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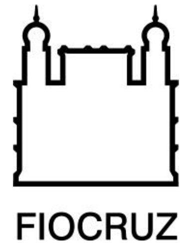

CURSO DE PÓS-GRADUAÇÃO EM PATOLOGIA

TESE DE DOUTORADO

CORPÚSCULOS LIPÍDICOS E EICOSANOIDES NOS MOMENTOS INICIAIS DA INFECÇÃO COM *Leishmania infantum chagasi*

Théo de Araújo Santos

Salvador-Ba

2013

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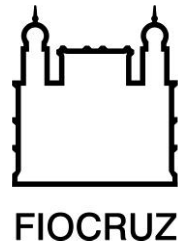

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Tese apresentada ao Colegiado do Curso de Pós-graduação em Patologia como requisito para obtenção do grau de Doutor em Patologia Experimental.

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"CORPÚSCULOS LIPÍDICOS E EICOSANÓIDES NOS MOMENTOS INICIAS DA INFECÇÃO COM *Leishmania infantum chagasi*"

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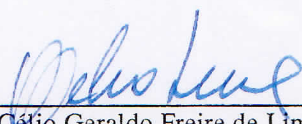

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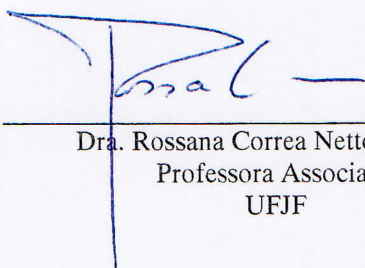

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Dedico este trabalho a

Carla, minha amada companheira

Letícia, meu tesouro amado

Meus pais Virgínia e Edielson, sempre presentes pelo exemplo

Lia e João, meus queridos irmãos

Cláudio Emanuel e Dona Del, meus pais postiços

A Deus que está acima de todas as coisas e sempre será meu eterno amigo e companheiro

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RESUMO

ARAÚJO-SANTOS, THÉO. CORPÚSCULOS LIPÍDICOS E EICOSANOIDES NOS MOMENTOS INICIAIS DA INFECÇÃO COM *Leishmania infantum chagasi.* Tese (Doutorado) – Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, 2013.

Corpúsculos lipídicos são organelas citoplasmáticas envolvidas na produção de eicosanoides em leucócitos. Eicosanoides como as prostaglandinas têm sido envolvidos no controle da resposta inflamatória e imunológica. A saliva de *Lutzomyia longipalpis* participa do estabelecimento e desenvolvimento da doença pela modulação das respostas hemostática, imunológica e inflamatória do hospedeiro favorecendo a infecção. Entretanto, o papel dos eicosanoides nos momentos iniciais da infecção por *Leishmania* ainda não foi esclarecido, assim como a participação da saliva neste contexto. Aqui, nós investigamos o papel dos eicosanoides induzidos pela saliva de *L. longipalpis* e produzidos pela *Leishmania infantum chagasi* na infecção. O sonicado de glândula salivar (SGS) de *L. longipalis* induziu um aumento no número de CLs em macrófagos de maneira dose e tempo dependente, o qual esteve correlacionado com o aumento de PGE_2 nos sobrenadante de cultura. As enzimas COX-2 e PGE-sintase foram co-localizadas nos CLs induzidos pela saliva e a produção de PGE_2 foi reduzida pelo tratamento com NS-398, um inibidor de COX-2. Nós verificamos que o SGS rapidamente estimulou a fosforilação de ERK-1/2 e PKC-α e a inibição farmacológica dessas vias inibiu a produção de PGE_2 pelos macrófagos estimulados com SGS. Em seguida, nós avaliamos o efeito da saliva de *L. longipalpis* sobre a produção de eicosanoides durante a infecção por *L. i. chagasi* no modelo peritoneal murino. Nós observamos que a saliva aumentou a viabilidade intracelular de *L. i. chagasi* tanto em neutrófilos como em neutrófilos recrutados para a cavidade peritoneal. As células recrutadas para cavidade peritoneal apresentaram maiores níveis da relação PGE_2/LTB_4 e o pré-tratamento com NS-398 reverteu o efeito da saliva sobre a viabilidade intracelular dos parasitas. Parasitas como *Leishmania* são capazes de produzir PGs utilizando uma maquinaria enzimática própria. Neste estudo nós descrevemos a dinâmica de formação e a distribuição celular dos CLs em *L. i. chagasi* bem como a participação desta organela na produção de PGs. A quantidade de CLs aumentou durante a metaciclogênese assim como a expressão de $PGF_{2\alpha}$ sintase (PGFS), sendo esta enzima co-localizada nos CLs. A adição de ácido araquidônico AA à cultura de *L. i. chagasi* aumentou a quantidade de CLs por parasita, bem como a secreção de $PGF_{2\alpha}$. A infecção com as diferentes formas de *L. i. chagasi* não foi capaz de estimular a formação de CLs na célula hospedeira. Por outro lado, os parasitas intracelulares apresentaram maiores quantidades de CLs. A infecção estimulou uma rápida expressão de COX-2, mas não foi detectado aumento na produção de $PGF_{2\alpha}$ nos sobrenadantes. Por fim, nós verificamos a presença do receptor de $PGF_{2\alpha}$ (FP) nos vacúolos parasitóforos de macrófagos infectados com *L. i. chagasi.* O pré-tratamento das células com um antagonista do receptor FP inibiu os índices de infecção de forma dose-dependente. Em conjunto, nossos dados apontam que os eicosanoides desempenham um papel crucial para evasão da resposta imune durante os momentos iniciais da infecção por *L. i. chagasi* com diferentes contribuições do parasita, do vetor e da célula hospedeira neste contexto.

Palavras-Chave: Corpúsculos Lipídicos; Eicosanoides; *Leishmania*; *Lutzomyia longipalpis*; Saliva.

ABSTRACT

ARAÚJO-SANTOS, THÉO. LIPID BODIES AND EICOSANOIDS IN THE EARLY STEPS OF *Leishmania infantum chagasi* INFECTION. Tese (Doutorado) – Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, 2013.

Lipid bodies (LB) are cytoplasmic organelles involved in eicosanoid production in leukocytes. Eicosanoids as prostaglandins (PG) have been implicated in the inflammatory and immune response control. Sand fly saliva participates of the establishment and development of the disease by modulation of haemostatic, inflammatory and immunological response of the host favoring the infection. However, the role of eicosanoids in the early steps of the infection remains to be investigated as well as the role of the sand fly in this context. Herein, we investigated the role of eicosanoids trigged by *L. longipalpis* saliva and produced by *Leishmania infantum chagasi* during infection. *L. longipalpis* salivary gland sonicate (SGS) induced an increase of LB number in the macrophages of a dose and time dependent manner, which was correlated with an increase of PGE_2 release in the culture supernatants. Furthermore, COX-2 and PGE-synthase co-localized within the LBs induced by *L. longipalpis* saliva and PGE_2 production was abrogated by treatment with NS-398, a COX-2 inhibitor. We verified SGS rapidly triggered ERK-1/2 and PKC-α phosphorylation, and blockage of the ERK-1/2 and PKC-α pathways inhibited the SGS effect on PGE_2 production by macrophages. Next, we evaluated the effect of the *L. longipalpis* saliva in the eicosanoid production during *L. i. chagasi* in the murine peritoneal model. We observed SGS increased parasite viability inside recruited monocytes and neutrophils. In this regarding, SGS-recruited cells to peritoneal cavity displayed an increase in the levels of PGE_2/LTB_4 and the pre-treatment with NS-398 abrogated the sand fly saliva effect on parasite viability. Parasites as *Leishmania* are capable to produce PGs using enzymatic machinery itself. Parasite LBs amounts increased during metacyclogensis as well as the $PGF_{2\alpha}$ synthase (PGFS) expression and this enzyme was co-localized on LBs. Exogenous addition of aracdonic acid in the *Leishmania* cultures increased LB number per parasite and $PGF_{2\alpha}$ release. Macrophage infection with different forms of *L. i. chagasi* was not able to stimulate LB formation in the host cell. Notwithstanding, *Leishmania* infection upregulated COX-2 expression but this was not followed by $PGF_{2\alpha}$ release by macrophages. We detected $PGF_{2\alpha}$ receptor (FP) on the *Leishmania* PV surface. In addition, the pre-treatment of the host cells with a selective antagonist of FP, dramatically hampered *Leishmania* infection in a dose dependent manner. In set, our data point out a crucial role for eicosanoids to immune response evasion during early steps of *L. i. chagasi* infection with different contributions of parasite, vector and host cells in this context.

Keywords: Lipid bodies; Eicosanoids; *Leishmania infantum chagasi; Lutzomyia longipalpis*; Saliva.

LISTA DE ABREVIATURAS

AA	-	Ácido Aracdônico
BODIPY	-	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, sonda fluorescente utilizada para marcação de corpúsculos lipídicos.
CL	-	Corpúsculo Lipídico
COX	-	Ciclooxigenase
CyPGs	-	Cis-Prostaglandinas
EIA	-	Ensaio Imunoenzimático
GPCR	-	Receptor acoplado a proteína G
HBSS -/-	-	Solução Salina Balanceada de Hank sem Ca2+ e Mg2+ do inglês *Hank´s Balanced Salts Solution without Ca2+ and Mg2+*
HBSS +/+	-	Solução Salina Balanceada de Hank com Ca2+ e Mg2+ do inglês *Hank´s Balanced Salts Solution with Ca2+ and Mg2+*
IFN-γ	-	Interferon-γ
IL	-	Interleucina
LDK	-	Quinase ligada a Corpúsculos Lipídicos em *Trypanossoma brucei* do inglês *Lipid Droplet Kinase*
LO	-	Lipoxigenase
LPS	-	Lipopolissacarídeo
LSH	-	*Leishmania*
LTB_4	-	Leucotrieno B_4
LV	-	Leishmaniose Visceral

MCP-1	-	Proteína quimiotática de Macrófagos do inglês *Monocyte Chemoattractant Protein-1*
MET	-	Microscopia Eletrônica de Transmissão
MIP	-	Proteína inibidora de Macrófago do inglês *Macrophage Inhibitory Protein*
NO	-	Óxido Nítrico
NS-398	-	Meta-sulfonamida N-[ciclohenilona)] 4-nitrofenil, inibidor seletivo de COX-2
PACAP	-	Peptídeo de ativação da adenilato-ciclase pituitária do inglês *Pituitary Adenylate Cyclase-Activating Peptide*
PAF	-	Fator de Ativação Plaquetária do inglês *Platelet-Activanting Factor*
PGE_2	-	Prostaglandina E_2
PGFS	-	Prostaglandina $F_{2\alpha}$ sintase
$PGF_{2\alpha}$	-	Prostaglandina $F_{2\alpha}$
FP	-	Receptor da Prostaglandina $F_{2\alpha}$
EP	-	Receptor da Prostaglandina $_{E2}$
PLA_2	-	Fosfolipase A_2
ROS	-	Espécies Reativas de Oxigênio
SGS	-	Sonicado de Glândula Salivar de *Lutzomyia longipalpis*
TGF-β	-	Fator de Crescimento Transformante Beta do inglês *Transforming Growth Factor Beta*
TNF-α	-	Fator de Necrose Tumoral alfa do inglês *Tumoral Necrosis Factor Alpha*

VP	-	Vacúolo parasitóforo
ΔMFI	-	Diferença da Intensidade Média de Fluorescência do inglês *Difference of Media Fluorescence Intensity*

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1. INTRODUÇÃO

1.1. Aspectos gerais da Leishmaniose Visceral

A leishmaniose é considerada uma das principais endemias do Mundo e o seu controle é uma das prioridades da Organização Mundial de Saúde. Estima-se que cerca de 2 milhões de novos casos sejam registrados a cada ano, sendo 500 mil de leishmaniose visceral (LV) (WHO 2010).

A LV tem ampla distribuição, ocorrendo na África, Ásia, Europa, Oriente Médio e nas Américas. O Brasil está entre os países mais acometidos com a leishmaniose em seus variados aspectos clínicos. Na América do Sul, 90% dos casos registrados de LV estão no Brasil, onde anualmente são registrados 3.156 casos, em média, ao longo dos últimos onze anos, tendo a incidência da LV aumentado de 1,7 para 2,7 casos por 100.000 habitantes entre 1993 e 2003. Atualmente, a LV é observada em 19 dos 27 estados da federação, com aproximadamente 1.600 municípios envolvidos, sendo 77% dos casos registrados encontrados na região Nordeste (CHAPPUIS et al., 2007; COSTA, 2005).

A LV tem como agentes etiológicos os parasitos *Leishmania donovani* e *Leishmania infantum.* Na América do Sul o agente etiológico é a *Leishmania chagasi*. O genoma das espécies *L. infantum e L. chagasi* é idêntico, sendo então, essa nomenclatura utilizada como sinonímia (WHO 2010). Durante esta tese utilizaremos o termo *Leishmania infantum chagasi* para distinguir a espécie trabalhada aqui daquela que ocorre na Europa.

A LV é uma infecção crônica que apresenta altas taxas de morbidade e mortalidade em muitos países em desenvolvimento. Os sintomas mais prevalentes são febre alta, substancial perda de peso, esplenomegalia e hepatomegalia. Quando não tratada, a doença pode ter uma taxa de letalidade próxima a 100% dentro do período de

dois anos (WHO 2010). A resposta imune durante a LV humana é caracterizada por uma resposta mista Th1 e Th2 e linfoproliferação *in vitro* diminui com a gravidade da doença (WHO 2010). Altos níveis de mortalidade estão normalmente associados com uma co-infecção com HIV (WHO 2010) e/ou bactérias e hemorragia (ABDELMOULA et al., 2003; SAMPAIO et al., 2010). No Brasil, a maioria dos casos ocorre em crianças com menos de 10 anos de idade e as formas assintomáticas e moderadas da doença são mais frequentes (WHO 2010). Um trabalho recém-publicado, mostrou que a gravidade em casos pediátricos de LV está associada com altos níveis de citocinas pro-inflamatórias séricas (COSTA et al., 2013), entretanto o perfil de eicosanoides na doença permanece por ser estabelecido.

Poucos estudos tem investigado preditores específicos de gravidade da doença. No Brasil, muitos esforços têm sido feitos para atender esta demanda e em 2006 foi proposto um manual para o tratamento de LV grave (Manual de Vigilância da Leishmaniose Visceral Grave, 2006). Recentemente um estudo propôs um escore de prognóstico para LV em crianças, o qual foi composto por seis fatores preditores de risco de morte por LV: sangramento de mucosa, icterícia, dispneia, infecções bacterianas, neutropenia e trombocitopenia (SAMPAIO et al., 2010). Entretanto, os possíveis mecanismos associados ao aumento da gravidade ainda são desconhecidos, mas aparentemente a inflamação sistêmica desempenha um papel central. A descrição de fatores específicos ligados a imunopatogênese da LV pode levar a descrição de potenciais biomarcadores para a gravidade da doença. Por sua vez, a avaliação desses biomarcadores pode favorecer o desenvolvimento de novos alvos terapêuticos e uma melhor condução clínicas dos casos.

A LV humana pode ser parcialmente reproduzida no modelo experimental murino, uma vez que camundongos infectados não apresentam o desfecho letal da

doença. Em camundongos C57BL/6 e BALB/c, a injeção intravenosa de *L. i. chagasi* leva ao aumento do baço e do fígado, resultando em um aumento da carga parasitária nestes órgãos, nos quais ocorre o desenvolvimento de uma imunidade órgão-específica (LIESE; SCHLEICHER; BOGDAN, 2008). O fígado é o sítio de resolução da infecção aguda associada com o desenvolvimento de granulomas inflamatórios circundados por células de Kupffer infectadas e resistência a reinfecção. O baço, embora seja um sítio inicial para a produção da resposta imune mediada por célula, se torna um sítio de persistência da infecção com mudanças imunopatológicas associadas. O progresso da doença é caracterizado pelo imunocomprometimento do hospedeiro associado com altos níveis de TNF e IL-10 (STANLEY; ENGWERDA, 2007).

O tratamento da LV é realizado pelo uso de antimoniais pentavalentes, entretanto a resistência a medicamentos tem aumentado, chegando a 50% dos casos na Índia (CHAPPUIS et al., 2007). A ausência de uma vacina eficaz contra a doença tem incentivado pesquisas por antígenos que possam ser utilizados como novos candidatos vacinais. Neste sentido, foram obtidos alguns sucessos com vacinas utilizando proteínas do parasita ou da saliva do vetor em modelos experimentais em hamsters e camundongo (GOMES et al., 2008). Entretanto, a busca por novos alvos terapêuticos ainda se faz necessária.

1.2. Ciclo biológico da *Leishmania*

Leishmania é um parasita digenético, caracterizado por uma forma promastigota, extracelular e uma forma amastigota, intracelular. A forma promastigota é encontrada no trato intestinal de *Diptera* da família *Psicodidae*, onde passam por diversos estágios de diferenciação até chegar à forma promastigota metacíclica ou infectiva, em um processo denominado metaciclogênese (figura 1).

Durante o repasto sanguíneo, o flebotomíneo consegue o sangue do hospedeiro pela introdução de suas peças bucais na pele do hospedeiro vertebrado, dilacerando tecidos, rompendo capilares e criando um lago hemorrágico no qual se alimenta. Durante este processo, os flebotomíneos precisam inibir várias respostas hemostáticas do hospedeiro, tais como a ativação das cascatas de coagulação, vasoconstricção, agregação plaquetária e resposta imune (ANDRADE et al., 2005). Neste ambiente, flebotomíneos evoluíram um conjunto de componentes farmacológicos potentes com atividades redundantes e sinérgicas que subvertem a resposta fisiológica do hospedeiro favorecendo o repasto sanguíneo (ANDRADE et al., 2007). Vários estudos utilizando técnicas avançadas de análise têm sido conduzidos para identificar fatores salivares e suas atividades biológicas.

