UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO GONÇALO MONIZ

UFBA **FIOCRUZ**

Curso De Pós-Graduação em Patologia Humana

DISSERTAÇÃO DE MESTRADO

OBTENÇÃO E CARACTERIZAÇÃO DE *LEISHMANIA INFANTUM* DEFICIENTE PARA O GENE *LPG2*

FLÁVIO HENRIQUE DE JESUS SANTOS

Salvador – Bahia
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Dissertação apresentada ao Curso de Pós-Graduação em Patologia para a obtenção do grau de Mestre.

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"OBTENÇÃO E CARACTERIZAÇÃO DE LEISHMANIA INFANTUM DEFICIENTE PARA O GENE LPG2 ".

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FONTES DE FINANCIAMENTO

"O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001"

DEDICATÓRIA

Dedico este trabalho a meus pais.

AGRADECIMENTOS

Ao Instituto Gonçalo Moniz e ao Laboratório Integrado de Laboratório de Inflamação e Biomarcadores (LIB) por toda a infraestrutura que possibilitou o desenvolvimento das atividades deste trabalho.

À CAPES pela bolsa de estudos que possibilitou a realização deste trabalho.

À Dra. Valéria de Matos Borges e ao Dr. Leonardo Paiva Farias, pelos ensinamentos, confiança e paciência, sendo essenciais para o desenvolvimento deste trabalho e para a minha formação profissional.

Ao Dr. Jonilson Berlink e ao Dr. Albert Descoteaux pelo apoio e ajuda em algumas fases do desenvolvimento deste trabalho.

Ao IGM e seus funcionários, especialmente a Coordenação de Ensino da Pós-Graduação em Patologia Humana e a Biblioteca.

Aos alunos (as) do LIB e a todas as pessoas que ajudaram direta ou indiretamente para a realização desse trabalho.

Aos meus pais, a minha família, a minha noiva e aos meus amigos, pelo companheirismo, incentivo e apoio o que contribuiu bastante para a minha formação.

SANTOS, Flávio Henrique. Obtenção e caracterização de *Leishmania infantum* deficiente para o gene *LPG2*. 53 f. il. Dissertação (Mestrado em Patologia Humana) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2019.

RESUMO

INTRODUÇÃO: O glicoconjugado LPG é uma das moléculas dominantes na superfície do estágio de promastigota de *Leishmania*. Este fator de virulência multifuncional participa em uma variedade de processos durante o estabelecimento da infecção no hospedeiro mamífero. Além do LPG, esses protozoários possuem em sua superfície outras moléculas como os PPGs, PGs e sAP que contribuem para a sobrevivência da *Leishmania* dentro da célula hospedeira, Umas das enzimas essenciais para síntese do LPG e destas outras moléculas contendo fosfoglicanos (PGs) em sua estrutura é a GDP-manose transferase que é codificada pelo gene *LPG2*. **OBJETIVOS:** Caracterizar a estrutura do gene *LPG2* no genoma de *L. infantum* (MCAN/BR/89/BA262). Obter parasitas *lpg2*$^{-/-}$ e caracteriza-los molecularmente e avaliar sua virulência em ensaios de infecção de macrófagos *in vitro*. **MÉTODOS:** A edição do genoma foi realizada utilizando o método clássico de recombinação homóloga com marcadores de resistência a antibióticos, e posteriormente com o sistema CRISPR/Cas9. Os parasitas nocautes foram selecionados utilizando ensaios de aglutinação com anticorpo CA7AE e com a lectina (RCA 120). **RESULTADOS:** O processo de obtenção dos parasitas nocautes por recombinação homóloga se demonstrou inviável devido a presença de uma cópia adicional do gene *LPG2* no genoma de *L. infantum* que não havia sido descrita anteriormente. Esse resultado nos levou a utilizar o sistema CRISPR/Cas9 para a interrupção do gene *LPG2* nesta espécie. Cinco clones foram isolados após as etapas de seleção utilizando o anticorpo CA7AE e posteriormente a lectina (RCA 120). Os resultados obtidos com o sequenciamento mostraram a ocorrência da edição esperada no genoma em todos os clones. Além disso, o resultado do Western blot mostrou a perda completa da expressão do LPG e de PGs. Os resultados de curvas de crescimento mostram que todos os clones *lpg2*$^{-/-}$ apresentam um fenótipo (morfologia) e uma taxa de crescimento similar, sugerindo a ausência de efeitos de edição inespecíficos "off-targets". Ensaios preliminares de infecção de macrófagos murinos *in vitro* utilizando os parasitas *lpg2*$^{-/-}$, demonstram uma redução na taxa de infecção (4h - 34,5% e 72h - 34,6%) além de uma diminuição na carga parasitária (4h - 46,2% e 72h - 49,9%). **CONCLUSÃO:** O gene *LPG2* está duplicado em *L. infantum* e foi nocauteado com sucesso utilizando o sistema CRISPR/Cas9. Os dados dos ensaios de infecção reforçam a importância do LPG e outros PGs como fatores de virulência na interação parasito-hospedeiro.

Palavras chaves: CRISPR/Cas9, *L. infantum*, lipofosfoglicano, LPG2.

SANTOS, Flávio Henrique. Obtaining and characterization of *Leishmania infantum* deficient for the *LPG2* gene. 53 f. il. Dissertação (Mestrado em Patologia Humana) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2019.

ABSTRACT

INTRODUCTION: Lipophosphoglycan glycoconjugate (LPG) is one of the dominant molecules on the surface of the *Leishmania* promastigote stage. This multifunctional virulence factor participates in a variety of processes during the establishment of infection in the mammalian host. In addition to LPG, these protozoa have on their surface other molecules, e.g. Proteophosphoglycans (PPGs), free phosphoglycan polymers (PGs) and acid phosphatases (sAP), which contribute to the survival of *Leishmania* within the host cell by modulating signaling of macrophages. One essential enzymes for the synthesis of LPG and other phosphoglycan (PG) containing molecules is the GDP-mannose transferase, which is encoded by the *LPG2* gene. **OBJECTIVES**: Characterize the genomic structure the *LPG2* gene in *L. infantum* (MCAN/BR/89/BA262). Generate *lpg2*$^{-/-}$ parasites, provide their molecular characterization and finally assess their virulence in vitro macrophages infection assays. METHODS: Genome editing was performed using the classical homologous recombination procedures by using antibiotic resistance genes as markers, and later on with the CRISPR/Cas9 system. Knockouts parasites were screened by agglutination assays using CA7AE antibody and subsequently the lectin (RCA 120). **RESULTS**: The process of obtaining knockout parasites by homologous recombination was unsuccessful due to the presence of an additional copy of the *LPG2* gene in the *L. infantum* genome, which has not been previously described. This result led us to use the CRISPR/Cas9 system for *LPG2* gene disruption in this species. Six clones were isolated following the screening steps using CA7AE antibody and then lectin (RCA 120). The sequencing results demonstrated the occurrence of the expected genome edition in all clones. In addition, the Western blot results revealed complete loss of LPG and PG expression. Growth curve assays demonstrated that all *lpg2*$^{-/-}$ clones present similar growth rates, suggesting the absence of off-target effects. Preliminary assays of murine macrophages infection by *lpg2*$^{-/-}$ parasites demonstrated a reduction in infection rate (4h - 34,5% e 72h - 34,6%), as well as in parasite load (4h - 46,17% e 72h - 49,88%). **CONCLUSION:** The gene *LPG2* is duplicated in *L. infantum* and was successfully knocked out using the CRISPR/Cas9 system. The results obtained herein reinforce the importance of LPG and other PGs as virulence factors in host-parasite interactions.

Key words: CRISPR/Cas9, *L. infantum*, Lipophosphoglycan, LPG2.

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LISTA DE ABREVIATURAS

Cas9	Sistema da nuclease 9
CRISPR	Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas
GIPLs	Glicoinositolfosfolípidios
HDR	Reparo dirigido por homologia
LC	Leishmaniose cutânea
LPG	Lipofosfoglicano
LV	Leishmaniose visceral
MCL	Leishmaniose mucocutânea
MMEJ	Junção das extremidades mediada por micro-homologia
NHEJ	Reparo por junções de extremidades não homologas
NTD	Doença Tropical Negligenciada
PAM	Protospacer Adaptor Motif
PCR	Reação em cadeia da polimerase
PGs	Fosfoglicano
PKDL	Leishmaniose dérmica pós-calazar
PPGs	Proteofosfoglicano
sAP	Fosfatases ácidas secretadas
TALENs	Nucleases efetoras do tipo ativador de transcrição
ZFNs	Nucleases de dedos de zinco

SUMÁRIO

1. INTRODUÇÃO

1.1 LEISHMANIOSE

A leishmaniose é uma enfermidade classificada como uma doença tropical negligenciada (NTD), provocada por protozoários do gênero *Leishmania* com distribuição mundial, sendo considerada endêmica em 98 países (ANVERSA, 2018). Essa doença apresenta 4 formas clinicas principais: leishmaniose visceral (LV, também conhecida como calazar); leishmaniose dérmica pós-calazar (PKDL); leishmaniose cutânea (LC); e leishmaniose mucocutânea (MCL). A forma cutânea é considerada a mais comum, e a forma visceral a mais grave sendo quase sempre fatal se não tratada a tempo (WHO et al., 2017). A LC é endêmica em 18 países das Américas e 70% dos casos mundiais ocorreram em 10 países: Afeganistão, Argélia, Brasil, Colômbia, Costa Rica, Etiópia, República Islâmica do Irã, Peru, Sudão e República Árabe da Síria (FOCUS, 2016). A *Leishmania amazonensis* e a *Leishmania braziliensis* são os principais agentes etiológicos desta forma da doença no Brasil (SCORZA; CARVALHO; WILSON, 2017). A LC caracteriza-se por inchaço local, presença de uma pápula eritematosa com o tamanho variando de 1 a 10 mm de diâmetro, ocorrendo também a formação de úlceras e adenopatia regional (TORRES-GUERRERO et al., 2017).

A LV está presente em 76 países. Nas Américas ela é endêmica em 12 países e 96% dos casos que ocorrem nesta região estão concentrados no Brasil (OPAS, 2018) (Figura 1). No período entre 2001-2016, pouco mais de 55 mil casos humanos de LV foram reportados nas Américas, sendo que no Brasil as regiões que se destacam são a Norte, Nordeste e Sudeste (OPAS, 2018). Essa forma da doença é caracterizada por febre, perda de peso, aumento no volume do baço, do fígado e anemia, podendo os pacientes desenvolver coinfecções como pneumonia e tuberculose. O principal agente etiológico dessa forma da doença na América Latina é a *Leishmania infantum* (SAPORITO et al., 2013).

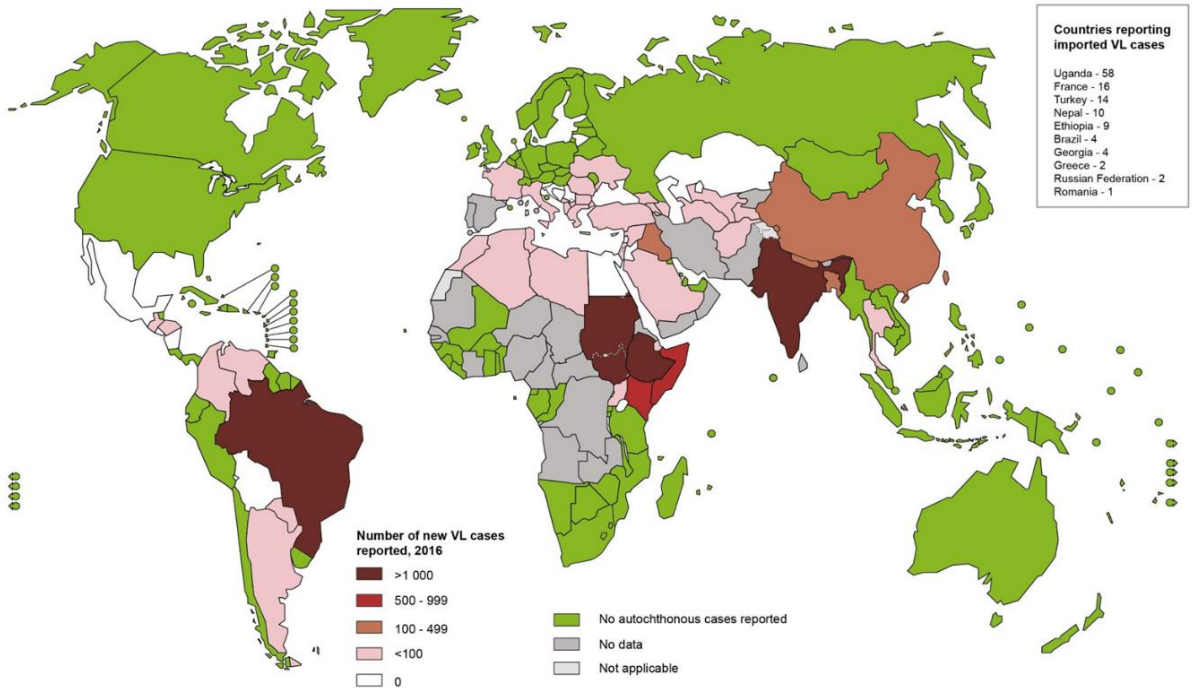

Figura 1: **Distribuição endêmica da leishmaniose visceral (LV) no mundo**. Mapa demostrando os números de novos casos de LV reportados no ano de 2016 (WHO, 2016).

1.2 MORFOLOGIA E CICLO BIOLÓGICO DA *Leishmania* sp.

Os parasitos do gênero *Leishmania* apresentam um ciclo digenético que envolve tanto um hospedeiro vertebrado quanto um hospedeiro invertebrado (inseto vetor). Esses parasitas apresentam diferentes morfologias que são adaptadas aos respectivos hospedeiros. Eles podem ser caracterizados por uma forma promastigota extracelular que possui um formato alongado, sendo essa a forma que é inoculada no hospedeiro vertebrado durante o repasto sanguíneo, além de possuírem um flagelo que é utilizado para fixação no intestino do flebótomo e para sua mobilidade. Já a forma amastigota é intracelular obrigatória, possui um formato arredondado e flagelo reduzido, sendo encontrada principalmente em células fagocitárias do hospedeiro vertebrado (SACKS; KAMHAWI, 2001; SUNTER; GULL, 2017), além de outros tipos celulares (Cavalcante-Costa et la., 2019) (Figura 2).

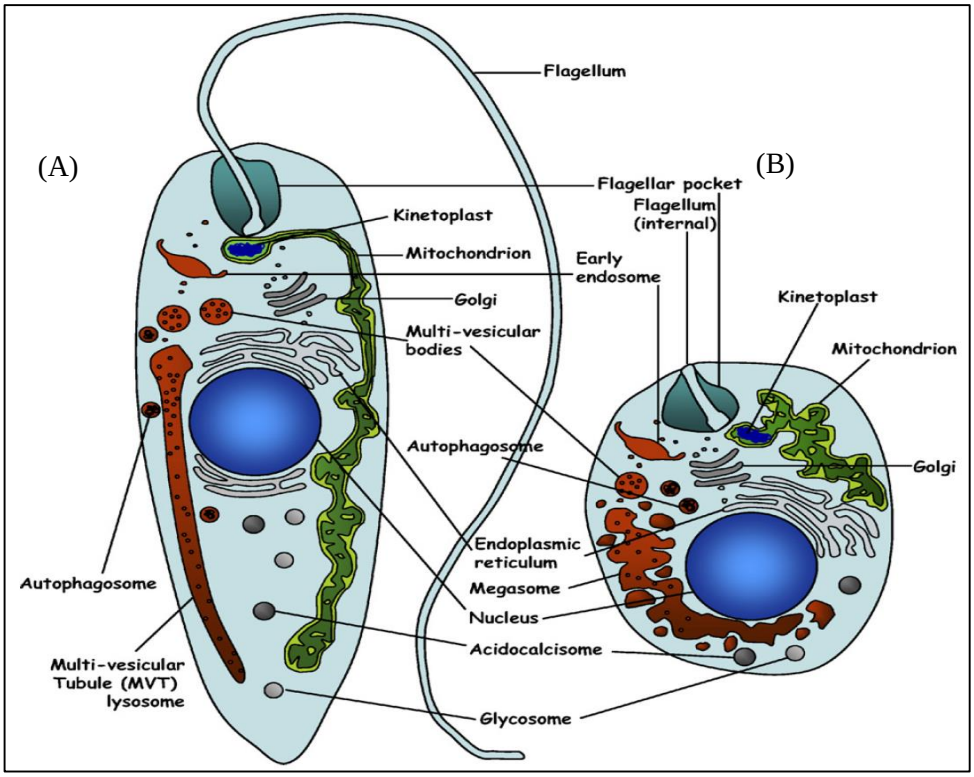

Figura 2: Representação esquemática da ultraestrutura de uma *Leishmania.* Formas promastigota **(A)** e amastigota **(B)**. (extraído e modificado de WILLIAMS; COOMBS; MOTTRAM, 2007).

A transmissão desses parasitas ocorre durante o repasto sanguíneo feito pela fêmea do flebotomíneo infectada podendo ser do gênero *Phlebotomus* no Velho Mundo ou *Lutzomyia* no Novo Mundo. Quando presente no trato intestinal do flebótomo, a forma promastigota passa por diversos estágios de diferenciação até alcançar a forma infecciosa (metacíclica), sendo esse processo denominado metaciclogênese (BATES, 2007).

Após a transmissão para o hospedeiro vertebrado esses parasitas são fagocitados por neutrófilos e posteriormente por macrófagos onde irá ocorrer a diferenciação na forma amastigota. Sendo a diminuição do pH no fagossomo e o aumento da temperatura alguns dos gatilhos para que ocorra essa mudança na forma do parasita que passa a se multiplicar por divisão binária até que ocorra o rompimento da célula hospedeira o que possibilita a infecção de novas células. Por fim, durante um novo repasto sanguíneo em um hospedeiro infectado os

flebótomos acabam ingerindo os parasitas na forma amastigota o que leva a renovação do ciclo biológico (CDC, 2013) (Figura 3).

