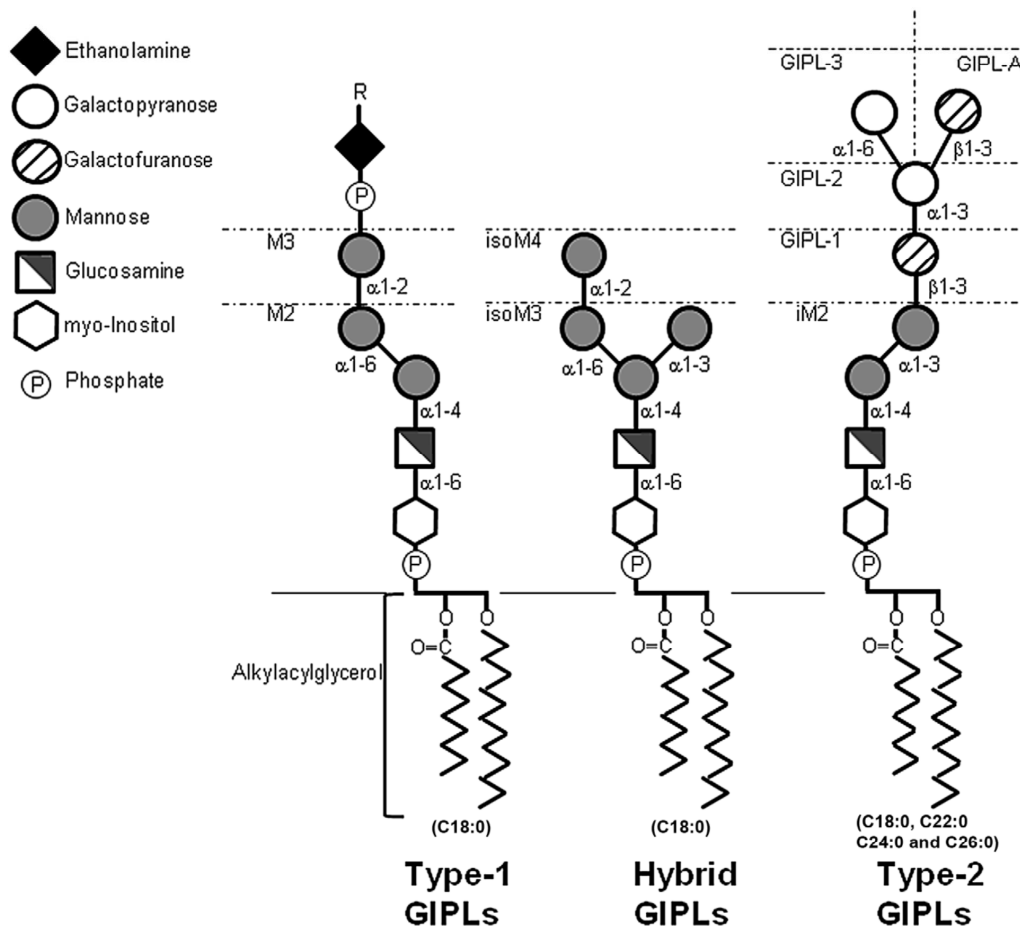


Figure 1. Types of GIPLs. For information on M2, M3, iM2, GIPL-A, isoM3 and isoM4, see introduction. Fatty acid chains vary in different GIPL species: The predominant type fatty acid in Type-1 and Hybrid GIPLs is C_{18:0}, in type-2 GIPLs the predominant lipids are C_{18:0}, C_{22:0}, C_{24:0} and C_{26:0}. "R" in Type 1 GIPLs represent a protein linked to the GIPL structure by a ethanolamine phosphate residue (e.g. gp63 surface metalloprotease) [31,78]. doi:10.1371/journal.pntd.0001543.g001

serum. RPMI supplemented with 10% FBS was added with (for primed macrophages) or without (for non-primed macrophages) the addition of IFN- γ (3 IU/mL) [44] and incubated for 18 h (37°C, 5% CO₂). GIPLs (25 mg/mL) and LPS (100 ng/mL) were added and incubated for 48 h. Supernatants were collected and stored at 270°C and cytokines (IL-1- β , IL-2, IL-4, IL-5, IL-10, IL-12p40, IFN- γ and TNF- α) were determined using the BD CBA Mouse Cytokine assay kits according to the manufacturer's specifications (BD Biosciences, CA, USA). Flow cytometric measurements were performed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). 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, USA) was used for data analysis. A total of 1,800 events were acquired for each preparation. Results are representative of two experiments in duplicate.

For inhibition studies, cell suspensions were washed with RPMI and enriched by plastic adherence for 18 h as described

above without the addition of IFN- γ . Cells were pre-incubated with GIPLs (15 min) prior to stimulation with LPS or IFN- γ . Supernatants were collected after 24 h for NO, TNF- α and IL-12 measurements. When used, LPS or IFN- γ were added 15 min after the addition of GIPLs. Culture supernatants were collected and nitrite concentrations determined by Griess reaction [45] and TNF- α and IL-12 concentrations were determined using ELISA (BD). Results are representative of two experiments in triplicate.

PI-specific phospholipase-C treatment (PI-PLC)

To evaluate whether intact GIPL structure is required for activity. Purified GIPLs were resuspended in 150 ml CHAPS buffer (298 mg HEPES, 47 mg EDTA and 50 mg CHAPS in 50 ml endotoxin-free water) and 2 U of PI-PLC (Sigma) (37°C, 16 h). Peritoneal macrophages were plated and stimulated with intact and PI-PLC treated GIPLs as described above. Nitrite content was measured on the supernatants by Griess reaction [45].

Preparation of cell lysates and immunoblotting

Stimulated cells (3×10^6 /sample) were washed with ice-cold PBS, lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 10% Glycerol (v/v), 0.5 mM dithiothreitol (DTT) and protease inhibitor cocktail from SigmaH). Cells were harvested with a plastic scraper and centrifuged at 13,000g (4°C, 10 min). Supernatants were transferred to fresh tubes and stored at -220°C until used. Cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and blocked (5% milk in TBS-0.1% Tween 20) for 1 h. Primary Abs (anti dually phosphorylated ERK, dually phosphorylated p38 and Total ERK, 1:1,000) 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 anti-mouse IgG conjugated with peroxidase (1:10,000). The reaction was visualized using luminol.

Nitrous acid deamination

Purified GIPLs were delipidated by nitrous acid deamination (300 ml of 0.5 M sodium acetate and 300 ml of 0.5 M NaNO_2) for 16 h at 37°C [40]. Samples were dried, resuspended in 0.1N HAc/0.1M HCl and applied to a phenyl-sepharose column (1 mL). The sugar headgroups were eluted using 0.1N HAc/0.1M HCl. After washing column with 2 volumes of water, lipids and unreacted GIPLs were eluted using Solvent E (H_2O /ethanol/diethyl ether/pyridine/ NH_4OH ; 15:15:5:1:0.017) [46].

Gel filtration

To desalt, deaminated GIPLs glycan headgroups were applied to Sephadex G-25 (165 cm) columns equilibrated with 10 ml of water. Eluted deaminated glycan headgroups were collected in 0.5 ml fractions, checked for the presence of salt using silver nitrate and dried in Speed-Vac [40].

Strong acid hydrolysis

To obtain depolymerized neutral monosaccharides, deaminated glycan headgroups were subjected to strong acid hydrolysis (2N trifluoroacetic acid, 3 h, 100°C) and dried in Speed-Vac. To remove acid, 500 ml of toluene were added to samples, homogenized using vortex and evaporated twice under N_2 . Samples were resuspended in 500 ml of water and desalted by ion exchanging chromatography.

Ion exchange chromatography

To remove salt from neutral monosaccharides, dried depolymerized neutral monosaccharides were diluted in 500 ml of H_2O and applied onto a column containing AG1-X8 acetate form over AG50W-X12 resins. Samples were eluted with 5 mL of water and dried in a Speed-Vac instrument [47].

Thin layer chromatography (TLC)

Intact and deaminated GIPLs were chromatographed on TLC Silica Gel 60 plates (Merck). To compare rough GIPL content of *L. braziliensis*, *L. infantum* and as reference *L. donovani*. Intact GIPLs were chromatographed in 1-butanol:methanol:water (4:4:3 v/v) for 20 h. To access Deamination by nitrous acid sensitivity, GIPLs were subjected to nitrous acid deamination as described above and resolved in chloroform:methanol:13M ammonium hydroxide:1M ammonium acetate:water (180:140:9:23 v/v) for 20 h. Bands were visualized as described above [46,48].

Fluorophore-assisted carbohydrate electrophoresis (FACE)

To access the oligosaccharide composition, deaminated GIPLs headgroups were fluorescently labeled with 0.05 N ANTS (8-aminonaphthalene-1,3,6-trisulfate) and 1 M cyanoborohydride (37°C, 16 h). To determine the monosaccharide composition of the GIPLs, depolymerized and desalted monosaccharides were fluorescently labeled with 0.1 M AMAC (2-aminoacridone) in 5% acetic acid and 1 M cyanoborohydride. Labeled sugars were subjected to FACE and the gel was visualized under UV light. Oligoglucose ladders (G_1 – G_7) and monosaccharides (D-galactose, D-glucose and D-mannose) (Sigma) were used as standards for oligosaccharides and monosaccharide gels, respectively [47,49].

HPLC

Desalted monosaccharides were separated using a DX-500 HPLC (Dionex Corp.) with ED40 electrochemical detection. Samples were run on a CarboPac PA10 column (46250 mm) in the presence of 18 mM NaOH (flow rate 1 mL/min, 2000 psi). D-galactose, D-glucose and D-mannose (100 mg/mL) were used as standards.

Statistical analyses

For nitrite and cytokine measurements, the Shapiro–Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution [50]. 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. Data were analysed using GraphPad Prism 5.0 software (Graph Prism Inc., San Diego, CA) and $P < 0.05$ was considered significant.

Results

Nitrite and cytokine production

To determine whether GIPLs from both *L. braziliensis* and *L. infantum* are able to induce the production of nitrite, peritoneal macrophages were incubated with live promastigotes (10:1) or treated with different concentrations of GIPLs (1 to 25 mg/mL) with IFN- γ serving as positive control (100 IU/mL). Neither of the purified GIPLs could induce any detectable increase in the production of nitric oxide (NO) in primed BALB/c macrophages (Figure 2) nor the production of the cytokines tested (IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p40, IFN- γ and TNF- α) in non-primed macrophages in all other mice lineages (data not shown). No NO production was detected in non-primed macrophages of BALB/c, C57BL/6, TLR2 Δ 2/2 and TLR4 Δ 2/2 mice (data not shown) and in BALB/c primed macrophages (Figure 3A). A higher NO production was detected on C57BL/6 IFN- γ -primed macrophages stimulated with GIPLs and live promastigotes when compared to BALB/c mice ($P < 0.001$). There was a significant NO production in primed C57BL/6 and TLR2 (2/2) macrophages stimulated with GIPLs in comparison to TLR4 (2/2) ($P < 0.01$) (Figure 3A) suggesting the involvement of TLR4 in this activation. Also, a slight reduction of NO production was noticed in macrophages from TLR2 (2/2) mice stimulated with live promastigotes when compared to C57BL/6 ($P < 0.04$). This reduction may indicate the participation of other parasite molecules that are recognized by TLR2 such as the LPG. The LPG is known to be a potent agonist of TLR2 and is capable of inducing the production of cytokines (IL-12, IFN- γ and TNF- α) in macrophages and NK cells [26,27]. Differently from NO, TNF- α production was higher in BALB/c

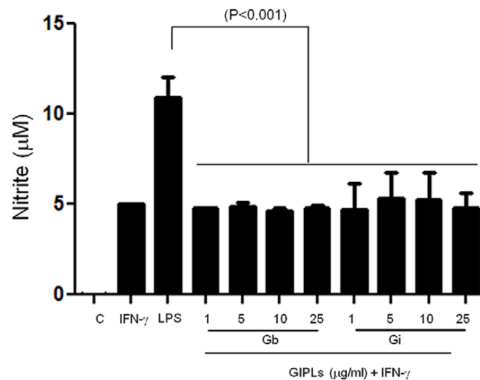


Figure 2. Nitrite production by BALB/c primed macrophages after stimulation with different concentrations of GIPLs. C, negative control; IFN- γ , gamma-interferon; LPS, lipopolysaccharide; Gb, *L. braziliensis* GIPLs; Gi, *L. infantum* GIPLs. Cells were primed with IFN- γ (3 IU/ml) for 18 h prior to the addition of the GIPLs or LPS (positive control). Non primed cells and primed cells without the addition of a new stimulus were also used as controls. ANOVA test was performed and $P_{0.05}$ was considered significant. Results are the representation of three experiments in triplicate.
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mice than in C57BL/6 ($P_{0.05}$) in response to the stimulation of GIPLs from both species. Similarly this production was higher in TLR2 (2/2) than TLR4 (2/2) ($P_{0.02}$). This data also indicate

a slight TLR4 involvement in TNF- α production. In both WT macrophages, the TNF- α production was higher after stimulation with GIPLs in comparison to live promastigotes (Figure 3B) ($P_{0.01}$). A lower TNF- α production was noticed in TLR2 (2/2) suggesting the involvement of TLR2 in this process.

GIPLs did not induce the production of any of the cytokines tested (IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p40 and IFN- γ) in BALB/c, C57BL/6, TLR2 (2/2) and TLR4 (2/2) mice (data not shown). In all experiments, live parasites from both species induced cytokine production close to background levels (Figure 3B and data not shown). These results suggest that GIPLs are able to activate NO in C57BL/6 mice and TNF- α in either BALB/c or C57BL/6 during the early steps of infection, and were not able to stimulate most of the cytokines assayed.

Inhibition of nitrite and IL-12 production in BALB/c macrophages pre-exposed to GIPLs

Compared to LPG, GIPLs had a less potent agonistic activity to stimulate nitrite and cytokine production in previous studies [27]. To test if this pattern was due to inhibition and/or lack of activation, thioglycollate elicited peritoneal macrophages were pre-incubated with GIPLs prior to stimulation with IFN- γ or LPS. A strong inhibition (approx. 42%) of NO production stimulated by IFN- γ was observed for *L. infantum* GIPLs and was almost completely abolished for *L. braziliensis* ($P_{0.01}$) (Figure 4A). A similar response was observed for LPS and this inhibition was more pronounced in *L. braziliensis* ($P_{0.001}$) (Figure 4B). Pre-incubation with GIPLs was also able to inhibit approximately 65% of IL-12, but not TNF- α production (Figures 4C and D). These results indicate an inhibitory role of GIPLs.

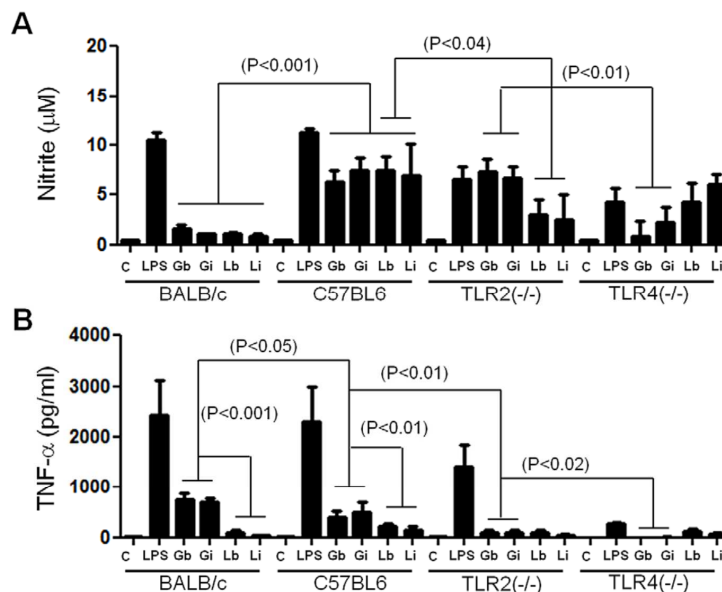


Figure 3. Nitrite and TNF- α production by primed macrophages after stimulation with GIPLs and parasites. C, negative control; Gb, *L. braziliensis* GIPLs; Gi, *L. infantum* GIPLs; Lb, *L. braziliensis* live promastigotes and Li, *L. infantum* live promastigotes. Cells were pre-incubated with IFN- γ (3 IU/ml) for 18 h then 25 mg/mL of GIPLs or 100 ng/mL of LPS was added. Supernatants were collected 48 hours later, in (A) NO concentrations were measured by Griess reaction and in (B) TNF- α concentrations determined by flow cytometry. ANOVA test was performed and $P_{0.05}$ was considered significant.
doi:10.1371/journal.pntd.0001543.g003

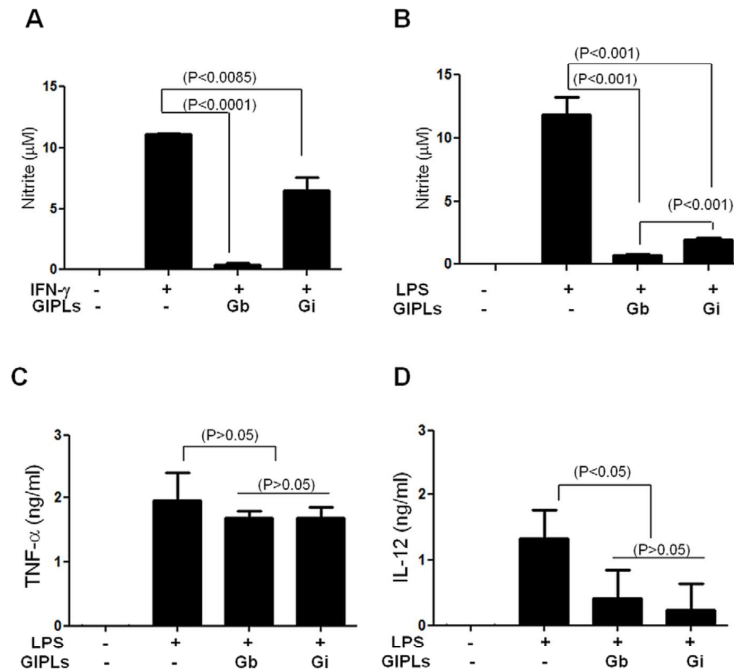


Figure 4. Modulation of nitrite, TNF- α and IL-12 production by *Leishmania* GIPLs in BALB/c macrophages. Cells were incubated with GIPLs (25 mg/ml) from *L. braziliensis* (Gb) and *L. infantum* (Gi) for 15 min prior to stimulation with IFN- γ (100 IU/ml) (A) or LPS (100 ng/mL) (B). Nitrite content was measured by Griess reaction; TNF- α and IL-12 concentrations were measured by ELISA. P, 0.05 was considered significant. Results are the representation of three experiments.
doi:10.1371/journal.pntd.0001543.g004

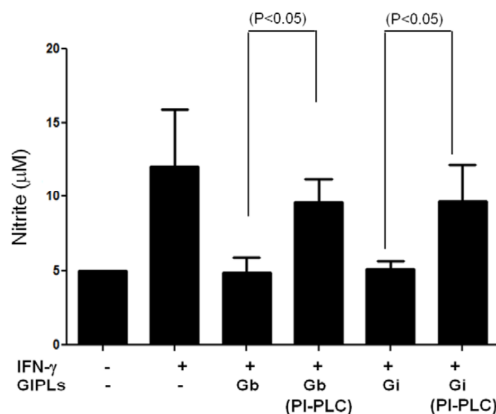


Figure 5. Modulation of nitrite production by macrophages stimulated with intact and PI-PLC treated GIPLs. Mouse peritoneal macrophages were incubated with GIPLs (25 mg/ml) from *L. braziliensis* (Gb), *L. infantum* (Gi), PI-PLC treated *L. braziliensis* GIPLs (Gb PI-PLC) and *L. infantum* PI-PLC treated GIPLs (Gi PI-PLC) for 15 min prior to stimulation with IFN- γ (100 IU/ml). Nitrite content was measured by Griess reaction on the supernatants after 24 h. Student "t" test was performed and P, 0.05 was considered significant. Results are the mean of two experiments.
doi:10.1371/journal.pntd.0001543.g005

Also, to test whether the intact structure of GIPLs is required for its inhibitory activity Macrophages were incubated with intact and PI-PLC treated GIPLs. As shown on Figure 5 PI-PLC treated GIPLs failed to inhibit NO production by IFN- γ stimulated cells.

Activation of MAPKs

Since GIPLs were strong inhibitors of cytokine production, we investigated whether those molecules could modulate MAPKs activation. Mouse peritoneal macrophages were previously incubated with GIPLs and MAPK activation was detected using western blot. No significant activation of p38 and only a minimal induction of ERK were observed. Also when cells were preincubated with GIPLs prior to stimulation with LPS, there was a reduction on the phosphorylation of both ERK and p38 (Figure 6). Densitometer analysis normalized by total-ERK expression detected an 18% and 17.5% decrease on ERK activation for *L. braziliensis* and *L. infantum*, respectively. For p38 this inhibition was 16.5% and 33%, respectively.