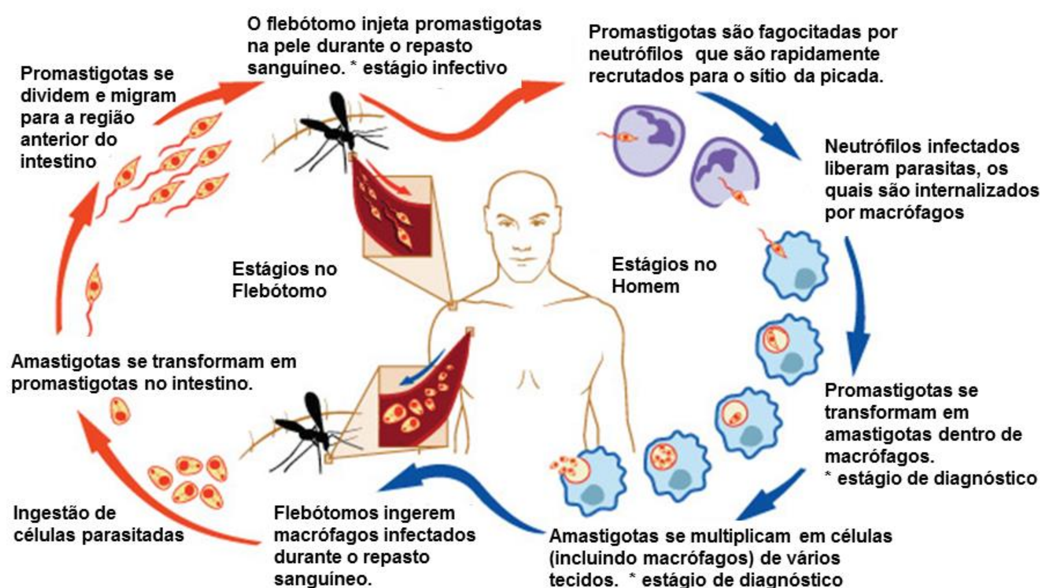

Figura 1. Ciclo biológico da *Leishmania* (traduzido e adaptado de http://www.niaid.nih.gov/topics/leishmaniasis/pages/lifecycle).

Lutzomyia longipalpis é o principal vetor da LV na América do Sul e a sua saliva tem sido extensivamente estudada. Durante a resposta inflamatória, a saliva de *L. longipalpis* induz o recrutamento celular, modula tanto a produção de anticorpos quanto a formação de imunocomplexos (SILVA et al., 2005; VINHAS et al., 2007), regula a atividade de linfócitos T e inibe células fagocíticas, tais como neutrófilos (Prates et al.,

2011), células dendríticas (COSTA et al., 2004) e macrófagos (ZER et al., 2001). Entretanto, o papel da saliva na indução de eicosanoides, bem como sua associação a biogênese de corpúsculos lipídicos ainda não havia sido investigado até o presente estudo.

1.3. Papel da saliva do vetor durante os estágios iniciais da infecção por *Leishmania*

As leishmanioses têm como vetores, dípteros pertencentes à ordem *Phlebotominae*, sendo os principais gêneros de importância médica *Phlebotomus* e o *Lutzomyia*, endêmicos do Velho Mundo e das Américas, respectivamente (SOARES; TURCO, 2003). No Brasil, o agente etiológico da LV é a *Leishmania infantum chagasi,* que é transmitida principalmente pelo flebotomíneo *Lutzomyia longipalpis*.

Durante o repasto de fêmeas de flebotomíneos, os capilares epiteliais são lacerados formando um lago sanguíneo, onde a *Leishmania* é inoculada juntamente com a saliva do vetor. Componentes salivares do flebótomo afetam a atividade hemostática do hospedeiro, facilitando a formação do lago sanguíneo pela inibição da coagulação, aumento da vasodilatação e atração de leucócitos para o local da picada (CHARLAB et al., 1995; RIBEIRO, 1987). Este cenário favorece a infecção do hospedeiro vertebrado pela *Leishmania* (ANDRADE et al., 2005, 2007).

Dentre as propriedades da saliva de *L. longipalpis* está a capacidade de estimular o recrutamento celular. Utilizando o modelo de bolsão inflamatório, Teixeira e cols. (2005) demonstraram experimentalmente que o sonicado de glândula salivar de *L. longipalpis* foi capaz de induzir um aumento no recrutamento de macrófagos após 12 horas de estímulo em camundongos BALB/c, mas não em camundongos C57BL/6. Este aumento foi correlacionado a expressão de CCL2/MCP-1 e seu receptor CCR2

(TEIXEIRA et al., 2005). A saliva de *Phlebotomus dubosqi* atrai monócitos *in vitro* (ANJILI et al., 1995) e a saliva de *P. papatasi,* não só atrai macrófagos como também favorece a infecção por *Leishmania donovani* nestas células, aumentando a carga parasitária (ZER et al., 2001). Além de induzirem o recrutamento de macrófagos, os componentes salivares de *L. longipalpis* inibem uma resposta pró-inflamatória em monócitos humanos estimulados com LPS (Costa et al., 2004). O tratamento com a saliva de *L. longipalpis* desabilita macrófagos estimulados com LPS à produção de citocinas como TNF-α e IL-10, ao passo que aumenta a capacidade produção de IL-6 nestas células (Costa et al., 2004). A saliva de *L. longipalpis* inibe a capacidade de macrófagos de apresentar antígenos de *Leishmania* a linfócitos T (THEODOS; TITUS, 1993). Foi demonstrado também que a saliva de *P. papatasi* é capaz de inibir a apresentação de antígeno e a produção de óxido nítrico em macrófagos infectados por *Leishmania major*, importante mecanismo microbicida no controle da infecção (BOGDAN; ROLLINGHOFF; DIEFENBACH, 2000; HALL; TITUS, 1995; THEODOS; TITUS, 1993).

A saliva de *L. longipalpis* também foi capaz de estimular o influxo de neutrófilos no modelo peritonial murino, o qual foi aumentado durante a infecção por *L. major* (MONTEIRO et al., 2007). Dados do nosso grupo revelaram que a saliva de *L. longipalpis* induziu um rápido edema com acúmulo de neutrófilos quando inoculada intradermicamente na orelha de camundongos previamente expostos à picada natural do flebotomíneo (SILVA et al., 2005). Peters e cols. (2008) demonstraram em tempo real que a picada do *Phlebotomus duboscqi* foi capaz de induzir o rápido influxo de neutrófilos para o local da picada. Recentemente, o nosso grupo desmonstrou que a saliva *L. longipalpis* é capaz de induzir apoptose de neutrófilos relacionada com a supressão da produção de ROS (Prates et al., 2011). Além disso, nós demonstramos que

neutrófilos estimulados com a saliva de *L. longipalpis* produzem fatores quimiotáticos para neutrófilos e macrófagos (Prates et al., 2011), o que poderia contribuir para a transmissão da *Leishmania* após a picada.

As proteínas da saliva de *L. longipalpis* foram purificadas e tiveram seus cDNAs descritos (ANDERSON et al., 2006). Dentre os componentes da saliva identificados que já tem atividade bem caracterizada na literatura estão: maxadilan (6,5 kDa), peptídeo com potente atividade vasodilatadora (Lerner et al., 1991; Svensjö et al., 2009); apirase (35,07 kDa), enzima com a ação anti-agregação plaquetária e anti-inflamatória que hidrolisa ADP e ATP a AMP e ortofosfato; hialuronidase (42,28 kDa), enzima que auxilia na difusão de agentes farmacológicos da própria saliva na pele (CERNA; MIKES; VOLF, 2002); adenosina desaminase (52 kDa), enzima que hidrolisa a adenosina em inosina, que possui efeitos anti-inflamatórios (CHARLAB; ROWTON; RIBEIRO, 2000); adenosina e AMP, envolvidos na vasodilatação e anti-agregação plaquetária, substâncias que inibem a síntese de óxido nítrico e a função de linfócitos (KATZ et al., 2000); alfa-amilase (54,02 kDa), enzima responsável pela digestão de carboidratos (RIBEIRO; ROWTON; CHARLAB, 2000); 5'-nucleotidase (60,62 kDa), pertencente a família das apirases, essa enzima degrada AMP à adenosina, uma proteína com atividade vasodilatadora, anti-agregante plaquetária e imunossupressora (CHARLAB et al., 1999); a proteína LJM11 da família yellow exerce uma função kratagonista, ou seja atua como quelante, neste caso de amina biogênicas (XU et al., 2011); além de proteínas com função ainda desconhecida, como as proteínas da família D7 (15,5 a 36,3 kDa), apesar de estarem expressas em grande quantidade na saliva de flebotomíneos (VALENZUELA et al., 2004) e a família antígeno-5 (28,8 kDa) (VALENZUELA et al., 2001).

Apesar do conhecimento sobre a ação de alguns componentes da saliva de *L. longipalpis,* pouco é conhecido sobre o seu efeito na indução da produção de mediadores lipídicos. Apenas o maxadilan, proteína presente na saliva de *L. longipalpis*, foi implicado em ativar a produção de PGE_2 em macrófagos murinos através de um receptor que reconhece um neuropeptídio, o PACAP. Este efeito induzido maxadilan parece estar associado com um perfil anti-inflamatório, pois concomitante à produção de PGE_2 foi observado um aumento de IL-6 e IL-10 e a redução da produção de TNF-α (BOZZA et al., 1998; SOARES et al., 1998; SVENSJÖ et al., 2009). Recentemente, nós demonstramos que a saliva de *L. longipalpis* é capaz de beneficiar a infecção por *L. i. chagasi* pela indução de apoptose em neutrófilos associada com o aumento da produção de PGE_2 e diminuição da produção de ROS por essas células (PRATES et al., 2011 – Ver apêndice).

1.4. Eicosanoides na resposta inflamatória

Os mediadores lipídicos desempenham um papel importante nos estágios iniciais da inflamação, bem como nas etapas de resolução do processo inflamatório. Após a lesão tecidual, a produção de prostaglandinas e leucotrienos está associada ao processo de vasodilatação, aumento da permeabilidade vascular e recrutamento celular de neutrófilos, gerando uma resposta pró-inflamatória, característica dos primeiros estágios da resposta inflamatória aguda. Já nos estágios tardios, a fagocitose de neutrófilos apoptóticos por macrófagos recrutados para o sítio inflamatório induz uma mudança na categoria de mediadores lipídicos para um perfil anti-inflamatório e, consequentemente, há uma redução no influxo de células ao local da lesão associado ao processo de resolução da inflamação (figura 2) (LAWRENCE; WILLOUGHBY; GILROY, 2002).

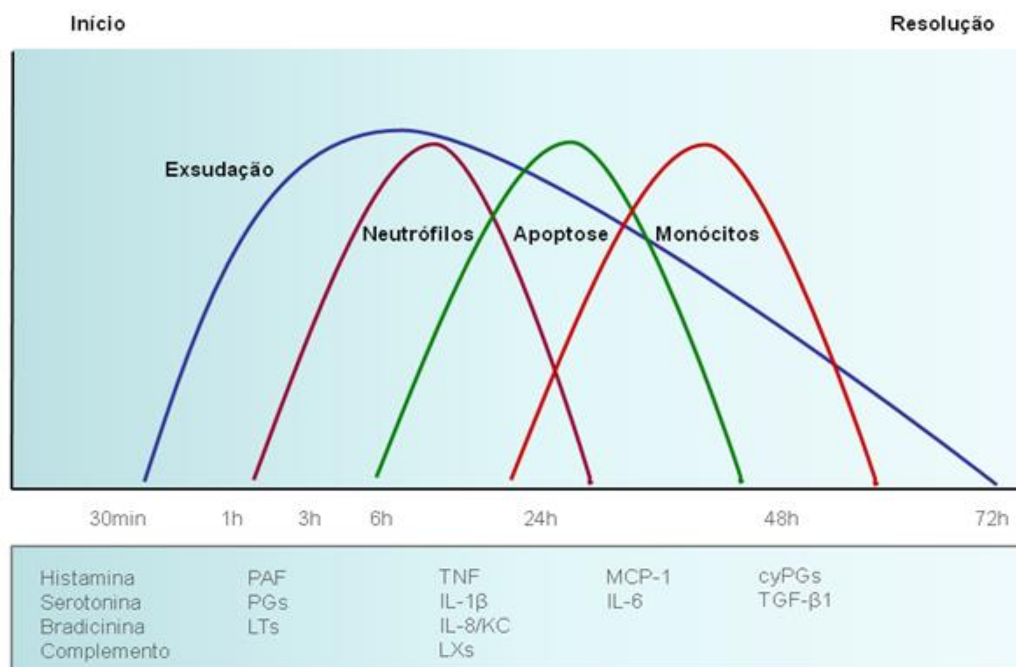

Figura 2. Representação esquemática da cinética da resposta inflamatória. O painel abaixo da figura mostra os principais mediadores inflamatórios produzidos ao longo dessa cinética (adaptado de Lawrence et al., 2002).

Mediadores lipídicos da inflamação são moléculas orgânicas biologicamente ativas que são liberadas no decorrer da resposta inflamatória. Os mediadores lipídicos mais estudados são os eicosanoides, uma família de metabólitos derivados da oxidação do ácido araquidônico (AA), uma molécula de 20 carbonos. O AA faz parte dos ácidos graxos que se encontram na porção *sn*-2 dos fosfolipídios de membrana e sua disponibilidade depende da capacidade relativa de enzimas de realizarem sua remoção ou reinserção nos fosfolipídios (BROCK; PETERS-GOLDEN, 2007). O processo de desacilação ou liberação do AA dos fosfolipídios de membrana está associado à atividade da enzima fosfolipase A_2 (PLA_2), a qual possui três famílias: a secretória e a citosólica, ambas dependentes de Ca^{2+} e a $iPLA_2$, independente de cálcio. A PLA_2 citosólica ($cPLA_2$) está envolvida no processo de síntese de eicosanoides e sua ação

pode ser estimulada por uma série de estímulos exógenos, como citocinas, hormônios ou microrganismos (BROCK; PETERS-GOLDEN, 2007).

O AA liberado pela estimulação da PLA_2, por sua vez, pode ser metabolizado principalmente por duas classes de enzimas: as ciclooxigenases (COX) e a lipoxigenases (LO) (figura 3).

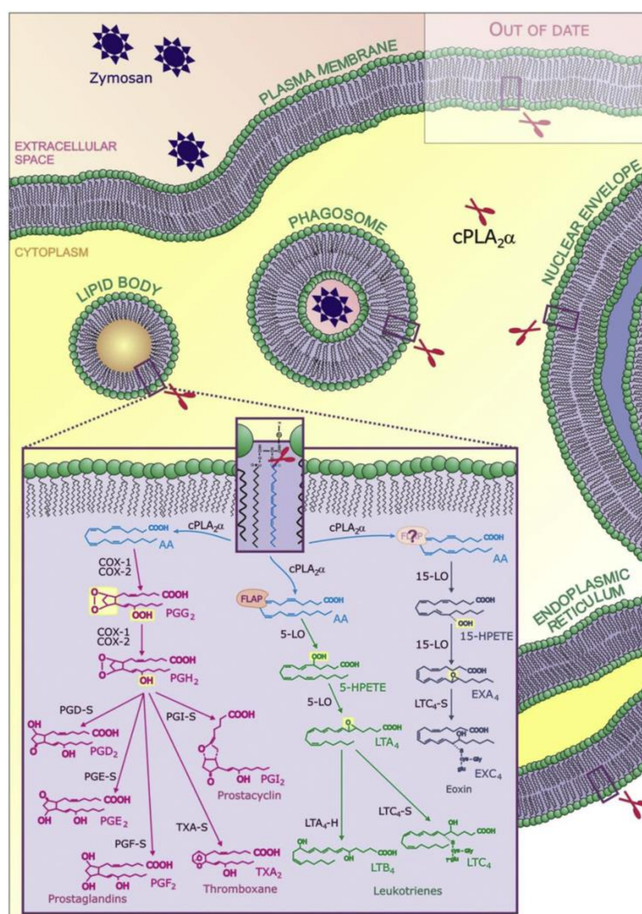

Figura 3. Representação esquemática das vias de produção dos principais eicosanoides (retirado de Bozza et al. 2011).

As COXs são isoenzimas que catalisam, a partir do AA, a formação de prostaglandina H_2, a qual pode ser convertida pela ação de PG sintases célula-específica em diversas moléculas biologicamente ativas, tais como: PGE_2, $PGF_{2\alpha}$, PGI_2, PGD_2 e tromboxano A_2 (TXA_2), coletivamente conhecidos como prostanóides (FUNK, 2001). A COX-1 tem expressão constitutiva, sendo a enzima responsável pela síntese basal de prostanóides, enquanto que a COX-2 é importante em vários processos inflamatórios

devido a sua expressão ser induzível (FUNK, 2001). Existe ainda a COX-3, a qual é um produto do *splicing* alternativo da COX-1 (CHANDRASEKHARAN et al., 2002). No contexto da infecção com microrganismos, a produção de prostaglandina E_2 tem sido associada ao aumento da produção de cAMP e supressão da resposta imune do hospedeiro com a inibição da produção de citocinas pró-inflamatórias, tais como: IFN-γ, TNF-α, IL-12, IL-2 e IL-1β. Em contrapartida, a PGE2 é capaz de induzir a produção de citocinas de perfil Th2, bem como IL-10, IL-4 e imunoglobulinas do tipo IgE e IgG1 (HARRIS et al., 2002).