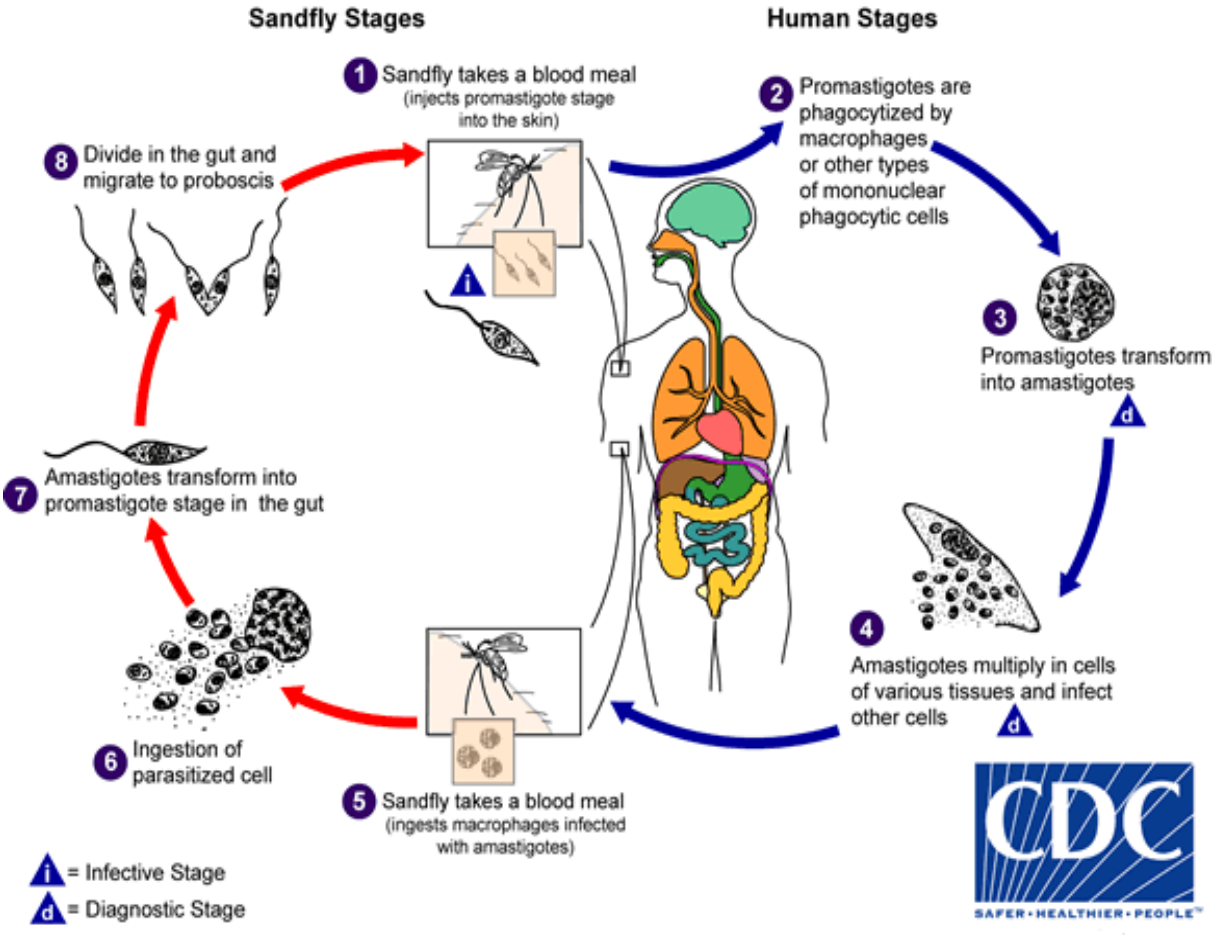

Figura 3: Representação esquemática do ciclo biológico de *Leishmania sp*. Esquema exemplificando as etapas que ocorrem no ciclo de *Leishmania sp* tanto no vetor invertebrado (flebotomíneo) quanto no hospedeiro vertebrado (mamífero). (Adaptado de https://www.cdc.gov/parasites/*Leishmania*sis/biology.html).

1.3 LIPOFOSFOGLICANO (LPG)

Uma característica chave de muitos microorganismos patogênicos é a presença de um denso glicocálice em sua superfície, composto por glicoprotéinas ancoradas por lipídeos e polissacarídeos. Semelhante a todos os parasitas da família *Trypanosomatidae* os protozoários do gênero *Leishmania* apresentam um glicocálice que cobre toda sua superfície. O lipofosfoglicano (LPG) é o glicoconjugado de superfície da *Leishmania* mais estudado sendo

dominante nas fases promastigotas procíclicas e metacíclicas desses parasitas com cerca de $5x10^6$ moléculas por célula cobrindo toda superfície do parasita incluindo o flagelo. Em contraste na forma amastigota esta molécula apresenta baixa expressão. Esse glicoconjugado vem sendo bastante caracterizado bioquimicamente e os polimorfismos presentes em sua estrutura mostraram-se críticos na especificidade da *Leishmania* para os diferentes vetores (DESCOTEAUX; TURCO, 1999; RAMIRO et al., 2012).

O LPG de *Leishmania* é uma macromolécula complexa composta por quatro domínios distintos. Uma ancora de GPI, que consiste de um 1-*O*-alquil-2-liso-fosfatidilinositol contendo uma cadeia alifática saturada única. Um núcleo glicano (“core”) que é um heptassacarídeo composto por dois galactopiranosídeos, um galactofuranosídeo, dois manosídeos e um resíduo de glucosamina ligado ao inositol. Esse núcleo glicano está ligado a um fosfoglicano (PG) linear que contém de 15-40 unidades repetidas de fosfodissacarídeo (Galβ1, 4Manα1-PO4). Podendo ser terminada (“cap”) por um di- tri- ou tetrasacarídeo que é formado por galactose e manose montados da seguinte forma: Manα1, 2Manα1 ou Galβ1, 4 (revisado em FORESTIER; GAO; BOONS, 2015) (Figura 4). Estudos bioquímicos já demonstraram que as diferentes espécies de *Leishmania* apresentam um LPG que possui tanto a âncora lipídica quanto o núcleo glicano idênticos, porém as cadeias laterais e o “cap” terminal apresentam polimorfismos (DESCOTEAUX; TURCO, 1999). Na espécie de *L. infantum* as substituições nas cadeias laterais variam dependendo da cepa, sendo encontrado três conjuntos diferentes de cadeias laterais: Tipo I, Tipo II e Tipo III (COELHO-FINAMORE et al., 2011; IBRAIM et al., 2013).

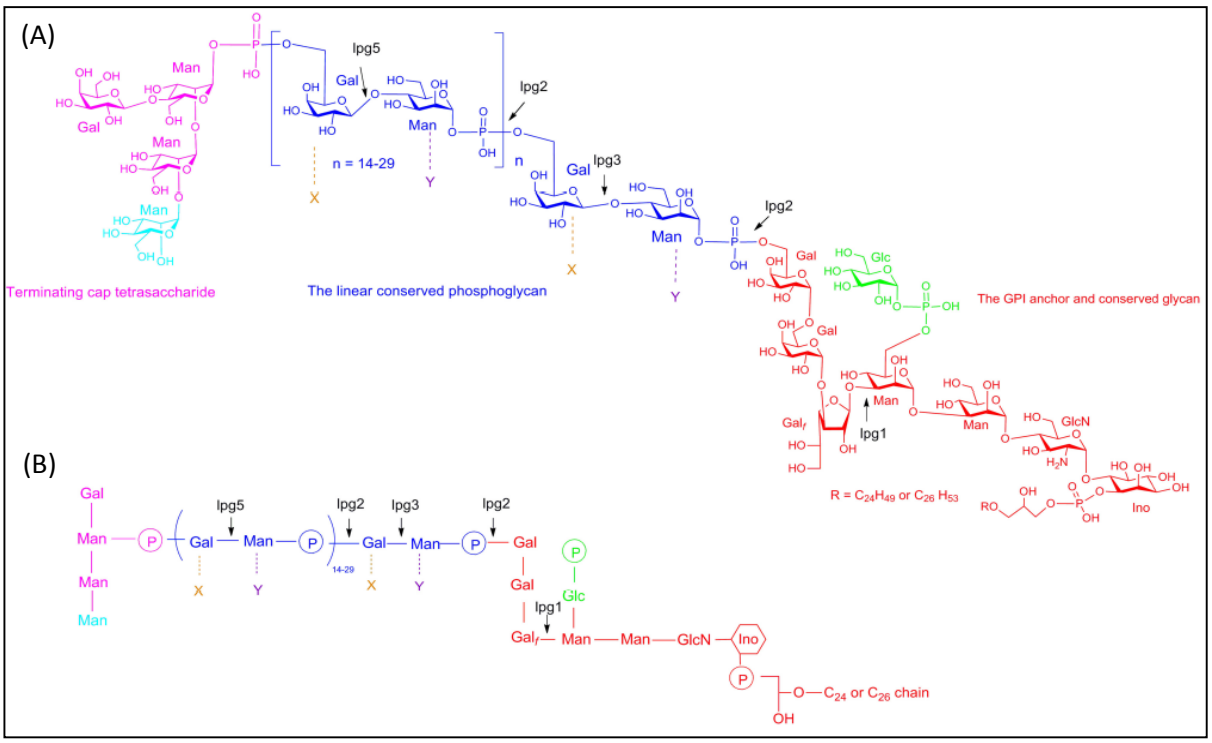

Figura 4: Estrutura do LPG de *Leishmania*. (A) Detalhamento da estrutura molecular constituída de quatro domínios. Uma âncora GPI e um núcleo glicano que estão representados em vermelho. Uma sequência linear de fosfoglicano, contendo de 15-40 repetições, representada em azul. Uma sequência terminadora "cap" representada em magenta. Em cada domínio os resíduos que não são conservados nas diferentes espécies de *Leishmania* estão representados com cores diferentes (Man, X, Y e Glc). Os genes responsáveis pelas diferentes etapas de montagem da molécula estão assinalados como (lpg1, lpg2, lpg3 e lpg5). Gal, galactose; Man, mannose; Glc, glucose; Galf, galactofuranose; GlcN, glucosamine; Ino, Inositol. **(B)** Representação simplificada dos diferentes domínios (extraído e modificado de Forestier 2015).

Estudos anteriores já demostraram a importância da molécula de LPG na interação com o hospedeiro invertebrado, na sobrevivência do parasita e no estabelecimento da infecção no hospedeiro vertebrado (SCHLEIN; SCHNUR; JACOBSON, 1990; SPÄTH et al., 2003a). Esses estudos demostraram que o LPG é importante para a ligação de promastigotas de algumas espécies de *Leishmania* como, *L. major* no intestino médio do flebótomo, além de ser importante para a proteção contra as enzimas hidrolíticas e na inibição da liberação de proteases no intestino médio do flebótomo (revisado em FRANCO et al., 2012). Durante a interação com o hospedeiro vertebrado a molécula de LPG revelou-se importante para o estabelecimento da infecção pela *Leishmania*. Ele confere proteção a lise mediada pelo complemento (JOINER,

1990). Além disso interage com os receptores do tipo Toll-like (TLR), suprime a fosforilação da p38 MAP quinase, modula a produção de espécies reativas de oxigênio e óxido nítrico e modula a produção de citocinas (CHANDRA; NAIK, 2008; KAVOOSI; ARDESTANI; KARIMINIA, 2009).

1.4 ESTRATÉGIAS PARA AVALIAR AS FUNÇÕES DO LPG

O envolvimento do LPG de *Leishmania* na virulência foi confirmado tanto com a utilização de moléculas de LPG purificadas em ensaios com macrófagos *in vitro*, quanto com a utilização de parasitas geneticamente modificados para genes específicos da via biosintética do LPG (revisado em FRANCO et al., 2012). A utilização do LPG purificado foi importante para caracterizar suas funções, porém essa abordagem apresenta algumas desvantagens, dentre as quais podemos citar o processo de purificação complexo e a dificuldade de obtenção de preparações sem contaminantes. Além disso, a dose utilizada não reflete as condições fisiológicas presentes no momento da interação parasito-hospedeiro. Outro desafio associado é a similaridade estrutural do LPG com outros glicoconjugados (contendo PGs) ancorados na superfície do parasita que podem gerar dúvidas sobre respostas de reatividade cruzada durante estes ensaios. Sendo assim, o uso de abordagens genéticas que se baseiam na identificação e disrupção dos genes que codificam as enzimas envolvidas com a síntese do LPG podem fornecer dados em um contexto biológico mais relevante (revisado em FORESTIER; GAO; BOONS, 2014).

A descoberta dos genes envolvidos com a via Biosintética da molécula de LPG em *Leishmania* permitiu a geração de cepas deficientes para esta molécula de superfície por meio da disrupção gênica dos genes (*LPG1*, *LPG2*, *LPG3*, e *LPG5*) envolvidos com a síntese dos diferentes motivos de polissacarídeos (revisado em FORESTIER; GAO; BOONS, 2014) (Tabela 1).

TABELA 1 *Leishmania* nocautes geradas para investigação dos genes envolvidos na síntese do LPG.

Gene	Enzima	Função	Nocaute/Referência
LPG1	Galactofuranosiltransferase	Síntese do núcleo glicano do LPG	*L. major* SPÄTH et al., 2003a *L. donovani* *L. mexicana* ILG, 2000 *L. infantum* LÁZARO-SOUZA et al., 2018
LPG2	Golgi GDP-manose transferase	Síntese do domínio PG comum ao LPG, PPGs, PGs e sAP.	*L. major* DESCOTEAUX et al., 1995 *L. donovani* GAUR et al., 2009 *L. mexicana* ILG; DEMAR; HARBECKE, 2001
LPG3	Golgi UDP-Gal transferase	Síntese de PG	*L. donovani* (DESCOTEAUX et al., 2002)
LPG5	Enzima homóloga de uma chaperona do reticulo endoplasmático de mamíferos (GRP94).	Síntese completa de PG	*L. major*(CAPUL et al., 2007)

O gene *LPG1* codifica a enzima galactofuranosiltransferase que está envolvida na síntese do núcleo glicano da molécula de LPG. Estudos que utilizaram cepas de *Leishmania* nocautes para este gene demonstraram o importante papel dessa molécula no processo de interação do parasito com o flebótomo e com seu hospedeiro vertebrado (LODGE; DIALLO; DESCOTEAUX, 2006; SPÄTH et al., 2003a; SVÁROVSKÁ et al., 2010). Esses estudos demonstraram que *L. major* e *L. donovani* nocautes para o gene *LPG1* (*lpg1*$^{-/-}$) são extremamente sensíveis a lise mediada pelo sistema complemento, não conseguem inibir a fusão do fagossomo com o lisossomo e são sensíveis ao estresse oxidativo (revisado em FORESTIER; GAO; BOONS, 2014; FRANCO et al., 2012). Contudo poucos estudos analisaram o papel do LPG de espécies de *Leishmania* do Novo Mundo, com exceção de *L. mexicana* (ILG, 2000a; ILG; DEMAR; HARBECKE, 2001) a maior parte dos estudos de glicobiologia foi realizada em *L. major* e *L. donovani* de forma que a variabilidade estrutural destas moléculas pode revelar diferenças fundamentais na patogênese estabelecida por cada espécie. Neste contexto, recentemente nosso grupo desenvolveu em colaboração com o Dr.

Albert Descoteaux uma cepa de *L. infantum* MCAN/BR/89/BA262 nocaute para o gene *LPG1* (Anexo 1). Estes parasitas se demonstraram menos virulentos em infecções de macrófagos, provavelmente devido à ativação do promotor da iNOS de maneira dependente do NF-κB. Entretanto, essa mutação não previne que as unidades repetitivas de dissacarídeos fosforilados sejam adicionadas em moléculas secretadas, incluindo PPGs, PGs e sAP (Figura 5) (LÁZARO-SOUZA et al., 2018).

1.5 OUTRAS MOLÉCULAS DE SUPERFÍCIE DA *LEISHMANIA*

Além do LPG esses protozoários apresentam em sua superfície outras moléculas que estão envolvidas com o processo de interação parasito-vetor e parasito-hospedeiro. Entre elas estão os glicosilinositolfosfolipídeos (GIPLs) que são uma família muito importante de glicolipídios com baixo peso molecular e que não possuem ligações com proteínas ou polissacarídeos. Eles são expressos em um número alto de cópias na superfície dos promastigotas e amastigotas e assim como a molécula de LPG também apresentam alguns polimorfismos estruturais. Os GIPLs já foram relacionados com a inibição da síntese de óxido nítrico em macrófagos infectados com *L. major* (revisado em DE ASSIS et al., 2012; GUHA-NIYOGI; SULLIVAN; TURCO, 2001). Outra importante molécula presente na superfície desses parasitos é a gp63 uma proteína com massa molecular de 63kDa, que possui dependência de zinco e é a principal glicoproteína em promastigotas sendo responsável por cerca de 1% das proteínas totais nessa fase do parasita. Na forma amastigota essa metaloprotease é expressa em níveis mais baixos e está mais localizada na porção da bolsa flagelar. Ela é ancorada a membrana celular através de uma âncora GPI e já foi relacionada com alguns processos durante a interação com o hospedeiro vertebrado (revisado em GUHA-NIYOGI; SULLIVAN; TURCO, 2001; YAO; DONELSON; WILSON, 2003).

Além dos GIPLs e gp63 os parasitas do gênero *Leishmania* apresentam em sua superfície outras moléculas que possuem porções estruturais semelhantes a molécula de LPG, com a presença das unidades repetitivas de fosfoglicanos (revisado em DE ASSIS et al., 2012), sendo elas, os proteofosfoglicanos (PPGs), os polímeros livres de fosfoglicanos (PGs) e as fosfatases ácidas secretadas (sAP). Esses glicoconjugados estão envolvidos com a interação e sobrevivência da *Leishmania* no interior da célula hospedeira, sendo importantes fatores de virulência juntamente com a molécula de LPG. Sua síntese é igualmente afetada pela deleção do gene *LPG2* que codifica uma GDP-manose transferase, responsável pelo transporte de GDP-manose no aparelho de Golgi, processo esse necessário para a síntese do domínio de fosfoglicano (PG) comum ao LPG e a essas moléculas (Figure 5) (MA et al., 1997; SPÄTH et al., 2003b). Alguns trabalhos demonstraram como a ausência do gene *LPG2* (*lpg2*$^{-/-}$) afeta esses parasitas. *L. donovani lpg2*$^{-/-}$ não é capaz de replicar em macrófagos murinos e induz níveis menores de infecção em baços e fígados de camundongos (GAUR et al., 2009). Já *L. major lpg2*$^{-/-}$ não é capaz de desenvolver lesões cutâneas e induz uma menor produção de IL-4, IL-10 e IFN-γ em células de camundongos infectados (UZONNA et al., 2004). Devido a essas características e atrelado ao fato de conferirem proteção frente a um desafio experimental com parasitas selvagens, foi proposto que *L. major lpg2*$^{-/-}$ poderia ser explorado como uma vacina atenuada (SPÄTH et al., 2003b; UZONNA et al., 2004).

Em contraste a esse cenário de atenuação observado nos parasitas de *L. major* e *L. donovani*, os nocautes para os genes *LPG1* e *LPG2* de *L. mexicana* não apresentaram diminuição da infectividade em ensaios com macrófagos ou camundongos (ILG, 2000a; ILG; DEMAR; HARBECKE, 2001). Esses dados observados em *L. mexicana* levantam a questão de que esses fatores de virulência podem desempenhar papéis distintos nas diferentes espécies de *Leishmania*. Vale ressaltar que pouco se sabe soube o papel dos gliconjugados de espécies de *Leishmania* do Novo Mundo (e.g. *L. amazonensis*, *L. braziliensis* e *L. infantum/L. chagasi*).

Sendo assim, visando contribuir para a melhor compreensão do papel desempenhado por esses gliconjugados na cepa de *L. infantum* seria importante gerar um nocaute para o gene *lpg2* que perderia todos os PG das moléculas de LPG, PPGs, PGs e sAP (Figura 5).