Preliminary characterization of *L. braziliensis* and *L. infantum* GIPLs

Due to the interspecific differences in the intensity of NO and IL-12 production inhibition (Figures 4) and MAPKs activation (Figure 6), we examined whether those variations could be due to polymorphisms in GIPLs structure and composition. Intact GIPLs were resolved on TLC plates and the GIPL profile differed between the two species (Figure 7A). *Leishmania braziliensis* exhibited slower migrating GIPLs compared to *L. infantum*, whose

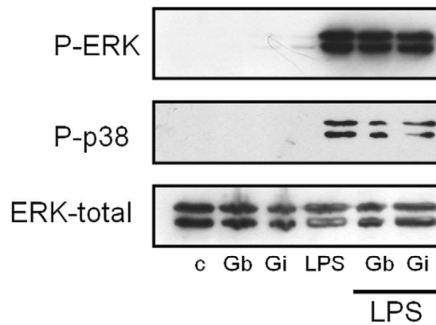


Figure 6. Activation of MAPKs (ERK and p38) by *Leishmania* GIPLs in BALB/c peritoneal macrophages. Mouse peritoneal macrophages were stimulated for 30 min with 25 mg/mL of GIPLs. Dually phosphorylated MAPKs were detected by western blot. C, negative control; Gb, *L. braziliensis* GIPLs and Gi, *L. infantum* GIPLs. Also cells were incubated with GIPLs prior to stimulation with LPS; total ERK content as a normalizing protein.
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profile was very similar to *L. donovani* [32] with three main bands co-migrating with isoM2, isoM3 and isoM4. In *L. braziliensis*, the three faster bands co-migrated with bands isoM2, isoM3 and isoM4 of *L. donovani*. All bands were susceptible to nitrous acid deamination, and this is consistent with the presence in the GIPLs of a non-N-substituted glucosamine residue (Figure 7B), a hallmark of *Leishmania* GIPLs anchors [51].

To better determine sizes of the glycan portions, purified GIPLs were deaminated and desalted. The carbohydrate portions were reductively labeled with a fluorophore and then subjected to FACE. Consistent with the TLC data (Figure 7), the carbohydrate portions of the GIPLs from *L. braziliensis* were larger exhibiting up to 8–9 sugars while those from *L. infantum* and *L. donovani* consisted of up to 4–5 sugars (Figure 8).

To access sugar composition, GIPLs were subjected to strong acid hydrolysis and the resulting monosaccharides were analysed

by FACE and HPLC (Figure 9A and B). Consistent with the TLC data (Figure 7A), the monosaccharide composition of *L. infantum* GIPLs was very similar to the GIPLs from *L. donovani* (Figure 9A). The relative amounts of galactose, glucose and mannose (calculated by the relative peak areas on HPLC) were determined (Figure 9B). Supporting our other findings and GIPL assignments, the GIPLs from *L. infantum* had higher concentrations of mannose (82%), followed by galactose (12%) and glucose (6%). This indicates that these are mostly Type I or hybrid GIPLs, whose structure bears a terminal mannose, but a small proportion of Type II GIPLs (terminated in galactose) is probably present. On the other hand, *L. braziliensis* GIPLs had higher galactose content (42%), followed by, mannose (30%) and glucose (28%), thus suggesting a Type II GIPL structure.

Discussion

Infection with protozoan parasites remains a prominent problem in different parts of the world having a major impact on public health in the developing countries. Leishmaniasis are considered by World Health Organization [52] as one of the major six important infectious diseases worldwide. This class of parasitic diseases currently affects over 12 million people all around the world, up to 1.5 million new individuals developing the visceral and tegumentar disease respectively each year. In Brazil, most of those cases are caused by *L. infantum* and *L. braziliensis*, respectively.

The question of how parasites interact with hosts cells to promote infection and survival has been the focus of interest for a long time. In order to survive in the macrophage cells, *Leishmania* has to prevent or inhibit a variety of intracellular mechanisms of parasite killing, one of which is dependent on ROS and RNI [53,54]. However, RNI alone is effective for controlling visceral Leishmaniasis [55].

Parasite surface molecules, especially the LPG, have long been known to play an important role in the host parasite interactions [17,27,56]. In this work, we focused on another class of glycoconjugates, the GIPLs in two New World species of *Leishmania* with different known immunopathologies. These molecules are abundantly present on the parasite surface in

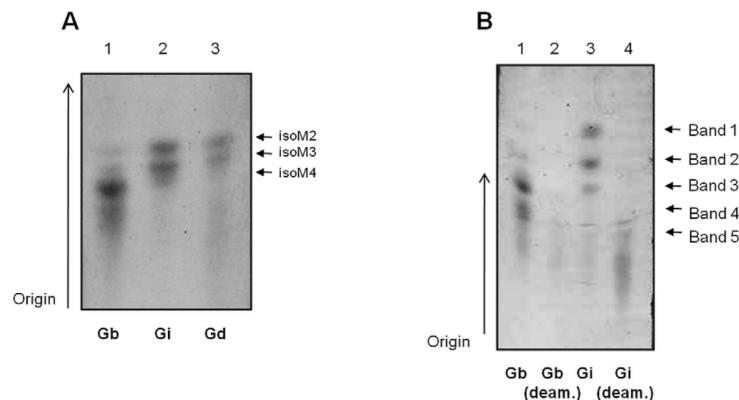


Figure 7. Thin layer chromatography (TLC) of *Leishmania* glycoinositolphospholipids (GIPLs). (A) Purified intact GIPLs: Lane 1, *L. braziliensis* GIPLs (Gb); lane 2, *L. infantum* GIPLs (Gi) and lane 3, *L. donovani* GIPLs (Gd). The assignments for *L. donovani* structures are: isoM2 as Mana1-3Mana1-4GlcN-PI; isoM3 as Mana1-6(Mana1-3)Mana1-4GlcN-PI and isoM4 as Mana1-2Mana1-6(Mana1-3)Mana1-4GlcN-PI [32]. LPG, lipophosphoglycan; GPI, glycosyl phosphatidylinositol. (B) Deaminated GIPLs. Lane 1, *L. braziliensis* untreated GIPLs (Gb); lane 2, deaminated *L. braziliensis* GIPLs (Gb deam.); lane 3, *L. infantum* untreated GIPLs (Gi) and Lane 4, deaminated *L. infantum* GIPLs (Gi deam.).
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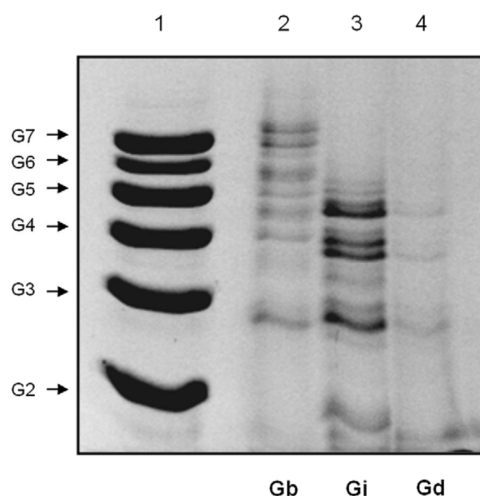


Figure 8. Fluorophore-assisted carbohydrate electrophoresis (FACE) of *Leishmania* GPIs. Lane 1, oligoglucose ladder represented by G2-G7; lane 2, *L. braziliensis* GPIs (Gb) and lane 3, *L. infantum* GPIs (Gi) and lane 4, *L. donovani* GPIs (Gd).
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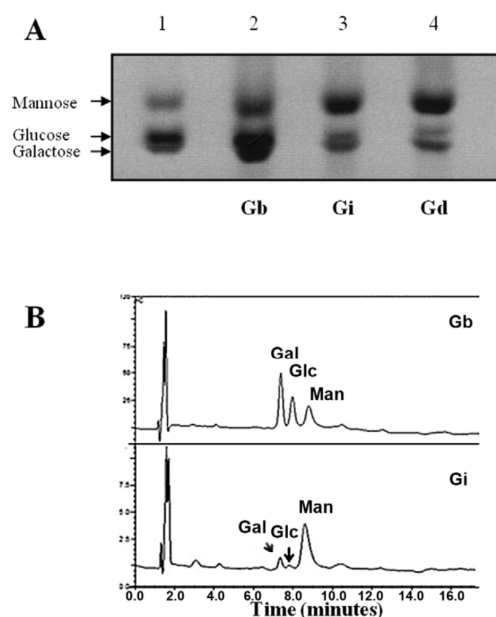


Figure 9. Monosaccharide profile of *Leishmania* glycoinositol-phospholipids (GPIs). (A) Fluorophore-assisted carbohydrate electrophoresis (FACE). Lane 1, standards represented by galactose, glucose and mannose (100 mg/ml); lane 2, *L. braziliensis* GPIs (Gb); lane 3, *L. infantum* GPIs (Gi) and Lane 4, *L. donovani* GPIs (Gd). (B) High performance liquid chromatography (HPLC). Gal, galactose; Glc, glucose and Man, mannose.
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numbers great than 10^7 . Recently, they have been found associated to lipid rafts, essential for parasite infectivity and selective modulation of the host cell response [39]. In fact, there are several indications that GPIs and other GPI-anchored molecules participate in cell signaling and are involved in the assembly of the NADPH oxidase complex, NO production [16,57,58,59,60] and inhibition of LPS and TNF- α induced c-fos gene expression by macrophages [61]. Also synthetic LPG, whose GPI anchor is structurally similar to GPIs, can stimulate ERK activation and therefore inhibit IL-12 synthesis by macrophages [9].

Previous studies have demonstrated GPIs antigenicity in chronic patients infected with *L. major* [38,62]. However, information concerning the biological relevance of GPIs at early steps of infection in the innate immune compartment was still limited. Here, we demonstrated that GPIs from both New World species were not able to activate the production of NO in non-primed macrophages, which was similar to published data from Old World species [59,60]. In primed macrophages an initial NO and TNF- α production was detected. Further, GPIs differentially inhibited NO production even in the presence of IFN- γ and LPS, two major NO inducers. Previous studies indicated that LPG was a more potent agonist than GPIs for the induction of pro-inflammatory cytokines [26,27]. In general, in comparison to LPS, GPIs induced a lower production of NO and TNF- α . Also, they exhibited a strong inhibitor pattern during NO and cytokine induction, especially IL-12.

Similar strategy was demonstrated using crude extracts of the rat tapeworm *Hymenolepis diminuta*, although using different pathways. As shown by Johnston et al. (2010) [63], crude extracts of this tapeworm could inhibit the production of TNF- α and IL-6 by mouse and human macrophages stimulated with TLR agonists poly(I:C) and Flagellin. These extracts also protected mice from experimental colitis accompanied by enhanced IL-10 and IL-4 production.

In vivo studies using Old World species of *Leishmania* have demonstrated the importance of TLRs and other components of the innate immune system during infection. MyD88 is the most common adaptor molecule for the activation of NF- κ B in most TLRs [28]. Also many studies using gene knockout have shown the importance of TLR and MyD88 adaptor molecule for cytokine production [29], IL-1 promoter activation [64], IFN- γ and IL-12 production [65].

NF- κ B activation through TLR2 [26], elastase dependent neutrophil control of *L. amazonensis* promastigotes [66], and ultimately parasite control and lesion healing [27,65,67].

Indeed, in primed macrophages, GPIs from both New World species were able to stimulate the production of NO, and this induction was mostly via TLR4 and to a lesser extent TLR2 (Figure 3A). However, no difference was observed while stimulating with live parasites.

Interestingly, in the *L. braziliensis* model, the TLR2 receptor plays a much more regulatory role in dendritic cells, repressing IL-12p40 and promoting IL-10 expression. This observation is correlated with sustained IFN- γ production and enhanced parasite control in TLR2 (2/2) mice [68]. However, in macrophages exposed to GPIs, this difference in NO expression between TLR2 (2/2) and TLR4 (2/2) strains was not due to IL-12, IFN- γ or IL-10 production (Figures 3B and 4). Also this induction was more pronounced in C57BL/6 than in BALB/c this was expected since C57BL/6 derived macrophages tend to be more responsive to stimuli than BALB/c macrophages [69]. These data are in accord with previous studies showing that related GPIs from *Trypanosoma cruzi* are able to activate TLR4 [70] and studies with

Old World species of *Leishmania* being able to activate TLR2, TLR3, TLR4 and TLR9 [28]. With exception to TNF- α , GPIs and live parasites from *L. braziliensis* and *L. infantum* were not able to induce the other cytokines studied (IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p40 and IFN- γ) in primed and non-primed macrophages (data not shown). Thus, we conclude that the GPIs from these two New World species are less potent agonists or strong inhibitors for macrophages and the data presented here supports that the later might be true.

When pre incubated with GPIs, a strong inhibition of both NO and IL-12 production was observed (Figures 4C and D). This inhibitory effect seems to be in specific pathways since no significant inhibition was detected for TNF- α (Figure 4C). This inhibition is dependent on the intact structure of GPIs since PI-PLC digested GPIs that have its glycan core detached from its lipid anchor, failed to inhibit NO production by IFN- γ stimulated macrophages (Figure 5). Also, regarding TNF- α , only WT mice were able to trigger the production of this cytokine and this production was very low for TLR2 (2/2) and completely absent in TLR4 (2/2) (Figure 3B). These data supports the premise that NF- κ B translocation is not affected by GPIs exposure [71]. It is noteworthy that the inhibition of IL-12 is not due to production of IL-10, because we observed no IL-10 production either in unprimed (data not shown) or in primed macrophages incubated with GPIs (Data not shown).

In TLR signaling, the most common adaptor molecule is MyD88 but other adaptor molecules may be involved in NF- κ B translocation such as mitogen-activated protein kinases (JNK or p38) [72]. Early studies showed that the *Leishmania* LPG can inhibit IL-12 without affecting NF- κ B translocation to the nucleus [9]. For maximal downstream activation and GPI-induced gene expression, a full activation and cooperation Protein Tyrosine Kinase (PTK) and Protein Kinase C (PKC) are required. Although iM4 *L. mexicana* GPIL stimulated rapid PTK phosphorylation it failed in activating PKC [16]. In fact the unusual glycolipid composition (mostly alkyl-acyl-glycerol) of *Leishmania* GPIs inhibits the activations of PKC [58,73]. This is in accordance with our observations that GPIs not only fail on inducing a pro-inflammatory response in non-macrophages but also that the GPIs inhibit the productions of IL-12 and NO.

Also we tested whether GPIs from both New World species were able to modulate the phosphorylation of MAPKs. We observed that the GPIs activate only ERK, whereas LPS activated both ERK and p38 (Figure 6). Also we observed that the GPIs can prevent the phosphorylation of both ERK and p38 MAPKs stimulated by LPS. However, ERK activation was too low to provide evidence for any further effect on IL-12 production. It is likely that *L. braziliensis* and *L. infantum* GPIs have a profound effect on macrophage cell signaling affecting PTKs, PKCs and MAPKs, and that GPIs from both species use similar pathways but differ in the intensity in which they modulate NO and IL-12 production.

In this work, GPIs interacted with primed macrophages resulting only in the production of NO and TNF- α . GPIs are abundant in the amastigote stage of *Leishmania* and are associated to highly specialized microdomains [39] and the participation of each kind of GPI on the process is still under debate [74,75,76]. Also it is possible that the dependency on a particular glycolipid may vary throughout species and life cycle stage. The data presented here clearly supports the hypothesis that *Leishmania* GPIs, differently from other trypanosomatids, may contribute to build a safer environment to promote infection by manipulating macrophage function and by disrupting the polarization of TH1/TH2 response, through inhibiting IL-12 production during the

initial stages of infection and manipulate macrophage for parasite survival.

In general, LPGs and GPIs share similar lipid anchor moieties among the various species of *Leishmania* and the integrity of this portion is important for TLR2 activation [27]. To ascertain if the differences in the inhibition of NO and IL-12 production could be related to polymorphisms in GPI structure, we analyzed the carbohydrate core of *L. braziliensis* and *L. infantum* GPIs. Previous studies from our group showed that the phosphoglycan domains of LPGs from *L. braziliensis* and *L. infantum* differ in structure and composition [40,41] and differences in glycan portions of GPIs were also observed in this study. The iM2 species of GPIs possesses the structure Mana1-3Mana1-4GlcN-P similar to LPG core region, and isoM3 has a hybrid glycan in GPIs (substitutions on both the third and sixth carbons of the distal mannose) with the structure of Mana1-6(Mana1-3Mana1-4GlcN-P). Our structural observations indicated that the GPIs from *L. infantum* are similar to the known structures in *L. donovani* [32] and are composed mainly of mannose residues. This data suggests that the majority of these GPIs as Type I GPIs and Hybrid GPIs. On the other hand, *L. braziliensis* GPIs shows a different profile of sugar composition and different bands distinguishable on TLC (Figure 7A). We determined that there was a stoichiometric ratio of galactose and mannose in the glycan portion of these GPIs. This data suggest that these GPIs are similar to the closely related species *L. panamensis* [36], which have a common Gal β 1-3Mana1-3Mana1-4GlcN-myoinositol glycan headgroup and a structurally related to LPG lipid anchor, suggestive of Type II GPIs. Type II GPIs can be very diverse and substitutions on the 3rd carbon of the Gal β residue by Gala-1, Gala1-3gala1, and even longer saccharides like Mana1-PO $_4$ -6Gala1-6Gala1 can be detected in other species like *L. major* [31]. These substitutions can lead to large GPIs containing up to 7, 8 or even more hexoses [34,36], which we observed from the *L. braziliensis* GPIs as seen on Figure 8.

In conclusion, GPIs from both New World species *L. infantum* and *L. braziliensis* have a strong inhibitory potential during intracellular *Leishmania* infection of the mammalian host. Only an initial production of NO and TNF- α was detected after stimulation by GPIs. Due to their importance in modulating NO and cytokine production, these molecules could be possible targets to alternative immunological and chemotherapeutic control methods. The preliminary qualitative analysis of GPIs from these two species showed that they differ in composition and structures thus, suggesting that the structural distinctions could be responsible for differential NO and IL-12 inhibition in macrophages. Also, GPIs were also capable of affecting macrophage ability to produce NO in the presence of IFN- γ and LPS. These data, together with already published data from other groups, suggest that GPIs may be involved in the interaction with the macrophage triggering a minimal pro-inflammatory response in the host and to the benefit of the parasite. Glycoconjugate interspecies polymorphisms, not only in the GPIs, but also in LPG, gp63 and other GPI-anchored molecules could be important for differential establishment of infection. These polymorphisms could result in different clinical outcomes, such as those shown by *L. infantum* and *L. braziliensis*, causative agents of a visceral and tegumentary forms, respectively [77].

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Author Contributions

Conceived and designed the experiments: RPS SJT FSN. Performed the experiments: RRA ICI. Analyzed the data: RRA ICI FSN SJT RPS.

Contributed reagents/materials/analysis tools: RPS SJT. Wrote the paper: RRA ICI FSN SJT RPS.

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5.1.2. Segundo artigo:

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Review

Glycoconjugates in New World species of *Leishmania*: Polymorphisms in lipophosphoglycan and glycoinositolphospholipids and interaction with hosts [☆]

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ABSTRACT

Background: Protozoan parasites of the genus *Leishmania* cause a number of important diseases in humans and undergo a complex life cycle, alternating between a sand fly vector and vertebrate hosts. The parasites have a remarkable capacity to avoid destruction in which surface molecules are determinant for survival. Amongst the many surface molecules of *Leishmania*, the glycoconjugates are known to play a central role in host–parasite interactions and are the focus of this review.

Scope of the review: The most abundant and best studied glycoconjugates are the Lipophosphoglycans (LPGs) and glycoinositolphospholipids (GIPLs). This review summarizes the main studies on structure and biological functions of these molecules in New World *Leishmania* species.

Major conclusions: LPG and GIPLs are complex molecules that display inter- and intraspecies polymorphisms. They are key elements for survival inside the vector and to modulate the vertebrate immune response during infection.

General significance: Most of the studies on glycoconjugates focused on Old World *Leishmania* species. Here, it is reported some of the studies involving New World species and their biological significance on host–parasite interaction. This article is part of a Special Issue entitled Glycoproteomics.

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1. Leishmaniasis

Leishmaniasis is an infectious disease that afflicts millions of people worldwide caused by parasites of the genus *Leishmania*. In accordance with the species involved and the immunological status of the host, this disease exhibits a spectrum of clinical manifestations: cutaneous (CL), mucocutaneous (ML), and visceral leishmaniasis (VL), also known as kala-azar [1].

Abbreviations: CL, cutaneous Leishmaniasis; ML, mucocutaneous Leishmaniasis; DCL, diffuse cutaneous leishmaniasis; VL, visceral Leishmaniasis; GPI, glycosylphosphatidylinositol; LPG, lipophosphoglycan; GIPLs, Glycoinositolphospholipids; sAP, secreted acid phosphatase; sPPGs, secreted proteinophosphoglycans; PSG, promastigote secretory gel; TLR, toll-like receptors; MyD88, Myeloid differentiation primary response gene 88; PKC, protein kinase C; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TNF- α , tumor necrosis factor α ; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; ERK, extracellular-signal-regulated kinases; PKR, protein kinase R; PTK, protein-tyrosine kinases; Man, mannose; Glc, glucose; Gal, galactose; Gal β , galactofuranose; PO $_4$, phosphate; GalNAc, N-acetyl-galactosamine; DC, dendritic cell; NK, natural killer cell; PAMPs, pathogen associated molecular patterns; NETs, neutrophil extracellular traps

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The most common syndrome form is CL, characterized by ulcerative skin lesions generally self-heal that develop at the site of the bite of the sand fly. CL is most frequently caused by *Leishmania major*, *Leishmania aethiopica* and *Leishmania tropica* in the Old World and by *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, *Leishmania peruviana*, *Leishmania mexicana* and related species in the New World America [2].