As lipoxigenases constituem a outra via de metabolismo do AA, dentre as quais a 5- lipoxigenase (5-LO) se destaca pela produção de leucotrienos (LTs) e lipoxinas (LXs). A expressão da 5-LO está correlacionada a eventos de inflamação da fase aguda, com a produção de citocinas pró-inflamatórias e radicais de oxigênio. Entre os produtos da via da 5-LO se destacam o LTB_4 em doenças infecciosas e os chamados cistenil-leucotrienos LTC_4, LTD_4 e LTE_4, envolvidos na resposta alérgica (Peters-Golden et al., 2007). O LTB_4 está correlacionado com o aumento da produção de citocinas pró-inflamatórias e diminuição da infecção em diversas patologias, associado ao aumento da produção de óxido nítrico (PETERS-GOLDEN et al., 2005; ROGERIO; ANIBAL, 2012).

Os eicosanoides se ligam a receptores associados à proteína G (GPCRs). A ação dos eicosanoides na resposta inflamatória está intimamente associada à cascata de transdução do sinal ativada pelos receptores aos quais eles se ligam. Dentre os eicosanoides, a PGE_2 é a molécula que apresenta uma maior variedade de resposta durante a ativação por se ligar a quatro diferentes receptores: EP1, EP2, EP3 e EP4. Os eicosanoides e seus respectivos receptores, bem como o efeito da inter-relação entre estes estão listados na tabela abaixo:

Eicosanoide	Receptor	Ativação		
LTB_4	BLT1 e 2	Gqi	↑ Ca^{2+}	↓cAMP
LTC_4, LTD_4, LTE_4	Cys-LT1 e 2			
$PGF_{2\alpha}$*	FP			
PGD_2	DP1	Gqi / Gs	-	-
	DP2	Gs	-	↑ cAMP
PGE_2	EP1	Gqi	↑ Ca^{2+}	↓cAMP
	EP3	Gi	-	↓cAMP
	EP2/4	Gs	-	↑ cAMP
PGI	IP			
TXA_2	TP	Gq	↑ Ca^{2+}	-

Tabela 1. Eicosanoides e seus respectivos receptores. São mostrados na tabela os desfechos da ativação quanto ao tipo de proteína G ativada, produção de Ca^{2+} e cAMP (BOS et al., 2004; Peters-Golden, 2007; Medeiros et al., 2012; PETERS-GOLDEN; HENDERSON JR.; HENDERSON, 2007). *$PGF_{2\alpha}$ pode se ligar também aos receptores EP1 e EP3 (BOS et al., 2004).

1.5. Corpúsculos lipídicos e a síntese de eicosanoides

Corpúsculos lipídicos (CLs) são organelas citoplasmáticas compostas de um conjunto de lipídios neutros, tais como diacilglicerol, triacilglicerol, caveolina e ésteres de colesterol circundados por uma hemi-membrana composta de fosfolipídios (BOZZA et al., 2011). Os CLs estão envolvidos no estoque e processamento de lipídios e estão presentes em todos os organismos. No entanto, apenas recentemente, os corpúsculos lipídicos foram reconhecidos como organelas (FARESE; WALTHER, 2009), uma vez que participam em diversos processos celulares como sinalização, tráfico de membranas e síntese de mediadores inflamatórios (BOZZA et al., 2011).

Os CLs apresentam uma grande quantidade de AA, o principal substrato utilizado na síntese de eicosanoides. Os CLs também possuem uma grande quantidade de proteínas relacionadas com o processo de sinalização celular e endereçamento de vesículas (WAN et al., 2007). Além disso, os CLs podem apresentar enzimas

diretamente relacionadas à síntese de eicosanoides, as COXs e LOs (BOZZA et al., 2011).

Tem sido demonstrado que os CLs podem ser os principais sítios intracelulares de produção de eicosanoides, uma vez que possuem todo o aparato enzimático e de substrato. O ambiente hidrofóbico dos CLs é ideal para o funcionamento da maquinaria responsável pela síntese de mediadores lipídicos. Foi demonstrado que a formação de CLs, sua constituição lipídica e o seu engajamento na produção de mediadores lipídicos específicos estão diretamente correlacionados ao estímulo inflamatório envolvido (figura 4). Neste sentido, a formação de CLs em leucócitos teria um importante papel durante a resposta inflamatória em diversos processos patogênicos (D’AVILA; MAYA-MONTEIRO; BOZZA, 2008)

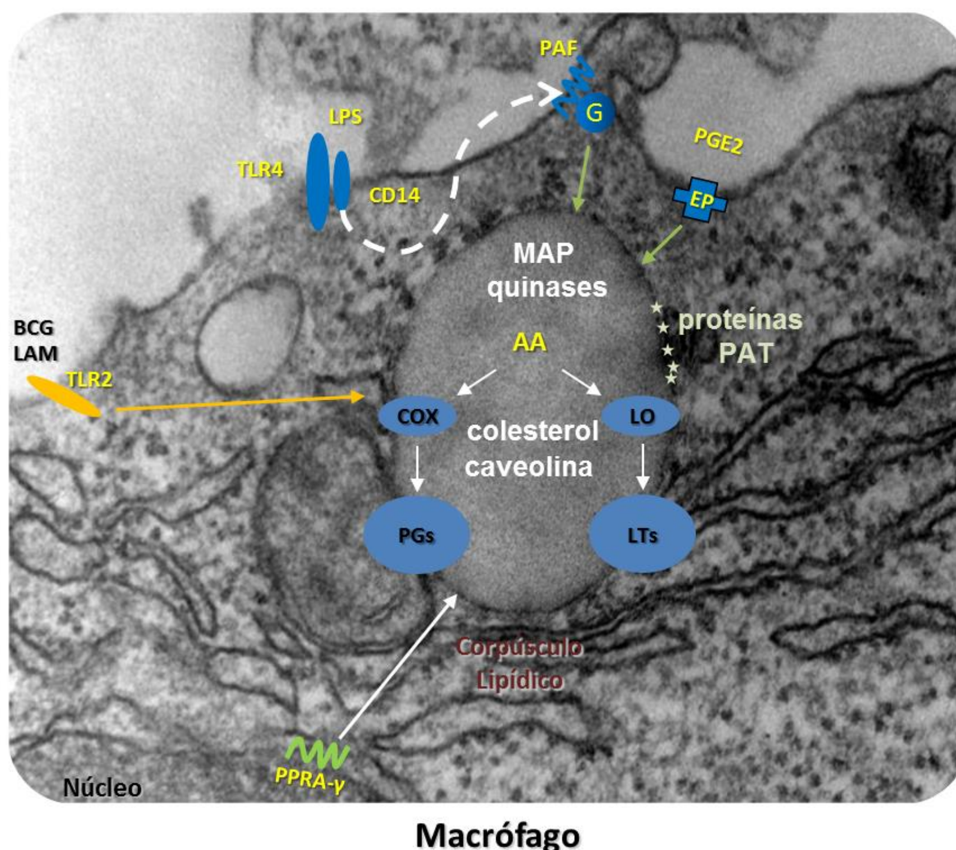

Figura 4. Representação esquemática sobre micrografia eletrônica de um corpúsculo lipídico. Na imagem são ilustrados alguns aspectos moleculares da organela bem como algumas vias de sinalização envolvidas na sua formação.

No contexto da infecção por patógenos, tem sido mostrado que estas organelas participam ativamente da produção de mediadores durante a infecção. Pacheco e cols. (2002) mostraram que LPS é capaz de induzir a formação de CLs de maneira dose e tempo dependente e identificou nestas organelas enzimas das vias de produção de leucotrienos e prostaglandinas, o que esteve associado com a produção destes mediadores *in vivo* (PACHECO et al., 2002). Componentes isolados da membrana de microrganismos tais como de *M. bovis* aumentaram a quantidade de corpúsculos lipídicos em macrófagos, o que esteve associado com um aumento na produção de PGE2 (D'AVILA et al., 2008). Ainda neste contexto, Melo e cols. (2003) mostraram que durante a infecção em ratos por *Trypanosoma cruzi* houve uma intensa formação de CLs em macrófagos peritoneais, o que esteve correlacionada com a produção de PGE2 no sítio inflamatório (MELO et al., 2003; MELO; SABBAN; WELLER, 2006). Durante a infecção por *T. cruzi* a presença no tecido cardíaco de corpúsculos lipídicos em macrófagos infectados é um indício de ativação celular (MELO, 2008).

Diferentes patógenos intracelulares se beneficiam da formação de CLs nas células hospedeiras. A formação dessas organelas e sua associação com os vacúolos parasitóforos foram demonstradas em infecções por *Trypanossoma cruzi* (D'AVILA et al., 2011), *Toxoplasma gondii* (CHARRON; SIBLEY, 2002) e *Plasmodium falciparum* (JACKSON et al., 2004). A distribuição dessas organelas próxima aos fagolisossomos sugere a possibilidade do corpúsculo lipídico servir como fonte de nutriente para o patógeno. Esses achados sugerem então, que a indução da formação de corpúsculos lipídicos por patógenos intracelulares pode ser uma via de inibição da resposta do hospedeiro.

1.6. Corpúsculos e mediadores lipídicos na infecção por *Leishmania*

Os eicosanoides desempenham um papel crucial na infecção por *Leishmania*. A maioria dos estudos que investigaram a participação dos eicosanoides na leishmaniose utilizaram *L. amazonensis* como modelo experimental. Durante a infecção de macrófagos por *L. amazonensis*, PAF (LONARDONI et al., 2000) e LTB4 (SEREZANI et al., 2006) induziram a morte do parasito. Recentemente, o nosso grupo também demonstrou participação de LTB_4 na morte de *L. amazonensis* em neutrófilos pela indução da produção de ROS e ativação da NFκB (Machado et al. 2013, manuscrito em preparação).

A outra via de processamento do AA é a das COXs. Diversos trabalhos têm demonstrado que a ativação de COX beneficia a infecção por *L. amazonensis* pela produção de PGE_2 (AFONSO et al., 2008; LONARDONI et al., 2000; PINHEIRO et al., 2008). A interação entre macrófagos humanos infectados e neutrófilos apoptóticos no modelo experimental humano (AFONSO et al., 2008) e murino (RIBEIRO-GOMES et al., 2005) resultou no sucesso da infecção por *Leishmania* e aumento da carga parasitária por um mecanismo de supressão da resposta imune dependente da produção de PGE_2 e TGF-β.

Um fator crucial para resposta induzida pelos eicosanoides é o receptor envolvido na ativação da célula hospedeira. A PGE_2 pode desempenhar tanto um papel anti-inflamatório como pró-inflamatório a depender dos receptores expressos pela célula alvo (HARRIS et al., 2002). A PGE_2 possui 4 receptores diferentes que são diferencialmente expressos em macrófagos, são eles EP1, 2, 3 e 4 (HARRIS et al., 2002). Os receptores EP1 e EP3 estão associados com a resposta pro-inflamatória com ativação de PKC e diminuição de cAMP, respectivamente. Já os receptores EP2 e EP4

estão associados à resposta anti-inflamatória, pela ativação de proteína G estimulatória com aumento dos níveis de cAMP. Recentemente, foi demonstrado que a infecção por *L. major* induz a expressão de EP1 e EP3 e, que a ativação desses receptores está associada com o aumento da carga parasitária, enquanto que a ativação de EP2 e EP4 induziu a redução da carga parasitária (PENKE et al., 2013).

A indução da produção de PGE_2 também foi demonstrada para espécies que causam leishmaniose visceral, tais como *L. donovani* (REINER; NG; MCMASTER, 1987) e *L. infantum* (MATTE et al., 2001; PANARO et al., 2001). Entretanto, o papel do PGE_2 na infecção por *L. infantum* permanece por ser determinado. Foi demonstrado que macrófagos murinos infectados por *L. donovani* tem o metabolismo de AA direcionado à produção de PGE_2 (REINER; MALEMUD, 1984, 1985; REINER; SCHULTZ; MALEMUD, 1988). Matte e cols. (2001) demonstraram que *L. donovani* é capaz de induzir a expressão de COX-2 e produção de PGE_2, entretanto Panaro e cols. (2001) demonstraram que macrófagos humanos tratados com PGE_2 eliminam melhor os parasitas internalizados. A infecção por *L. donovani* de macrófagos induziu uma maior expressão de COX e PGE sintase quando comparada a infecção por *L. major*, o que sugere haver a indução de respostas distintas a depender da espécie de *Leishmania* (GREGORY et al., 2008).

Apesar de existirem vários trabalhos mostrando a importância dos eicosanoides para infecção por *Leishmania,* os dados sobre a formação de CLs lipídicos em células infectadas são escassos. Pinheiro e cols. (2008) mostraram que a infecção por *L. amazonensis* só foi capaz de induzir a formação de CLs em células de camundongos Balb/c privadas de nutrientes, e esta formação esteve associada com a produção de PGE2. Durante a infecção por *L. major* foi observado a formação de CLs em macrófagos derivados de medula, mas não foi observada uma produção de PGE_2

associada a essa formação (RABHI et al., 2012). Desta forma, o papel dos CLs na infecção por *Leishmania*, bem como por *L. i. chagasi* permanece por ser estudado.

1.7. Eicosanoides e Corpúsculos lipídicos de *Leishmania*

O estudo de CLs em diversos parasitas tem sido direcionado à participação destas organelas no estoque e metabolismo de lipídios. Em *Toxoplasma gondi* estas inclusões têm sido implicadas no armazenamento de lipídios “seqüestrados” da célula hospedeira, embora o mecanismo pelo qual o parasito obtém os lipídeos intracelularmente aindam não sejam bem compreendidos (NISHIKAWA et al., 2005; QUITTNAT et al., 2004).

CLs também foram caracterizadas ultraestruturalmente em *Leishmania donovani* (CHANG, 1956). Pimenta e cols. (1991) correlacionaram o aumento do número de inclusões lipídicas em promastigotas *Leishmania* com o processo de metaciclogênese, produção e endereçamento de LPG à membrana plasmática do parasita (PIMENTA; SARAIVA; SACKS, 1991). O aumento dos CLs em *Leishmania* esteve correlacionado com o tratamento com drogas leishmanicidas que afetavam a via de síntese de ergosterol, importante componente estrutural da membrana plasmática dos parasitas (VANNIER-SANTOS et al., 1995).

Apesar da semelhança morfológica entre os CLs dos leucócitos e os de células de outros organismos, a função de CLs de parasitas e a produção de eicosanoides por estes CLs ainda não foi demonstrada. Genes homólogos a COX e proteínas análogas não existem em organismos da Ordem *Trypasomatidae*, contudo parasitas tais como *Leishmania* são capazes de metabolizar ácido araquidônico a PGs (KUBATA et al., 2007). A produção de PGs por *Leishmania* é possível, por que estes parasitas possuem uma enzima chamada prostaglandina $F_{2\alpha}$ sintase (PGFS), a qual é responsável pela

produção de PGF2α (KABUTUTU et al., 2003). Os sítios de produção intracelular bem como a participação dos CLs na síntese de $PGF_{2\alpha}$ eram desconhecidos até o presente estudo. Além disso, não existe dado na literatura sobre a participação da $PGF_{2\alpha}$ na resposta imune, o que torna este campo atraente para investigação científica.

2. JUSTIFICATIVA

A saliva total e as frações proteicas de *L. longipalpis* têm sido cogitadas como antígenos vacinais devido à importância deste componente na transmissão por *Leishmania*. Apesar de existirem trabalhos na literatura sobre a importância de eicosanoides para a infecção por *Leishmania*, não existiam dados sobre o papel dos eicosanoides nos estágios iniciais da doença até o presente estudo. Este trabalho contribuiu neste sentido, mostrando que a saliva de *Lutzomyia longipalpis* é capaz de beneficiar a infecção por *L. i. chagasi* por modular a produção de eicosanoides. Além disso, a capacidade de produção de eicosanoides pelos parasitas e essa característica como um fator de virulência é negligenciada pela literatura. O estudo sobre os mecanismos de produção de eicosanoides por *L. i. chagasi* traz novas perspectivas para o entendimento da biologia celular da *Leishmania* e suas implicações com a célula hospedeira.

3. OBJETIVOS

3.1. Geral

Investigar o papel dos corpúsculos lipídicos e eicosanoides produzidos durante os momentos iniciais da infecção por *Leishmania infantum chagasi*

3.2. Específicos

- Avaliar o efeito da saliva de *L. longipalpis* na ativação celular quanto à formação de corpúsculos lipídicos e produção de eicosanoides *in vivo* e *in vitro*;
- Investigar vias de sinalização celular envolvidas no processo de ativação da produção de eicosanoides induzidos pela saliva de *L. longipalpis in vitro*;
- Avaliar o efeito da saliva de *L. longipalpis* na produção de eicosanoides durante a infecção por *L. i. chagasi in vivo* e *ex vivo;*
- Investigar o envolvimento dos corpúsculos lipídicos na capacidade de produção de eicosanoides por *L. i. chagasi;*
- Avaliar a contribuição de eicosanoides produzidos pela *L. i. chagasi* como fator de virulência e na infecção *in vitro*.