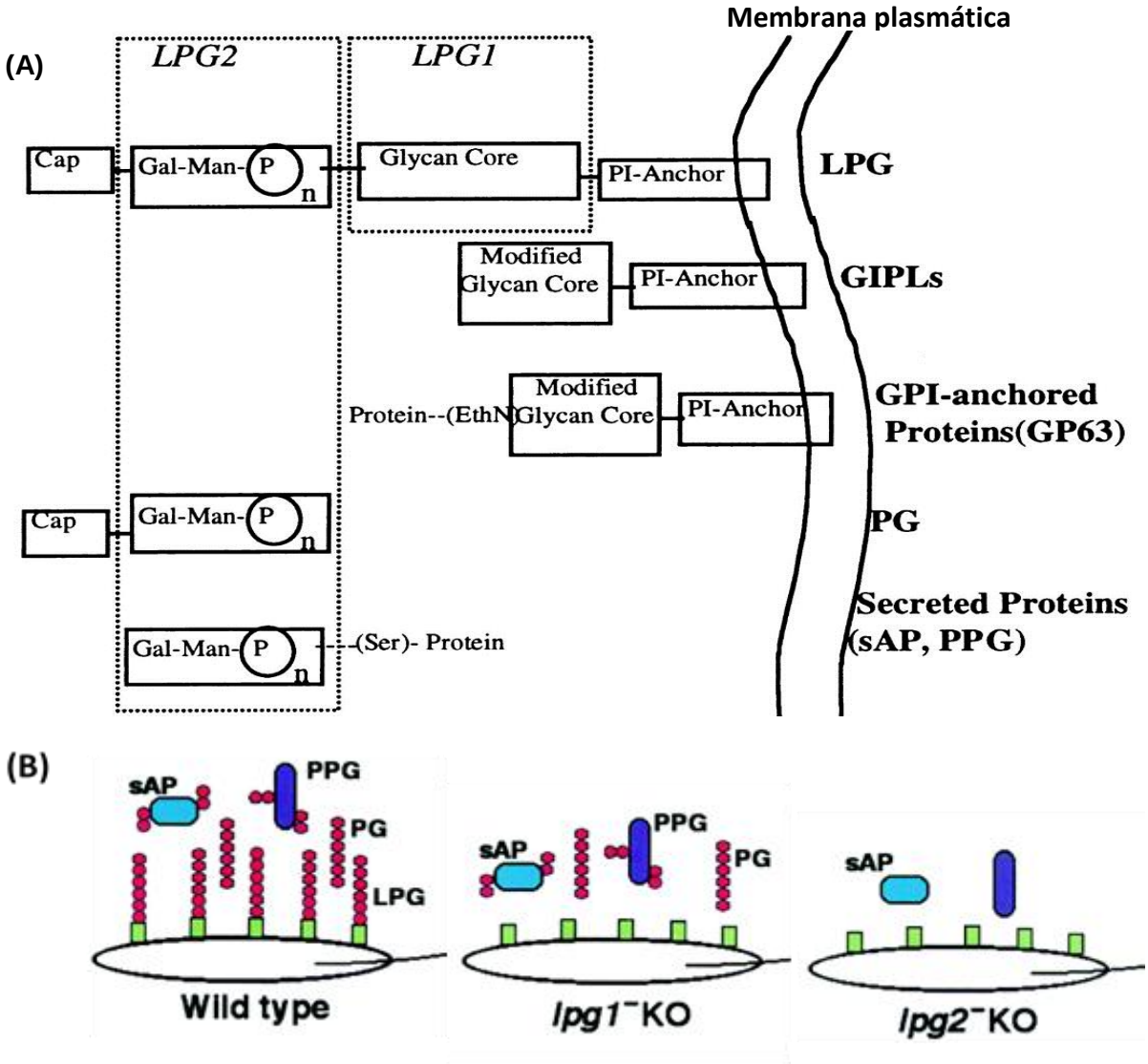

Figura 5: Representação esquemática de glicoconjugados de superficie contendo PG para o tipo selvagem e mutantes *LPG1* e *LPG2*. (A) As estruturas incluídas nas caixas tracejadas designam os domínios especificamente afetados pelas mutações em *LPG1* e *LPG2*. **(B)** Estrutura esquemática de formas promastigotas de *Leishmania* selvagem e dos mutantes (*lpg1*$^{-/-}$ e *lpg2*$^{-/-}$). Em verde a âncora de GPI do LPG e em vermelho às repetições de dissacarídeo-fosfato. sAP, fosfatase ácida secretada; PPG, proteofosfoglicano; PG, fosfoglicano (extraído e modificado de Sacks et al., 2000 e Robert Lodge, Albert Descoteaux 2005).

1.6 MECANISMOS DE EDIÇÃO GÊNICA

A capacidade de editar o DNA genômico com precisão em locais específicos possui um grande valor para a biologia molecular, biotecnologia e outras áreas relacionadas. Assim, técnicas para a edição precisa do genoma de organismos eucariotos tem sido um dos grandes objetivos dos biólogos moleculares e biotecnologistas ao longo dos anos. No final da década de 80 e início da década de 90 um trabalho inovador dos cientistas Capecchi e Smithies utilizando células de mamíferos demostrou que essas células eram capazes de incorporar uma cópia de DNA exógeno ao seu próprio DNA por meio de um processo conhecido como recombinação homóloga (Figura 6), porém esse método apresentava algumas limitações como baixa taxa de integração, dependência do tipo celular utilizado e risco de integração aleatória em regiões genômicas indesejáveis (CAPECCHI, 1989; SMITHIES et al., 1985; THOMAS; POLGER; CAPECCHI, 1986). Nas décadas seguintes pesquisadores buscaram alternativas para superar as limitações observadas com esse processo de recombinação homóloga, e então diversos sistemas surgiram com o intuito de permitir a edição gênica de maneira efetiva e especifica. Com relação ao mecanismo básico, as principais tecnologias desenvolvidas a partir de então se baseiam no princípio de que deve ocorrer uma quebra da dupla fita de DNA seguida do reparo por junções de extremidades não homologas (NHEJ) ou reparo dirigido por homologia (HDR). A seguir serão abordados alguns aspectos dos sistemas de “Nucleases Dedos de Zinco (ZFNs)”; “Nucleases efetoras semelhantes a ativadores de transcrição (TALENs); e o “sistema da nuclease 9 (CAS9)” associada a “Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas (CRISPR)” (Figura 6).

Inicialmente Jasin e colaboradores (1994) descreveram que a utilização de meganucleases específicas para gerar quebras de dupla fita na molécula de DNA aumentava a frequência de integração do DNA exógeno no local desejado, no entanto essa técnica era restrita a utilização de endonucleases raras que possuem sítios únicos de reconhecimento (JASIN,

1994). Ainda na busca por melhorar as técnicas e a precisão da edição gênica os pesquisadores chegaram ao desenvolvimento de moléculas artificiais geradas pela fusão de uma região de ligação ao DNA (dedo de zinco) e uma endonuclease do tipo *Fok* I essas moléculas conhecidas como "nucleases de dedo de zinco (ZFNs)" podem ser projetadas para ligar a uma sequência particular do genoma permitindo assim que o domínio *Fok* I gere uma quebra de dupla fita na molécula de DNA (CHANDRASEGARAN, 1996). Outra metodologia aplicada na geração de quebras de dupla fita na molécula de DNA é a utilização das "nucleases efetoras do tipo ativador de transcrição (TALENs)" que também consiste na fusão de um domínio de ligação ao DNA com a endonuclease *Fok* I, porém ao contrário dos domínios de ligação ao DNA das enzimas ZFNs que reconhecem uma sequência de 3 pb do DNA o domínio presente nas TALENs reconhece somente uma base por vez (CHRISTIAN et al., 2010).

Embora a utilização das ZFNs e TALENs tenham aumentado a eficácia de edição gênica essas metodologias ainda apresentam algumas limitações, com relação aos custos, ao desenvolvimento, eficiência e uso limitado a alguns organismos. Por outro lado, recentemente um sistema presente em bactérias e Archaea tornou-se uma ferramenta de edição genômica relativamente barata, fácil de programar e com alta eficiência. Esse sistema ficou conhecido como CRISPR/Cas9 e em sua essência funciona como uma espécie de sistema imune adaptativo em cerca de 40% das bactérias e 90% das Archaeas que tiveram seu genoma sequenciado até o momento. Esse mecanismo atua protegendo esses organismos de bacteriófagos invasores.

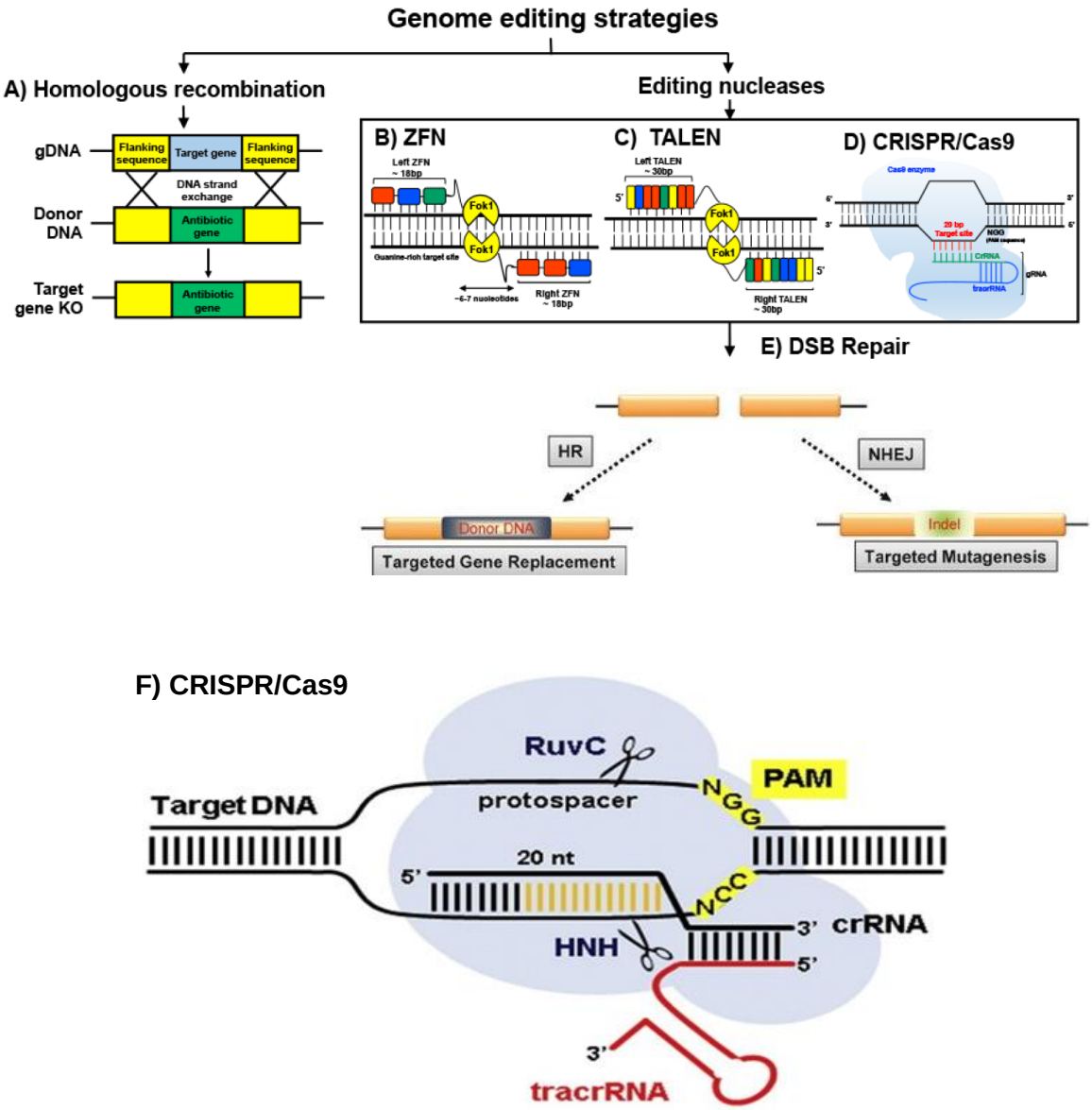

Figura 6: **Estratégias empregadas para edição genômica**. Subdivididas em não enzimática **(A)** Recombinação Homóloga. E enzimáticas **(B)** Nucleases de dedo de zinco (ZFNs). **(C)** Nucleases efetoras do tipo ativador de transcrição (TALENs). **(D)** Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas (CRISPR/Cas9). **(E)** Esquematização da quebra de dupla fita do DNA (DSB) e dos mecanismos de reparo. **(F)** Detalhamento do funcionamento da ferramenta CRISPR/Cas9 para edição genômica. HR – reparo por recombinação homologa, NHEJ - Reparo por junções de extremidades não homologas (extraído e modificado de (BORTESI; FISCHER, 2015; HO et al., 2018; KHALILI et al., 2016).

Atualmente o sistema CRISPR/Cas9 pode ser dividido em duas classes. Segundo KOONIN e colaboradores (2018) a classe 1 possui os sistemas do tipo I e III que são comumente encontrados em Archaeas, e a classe 2 que possui os sistemas do tipo II, IV, V e VI. Dentre

esses sistemas o mais utilizado é o CRISPR/Cas9 do tipo II de *Streptococcus pyogenes*, por ser um dos mais bem caracterizados e possuir requisitos simples para o seu funcionamento como a necessidade de uma sequência PAM do inglês "Protospacer Adaptor Motif" simples (codificada pelas bases NGG) (JINEK et al., 2012).

Esse sistema CRISPR/Cas9 possui dois principais componentes, a endonuclease Cas9 e um RNA guia (gRNA) que atua direcionando a enzima Cas9 para seu alvo especifico no DNA. O gRNA é constituído por uma sequência correspondente ao tracrRNA ("transactivating crispr RNA") que é necessário para formar um complexo com a enzima Cas9 e o crRNA ("CRISPR RNA") uma sequência de 20 nucleotídeos que são complementares a sequência do DNA alvo que é seguida pelos nucleotídeos da sequência PAM (NGG) que são indispensáveis para a ligação da enzima Cas9 ao local de cisão. Após ser guiada para o local especifico de clivagem pelo gRNA a enzima Cas9 gera uma quebra na dupla fita de DNA 3 pb a montante da sequência NGG utilizando seus domínios conservados HNH e RuvC (Figura 6B). Após a clivagem a célula hospedeira promove o reparo das fitas de DNA utilizando diferentes mecanismos de reparo, os mais comuns são a junção de extremidades não homólogas (NHEJ do inglês "Non-homologous end joining") que é propenso a erros podendo gerar inserções ou deleções que levam a mutações de quebra do quadro de leitura ("ORF frame shift") interrompendo assim o funcionamento normal do gene alvo, ou, o DNA pode ser reparado por meio do reparo dirigido por homologia (HDR do inglês "Homologous direct repair") sendo a forma mais comum a recombinação homóloga que utiliza um DNA molde homologo a região da ruptura para reparar o dano sem gerar erros. Além desses dois métodos de reparo outro mecanismo utilizado para o reparar o DNA é o MMEJ do inglês "Microhomology-mediated end joining" que utiliza sequências micro homólogas para alinhar as fitas danificadas antes da adesão, esse método de reparo também é propenso a erros, sendo assim, útil para gerar mutações após a ruptura gerada pela Cas9 (ADLI, 2018; JINEK et al., 2012).

1.7 MECANISMOS DE EDIÇÃO GÊNICA EM *LEISHMANIA*

Os parasitas do gênero *Leishmania* possuem um amplo genoma com uma grande variedade de genes que apresentam funções desconhecidas. Desde o início da década de 90, a metodologia de obtenção de nocautes por recombinação homóloga, utilizando marcadores de resistência a antibióticos, contribuiu enormemente para a compreensão da função de uma série de genes envolvidos com o processo de patogênese da *Leishmania* (CRUZ; COBURN; BEVERLEYT, 1991; GAUR et al., 2009; MATTE et al., 2018). Entretanto essa metodologia apresenta algumas limitações, como a utilização mais efetiva a genes cópia única, é um processo lento e trabalhoso que depende da utilização de genes marcadores de resistência os quais podem limitar a utilização desses parasitos em modelos experimentais *in vivo*. Sendo assim, a utilização do sistema CRISPR/Cas9 que foi recentemente adaptado para ser utilizado em parasitas do gênero *Leishmania* (BENEKE et al., 2017; ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017; ZHANG; MATLASHEWSKI, 2015), pode contribuir enormemente na compreensão e identificação de genes envolvidos em diferentes aspectos da biologia do parasita.

2. JUSTIFICATIVA E HIPÓTESE

Pouco se sabe sobre o papel do LPG de espécies de *Leishmania* do Novo Mundo (e.g. *L. amazonensis* e *L. infantum*). A maior parte dos estudos envolvendo nocautes foram realizados em *L. major* e *L. donovani* de forma que a variabilidade estrutural destas moléculas pode revelar diferenças fundamentais na patogênese estabelecida por cada espécie. Recentemente em colaboração com o Dr. Albert Descoteaux foi produzida uma cepa de *L. infantum* deficiente para o gene *LPG1* (*lpg1*$^{-/-}$). Estes parasitas se demonstraram menos virulentos em infecções de macrófagos, provavelmente devido à ativação do promotor da iNOS de maneira dependente do NF-kB(LÁZARO-SOUZA et al., 2018). Entretanto, essa mutação não previne que as unidades repetitivas de dissacarídeos fosforilados sejam adicionadas em moléculas secretadas, incluindo PPGs, PGs e sAP. Neste contexto, seria de grande valia a construção de uma linhagem nocaute de *Leishmania infantum* para o gene *LPG2*. Nossa hipótese é que este parasita perderia a expressão de todos os domínios de fosfoglicanos incluindo aqueles do LPG e de proteofosfoglicanos ligados a membrana (e.g. PPGs, PGs e sAP).

3. OBJETIVOS

3.1. OBJETIVO GERAL

Obtenção e caracterização do parasita nocaute (*lpg2*$^{-/-}$) de *Leishmania infantum* deficiente para fosfoglicanos (PGs).

3.2. OBJETIVOS ESPECÍFICOS

• Gerar parasitas de *L. infantum* nocautes para o gene *LPG2* através da técnica de recombinação homóloga utilizando genes marcadores de resistência a antibióticos.

• Estabelecer e padronizar o sistema CRISPR/Cas9 para geração de parasitas nocautes para o gene *LPG2* do gênero *Leishmania* sem o uso de genes marcadores de resistência a antibióticos.

• Estabelecer um protocolo de seleção dos parasitas *lpg2*$^{-/-}$ através de ensaios de aglutinação utilizando anticorpos e lectinas.

• Estabelecer as condições de crescimento dos parasitas nocautes e avaliar sua virulência em ensaios de infecção *in vitro*.

4. MANUSCRITO

4.1 MANUSCRITO 1

Título: Editing the *LPG2* gene duplication in *Leishmania infantum*: a case for CRISPR-CAS9

Neste trabalho caracterizamos a duplicação do gene *LPG2* em *Leishmania infantum* e obtivemos os parasitas nocaute (*lpg2*$^{-/-}$) utilizando a ferramenta CRISPR/Cas9.

Situação: Fase final de preparação para ser submetido a **Frontiers Microbiology** na forma de Brief Research Report

Resumo: Neste trabalho demostramos que diferente de outras espécies (*L. major*, *L. donovani* e *L. mexicana*) o gene *LPG2* que codifica uma GDP-manose transferase está duplicado na espécie *L. infantum* o que inviabilizou a utilização do método clássico de recombinação homóloga para sua disrupção gênica. Para contornar essa limitação implantamos e padronizamos com sucesso o sistema CRISPR/Cas9. Os resultados demonstraram que a edição do gene *LPG2* em *L. infantum* foi alcançada com sucesso utilizando o sistema CRISPR/Cas9. Os resultados de curvas de crescimento mostram que todos os clones *lpg2*$^{-/-}$ apresentam um fenótipo (morfologia) e uma taxa de crescimento similar, sugerindo a ausência de efeitos de edição inespecíficos "off-targets". Ensaios preliminares de infecção de macrófagos murinos *in vitro* utilizando os parasitas *lpg2*$^{-/-}$, demonstram uma redução na taxa de infecção (4h - 34,5% e 72h - 34,6%) além de uma diminuição na carga parasitária (4h - 46,17% e 72h - 49,88%) reforçando a importância do LPG e outros PGs como fatores de virulência na interação parasito-hospedeiro.

Editing the *LPG2* gene duplication in *Leishmania infantum*: a case for CRISPR-CAS9

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Keywords: GDP-mannose transporter, Lipophosphoglycan, *Leishmania infantum*, gene targeting, CRISPR/CAS9

Abstract

On the surface of the *Leishmania* promastigote, phosphoglycans (PG) such as lipophosphoglycan (LPG), proteophosphoglycan (PPG), free phosphoglycan polymers (PGs) and acid phosphatases (sAP), are dominant and contribute to the invasion and survival of *Leishmania* within the host cell by modulating macrophage signaling and intracellular trafficking. Phosphoglycan synthesis depends on the Golgi GDP-mannose transporter encoded by the *LPG2* gene. Aiming to investigate the role of PG-containing molecules in *Leishmania infantum* infection process, herein we describe the generation and characterization of *L. infantum LPG2*-deficient parasites. This gene was unexpectedly identified as duplicated in the *L. infantum* genome, which impaired gene targeting using the classical homologous recombination approach. This limitation was circumvented by the use of CRISPR/Cas9 technology. Knockout parasites were selected by agglutination assays using CA7AE antibodies followed by a lectin (RCA 120). Five clones were isolated and molecularly characterized, all revealing the expected edited genome, as well as the complete absence of LPG and PG-containing molecule expression. No differences in phenotype or growth rate were observed in the five isolated clones, suggesting the absence of off-target sites during Cas9 genome editing. Finally, the deletion of *LPG2* was found to impair the outcome of infection in murine bone marrow-derived macrophages, as demonstrated by a reduced infection rate (34.6%) and lower parasite load (49.88%) in comparison to wild-type parasite infection. The results obtained herein reinforce the importance of LPG and other PGs as virulence factors in host-parasite interactions.