A variant form of cutaneous leishmaniasis, called mucocutaneous leishmaniasis (ML), is caused by *L. braziliensis*, which has a tropism for macrophages of the oronasopharyngeal region and produces a mucosal granuloma that eventually destroys the nose and mouth. *L. panamensis*, *L. guyanensis* and *Leishmania amazonensis* have also been associated with ML [3,4]. Another type of CL is diffuse cutaneous leishmaniasis (DCL), caused by *L. amazonensis*. It is characterized by a chronic, progressive, polyparasitic variant and is manifested by disseminated non-ulcerative skin lesions [1].

Finally, VL is a human systemic disease and represents the most severe clinical manifestation of *Leishmania* infection. *Leishmania donovani* and *Leishmania infantum* (syn. *Leishmania chagasi*) in the Old and New World are the species that are implicated in this disease [5]. The parasite disseminates and infects macrophages of the liver, the spleen and the bone marrow and may be fatal when untreated [6].

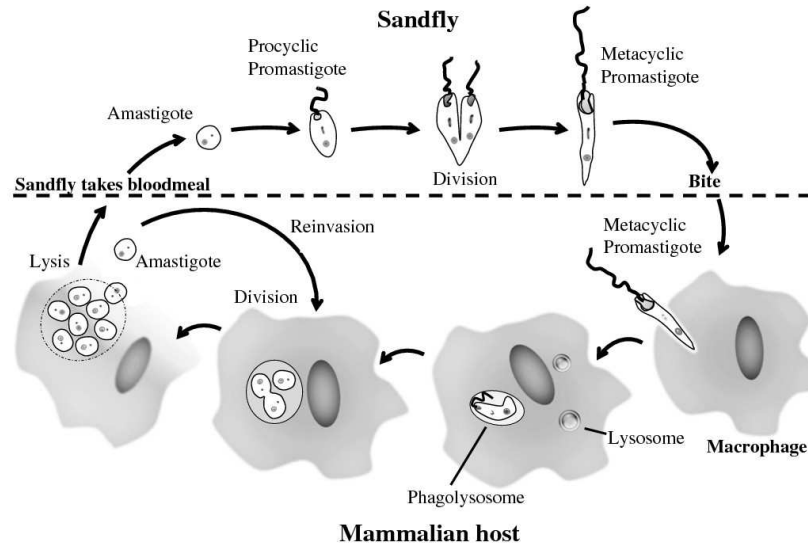


Fig. 1. Life cycle of *Leishmania* parasites. Inside the vector insect, *Leishmania* varies from replicative procyclic promastigotes that, through the metacyclogenetic cycle, differentiates into the infective metacyclic promastigote that is injected into the vertebrate host when the insect takes a blood meal. Inside the vertebrate host, the parasite is phagocytized, mainly by neutrophils, macrophages and dendritic cells where they differentiate into the amastigote form. The amastigotes can either infect other cells, or be ingested by another phlebotomine in the blood meal.

The genus *Leishmania* is sub-divided in two subgenus according to the behavior in the sand fly gut. In the subgenus *Leishmania*, the parasites are mainly found in the midgut and foregut, whereas in the subgenus *Viannia* they are attached to the hindgut prior to migration to anterior parts [7].

2. The life cycle of *Leishmania*

The parasites have a notable capacity to avoid destruction in the hostile environments encountered in their life cycle, alternating between two stages: flagellated promastigotes in the digestive tract of the sand fly vectors and non-motile amastigotes that proliferate within macrophages of the mammalian host. Promastigotes can be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as the infective non-dividing metacyclic promastigotes, which detach from the gut epithelial cells and migrate towards the anterior end of the digestive tract [8]. Transmission of the parasite in the mammalian host occurs during the bite of sand fly vector, of either

the genus *Phlebotomus* (Old World) or the genus *Lutzomyia* (New World) [9] (Fig. 1).

3. Major *Leishmania* glycoconjugates

3.1. Overview

To survive successfully and multiply within these two hostile environments, the parasites must undergo profound biochemical and morphological adaptations, including the expression of glycoconjugates composed largely of molecules attached by glycosylphosphatidylinositol (GPI) anchors [10]. GPI molecules are typically considered as anchors of surface molecules such as proteins [11,12] present in most if not all eukaryotes. In *Leishmania*, however, these GPI-anchored molecules include lipophosphoglycan (LPG), glycoinositolphospholipids (GIPLs), glycoproteins 63 (gp63) and the proteophosphoglycan (PPG), or they are secreted as protein-containing phosphoglycans (PGs), including the secreted proteophosphoglycan (sPPG) and a secreted acid phosphatase (sAP) [13]. In

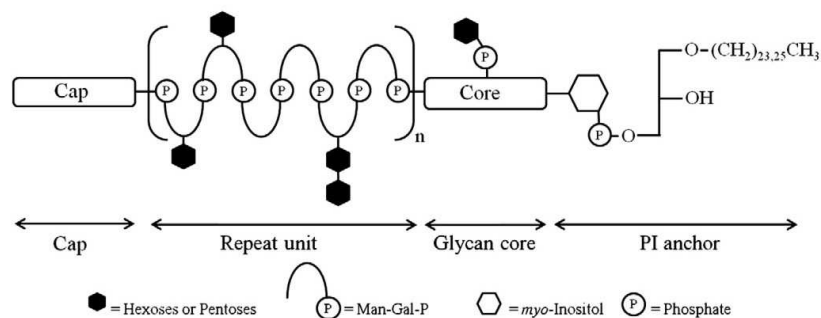


Fig. 2. Structure of LPG. The LPG has four domains, the phosphatidylinositol-linked lyso-alkylglycerol lipid anchor, the conserved glycan core, the repeat units and the cap. The structure and number of phosphoglycan repeat units vary and depend on the stage of differentiation and the species of *Leishmania*. Gal, galactose; Man, mannose; Core, hexasaccharide glycan core; Cap, neutral oligosaccharide.

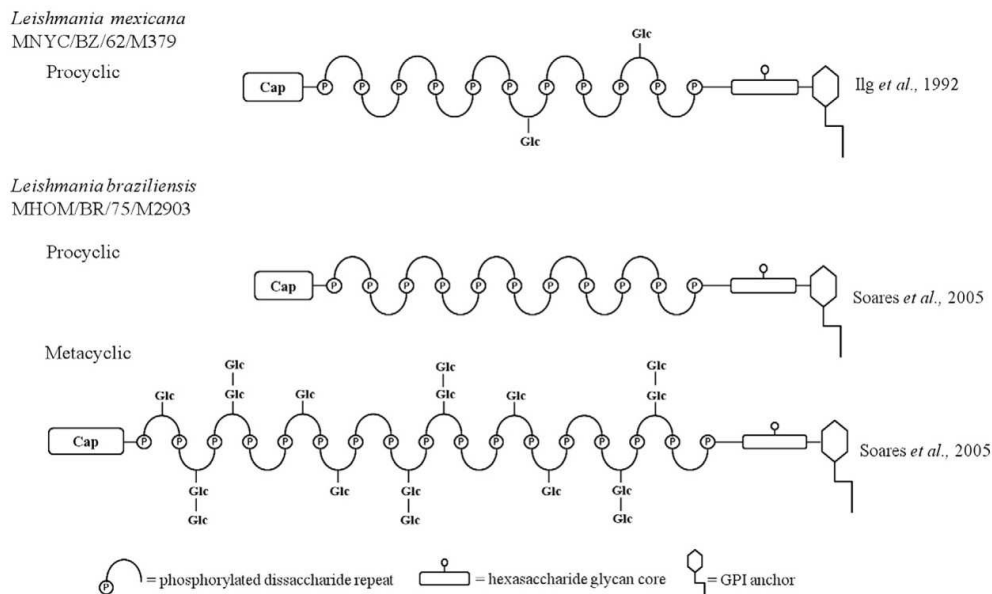


Fig. 3. Schematic diagram of LPG from procyclic and metacyclic *L. braziliensis* and procyclic *L. mexicana*. During metacyclogenesis, the LPG can increase in number of repeating units and the nature of side chain substitutions. In *L. braziliensis*, 1–2 glucose residues are added after metacyclogenesis. The metacyclic structure of *L. mexicana* LPG has not been characterized yet [31,34].

this review, we will focus on LPG and GPIs on New World species of *Leishmania*. For information on gp63 see references [14–17].

3.2. Interspecies and intraspecies variation in Lipophosphoglycan

The most studied surface glycoconjugate is LPG, which forms a dense glycocalyx covering the entire surface of the parasite and the flagellum [10]. LPG is predominantly expressed in promastigotes and is virtually absent in the intracellular amastigotes [18–20]. LPG has been biochemically characterized and polymorphisms in its structure are critical in the specificity of *Leishmania* to different vectors. The recognition of binding sites in the epithelium by the LPG is a crucial step preventing loss of the parasite during the excretion of the digested blood meal [21,22]. During the differentiation process of metacyclogenesis, LPG undergoes crucial changes in structure (discussed below) [23]. Understanding variations and the LPG structures are crucial for the comprehension of the mechanisms of how parasites survive under extremely adverse conditions.

In *Leishmania*, the basic LPG structure consists of four domains (Fig. 2): (1) a lipid anchor characterized by a 1-O-alkyl-2-lyso-phosphatidylinositol containing either C24 or C26 as the aliphatic substituent, (2) a glycan core, consisting of the structure $\text{Gal}(\alpha 1,6)\text{Gal}(\alpha 1,3)\text{Gal}_t(\beta 1,3)[\text{Glc}(\alpha 1)-\text{PO}_4]\text{Man}(\alpha 1,3)\text{Man}(\alpha 1,4)-\text{GlcN}(\alpha 1)$, (3) a backbone repeat units ($\text{Gal}(\beta 1,4)\text{Man}(\alpha 1)-\text{PO}_4$), and (4) an oligosaccharide cap structure [10,24]. Structural analysis of LPG from different species revealed complete conservation of the lipid anchor and the glycan core. The polymorphisms among *Leishmania* species are in the sugar composition and sequence of branching sugars attached to the repeat units and in the cap structure [25].

The pioneering studies involving LPG characterization included mainly Old World Species such as *L. donovani*, *L. major*, *L. tropica* and *L. aethiopica* [26–30]. In contrast, many aspects on the glycobiology in New World *Leishmania* are still unknown. The first LPG characterized in a New World species was from *L. mexicana* procyclic promastigotes, where the C3 hydroxyl of the repeat unit galactose is partially

substituted with β -Glc residues in approximately 20% of the repeat units [31] (Fig. 3). This species exhibits three types of terminally mannosylated caps including $\text{Man}\alpha 1-2\text{Man}$, $\text{Man}\alpha 1-2\text{Man}\alpha 1-2\text{Man}$ or $\text{Man}\alpha 1-2(\text{Gal}\beta 1-4)\text{Man}$. For this species, the structure of metacyclic promastigotes LPG is still unknown so that there is no evidence that it changes during metacyclogenesis. However, a large number of studies have looked at the interaction between this species and the vector *L. longipalpis* that will be described in the next chapter.

Ten years later after the description of *L. mexicana* LPG, the LPG of *L. infantum* (strain PP75) was characterized [32] (Fig. 4). Similar to *L. mexicana*, it also possesses β -glucose residues in approximately 1/3 of the repeat units. The expression of these glucose residues are down-regulated in the metacyclic stage after metacyclogenesis. Differently from *L. mexicana*, *L. infantum* caps are terminally galactosylated and glucosylated, represented by $\text{Gal}(\beta 1,4)\text{Man}$ with lesser amounts of the trisaccharide $\text{Glc}(\beta 1,3)\text{Gal}(\beta 1,4)\text{Man}$, respectively. The expression of β -Glc residues in both the caps and the side chains of LPG were suggestive of being determinant for the binding to *L. longipalpis* midguts. The number of repeat units in procyclic and metacyclic LPGs was determined using capillary electrophoresis (CE). The repeat unit numbers were 19 and 34 for procyclic and metacyclic LPGs, respectively [33]. In conclusion, similar to Old World species, *L. infantum* LPG also increases in size after metacyclogenesis due to an approximate doubling in the number of repeat units.

Following the description of *L. infantum* LPG, the first LPG structure from a *Viannia* species was provided [34] (Fig. 3). In *L. braziliensis* LPG, a different mechanism in the carbohydrate regulation in the LPG side-chains was observed. The *L. (V.) braziliensis* LPG from the procyclic form is similar to the *L. donovani* (Sudan), being devoid of side chains that branch off the Gal-Man-P disaccharide backbone [29], while in the metacyclic stage it contains one or two β -Glc residues as side chains. This up-regulation is the opposite that is observed for some species belonging to the subgenus *Leishmania*. For example, in *L. donovani* (India) [26] and *L. infantum* (Brazil) [32], down-regulation of β -Glc residues occur during differentiation into metacyclics. The caps

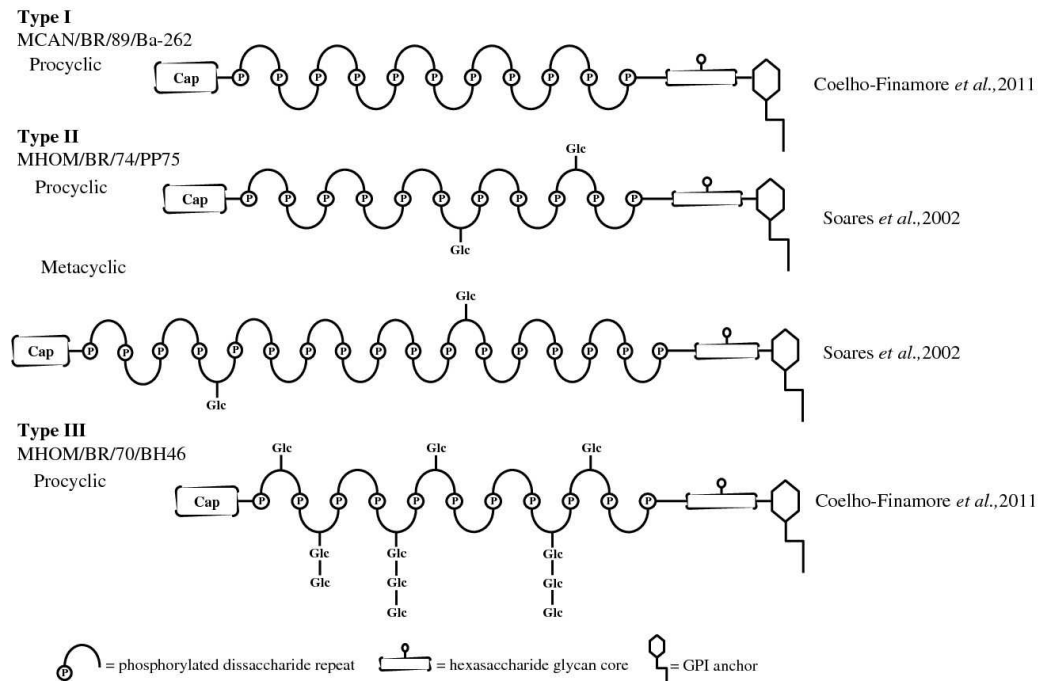


Fig. 4. Schematic diagram of different types of LPG (I, II and III) from New World *Leishmania infantum* strains. The main difference observed on the repeating units from *L. infantum* strains is the degree of $\beta(1,3)$ glucosylation that categorizes these LPGs into three classes: Type-I LPGs with no side chain substitutions; Type-II LPGs with one glucose side chain substitutions on the repeating units and Type-III LPGs with two or more glucose side chain substitutions [32,35].

of *L. braziliensis* were not fully elucidated, but qualitative analysis of monosaccharides using CE indicated the presence of mannose, glucose and galactose (3:1:3) [34].

An unknown aspect of the glycobiology not only in the New World, but also in Old World species, was the level of intraspecies polymorphisms in the LPG structures. Recently, the repeat units of 16 *L. infantum* strains from Brazil, Africa and Europe were evaluated. The results indicated that intraspecies polymorphism in *L. infantum* LPG is very low and most of the strains (~90%) are devoid of side chains. One strain (PP75) had a β -Glc substitution in the side-chains, whereas strain BH46 had up to three glucose side chains.

The latter was the first reported example of a poly-glucosylated LPG [35] (Fig. 4). LPG and GPIs structures described are represented in Table 1.

3.3. Interaction with the sand fly

It has been postulated that inter- and perhaps intraspecies-specific polymorphisms in the phosphoglycan domains of LPG might be crucial for *Leishmania* specificity to a given vector [9,21,22,36]. Many studies have addressed this issue which has led to the intense discussion of permissive and specific vectors. Although some vectors may be infected and sustain infection by different *Leishmania* species, some results have been controversial regarding the role of LPG in this process. For example, *L. major* can only infect and sustain infection in *Phlebotomus papatasi* [22]. On the other hand, many species can interact with different sand fly midguts and even *L. mexicana* LPG-deficient mutants could sustain infection in permissive vectors [37]. More importantly, some conclusions were derived from the in vitro model, which has limitations as recently demonstrated [38] and many challenges and questions still remain to be elucidated [39].

The first study using the in vitro binding system in *L. longipalpis* and *L. infantum* showed similar results to Old World species *L. donovani* and *L. major*. Differently from metacyclic PGs where the lipid anchor was removed by phospholipase C treatment, procyclic PGs were able to attach and inhibit parasite adhesion [32]. In spite of having a strong evidence for the existence of a midgut receptor, no available information exists for this fly receptor in *L. longipalpis*. Only for *L. major*, a galectin receptor has been found in the midgut of *P. papatasi* [40]. Four galectins (A-D) have been reported in the transcriptome of *L. longipalpis* [41] and their role as putative ligands for LPG and parasite binding should be explored. Since this species is thought to be very permissive [42], it is still

Table 1
Studies on LPGs and GPIs from New and Old World *Leishmania* species.

Glycoconjugate	Refs.
Lipophosphoglycan	
<i>L. donovani</i>	[18,29,155]
<i>L. major</i>	[19,27,28,156]
<i>L. tropica</i>	[28,30]
<i>L. aethiopica</i>	[28]
<i>L. mexicana</i>	[31]
<i>L. infantum</i>	[32,35]
<i>L. braziliensis</i>	[34]
Glycosylated phospholipid	
<i>L. donovani</i>	[18]
<i>L. major</i>	[139,140]
<i>L. aethiopica</i>	[142]
<i>L. tropica</i>	[142]
<i>L. mexicana</i>	[141,157]
<i>L. panamensis</i>	[136]
<i>L. infantum</i> and <i>L. braziliensis</i>	[154]

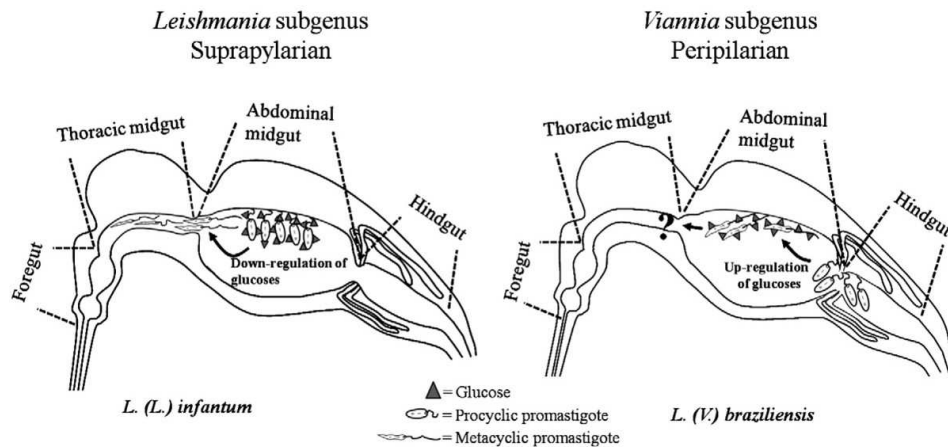


Fig. 5. The different developmental patterns of *Leishmania* within the sand fly gut. The *Leishmania* subgenera confine their development to the midgut and foregut of their sand fly vectors. In the subgenus *Leishmania*, parasites are attached to the midgut through LPG. In *L. (L.) infantum* (strain PP75), after metacyclogenesis, the down-regulation of β -glucoses enables its detachment and migration to anterior parts. In the case of the members of the *Viannia* subgenus, such as *L. (V.) braziliensis*, parasites are also seen attached to the hindgut lining by flagellar hemidesmosomes. An up-regulation of glucoses occurs during their migration to the midgut. However, how the detachment occurs prior to migration to the foregut is a missing step in this species [32,34,46].

unknown if the existence of a receptor could be similar as that observed for *P. papatasi*. It was demonstrated that LPG-independent mechanisms may exist in permissive sandflies such as glycoproteins bearing N-acetylgalactosamine (GalNAc) residues in the midgut of *Phlebotomus arabicus* [43]. In this work, *lpg1*-mutants (unable to synthesize LPG) of *L. major* were able to sustain infection in this permissive vector and *L. longipalpis*. However, those combinations are not the ones occurring in nature, but the development of *L. infantum* mutants could help to solve this gap involving the permissiveness of *L. longipalpis*. Another interesting example is the finding of *L. major/L. infantum* hybrids. Those hybrids expressed mostly *L. major* LPG, which enabled their infection in *P. papatasi*. Although both species could survive separately in the permissive *L. longipalpis*, *L. infantum* was not able to survive in *P. papatasi* [44]. It was recently demonstrated the occurrence of intraspecies polymorphism in *L. infantum* LPG. The biological role of the three types of LPG (I, II and III) was studied during the interaction with the vector *L. longipalpis*. All strains could successfully sustain infection in this vector demonstrating no apparent effect of LPG polymorphisms in this process. Even the strain from Portugal (IPT1) developed very well in *L. longipalpis*; consistent with the idea that introduction of *L. infantum* in the Americas was made possible by the presence of this permissive vector [43].