4. MANUSCRITOS

4.1. MANUSCRITO I

***Lutzomyia longipalpis* Saliva Triggers Lipid Body Formation and Prostaglandin E_2 Production in Murine Macrophages**

A Saliva de *Lutzomyia longipalpis* Induz a Formação de Corpúsculos Lipídicos e a Produção de Prostaglandina E_2 em Macrófagos Murinos

Este trabalho avalia o efeito da saliva de *L. longipalpis* na ativação celular de macrófagos quanto à formação de corpúsculos lipídicos e a produção de eicosanoides associada a essas organelas, bem como vias de sinalização envolvidas neste processo.

Resumo dos resultados: Neste estudo vimos que o sonicado de glândula salivar (SGS) de *L. longipalpis* induziu o recrutamento de neutrófilos e macrófagos para a cavidade peritoneal com cinética distinta para ambos os tipos celulares. A saliva do flebotomíneo induziu a produção de PGE_2 e LTB_4 em leucócitos após a estimulação com ionóforo de cálcio *ex vivo*. Após três e 6 horas de inoculada, a saliva induziu o aumento de CLs em macrófagos, mas não em neutrófilos quando comparados ao grupo controle que recebeu solução salina. Além disso, macrófagos peritoneais residentes quando estimulados com SGS *in vitro* tiveram um aumento no número de CLs de maneira dose e tempo dependente, o qual esteve correlacionado com o aumento de PGE_2 nos sobrenadante de cultura. As enzimas COX-2 e PGE-sintase foram co-localizadas nos CLs induzidos pela saliva e a produção de PGE_2 foi reduzida pelo tratamento com NS-398, um inibidor de COX-2. Por fim, nós verificamos que o SGS rapidamente estimulou a fosforilação de

ERK-1/2 e PKC-α e a inibição farmacológica dessas vias inibiu a produção de PGE_2 induzida pela saliva.

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Lutzomyia longipalpis Saliva Triggers Lipid Body Formation and Prostaglandin E_2 Production in Murine Macrophages

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Abstract

Background: Sand fly saliva contains molecules that modify the host's hemostasis and immune responses. Nevertheless, the role played by this saliva in the induction of key elements of inflammatory responses, such as lipid bodies (LB, also known as lipid droplets) and eicosanoids, has been poorly investigated. LBs are cytoplasmic organelles involved in arachidonic acid metabolism that form eicosanoids in response to inflammatory stimuli. In this study, we assessed the role of salivary gland sonicate (SGS) from *Lutzomyia* (*L.*) *longipalpis*, a *Leishmania infantum chagasi* vector, in the induction of LBs and eicosanoid production by macrophages *in vitro* and *ex vivo*.

Methodology/Principal Findings: Different doses of *L. longipalpis* SGS were injected into peritoneal cavities of C57BL/6 mice. SGS induced increased macrophage and neutrophil recruitment into the peritoneal cavity at different time points. Sand fly saliva enhanced PGE_2 and LTB_4 production by harvested peritoneal leukocytes after *ex vivo* stimulation with a calcium ionophore. At three and six hours post-injection, *L. longipalpis* SGS induced more intense LB staining in macrophages, but not in neutrophils, compared with mice injected with saline. Moreover, macrophages harvested by peritoneal lavage and stimulated with SGS *in vitro* presented a dose- and time-dependent increase in LB numbers, which was correlated with increased PGE_2 production. Furthermore, COX-2 and PGE-synthase co-localized within the LBs induced by *L. longipalpis* saliva. PGE_2 production by macrophages induced by SGS was abrogated by treatment with NS-398, a COX-2 inhibitor. Strikingly, SGS triggered ERK-1/2 and PKC-α phosphorylation, and blockage of the ERK-1/2 and PKC-α pathways inhibited the SGS effect on PGE_2 production by macrophages.

Conclusion: In sum, our results show that *L. longipalpis* saliva induces lipid body formation and PGE_2 production by macrophages *ex vivo* and *in vitro* via the ERK-1/2 and PKC-α signaling pathways. This study provides new insights regarding the pharmacological mechanisms whereby *L. longipalpis* saliva influences the early steps of the host's inflammatory response.

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Introduction

To obtain a blood meal, sand flies locate blood by introducing their mouthparts into the vertebrate host's skin, tearing tissues, lacerating capillaries and creating hemorrhagic pools upon which they feed. During this process, sand flies need to circumvent a number of the host's homeostatic responses, such as activation of blood coagulation cascades, vasoconstriction, platelet aggregation and immune responses [1,2]. In this environment, sand flies evolved an array of potent pharmacologic components with redundant and synergistic activities that subvert the host's physiological responses and favor the blood meal. Intense research using high-throughput analyses has been conducted to identify salivary factors and their biological activities. *Lutzomyia* (*L.*) *longipalpis*, the main vector of visceral leishmaniasis in South America, has been extensively studied. During the inflammatory response, *L. longipalpis* saliva induces cellular recruitment, modulates both antibody production and the formation of immunocomplexes [3,4], regulates T cell activities and inhibits dendritic cells and macrophages, the latter being preferential host cells for

Author Summary

After the injection of saliva into the host's skin by sand flies, a transient erythematous reaction is observed, which is related to an influx of inflammatory cells and the release of various molecules that actively facilitate the blood meal. It is important to understand the specific mechanisms by which sand fly saliva manipulates the host's inflammatory responses. Herein, we report that saliva from *Lutzomyia (L.) longipalpis*, a widespread *Leishmania* vector, induces early production of eicosanoids. Intense formation of intracellular organelles called lipid bodies (LBs) was noted within those cells that migrated to the site of saliva injection. *In vitro* and *ex vivo*, sand fly saliva was able to induce LB formation and PGE_2 release by macrophages. Interestingly, PGE_2 production induced by *L. longipalpis* saliva was dependent on intracellular mechanisms involving phosphorylation of signaling proteins such as PKC-α and ERK-1/2 and subsequent activation of cyclooxygenase-2. Thus, this study provides new insights into the pharmacological properties of sand fly saliva and opens new opportunities for intervening with the induction of the host's inflammatory pathways by *L. longipalpis* bites.

Leishmania [5,6]. There is also evidence that maxadilan, a *L. longipalpis* salivary protein with vasodilator properties, down-regulates LPS-induced TNF-α and NO release through a mechanism dependent on PGE_2 and IL-10 [7].

PGE_2 is an eicosanoid derived from arachidonic acid (AA) metabolism by the enzyme cyclooxygenase (COX). Prostanoids and leukotrienes can be intensely produced by macrophages during inflammatory responses [8], and these mediators are implicated in cellular recruitment and activation. Among the eicosanoids, LTB_4 induces neutrophil recruitment [9], whereas PGE_2 and PGD_2 attract mainly macrophages [10]. Previous studies used different experimental models to show that *L. longipalpis* saliva induces an influx of neutrophils [11] and macrophages [12], but neither the role of saliva in LTB_4 and PGE_2 release nor the involvement of these mediators in this process has been fully addressed.

Under inflammatory and infectious conditions, prostaglandins and others lipid mediators are mainly produced by cytoplasmic organelles called lipid bodies (LB) [13]. Intense research over the past few years has defined lipid bodies as dynamic cytoplasmic organelles. It has been demonstrated that lipid bodies compartmentalize enzymes involved in the biosynthesis, transport and catabolism of lipids, proteins involved in membrane and vesicular transport and proteins involved in cell signaling and inflammatory mediator production, including eicosanoid-forming enzymes, phospholipases and protein kinases. All of these molecules can be localized into lipid bodies in various cells under a range of activation conditions, suggesting a wide role for lipid bodies in the regulation of cellular lipid metabolism and signaling [13].

Herein, we evaluated the effect of *L. longipalpis* salivary gland sonicate (SGS) on the induction of LB formation as well as PGE_2 and LTB_4 production *in vitro* and *ex vivo*. Moreover, we explored the role of peritoneal macrophages in the production of these lipid mediators in response to *L. longipalpis* SGS *in vitro*. Finally, we found that the PGE_2 production induced by *L. longipalpis* saliva is dependent on intracellular mechanisms involving the phosphorylation of signaling proteins such as PKC-α and ERK-1/2 and subsequent activation of COX-2.

Methods

Antibodies and Reagents

Dimethylsulfoxide (DMSO) was purchased from ACROS Organics (New Jersey, NJ). RPMI 1640 medium and L-glutamine, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). Nutridoma-SP was from Roche (Indianapolis, IN). A23187 calcium ionophore, was from Calbiochem/Novabiochem Corp. (La Jolla, CA). NS-398, PGE_2 and LTB_4 enzyme-linked immunoassay (EIA) Kits, anti-murine COX-2 and PGE-synthase antibodies were all from Cayman Chemical (Ann Arbor, MI). 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503) was obtained from Molecular Probes (Eugene, OR). Osmium tetroxide (OsO4) was obtained from Electron Microscopy Science (Fort Washington, PA). Aqua Polymount was from Polysciences (Warrington, PA). Thiocarbohydrazide, Ca^{2+}-Mg^{2+}-free HBSS($^{-/-}$), HBSS($^{+/+}$) with Ca^{2+}-Mg^{2+}, LPS from *Escherichia coli* (serotype 0127:b8), and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-mouse kinase proteins were from Santa Cruz Biotechnology (Santa Cruz, CA). PD 98059, 2′-Amino-3′-methoxyflavone and Bisindolylmaleimide-I, 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide were obtained from Merck-Calbiochem (Darmstadt, Hessen).

Mice

Inbred male C57BL/6 mice, age 6–8 weeks, were obtained from the animal facility of Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (CPqGM-FIOCRUZ, Bahia, Brazil). All experimental procedures were approved and conducted according to the Animal Care and Using Committee of the FIOCRUZ.

Sand flies and preparation of salivary glands

Adult *Lutzomyia longipalpis* captured in Cavunge (Bahia, Brazil) were reared at the Laboratório de Imunoparasitologia/CPqGM/FIOCRUZ (Bahia, Brazil) as described previously [3]. Salivary glands were dissected from 5- to 7-day-old *L. longipalpis* females under a Stemi 2000 Carl Zeiss stereoscopic microscope (Göttingen, Germany) and stored in groups of ten pairs in 10 μL of endotoxin-free PBS at −70°C. Immediately before use, the glands were sonicated with a Branson Sonifier 450 (Danbury, CT) and centrifuged at 10,000× g for four minutes. The supernatant from salivary gland sonicate (SGS) was used for experiments. The level of LPS contamination of *L. longipalpis* SGS preparations was determined using a commercially available LAL Chromogenic Kit (Lonza Bioscience, Walkersville, MD); negligible levels of endotoxin were found in the salivary gland supernatant (0.1 ηg/mL). We measured 0.7 micrograms of protein in an amount equivalent to 0.5 pair of salivary glands and used SGS dilutions (2.0–0.2 pairs) in our experiments [14].

Leukocyte recruitment to the peritoneal cavity

To assess the leukocyte recruitment induced by *L. longipalpis* SGS, we used the well-established peritoneal model of inflammation because the peritoneal cavity is a self-contained and delineated compartment and thus provides a large number of post-stimulus leukocytes. As previously established in the air pouch murine model [12] and peritoneal cavity (unpublished data), a 0.5-pair dose of SGS was used for the leukocyte recruitment assay. C57BL/6 mice were inoculated i.p. with 0.1 mL of *L. longipalpis* SGS (0.5 pair/cavity), endotoxin-free saline (negative control) or 0.1 mL of LPS (20 μg/mL, positive control). At 1, 3 and 6 h post-stimulus, leukocytes inside the peritoneal cavity were harvested by

injection and recovery of 10 mL of endotoxin-free saline. Total counts were performed on a Neubauer hemocytometer after staining with Turk's solution. Differential cell counts (200 cells total) were carried out microscopically on cytospin preparations stained with Diff-Quick.

Lipid body staining and quantification

Cells harvested by peritoneal lavage 1, 3, 6 or 24 h after i.p. injection of 0.1 mL of *L. longipalpis* SGS (0.5 pair/cavity), endotoxin-free saline or LPS (20 µg/mL) were centrifuged at 400× *g* and the lipid bodies within the leukocytes were stained with BODIPY 493/503 (5 ug/mL) according to Plotkowisk *et al.* [15]. Samples were analyzed using a FACSort flow cytometer from Becton Dickinson Immunocytometry Systems (San Jose, CA) and by fluorescence microscopy.

Macrophages adhered to coverslips within 24-well plates were fixed with 3.7% formaldehyde and stained with osmium tetroxide as described previously [16]. The morphology of the fixed cells was observed, and lipid bodies were counted by light microscopy with a 100x objective lens in 50 consecutively scanned macrophages.

Resident peritoneal macrophage harvesting and treatments

For *in vitro* assays, macrophages were obtained by peritoneal lavage with cold RPMI 1640. Then, cells were centrifuged at 400× g for 10 minutes. Macrophages (3×10^5/well) were cultured in 1 mL of RPMI 1640 medium supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in 24-well plates for 24 hours. Next, the macrophages were stimulated with different doses of *L. longipalpis* SGS (0.2, 0.5, 1.0, 1.5, 2.0 pairs/well). In some experiments, LPS (500 ng/well) was used as a positive control. One, 6, 24, 48 and 72 hours after stimuli, supernatants were collected and cells were fixed with 3.7% formaldehyde. For inhibitory assays, macrophages were pretreated for one hour with 1 µM NS-398, a COX-2 inhibitor; 20 ηM BIS, a PKC inhibitor; or 50 µM PD98059, an ERK-1/2 inhibitor. Then, the cells were stimulated with SGS (1.5 pairs/well) or medium containing vehicle (DMSO) for 24 hours, and the supernatants were collected for eicosanoid measurement. Cell viability as assessed by trypan blue exclusion was always greater than 95% after the end of treatment.

Immunofluorescence for COX-2 and PGE-synthase

Resident peritoneal macrophages were cultured on coverslips in the presence of *L. longipalpis* SGS (1.5 pair/well) as described above. After 24 h, the cells were washed twice with 500 µl of $HBSS^{-/-}$ and immediately fixed with 500 µL of water-soluble EDAC (1% in $HBSS^{-/-}$), used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins. After 15 min of incubation at room temperature (RT) with EDAC to promote both cell fixation and permeabilization, macrophages were then washed with $HBSS^{-/-}$ and incubated with 1 µM BODIPY 493/503 for 30 min. Then, the cover slips were washed with $HBSS^{-/-}$ and incubated with mouse anti-COX-2 (1:150) or anti-PGE-synthase (1:150) for 1 h at RT. MOPC 21 (IgG1) was used as a control. After further washes, cells were incubated with biotinylated goat anti-rabbit IgG secondary Ab, washed twice and incubated with avidin conjugated with PE for 30 min. The cover slips were then washed three times and mounted in Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA). The samples were observed by fluorescence microscopy and images were acquired using the software Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Western blotting analysis

Macrophages were treated or not with SGS (1.0 pair/well) for 40 min. Next, the cells were washed once with phosphate-buffered saline, homogenized in lysis buffer containing phosphatase inhibitors (10 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 0.5% v/v Nonindet-P40, 10% v/v glycerol, 1 mM DTT, 0.1 mM EDTA, 1 mM sodium orthovanadate, 25 mM NaF and 1 mM PMSF) and a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentrations were determined using the method of Lowry *et al.* [17] with BSA as the standard. Total proteins (20 µg) were then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously [18] and transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TT) plus 5% BSA for 1 h before incubation overnight in the primary rabbit anti-mouse PKC-α and anti-ERK-1/2 (1:1,000) antibodies. After removal of the primary antibody and washing five times in TT, the membranes were incubated in the secondary antibody conjugated to peroxidase (1:10,000) for 1 h.

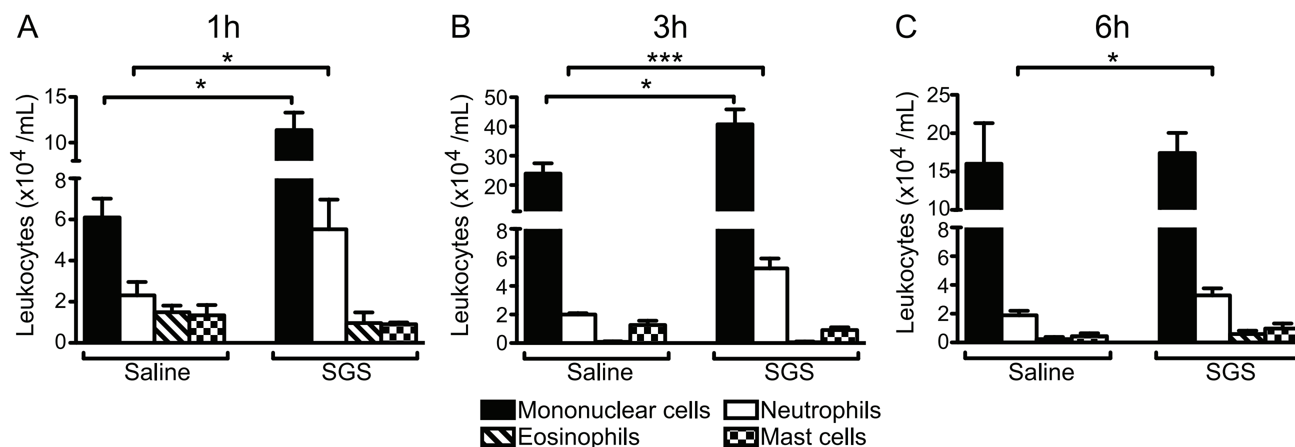

Figure 1. Leukocyte influx into the peritoneal cavity of C57BL/6 mice in response to *L. longipalpis* SGS. Mice were injected i.p. with endotoxin-free saline or SGS (0.5 pair/cavity). One (A), 3 (B) and 6 (C) hours after stimulation, cells were harvested by peritoneal lavage and differential leukocyte counts were performed on Diff-quick stained cytospin preparations. The data are the means and SEM from an experiment representative of three independent experiments. Groups were compared using Student's *t* test at each time point. *, $p<0.05$ and ***, $p<0.001$.
doi:10.1371/journal.pntd.0000873.g001

Washed blots were then incubated with an ECL chemiluminescence kit (Amersham, UK). The membranes were discharged and immunoblotted again using primary rabbit anti-mouse phosphorylated-PKC-α and ERK-1/2 (1:1,000) antibodies according to the manufacturer's instructions (Amersham, UK).