Introduction

Leishmania promastigotes are coated by a thick glycocalyx consisting of glycoconjugates crucial to parasite pathogenesis. Lipophosphoglycan (LPG), proteophosphoglycan (PPG) and glycophosphatidylinositol lipids (GIPL), as well as gp63 metalloproteinase, comprise the vast majority of these molecules. *Leishmania* also secrete protein-linked phosphoglycans (PG) (e.g. secreted proteophosphoglycan (sPPG) and secreted acid phosphatase (sAP) (reviewed in (FORESTIER; GAO; BOONS, 2014; FRANCO et al., 2012; GUHA-NIYOGI; SULLIVAN; TURCO, 2001)). In promastigotes, LPG plays an important role in parasite survival inside the sand fly vector, in addition to macrophage infection (BALARAMAN et al., 2005; SACKS et al., 2000) (Moradin&Descoteaux, 2012). Moreover, intracellular survival and the multiplication of amastigotes in macrophages is enhanced by other PG-containing molecules (e.g. PPG and sAP), which are highly expressed on the surface of amastigotes (GAUR et al., 2009).

LPG is organized into four domains: a conserved 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol membrane anchor, a conserved diphosphoheptasaccharide core structure, a polymer that consists of repeating phosphodisaccharide units (phosphoglycan or PG) and carries species-specific side chains, as well as and variable mannose-rich cap structures (reviewed in MCCONVILLE; FERGUSON, 1993; Descoteaux&Turco, 1999). The PPGs comprise a heterogeneous family of cell surface and secreted proteins containing Ser-Thr rich regions to which phosphodisaccharide repeating units (Manα1-PO4 residue) (PG) are covalently linked, similarly to the LPG molecule (reviewed in ILG, 2000; RAMIRO et al., 2012). This type of phosphoglycosylation is the most abundant type of protein glycosylation found in *Leishmania*. Investigations focused on the synthesis of LPG and PG-containing molecules (PPG, PGs, sAP) have attracted considerable interest, and several enzymes and

transporters involved in this process have been identified either biochemically, genetically, or both (DESCOTEAUX et al., 1995, 1998, 2002; RYAN et al., 1993).

An enzyme critical for the synthesis of LPG and PG-containing molecules is the Golgi GDP-mannose transporter (encoded by *LPG2*) (DESCOTEAUX et al., 1995) which contains up to nine transmembrane domains and presents a TPT domain, which is found in many transporters with affinity for triose phosphate. In *Leishmania*, this gene is required for the addition of disaccharide-phosphate units to lipophosphoglycan and related glycoconjugates (HONG et al., 2013; MA et al., 1997; SAHOO et al., 2009; SEGAWA et al., 2005).

Over the past decades, the development of *Leishmania* mutants deficient in LPG or other PG-containing molecules has provided researchers with powerful tools to analyze the function of these structures/molecules (BUTCHER et al., 1996; DESCOTEAUX et al., 1995; MCNEELY et al., 1990). *L. major* and *L. donovani lpg2*$^{-/-}$ mutants failed to survive in the midgut of the sand fly vector and were unable to establish infection in macrophages. In an animal infection model, *L. major* parasites were found to exhibit persistence without causing pathological manifestations, with parasites persisting at low levels throughout the life of the infected animals (SPÄTH et al., 2003b). However, whether LPG and PGs are necessary for parasite survival in all *Leishmania* species has been contested based on *L. mexicana* studies. Although *lpg1*$^{-/-}$ and *lpg2*$^{-/-}$ parasites of this species exhibit complement sensitivity, no decreases in infectivity were observed *in vitro* in macrophages or *in vivo* in mice (GAUR et al., 2009; ILG, 2000a; ILG; DEMAR; HARBECKE, 2001). Our group recently generated an LPG-deficient mutant of *L. infantum* through the deletion of the putative galactofuranosyl transferase gene (*LPG1)* involved in the synthesis of the LPG glycan core. Phenotypically, this deletion impaired the outcome of infection in murine bone marrow-derived macrophages, likely due to the activation of the iNOS promoter in an NF-kB-dependent manner (LÁZARO-SOUZA et al., 2018). While these parasites expressed a truncated LPG molecule lacking the PG domain, they

were still able to assemble and secrete other PG-containing molecules (e.g. PPG, sAP and other PGs) (DERMINE et al., 2000; SPATH et al., 2000).

In an effort to investigate the role of PG-containing molecules in the *Leishmania infantum* infection process, we applied gene targeting by homologous recombination and also CRISPR/Cas9 technology to generate an *L. infantum* mutant lacking the Golgi GDP-mannose transporter gene (*lpg2*$^{-/-}$). Our results demonstrate both the value and the caveats of using of both systems for genome editing. The generated mutant produced distinct infection outcomes in comparison to WT parasites, and can therefore be usefully applied to investigate the role of *L. infantum* PG-containing molecules in host-parasite interactions.

Methods

Ethics Statement

All experiments were conducted in strict accordance with good practices as defined by the Animal Experimentation Committee of the Gonçalo Moniz Institute (IGM-FIOCRUZ, Salvador, Bahia-Brazil) under license no. 021/2015.

Animals

Inbred male C57BL/6 mice, aged 6–8 weeks, were obtained from the animal care facility of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (IGM-FIOCRUZ).

Parasite Cultures

Leishmania infantum Ba262 (MCAN/BR/89/BA262) promastigotes were cultured in HOMEM medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin,

100μg/mL streptomycin and 2mM L-glutamine in 25 cm^2 flasks at 25□C until late log-phase. The number of promastigotes was determined by counting in a Neubauer chamber.

LPG2 gene targeting by homologous recombination

Initially, we attempted to obtain an *L. infantum* LPG-deficient mutant (*lpg2*$^{-/-}$) by homologous recombination, using a previously described strategy (SCIANIMANICO et al., 1999) with modifications. Briefly, the gene sequences from the resistance markers Hygromycin and Neomycin were amplified by PCR using specific oligonucleotides (Supplementary Table 1) and ligated in the pUC18 vector (Sigma, USA), thusly generating the pUC18-Hyg and pUC18-Neo constructs. Next, the 5´ and 3´ UTR *LPG2* gene regions, consisting of 838 bp and 858 bp fragments respectively, were amplified by PCR from *L. infantum* Ba262 (MCAN/BR/89/BA262) genomic DNA using a specific oligonucleotide design based on the 19 kb genomic contig (GenBank accession CACT01000040.1), which was predicted to encode the *LPG2* gene (LinJ_34_4290) (Supplementary Table 1). The respective fragments were then digested with *Eco*RV/*Kpn*I and *Xba*I/*Hind*III enzymes, and ligated in the pUC18-Hyg and pUC18-Neo constructs. The final constructs were sequenced for confirmation and designated p*LPG2*-Hyg and p*LPG2*-Neo. Log-phase WT *L. infantum* promastigotes were electroporated in two steps with 10 μg of purified fragments (p*LPG2*-Neo and p*LPG2*-Hyg) using 0.4 cm cuvettes in a Gene Pulser II (BIO-RAD) under electroporation conditions based on a previously described high voltage protocol (ROBINSON; BEVERLEY, 2003). Briefly, two pulses were applied at 10-second intervals (25 μF, 1500 V). Following electroporation, promastigotes were incubated for 24 h at 25°C in drug-free medium, and the neo-resistant parasites were subsequently selected in the presence of 70 μg/mL G418 for 22 days at 25°C. The resistant parasites (*lpg2*$^{+/-}$) were isolated and submitted to a second round of electroporation using the p*LPG2*-Hyg fragment to disrupt the second allele of the *LPG2* gene. Double knockout (KO)

lpg2$^{-/-}$ parasites were then selected in the presence of both 70 μg/mL G418 and 50 μg/mL Hygromycin B for 22 days at 25°C. The absence of the *LPG2* gene in the resulting double drug-resistant promastigotes was verified by PCR following DNA extraction using "NucleoSpin tissue" kit (Macherey Nagel).

gRNA design and cloning into pLdCN

The present procedures involving the CRISPR/Cas9 system were based on pioneering work previously performed by Greg Matlashewski and colleagues (ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017; ZHANG; MATLASHEWSKI, 2015) with minimal modifications. Two gRNA-targeting sequences and the respective oligonucleotide donors (oligodonors) were selected using the gRNA designer tool (http://grna.ctegd.uga.edu/) and then manually inspected (Supplementary Table 1). The complementary guide sequence oligonucleotides were first phosphorylated in T4 DNA ligase buffer with T4 polynucleotide kinase and then annealed in a thermocycler (MJ Research: PTC-200, DNA Engine) (program: 95˚C for 5 min, ramp to 25˚C at a rate of -0.1˚C/sec). The annealed guide sequence was then cloned into the pLdCN plasmid (Addgene plasmid #84290; http://n2t.net/addgene:84290; RRID: Addgene_84290) previously digested with *Bbs*I.

Parasite transfection (CRISPR/Cas9)

To obtain *L. infantum* parasites expressing Cas9, log-phase WT *L. infantum* promastigotes were electroporated with purified pLdCN (gRNA440 and gRNA516) under conditions described in the section above (***LPG2* gene targeting by homologous recombination)**. After selection with G418 (70 μg/mL), four rounds of transfection were performed using 100 μM single-stranded oligodonors at 3-day intervals (ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017).

LPG2 knockout selection and limiting dilution assay

After transfection with the oligodonors, the putative *lpg2*$^{-/-}$ parasites were selected by consecutive rounds of agglutination with the CA7AE monoclonal IgM antibody (MediMabs) (1:2000 dilution), which recognizes the Gal(β1,4)Man(α1-PO4) repeating units contained in LPG and PG-containing molecules (DESCOTEAUX et al., 1998). Briefly, parasites were incubated for 2h with the antibody and then centrifuged at 100 x g for 7 minutes to remove any agglutinated promastigotes. The supernatant was then transferred to a new tube and centrifuged at 1,300 x g for 7 minutes to sediment non-agglutinated parasites (theoretically *lpg2*$^{-/-}$). Subsequently, these non-agglutinated parasites were incubated in fresh culture medium containing CA7AE (1:2000) for 3 days, followed by five rounds of the above-described agglutination steps. As a further selection step, non-agglutinated parasites were selected with 100 μg/ml of Ricin 120 (Vector Laboratories, USA) as described in (KING; TURCO, 1988). Finally, a limiting dilution assay was performed for clonal isolation using 96-well plates with culture medium containing 100 μg/mL of ricin, incubated for 3 weeks at 25°C with medium supplementation when necessary. Subsequently, genomic DNA was extracted from the last dilution in which parasite growth was observed.

Sequencing analysis

The sequence corresponding to the 5'UTR-Neo/Hyg/*LPG2*-3'UTR genomic DNA region was amplified with specific oligonucleotides (Supplementary Table 1) by PCR. The PCR products were purified on agarose gels (Wizard® SV Gel and PCR Clean-Up System) and sequenced using the Gonçalo Moniz Institute sequencing platform.

Western blotting

Late log-phase promastigotes at a concentration of 2 x 10^7 cells were centrifuged and resuspended in 100 µl of RIPA Lysis Buffer (150 mM NaCl, 0.5 M EDTA, pH 8.0, 1 M Tris, pH 8.0, 1%NP-40, 1% sodium deoxycholate, 0.1% SDS and dH_2O) containing a protease inhibitor (Sigma, cat. No. P-2714). The extract was incubated on ice and vortexed for approximately 5 minutes. The lysated material was separated by SDS-PAGE on a 12% polyacrylamide gel and Western blotting was performed as previously described (LÁZARO-SOUZA et al., 2018).

Confocal Immunofluorescence Microscopy

Late log-phase promastigotes were adhered on Poly-L-Lysine-coated glass coverslips (BD Biosciences, San Jose, CA) by centrifugation, fixed with 4% paraformaldehyde (Canemco and Marivac) for 20 min and simultaneously blocked and permeabilized for 20 min using a solution containing 0.1% Triton X-100, 1% BSA, 6% non-fat dry milk, 20% goat serum and 50% FBS. The distribution of LPG and other PGs containing the Gal(b1,4)Man(a1-PO4) repeating unit epitope was visualized using the CA7AE mouse monoclonal antibody (MediMabs, 1:2,000) after a 2 h incubation period, followed by an additional 30 min incubation with Alexa Fluor 488 goat anti-mouse IgM (Molecular Probes) at 1:500. Parasite nuclei were stained with DAPI (Molecular Probes) at 1:17,000. All steps were performed at room temperature. The coverslips were then mounted in Fluoromount-G (Interscience) and sealed with nail polish. Parasites were observed under a Plan APOCHROMAT 63x oil-immersion DIC 1.4 NA objective on a Leica SP8 confocal microscope in reflective mode, using a 488 nm laser, with an LP505 filter.

Bone Marrow-Derived Macrophages and Infection procedures

Bone marrow-derived macrophages (BMDM) were obtained from C57BL/6 mice as previously described (LÁZARO-SOUZA et al., 2018). Briefly, cells were collected from femurs and differentiated in RPMI 1640, 10% inactivated FBS, 30% L929 cell-conditioned media (LCCM) containing 2mM L-glutamine, 100 U/mL Penicillin and 100μg/mL Streptomycin at 36ºC under 5% CO_2. BMDMs were collected after 7 days and seeded on tissue culture plates in RPMI 1640 media, 10% inactivated FBS, 5% LCCM and 2mM L-glutamine. Cells (2×10^5) adhered on coverslips were then infected with either WT or *lpg2*$^{-/-}$ parasites at 10:1. After 4 or 72 h of infection, coverslips were fixed and stained with DiffQuik (Wright-Giemsa). Intracellular parasites were counted under light microscopy to determine the infection index under each experimental condition.

Statistical Analysis

BMDM cell infection assays were performed in quadruplicate in at least three experimental replicates. Data are presented as means and SE (standard error) of representative experiments. GraphPad Prism 5.0 software (GraphPad Software) was used for data analysis. Wild-type and knockout groups were compared using the Student's t-test. Differences were considered statistically significant when $p \leq 0.05$.

Results

LPG2* is duplicated in *L. infantum

After selection of double drug-resistant (Neo/Hyg) parasites, the absence of the *LPG2* gene was assessed by PCR. Electrophoresis results confirmed the expected occurrence of homologous recombination and the successful integration of the resistance markers (Neo and Hyg).

However, surprisingly, the coding region of the *LPG2* gene was still detected following amplification (Figure 1A).

This effectively indicated that either homologous recombination did not occur, or that the *LPG2* gene is duplicated in this species. No available evidence suggested the latter possibility, since the *L. infantum* JPCM5 genome assembly published at the time of the initial recombination experiments showed *LPG2* as a single-copy gene located in an unplaced 19 kb contig (GenBank accession CACT01000040.1). However, a more recent resequencing of the JPCM5 genome, which employed a hybrid sequencing approach with PacBio long reads and Illumina short reads, yielded a more robust assembly containing 36 scaffolds accounting for the 36 chromosomes present in this species (FUENTE et al., 2017) (EMBL accession GCA_900500625.1). BLAST searches of the 19 kb contig as a query against this novel assembly revealed that the entire contig, including the *LPG2* gene, was duplicated in a tandem array in chromosome 34 of *L. infantum* JPCM5 (Figure 1B). This finding highlights a limitation associated with the obtainment of knockout parasites using classical homologous recombination procedures.

Generation of *L. infantum* expressing Cas9

As a first step to perform *LPG2* gene editing, we developed *L. infantum* parasites expressing the Cas9 enzyme. WT parasites were transfected with the pLdCN plasmid and selected in G418 culture medium to generate the strain constitutively expressing the Cas9 nuclease. The expression of the Cas9 enzyme was confirmed in G418-resistant parasites by Western blotting (Figure 2A). The growth curves of promastigotes revealed that the expression of Cas9-gRNAs influenced parasite growth. A delay in the replication capability of the Cas9-gRNA expressing parasites was observed in comparison to WT. In addition, the former reached stationary phase by day 6, with a cell density of ~$5x10^7$, while the latter reached this phase two days later, with a cell density of ~$3x10^7$ (Figure 2B). *L infantum* expressing Cas9 were submitted to four rounds

of transfection using single-stranded oligodonors to improve *LPG2* gene disruption and knockouts were subsequently selected.

Selection of *L. infantum lpg2*$^{-/-}$

Five rounds of selection using the CA7AE antibody were unexpectedly unsuccessful in completely removing WT parasites by agglutination (Figure 2C). This was circumvented through the use of Ricin 120, after which complete depletion of WT *L. infantum* and the selection of *lpg2*$^{-/-}$ was observed (Figure 2D).

Characterization of *L. infantum* *lpg2*$^{-/-}$

To examine the types of mutations created by the CRISPR-Cas9 system at the *LPG2*-targeting sites, genomic DNA was extracted from these non-agglutinating parasites after clonal isolation (clones denominated E3, E4, F1, G2 and G6). PCR was performed with primers designed to amplify the *LPG2* gene, followed by DNA sequencing of the targeted sites (Figure 3A). All five sequenced clones revealed the expected genome editing with oligodonor insertions, and no random insertions/deletions were observed within the sequenced region (nucleotides 15-870, data not shown).

To characterize these five knockout clones, growth curves were constructed and compared to wild-type parasites (Figure 3B). This assay revealed no significant differences in the growth profile of the five *lpg2*$^{-/-}$ clones, suggesting the absence of off-target editing. In addition, although the *lpg2*$^{-/-}$ parasites reached stationary phase at different cell densities, no significant delays in the time required to reach this phase were seen in comparison to wild-type parasites (Figure 3B).

Next, the loss of LPG expression in *L. infantum lpg2*$^{-/-}$ was demonstrated in promastigotes of all five isolated clones by Western blot and by confocal immunofluorescence microscopy for

clone E4 (Figure 3C and D). Together, these data indicate that the *LPG2* gene was successfully edited in the *lpg2*$^{-/-}$ mutants, resulting in the generation of a parasite deficient in LPG and PG-containing molecules (e.g. PPGs).

lpg2$^{-/-}$ parasites exhibit limited survival in macrophages

To evaluate differences in parasite survival between WT and genome-edited parasites *in vitro*, BMDMs were infected for 4 or 72 h. *lpg2*$^{-/-}$ parasites exhibited a reduced infection index at 4 h and 72 h after infection (Figure 4A), as well as reduced survival within macrophages at these same time points (Figure 4B).

Discussion

We recently confirmed the involvement of LPG as a virulence factor in *L. infantum* through the use of parasites that had the *LPG1* gene knocked out (LÁZARO-SOUZA et al., 2018). However, other phosphoglycan-containing molecules (PPGs, PGs and sAPs) may also play a role in parasite survival within host cells (GAUR et al., 2009; SPÄTH et al., 2003b). Here we developed an *L. infantum* species parasite knocked-out for the *LPG2* gene, which did not express LPG or other PG-containing molecules (PPG, PGs, sAP).

Initially, we sought to obtain knockouts using the traditional method of homologous recombination involving resistance marker genes. Although it presents some limitations, this method has been useful for single gene disruption and was shown to successfully knock out the *lpg2*$^{-/-}$ gene in *L. major*, *L. donovani* and *L. mexicana* (DESCOTEAUX et al., 1995; GOYARD et al., 2003; ILG, 2000a; ILG; DEMAR; HARBECKE, 2001; SPÄTH et al., 2003a). . After performing two transformation rounds and selecting for the potential *lpg2*$^{-/-}$ parasites, the presence of an integral allele of the *LPG2* gene was still observed in genomic DNA, suggesting

that either the homologous recombination process was inefficient, or that this gene was possibly duplicated in the *L. infantum* genome.