Only two studies have addressed the presence of midgut binding sites for *L. braziliensis* in its sand fly vectors. This species belongs to the subgenus *Viannia* [7], known to start its development in the pyloric triangle prior to anterior migration to mouth parts (Fig. 5). In this sense, heparin binding proteins (HBPs) from *L. braziliensis* promastigotes were able to recognize proteins extracted from the midguts of *Lutzomyia intermedia* and *Lutzomyia whitmani*. This class of proteins was suggested to have importance during the interaction with the invertebrate host but inhibitions assays were not performed to confirm this idea [45]. On the other hand, using the in vitro binding model, not only PGs form procyclic but also metacyclic parasites were able to bind and inhibit parasite adhesion in the midguts of *L. intermedia* and *L. whitmani*. This may suggest that PGs are necessary not only for attachment, but also for their migration towards the insect's mouth parts [46]. The unusual pattern of attachment by metacyclic PG in this species might be a result of its peripylarian behavior and should be more explored. A missing step in this interaction is how metacyclic parasites could detach. A "second" metacyclic stage has been suggested [36,46] and a model is proposed (Fig. 5).

3.4. Secreted glycoconjugates (sAPs and PPGs)

All species of *Leishmania*, except *L. major*, abundantly secrete acid phosphatases (sAPs). These highly glycosylated proteins are released by promastigotes not only in their life cycle in the sand fly but also under axenic culture conditions. Similar to LPG, they are very polymorphic and also increase in size due to changes in phosphoglycosylation during *Leishmania* metacyclogenesis. They can be found in monomers or polymers depending on the species. The sAP of *L. donovani* is secreted as monomers and oligomers of the phosphoglycoprotein, whereas in *L. mexicana*, *L. braziliensis* and *L. amazonensis*, sAP consists of extended filaments that contain multiple units [47–52]. The sAP structure in *L. infantum* is not known, but it does not seem to increase in size after metacyclogenesis [32].

More recently, the role of other glycoconjugates such as secreted proteophosphoglycans (sPPGs) have been studied using the model *L. infantum* and *L. mexicana* in *L. longipalpis* shedding new light on the mechanisms of parasite transmission during the bite of the sand fly. This glycoconjugate is part of the promastigote secretory gel (PSG) that blocks the anterior part of the sand fly gut and is released during the bite having important consequences on parasite transmission and establishment in the skin of the mammalian host [53–56].

3.5. Glycoconjugates in vertebrate host–*Leishmania* interactions

3.5.1. Overview

Leishmaniasis comprise a wide spectrum of clinical manifestations depending on the species involved. However, it is not known to what extent variations in surface glycoconjugates may drive polarization of TH1/TH2 responses and determine the outcome and immunopathology of the disease.

LPG is crucial not only for the interaction with the invertebrate host as reviewed above, but also for the early steps during establishment of the infection. In *L. major*, after internalization and differentiation into amastigotes within the parasitophorous vacuoles within macrophages, LPG expression is significantly reduced with greater expression of proteophosphoglycans (PPGs) [57] which share common glycan features with LPG. Another class of related glycoconjugates is the glycoinositol-phospholipids (GIPLs), which are present either in promastigotes or amastigotes, the replicating form during the course of infection in the vertebrate host.

In general, LPGs from either New or Old *Leishmania* species have been reported to participate in a variety of processes during the establishment of infection in the vertebrate host. These processes include: resistance to the lytic action of the complement system, attachment and entry into macrophages, protection from proteolytic damage within acidic vacuoles [58], inhibition of phagosomal maturation [59], modulation of nitric oxide (NO) and IL-12 production [60–62], inhibition of protein kinase C [63], induction of neutrophil extracellular traps (NETs) [64] and induction of protein kinase R (PKR) [66].

Upon inoculation by the sand fly in the host skin, several cellular and molecular events take place in the site of the bite. Cellular components of the innate immune system are determinant during early steps of infection not only in *Leishmania* but also in other Protozoa. Dendritic cells (DC), natural killer (NK), neutrophils and macrophages are some of the key elements attracted in response to the parasite. The activation of these cells is essential for the development of polarized Th1 lymphocytes and the establishment of a solid acquired cell-mediated immunity. The ability of the cellular innate immune compartment in orchestrating an initial effective immune response will be important during subsequent steps of infection, such as chronic, re-infection and immunopathology [67].

The mechanisms underlining the ability of *Leishmania* to circumvent the host defenses to get access and multiply intracellularly are surprisingly complex and diverse. The infection outcome depends not only on the species and virulence of the parasite but also on the macrophage subpopulation and their state of activation. *Leishmania* parasites are able to recognize and gain access inside the macrophage through several phagocytic receptors including mannose-fucose receptor (MFR) [68–70], complement receptors CR1 and CR3 [71,72] and also exploiting Fc receptor [73,74]. The most common pathogen-associated molecular patterns (PAMPs) in *Leishmania* are LPG and GPIs. They are recognized by toll-like receptors (TLRs) by different cell types, interfering with signaling transduction pathways [75–78]. Some of those survival strategies will be discussed here and in the next sections.

3.5.2. LPG interaction with cellular components

The evidence that LPG is a multivirulence factor was based primarily on *in vitro* studies using the purified molecule. Some studies have demonstrated its importance and diversity during the host response to *Leishmania* species. Old World species such as *L. major* LPG mutants fail to infect and sustain infection in macrophages, whereas in *L. mexicana* the parasites could develop normally [79–81].

It has been shown for *L. major* that neutrophils are the first line defense against infection arriving at the site of infection before inflammatory macrophages, the primary targets for parasite replication [82]. The importance of *Leishmania* LPG interaction with neutrophils has been also demonstrated on *L. donovani* *lpg1*(–/–) and *lpg2*(–/–) gene mutants. It was demonstrated that LPG drives the persistence of the parasite on tight, non-lytic, compartments inside neutrophils, and both mutants lacking LPG were unable to prevent the fusion of the phagosome with lysosomes and the formation of a lytic environment, followed by parasite killing [83].

In *L. amazonensis*, the role of neutrophils in the initial steps of infection has been recently demonstrated using a phagocytosis-independent killing mechanism mediated by neutrophil extracellular traps (NETs). Those structures are webs composed by chromatin and granular proteins having microbicidal effect against pathogens including fungi and bacteria [84]. Not only the parasites, but also purified *L. amazonensis* LPG, were able to induce NETosis in a cell- and dose-dependent manner [64]. On the other hand, the Old World species *L. donovani* seems to induce NETosis independently from the presence of LPG and gp63, also LPG knockout mutants display an enhanced susceptibility to NETs dependent killing, which indicates a protective role of LPG on this species [85].

3.5.3. Inhibition of phagosome–endosome fusion and oxidative burst by LPG

Most of the studies of phagosome maturation are restricted to Old World species of *Leishmania*. In general, at the onset of infection, promastigotes of *Leishmania* are internalized by macrophages into phagosomes, also named parasitophorous vacuoles. These structures undergo a sequential series of fusions with endocytic organelles and lysosomes generating a potent microbicidal compartment. *Leishmania* promastigotes developed strategies to survive intracellularly in the lytic environment of phagolysosomes [59,86]. It was demonstrated that LPG inhibits phagolysosomal biogenesis, thus protecting invading promastigotes from hydrolytic degradation and providing an environment propitious for their differentiation into amastigotes [86]. Furthermore, there is evidence that phagosomes containing *Leishmania* promastigotes display low fusogenic properties toward endocytic organelles. Also vacuoles formed around *L. donovani* *lpg*-defective mutants promote, during the early phase of macrophage infection, complete destruction of the parasite caused by extensive endosomes and lysosomes fusion [59]. Russell et al. [87] have shown that phagosome–endosome fusion can be restored in phagosomes containing *L. mexicana* by a down-regulation of LPG expression, facilitating transformation of promastigotes into amastigotes.

The molecular mechanisms by which LPG inhibits phagosome–endosome fusion in New World species are still poorly understood. Many reports have evaluated the role of protein kinase C α (PKC α), one of the components involved in the process of *L. donovani* infections. PKC α is associated with the phagosomal membrane and phosphorylates the myristoylated alanine-rich C kinase substrate (MARCKS), a membrane protein associated with actin-based motility and with membrane trafficking. This pathway leads to the movement of both lysosomes and phagosomes on microtubules [88,89]. In macrophages, the inhibition of PKC α by *L. donovani* LPG leads to the inhibition of F-actin depolymerization at the phagosomal membrane, avoiding the fusion events for the delivery of endosomal contents into the parasitophorous vacuoles [90,91].

The inhibition of PKC α by LPG was also observed in *L. mexicana*. Interestingly, *L. mexicana* LPG was able to inhibit PKC α activation in susceptible BALB/c mice but not on the resistant C57BL/6 mice. This was also accompanied by oxidative burst inhibition on BALB/c mice and not on C57BL/6 [92].

In addition to phagosome–endosome fusion inhibition, it has been shown that LPG is able to insert into the surface of the lipid bilayer after internalization of promastigotes [93,94]. By inserting into the membrane lipid bilayer, the LPG modifies its physical organization and disrupts specialized membrane microdomains (lipid rafts) and normal fusogenic properties. This close interaction with the cellular membrane causes a reduction of promastigote phagocytosis, oxidative burst inhibition by preventing the assembly of the NADPH oxidase complex, normal cellular signaling and phagosome acidification [95–100].

3.5.4. Modulation of inducible nitric oxide synthase (iNOS)

Host invasion by pathogens frequently induces activation of many immunological mediators, including chemokines, cytokines, adhesion molecules, and enzymes that produce secondary inflammatory mediators such as NO. In macrophages, iNOS expression is activated by a number of immunological stimuli, such as IFN- γ , TNF- α and lipopolysaccharide (LPS) [101] and catalyzes the synthesis of high concentrations of NO [102]. *Leishmania* has mechanisms that interfere with NO production and thus facilitate survival inside the cells. It is also able to inhibit many of the cytokine-inducible macrophage functions necessary for the development of an effective immune response [103].

Early studies demonstrated that *L. major* LPG induced the production of IFN- γ , and the expression of iNOS, resulting in host protection and parasite resistance in the mouse model [104,105]. The importance of iNOS was further confirmed when mice lacking the enzyme

were unable to control infection in vivo and their macrophages did not eliminate promastigotes in vitro [106]. Tumor necrosis factor- α (TNF- α) is a Th1-type proinflammatory cytokine crucial for NO generation in IFN- γ -primed macrophages infected with *L. major* [107]. The importance of TNF- α and NO production was studied in mice infected with *L. major*, where mutants for this cytokine were not able to resist to infection [108]. In another study, *L. major* PGs synergized with IFN- γ stimulating the macrophages to express high levels of iNOS and this induction was correlated with the complexity of PG structures [61]. Similarly, in the New World species, in vitro studies showed that IFN- γ primed macrophages exposed to different types of *L. infantum* LPG triggered higher NO production by more complex type II and III LPGs [35]. On the other hand, *L. amazonensis* promastigotes inhibited the NO production induced by LPS in J774-G8 macrophages [109]. Furthermore, *L. mexicana* LPG was able to induce TNF- α via TLR2 and this production was dependent on the integrity of the lipid anchor [75]. Thus, either for Old or New World species of *Leishmania*, LPG was not able to induce NO in unprimed macrophages.

3.5.5. Cellular signaling and cytokine production

There are multiple ways by which intracellular pathogens like *Leishmania* take advantage of the host cell's machinery in order to survive and replicate. Interference with the host macrophages signaling transduction pathways is one of the survival strategies used by the parasites [78]. Those mechanisms occur due to events of phosphorylation and dephosphorylation in cascades of kinases and phosphatases. Mitogen-activated protein kinases (MAPKs), NF- κ B pathways triggered via TLRs and CD40 have been implicated in *Leishmania* infection (reviewed in [110]). *Leishmania* LPG has been reported to activate innate immune signaling pathways in macrophages and NK cells through TLR2 [75,76]. In vivo studies with knock-out mice have further demonstrated the importance of other TLRs during *Leishmania* infection (reviewed in [111]). In *L. major* and *L. donovani* the roles of MyD88, TLR2, TLR3, TLR4 and TLR9 have

been demonstrated [75,77,112,113]. In *L. infantum*, TLR9, IL-12 and myeloid DCs are important for the activation of NK cells in controlling the parasite [114]. On the other hand, the development of a protective immune response to *L. mexicana* in the murine model does not appear to require IL-12 secretion [115], possibly contributing to the dispensability of LPG in this species. In contrast, in vitro studies demonstrated that dendritic cells mount a LPG-dependent proinflammatory transcriptional response to *L. mexicana* [116]. In another study, *L. mexicana* LPG was able to differentially impair the nuclear translocation of NF- κ B in monocytes with a subsequent decrease in the IL-12 production [117]. In *L. braziliensis*, IL-12, TNF- α and iNOS were critical in the outcome of the disease in the murine model [118]. Furthermore, in this species the role of MyD88 was considered indispensable for the generation of protective immunity, whereas TLR2 had a regulatory role during the infection [119]. In *L. amazonensis*, disruption of the CD40/CD40L interaction enhanced the infection of the parasite, resulting in low production of IFN- γ , lymphotoxin-TNF and NO [120]. In bone marrow-derived mast cells (BMMCs), the TLR2 expression was higher in C57BL/6 than in BALB/c mice. This also resulted in increased levels of TNF- α , IL-10 and MIP-1 α after in vitro stimulation with *L. mexicana* LPG [121]. Altogether, these studies indicate that the essential components of a protective immune response may vary depending on the *Leishmania* species and the mouse subset from where the cells were derived. Some of those mechanisms are represented in Fig. 6.

The MAP kinases are an important group of serine/threonine signaling kinases that are activated by dual phosphorylation in response to diverse extracellular stimuli, linking transmembrane signaling with gene induction events in the nucleus [122,123]. The first in vitro study using *L. major* metacyclic promastigotes and synthetic PG indicated that LPG differentially regulated IL-12 and NO production in macrophages. This occurred independently of NF- κ B activation and the suppressive effect observed on IL-12 induction was resulting of ERK1/2 MAPKs activation [124]. The role of LPG in the activation of

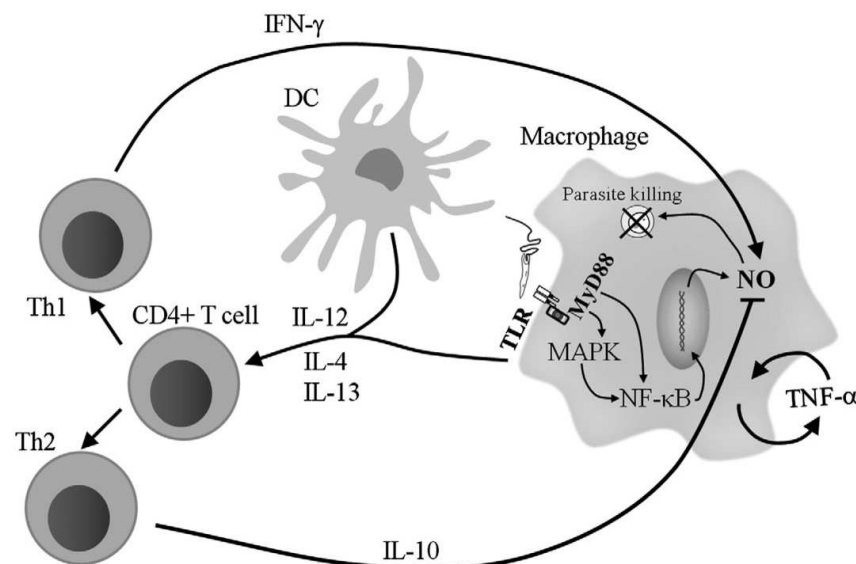


Fig. 6. Immunological determinants influencing *Leishmania* infection. The *Leishmania* promastigote is phagocytosed by the macrophages. Activated dendritic cells (DC) and macrophages produce IL-12, which drives Th1 cell differentiation, proliferation and IFN- γ production, which activate macrophages to produce NO and kill the parasites. In contrast, the production of IL-4 and IL-13 drives T cells differentiation into Th2, capable of producing IL-10 and additional IL-4, which inhibit NO production. This is associated with parasite survival and persistence of infection.

MAPKs was confirmed by *lpg1*- and *lpg2*- mutants of *L. donovani*. Those parasites were able to activate higher levels of ERK1/2 MAPKs and could not sustain infection [125]. In another study, *Leishmania* LPG stimulated the activation of ERKs, p38 and JNK, and this appeared to be necessary for AP-1 activation, IL-12 and NO production [126].

Another important group involved in cell signaling is protein kinases C (PKCs). They are a family of at least 12 serine-threonine kinases activated by membrane phospholipids and are key kinases for activating effector genes and mount a protective response against aggressor pathogens [127]. Respiratory burst activity and NO production are regulated by phosphorylation events mediated by PKC. Not only *L. donovani* parasites, but also purified LPG is able to inhibit the oxidative burst through PKC in macrophages and increase intracellular survival of the parasites. The two mechanisms proposed are that LPG might act as a competitive inhibitor of PKC, or it could act as a chelator of Ca^{2+} that is required for PKC activity [94,128–131]. Regarding New World *Leishmania* species, *L. mexicana* purified LPG inhibited PKC α activity in BALB/c macrophages, reducing the oxidative burst and increasing parasite survival [92]. Another group involved in cell signaling is protein kinases R (PKRs). They are activated by a number of stimuli, such as dsRNA, TNF- α , IL-1 β , IFN- γ , and LPS [132–134]. In *L. amazonensis* infection, an increase in the activation of double-stranded RNA (dsRNA)-dependent in RAW cells was observed. The parasites could negatively modulate NF- κ B activation and the subsequent NO production; this effect was correlated to the suppressive IL-10 production [65]. More recently, LPG from this species was found to increase the PKR transcript levels and this effect was via TLR2 [66].

In conclusion, the mechanism of LPG influencing signaling events is still a ripe area to study parasite–host interactions with New World species of *Leishmania*.

4. Glycoinositolphospholipids (GIPLs)

4.1. Interspecies and intraspecies variation in GIPLs structure

Besides LPG, another important class of GPI-anchored molecules in *Leishmania* is GIPLs. They are the most abundant glycolipid component of the cell membrane and are structurally analogous to protein and LPG anchors [11]. Due to their own structural features such as side chain modifications and fatty acids substitutions, they can be classified as a metabolically distinct family of molecules rather than a precursor or byproduct of GPI anchors [135,136]. Similar to LPG, GIPLs are also polymorphic in both glycan and lipid structures, although a conserved basic core is present in all *Leishmania* species studied to date. It consists of a Man α 1-4GlcN core linked to an alkyl-acylglycerol or a lyso-alkylglycerol through a phosphatidylinositol (PI). Different from other trypanosomatids, there is no unsaturated fatty acid substitution of the lipid moiety [137].

These GIPLs structures are commonly divided into three distinct groups depending on whether the R-Man α 1- substitution occurs on the 3rd, 6th or both carbons. When the R-Man α 1- addition occurs on the 6th carbon of the proximal mannose, the resulting structures are classified as type-I GIPLs (mainly M2 and M3 GIPLs) which are structurally related to protein GPI anchors. When the substitution occurs on the 3rd carbon, the resulting GIPLs are classified as Type-II GIPLs (mainly iM2, GIPL-1, GIPL-2, GIPL-3 and GIPL-A) which are structurally related to LPG anchor. The third type is named Hybrid GIPLs in which there is R-Man α 1-substitutions to both 3rd and 6th carbons of the proximal mannose residue [11] (Fig. 7).