Quantification of the level of proteins in the western blotting membranes was determined by densitometry. Briefly, bands were scanned and processed using Adobe Photoshop 5.0 software (Adobe Systems Inc.), and arbitrary values for protein density were estimated. Ratios between phosphorylated and unphosphorylated proteins were obtained to calculate the difference between groups.

PGE_2 and LTB_4 measurement

C57BL/6 mice were inoculated i.p. with 0.1 mL of *L. longipalpis* SGS (0.5 pair/cavity), endotoxin-free saline or 0.1 mL of LPS (500 ηg/mL). At 1, 3 and 6 h post-stimulus, leukocytes were harvested by peritoneal washing with $HBSS^{-/-}$ and 1×10^6 cells/mL were resuspended in $HBSS^{+/+}$ and stimulated with A23187 (0.5 μM) for 15 min [16]. The reactions were stopped on ice, and the samples were centrifuged at 500× g for 10 min at 4°C. Supernatants from leukocytes re-stimulated *ex vivo* or those of *in vitro* assays were collected for measurement of PGE_2 and LTB_4 by enzyme-linked immunoassay (EIA) according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Statistical analysis

The *in vivo* assays were performed using at least five mice per group. Each experiment was repeated at least three times. Data are reported as the mean and standard error of representative experiments and were analyzed using GraphPad Prism 5.0 software. Disparities in leukocyte recruitment, lipid bodies and lipid mediator quantification were explored using Student's *t* test. Means from different groups from the *in vitro* assays were compared by ANOVA followed by Bonferroni's test or a post-test for linear trends. Differences were considered statistically significant when $p \leq 0.05$.

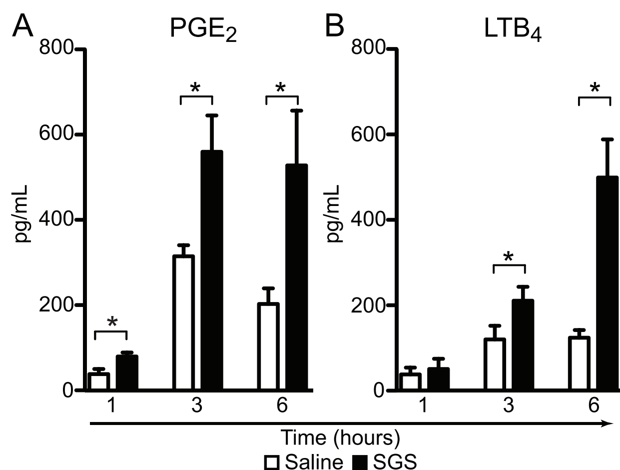

Figure 2. Kinetics of eicosanoid production in response to *L. longipalpis* SGS *ex vivo*. C57BL/6 mice were injected i.p. with saline or SGS (0.5 pair/cavity). One, 3 and 6 hours after stimulation, peritoneal cavities were washed and cells were harvested. The cells were then incubated with A23187 (0.5 μM) for 15 min at 37°C to evaluate LTB_4 and PGE_2 production. The concentrations of PGE_2 (A) and LTB_4 (B) in the supernatant were measured by ELISA. The data are the means and SEM from an experiment representative of three independent experiments. Groups were compared using Student's *t* test at each time point. *, $p<0.05$.
doi:10.1371/journal.pntd.0000873.g002

Results

Lipid bodies and eicosanoids in leukocytes recruited by *L. longipalpis* SGS

To measure the leukocyte recruitment induced by SGS, we injected 100 μL of saline or SGS (0.5 pair/cavity), and 1, 3 and 6 hours after injection, we enumerated total leukocytes recruited to the peritoneal cavity. Most of the cells recruited were mononuclear cells and neutrophils (Figure 1). In this context, SGS induced mononuclear cell recruitment for 3 hours (Figure 1 A and B) and neutrophil recruitment for over 6 hours (Figure 1A–C) of stimulation when compared with the saline group. Other cell populations (eosinophils and mast cells) were not altered after SGS stimulation, and there was no variation in these numbers over time (Figure 1). The peritoneal cell population in unstimulated animals (time zero) was composed of mononuclear cells ($2.985 \times 10^4 \pm 0.027$) and negligible amounts of neutrophils ($0.018 \times 10^4 \pm 0.027$). At this time, macrophages are the major cells within

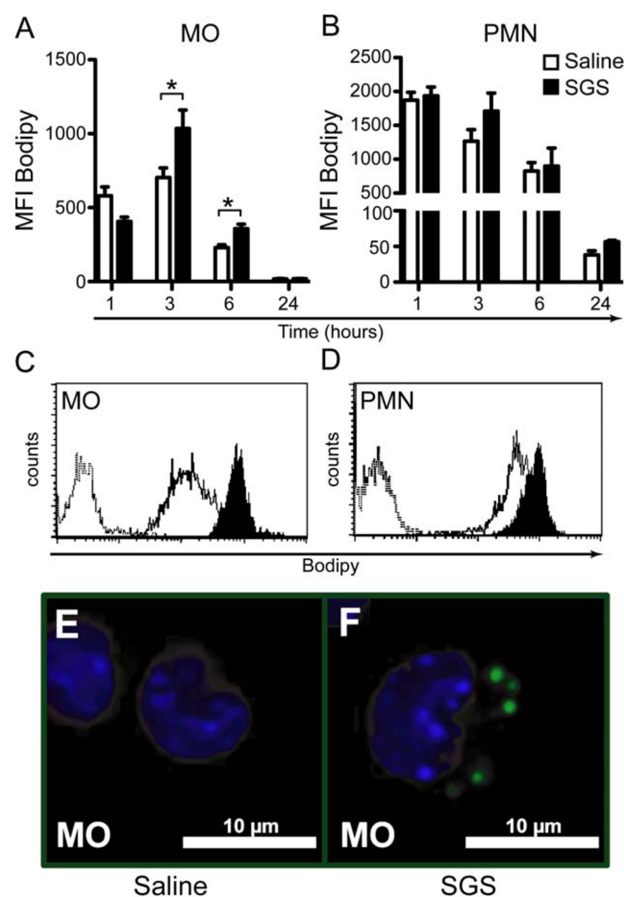

Figure 3. Lipid body formation induced by SGS *in vivo*. C57BL/6 mice were injected i.p. with saline or SGS (0.5 pair/cavity). One, 3, 6 and 24 hours after stimulation, cells were harvested from the peritoneal cavity and stained with the neutral lipid probe BODIPY 493/503. Kinetics of LB formation in mononuclear (A) and polymorphonuclear (B) cells. Mean fluorescence intensity (MFI) histograms of mononuclear (C) and polymorphonuclear (D) cell populations at the 3-hour time point. Dotted lines indicate unstained cells, full lines indicate stained cells from the saline group (empty curves) and from the SGS-treated group (filled curves). LBs in mononuclear cells stimulated with saline (E) or SGS (F) for 3 h detected by fluorescence microscopy, nuclei stained with DAPI. Groups were compared using Student's *t* test at each time point. *, $p<0.05$. MO, mononuclear; PMN, polymorphonuclear.
doi:10.1371/journal.pntd.0000873.g003

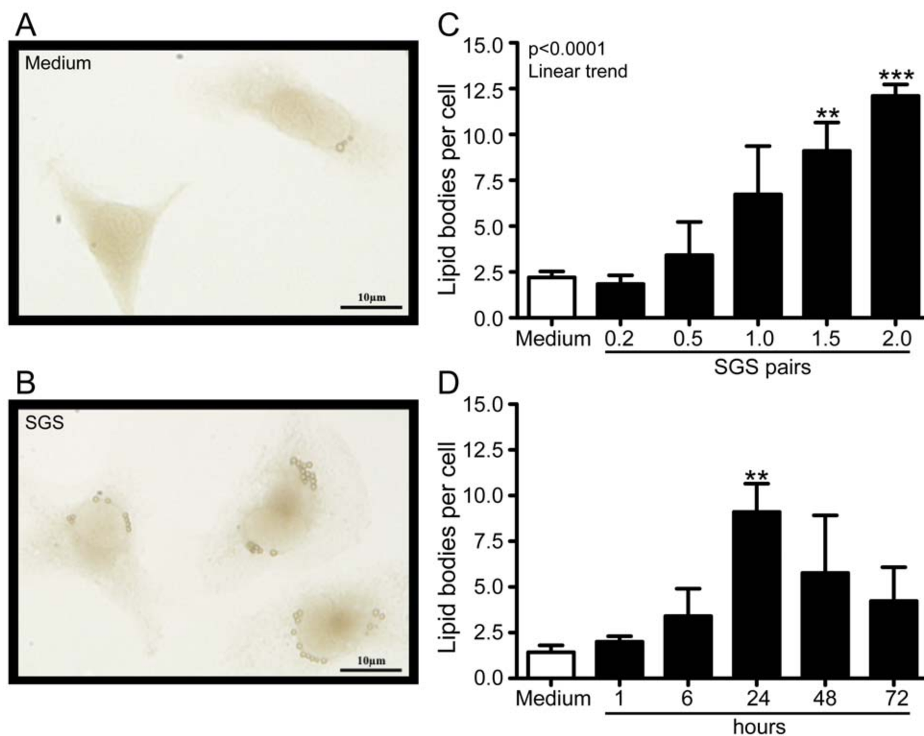

Figure 4. Effect of *L. longipalpis* SGS on lipid body formation in peritoneal macrophages *in vitro*. Representative image of peritoneal macrophages untreated (A) or stimulated with SGS (1.5 pair/well) (B) for 24 hours. Dose-response (C) and kinetics (D) of lipid body formation induced by SGS in peritoneal macrophages. **, $p<0.01$ and ***, $p<0.001$ compared with unstimulated cells.
doi:10.1371/journal.pntd.0000873.g004

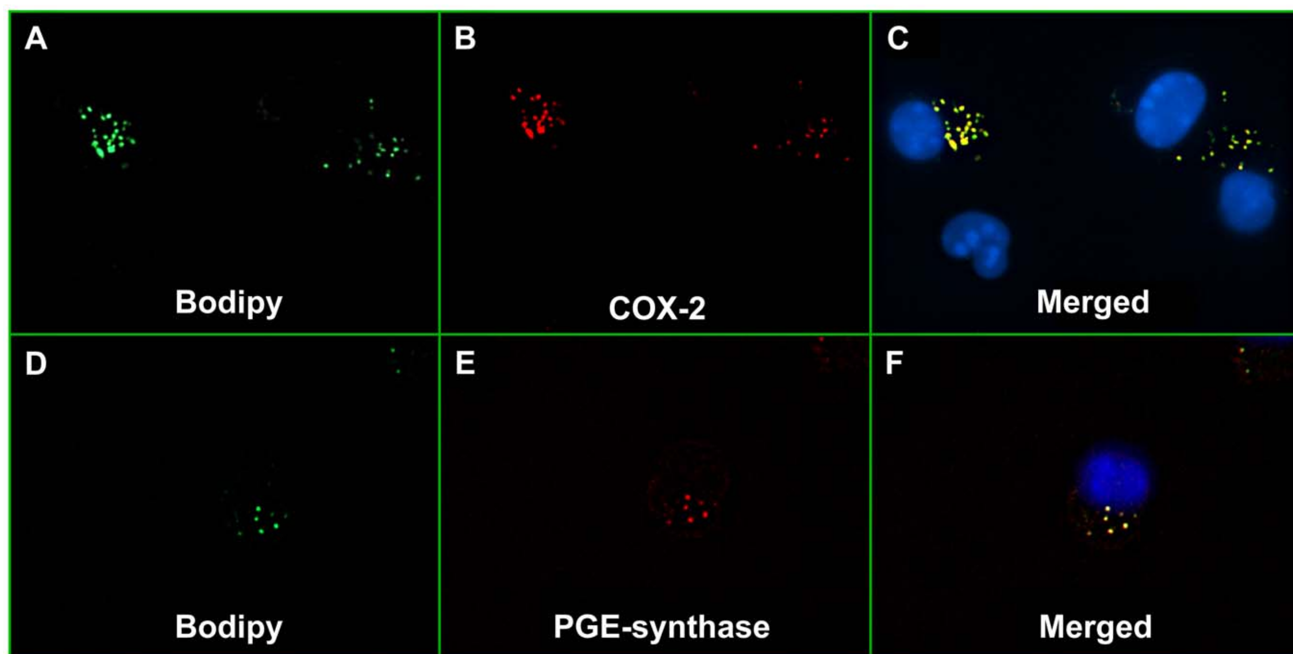

Figure 5. COX-2 and PGE-synthase co-localize within lipid bodies induced by *L. longipalpis* SGS. Peritoneal macrophages were stimulated with SGS (1.5 pair/well) for 24 hours. BODIPY probe-labeled lipid bodies were visualized as green punctuate intra-cytoplasmic inclusions (A and D). COX-2 (B) and PGE-synthase (E) were localized with anti-COX-2 and anti- PGE-synthase antibodies, respectively. Merged images show co-localization of COX-2 (C) and PGE-synthase (F) within lipid bodies.
doi:10.1371/journal.pntd.0000873.g005

the mononuclear population in the peritoneal cavity besides lymphocytes, which represent ~10% of mononuclear cells (data not shown). As shown in Figure 2, SGS administration led to enhanced PGE_2 (Figure 2A) and LTB_4 (Figure 2B) release within those cells recruited to the peritoneal cavity.

Because LBs are sites of eicosanoid production [19], we evaluated LB formation in leukocytes recruited to the peritoneal cavity by FACs using the neutral lipid probe BODIPY 493/503. The kinetics of LB formation was evaluated at 1, 3, 6 and 24 hours after SGS stimulation by measuring mean fluorescence intensity (MFI). SGS increased MFI in mononuclear but not in polymorphonuclear cells after 3 and 6 hours, (Figure 3A and B) compared with the saline group. Histograms (Figure 3C and D) and fluorescence microscopic images (Figures 3E and F) at the 3-hour time point confirmed these effects of SGS on macrophages.

L. longipalpis SGS triggers LB biogenesis in peritoneal macrophages *in vitro*

To assess the role of SGS in lipid body formation in resident macrophages, we stimulated these cells with different doses of SGS (0.2–2.0 pairs/well) for different time periods (1, 6, 24, 48 and 72 hours). At 24 hours post-stimulus, SGS strongly induced LB formation compared with the untreated group (Figure 4A–D). LB formation was induced in a dose-dependent manner, and the maximum of LBs per macrophage was observed at a dose of 2.0 pairs/well (Figure 4C). Because LB formation induced by SGS (1.5 pairs/well) was more evident at 24 hours (Figure 4D), we selected this time point to perform further experiments.

L. longipalpis SGS induces macrophage PGE_2 production via the COX-2 enzyme

Prostaglandins are produced by cyclooxygenases, which occur in constitutive (COX-1) and inducible (COX-2) forms [20]. We investigated the expression and subcellular localization of COX-2 within SGS-stimulated macrophages. Immunofluorescence microscopy revealed the presence of COX-2 (Figure 5A–C) and PGE-synthase (Figure 5D–F) within LBs in macrophages stimulated with SGS.

Next, we measured PGE_2 and LTB_4 production in the supernatant of macrophage cultures. SGS induced PGE_2 production starting at 1.0 pair/well (Figure 6A), whereas LTB_4 was not detectable under any conditions (data not shown). As expected, PGE_2 production by macrophages stimulated with SGS was reduced to basal levels when the cells were pre-incubated with NS-398, a COX-2 inhibitor (Figure 6B). Thus, the PGE_2 production in peritoneal macrophages induced by SGS occurs in newly formed lipid bodies and is dependent on COX-2.

SGS induces PGE_2 production via PKC-α and ERK-1/2

Multiple pathways are involved in the signaling for PGE_2 production [13]. Recently, ERK and PKC-α were shown to be involved in COX-2 activity [21]. We observed that SGS activated both ERK (Figure 7A and C) and PKC-α phosphorylation (Figure 7B and D), but it did not alter the levels of the unphosphorylated proteins. To investigate whether these kinases are involved in the induction of PGE_2 production by SGS, we pretreated macrophages with bisindolylmaleimide I (BIS I) and PD98059, PKC-α and ERK-1/2 inhibitors, respectively (Figure 8A–B). Inhibition of both enzymes completely abrogated PGE_2 production induced by SGS (Figure 8A–B). In sum, these results suggest that PKC-α and ERK-1/2 are involved in the PGE_2 production induced by SGS.