The homologous recombination gene disruption strategy was designed based on information present in an unplaced 19 kb genomic contig of *L. infantum* JPCM5 (GenBank accession CACT01000040.1), in which the *LPG2* gene was originally mapped (LinJ_34_4290). It is important to note that although this contig was described in association with chromosome 34, it was not physically mapped to this chromosome, which raised doubts regarding the consistency of this contig. Subsequent analysis in a new assembly of the *L. infantum* JPCM5 genome (FUENTE et al., 2017) indicated that this 19Kb contig (including the *LPG2* gene) is duplicated in a tandem array located in chromosome 34. This new assembly, performed using PacBio and Illumina technology, was more robust, as evidenced by fewer gaps and increased identification of duplicate regions (tandem arrays). Interestingly, the duplication of the *LPG2* gene in the tandem array was not mentioned by these authors in their list of duplicated genes.

The fact that duplication of *LPG2* was not initially identified highlights limitations regarding the way *Leishmania* genomes are traditionally assembled, which usually entails the use of low-coverage whole-genome sequencing using short reads, with subsequent assembly performed using a reference genome obtained from a previously sequenced genome that can even belong to a different *Leishmania* species (PEACOCK et al., 2007). The presence of two copies of *LPG2* in *L. infantum* limited our ability to perform genetic manipulation via homologous recombination in this parasite species, which emphasizes the need to improve the overall quality of *Leishmania* genomes, i.e. completeness and contiguity, preferentially through the use of recently employed hybrid sequencing strategies (short and long reads) (GONZÁLEZ-DE LA FUENTE et al., 2017, 2019; LYPACZEWSKI et al., 2018). In the end, we overcame this limitation through the use of the CRISPR/Cas9 system for genome editing.

The CRISPR/Cas9 system proved more flexible in producing *Leishmania* knockouts without the use of genetic resistance markers for selection, thereby allowing for more direct comparisons with WT parasites. In addition, the use of oligodonors containing stop codons permitted more specific editing of the target gene as opposed to random insertions/deletions (ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017).

The generated *L. infantum* expressing CAS9-gRNA presented delayed growth, which could be attributed to the presence of the antibiotic (G418) required for expression of the plasmid (pLdCN). Parasites at this initial stage does not present any evidence of LPG2 gene editing as evidenced by sequencing (Supplementary Figure 1).

For the selection and clonal isolation of the *lpg2*$^{-/-}$ parasites, we initially used the CA7AE monoclonal antibody that recognizes the Gal (β1,4)Man(α1-PO4) repeat region of the LPG molecule (DESCOTEAUX et al., 1998). However, after five rounds of agglutination, there was reason to believe that wild-type parasites could still be present in knockout cultures, due to the fact that it was not possible to completely eliminate wild-type parasites in control cultures. These results highlight a limitation associated with the use of this antibody to select *lpg2*$^{-/-}$ parasites without the additional presence of a resistance gene marker. To overcome this limitation, we chose to employ a lectin (RCA 120), which recognizes the terminal β-galactose residues, that was shown to be cytotoxic to wild-type *L. donovani* and *L. major* parasites, but did not affect parasites that do not present LPG and other PG-containing molecules (CAPPAI et al., 1994; KING; TURCO, 1988; OPAT et al., 1996). After two rounds of agglutination, the use of this lectin resulted in wild-type parasite death and complete elimination. Despite the absence of resistance markers to select knockout parasites, the CRISPR/Cas9 system, coupled to oligodonors, demonstrated high efficiency in target gene editing, as evidenced by the analysis of sequencing data from five of the generated clones, which all presented the expected result at the site of Cas9 cleavage. In addition, Western blotting and confocal microscopy demonstrated

the complete elimination of PG-repeating units in both LPG and other PG-containing molecules, which is consistent with the literature (DERMINE et al., 2000; DESCOTEAUX et al., 1995; LÁZARO-SOUZA et al., 2018; LYE et al., 2004; SPÄTH et al., 2003b)After selecting the *lpg2*$^{-/-}$ parasites, Cas9 protein expression was eliminated by removing the selection marker for the pLdCN vector, which was achieved after 5-6 passages (data not shown). No differences in phenotype or growth rate were observed in the five isolated clones, suggesting the absence of off-target sites during Cas9 genome editing. Although low cell density was observed when the *lpg2*$^{-/-}$ parasites reached stationary phase, the time required to reach this phase was not significantly delayed. This finding stands in contrast to delays seen in achieving stationary phase when knocked-out parasites are obtained by homologous recombination using resistance markers, which likely occurs due to the presence of antibiotics in culture medium (LÁZARO-SOUZA et al., 2018). In addition, we did not observe any significant morphological or ultrastructural changes, indicating that the deletion of the *LPG2* gene did not interfere with the cellular biology of *L infantum* promastigotes.

Finally, to evaluate the virulence of *L. infantum lpg2*$^{-/-}$ parasites *in vitro*, murine macrophages infected for 4 h and 72 h exhibited a significant reduction in infection rate and parasite load in comparison to WT. Our results indicate that, similarly to what is observed in other *Leishmania* species, such as *L. donovani* and *L. major* (LODGE; DIALLO; DESCOTEAUX, 2006; SPATH et al., 2000) LPG and other PG-containing glycoconjugates participate in host-parasite interaction in *L. infantum* infection.

In sum, the results presented herein provide convincing evidence of the unexpected duplication of the *LPG2* gene in the *L. infantum* genome. Both copies of this gene were successfully edited using the CRISPR/Cas9 system, highlighting the potential for the use of these KO parasites to assess the signal transduction pathways elicited by PG-containing molecules from different *Leishmania* species in different immune cells. In addition, the success

of *LPG2* gene editing using CRISPR exemplifies the potential of this technique, which can be applied to turn off other virulence factors sequentially, paving the way for the development of new vaccines.

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ACKNOWLEDGEMENTS

The authors would like to thank the Rede de Plataformas Tecnológicas (FIOCRUZ-Bahia) for the use of its DNA Sequencing Platform, Dr. Ricardo Khouri (FIOCRUZ-Bahia) for technical help with chemicals and reagents and Andris K. Walter for English language revision and manuscript copyediting assistance (FIOTEC-Programa de Capacitação).

AUTHOR CONTRIBUTIONS

FHJS, PIR, AD, JBL, VMB and LPF conceived and designed the study and contributed to data analysis. FHJS, JBL, JLS and LPF performed the experiments. FHJS, JBL, PIR, AD, JLS, VMB and LPF wrote and critically revised the manuscript. All authors have read and approved the final version of this manuscript.

FUNDING

This work was supported by the Brazilian National Research Council (CNPq) (Grant number: 431857/2018-0) and the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (Grant

number: 04/2015) to VMB. VMB is a senior investigator funded by CNPq. AD is the holder of the Canada Research Chair on the Biology of intracellular parasitism. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) –Finance Code 001.

CONFLICT OF INTEREST STATEMENT

The authors declare that all research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

5. DISCUSSÃO

O LPG é uma molécula dominante na superfície de promastigotas de todas as espécies de *Leishmania* essa molécula possui papel critico na ligação das formas promastigotas no intestino do flebótomo, confere proteção a lise mediada pelo sistema complemento inibe a maturação do fagossomo e modula a produção de citocinas e NO. Além do LPG esses parasitas também apresentam em sua superfície outras moléculas contendo fosfoglicanos em suas estruturas (PPGs, PGs e sAP) que também contribuem para a sobrevivência do parasito no interior da célula hospedeira através da modulação da sinalização dos macrófagos e produção de citocinas. O envolvimento dessas moléculas durante a etapa do ciclo que ocorre no flebótomo e a que ocorre no hospedeiro vertebrado foi confirmada tanto em condições não fisiológicas com a utilização de LPG purificado quanto com a utilização de parasitas deficientes em passos específicos das vias biossintéticas dessas moléculas (revisado em FRANCO et al., 2012).

Inicialmente buscamos obter o nocaute *lpg2*$^{-/-}$ em *L. infantum* utilizando o mecanismo tradicional de recombinação homóloga com genes marcadores de resistência. Mesmo apresentando algumas limitações esse método ainda pode ser útil para a disrupção de genes cópia única e foi utilizado com sucesso para a obtenção dos nocautes *lpg1*$^{-/-}$ de *L. major*, *L. donovani*, *L. mexicana* e *L. infantum* (GOYARD et al., 2003; ILG, 2000a; ILG; DEMAR; HARBECKE, 2001; LÁZARO-SOUZA et al., 2018; SPÄTH et al., 2003a). Após realizarmos as duas rodadas de transformação e selecionar os potenciais parasitas *lpg2*$^{-/-}$ ainda observarmos a presença de um alelo integro do gene *LPG2 no* DNA gnômico, o que sugeria que o processo de recombinação homóloga havia sido ineficiente ou que havia uma possível duplicação para esse gene em *L. infantum* (Figura 1A).

Nossa estratégia de disrupção gênica inicial foi desenhada com base nas informações de um Contig genômico de 19 kb de *L. infantum* (Linj34_V3.4290) onde o gene *LPG2* estava mapeado (https://www.genedb.org/#/species/Linfantum). É importante frisar que este Contig era reportado como associado ao cromossomo 34, porém não havia sido mapeado fisicamente neste cromossomo, levantando dúvidas sobre a consistência deste Contig. Visando esclarecer esta questão em dezembro de 2017, realizando novas buscas nos bancos de dados identificamos uma nova montagem do genoma de *L. infantum*. Neste trabalho, os autores utilizaram duas plataformas diferentes de sequenciamento PacBio (com "reads" até 40 Kb) e Illumina (com "reads" de ~150 pb) para gerar um nova montagem do genoma de *L. infantum* (cepa de referência JPCM5 (MCAN/ES/98/LLM-724) (FUENTE et al., 2017). Esta montagem se apresentava mais completa com redução do número de "gaps" e identificação mais precisa de regiões duplicadas ("tandem arrays"). Novas análises do Contig de 19kb contra essa nova montagem indicaram que este Contig (incluindo o gene *LPG2*) encontrava-se duplicado em um "tandem array" (Figura 1B). Esse caso especifico do gene *LPG2* evidencia um problema comum nas montagens de genomas de *Leishmania*, ocasionado principalmente pela baixa cobertura utilizando "reads" curtos e com anotação de referência baseada em genomas de outras espécies de *Leishmania* já sequenciadas.

Sabe-se que os parasitas do gênero *Leishmania* exibem uma alta plasticidade genômica, sendo capazes de modificar o seu material genético em resposta a modificações das condições ambientais e resistência a drogas (LAFFITTE et al., 2016; SACKS; KAMHAWI, 2001; STERKERS et al., 2012). Além disso, esses parasitas apresentam um mecanismo incomum de controle transcricional e possuem um genoma que está organizado em agrupamentos direcionais de genes que podem incluir centenas de genes sendo que a transcrição ocorre de maneira policistrônica. Outra característica estrutural do genoma de *Leishmania* é a presença de genes duplicados em série levantando a hipótese de que isso permita o aumento da expressão

gênica. Neste contexto, a duplicação do gene *LPG2* em *L. infatum* pode possuir implicações no fenótipo/adaptações apresentado por esses parasitos no curso da infecção, podendo indicar a necessidade do aumento da expressão desse gene (CLAYTON; SHAPIRA, 2007; ROGERS et al., 2011). Adicionalmente, essa plasticidade genômica de *Leishmania* e a presença de genes com mais de uma cópia dificulta a manipulação genética desses parasitas. Visando superar esta limitação na obtenção do parasita *lpg2*$^{-/-}$ em *L. infantum*, estabelecemos em nosso laboratório o sistema CRISPR/Cas9 para edição do genoma de *Leishmania*. Buscando desenvolver um parasita que permitisse comparações mais diretas com o parasita selvagem optamos por gerar uma *Leishmania* nocaute sem a utilização de genes marcadores de resistência para a seleção. Além de não submeter os parasitas nocautes a pressão seletiva de antibióticos optamos por utilizar "oligodonors" contendo códons de parada de modo a obter uma edição mais especifica do gene alvo ao invés de inserções/deleções aleatórias. Adicionalmente, com esta estratégia teoricamente seria possível acelerar a obtenção dos parasitos nocautes em cerca de 25 vezes conforme descrito por (ZHANG; LYPACZEWSKI; MATLASHEWSKI, 2017).

Inicialmente para viabilizar a edição do gene *LPG2* desenvolvemos um parasita de *L. infantum* que expressa a enzima Cas9 juntamente com o gRNA. Após a confirmação da expressão da enzima Cas9 por Western blot (Figura 2A, anexo), avaliamos a taxa de crescimento desses parasitas em cultura. A diferença observada na curva de crescimento (Figura 2B, anexo) pode ser atribuída a presença do antibiótico (G418) necessário para a expressão do plasmídeo (pLdCN) e principalmente devido a ação da enzima Cas9/gRNA que em células de mamífero já demostrou induzir um atraso no ciclo celular (GEISINGER; STEARNS, 2019). Na etapa de seleção e isolamento clonal dos parasitas, devido à ausência dos marcadores de resistência, tivemos que buscar estratégias alternativas para isolar os parasitas *lpg2*$^{-/-}$. Inicialmente optamos por utilizar o anticorpo monoclonal CA7AE que reconhece a região de repetição de Gal(β1,4) Man(α1-PO4) da molécula de LPG. Contudo mesmo após 7 rodadas de

aglutinação acreditávamos ainda existir uma porcentagem de parasitas selvagens na cultura dos nocautes. Isto pode ser afirmado ao observarmos que não foi possível depletar completamente os parasitas selvagens de culturas controle (contendo somente parasitas selvagens) (FIGURA 2C em anexo). Esses resultados demostraram assim uma limitação na utilização desse anticorpo para a seleção destes parasitas. Visando superar esse entrave na seleção dos parasitas *lpg2*$^{-/-}$ optamos por utilizar uma lectina (RCA 120) que em estudos anteriores havia se mostrado citotóxica para *L. donovani* e *L. major* selvagens, porém não afetava os parasitos que não apresentam LPG e outros PGs em sua superfície (CAPPAI et al., 1994; KING; TURCO, 1988; OPAT et al., 1996). Essa lectina possui especificidade pela porção de galactose e na concentração de 100 µg/ml foi capaz de levar em 2 rodadas de aglutinação os parasitas selvagens a morte permitindo assim a seleção mais eficaz dos parasitas *lpg2*$^{-/-}$ (FIGURA 2D em anexo). O processo de seleção dos parasitas nocautes com essas duas estratégias levou cerca de 3-4 meses, porém acreditamos que se tivéssemos utilizado diretamente a Ricin, ele poderia ser abreviado a 1 mês.

Apesar da ausência dos marcadores de resistência para a seleção o sistema CRISPR/Cas9 apresentou uma alta eficiência na edição do gene alvo, como observado pelos dados de sequenciamento de 5 clones (isolados por diluição limitante) que apresentaram a edição esperada no sitio de clivagem da enzima Cas9 (FIGURA 3A em anexo) Além disso os resultados do Western blot mostraram que ocorreu a eliminação completa da expressão tanto do LPG quanto de outros PGs. Esse resultado está de acordo com o que é observado na literatura, onde os parasitas *lpg2*$^{-/-}$ apresentam uma perda completa na expressão do LPG e PGs (LYE et al., 2004) ao passo que os parasitas *lpg1*$^{-/-}$ ainda expressam outros PGs.

Após as etapas de seleção e caracterização dos parasitas *lpg2*$^{-/-}$ optamos por retirar o antibiótico da cultura visando a eliminação do plasmídeo (pLdCN) e consequentemente perda da expressão da proteína Cas9. Foi possível observar que após 5-6 passagens não observamos

expressão da proteína Cas9 em 2 dos 5 clones selecionados (dados não apresentados). Acreditamos que com o aumento do número de passagens todos os clones devem perder a expressão da Cas9. Após essas etapas realizamos uma curva de crescimento visando comparar os cinco clones obtidos e observamos que não houve uma diferença fenotípica acentuada no crescimento desses parasitas, sugerindo que provavelmente não ocorreram eventos de edição inespecíficos "off-targets". Quando comparado com crescimento dos parasitas selvagens existe uma diferença na densidade celular em que os nocautes atingem a fase estacionária, porém não há um atraso significativo no tempo em que os nocautes chegam a esta fase. Esses dados diferem daqueles observados quando os parasitas nocautes são obtidos por recombinação homóloga utilizando marcadores de resistência (LÁZARO-SOUZA et al., 2018), o que provavelmente se deve a presença dos antibióticos na cultura.

Como uma primeira etapa para avaliarmos a virulência dos parasitas *lpg2*$^{-/-}$ *in vitro*, infectamos macrófagos murinos por 4 h ou 72 h e observamos que houve uma redução significativa na taxa de infecção (4h - 34,5% e 72h - 34,6%) e na carga parasitaria (4h - 46,2% e 72h - 49,9%) com estes parasitas quando comparado com o selvagem. Esses dados reforçam a importância do LPG e outros PGs na manutenção da infecção *in vitro* por *L. infantum*. Esses resultados indicam que de modo similar ao que foi observado com os parasitas de *L. donovani* (*lpg1*$^{-/-}$ e *lpg2*$^{-/-}$) o LPG e outros PGs são necessários para o estabelecimento da infecção em macrófagos *in vitro* (GAUR et al., 2009). As metodologias aqui padronizadas, tanto as relacionadas ao sistema CRISPR/Cas9, quanto as referentes a seleção de nocautes por aglutinação deverão ser utilizadas para a obtenção de *L. amazonensis* nocautes.

6. CONCLUSÃO

O gene *LPG2* está duplicado em *L. infantum*. Conseguimos implementar a ferramenta CRISPR/Cas9 para edição genômica dessa espécie de *Leishmania* e a infecção *in vitro* reforça a importância do LPG e outros PGs na manutenção da *L. infantum* no hospedeiro vertebrado.

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ANEXOS

Anexo 1

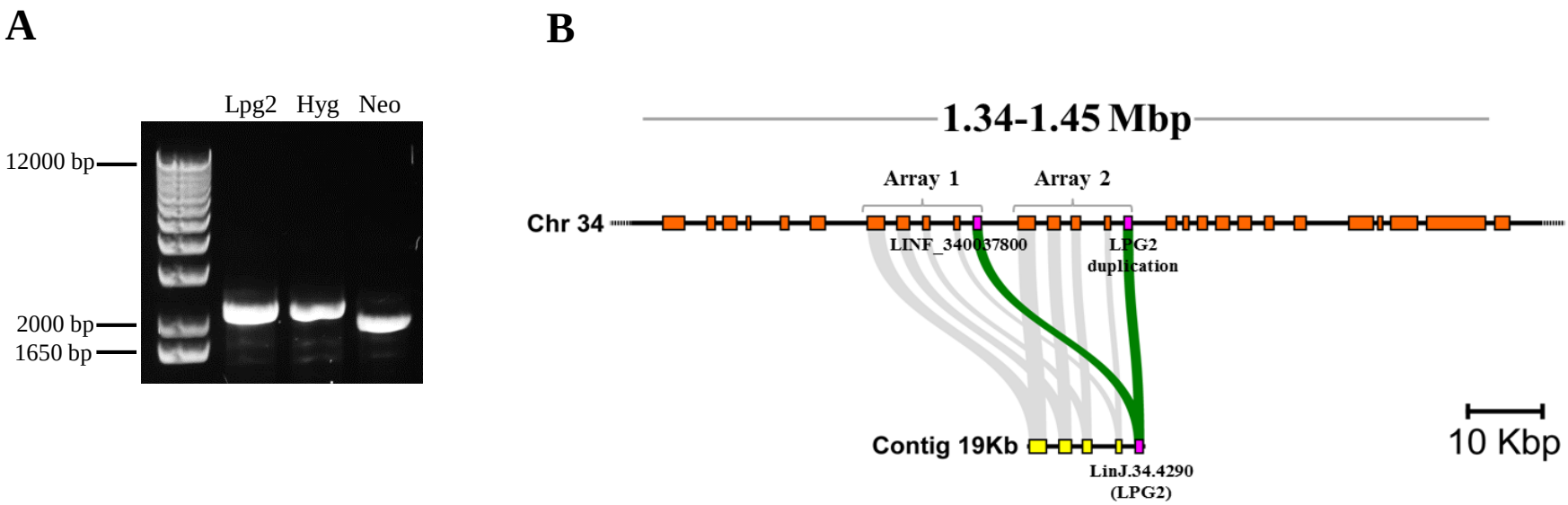

Figure 1: Evidence of *LPG2* duplication in *L. infantum*. (A) Genomic DNA from *L. infantum* Hyg/NeoR strain was amplified by PCR using specific oligonucleotides (Supplementary Table 1) to verify *LPG2* gene targeting by homologous recombination. **(B)** Comparison of the genomic contexts where *LPG2* was originally located (in an unplaced 19 kbp contig shown at the bottom) to the recent resequencing and reassembly of the *L. infantum* JPCM5 genome (FUENTE et al., 2017), where the duplication of the *LPG2* gene array is evidenced in chromosome 34 (top). Gray connecting segments indicate sequence conservation, and the coordinates at the top refer to the novel chromosome 34 assembly.