The first GIPL structure was reported from *L. major*. This early study showed a highly galactosylated glycan moiety linked to the lipid anchor through a non-N-acetylated glucosamine residue varying

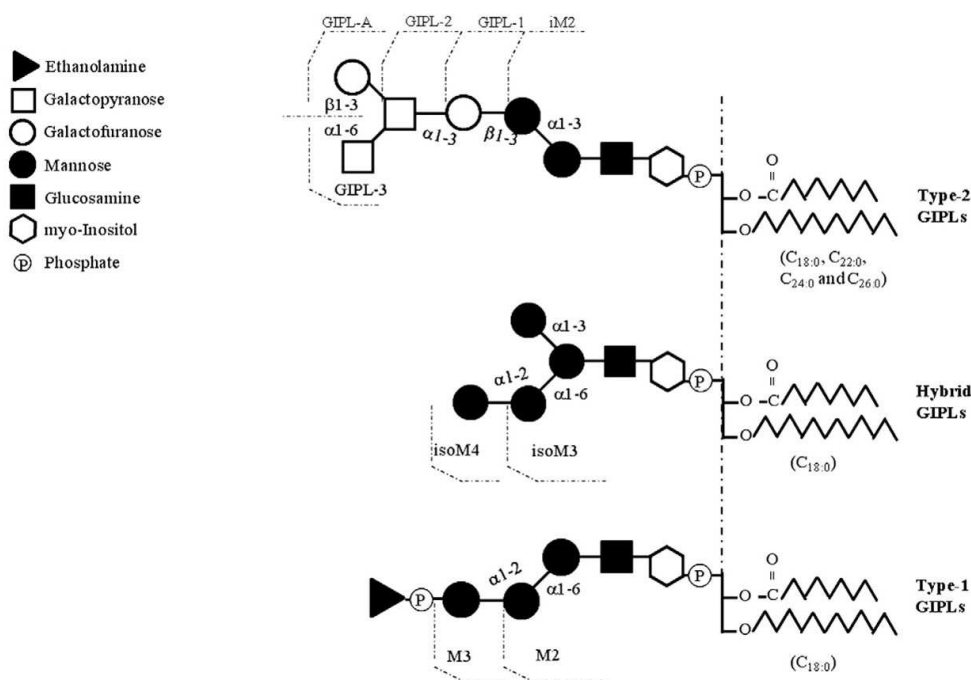


Fig. 7. Schematic representation of different types of glycoinositolphospholipids (GIPLs). *Leishmania* GIPLs are divided into three distinct groups: Type-I GIPLs are characterized by a R-Man α 1- addition to the sixth carbon of the proximal mannose residue; Type-II GIPLs have a R-Man α 1- substitution to the third carbon of the proximal mannose; Hybrid GIPLs have a R-Man α 1- substitution on both third and sixth carbons of the proximal mannose residue [11].

in length and polarity [138]. A more complete structure came later when it was shown that *L. major* expresses a myriad of GIPLs with a general structured expressed as R-Gal β 1-3Man α 1-3Man α 1-4GlcN-PI [139]. This novel class of molecules ranges from simple Gal β 1-3Man α 1-3Man α 1-4GlcN-PI (GIPL-1) structures to more complex Gal β 1-3Gal α 1-3Gal α 1-6Gal β 1-3Man α 1-3Man α 1-4GlcN-PI (GIPL-A) and Gal α 1-6Gal α 1-3Gal α 1-6Gal β 1-3Man α 1-3Man α 1-4GlcN-PI (GIPL-3). Also these GIPLs can be represented by its lyso-alkylglycerol counterparts or even have a Glc(α 1-6) residue substitution onto the distal mannose [139,140].

Following the structural analysis of the *L. major* GIPLs, the structure of *L. donovani* GIPLs was elucidated. Chemical analysis and enzymatic sequencing revealed a new GIPL profile that is different from *L. major* by being highly mannoseylated. In *L. donovani*, the predominant GIPLs had substitutions on both 3rd and 6th carbon of the proximal mannose of the Man α 1-4GlcN motif with mainly C_{14:0}, C_{16:0} and C_{18:0} fatty acids. The main GIPLs on this species are Man α 1-3Man α 1-4GlcN-PI (isoM2) Man α 1-6(Man α 1-3)Man α 1-4GlcN-PI (isoM3 GIPLs) and Man α 1-2Man α 1-6(Man α 1-3)Man α 1-4GlcN-PI (isoM4 GIPLs) [18].

In 1993, the first New World species GIPLs were characterized. *L. mexicana* GIPLs structure shared close relation to *L. major* GIPLs with a high degree of galactosylation and R—Man α 1- substitution to the 3rd carbon of the proximal mannose expressing mainly Gal α 1-6Gal α 1-3Gal α 1-6Gal β 1-3Man α 1-3Man α 1-4GlcN-PI (GIPL-3), Gal α 1-3Gal α 1-6Gal β 1-3Man α 1-3Man α 1-4GlcN-PI (GIPL-2) and Man α 1-3Man α 1-4GlcN-PI (iM2 GIPLs) [141].

While there is a wide polymorphism on GIPL composition throughout different species, intraspecific polymorphisms are unknown. The structure of three different *L. major* strains together with other two Old World species, *L. aethiopica* and *L. tropica* was analyzed [142]. Interestingly, the intraspecific variance of the GIPL composition was shown to be based mainly on relative quantities of each GIPL species as all strains of *L. major* was shown to express GIPL-1, GIPL-2, GIPL-3 with or without their phosphorylated counterparts (addition to a P-Glc to the Man α 1-6 residue). *L. aethiopica* and *L. tropica* were shown to have mainly mannoseylated GIPLs much like *L. donovani* also differing on their abundance.

The first and only study addressing a *Viannia* subgenus species was in *L. panamensis*. It was shown that *L. panamensis* GIPLs had some unusual glycan and lipid structures. Chemical analysis showed that *L. panamensis* GIPLs comprise mainly two glycan cores, the type-II GIPLs Man α 1-3Man α 1-4GlcN-PI and the Hybrid GIPLs Man α 1-2Man α 1-6(Man α 1-3)Man α 1-4GlcN-PI with the unusual extensions of Gal α 1-2Gal β 1, Gal α 1-2Gal α 1-2Gal β 1 or Gal α 1-3Gal α 1-2Gal β 1, never observed in any other *Leishmania* species. Another feature of *L. panamensis* GIPLs, that is unusual compared to the other species, is the presence of diacylglycerol lipid moieties rather than alkyl-acylglycerol or 1-*o*-lyso-alkyl-glycerol. These are novel features of *Leishmania* GIPLs, not yet observed in any other species [136].

Given that these molecules are expressed in high copy numbers on the surfaces of both promastigote and amastigote forms, understanding their role on *Leishmania* biology is crucial to understand how these parasites interact with their hosts. This may lead to alternative strategies to control *Leishmaniasis*.

4.2. GIPL interactions with sand fly and the vertebrate host

Although GIPLs are expressed in a high copy number, their function on *Leishmania* biology is still unclear. Since these molecules share a common cellular enzyme machinery to GPI anchors, it is difficult to experimentally determine their specific relevance in the overall *Leishmania* biology. In contrast to LPG, there are no available studies involving GIPLs and sand fly interaction and this is an open field to be explored.

The first reports on *Leishmania* GIPLs were purely structural, and little was known about their biological significance. The first time

Leishmania GIPLs were linked to a possible role on infection was in 1990 through the demonstration that GIPLs could interact with humoral factors on the vertebrate host. *L. major* infected patients had high titers of anti α -galactosyl antibodies [139]. This was later confirmed in other species such as *L. donovani*, *L. mexicana* and *L. braziliensis* [143].

As was conducted experimentally with LPG, several studies with glycosylation mutants of *Leishmania* have tried to determine the importance of GIPLs biology on the innate cellular compartment. Most evidence points to a modulatory effect by down regulating classical macrophage activation thus promoting a silent, non-destructive entry into the host cell. *L. amazonensis* and *L. mexicana* mutants lacking some or all GIPLs species have been generated, either by expressing heterologous phospholipases that can deplete mature GIPLs and their precursors or by knocking out genes responsible for the addition of mannose to their structure. Although these mutants had little or no impact on promastigote growth in culture, a significant reduction was observed in vitro on *L. amazonensis* amastigote survival in macrophages and a significant impact on *L. mexicana* infectivity both in vitro and in vivo [144–147]. In a recent study with *L. braziliensis* GIPLs, it was reported that they are not randomly distributed throughout the plasma membrane but are rather associated with high specialized microdomains. Depletion of these structures in this species reduced in vitro macrophage infectivity [148].

Several molecular mechanisms triggered by GIPLs have been reported. It has been reported that TLR4 receptors are recognized by *Trypanosoma cruzi* GIPLs [149] and although *Leishmania* GIPLs are able to interact with cells, it is not clear if this occurs via TLRs. It has been shown that GIPLs from *L. major* could inhibit the release of NO [150]. Later, it was demonstrated that *L. mexicana* GIPLs could rapidly activate protein-tyrosine kinases (PTKs), but with no cell activation and cytokine release [151]. Interestingly, the PTK activation also required the minimal structure of Man α 1-2Man α 1-6Man α 1-4GlcN-PI (M3 and isoM4 forms of *L. mexicana*) [137] and *L. donovani* [18]. This glycan structure can accommodate minor side chains additions without affecting its activity. On the other hand, *L. mexicana* GIPLs were unable to activate PKC [151]. Consistent with this information, it was demonstrated that *L. major* GIPLs and its synthetic lipid moiety also inhibited PKC [152,153]. Recently, GIPLs from *L. infantum* and *L. braziliensis* were found to differentially inhibit NO production by mouse macrophages stimulated with IFN- γ and LPS, with no effect on TNF- α release [154]. This observation is important evidence that *Leishmania* GIPLs impair regulatory signals that can modulate the host cell response. Although the structures of the *L. infantum* and *L. braziliensis* GIPLs are not fully elucidated, preliminary qualitative analysis on monosaccharide composition indicates that the former is very similar to type I or hybrid, whereas the latter is consistent of a type II GIPLs [154].

5. Concluding remarks

A great number of studies have implicated the importance of glycoconjugates in *Leishmania* biology either in New or Old World species of *Leishmania*. The structures of those molecules have exhibited a surprising polymorphism not only intra- but also interspecifically. Additionally, the expression of those molecules varies according to stage depending on the host, being able to modulate a variety of mechanisms to avoid parasite destruction. Although there are many questions still unanswered, especially regarding New World species, the lack of mutants and a precise host cells model warrant further investigations. It is intriguing how the structure and biology of those molecules has evolved in *Leishmania*, especially in the subgenus *Viannia*, where unique mechanisms of interaction occur in the sand fly and vertebrate host. Overall, understanding the mechanisms of how these parasite-derived molecules modulate important parasite–host interactions may result in alternative control strategies.

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5.2. Artigo Submetido:

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- Co-autoria entre Ibraim CI e Assis RR.

Two biochemically distinct Lipophosphoglycans from *Leishmania braziliensis* and *Leishmania infantum* trigger different innate immune responses in murine macrophages

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Abstract

Background: The dominant, cell surface lipophosphoglycan (LPG) of *Leishmania* is a multifunctional molecule involved in the interaction with vertebrate and invertebrate hosts. Although the role of LPG on infection has been extensively studied, it is not known if LPG interspecies variations contribute to the different immunopathologies of leishmaniasis. To investigate the issue of interspecies polymorphisms, two *Leishmania* species from the New World that express structural variations of side chains of LPG repeat units were examined. In this context, the procyclic form of *L. braziliensis* LPG (strain M2903), is devoid of side chains, while the *L. infantum* LPG (strain BH46) has with up to three glucose residues in the repeat units.

Methods: Mice peritoneal macrophages from Balb/c, C57BL/6 and knock-out (TLR2 $-/-$ e TLR4 $-/-$) were primed with IFN- γ and stimulated with purified LPG from both species. Nitric oxide and cytokine production, MAPKs (p38 and ERK) and NF- κ B activation were evaluated.

Results: Macrophages stimulated with *L. braziliensis* LPG, had a higher TNF- α , IL-1 β , IL-6 e NO production than those stimulated with that of *L. infantum*. Furthermore, the LPGs from the two species resulted in differential kinetics of signaling via MAPK activation. *L. infantum* LPG exhibited a gradual activation profile, whereas *L. braziliensis* LPG showed a sharp but transient activation. *L. braziliensis* LPG was able to activate NF- κ B.

Conclusion: These data suggest that two biochemically distinct LPGs were able to differentially modulate macrophage functions.

Keywords: *Leishmania infantum*, *Leishmania braziliensis*, Lipophosphoglycan (LPG), Macrophage modulation

Background

Leishmaniasis are a wide spectrum of diseases widely distributed in the Americas (Grimaldi *et al.*, 1989). In Brazil, *Leishmania braziliensis* and *Leishmania infantum* are the causative agents of cutaneous (CL) and visceral leishmaniasis (VL), respectively (Herwaldt, 1999). At the early steps of infection in the vertebrate host, the parasite must survive the production of inflammatory mediators such as reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and cytokines (Evans *et al.*, 1993; Gazzinelli *et al.*, 2004).

In trypanosomatids, many GPI-anchored molecules are known to be closely associated with cell signaling, acting as agonists and second messengers in response to cytokines and other stimuli (Feng *et al.*, 1999; Merida *et al.*, 1990; Saltiel, 1991; Soares *et al.*, 2012; Tachado *et al.*, 1997). In *Leishmania* Lipophosphoglycan (LPG) has been extensively studied and is known to be a multifunctional virulence factor with functions that include: attachment to the sand fly vector midgut (Kamhawi *et al.*, 2004), attachment and entry into macrophages (Descoteaux & Turco, 1999), induction of neutrophil extracellular traps (NETs) (Guimaraes-Costa *et al.*, 2009), inhibition of protein kinase C (PKC) dependent cell activation (Descoteaux *et al.*, 1991; Olivier *et al.*, 1992), retardation of phagosome maturation (Winberg *et al.*, 2009), disruption of NADPH oxidase assembly at the phagosome membrane (Lodge & Descoteaux, 2006) modulation of NO production (Brittingham & Mosser, 1996) and induction of protein kinase R (Luz *et al.*, 2012; Vivarini Ade *et al.*, 2011). Although the lipid anchor is conserved (Assis *et al.* 2012), previous studies have shown that changes in the carbohydrate structure of procyclic LPG and GIPLs can account for variations in macrophage modulation (Assis *et al.*, 2012; Coelho-Finamore *et al.*, 2011; Proudfoot *et al.*, 1996).

Structurally, LPG has four distinct domains: (i) a well conserved GPI anchor composed of 1-*O*-alkyl-2-lyso-phosphatidylinositol (PI); (ii) a core composed of Gal(α 1-6)Gal(α 1-3)Gal(β 1-3)[Glc(α 1)PO₄]Man(α 1-3)Man(α 1-4)-GlcN(α 1) heptasaccharide; (iii) a portion of disaccharide repeats of the Gal(β 1-4)Man(α 1)PO₄ units and (iv) a terminal neutral oligosaccharide (“cap”) (Descoteaux & Turco, 1999). In *L. infantum*, the repeat units of LPG display the addition of β Glc to the C3 carbon in the Gal residues (Soares 2002). These side chain substitutions can vary between different strains of *L. infantum* which can have three different sets of side chains: Type I which has no side chain substitutions; Type II which has only one β Glc addition to the Gal residue of the REPs and Type III which contains two or three β Glc residue substitutions on the Gal residue (Coelho-Finamore *et al.*, 2011) (Fig. 1). Interestingly, in the metacyclic promastigote form of LPG in this species there are no sugar substitutions in the repeat unit backbone (Soares *et al.*, 2002). Elucidation of the *L. braziliensis* LPG structure showed that procyclic promastigote forms are devoid of side-chains, whereas metacyclic LPG displays up to two β Glc residues linked to the Gal residue of the repeat unit backbone (Soares *et al.*, 2005). It is not known to what extent interpecies variations in New World species of *Leishmania* can differentially activate the host’s immune system. Recently, the structural composition of *L. braziliensis* and *L. infantum* GIPLs revealed that the former are galactose rich and its structure is suggestive for the predominance of type II GIPLs having a Man α 1-3 substitution to the proximal mannose and are mainly galactose terminating structures. On the other hand, *L. infantum* GIPLs are mainly of type I and/or Hybrid GIPLs possessing Man α 1-6 and Man α 1-3[Man α 6] substitutions to the proximal mannose, respectively (Assis *et al.*, 2012; McConville & Ferguson, 1993). Those variations were implicated in a higher inhibitory activity in NO and cytokine

production by *L. braziliensis* GIPLs compared to *L. infantum*. However, the role of LPGs from those species in this process has not been investigated.

This work is part of a wider study on the glycobiology of New World species of *Leishmania*. In previous studies, we reported on the LPG and GIPLs biochemical structures of *L. braziliensis* and *L. infantum* (Assis *et al.*, 2012; Soares *et al.*, 2005; Soares *et al.*, 2002) and showed that the differences in GIPLs structures were relevant in the parasite biology (Assis *et al.*, 2012). In this study, we expanded those findings and show that the LPG of these two New World *Leishmania* species also differentially modulated the activation of mouse peritoneal macrophages.

Methods

Ethics statement

All animals were handled in strict accordance with animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CEUA) of Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte (BH), Minas Gerais (MG), Brazil (Protocol P-0297-06). Knock-out mice handling protocol was approved by the National Commission of Biosafety (CTNBio) (protocol #01200.006193/2001-16).

Parasites

World Health Organization Reference strains of *L. braziliensis* (MHOM/BR/1975/M2903) and *L. infantum* (MHOM/BR/1970/BH46) were used. Promastigotes were cultured in M199 medium supplemented with 10% heat-inactivated 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 (Soares *et al.*, 2012) until late log phase.

Extraction and purification of LPG

For optimal LPG extraction, late log phase cells were harvested and washed twice with PBS prior to extraction of LPG. The LPG extraction was performed as described elsewhere with solvent E (H₂O/ethanol/diethylether/pyridine/NH₄OH; 15:15:5:1:0.017) after a sequential organic solvent extraction (Orlandi & Turco, 1987). For purification, the solvent E extract was dried under N₂ evaporation, resuspended in 2ml of 0.1 N acetic acid/0.1 M NaCl, and applied onto a column with 2ml of phenyl-Sepharose, equilibrated in the same buffer. The column was washed with 6 ml of 0.1 N acetic acid/0.1 M NaCl, then 1ml of 0.1 N acetic acid and finally 1ml of endotoxin free water. The LPG was eluted with 4ml of solvent E then dried under N₂ evaporation. LPG concentration was determined as described elsewhere (Dubois *et al.*, 1951). Prior to use on *in vitro* macrophage cultures, LPG was diluted in fresh RPMI. All solutions were prepared in sterile, LPS-free distilled water (Sanobiol, Campinas, Brazil).

Purification of murine peritoneal macrophages and cell culture

Thioglycollate-elicited peritoneal macrophages were extracted from BALB/c, C57BL/6 and C57BL/6 (TLR2 ^{-/-} and TLR4 ^{-/-} knockouts) by peritoneal washing with ice cold serum-free RPMI and enriched by plastic adherence for 1 h at 37 °C/5% CO₂. Cells (3 X 10⁵ cells/well) were washed with fresh RPMI then 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% CO₂). Cells were primed with gamma interferon (IFN-γ) (3 IU/mL) (Kolodziej *et al.*, 2008) for 18h prior to incubation with LPG or live stationary *Leishmania* parasites (10:1), LPG (10 µg/mL) or lipopolysaccharide (LPS) (100 ng/mL).

Chinese Hamster Ovary (CHO) cell lines

The CHO reporter cell lines (TLR2-TLR4-, which do not express TLR2 nor TLR4; TLR2+, expressing TLR2; TLR4+, expressing TLR4 ([Lien et al., 1999](#)) were maintained as adherent monolayers in Ham's F-12/DMEM supplemented with 5% FBS, at 37°C, 5% CO₂, and antibiotics. All of the cell lines are derived from clone 3E10, that has been stably transfected with a reporter construct containing the structural gene for CD25 under the control of the human E-selectin promoter.

This promoter contains a NF- κ B binding site; CD25 surface expression is completely dependent upon NF- κ B translocation to the cell nucleus ([Delude et al., 1998](#)).

Cytokine and nitrite measurements

For CBA multiplex cytokine detection, cells were plated, primed as described above and incubated for 18 h (37°C, 5% CO₂). LPG (10 μ g/mL) and live stationary promastigotes (10:1 ratio) were added and incubated for 48 h, LPS (100 ng/mL) was added as a positive control. For negative controls fresh medium was added. Supernatants were collected and cytokines (IL1- β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IFN- γ and TNF- α) were determined using the BD CBA Mouse Cytokine assay kits according to the manufacturer's specifications (BD Biosciences, CA, USA). Flow cytometry measurements were performed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). 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 of 1,800 events were acquired for each preparation ([Assis et al., 2012](#)). Results are representative of two experiments in duplicate. Nitrite concentrations determined by Griess reaction ([Drapier et al., 1988](#)).

For the inhibition assay, purified cells were primed for 6h with 3 IU/ml of IFN- γ prior to stimulation with LPG (10 μ g/ml). Cells were incubated for 18h at 37 °C/5% CO₂, then LPS (100

ng/ml) was added to the medium and incubated for another 24h at 37 °C/5% CO₂. Supernatants were collected and nitrite concentrations determined by Griess reaction. Results shown are the mean of two experiments in triplicate ([Assis et al., 2012](#)).