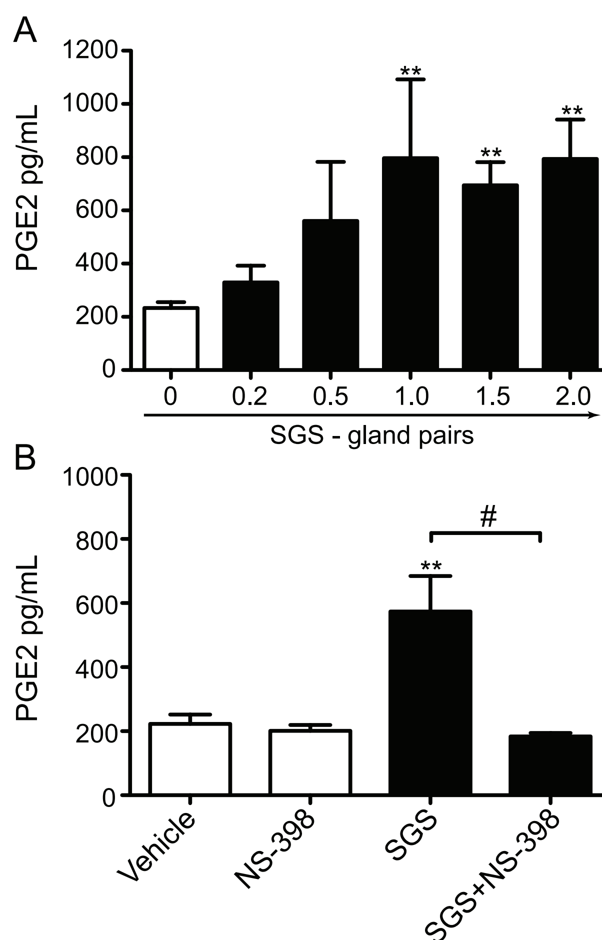

Figure 6. *L. longipalpis* SGS induces PGE_2 production via COX-2. A, Dose-response of PGE_2 production induced by SGS in peritoneal macrophages. B, Macrophages were pre-treated for 1 hour with the COX-2 inhibitor N-398 before incubation with SGS (1.5 pair/well). Twenty-four hours after stimulation, PGE_2 was measured in the supernatant. The data are the means and SEM from a representative experiment of three independent experiments. **, $p<0.01$ and #, $p<0.05$.
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Discussion

Sand fly saliva triggers an inflammatory response characterized by cellular influx followed by hemostatic and immune mechanism suppression. Nevertheless, the role of sand fly saliva in eicosanoid production during the early steps of the innate immune response is poorly understood. In inflammatory conditions, eicosanoids are mostly produced in cytoplasmic organelles called lipid bodies (LBs), which are formed in leukocytes and other cells involved in the inflammatory and infectious responses to several stimuli [13]. Herein, we showed that *L. longipalpis* saliva induces lipid body formation and PGE_2 production in peritoneal macrophages *ex vivo* and *in vitro* via kinase phosphorylation and COX-2 activation.

Previous investigations have demonstrated that sand fly saliva plays an important role in cellular recruitment in multiple experimental models [3,9,11,12], including *in vivo* sand fly bites [22]. Herein, we confirmed previous reports that *L. longipalpis* SGS induces an inflammatory infiltration composed mainly of macrophages and neutrophils. Moreover, we showed that the cellular recruitment induced by *L. longipalpis* saliva is concomitant with PGE_2 and LTB_4 production. In this scenario, lipid mediators

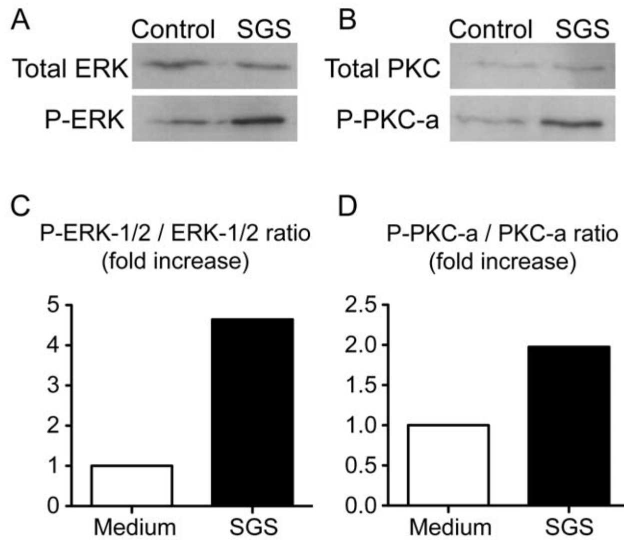

Figure 7. *L. longipalpis* SGS induces PKC-α and ERK phosphorylation. Peritoneal macrophages were incubated in the absence (control) or presence of SGS (1.5 pair/mL) for 40 min. The cells were lysed and immunoblotted using polyclonal anti-ERK-1/2 (A) or anti-PKC-α (B) antibodies. The membranes was discharged and immunoblotted using polyclonal anti- phospho-ERK-1/2 (A) or anti- phosphor-PKC-α (B) antibodies. Quantification of phosphorylated-ERK-1/2 (C) and phosphorylated-PKCα (D) was determined by densitometry. The data show the fold increase in the phosphorylated/unphosphorylated kinase ratio of the SGS group relative to the control group. P-, phosphorylated.
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could be triggering cellular recruitment. Secretion of LTB_4 by resident macrophages plays an important role in neutrophil migration [23]. In addition, lipopolysaccharides induce macrophage migration via prostaglandin D_2 and prostaglandin E_2 [10].

Prostaglandin E_2 is an abundant eicosanoid produced by inflammatory cells, and it is known to exert anti-inflammatory and vasodilator effects. PGE_2 is found in *Ixodes scapularis* saliva and is also implicated in the immunomodulatory activity of tick saliva on dendritic cell and macrophage activation [24]. Furthermore, previous studies using saliva from several *Phlebotomus* species have suggested that the anti-inflammatory properties of sand fly saliva could be attributed to PGE_2 and IL-10 released by dendritic cells [9,25]. In these studies, the cellular recruitment induced by OVA stimulation was abrogated by saliva from various sand fly species [9,25], which was associated with an anti-inflammatory profile dependent on the production of IL-10, IL-4 [25] and PGE_2 [9]. Intriguingly, maxadilan, a vasodilator peptide with immunomodulatory activities present in *L. longipalpis* saliva, is able to induce LPS-activated macrophages to release PGE_2 via COX-1, an enzyme that is constitutively active [7]. In the present study, we showed that *L. longipalpis* SGS triggers PGE_2 production in resident macrophages by an inducible pathway, since this effect was completely abrogated when the cells were incubated in the presence of NS-398, a COX-2 inhibitor. Nevertheless, whether sand fly saliva contains other molecules involved in PGE_2 production or pharmacological amounts of this mediator similarly to tick saliva remains unknown.

Our study is the first to establish a direct link between *L. longipalpis* saliva, eicosanoid production and lipid body formation. Under inflammatory and infectious conditions, lipid mediators are mainly produced within LBs, which compartmentalize both the substrate and the enzymatic machinery required for eicosanoid production [13]. In this regard, the enzymes COX and 5-LO have

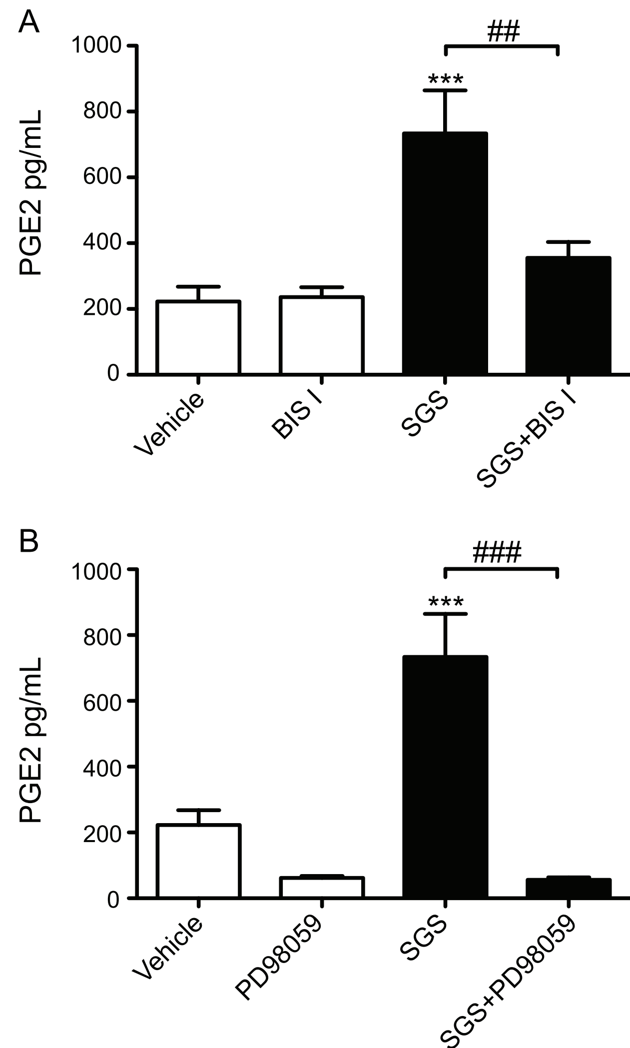

Figure 8. ERK and PKC kinase inhibitors abrogate PGE_2 production induced by *L. longipalpis* SGS. Peritoneal macrophages were pre-treated for 1 hour with BIS I (A) or PD98059 (B) before incubation with SGS (1.5 pair/well). Twenty-four hours after stimulation, PGE_2 was measured in the supernatant. The data are the mean and SEM from an experiment representative of three independent experiments. ***, $p<0.001$; ##, $p<0.01$ and ###, $p<0.001$. PD98059, ERK inhibitor; BIS-I, PKC inhibitor.
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been localized to lipid bodies in various inflammatory cells by the use of multiple techniques including fluorescence microscopy [13]. Previous studies have shown that various inflammatory and infectious stimuli are able to trigger LB formation in macrophages [13,19]. Our findings demonstrate that SGS induces LB formation in macrophages *in vivo* and *in vitro*, suggesting that *L. longipalpis* saliva acts directly on these cells, but not on neutrophils. Indeed, *L. longipalpis* SGS triggered LB formation in macrophages committed to PGE_2 production via COX-2 and PGE-synthase.

Data regarding the direct effects of sand fly salivary compounds on host signaling pathways cells are scarce. The extracellular signal-regulated kinases (ERKs) and protein kinase C (PKC) are among the key enzymes implicated in signaling pathways of diverse cellular responses, including eicosanoid production. The MAP kinases ERK1 and ERK2 induce activation of cPLA2, an enzyme that hydrolyzes arachidonic acid, which is metabolized to

prostaglandin H2 by COX [13]. Previous studies have demonstrated the compartmentalization of MAP kinases and cPLA2 at arachidonate-enriched lipid bodies [26,27], as well as COX-2 and PGE-synthase [16,28,29]. Herein, it is shown for the first time that *L. longipalpis* SGS triggers ERK-1/2 and PKC-α phosphorylation in macrophages. Other studies have shown that COX-2 activation and PGE_2 production in LPS stimulated-macrophages is dependent on the phosphorylation of protein kinases such as PKC-α [21] and ERK-1/2 [30]. We showed that the PGE_2 production induced by SGS is dependent on both ERK-1/2 and PKC. This association between the activation of kinases and the metabolism of eicosanoids within lipid bodies may serve to enhance rapid eicosanoid production in response to extracellular stimuli such as sand fly saliva. Of note, in addition to their role in regulating the host response to infection by modulating inflammatory mediator production, lipid bodies may also serve as rich sources of nutrients for intracellular pathogens, thus favoring intracellular pathogen replication [31,32].

In brief, the present work provides new insights into the mechanisms involved in macrophage responses to *L. longipalpis* saliva, including LB formation and the signaling pathways that trigger PGE_2 release. Although the roles of the newly formed LBs and PGE_2 induced by sand fly saliva in the pathogenesis of leishmaniasis have not yet been addressed, several studies have shown that PGE_2 is essential to the infection of macrophages [33,34] and parasite dissemination after infection [35]. The induction of PGE_2 production by sand fly saliva demonstrated herein can influence the initial steps of host infection by favoring less intense macrophage activation. Our group and others have been providing strong evidence that saliva components are immunogenic and have potential as markers of exposure to sand fly vectors [36–39]. Further studies are required to determinate if the immunization based on components of vector saliva interferes in eicosanoid production with consequences for the host's immune response and the transmissibility of the parasite.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TAS DBP BBA DON JC PFE CIB AB PTB VMB. Performed the experiments: TAS DBP BBA DON JC PFE. Analyzed the data: TAS DBP BBA DON JC PFE CIB PTB VMB. Contributed reagents/materials/analysis tools: ABC MACSN JCM PTB VMB. Wrote the paper: TAS DBP BBA PTB VMB.

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4.2. MANUSCRITO II

New Insights on the Inflammatory Role of *Lutzomyia longipalpis* Saliva in Leishmaniasis

Novas Ideias Sobre ao Papel Inflamatório da Saliva de *Lutzomyia longipalpis* na Leishmaniose

Este trabalho revisa os principais achados do nosso grupo sobre o papel da saliva na resposta inflamatória durante os momentos iniciais da infecção por *Leishmania*. Nesta revisão destacamos o efeito da saliva sobre macrófagos e neutrófilos no que tange a modulação da produção de PGE_2. A seção 4.1 intitulada "Eventos Inflamatórios Disparados pela Saliva de *L. longipalpis*" aborda dados preliminares que serão melhor discutidos no Manuscrito III desta tese. Na seção 5 intitulada "Resposta do Macrófago Hospedeiro à Saliva de *L. longipalpis*" encontramos um breve resumo dos dados apresentados no Manuscrito I desta tese, bem como uma discussão sobre os achados da literatura acerca do efeito da saliva sobre macrófagos. Por fim, na seção 6 intitulada "Neutrófilos e Saliva de *L. longipalpis*: Uma Interação Negligenciada sobre o Cenário da Infecção por *Leishmania*", nós abordamos nossos achados sobre o efeito da saliva na indução da apoptose de neutrófilos murinos e humanos. Os dados desta última seção são apresentados em uma publicação de minha co-autoria intitulada "*Lutzomyia longipalpis* saliva drives apoptosis and enhances parasite burden in neutrophils", a qual pode ser encontrada na seção Apêndice.

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Review Article

New Insights on the Inflammatory Role of *Lutzomyia longipalpis* Saliva in Leishmaniasis

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When an haematophagous sand fly vector insect bites a vertebrate host, it introduces its mouthparts into the skin and lacerates blood vessels, forming a hemorrhagic pool which constitutes an intricate environment of cell interactions. In this scenario, the initial performance of host, parasite, and vector "authors" will heavily influence the course of *Leishmania* infection. Recent advances in vector-parasite-host interaction have elucidated "co-authors" and "new roles" not yet described. We review here the stimulatory role of *Lutzomyia longipalpis* saliva leading to inflammation and try to connect them in an early context of *Leishmania* infection.

1. Introduction

Leishmaniasis remains a serious problem in public health, endemic in 88 countries on four continents, but most of the cases occur in underdeveloped or developing countries [1]. Visceral Leishmaniasis (VL) is a progressive infection with fatal outcome in the absence of treatment. Approximately 90% of the VL cases registered in the Americas occur in Brazil and are concentrated in the Northeast region. In the New World, *Lutzomyia longipalpis* is the principal vector of *Leishmania infantum chagasi*, the agent of American Visceral Leishmaniasis [2].

The causes related to development of distinct clinical manifestations in leishmaniasis are multifactorial and reflect the complexity at the vector-pathogen-host interface [3]. Protozoan parasites of the genus *Leishmania* are the causative agents of the disease and are transmitted to the mammalian hosts by the bite of female phlebotomine sand flies during blood repast. For blood meal obtainment, sand flies introduce their mouthparts into the skin, tearing tissues, lacerating capillaries, and creating haemorrhagic pools upon which they feed [4]. The presence of sand fly saliva in the blood pool, the environment where the parasite encounters host cells, influences the development and functions of several leukocytes. In recent years, the importance of the interaction between components of sand fly saliva and host immune mechanisms in regulating infectivity and disease progression has become clearer and suggests their consequences to disease outcome in leishmaniasis [5].

The aspects involved in immune response resulting in resistance or susceptibility widely depend on the first attempt of host's innate response to contain infection that may influence on the predominance of a pattern of future host's immune adaptive response against *Leishmania*. Many

studies have been performed to understand the mechanisms leading to protection or exacerbation of the disease however; relatively few studies have investigated the role of the sand-fly-derived salivary compounds in the innate immunity. In this paper we integrate the influence of sand fly bite with current ideas regarding the role of early steps of host inflammatory response against *Leishmania*.

2. Sand Fly Saliva: A Rich Field of Study

Sand fly vectors display a rich source of salivary biological active components to acquire blood from vertebrate hosts, a task not easy due the haemostatic, inflammatory and immune responses resultant from the bite [6]. Thus, it is not unexpected that many scientists have progressively investigated several aspects of sand fly saliva, concerning its composition and the range of mammalian response to it.