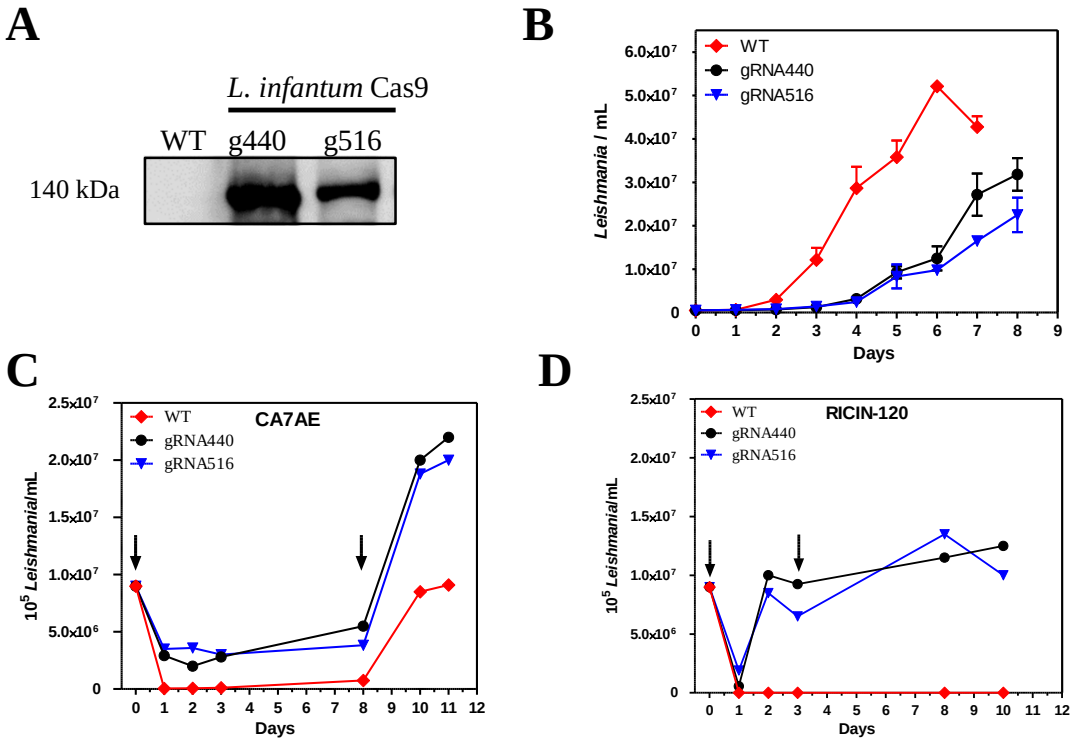

Figure 2: Generation of *LPG2* knockout using CRISPR/CAS9. **(A)** Western blot analysis of *L. infantum* promastigotes expressing Cas9 (gRNA440 and gRNA516). **(B)** Growth curves of *L. infantum* wild-type (WT) and *L. infantum*-Cas9 (gRNA440 and gRNA516) promastigotes. **(C)** Agglutination assay using the CA7AE monoclonal antibody and associated growth curves. **(D)** Agglutination assay using Ricin 120 lectin and associated growth curves, demonstrating the selection of *LPG2* knockouts.

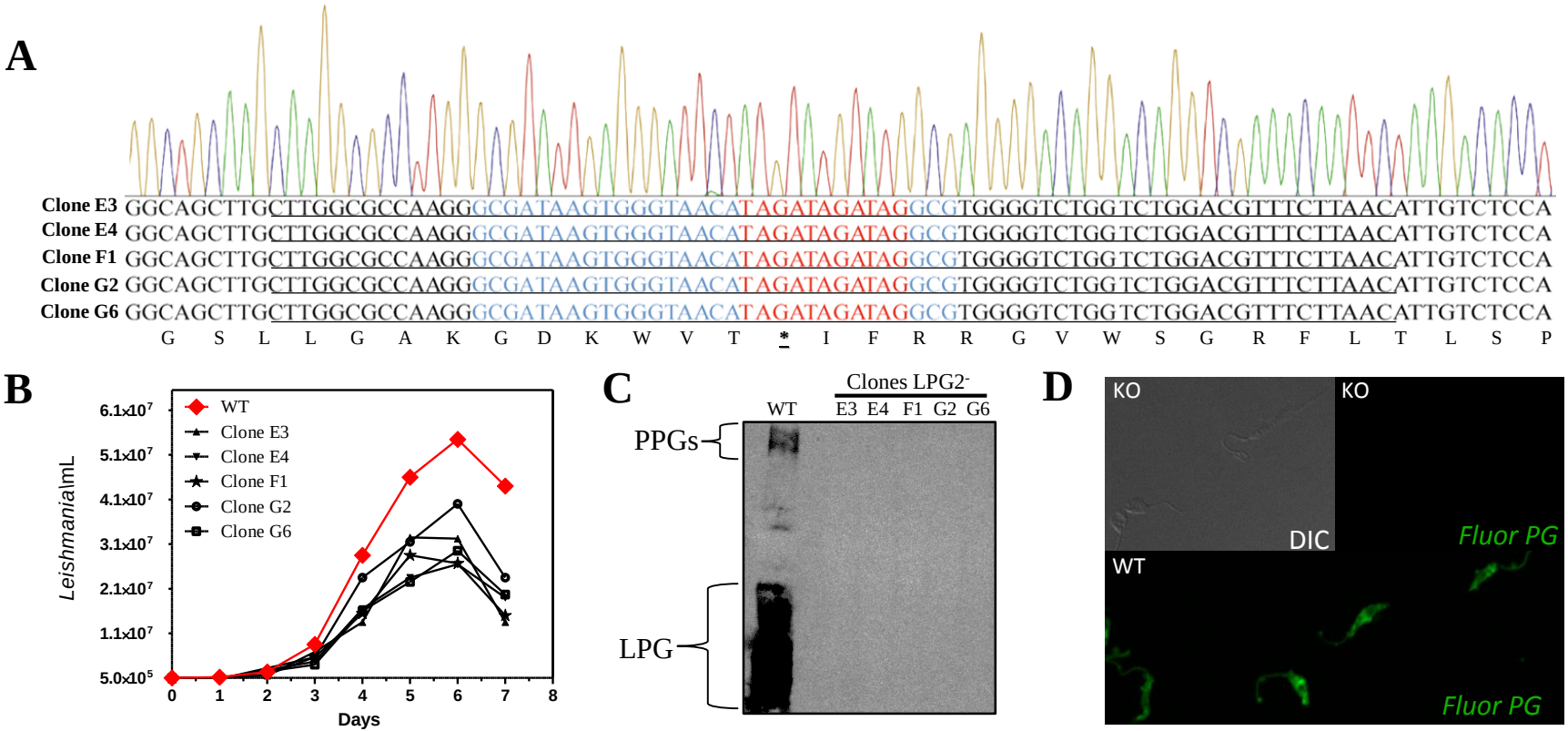

Figure 3: Molecular characterization of *lpg2*$^{-/-}$. (A) Chromatogram showing the region of the *LPG2* gene in which the precise insertion of a stop codon (denoted by an *) occurred by homologous recombination at the cleavage site of the Cas9 enzyme (in red). The oligodonor sequence is underlined and the gRNA440 sequence is highlighted in blue. **(B)** Growth curve of *L. infantum* wild-type (WT) promastigotes and clones of *L .infantum lpg2*$^{-/-}$. **(C)** Western blot analysis of the expression of LPG and PPGs in *L. infantum* WT and *lpg2*$^{-/-}$ promastigotes. **(D)** Confocal immunofluorescence analysis of WT and *lpg2*$^{-/-}$ parasites.

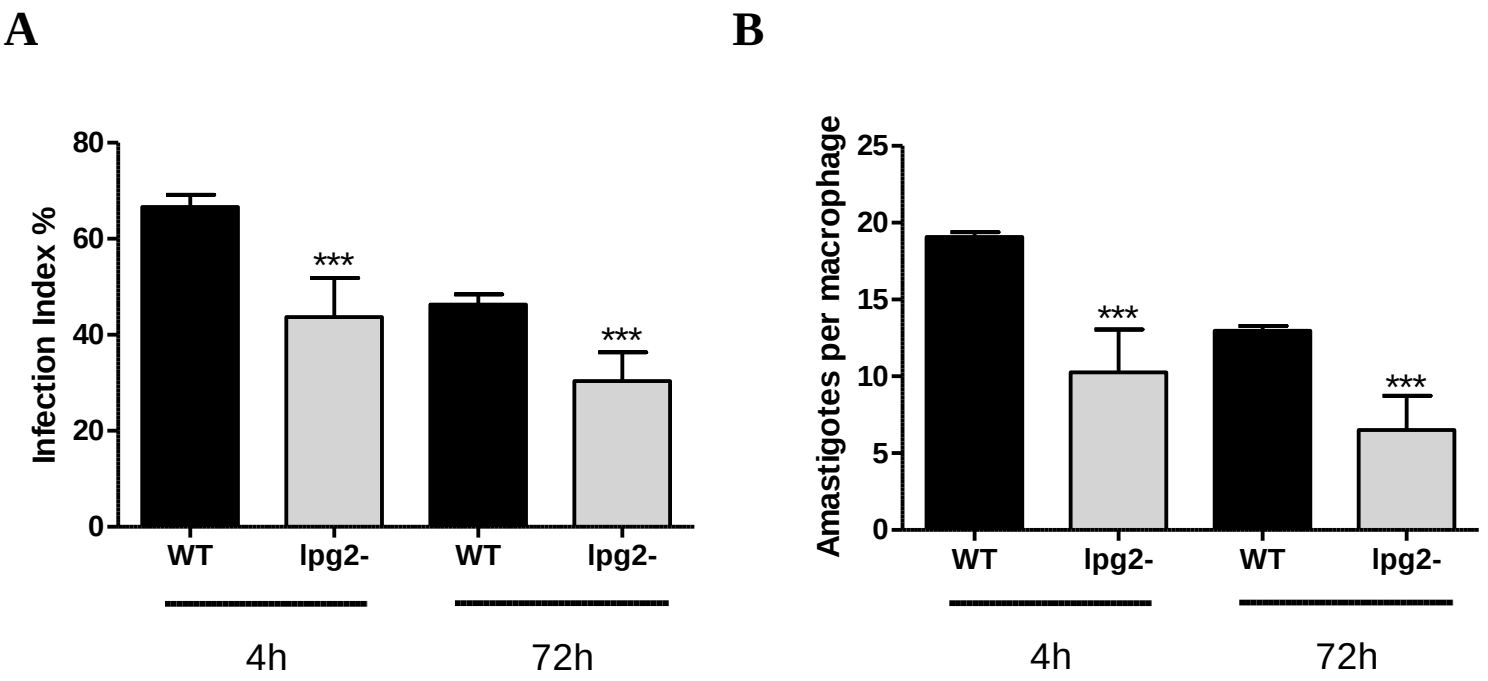

Figure 4: Reduced virulence of *lpg2*$^{-/-}$ parasites in macrophages infected *in vitro*. C57BL/6 mouse macrophages were infected with *L. infantum* Ba262 wild-type and *lpg2*$^{-/-}$ knockout promastigotes at 4 h and 72 h. Coverslips were analyzed by optical microscopy. **(A)** Macrophage infection rate. **(B)** Parasite load determined by amastigote quantification in macrophages. Data are representative of means and SE from at least three independent experiments performed in quadruplicate for each condition. *$p < 0.05$.

Anexo 2

Supplementary Table 1- Oligonucleotides sequences used to assemble the fragments p*LPG2*-Hyg and p*LPG2*-Neo for gene targeting of LPG2 by homologous recombination and CRISPR/Cas9.

Homologous Recombination

Fragment	**Forward Primer (5' – 3')** **Reverse Primer (5' – 3')**	**Restriction sites**
5UTR'-LPG2-3'UTR	GATATCGCTTCCATCTGAAATGTGCTG TCGCATGGTGCGATGCAGCTGTAG	-
LPG2 5'UTR	CTGGAGCTCGATATCGCTTCCATCTGA CATGGTACCGGCAAATGCTGATGCAATCC	EcoRV/SacI KpnI
LPG2 3'UTR	GTGTCTAGAAGTAGTCACTGCTGTTAGCAG CAGAAGCTTATGGTGCGATGCAGCTGTAG	XbaI HindIII
LPG2	GTAAAGCTTATGAACCATACTCGCTCTG GTAAAGCTTCTACTCAGATTT*GGAGGT*G	HindIII HindIII
Hyg	GCCGGATCCATGAAAAAGCCTGAACTCA GTCGGATCCCTATTCCTTTGCCCTCGG	BamHI BamHI
Neo	AAGGGATCCATGGGATCGGCCATTGAA CTGGGATCCTCAGAAGAACTCGTCAAGAA	BamHI BamHI
	CRISPR/Cas9	
gRNA440	TTGTGCGATAAGTGGGTAACAGCG AAACCGCTGTTACCCACTTATCGC	-
gRNA516	TTGTGTACAGCGTGTACGAGACGG AAACCCCTCTGCTACACGCTGTAC	-
OD440	5'CTTGGCGCCAAGGGCGATAAGTGGGTAACATAGATAGATA GGCGTGGGGTCTGGTCTGGACGTTTCTTAAC 3'	-
OD516	5´AAGGACAGCCTTCATGTACAGCGTGTACGACTATCTATCTA GACGGTGGAGACAATGTTAAGAAACGTCCA 3'	-
		-

Red – STOP codons

Anexo 3

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ORIGINAL RESEARCH
published: 05 April 2018
doi: 10.3389/fmicb.2018.00626

Leishmania infantum Lipophosphoglycan-Deficient Mutants: A Tool to Study Host Cell-Parasite Interplay

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Lipophosphoglycan (LPG) is the major surface glycoconjugate of metacyclic *Leishmania* promastigotes and is associated with virulence in various species of this parasite. Here, we generated a LPG-deficient mutant of *Leishmania infantum,* the foremost etiologic agent of visceral leishmaniasis in Brazil. The *L. infantum* LPG-deficient mutant (Δ*lpg1*) was obtained by homologous recombination and complemented via episomal expression of *LPG1* (Δ*lpg1* + *LPG1*). Deletion of *LPG1* had no observable effect on parasite morphology or on the presence of subcellular organelles, such as lipid droplets. While both wild-type and add-back parasites reached late phase in axenic cultures, the growth of Δ*lpg1* parasites was delayed. Additionally, the deletion of *LPG1* impaired the outcome of infection in murine bone marrow-derived macrophages. Although no significant differences were observed in parasite load after 4 h of infection, survival of Δ*lpg1* parasites was significantly reduced at 72 h post-infection. Interestingly, *L. infantum* LPG-deficient mutants induced a strong NF-κB-dependent activation of the inducible nitric oxide synthase (iNOS) promoter compared to wild type and Δ*lpg1* + *LPG1* parasites. In conclusion, the *L. infantum* Δ*lpg1* mutant constitutes a powerful tool to investigate the role(s) played by LPG in host cell-parasite interactions.

Keywords: Lipophosphoglycan, *Leishmania infantum*, gene targeting, lipid droplets, macrophage

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

Received: *13 December 2017*
Accepted: *19 March 2018*
Published: *05 April 2018*

Citation:
Lázaro-Souza M, Matte C, Lima JB, Arango Duque G, Quintela-Carvalho G, de Carvalho Vivarini Á, Moura-Pontes S, Figueira CP, Jesus-Santos FH, Gazos Lopes U, Farias LP, Araújo-Santos T, Descoteaux A and Borges VM (2018) Leishmania infantum Lipophosphoglycan-Deficient Mutants: A Tool to Study Host Cell-Parasite Interplay. Front. Microbiol. 9:626. doi: 10.3389/fmicb.2018.00626

INTRODUCTION

Lipophosphoglycan (LPG) is one of the most abundant components of *Leishmania* membranes (Turco and Descoteaux, 1992). In the course of parasite interaction with invertebrate hosts, LPG binds to the midgut epithelium of specific species of the sandfly vectors (Sacks et al., 2000), and protects parasites against the digestive enzymes present in the peritrophic matrix following blood feeding (Sacks and Kamhawi, 2001). In vertebrate hosts, LPG contributes to virulence by shielding *Leishmania* against the complement system (Spath et al., 2003) and by inhibiting phagolysosomal

biogenesis (Desjardins and Descoteaux, 1997; Vinet et al., 2009; Moradin and Descoteaux, 2012). Purified LPG has been considered as a pathogen-associated molecular pattern molecule (PAMP) that triggers Toll-like receptors (TLR) and is also known to interfere with pro-inflammatory and signaling pathways in host cells (Descoteaux et al., 1991; Descoteaux and Turco, 1993; Becker et al., 2003; de Veer et al., 2003; Kavoosi et al., 2009; Rojas-Bernabé et al., 2014; Tavares et al., 2014; Lima et al., 2017).

This complex glycolipid is organized in four domains: a conserved 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol membrane anchor, a conserved diphosphoheptasaccharide core structure, a polymer of repeating phosphodisaccharide units (phosphoglycan or PG) carrying species-specific side chains and variable, often mannose-rich cap structures (Turco and Descoteaux, 1992; McConville and Ferguson, 1993). Although the biosynthesis of LPG has attracted considerable interest, to date only few enzymes and transporters involved in this process have been identified either biochemically, genetically, or both (Ryan et al., 1993; Descoteaux et al., 1995, 1998, 2002).

One of the key enzymes in the biosynthesis of LPG is *LPG1*, a putative galactofuranosyl transferase specifically involved in the synthesis of the LPG glycan core (Ryan et al., 1993). Consequently, parasites lacking the *LPG1* gene (Δ*lpg1*) express a truncated LPG without the PG domain; they nonetheless assemble and secrete other PG-containing molecules (Dermine et al., 2000; Späth et al., 2000). Both *L. major* and *L. donovani* require *LPG1* for the establishment of infection within macrophages, as evidenced by the elimination of *LPG1*-null mutants following phagocytosis; yet, restoration of LPG expression by genetic complementation restored the capacity to replicate within macrophages (Späth et al., 2000; Lodge et al., 2006). Interestingly, phosphoglycan synthesis does not seem to be an absolute requirement for virulence in all *Leishmania* species, since *L. mexicana* phosphoglycan-deficient parasites were found to be similarly virulent to their wild-type (WT) counterparts (Ilg et al., 1999, 2001; Ilg, 2000). This difference in LPG requirement for the establishment of infection within macrophages may be related to the fact that *L. mexicana* resides in large fusogenic communal vacuoles, as opposed to the non-fusogenic, tight individual vacuoles in which *L. major* and *L. donovani* replicate. The role played by *LPG1* in *L. infantum* infectivity in mammals remains to be established.