Flow cytometry analysis

In order to evaluate the activation of NFκB by LPG, CHO reporter cells were plated at a density of 1×10^5 cells/well in 24-well tissue culture dishes. The following day, either molecule or bacteria (*Staphylococcus aureus* [1000 bacteria/well], positive control of TLR2; LPS (200ng/well), positive control of TLR4; or LPG (0,2ug or 0,02ug/well) from *Leishmania brasiliensis* or from *Leishmania infantum* was added as indicated, for 18 h. The cells were then harvested with trypsin-EDTA and washed once with medium and again with PBS. Subsequently, the cells were counted and 1×10^5 cells stained with PE-labeled anti-CD25 (mouse mAb to human CD25, R-PE conjugate; Caltag Laboratories, Burlingame, CA) 1:200 in PBS, on ice, in the dark, for 30 min. After labeling, the cells were washed twice with 1 mM sodium azide in PBS, resuspended in 1 mM sodium azide in PBS, and examined by flow cytometry (BD Biosciences, San Jose, CA) for the expression of surface CD25 as described ([Lien et al., 1999](#)). Analyses were performed using CellQuest software (BD Biosciences).

Preparation of cell lysates and immunoblotting

Thioglycollate-elicited peritoneal macrophages were plated as above on 6 well tissue culture plates (3×10^6 /well) for 18h prior to assay. The cells were washed with warm RPMI and incubated with LPG from both species for different times (5, 15, 30 and 45 min) or with medium (negative control) or LPS (100 ng/ml) as positive control. Cells were then washed with ice-cold PBS and lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 150 mM

NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 10 % glycerol (v/v), 0.5 mM dithiothreitol (DTT) and protease inhibitor cocktail from Sigma®). Cells were harvested with a plastic scraper and centrifuged at 13,000 X *g* (4 °C, 10 min). Supernatants were transferred to new tubes and stored at -20 °C until used for immune blotting. Cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and blocked (5% milk in TBS-0.1% Tween 20) for 1 h. Primary Abs (anti dually phosphorylated ERK, dually phosphorylated p38 and Total ERK, 1:1,000, total p38 primary antibody was used as a normalizer) were incubated for 16 h at 4°C. Membranes were washed (3 X 10 min) with TBS-0.1% Tween 20 and incubated 1 h with anti-mouse IgG conjugated with peroxidase (1:10,000). The reaction was visualized using luminol ([Assis et al., 2012](#)).

Statistical analyses

For nitrite and cytokine measurements, the Shapiro–Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution ([Shapiro & Wilk, 1950](#)). 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. Data were analyzed using GraphPad Prism 5.0 software (Graph Prism Inc., San Diego, CA) and $P < 0.05$ was considered significant.

Results

Nitrite and cytokine production

In order to evaluate the role of TLR2 and TLR4 in this process, IFN- γ primed peritoneal macrophages from BALB/c, C57BL/6, TLR2 (-/-) and TLR4 (-/-) mice were incubated with 10 μ g/ml of LPG and live promastigotes (10:1) from *L. braziliensis* and *L. infantum*. Nitrite and

cytokine concentrations determined on the supernatants after 48 h (Figures 2, 3 and 4) ([Assis et al., 2012](#)).

A higher NO production was detected on IFN- γ -primed C57BL/6 macrophages stimulated with both LPGs and live promastigotes when compared to BALB/c mice ($P < 0.001$) (Figure 2A). There was a significant decrease in NO production in IFN- γ -primed TLR4 (-/-) macrophages stimulated with LPG in comparison to TLR2 (-/-) and wild type C57BL/6 macrophages ($P < 0.01$) suggesting the involvement of TLR4 in this activation. No significant difference of NO production was noticed in macrophages from TLR2 (-/-) or TLR4 (-/-) mice stimulated with live promastigotes when compared to wild type C57BL/6 ($P < 0.05$) (Figure 2A).

In a multiplex flow cytometer approach, the concentrations of several cytokines were determined on the same supernatants used for nitrite measurement. No significant quantities of IL-2, IL-4, IL-5 and IFN- γ were detected (data not shown); on the other hand, a significant production of TNF- α , IL-1 β and IL-6 was observed (Figure 2B and Figure 3A and B). As for nitrite production, these cytokines production, in BALB/c and C57BL/6 macrophages, were higher for *L. braziliensis* LPG stimulated cells ($P < 0.05$). In all experiments, live parasites from both species induced cytokine production close to background levels (Figure 2B, 3A and 3B). As expected, LPG was able to induce these three cytokines in wild type and TLR2 (-/-) mice but not in TLR4 (-/-) mice. A reduction in these cytokines production in TLR2 (-/-) macrophages was demonstrated, indicating the importance of this receptor in LPG recognition and signaling. A complete abrogation of these cytokines production was observed in TLR4 (-/-) mice suggesting also the participation of TLR4 in LPG recognition and signaling. There was no significant production of IL-10, IL-12p40 (figure 4) or IFN- γ , IL-2, IL-4 and IL-5 after LPG or live promastigote incubation in none of the mouse strains tested (data not shown).

Activation of MAPKs

To better access the signaling events around LPG recognition and macrophage activation, BALB/c macrophages were incubated with *L. braziliensis* and *L. infantum* LPG and MAPK activation was accessed as a function of time. A strong ERK activation was observed in *L. braziliensis* in LPG stimulated macrophages after 15 min incubation but no activation was detected before or after this time interval. In contrast, no ERK activation by *L. infantum* LPG was found. *L. braziliensis* LPG stimulated a sharp p38 and ERK phosphorylation beginning in 5 minutes and peaking at 15 minutes (Figure 5). In a different activation profile, a progressive increase of p38 activation was observed for *L. infantum* LPG stimulated macrophages (Figure 5). Together, these data suggests that the LPG from these two species differentially activates MAPK which may account for the differences in macrophage nitrite and cytokine productions and maybe the differential pathogenesis caused by these two species.

LPGs from *Leishmania infantum* and from *Leishmania brasiliensis* are agonists of TLR2, inducing *ex vivo* the nuclear translocation of NFκB.

In order to better assess the role of TLR2 and TLR4 in the recognition of LPG and activation of NFκB. CHO reporter cells were treated with LPG for 18h and reporter protein CD25 expression evaluated by flow cytometry. As showed in Figure 7, after exposure of CHO cells expressing TLR2 (TLR2+) to 0,2ug or to 0,02ug of LPGb, there was higher production of protein CD25, resulted from expression of gene reporter, in compare with the CHO cells without that exposition, meaning that LPGb is a stark agonist of TLR2, inducing the nuclear translocation of NFκB. After the exposition of TLR4+ cells to LPGb or of TLR4+ or TLR2+ to LPGi, there was a slightly higher production of the protein codified by the gene reporter than the production

in the nonstimulated cells, meaning that the LPGb and LPGi are weak agonists of TLR4 and that LPGi is a weak agonist of TLR2.

Inhibition of nitrite production in BALB/c macrophages by *Leishmania* LPG

Previously, it was shown that GIPLs from both species were able to inhibit NO production in LPS stimulated mice peritoneal macrophages ([Assis et al., 2012](#)). To determine whether LPG would also result in inhibitory activity, BALB/c macrophages were incubated in presence of LPG for 18h prior to LPS exposure. A strong inhibition of NO production stimulated by LPS was observed after LPG incubation from both species. The inhibition was over 70% ($P < 0.01$) for LPG from both species (Figure 6).

Discussion

Leishmaniasis is considered by the World Health Organization as one of the six major infectious diseases in the whole world ([WHO, 2012](#)) and affects over 1.5 million people every year worldwide. In Brazil, the majority visceral and tegumentary cases are due to *L. infantum* and *L. braziliensis*, respectively. Parasite glycoconjugates have long been incriminated in a variety of events during parasite-host interactions modulating important host cell functions ([Assis et al., 2012](#); [de Assis et al., 2012](#); [de Veer et al., 2003](#); [Descoteaux & Turco, 1999](#)). Among these glycoconjugates, LPG is the best studied especially in Old World species of *Leishmania*. In this study, the role of the LPGs from two epidemiologic important *Leishmania* species in Brazil in interfering in signaling mechanisms was assessed in murine macrophages.

Since it was not observed a significant difference between procyclic and metacyclic *L. major* LPG NK cell activation [40] and that the conserved GPI anchor is important for LPG activity [32] this work used stationary phase LPG. Although metacyclic promastigotes can be readily prepared from culture by several methods, only a small percentage of parasites, less than 5% [37], differentiates into these forms. For this reason, all experiments described here could not have been done with metacyclic LPG.

One of the most important events in the initial steps of *Leishmania* infection is the production of NO by macrophages. In many models, its production is dependent on a combination of IFN- γ and TNF- α via TLR mechanisms ([Mosser & Edwards, 2008](#)). Our results indicated that LPG from both species could induce the production of NO in IFN- γ -primed macrophages. Its production was higher by macrophage stimulated with *L. braziliensis* LPG than that of *L. infantum*. C57BL/6 macrophages incubated with both LPGs showed a higher production of NO, IL-1 β and IL-6 than BALB/c. On the other hand, TNF- α production after

stimulation by *L. braziliensis* LPG was higher in BALB/c macrophages (Figures 2 to 4). Similar results were also observed for GIPLs (Assis *et al.*, 2012), these differences of activation between C57BL/6 and BALB/C mice may be due to the genetic background of mouse strains (Silveira *et al.*, 2009; Watanabe *et al.*, 2004). In the present study, no macrophage activation was observed in WT, TLR2(-/-) and TLR4(-/-) murine cells after incubation with live promastigotes.

Previous reports have shown that *in vivo*, pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , as well as chemokines, are induced in the initial events of *L. major* and *L. donovani*, causative agents of cutaneous and visceral leishmaniasis infection in the Old World, respectively. Similar results were observed here using *L. braziliensis* and *L. infantum* LPGs since macrophages stimulated with *L. braziliensis* LPG exhibited higher cytokine and NO production compared to that of the visceral form *L. infantum* (figures 2 and 3). This finding was confirmed after incubation of the LPGs with CHO cells demonstrating that *L. braziliensis* LPG was able to induce NF-kB translocation. These data reflect a similar stimulation pattern between Old World and New World species that causes similar disease outcomes. More importantly, the lack of IL-10, IL-12 production, persistent MAPKs activation and the lack of NF-kB translocation via TLR4 ensure that no traces of endotoxins were present in our preparations.

Because most *Leishmania* glycoconjugates are on the external surface of the cell plasma membrane or secreted, they are able to modulate important functions in cell biology (Assis *et al.*, 2012). The interspecies variations observed in *L. infantum* and *L. braziliensis* may be dependent on the action and specificity of glycosyltransferases (Soares *et al.*, 2005; Soares *et al.*, 2002). For example, in *L. donovani* (MONGI strain) critical glycosyltransferases are down regulated in the metacyclic phases (Mahoney *et al.*, 1999). Such intra- and interspecies variability is likely to have implications in antigenicity enabling carbohydrates to be important sources of biological

diversity (Acosta-Serrano *et al.*, 2001). In this work, the differential pattern of macrophage activation might be due to carbohydrate polymorphisms in the LPG of these two species. Our results with the two New World species of *Leishmania* are consistent with the reports from many Old World *Leishmania* species and strains which showed that LPG with its varied structural polymorphisms induced different levels of NO and TNF- α in murine macrophages (Coelho-Finamore *et al.*, 2011; de Veer *et al.*, 2003; Proudfoot *et al.*, 1996).

No IL-1 β , IL-10 or IL-12 production was observed by cells stimulated with LPGs from both New World species. Similar observations can be made in the human visceral leishmaniasis where immune suppression and a mixed Th1/Th2 profile modulate most of the immune response (WHO, 2012). The lack of IL-12 production by cells stimulated with *Leishmania* GPI-anchored glycoconjugates was also observed elsewhere, where mouse peritoneal macrophages failed to produce IL-12 when co-incubated with *L. braziliensis* or *L. infantum* GIPLs and when also stimulated with IFN- γ or LPS (Vargas-Inchaustegui *et al.*, 2009). It is also important to note that the lack of IL-12 production was not due to IL-10 release, since we did not observe any production of this cytokine (figure 2B). This is similar to that observed when macrophages were treated with synthetic *L. major* LPG (Feng *et al.*, 1999) and *L. braziliensis* and *L. infantum* GIPLs (Assis *et al.*, 2012).

In the present work we also evaluated the role of TLRs on the recognition and signaling of LPG. TLR2 was first incriminated as the LPG receptor in macrophages and NK cells (Becker *et al.*, 2003; de Veer *et al.*, 2003). Additional *in vivo* experiments demonstrated the importance of TLR3, TLR4 and TLR9 in different *Leishmania* species (Tuon *et al.*, 2008). By using RNA interference methodologies, it was shown that both TLR2 and TLR3 were implicated in the recognition of *L. donovani* LPG in IFN- γ primed macrophages (Flandin *et al.*, 2006). *In vivo*, it

was shown that TLR4 deficient mice are more susceptible to *Leishmania* infection, failing to efficiently resolve the lesions (Kropf *et al.*, 2004) while TLR2 shows a more regulatory role in *L. braziliensis*-infected dendritic cells (Vargas-Inchaustegui *et al.*, 2009). Here, we demonstrated *in vitro* with macrophages and CHO cells that TLR2 and, in a lesser extent, TLR4 were recognized by LPGs from both species clearly suggesting their participation in the LPG signalling. The inability of *L. infantum* LPG to activate NF- κ B and ERK could be suggested as an evasion mechanism compared to *L. braziliensis* LPG.

Given that the LPGs were able to induce NO and cytokine production, we investigated whether activation of MAPK signalling was affected. In contrast to GPIs (which fail to activate MAPKs) (Assis *et al.*, 2012), LPG from both species activated MAPKs, but with different kinetics. *L. infantum* LPG was not able to activate ERK1/2. In contrast, *L. braziliensis* LPG strongly activated MAPK activity after 15 min. Interestingly, p38 and JNK activation exhibited a gradual and transient profile in *L. infantum* and *L. braziliensis*, respectively (Figure 5). Although a punctual MAPK activation was observed for *L. braziliensis* LPG after 5min, this may not be a sufficient stimulus for IL-12 production. p38 activation appears to be important for controlling *Leishmania* infection since anisomycin reduced parasite survival upon p38 activation (Junghae & Raynes, 2002). Feng *et al.* (Feng *et al.*, 1999) reported that ERK1/2 and p38 are important for NO and TNF- α production by macrophages stimulated with LPS. Consistent with those observations, our data suggests that the LPGs from New World species are also able to differentially activate MAPKs.

The data presented here demonstrated a differential production of NO, cytokines and MAPK activation profile by *L. braziliensis* and *L. infantum* LPG stimulated macrophages. The LPG from these species have a limited proinflammatory potential since they fail to activate

important proinflammatory cytokines such as IL-12 and only activates low amounts of IL-1 β while activation early inflammatory cytokines such as TNF- α and IL-6. Additionally, as seen in figure 6, preincubation with LPG prior to stimulation with LPS reduced the nitrite production to basal levels, indicating that the dynamics of infection must be well regulated and consistent with the long-known proposal that LPG acts as a multifunctional virulence factor for *Leishmania*.

Together with complex interface of interaction between parasite and host, glycoconjugate interspecies polymorphisms, not only in the LPG, but also in GPIs, gp63 and other GPI-anchored molecules are important for differential regulation of initial events of the immune response as well as establishment of infection. Our results with New World species are consistent with this issue of the importance of LPG polymorphisms; structural variations in LPG resulted in differential activation of macrophages (NO, cytokines and MAPKs). Those polymorphisms could result in different clinical outcomes, such as those shown by *L. infantum* and *L. braziliensis*, causative agents of a visceral and tegumentary forms, respectively (Singh *et al.*, 2006).

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Figure Legends:

Figure 1 Biochemical structures of LPG from *L. braziliensis* and *L. infantum*. Although *Leishmania* LPG shows a well conserved lipid anchor and oligosaccharide core structure, a wide polymorphism on the composition of the sugar branch on the repetitive units is well known. For *L. braziliensis* procyclic promastigote LPG, there is no substitutions on the repetitive units while in *L. infantum* LPG there are different sugar branch substitutions of glucose oligosaccharides that vary from one to three glucoses.

Figure 2 IL-10 (A) and IL-12 (B) production by IFN- γ primed macrophages stimulated with LPG and live parasites. C, negative control; LPGb, *L. braziliensis* LPG; LPGi, *L. infantum* LPG; Lb, *L. braziliensis* live promastigotes and Li, *L. infantum* live promastigotes. Cells were pre-incubated with IFN- γ (3 IU/ml) for 18 h then 10 μ g/mL of LPG. As a positive control, LPS (100 ng/mL) was added. Fresh medium alone was added to negative control cells. Supernatants were collected 48 hours later, cytokine concentrations determined by flow cytometry. ANOVA test was performed and $P < 0.05$ was considered significant.

Figure 3 Nitrite (A) and TNF- α (B) production by IFN- γ primed macrophages stimulated with LPG and live parasites. C, negative control; LPGb, *L. braziliensis* LPG; LPGi, *L. infantum* LPG; Lb, *L. braziliensis* live promastigotes and Li, *L. infantum* live promastigotes. Cells were pre-incubated with IFN- γ (3 IU/ml) for 18 h then 10 μ g/mL of LPG. As a positive control, LPS (100 ng/mL) was added. Fresh medium alone was added to negative control cells. Supernatants were collected 48 hours later, Nitrite concentration was measured by Griess reaction and cytokine concentrations determined by flow cytometry. ANOVA test was performed and $P < 0.05$ was considered significant.

Figure 4 IL-1 β (A) and IL-6 (B) production by IFN- γ primed macrophages stimulated with LPG and live parasites. C, negative control; LPGb, *L. braziliensis* LPG; LPGi, *L. infantum* LPG; Lb, *L. braziliensis* live promastigotes and Li, *L. infantum* live promastigotes. Cells were pre-incubated with IFN- γ (3 IU/ml) for 18 h then 10 μ g/mL of LPG. As a positive control, LPS (100 ng/mL) was added. Fresh medium alone was added to negative control cells. Supernatants were collected 48 hours later, cytokine concentrations determined by flow cytometry. ANOVA test was performed and $P < 0.05$ was considered significant.

Figure 5 Activation of MAPKs (ERK, p38 and JNK) by *Leishmania* LPG in BALB/c peritoneal macrophages. Mouse peritoneal macrophages were stimulated for 30 min with 10 µg/mL of LPG from *L. infantum* (**A, C and E**) and *L. braziliensis* (**B, D and F**). Dually phosphorylated MAPKs were detected by western blot. C, negative control; LPGb, *L. braziliensis* LPG and LPGi, *L. infantum* LPG. Total p38 (**E and F**) content was used as a normalizing protein.

Figure 6 Modulation of nitrite by *Leishmania* LPG in BALB/c macrophages. Cells were incubated with LPG (10 µg/ml) from *L. braziliensis* (LPGb) or *L. infantum* (LPGi) for 18h prior to stimulation with LPS (100ng/ml) combined with 10µg/ml of LPG. Cells were then incubated for another 24h and nitrite content was measured on the supernatants by Griess reaction.

Figure 7 LPGs purified from *Leishmania infantum* and *Leishmania braziliensis* induce translocation of NFκB through TLRs. CHO cells expressing TLR2 (TLR2+), TLR4 (TLR4+), or neither (TLR2-/TLR4-) were either untreated (gray line) or exposed (black line) to LPS, *Staphylococcus aureus* (SA), *L. infantum* LPG (LPGi) or *L. braziliensis* LPG (LPGb), as indicated. CD25 expression was measured by flow cytometry 18 h after stimulation. Percentage = percentage of CD25 expression on stimulated cells minus percentage of CD25 expression on non-stimulated cells.