Among the New World species of sand fly which are vectors of *Leishmania*, *L. longipalpis* and its salivary gland content are the best studied. One of the first components related to *L. longipalpis* salivary gland was maxadilan [7], the most potent vasodilator peptide known and one of the two phlebotomine salivary proteins more extensively studied. Maxadilan is recognized by causing typical erythema during the feeding of *L. longipalpis* [8]. Further, it was described that maxadilan is able to modulate the inflammatory response by inhibiting cytokines such as TNF-α, by inducing IL-6 production, and by stimulating hematopoiesis [9–11]. Charlab et al. (1999) reported nine full clones and two partial cDNA clones from salivary gland from *L. longipalpis* [12]. In that work, they reported for the first time a hyaluronidase activity from sand fly saliva, an activity not yet described on phlebotomine sand flies, helping the diffusion of other pharmacological substances through the skin matrix [13]. It was also described an apyrase activity on *L. longipalpis* saliva which hydrolyses ATP and ADP to AMP, functioning as a potent antiplatelet factor [12, 14]. Interestingly, a 5′-nucleotidase activity is also present in *L. longipalpis* saliva exert vasodilator and antiplatelet aggregation role by converting AMP to adenosine [12]. One of the most abundant protein found in the *L. longipalpis* saliva is the *Yellow*-related protein [12, 13, 15, 16]. Our group has demonstrated that this family of proteins are the most recognized in sera from children living in an endemic area of visceral Leishmaniasis in Brazil [17] and by normal volunteers exposed to laboratory-reared *L. longipalpis* bites [18]. Recently, Xu et al. (2011) described the structure and function of a *yellow* pro-tein LJM 11 [19]. In this report, the authors described that yellow proteins from *L. longipalpis* saliva act as binder of proinflammatory biogenic amines such as serotonin, histamine, and catecholamines [19]. One member of the D7 family of proteins (commonly found in dipterans saliva) is present in *L. longipalpis* [12]. The exact function of this protein in sand fly saliva is still unknown. However, its role on mosquito's saliva suggests that it could act as anticoagulant or binding biogenic amines avoiding host inflammatory events [12, 15].

Herein, we present some of the most studied proteins related to *L. longipalpis* saliva. (See [6, 15, 16, 20] for more details about this topic). Although many of them have been associated with blood-feeding, their biological functions remain undefined. Nevertheless, by modulating the host haemostatic and inflammatory response, this yet unreported sand fly salivary content remains as a research challenge, acting on host immunity to *Leishmania* during transmission and establishment of infection.

3. Immune Response to *Lutzomyia longipalpis* Saliva against *Leishmania*

There are several studies contributing to a better understanding of *L. longipalpis* saliva effects on host immunity to *Leishmania* infection. A brief exposition of these major contributions in the last 10 years is shown in Figure 1.

In mice, salivary products seem to exacerbate the infection with *Leishmania* and may, in fact, be mandatory for establishment of the parasite in vertebrate hosts. It has been shown that components of *L. longipalpis* or *Phlebotomus papatasi* salivary gland lysates mixed with *Leishmania major* resulted in substantially larger lesions compared to controls [21, 22]. Our group have shown that repeated exposure of BALB/c mice to *L. longipalpis* bites leads to local inflammatory cell infiltration comprised of neutrophils, macrophages and eosinophils [23]. Total IgG and IgG1 antibodies react predominantly with three major protein bands (45, 44, and 16 kD) from insect saliva by Western blot [23]. The injection of immune serum previously incubated with salivary gland homogenate induced an early infiltration with neutrophils and macrophages, suggesting the participation of immune complexes in triggering inflammation [23].

We have shown that in endemic areas natural exposures to noninfected sand fly bites can influence the epidemiology of the disease [17, 24]. We observed that people who presented antibodies against saliva of *L. longipalpis* also showed DTH anti-*Leishmania,* suggesting that the immune response against saliva of the vector could contribute to the induction of a protective immune response against the parasite. Recently, in a prospective study this data was reinforced by Aquino et al. (2010) evaluating 1,080 children from 2 endemic areas for VL [25]. There was a simultaneous appearance of antibodies anti-saliva and an anti-*Leishmania* DTH, or a cellular response against the parasite [25], sup-porting the idea that eliciting immunity against saliva could benefit the induction of a protective response against the parasite. The anti-sand fly antibodies can serve as epidemiological marker of vector exposure in endemic areas. In fact, we demonstrated that two salivary proteins, called LJM 17 and LJM 11, were specifically recognized by humans exposed to *L. longipalpis*, but not *Lutzomyia intermedia* [26]. We also evaluated the specificity of anti-*L. longipalpis* in a panel of 1,077 serum samples and verified that LJM 17 and LJM 11 together in an ELISA assay identified the effectiveness of these proteins for the prediction of positivity against salivary gland sonicate (SGS) [27]. In experimental model using C57BL/6 mice, immunization with LJM 11 triggered DTH response and decrease the diseased burden after *L. major* infection [19].

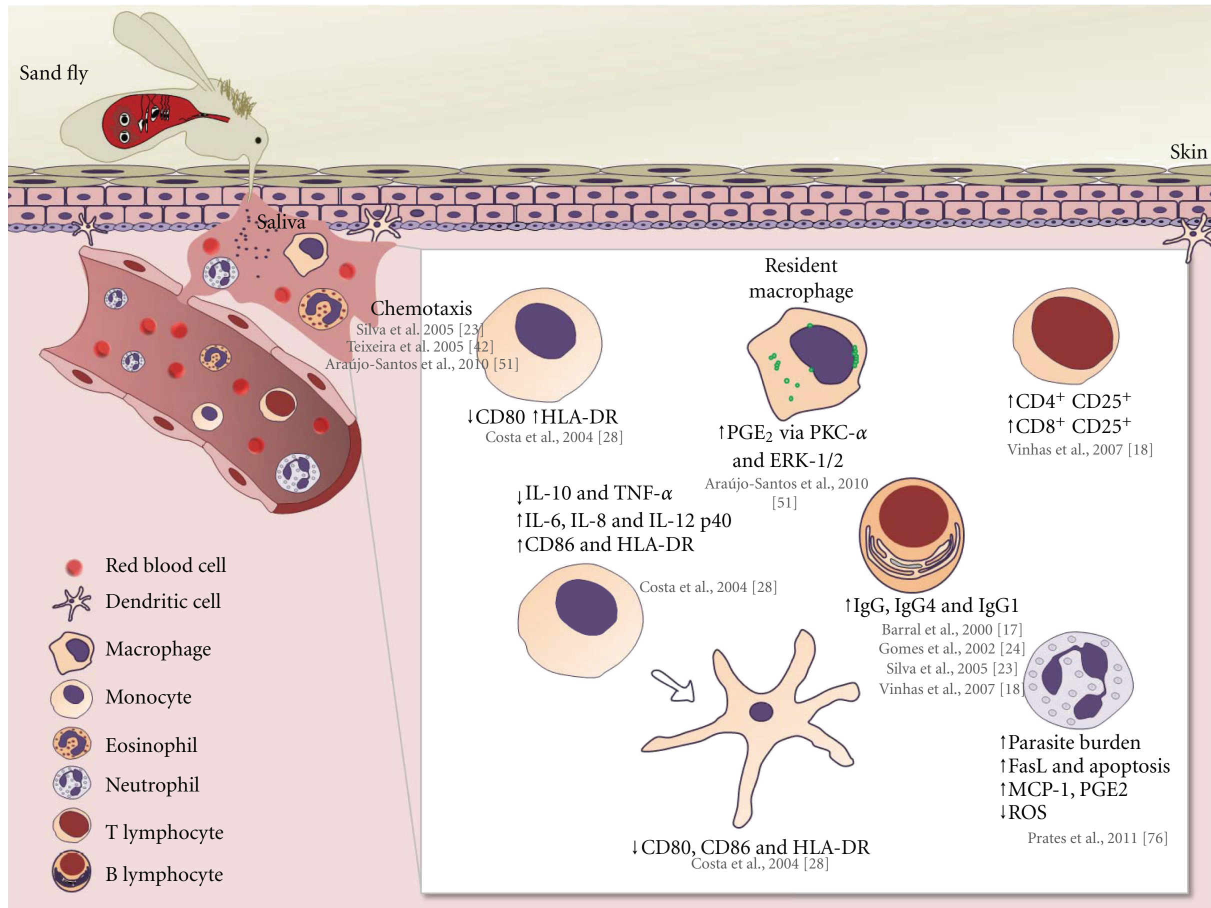

FIGURE 1: Roles of *Lutzomyia longipalpis* saliva in host immune response cell. After *L. longipalpis* saliva injection a set of events can be triggered in the host immune response. Herein, we summarized the roles of saliva on major cell populations involved in the host immune response against *Leishmania* infection.

We also characterized the immunological patterns following sand fly saliva exposure, using healthy volunteers exposed to laboratory-reared *L. longipalpis* [18]. We noticed high levels of IgG1, IgG4, and IgE antibodies anti-saliva. Furthermore, following *in vitro* stimulation with salivary gland sonicate, there was an increased frequency of CD4(+)CD25(+) and CD8(+)CD25(+) T cells as well as IFN-γ and IL-10 synthesis. Strikingly, 1 year after the first exposure, PBMC from the volunteers displayed recall IFN-γ responses that correlated with a significant reduction in infection rates using a macrophage-lymphocyte autologous culture. Together, these data suggest that human immunization against sand fly saliva is feasible and recall responses are obtained even 1 year after exposure, opening perspectives for vaccination in man [18].

Sand fly saliva also seems to exert a direct effect on human antigen presenting cells. *L. longipalpis* SGS inhibited IL-10 and TNF-α production but induced IL-6, IL-8, and IL-12p40 production by LPS-stimulated monocytes and dendritic cells [28]. Besides cytokine production, sand fly saliva also interfered with the expression of costimulatory molecules in macrophages (reduced CD80 and increased HLA-DR expression) and in monocytes (increased CD80 and HLA-DR expression). During dendritic cell differentiation induced by CD40L, a slight reduction in CD80, CD86, HLA-DR, and CD1a expression were also observed [28].

Whereas enhancement of *Leishmania* transmission by saliva is probably due to immunomodulatory components of sand fly saliva, an explanation of the anti-*Leishmania* effect resulting from host immunization against salivary antigen is not straightforward. Immunity in this system could derive from neutralization of salivary immunomodulators such as the peptide maxadilan from *L. longipalpis* (as reviewed in [22]). Alternatively, immunity could derive from a DTH reaction at the site of the bite generated by a cellular response to salivary antigens injected by the fly [29, 30]. This particular reaction could turn the lesion and its surroundings into an inhospitable site for the establishment of *Leishmania* infection in the new host, or it could modify the environment priming the initial events of the host immune reaction to *Leishmania*.

The disease exacerbative properties of saliva, often resulting from the bioactive property of one or more of its molecules, should not be confounded with antigenic molecules in saliva that induce an adaptive immune response in the host. This acquired immunity can be either protective

or exacerbative depending on the nature and dominance of the salivary components of a vector species. Exposure to uninfected bites of the sand fly *P. papatasi* induces a strong delayed-type hypersensitivity response and IFN-γ production at the bite site that confers protection in mice challenged by *L. major*-infected flies [29]. By contrast, acquired immunity to *L. intermedia* saliva results in disease exacerbation not protection [31]. Moreover, *P. papatasi* saliva, despite its overall protective property, contains molecules that alone induce a protective (PpSP15) or exacerbative (PpSP44) im-mune response in the host [32, 33]. It is likely that *L. intermedia* saliva also contains molecules with similar profiles despite the overall exacerbative effect of total saliva.

Recently, we developed a model for visceral Leishmaniasis (VL) in hamsters, using an intradermal inoculation in the ears of 100,000 *L. chagasi* parasites together with *L. longipalpis* saliva to mimic natural transmission by sand flies [34]. Hamsters developed classical signs of VL rapidly, culminating in a fatal outcome 5-6 months postinfection. Immunization with 16 DNA plasmids coding for salivary proteins of *L. longipalpis* resulted in the identification of LJM19, a novel 11-kDa protein that protected hamsters against the fatal outcome of VL. LJM19-immunized hamsters maintained a low parasite load that correlated with an overall high IFN-γ/TGF-β ratio and inducible NOS expression in the spleen and liver up to 5 months post-infection. Importantly, a delayed-type hypersensitivity response with high expression of IFN-γ was also noted in the skin of LJM19-immunized hamsters 48 h after exposure to uninfected sand fly bites. Induction of IFN-γ at the site of bite could partly explain the protection observed in the viscera of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-*Leishmania* immunity. Recently, Tavares et al. [35] showed that LJM19 was also able to protect hamsters against an infection composed by *Leishmania braziliensis* plus saliva of *L. intermedia*, the vector responsible for the transmission of this parasite in Brazil [35]. The immunization also induced a higher ratio of IFN-γ/TGF-β production in the cells from lymph nodes draining the infection site. Collin et al., (2009) immunized dogs using intradermal injections of DNA codifying salivary proteins of *L. longipalpis* (LJM17 and LJL 143), followed by injection of recombinant *Canarypox virus* containing the same genes [36]. They also observed a potential protective response against *Leishmania*, showing high concentrations of IFN-γ in PBMC stimulated with recombinant salivary proteins. Importantly, the bite of uninfected sand flies resulted in a strong DTH characterized by high amount of IFN-γ and low levels of TGF-β [36]. Together, these results point out the possibility to immunize against leishmaniasis using defined proteins of vector's saliva against *Leishmania*.

4. Early Steps of Host-Vector-*Leishmania* Interplay: Cell Recruitment Induced by Saliva

It is well established that the first steps in leishmaniasis are critical in determining the development of the disease. In order to understand this critical moment, several reports have investigated the early recruitment of cells induced by both *L. longipalpis* saliva alone or coinoculated with *L. chagasi*. Sand fly saliva is able to induce an inflammatory process in the host by recruiting different cells into the bite site. In fact, it was verified that *L. longipalpis* salivary gland lysate markedly modifies the inflammatory response to infection with *L. braziliensis* in BALB/c mice [37]. The saliva-associated lesions progressed to extensive accumulations of heavily parasitized epithelioid macrophages, with persistent neutrophilia and eosinophilia [37]. Eosinophilia has also been described in dogs intradermally inoculated with *L. longipalpis* saliva associated with *L. chagasi* promastigotes [38]. Interestingly, this inflammatory response was not observed in animals that received saliva or parasites alone [38]. The significance of this in the context of Leishmaniasis remains to be investigated. However, this phenomena is not exclusive to *L. longipalpis* saliva once eosinophils were described in the inflammatory course at the site of immunization of mice with the salivary recombinant 15-kDa protein from *P. papatasi*, the sand fly species vector of *Leishmania major* [32]. It is well established the abundant presence of eosinophils in both inflammatory site and allergic response. Activated eosinophils release lipid mediators as PAF, prostaglandins, leukotrienes, and lipoxins, as well as cytokines IL-10 and IL-8 that, in conjunct, trigger vasodilatation and leukocyte chemotaxis (reviewed in [39]). In the context of sand fly bite, this eosinophilic reaction could favor vector feeding but creates an unfriendly environment for *Leishmania* parasites.

Host cell infiltration induced by sand fly bite is the most physiologic approach to reinforce the inflammatory role of vector saliva. This event has been explored using *P. papatasi*, in which saliva-induced DTH response observed was associated to a possible fly adaptation to manipulate host immunity for the vector's own advantage [30]. Concerning *L. longipalpis* saliva, our group investigated the initial vertebrate reactions against sand fly saliva. We demonstrated that repeated exposures of BALB/c mice to *L. longipalpis* bites lead to an intense and diffuse inflammatory infiltrate characterized by neutrophils, eosinophils, and macrophages [23]. This response was observed by histological analysis of the ear dermis from exposed mice as early as 2 hours and was sustained up to 48 hours after challenge with the *L. longipalpis* salivary sonicate [23]. Moreover, the injection of immune serum previously incubated with salivary gland homogenate induced an early infiltration with neutrophils and macrophages, suggesting the participation of immune complexes in triggering inflammation [23]. An elegant and remarkable visual advance obtained by two-photon intravital imaging has recently demonstrated that the neutrophils represent the first cell population which is recruited to *Phlebotomus duboscqi* bite site [40]. Although the participation of vector salivary components had not been directly attributed to this inflammatory event by the authors, we could not discharge this possibility considering diverse data showing that saliva from different sand flies species exert chemotaxis. As neutrophils were observed on *L. longipalpis* bite site [23] the implications of its saliva on this cells will be further discussed in this paper.

In addition to *in vivo* models, cell chemotaxis induced by saliva has also been observed *in vitro*. This is of particular interest, indicating that *L. longipalpis* salivary components can act directly as inflammatory mediator. Using transwell system, Zer et al. (2001) showed the direct chemotatic effect of saliva on BALB/c peritoneal macrophages. In the same work, it was demonstrated that *L. longipalpis* saliva is able to both increase the percentage of macrophages that became infected with *Leishmania* in BALB/c and C3H/HeN mice and exacerbate the parasite load in these cells [41]. The authors discuss the possibility that, during natural transmission, saliva could reduce the promastigote exposure to the immune system by attracting host cells to the bite site and by accelerating the uptake of these parasites.

Exploring a straightforward and consistent model—the mouse air pouch—to investigate the inflammatory response induced by *L. longipalpis*, our group has described that *L. longipalpis* salivary gland sonicate was able to induce not only macrophages, but also neutrophil and eosinophil recruitment after 12 h in BALB/c [42]. The increased macrophage recruitment was linked to production of chemokine CCL2/MCP-1 and expression of its receptor CCR2 in the air pouch lining tissue. It was observed that *L. longipalpis* also synergizes with *L. chagasi* to recruit more inflammatory cells to the site of inoculation [42]. This is noteworthy because it increases the availability of "safe targets," the macrophages, for parasite evasion of the effector immune responses [43]. Interestingly, the recruitment profile observed in BALB/c was not observed in C57BL/6 mice, indicating that the same salivary components can induce diverse inflammatory effects depending on the host background [42]. However, because of limited number of cells that can be recovered on the air pouch model, some questions concerning early inflammatory events could not be investigated. Alternatively, the peritoneal cavity has been employed to this kind of study allowing the collection of high number of immigrating cells [44, 45]. In this regard, leukocyte recruitment into peritoneal cavity induced by *L. longipalpis* saliva has been evaluated in both BALB/c and C57BL/6 mouse strains [45]. In this work, significant neutrophil recruitment was observed six hours after administration of saliva, *L. major*, or saliva plus *L. major*. However, in BALB/c mice, all stimuli were able to induce more neutrophil migration than in C57BL/6 mice. Seven days later, it was observed that all stimuli were able to induce higher numbers of eosinophils and mononuclear cells in BALB/c when compared with C57BL/6 mice [45]. This study focused on the effect of saliva from *L. longipalpis* on adaptive immunity, evaluating CD4+ T lymphocyte migration and production of IL-10 and IFN-γ cytokines [45].