This report describes the disruption of *LPG1* in *L. infantum*, the main etiological agent of visceral leishmaniasis in Brazil. While deletion of *LPG1* did not alter parasite morphology *in vitro* or the presence of subcellular organelles, e.g., lipid droplets (LD), Δ*lpg1* parasites experienced distinct infection outcomes in comparison to WT parasites. Hence, the *L. infantum LPG1*-null strain described in the present study constitutes a powerful tool to investigate the role of LPG in host-parasite interactions.

METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of Institutional Review Board for Animal Experimentation (CEUA), Gonçalo Moniz Institute, Fundação Oswaldo Cruz. The protocol was approved by the Institutional Review Board for Animal Experimentation (CEUA), Gonçalo Moniz Institute, Fundação Oswaldo Cruz (Protocol No. 021/2015).

Animals

Inbred male C57BL/6 mice, aged 6–8 weeks, were obtained from the animal care facility of the Gonçalo Moniz Institute, Fundação Oswaldo Cruz (IGM-FIOCRUZ, Bahia, Brazil).

Targeted Deletion of the *LPG1* Gene and Complementation

The constructs for *LPG1* (beta galactofuranosyl transferase) gene targeting were designed based on the *L. infantum LPG1* gene sequence (GenBank accession No. GU233511). Homozygous *LPG1*-null mutants (Δ*lpg1*) were obtained using two targeting constructs (**Figures 1A,B**). For the *NEO* targeting construct, the entire *LPG1* gene was amplified by PCR from *L. infantum* BH46 (MCAN/BR/89/BH46) DNA using *Taq* DNA polymerase (New England Biolabs) and oligodeoxynucleotides AD-358 (forward) (5′-gtacaagcttccatATGGCGCCGCCTCGCTG-3′) and AD-359 (reverse) (5′-gctactcgagTTAGCTGGGGTCAACAG-3′). This fragment was digested with *Hind*III and *Xho*I, and then ligated with the *Hind*III-*Xho*I-digested pBluescript II SK$^-$ vector, yielding pBS-*LPG1*. The *NEO* resistance cassette from pLeishNeo (unpublished) was extracted with *Not*I and *Eco*RV, blunted and inserted in the *Msc*I site of pBS-*LPG1*, within the *LPG1* gene, yielding pBS-*LPG1::NEO*. For the *HYG* targeting construct, nucleotides 1–437 of the *LPG1* gene were amplified by RT-PCR from *L. infantum* BH46 mRNA using oligodeoxynucleotides AD-53 (forward) (5′-cgggatccatATGGCGCCGCCTCGCTG-3′) and AD-357 (reverse) (5′-ggaattcTCGGGGTGGTGAATG-3′). This fragment was digested with *Bam*HI and *Eco*RI, and then ligated with the *Bam*HI-*Eco*RI-digested pBluescript II SK$^-$ vector. A 467-bp fragment containing nucleotides 781–1,247 of the *LPG1* ORF was amplified by PCR from *L. infantum* BH46 genomic DNA using oligodeoxynucleotides AD-355 (forward) (5′-gcaagcttGGCATCTATTACACAGACCACAAGG-3′) and AD-356 (reverse) (5′-caggtcgacTGGCAGCGAATGTTTTCACC-3′). This fragment was digested with *Hind*III and *Sal*I, then ligated with the same vector, downstream of the first *LPG1* sequence, at the *Hind*III and *Sal*I restriction sites. The *HYG* resistance cassette from pX63-HYG was excised with *Sal*I and *Bam*HI, blunted and inserted between the two *LPG1* sequences, at the *Eco*RV restriction site, yielding pBS-*LPG1::HYG*. For genetic complementation of the Δ*lpg1* mutant, the entire *LPG1* ORF was amplified by PCR from *L. infantum* BH46 genomic DNA using Native *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and oligodeoxynucleotides AD-358 (forward) (5′-gtacaagcttccatATGGCGCCGCCTCGCTG-3′) and AD-359 (reverse) (5′-gctactcgagTTAGCTGGGGTCAACAG-3′). This fragment was digested with *Hind*III and *Xho*I, and then ligated with the *Hind*III-*Xho*I-digested pBluescript II SK$^-$ vector, yielding pBSII-*LPG1*. The absence of mutations in the amplified *LPG1* ORF was verified by Sanger sequencing (Génome Québec; GenBank accession No. GU233511). The *LPG1* gene was then excised from pBSII-*LPG1* with *Eco*RV and *Xho*I, blunted and

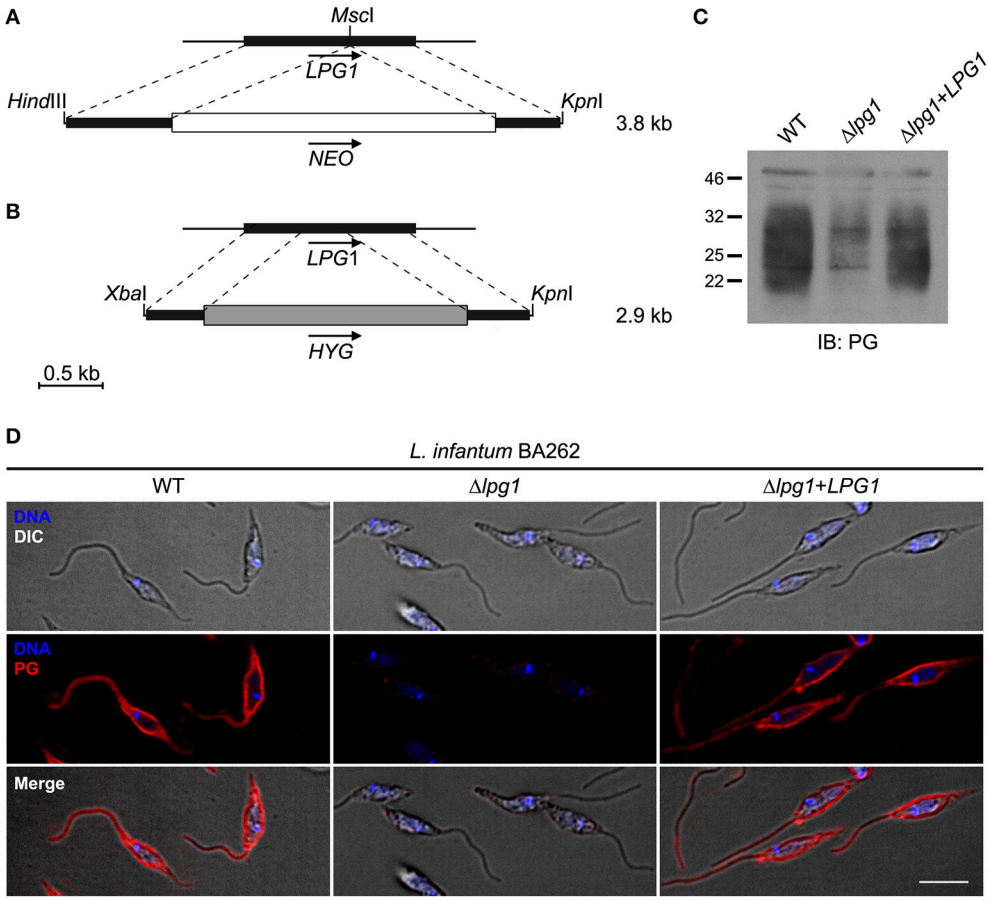

FIGURE 1 | Constructs for the targeted deletion and complementation of the *LPG1* gene in *Leishmania infantum*. **(A,B)** *LPG1*::*NEO* and *LPG1*::*HYG* targeting constructs for the disruption of *LPG1*. For the *LPG1*::*NEO* construct, the *NEO* resistance cassette (white box) was inserted in the *Msc*I site of the *LPG1* ORF (black rectangle). In the *LPG1*::*HYG* construct, portions of the *LPG1* ORF (black rectangles) corresponding to positions +1 to +437 and to positions +781 to +1247 downstream of the ATG translation initiation codon flank the HYG resistance cassette (shaded rectangle). Dashed lines delimit regions of recombination between the LPG1 gene and the targeting constructs. Arrows indicate gene orientation. **(C)** Western blot analysis of LPG expression in WT, Δ*lpg1*, and Δ*lpg1* + *LPG1* promastigotes. Parasite lysates were probed with the anti-phosphoglycan (PG) antibody CA7AE, as described in Materials and Methods. **(D)** Confocal immunofluorescence analysis of WT, Δ*lpg1* and Δ*lpg1* + *LPG1* parasites. Late log-phase promastigotes were adhered on Poly-L-Lysine-coated glass coverslips, fixed and incubated with DAPI to stain DNA (blue), and with the CA7AE antibody to visualize LPG and other Gal(β1,4)Man(α1-PO4) repeating unit-containing PGs (red), as described in Materials and Methods. Fluorescence staining images merged with differential interference contrast (DIC) are shown in the lower panels. Scale bar, 5 μm.

ligated with the *Eco*RV-digested pLeishZeo vector (unpublished), yielding pLeishZeo-*LPG1*.

Transfection and Selection of *L. infantum* Δ*lpg1* Promastigotes

Log-phase WT *L. infantum* BA262 (MCAN/BR/89/BA262) promastigotes were first electroporated with the purified *LPG1*::*HYG* targeting construct (excised as a 2.9-kb *Xba*I-*Kpn*I fragment from pBS-*LPG1*::*HYG*) using 0.2 cm electroporation cuvettes, at 0.45 kV and a high capacitance of 500 μF as previously described (Turco et al., 1994). Following electroporation, promastigotes were incubated for 24 h in drug-free, complete M199 medium and subsequently grown in the presence of 50 μg/mL Hygromycin B (Roche Diagnostics). To generate *LPG1*-null mutants, log-phase $lpg1^{+/HYG}$ heterozygous *L. infantum* BA262 promastigotes were electroporated with purified *LPG1*::*NEO* targeting construct (excised as a 3.8-kb *Hind*III-*Kpn*I fragment from pBS-*LPG1*::*NEO*) and grown after 24 h in the presence of both 50 μg/mL Hygromycin B and 70 μg/mL G418 (Life Technologies). Absence of LPG in the resulting double drug-resistant Δ*lpg1* promastigotes was verified by Western blot analysis and confocal immunofluorescence. To restore *LPG1* expression, log-phase *L. infantum* BA262 Δ*lpg1* cells were electroporated with pLeishZeo-*LPG1*. Complemented mutants (Δ*lpg1* + *LPG1*) were selected with 80 μg/mL Zeocin (in addition to G418 and Hygromycin B at concentrations specified above) and verified by Western blotting and confocal immunofluorescence.

Parasite Cultures

L. infantum promastigotes were cultured in HOMEM medium supplemented with 10% inactivated Fetal Bovine Serum (FBS),

100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine in 25 cm^2 flasks at 24°C until late log-phase. For *L. infantum* BA262 Δ*lpg1,* Hygromycin (50 μg/mL) and G418 (70 μg/mL) were added to the medium. For *LPG1*-complemented parasites (Δ*lpg1* + *LPG1*), Hygromycin (50 μg/mL), G418 (70 μg/mL), and Zeocin (100 μg/mL) were added to the medium.

Western Blotting

Late log-phase promastigotes were washed with ice-cold PBS containing 1 mM Na_3VO_4, then lysed in 50 mM Tris-HCl pH 8, 150 mM NaCl and 1% Nonidet P-40, containing complete protease inhibitors (Roche Applied Science) and phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF, 1.5 mM EGTA and 10 mM $Na_4P_2O_7$). Samples were sonicated briefly, and insoluble material was removed by centrifugation for 10 min at 4°C. Protein concentrations were determined using the Pierce BCA protein assay kit (Pierce). Proteins were separated by SDS-PAGE and then transferred to Hybond-LFP PVDF membranes (GE Healthcare Life Sciences) using a Trans-Blot SD Semi-Dry Transfer Cell apparatus (BioRad). Membranes were blocked with 5% BSA and incubated with the mouse monoclonal antibody CA7AE (MediMabs). For immunodetection, goat anti-mouse IgM Heavy Chain Secondary antibody conjugated with horseradish peroxidase (HRP), and enhanced chemiluminescence (ECL) detection reagents from GE Healthcare Life Sciences were used.

Confocal Immunofluorescence Microscopy

Late log-phase promastigotes were adhered on Poly-L-Lysine-coated glass coverslips (BD Biosciences, San Jose, CA) by centrifugation, fixed with 4% paraformaldehyde (Canemco and Marivac) for 20 min and simultaneously blocked and permeabilized with a solution of 0.1% Triton X-100, 1% BSA, 6% non-fat dry milk, 20% goat serum and 50% FBS for 20 min. The distribution of LPG and other PGs containing the Gal(β1,4)Man(α1-PO_4) repeating unit epitope was visualized using the mouse monoclonal antibody CA7AE (MediMabs, 1:2,000) after 2 h incubation followed by Alexa Fluor 568 goat anti-mouse IgM (Molecular Probes) at 1:500 for 30 min incubation. Parasite nuclei were stained with DAPI (Molecular Probes) at 1:17,000. All steps were performed at room temperature. Coverslips were then mounted in Fluoromount-G (Interscience) and sealed with nail polish. Promastigotes were observed with a Plan APOCHROMAT 63x oil-immersion DIC 1.4 NA objective on a Zeiss LSM780 confocal microscope equipped with a 30 mW 405 nm diode laser, 25 mW 458/488/514 argon multiline laser, 20 mW DPSS 561 nm laser and 5 mW HeNe 633 nm laser, coupled to a Zeiss Axio Observer Z1. Images were acquired in plane scanning mode, and were minimally and equally processed using Carl Zeiss ZEN 2011 software.

Electron Microscopy

Late log-phase promastigotes were fixed with 2% paraformaldehyde plus 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Next, parasites were processed for Transmission Electron Microscopy (TEM) by post-fixing in 1% osmium tetroxide (OsO_4) plus 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, then dehydrated in acetone at increasing concentrations of 50, 70, 90, and 100% followed by processing for resin embedding (PolyBed 812, Polysciences). Sections were mounted on uncoated 200-mesh copper grids and viewed under a TEM microscope (JEOL JEM-1230). Alternatively, parasites were processed for Scanning Electron Microscopy (SEM) by first fixing as described above, then adhered on Poly-L-Lysine-coated glass coverslips and post-fixed as described above. Samples were then submitted to critical point-drying under CO_2, coated with a 20 nm-layer of gold particles and examined under SEM (JSM-6390LV, JEOL).

Parasite Growth Curves

Early log-phase promastigotes (1×10^5/ml) were cultured and the number of viable promastigotes was determined by daily direct counting performed in a Neubauer chamber.

Lipid Droplets Staining and Quantification

Late log-phase promastigotes were fixed with 3.7% formaldehyde and stained with osmium tetroxide. Cell morphology was observed, and LD were counted by light microscopy using a 100X objective lens in 50 consecutively scanned parasites (Araújo-Santos et al., 2014).

Bone Marrow-Derived Macrophages (BMDM) Macrophage and Infection

Bone marrow-derived macrophages (BMDM) were obtained from C57BL/6 mice as previously described. Briefly, cells were collected from femurs and differentiated in RPMI 1640, 20% inactivated FBS, 30% L929 cell-conditioned media (LCCM), 2 mM L-glutamine, 100 U/mL Penicillin, and 100 μg/mL Streptomycin at 36°C under 5% CO_2. BMDMs were collected after 7 days and seeded on tissue culture plates in RPMI 1640 media, 10% inactivated FBS, 5% LCCM and 2 mM L-glutamine (Araújo-Santos et al., 2014).

Cells (2×10^5) adhered on coverslips were infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* parasites at a 10:1 multiplicity of infection (MOI). After 4 or 72 h of infection, coverslips were fixed and stained with DiffQuik (Wright-Giemsa). Intracellular parasites were counted under light microscopy to determine the infection index under each experimental condition (Araújo-Santos et al., 2014).

RAW 264.7 Cell Line, Culture, and Infection

The mouse macrophage leukemia cell line RAW 264.7 (TIB-71; American Type Culture Collection (ATCC), Manassas, VA, USA) was maintained in DMEM medium with high glucose (Vitrocell Embriolife, Campinas, SP, Brazil) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator at 37°C under 5% CO_2. RAW 264.7 cells were infected with either WT, Δ*lpg1* or Δ*lpg1*+*LPG1* parasites at a 10:1 multiplicity of infection (MOI). After 4 or 8 h of infection, cells were processed

for quantitative RT-PCR. For the luciferase reporter assay, cultures were washed 2 h post-infection and analyzed 24 h later.

RNA Extraction and RT-qPCR

For real time quantitative polymerase chain reaction analysis, total RNA of control and infected RAW 264.7 cells (1×10^6 cells) was extracted using an Invitrap® Spin Cell RNA mini kit (STRACTEC Molecular GmbH, Berlin, Germany). RNA extracts (2 μg) were reverse transcribed into first-strand cDNA with ImProm-II (Promega) and oligo(dT) primers in accordance with manufacturer instructions. The following primer DNA sequences were used to determine iNOS mRNA levels: Forward 5′-CAGCTGGGCTGTACAAACCTT-3′ and Reverse: 5′-CATTGGAAGTGAAGCGTTTCG- 3′, while GAPDH mRNA levels were quantified using: Forward 5′-TGCACCACCAACTGCTTAGC-3′ and Reverse 5′-GGCATGGACTGTGGTCATGAG-3′. Amplicon specificity was carefully verified by the presence of a single melting temperature peak in dissociation curves calculated following RT-qPCR, which was performed via the Applied Biosystems StepOne™ detection system (Applied Biosystems) using GoTaq® qPCR Master Mix (Promega Corp., Madison, WI, USA). All RT-qPCR analyses were performed in triplicate. RT-qPCR data was normalized using GAPDH primers as an endogenous control. All gene expression ratios were calculated by the ΔΔCt method using StepOne software version 2.0 (Applied Biosystems).

Transient Transfections and Luciferase Assays

To investigate NF-κB transcriptional activity, RAW 264.7 were plated in 48-well polystyrene plates (1×10^5 cells per well) and transfected with 1 μg of the p6kB-Luc luciferase reporter construct (kindly provided by Dr. Patrick Baeuerle, Munich University) in the presence of LIPOFECTAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA). pTK-3XNS luciferase reporter construct was used to measure iNOS promoter activity, provided by Dr. David Geller (University of Pittsburgh, Pennsylvania, EUA). Luciferase activity was normalized using 40 ng of pRL-CMV plasmid (Promega Corp., Madison, WI, USA). Transfected cells were infected with either WT, Δ*lpg1* or Δ*lpg1*+*LPG1* parasites at a 10:1 MOI. After 24 h of infection, cells were washed with PBS, lysed according to the Dual Luciferase System protocol (Promega Corp.), and analyzed in a GloMax®-Multi detection system (Promega Corp.). Positive controls consisting of cells stimulated with 1 μg/mL of LPS (Sigma-Aldrich) were used to induce the activation of iNOS gene expression.

Statistical Analysis

BMDM and RAW 264.7 cell infection assays were performed in triplicate, and each experiment was repeated at least three times. Data are presented as the mean and SE (standard error) of representative experiments, and GraphPad Prism 5.0 software (GraphPad Software) was used for data analysis. Means from different groups were compared by One-way ANOVA and comparisons between two groups were performed using the Student Newman-Keuls post-test. Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

Generation of a *L. infantum Lpg1*-Null Δ*lpg1* Mutant

To generate a *L. infantum* LPG-defective (Δ*lpg1*) mutant, WT *L. infantum* BA262 promastigotes were transfected with the *LPG1* targeting constructs (**Figures 1A,B**). The resulting *HYG*- and *NEO*-resistant Δ*lpg1* parasites were transfected with a *LPG1* expression vector to generate add-back LPG-expressing parasites (Δ*lpg1* + *LPG1*). Loss of LPG expression in the Δ*lpg1*, was determined by comparing LPG levels in WT, Δ*lpg1*, and Δ*lpg1* + *LPG1 L. infantum* promastigotes by Western blot and by confocal immunofluorescence microscopy (**Figures 1C,D**). Together, these data indicate that the *LPG1* gene was successfully deleted in the Δ*lpg1* mutants, resulting in the generation of a LPG-defective *L. infantum* mutant.