Figures:

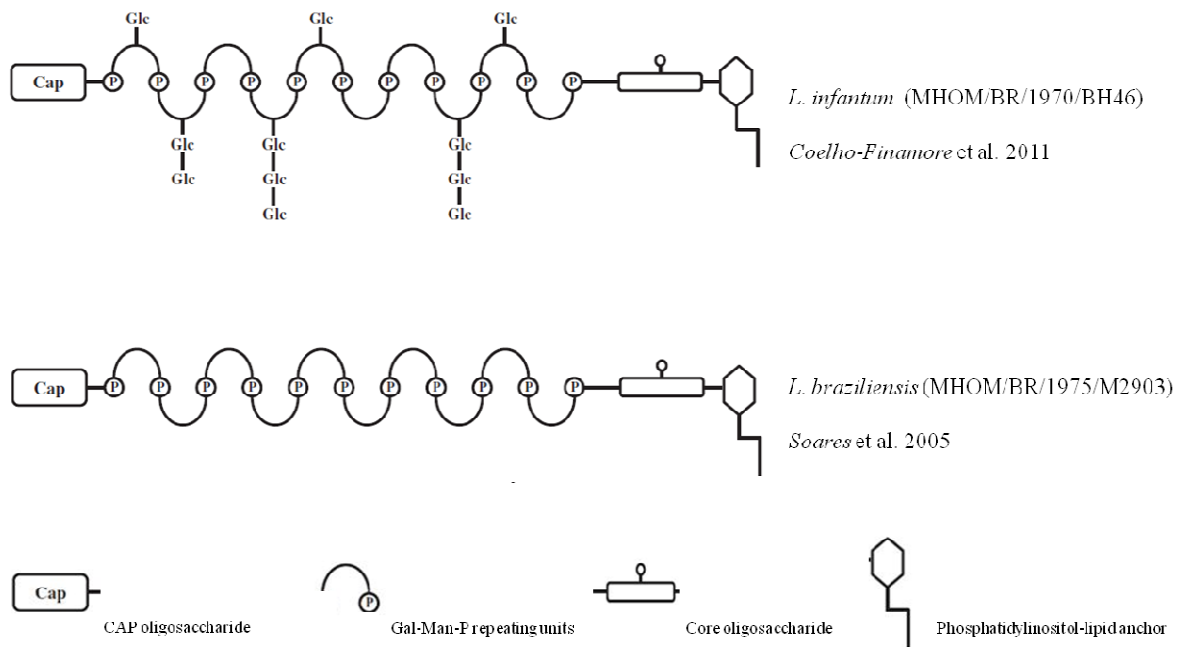
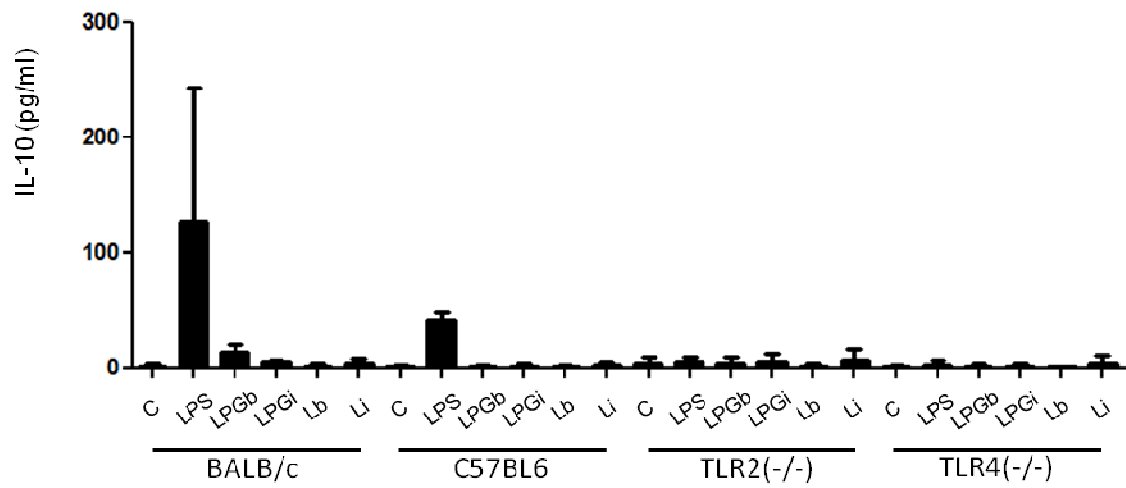


Figure 1

A



B

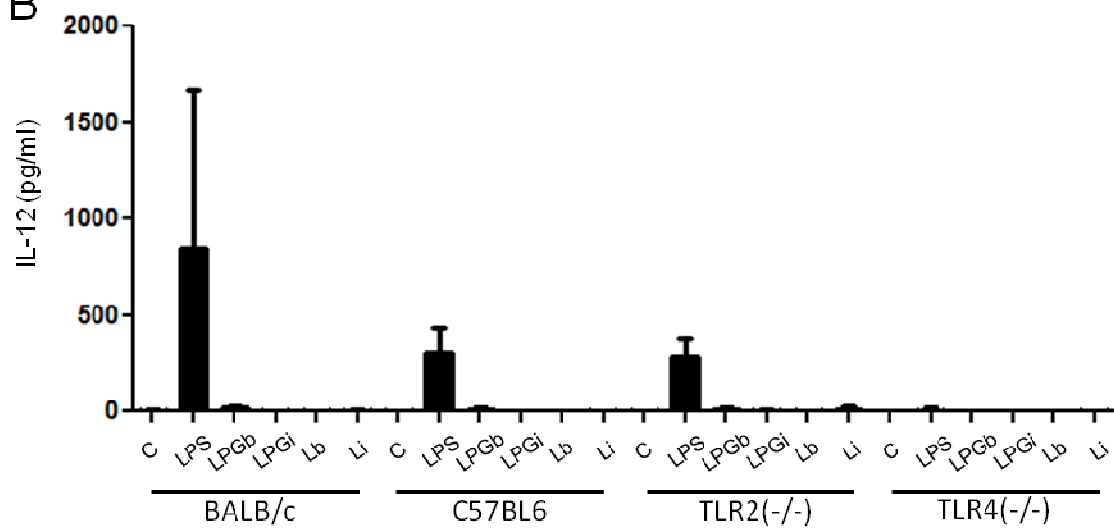


Figure 2.

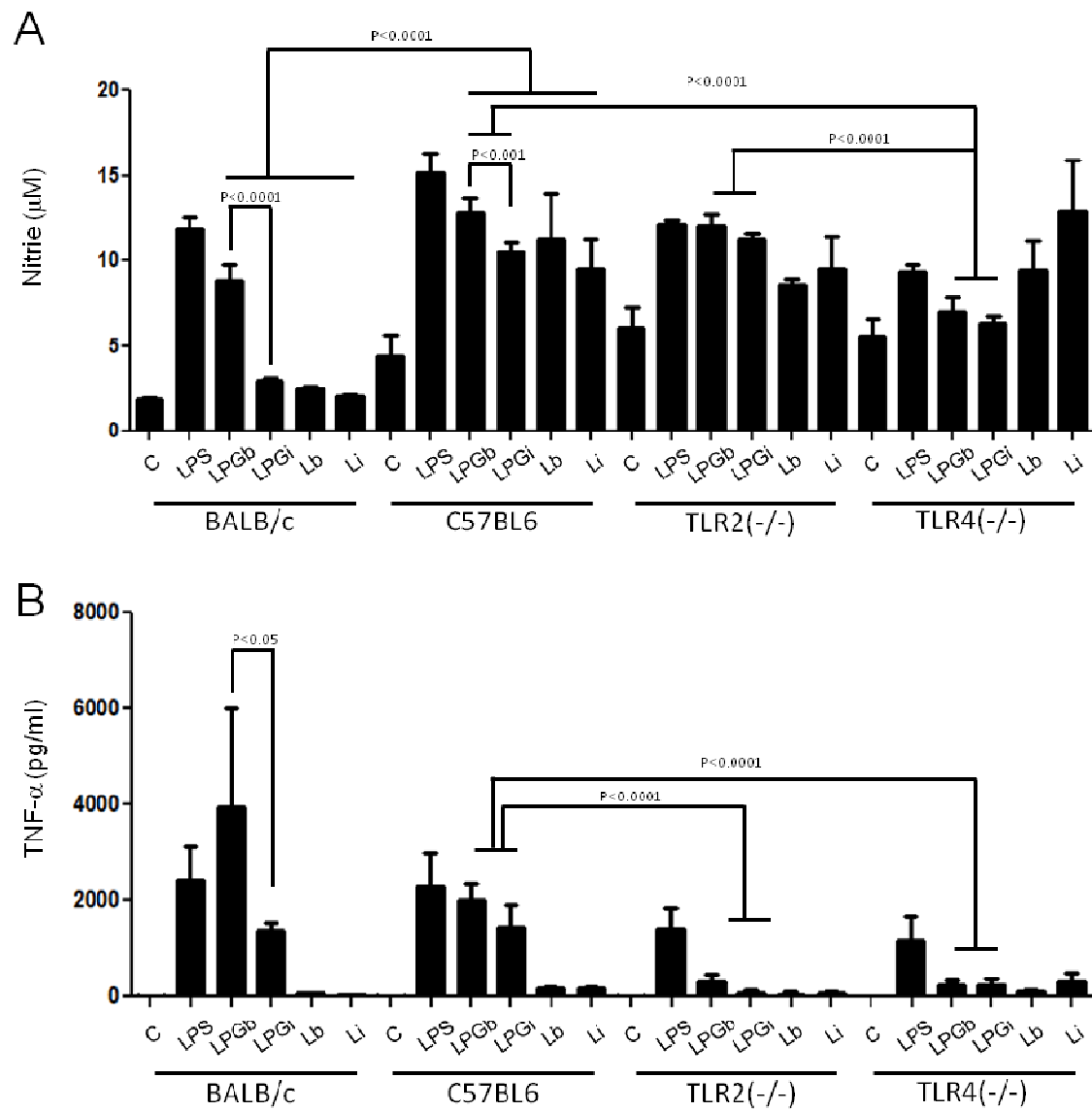


Figure 3.

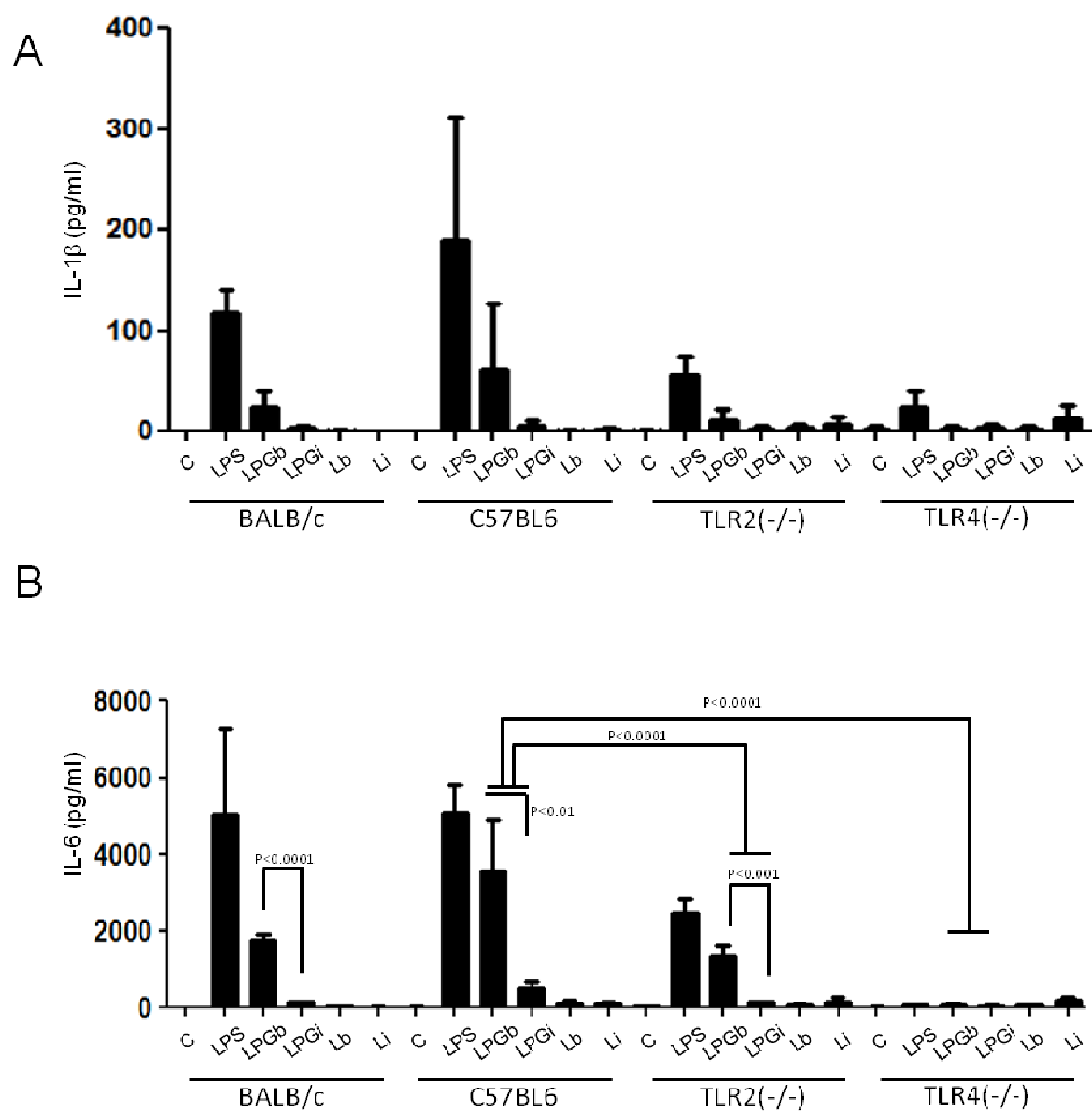


Figure 4.

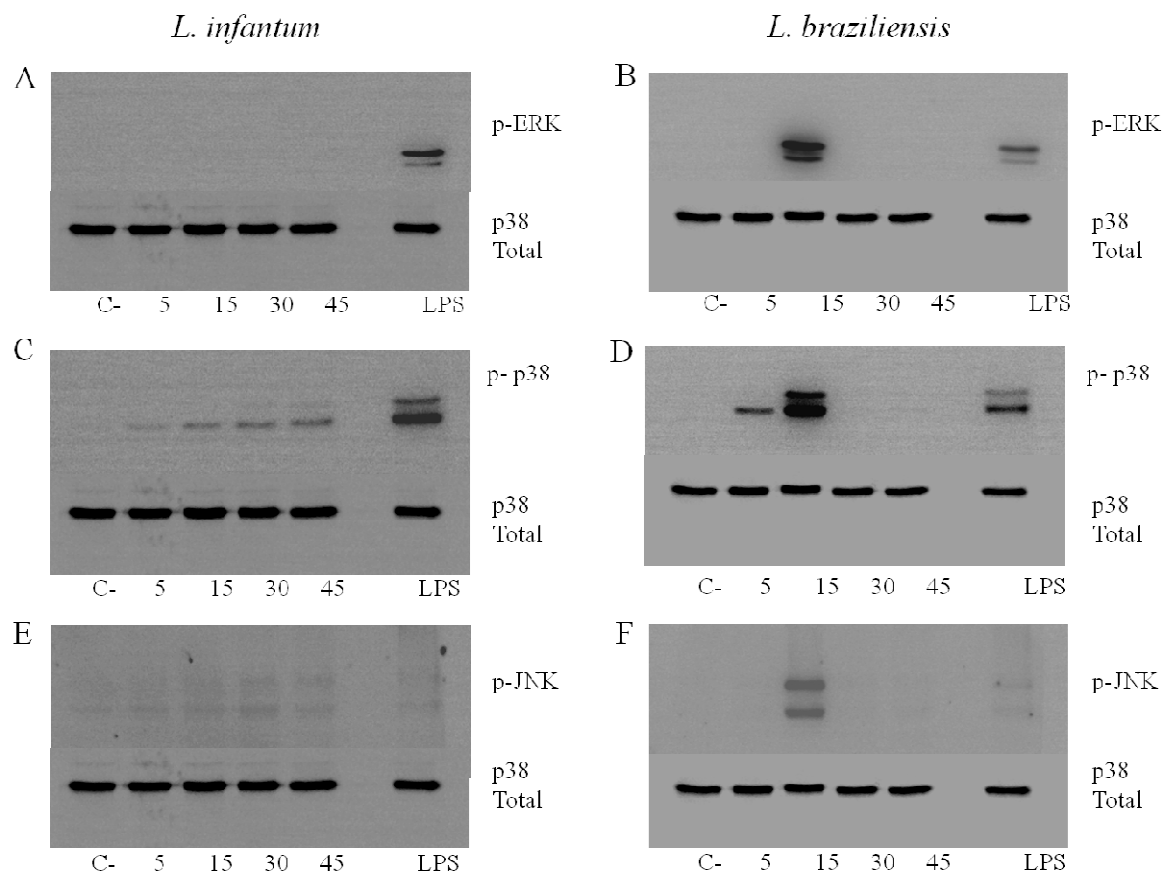


Figure 5.

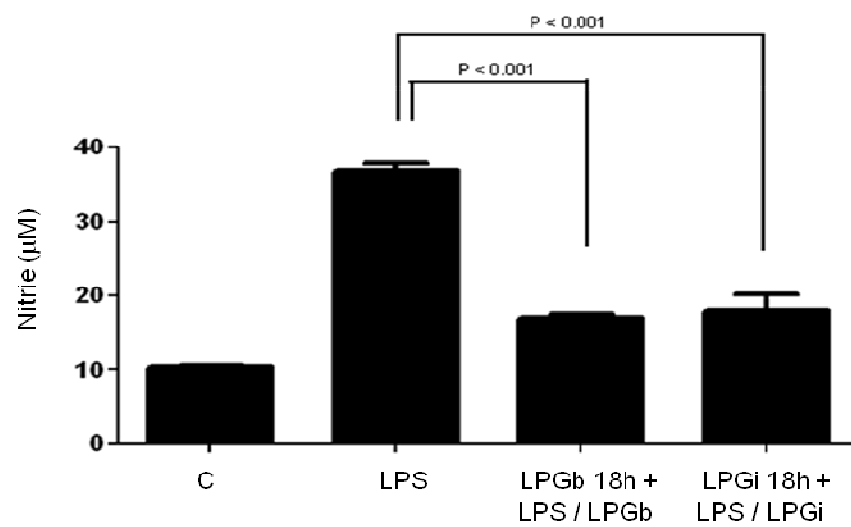


Figure 6.

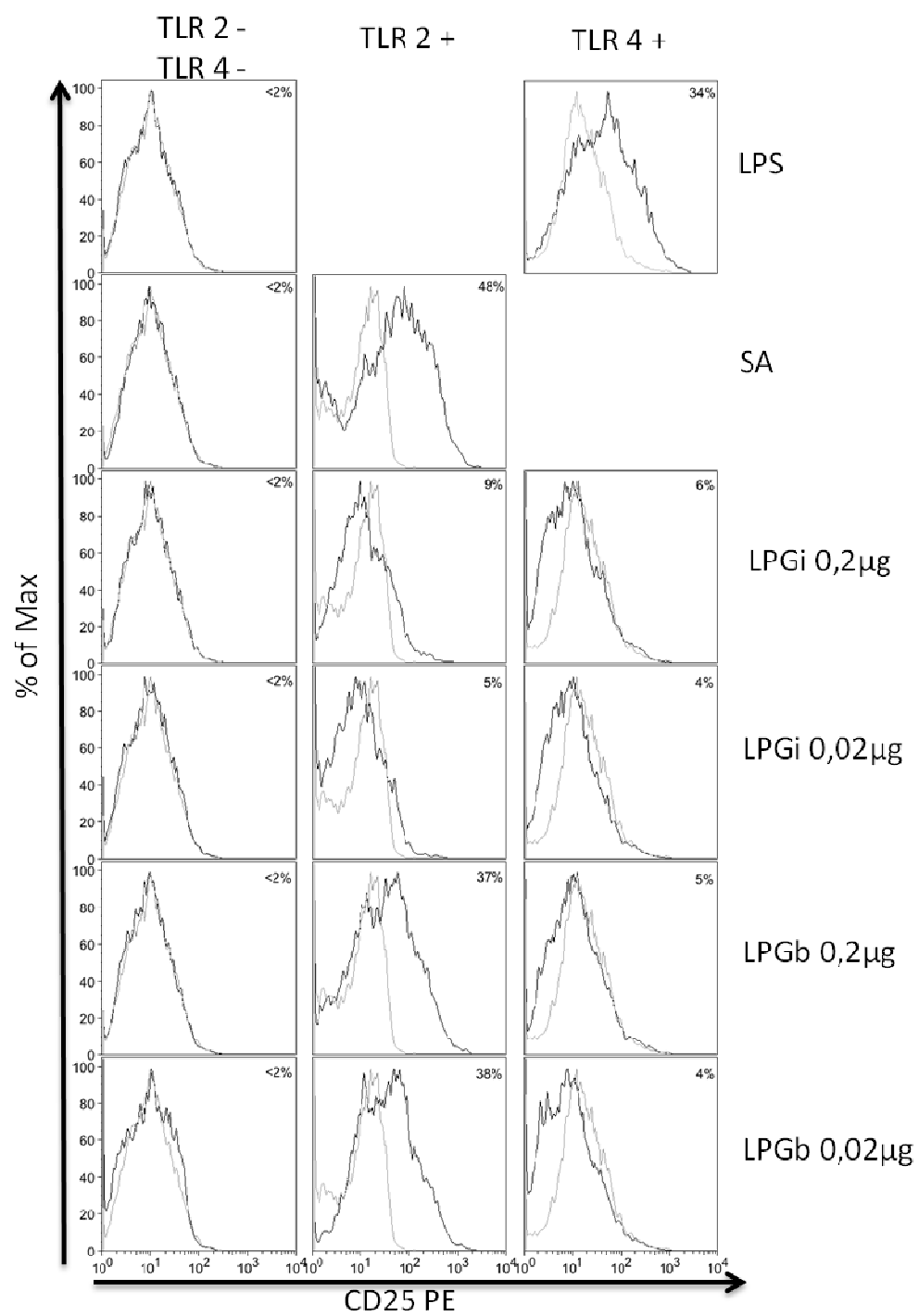


Figure 7.