LPG1-Null Mutants Retain *L. infantum* Viability and Morphology

To determine the effect of deleting *LPG1* on parasite growth and morphology, axenic cultures of the three isolates were monitored and counted daily for 10 days until reaching late log phase. A delayed replication capability of the Δ*lpg1* mutant parasites was noted in comparison to the WT and Δ*lpg1* + *LPG1* parasites (**Figure 2A**). Wild-type *L. infantum* presented regular growth for 7 days until reaching stationary phase, with a cell density of approximately 3–4 $\times 10^7$ parasites/ml, while the Δ*lpg1* mutant reached the same phase approximately 3 days later, with a cell density of 1–2 $\times 10^7$ parasites/ml (**Figure 2A**). The Δ*lpg1* + *LPG1* mutants presented an intermediate growth profile, reaching stationary phase shortly after the WT parasites. Area Under the Curve (AUC) analysis of the growth curve revealed a significant difference only when comparing WT and Δ*lpg1* parasites ($p < 0.05$), yet no differences were observed between WT and Δ*lpg1* + *LPG1* mutants (**Figure 2B**). Upon reaching stationary phase, parasites were examined by electron microscopy to assess the presence of morphological alterations. Under both SEM and TEM, no alterations in morphology (**Figure 2C**) or in ultrastructural characteristics (**Figure 3A**) were detected among WT, Δ*lpg1*, and Δ*lpg1* + *LPG1* promastigotes. In addition, the absence of *LPG1* had no impact on the number of lipid bodies present within the parasites (**Figure 3A**). Hence, whereas the *LPG1* gene had a limited impact on *L. infantum* promastigotes proliferation, it did not significantly alter morphological features of these parasites (**Figure 3**).

Lpg1-Null Mutants Exhibit Limited Survival in Macrophages

To evaluate differences in parasite survival among WT and transgenic parasites *in vitro*, BMDMs were infected for 4 or

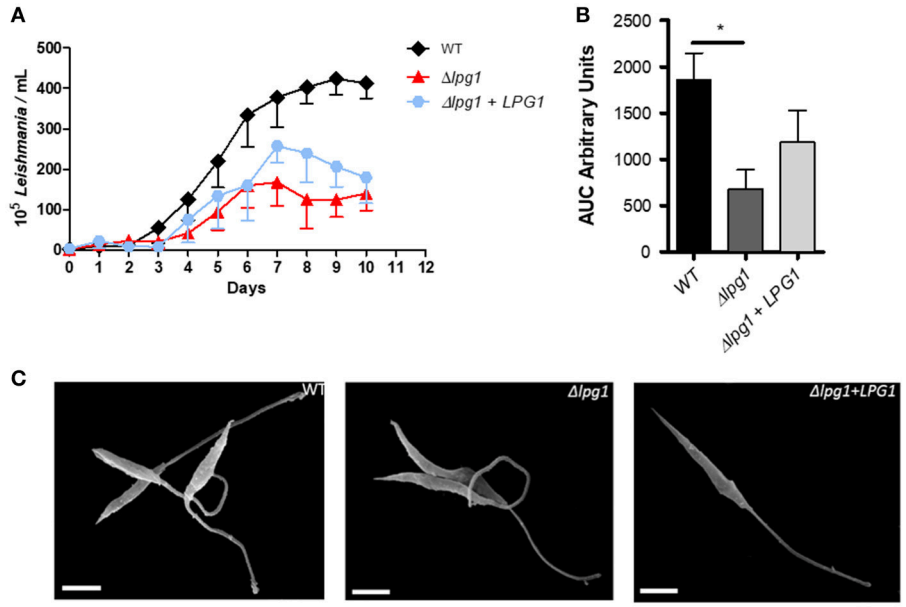

FIGURE 2 | Growth curve and morphology of the Δ*lpg1* mutant. WT, Δ*lpg1* and Δ*lpg1* + *LPG1* parasites were cultured at initial concentrations of 1 × 10^5/ml in HOMEM medium. **(A)** Axenic growth curve of late log-phase WT, Δ*lpg1* and Δ*lpg1* + *LPG1* parasites, as showed by the area under the curve (AUC) **(B)**. The number of viable parasites was evaluated by direct counting. Each point represents mean and SE. Data are representative of at least three independent assays and were collected in triplicate for each condition. *$p < 0.05$. **(C)** Parasites were processed for scanning electron microscopy (SEM) and photographed under a JEOL JSM-6390LV microscope at 6000x magnification **(C)**. Scale bar, 2 μm.

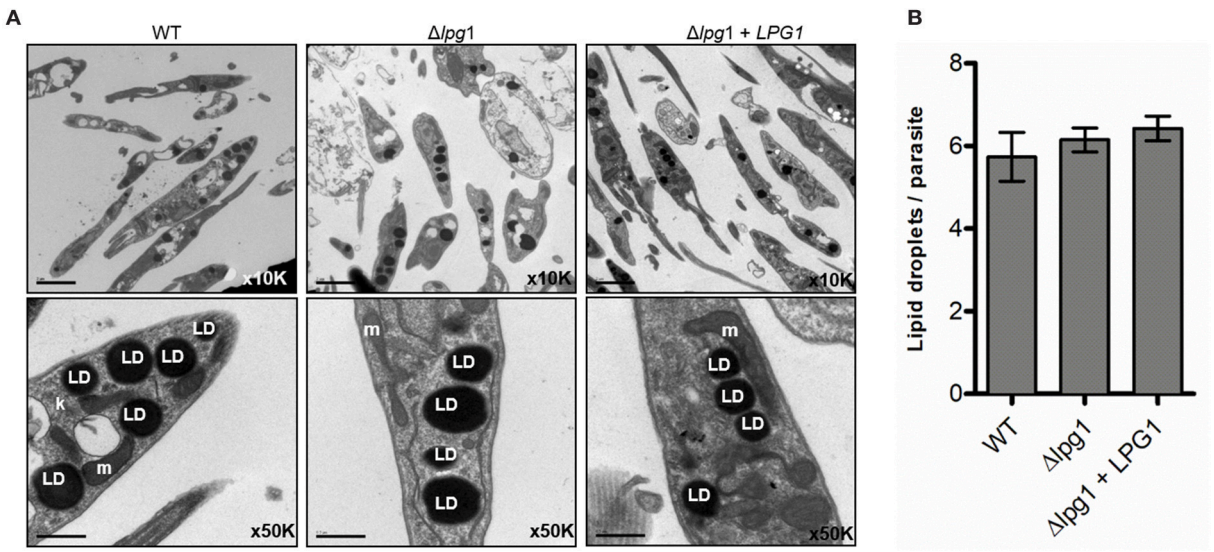

FIGURE 3 | Deletion of *LPG1* does not alter LD formation in *Leishmania infantum*. **(A)** Panels show stationary phase WT, Δ*lpg1* and Δ*lpg1* + *LPG1* promastigotes analyzed by transmission electron microscopy (TEM) and photographed under a JEOL 1230 microscope. **(B)** Bars represent the mean number of LD ± SE in WT, Δ*lpg1* or Δ*lpg1* + *LPG1* parasites stained with osmium tetroxide. k, kinetoplast; LD, lipid droplets; m, mitochondrion. Scale bar, 0.5 μm.

72 h. No differences were observed between the *Leishmania* parasites after 4 h of infection (**Figure 4**). However, at 72 h post-infection, WT parasites survived more efficiently than the Δ*lpg1* mutant. Expression of *LPG1* in the Δ*lpg1* mutant partially restored its capacity to survive and replicate within macrophages. These data reinforce the importance of LPG as a virulence factor in the successful maintenance of *Leishmania infantum* infection.

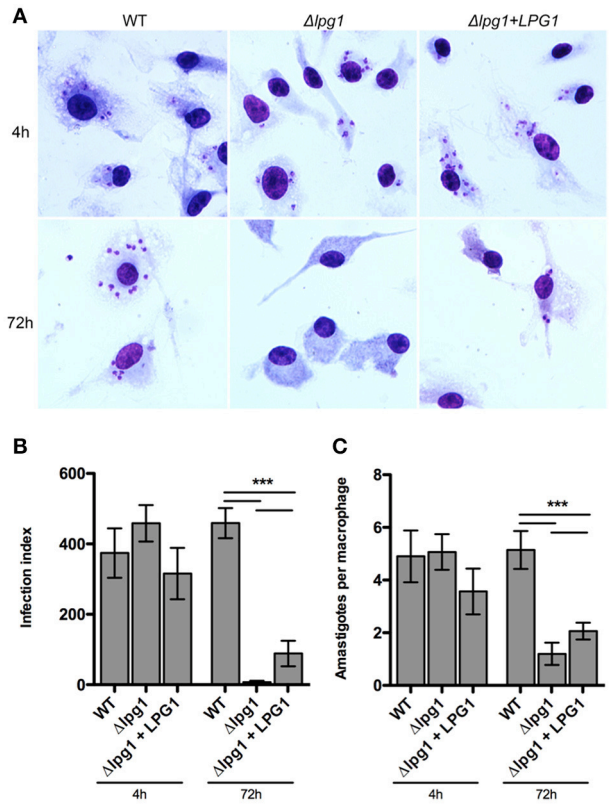

FIGURE 4 | *LPG1* promotes intraphagosomal survival in infected macrophages. C57BL/6 BMDMs were infected with WT, Δ*lpg1* and Δ*lpg1* + *LPG1* promastigotes as described under Materials and Methods. At 4 and 72 h post-infection, cells were fixed and Giemsa-stained. **(A)** Micrographs of infected BMDMs at 4 and 72 h post-infection. The infection index **(B)** and amastigotes per macrophage **(C)** were quantified by light microscopy. Arrows point to amastigotes inside a parasitophorous vacuole. Original magnification × 1000. Statistical differences were evaluated using the Student Newman-Keuls test. ***$p < 0.001$ compared to WT or between groups.

LPG1-Null Mutants Induce NF-κB-Dependent iNOS Expression in Macrophages

To assess the impact of LPG on the expression of inducible nitric oxide synthase by host cells, we first analyzed iNOS transcript levels in RAW 264.7 cells infected with WT or transgenic parasites. The Δ*lpg1* mutant induced a robust (3.5-fold increase) expression of iNOS compared to WT and Δ*lpg1* + *LPG1* promastigotes (**Figure 5A**). We next performed luciferase reporter assays to characterize the modulation of the iNOS promoter by *L. infantum* promastigotes. RAW 264.7 cells were transiently transfected with either the iNOS promoter reporter construct pTK-3XNS or the NF-κB consensus luciferase reporter construct (p6κB-Luc) prior to infection with either *L. infantum* WT, Δ*lpg1* or Δ*lpg1*+*LPG1*, or stimulation with LPS. As shown in **Figures 5B,C**, LPG-deficient promastigotes induced stronger activation of the iNOS promoter and of the NF-κB reporter. Collectively, these findings indicate that LPG contributes to the evasion of iNOS expression by *L. infantum* promastigotes.

DISCUSSION

Previous studies using purified LPG were important to unravel its impact on the activation of the immune system. Although purified LPG from different species can activate the release of inflammatory mediators, understanding the role of this response in the context of infection remains a challenge. While some groups have characterized Old World *LPG1*-defective *Leishmania* species, the behavior of these parasites when compared to their WT counterparts varies depending on the species under study (Spath et al., 2003; Capul et al., 2007; Forestier et al., 2014). Here, we generated for the first time a LPG-deficient mutant of *L. infantum,* a New World species cluster.

A comparison of parasite growth between axenic cultures containing each of the three isolates showed that the deletion of *LPG1* resulted in a delayed capability of the Δ*lpg1* mutant parasites to replicate in comparison to cultures of WT and Δ*lpg1* + *LPG1* parasites. While the deletion of the *LPG1* gene had a limited impact on *L. infantum* promastigote proliferation, no significant morphological or ultrastructural alterations were seen in these parasites, indicating that targeting the *LPG1* gene does not interfere with the intrinsic cell biology of *L. infantum*.

Recently, we have demonstrated that intact LPG from *L. infantum* promastigotes, but not its glycan and lipid moieties, induced a range of pro-inflammatory responses, including prostaglandin E_2 (PGE_2) and nitric oxide (NO) release, increased LD formation, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 expression (Lima et al., 2017). Consequently, a limitation of using purified LPG is that the physiological conditions present in host cell-parasite interactions are not accurately replicated. LDs are key cytoplasmic organelles involved in production of lipid mediators and pro-inflammatory cytokines in mammalian cells (Bozza et al., 2011). Several intracellular pathogens, including *Leishmania*, take advantage of LD formation in host cells (Rabhi et al., 2016). Moreover, LDs have also been described in trypanosomatids in association with arachidonic acid metabolism (Araújo-Santos et al., 2014). Our group previously reported an increase in LD formation during *L. infantum* metacyclogenesis, as well as in the intracellular amastigote form (Araújo-Santos et al., 2014). Here, we showed that the absence of the *LPG1* gene in *L. infantum* did not alter the biogenesis of LDs. In addition, our previous findings showed that parasite-derived $PGF_{2\alpha}$ produced inside LDs plays a critical role during macrophage infection (Araújo-Santos et al., 2014). We fully intend to comprehensively investigate the potential influence of *LPG1* on the release of $PGF_{2\alpha}$ in infected macrophages using this novel *L. infantum* Δ*lpg1* mutant.

In *L. major* and *L. donovani*, the specific loss of LPG through the ablation of *LPG1* galactofuranosyl transferase strongly impairs the ability of parasites to survive within the sandfly host, as well as to establish infection in mammalian macrophages and in mice (Sacks et al., 2000; Spath et al., 2003; Secundino et al., 2010). Hence, in these species, LPG impairs the microbicidal mechanisms associated with the biogenesis of phagolysosomes, including assembly of the NADPH oxidase and recruitment of the v-ATPase (Lodge et al.,

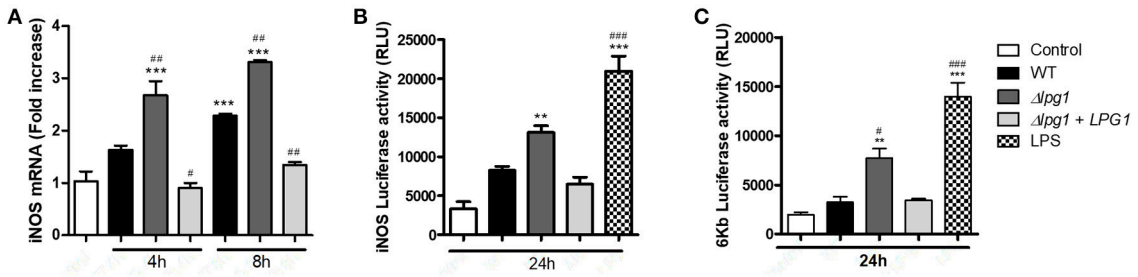

FIGURE 5 | *L. infantum* promastigotes evade NF-κB-dependent iNOS in an LPG-dependent manner in RAW 264.7 cells. RAW 264.7 cells were infected with either WT, Δ*lpg1* or Δ*lpg1* + *LPG1* promastigotes. After 4 or 8 h of infection, iNOS expression was determined by qPCR **(A)**. RAW 264.7 cells were transfected with either the iNOS promoter reporter construct pTK-3XNS, or the NF-κB consensus luciferase reporter construct (p6κB-Luc) prior to infection with either *L. infantum* WT, Δ*lpg1* or Δ*lpg1*+*LPG1*, or stimulation with LPS. At 24 h post-infection, activity of the iNOS promoter **(B)** and the NF-κB reporter **(C)** was measured by quantification of luciferase activity **(B)**. Bars represent means ± SE of three representative experiments performed in triplicate for murine cells. ***$p < 0.0001$; **$p < 0.001$ compared to control. ###$p < 0.0001$, ##$p < 0.001$ and #$p < 0.05$ compared to WT. $P < 0.0001$ compared with control group Student Newman-Keuls post-test.

2006; Vinet et al., 2009). Our results indicate that, similarly to these species, *L. infantum LPG1* is required for replication within macrophages. Whether LPG contributes to the ability of *L. infantum* to successfully infect macrophages through the impairment of phagolysosomal biogenesis remains to be investigated. Interestingly, we observed that our Δ*lpg1* mutant parasites induced robust NF-κB-dependent iNOS expression compared to parental WT *L. infantum* promastigotes. This seems to suggest that the reduced survival of Δ*lpg1* mutants in mouse macrophages may be related to higher levels of iNOS, which is responsible for the generation of leishmanicidal nitric oxide (Coelho-Finamore et al., 2011; Passero et al., 2015). Further study will involve investigating the contribution of nitric oxide production with respect to the reduced ability of Δ*lpg1* mutants to survive within macrophages. The underlying mechanism by which Δ*lpg1* mutant parasites induce high levels of NF-κB activation remains unknown, and thus represents an additional aspect of host cell-parasite interplay that we intend to further investigate.

With regard to the partial restoration of the WT phenotype observed in Δ*lpg1* + *LPG1* parasites, it has been well-documented that complemented parasites commonly do not fully recover virulence. The inappropriate regulation of *LPG1* expression by the episomal vector may be a possible explanation for this observation (Späth et al., 2000; Spath et al., 2003; Joshi et al., 2002). A previous study demonstrated that *L. major LPG1*-deficient mutant promastigotes present an attenuated virulence phenotype, as evidenced by the delayed formation of lesions *in vivo* (Späth et al., 2000). In addition, this delay was associated with a 100-fold decrease in parasite survival within macrophages *in vitro*. The data presented herein are consistent with these results, as well as with other reports in the literature (Privé and Descoteaux, 2000; Sacks et al., 2000; Zhang et al., 2004) propounding LPG as a virulence factor.

Taken together, the present findings support the importance of creating LPG-deficient mutants in various *Leishmania* spp. as a unique tool to investigate the specific impact and contribution of this abundant virulence factor in the complex host cell-*Leishmania* interplay. Hence, we are currently conducting studies to compare the responses of various immune cells to live *L. infantum* promastigotes in the presence or absence of surface-expressed LPG, since we feel it is important to thoroughly characterize these isolates to obtain a more comprehensive understanding regarding the role of *L. infantum LPG1* in future *in vitro* and *in vivo* studies.

AUTHOR CONTRIBUTIONS

ML-S, CM, JL, GA, GQ-C, AC, SM-P, UG, LF, TA-S, AD, and VB conceived and designed the study, contributed to the data analysis, drafted, and revised the manuscript. ML-S, CM, JL, GA, GQ-C, AC, SM-P, CF, FJ-S, and TA-S performed the experiments. ML-S, CM, JL, LF, TA-S, AD, and VB wrote and revised the manuscript. All authors read and approved the final version of this manuscript.

FUNDING

This work was supported by the Brazilian National Research Council (CNPq), (Grant number: 400135/2014-0 to VB), Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (Grant number: 04/2015 to VB), Science without Borders (SwB) (to VB and AD) and the Canadian Institutes of Health Research (CIHR) (grant MOP-125990 to AD). VB and UG are senior investigator funded by CNPq. AD is the holder of the Canada Research Chair on the Biology of intracellular parasitism. GA was partially supported by a CIHR Frederick Banting and Charles Best Doctoral Award. AC: received a fellowship from CNPq. The funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

The authors thank Andrezza Souza for technical and logistics support. The authors would like to thank Andris K. Walter for English language revision and manuscript copyediting assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LM declared a shared affiliation, with no collaboration, with two of the authors, AC and UG, to the handling Editor.