6. Discussão

Infecções parasitárias são um grave problema no mundo com um grande impacto na saúde pública de países em desenvolvimento. As Leishmanioses são consideradas pela Organização Mundial de Saúde (OMS) como uma das seis doenças parasitárias do mundo afetando cerca de 12 milhões de pessoas em todo o mundo. No Brasil a maior parte dos casos de das formas tegumentar e visceral são causadas pelas espécies *L. braziliensis* e *L. infantum*, respectivamente.

Entretanto, apesar de ter seu ciclo bem descrito, a questão de como estes protozoários interagem com as células hospedeiras para sobreviver e se desenvolver ainda é foco de muitos estudos, e muitas perguntas ainda não foram respondidas.

Para sobreviver no interior de macrófagos, os parasitos devem prevenir ou inibir uma grande variedade de mecanismos microbicidas como a produção de radicais reativos de oxigênio (ROS) e de nitrogênio (NO).

Moléculas de superfície de *Leishmania*, especialmente o LPG, tem sido o alvo de estudos a muitos anos, e já teve diversos mecanismos de interação com o hospedeiro descritos. (de Assis *et al.*, 2012; de Veer *et al.*, 2003; Descoteaux & Turco, 1999). Neste trabalho, o foco principal, foi em outra classe de glicoconjugados, os GIPLs, de duas espécies de *Leishmania* de importância epidemiológica no Brasil. Os GIPLs são moléculas abundantes, presentes na superfície dos parasitos em grande número (chegando a 10^7 moléculas por célula).

Recentemente, estas moléculas foram encontradas associadas a *Lipid Rafts*, estruturas essenciais para a infectividade do parasito e modulação da resposta da célula hospedeira (Yoneyama *et al.*, 2006). De fato existem diversas indicações de que os GIPLs, bem como outras

moléculas ancoradas por GPI, participam em importantes eventos de sinalização celular e estão envolvidos na montagem do complexo NADPH oxidase, produção de NO (Chawla & Vishwakarma, 2003; Lodge & Descoteaux, 2006; Proudfoot *et al.*, 1996; Proudfoot *et al.*, 1995; Tachado *et al.*, 1997) e inibição da expressão gênica dependente de *c-fos* estimulada por LPS ou TNF- α (Descoteaux *et al.*, 1992). Adicionalmente, LPG sintético, cuja estrutura molecular da âncora lipídica é similar aos GIPLs, pode estimular a ativação de ERK e inibir a produção de IL-12 em macrófagos (Feng *et al.*, 1999).

Estudos prévios demonstraram a antigenicidade de GIPLs em pacientes infectados por *L. major* (McConville & Blackwell, 1991; McConville *et al.*, 1990). Entretanto, informações sobre a relevância biológica dos GIPLs nos estágios iniciais da infecção são limitados. Aqui nós demonstramos que os GIPLs de ambas as espécies não ativam a produção de óxido nítrico em macrófagos não primados, dados semelhantes aos publicados com outras espécies (Proudfoot *et al.*, 1995; 1996). Em macrófagos primados, foi observada uma produção inicial de NO e TNF- α . Posteriormente demonstramos que pré incubando os macrófagos com os GIPLs, estas moléculas são capazes de inibir a produção de NO, tanto na presença de IFN- γ quanto LPS, dois grandes estimuladores da produção de NO.

Trabalhos anteriores demonstraram que o LPG é um agonista mais proeminente de TLR para a indução da produção de citocinas pro inflamatórias (Becker *et al.*, 2003; deVeer *et al.*, 2003). Em comparação com LPS e LPG, os GIPLs induzem uma produção menor de NO e TNF- α . Bem como são potentes inibidores da produção de IL-12 e NO.

Estudos *in vivo* com espécies de *Leishmania* do Velho Mundo tem demonstrado a importância dos TLRs, entre outros componentes do sistema imune inato durante a infecção. De

fato, em macrófagos primados, os GIPLs de *L. braziliensis* e *L. infantum* foram capazes de estimular a produção de NO, e esta produção foi primariamente via TLR4, e em segundo lugar TLR2. Entretanto, em macrófagos pré expostos aos GIPLs, foi observada uma inibição de IL-12. Esta inibição não foi devido a produção de IL-10.

Adicionalmente, pode-se observar uma produção maior de NO por camundongos C57, fenômeno esperado, uma vez que esta linhagem é mais responsiva.

Com o intuito de explorar melhor os mecanismos celulares envolvidos na capacidade inibitória dos GIPLs, nós testamos se os GIPLs modulam a ativação de MAPKs. Nós observamos que os GIPLs apenas ativam ERK, contudo, esta ativação foi branda e transitória enquanto que o LPS ativa tanto ERK quanto p38. Também observamos que os GIPLs foram capazes de inibir parcialmente a ativação de ERK e p38.

De forma geral, os GIPLs e o LPG, apresentam âncoras lipídicas bioquimicamente semelhantes e sua integridade molecular é importante para sua atividade biológica. Como foi observado, a remoção da âncora lipídica pelo tratamento com PI-PLC faz com que os GIPLs percam sua atividade inibitória.

Em trabalhos anteriores do nosso grupo, foi demonstrado que as porções glicídicas dos LPGs diferem em estrutura e composição e atividade biológica (Soares *et al.*, 2002; Soares *et al.*, 2005; Coelho-Finamore *et al.*, 2011). Neste trabalho, avaliamos a estrutura e composição da porção glicídica dos GIPLs de *L. braziliensis* e *L. infantum*.

Nossos dados estruturais demonstram que os GIPLs de *L. infantum* são semelhantes aos GIPLs de *L. donovani* e que são compostos primariamente de manose. Estes dados sugerem que estes GIPLs são, primariamente, GIPLs do tipo I ou híbridos. Por outro lado, os GIPLs de *L.*

braziliensis são ricos em galactose e estruturalmente semelhantes aos GIPLs de *L. panamensis* (Zawadzki *et al.*, 1998). Estes dados sugerem que estes GIPLs são primariamente GIPLs do tipo II.

Em conclusão, os GIPLs de ambas as espécies do Novo Mundo, possuem uma forte atividade inibitória em macrófagos murinos. Estas moléculas são capazes de afetar a ativação de macrófagos e inibir a produção de mediadores inflamatórios. Além disso, os dados moleculares qualitativos demonstram que estas moléculas diferem tanto em tamanho quanto em composição. Estes dados, em conjunto com dados publicados, sugerem que os GIPLs são importantes moléculas na interação de *Leishmania* com o sistema imune do hospedeiro, e desempenham um papel fundamental para o parasito facilitando a infecção e permitindo sua sobrevivência.

7. Considerações finais

Os primeiros trabalhos que apontaram para importância dos GIPLs na interação com macrófagos evidenciaram que estas moléculas possuem um papel protetor na infecção. Como demonstrado por Proudfoot *et al.* (1995), Os GIPLs de *L. major* eram capazes de inibir a produção de óxido nítrico por macrófagos murinos. Posteriormente foi demonstrado que estas moléculas eram capazes de ativar PTKs mas sem estimular a ativação de PKCs (Tachado *et al.* 1997). Posteriormente, Chawla e Vishwakarma (2003) demonstraram a capacidade de GIPLs sintéticos de inibir a ativação de PKC.

Neste trabalho ficou demonstrado que os GIPLs das espécies do Novo Mundo *L. braziliensis* e *L. infantum* também são capazes de inibir a produção de Óxido Nítrico em macrófagos murinos peritoneais não primados com IFN- γ (Figura 4A e 4B do primeiro artigo - pág 51). Esta capacidade inibitória foi também evidenciada pela capacidade destas moléculas de inibir a produção de IL-12 (Figura 4D do primeiro artigo - pág 51).

O mecanismo pelo qual os GIPLs exercem seu papel inibitório é ainda pouco conhecido e as evidências sugerem uma atuação multifatorial. Isso pode ser observado pelo fato de que apesar de que os como demonstrado nas figuras 3 e 4, do primeiro artigo (pág 51), GIPLs de *L. braziliensis* e *L. infantum* não são capazes de inibir a produção de TNF- α por macrófagos murinos não primados com IFN- γ bem como estimulam a produção desta citocina por macrófagos primados com IFN- γ .

Estas observações sugerem, em conjunto com os experimentos com células de camundongos *knockout* para receptores do tipo Toll 2 e 4 (Figura 3 do primeiro artigo - pág 51)) que estas moléculas podem ser reconhecidas por receptores e que estas moléculas não afetam a

viabilidade de NF κ B, uma vez que esta molécula é fundamental para a produção de TNF- α via TLR (Falvo *et al.*, 2010).

Outro ponto importante de ressaltar são as evidências, como exposto anteriormente, da interação dos GIPLs com as PTKs e PKCs. Adicionalmente aos dados apresentados nos artigos anexados, com o intuito de investigarmos os mecanismos pelos quais os GIPLs de *L. braziliensis* e *L. infantum* inibem a produção de NO e IL-12, macrófagos peritoneais de camundongos da linhagem BALB/c foram incubados com GIPLs (25 μ g/ml), LPS (100ng/ml), IFN- γ (3 U/ml) ou uma combinação de GIPLs + IFN- γ ou GIPLs + LPS. As células foram incubadas por 45 minutos e então lavadas com PBS e lisadas. A ativação de PTK foi avaliada pela técnica de *western blot*.

Como demonstrado na figura 8, diferente do que foi observado para GIPLs de *L. mexicana* (Tachado *et al.*, 1997). Os GIPLs de *L. braziliensis* e *L. infantum*, não foram capazes de ativar PTKs mas também não inibiram sua ativação quando as células foram estimuladas em conjunto com LPS ou IFN- γ .

Estes dados sugerem que o mecanismo pelos quais os GIPLs atuam nas células hospedeiras é independente de PTKs e que apesar de serem agonistas de TLR 2 e 4, sua capacidade de ativar estas vias é baixa ou que sejam capazes de ativar vias inibitórias.

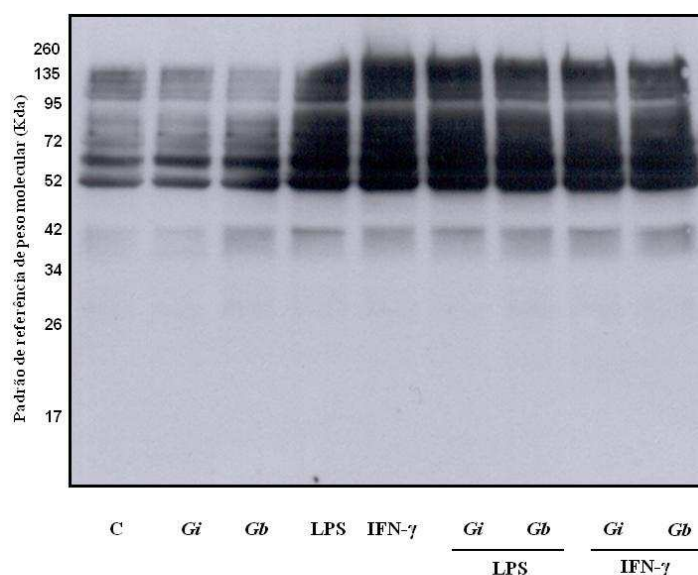


Figura 8. Ativação de proteínas tirosina cinases (PTKs) por GIPLs de *L. braziliensis* e *L. infantum*. Macrófagos peritoneais de Camundongos de linhagem BALB/c foram estimulados por 45 min com GIPLs (25µg/ml) ou uma combinação de GIPLs + IFN-γ ou GIPLs + LPS. A ativação de PTKs foi avaliada por *western blot*. C, controle negativo (células estimuladas apenas com meio); Gi, GIPLs de *L. infantum* e Gb, GIPLs de *L. braziliensis*.

Por este motivo, existe a necessidade de se investigar outras vias, como as vias de ativação de PKC e vias inibitórias como PKCζ e PI3K/Akt. Com o intuito de investigar a capacidade dos GIPLs de ativar PKC, macrófagos murinos (linhagem BALB/c) foram incubados com os GIPLs (25µg/ml) de ambas as espécies por 15, 30, 45 e 60 minutos. Após este tempo, as células foram lavadas e lisadas e a ativação de PKCα e PKCζ avaliada por *western blot*. Como proteína normalizadora, foi utilizada β-actina.

Como demonstrado na figura 9, os GIPLs de ambas espécies não foram capazes de ativar de forma significativa PKCα. Isso era esperado, uma vez que estas moléculas não apresentam

forte atividade estimulatória em macrófagos não primados. A PKC α , é uma cinase do grupo das PKCs clássicas, ativadas por diacilglicerol (DAG) de forma dependente de cálcio. Este isotipo é comumente associado a ativação da produção de Radicais reativos de Oxigênio e resposta pro inflamatória bem como ativação da resposta de macrófagos induzida por LPS.

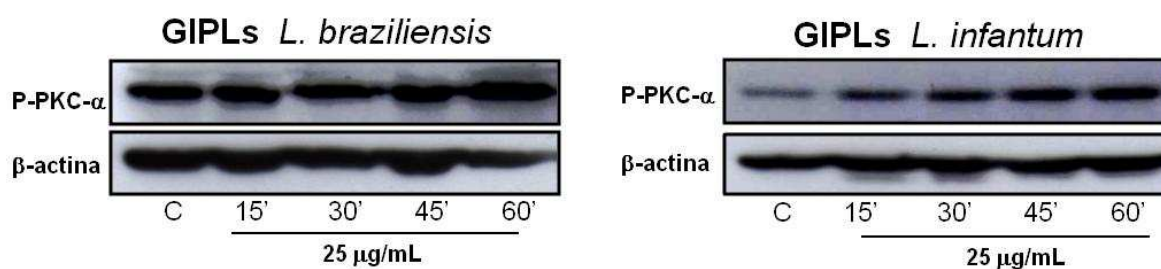


Figura 9. Ativação de proteína cinase C alfa (PKC α) por GIPLs de *L. braziliensis* e *L. infantum*. Macrófagos peritoneais de Camundongos de linhagem BALB/c foram estimulados por 15, 30, 45 e 60 min com GIPLs (25 μ g/ml). A ativação de PKC α foi avaliada por *western blot*. C, controle negativo (células estimuladas apenas com meio).

Por outro lado, como demonstrado na figura 10, os GIPLs de ambas as espécies foram capazes de ativar PKC ζ . A PKC ζ , é uma cinase do grupo das PKCs atípicas, ativadas por inositol-trifosfato e são insensíveis a DAG e independentes de cálcio. Este isotipo é comumente associado a produção de IL-10 citocina com importante papel imuno modulatório. A Ativação de PKC ζ , na infecção por leishmania, também é comumente acompanhado do aumento da produção de ceramida. O aumento da produção de ceramida leva a ativação de fosfotirosina-fosfatase (PTP) que consequentemente leva a inibição de MAPKs.. Isso, em parte, ajuda a

compreender o mecanismo pelo qual os GIPLs de *L. braziliensis* e *L. infantum* podem ter inibido parcialmente a ativação de p38 e ERK (figura 6 de Assis *et al.*, 2012a).

A ativação de PKC ζ ocorreu com dinâmicas um pouco diferente entre as espécies, sendo os GIPLs de *L. infantum*. Enquanto os GIPLs de *L. infantum* foram capazes de ativar esta via aos 15 minutos de incubação, esta ativação só ocorreu aos 30 minutos para *L. braziliensis*.

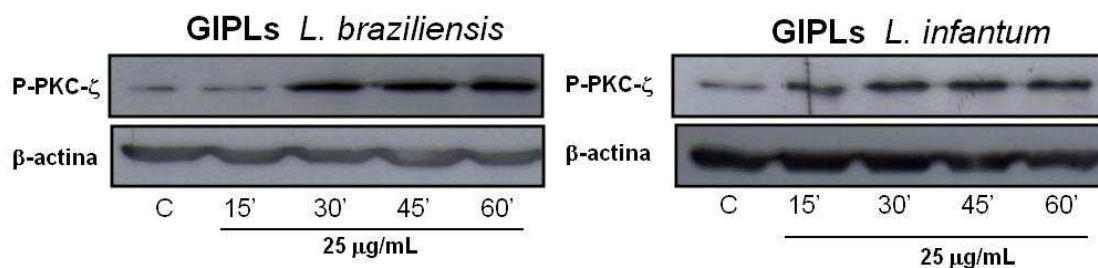


Figura 10. Ativação de proteína cinase C zeta (PKC ζ) por GIPLs de *L. braziliensis* e *L. infantum*. Macrófagos peritoneais de Camundongos de linhagem BALB/c foram estimulados por 15, 30, 45 e 60 min com GIPLs (25 μ g/ml). A ativação de PKC ζ foi avaliada por *western blot*. C, controle negativo (células estimuladas apenas com meio).

A ativação de PKC ζ é um fenômeno importante e deve ser mais explorado em trabalhos futuros. Em especial, os mecanismos de ativação de PKC pelos GIPLs. Estes mecanismos não foram explorados neste trabalho mas algumas considerações podem ser feitas. Como sugerido por Chawla *et al.* (2003), após o contato com a célula hospedeira, os GIPLs podem ser clivados por fosfolipases fazendo com que sua porção *fosfatidilinositol* possa ser reconhecida, ou sirva como substrato alternativo para PKC diretamente ou indiretamente a via PI3K/PKB, capaz de ativar PKC. Além disso, a interação com a membrana pela porção lipídica, pode interferir com a

organização molecular de domínios de membrana (*lipid rafts*) e consequentemente afetando a capacidade dos receptores de se organizarem e sinalizarem corretamente (Tachado *et. al.*, 1997).

Apesar de não terem sido ainda descritas, evidências diretas da ativação de PI3K/PKB por GIPLs, uma evidência indireta pode ser observada na figura 11. Neste experimento, os macrófagos foram tratados com os GIPLs por 15 minutos antes da adição da estimulação com LPS. Em seguida foram adicionados inibidores de MAPKs (SB203580 ou PD 98059) e as células incubadas por 24h. A concentração de TNF- α no sobrenadante foi avaliada por ELISA.

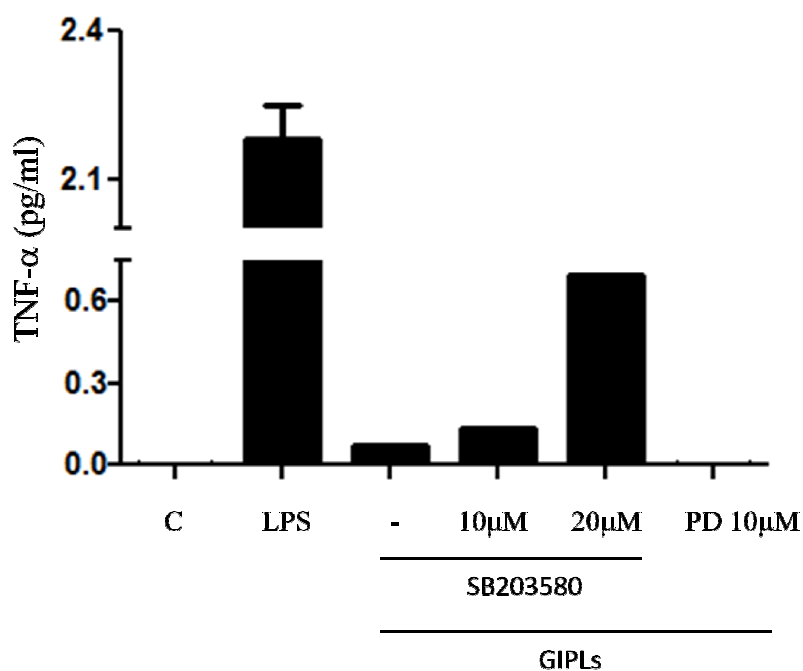


Figura 11. Produção de TNF- α por macrófagos murinos após estimulação com GIPLs e inibidores de MAPK. Os macrófagos foram tratados com GIPLs e diferentes concentrações de inibidores (SB203580 - inibidor de p38 e PD 98059 - inibidor de ERK).

Como descrito por (Lali *et al.*, 2000) o inibidor de p38 (SB203580) possui especificidade imitada para esta cinase e em concentrações mais elevadas é capaz de inibir também a via de PI3K/PKB (Lali *et al.*, 1999). A via de PI3K/PKB inibe a produção de TNF- α . Assim, como demonstrado na figura 11, o tratamento com concentrações altas de SB203580 levou a produção de TNF- α , de forma dose dependente. Este dado, indica indiretamente, que os GIPLs ativaram PI3K, que inibe a produção de TNF- α em células não primadas e tratadas com os GIPLs. A inibição de PI3K com os inibidor de p38 reduziu o efeito desta via na inibição da produção da citocina de forma dose dependente.

É importante ressaltar que estas são evidências indiretas. A implicação de PI3K/PKB de forma definitiva na sinalização estimulada por GIPLs de *Leishmania* depende de muitos outros experimentos. E sua relevância ainda precisa ser comprovada de forma direta.

De forma geral, os dados apresentados, demonstram que os GIPLs de *L. braziliensis* e *L. infantum* são capazes de interferir em importantes vias de sinalização celular e que possivelmente atuam de forma extremamente importante no processo de infecção ao contribuir com a construção de um contexto inflamatório favorável ao parasito e que estas moléculas merecem uma atenção maior no estudos dos mecanismos de evasão de *Leishmania*.

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