4.1. Inflammatory Events Triggered by L. longipalpis Saliva. Neutrophils rapidly accumulate at the inflammatory site (as reviewed in [46]) and have been described on the sand fly bite site [23, 40]. Focusing on inflammatory events triggered by *L. longipalpis* saliva using the peritoneal model, we could observe a distinct kinetic of neutrophil recruitment to the peritoneal cavity of BALB/c and C57BL/6 mice (Figure 2). A late neutrophil influx was observed in BALB/c mice (Figure 2(a)), whereas in C57BL/6 mice neutrophils were already evident in the first hours after *L. longipalpis* saliva inoculation compared to mice injected with endotoxin-free saline (Figure 2(b)).

The link between neutrophil recruitment induced by *L. longipalpis* saliva and other events which initiate and switch off the inflammatory response is an attractive field to be explored. Inflammation resolution is regulated by the release of mediators that contribute to an orchestrated sequence of events [47]. For simplicity, they result in predominance of neutrophils in the inflamed area which are later replaced by monocytes that differentiate into macrophages. During the resolution, inflammatory cells undergo apoptosis and are phagocytosed. Clearance of apoptotic cells by macrophages drives a response characterized by release of anti-inflammatory mediators [48]. Such safe removal of apoptotic cells has been implicated in exacerbation of *Leishmania* infection [49, 50]. The influence of *L. longipalpis* saliva in the time course of inflammation could be observed in cytospin preparations of the peritoneal cells from C57BL/6 mice. Neutrophils in contact with or phagocytosed by macrophages were observed at six hours (Figures 2(c) and 2(d)) and leukocyte phagocytosis by macrophages was an early event as well (Figure 2(e)). Moreover, apoptotic neutrophils were evident in C57BL/6 mice in the presence of saliva (Figure 2(f)). Therefore, components of sand fly saliva are able to both recruit and induce proapoptotic effects on neutrophils. These findings, in the scenario of anti-inflammatory clearance of apoptotic cells, add to the notion of beneficial effects of vector saliva on *Leishmania* transmission. Further work on mediators and mechanisms involved in this process is necessary.

5. Host Macrophage Response to *L. longipalpis* Saliva

Sand fly saliva displays an important role in the macrophage response by triggering the recruitment [42, 51] and suppressing the killing of parasites within macrophages [41, 52]. In this regard, *P. papatasi* saliva inhibits the NO production in macrophages treated with IFN-γ [52] and *L. longipalpis* saliva hampers *Leishmania* antigen presentation to T lymphocytes by macrophages [53] as well as upregulates the IL-10 production related with NO suppression in macrophages infected with *L. amazonensis* [54]. Moreover, pure adenosine from *P. papatasi* saliva decreases NO production in murine macrophages [55] and maxadilan peptide present in *L. longipalpis* saliva upregulates IL-6, IL-10, and TGF-β cytokine responses of LPS-activated macrophages and downregulates IL-12, TNF-α, and NO associated with *L. major* killing [56]. Despite this, few research reports cover the cellular pathways involved in sand fly saliva modulation of macrophage response. Previous study showed that maxadilan acts on PAC-1 receptor in LPS-activated macrophages and inhibits TNF-α production whereas it increases IL-6 and PGE_2 [11], and the authors suggest the participation of cAMP activation by maxadilan in this process.

Although the literature abounds with reports on the effects of sand fly saliva in the immune response and infection,

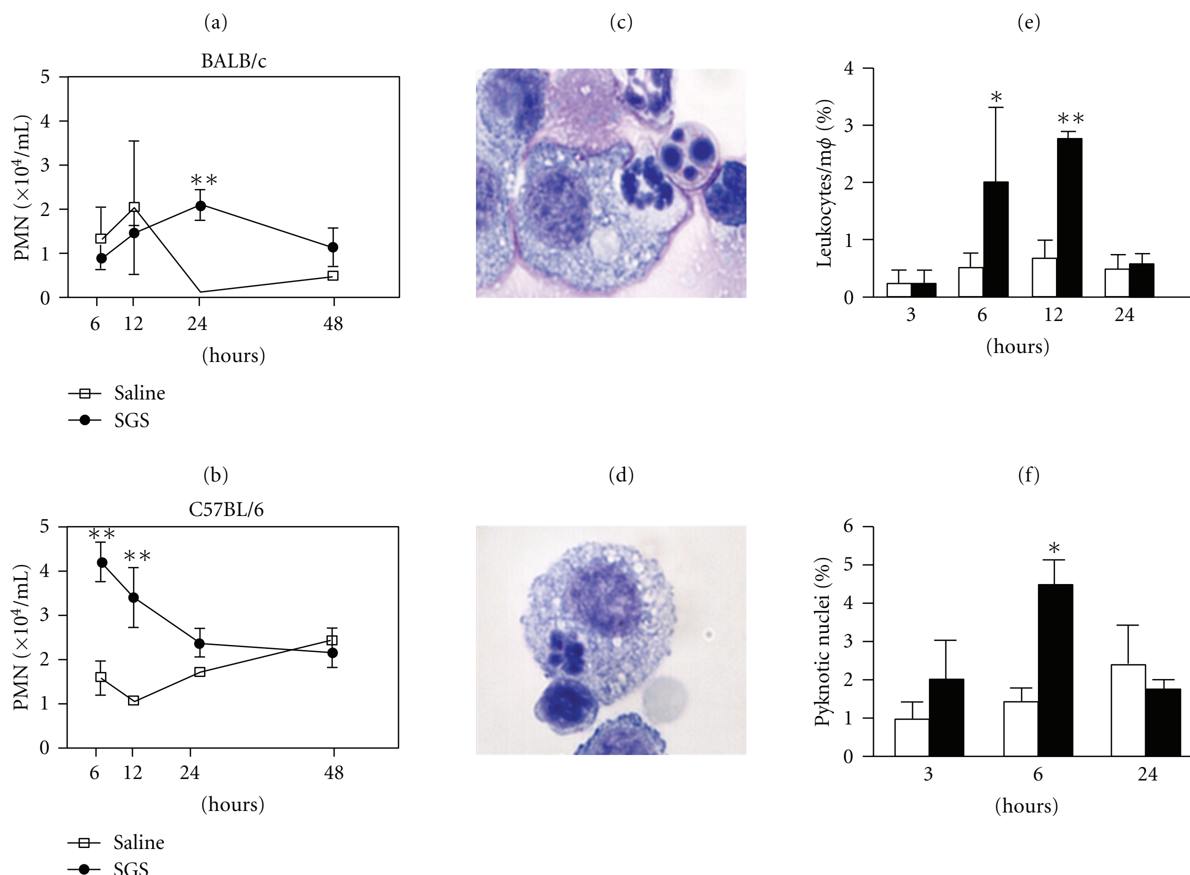

Figure 2: Neutrophil influx, apoptosis, and phagocytosis into BALB/c and C57BL/6 peritoneal cavity in response to *L. longipalpis* saliva. Mice were injected with endotoxin-free saline or *L. longipalpis* salivary gland sonicate (SGS) (0.5 pair/animal). After stimulation, peritoneal cavities were washed and differential cell counts were performed on Diff-Quik stained cytospin preparations. (a-b) Kinetics of neutrophil recruitment in BALB/c (a) and C57BL/6 (b) mice. (c-d) Representative events of C57BL/6 neutrophil phagocytosis by macrophages on Diff-Quik stained cytospin (magnification 1000x). (e-f) Phagocytosis of C57BL/6 leukocytes by macrophages (e) and neutrophil apoptosis (f) after stimulation with SGS (•) or saline (□). Data shown are from a single experiment representative of three independent experiments. Values represent means ± SEM of five mice per group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

the effect of whole sand fly saliva on macrophages is poorly understood. Recently, we showed that *L. longipalpis* saliva activates lipid body (LB) formation in resident macrophages committed with PGE_2 production by COX-2 enzyme (Figure 3) [51]. Lipid bodies are intracellular sites related with eicosanoid production, and their formation can be triggered by activation via different intracellular pathways (as reviewed in [57]). In this context, *L. longipalpis* saliva activated ERK-1/2 and PKC phosphorylation and the inhibition of both pathways resulted in blockade of saliva-induced PGE_2 production by macrophages [51]. PGE_2 modulates the macrophage response during *Leishmania* infection in macrophages [58, 59] and is related with parasite dissemination after infection; however, the role of saliva in the PGE_2 released by macrophages during *Leishmania* infection remains to be addressed. Further studies will be necessary to clarify the importance of eicosanoids stimulated by sand fly saliva in macrophage clearance of parasites and consequently in parasite transmission after sand fly bite.

6. Neutrophils and *L. longipalpis* Saliva: A Neglected Interaction on Scenery of *Leishmania* Infection

Looking to the neutrophils as a significant host-defense cell player in both innate and adaptive response of immune system, it is surprising that few works have attempted to investigate the consequences of vector's saliva and neutrophils interaction in the pathogenesis of leishmaniasis. The reasons to encourage this special attention rise from several lines of evidence showing that neutrophils participate in *Leishmania* immunopathogenesis, by uptaking promastigote forms, producing cytokines and inflammatory mediators or interacting with macrophages enhancing infection (as reviewed in [60, 61]).

Neutrophils are considered as an initial target of *Leishmania* infection [40, 62], and they are implicated in the immunopathogenesis of murine leishmaniasis [50, 63, 64]. Moreover, significant numbers of neutrophils are present at

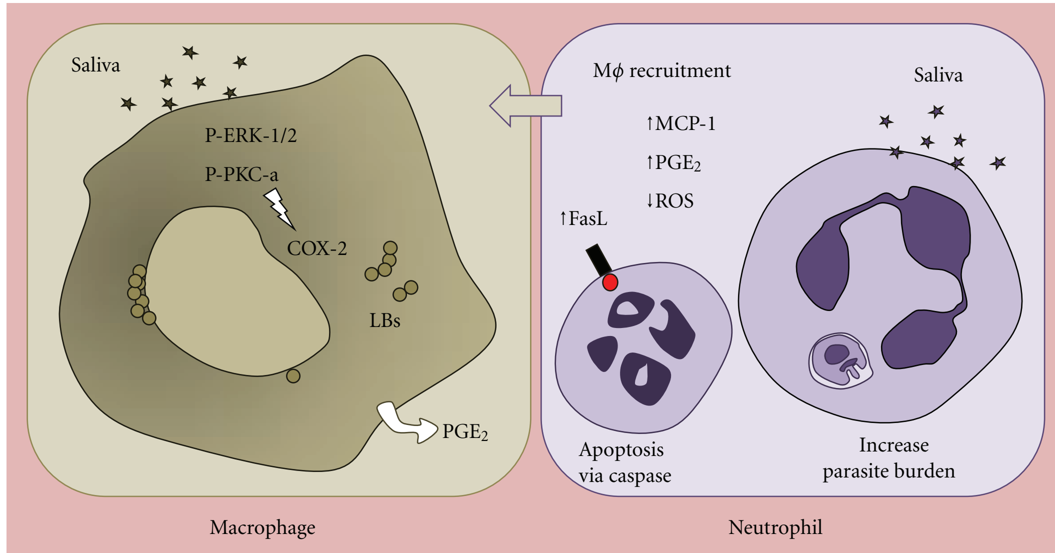

Figure 3: Effects of *Lutzomyia longipalpis* saliva on macrophage activation and neutrophil apoptosis. Macrophages and neutrophils are the first host cells to contact *Leishmania* after sand fly bite. Saliva triggers macrophages activation by lipid bodies formation committed with the PGE_2 production via COX-2 after phosphorilation of kinases. On the other hand, saliva induces neutrophil apoptosis by caspase and FasL activation. In addition, neutrophils activated by saliva become susceptible to *Leishmania chagasi* and release MCP-1, which is associated with macrophage recruitment. This scenario promoted by *L. longipalpis* saliva can contribute to *Leishmania* transmission in the early times of infection.

the inoculation site, lesions, and draining lymph nodes from *Leishmania*-infected mice [31, 63, 65–67]. In addition, *Leishmania* parasites undergo a silent entry into macrophages inside phagocytosed neutrophils, thus reinforcing the role of neutrophils on establishment of *Leishmania* infection [68]. *Leishmania donovani* inhibition of traffic into lysosome-derived compartments in short-lived neutrophils was suggested as a key process for the subsequent establishment of long-term parasitism [69]. On the other hand, neutrophils have also been implicated in parasite control. Phagocytosis of *L. major* by human neutrophils led to parasite killing [70]. Human neutrophils were capable to kill *L. donovani* by oxidative mechanisms [71], and, more recently, it was described the involvement of NET's (Neutrophil Extracellular Traps) on *L. amazonensis* destruction [72].

One elegant approach that reinforced the essential role for neutrophils in leishmaniasis revealed the presence of *Leishmania*-infected neutrophil on the sand fly bite site [40]. However, in that work, although the sustained neutrophil recruitment had been evident only in response to the sand fly bite, the authors did not attribute the neutrophil influx to vector salivary components. Surprisingly, besides neutrophil recruitment, there are no previous reports on further effects of sand fly saliva on neutrophil inflammatory response. Interestingly, studies performed with tick saliva disclose that the inhibition of neutrophil functions favors the initial survival of spirochetes [73–75].

Our group has recently shown the first evidence of direct effect of *L. longipalpis* salivary components on C57BL/6 mice neutrophils [76]. In summary, we described that saliva from *L. longipalpis* triggers apoptosis of inflammatory neutrophils obtained from C57BL/6 peritoneal cavity (Figure 3). The proapoptotic effect of saliva was due to caspase activation and FasL expression on neutrophil surface. Although salivary glands from blood feeding vectors have a variety of components [76], it seems that the proapoptosis compound in *L. longipalpis* saliva is a protein. However, further work is required to elucidate which protein or proteins act in this process. Additional helpful information from this study is that preincubation of *L. longipalpis* saliva with anti-saliva antibodies abrogated neutrophil apoptosis. This allows us to propose that proapoptotic component from *L. longipalpis* saliva could be target for the host's antibodies.

Moreover, neutrophil apoptosis induced by *L. longipalpis* saliva was also increased in the presence of *L. chagasi* [76]. This is particularly interesting by reinforcing the synergistic effect of both vector component and parasite on host inflammatory response, as have been observed in cell chemotaxis [42]. Interestingly, saliva from *L. longipalpis* enhanced *L. chagasi* viability inside neutrophils. This effect was attributed to modulation of neutrophil inflammatory response [76], as treatment of neutrophils with a pan caspase inhibitor (z-VAD) and a COX-2 inhibitor (NS-398) abrogated the increased parasite burden observed. Finally, we also described a novel inflammatory function of *L. longipalpis* saliva on neutrophils, stimulating MCP-1 production, able to attract macrophages *in vitro*. Even though chemotatic activity from *L. longipalpis* saliva has been previously reported, this is the first demonstration that saliva modifies directly the neutrophil inflammatory function, inducing the release of chemotatic factors by these cells.

7. Future Directions

In this paper, we explored the new inflammatory events induced by *L. longipalpis* in the recruitment and cellular function of leukocytes, as well as the repercussion to *L. chagasi* infection. The understanding of protective mechanisms regarding the initial steps of host's response to salivary molecules that can correlate with resistance or susceptibility to *Leishmania* has been poorly explored. Further investigation should address factors that determine the success of *Leishmania* infection. Identifying new escape mechanisms used by *Leishmania* associated to the pharmacological complexity of the sand fly saliva remains a challenge. In this scenario, phylogenetic implications between vector and *Leishmania* species can result in distinct action under host cells. The insights from the inflammatory scenery approached here, as lipid body induction in macrophages and apoptotic death of neutrophils, need to be investigated during the interaction between saliva from other sand fly and *Leishmania* species. Another important point is that these inflammatory effects were detected in salivary gland extract of sand fly vector. However, recombinants proteins from *L. longipalpis* saliva that presented known immunogenic role should be tested as inducers of these inflammatory events during infection by *Leishmania* sp. The studies discussed here suggest that saliva components can act on virulence factors from parasite surface in the first steps involved the recognition, resistance to oxidative mechanisms, and modulation of inflammatory mediators' produced by host cells. However, this finding seems to be part of a "large puzzle," since they are viewed in isolation, by methodological limitations. Recent emerging imaging technologies have opened the possibility to monitor the process of *Leishmania*-host cell interaction in real time from the first moment upon sand fly bite, allowing understanding of molecular and cellular mechanisms in *Leishmania* experimental infection. These advances will enable future integrated studies that may increase understanding of immunopathogenic mechanisms induced by saliva in this intricate and fascinating interaction.

Conflict of Interests

The authors have no financial or other conflicts to declare.

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4.3. MANUSCRITO III

***Lutzomyia longipalpis* Saliva Favors *Leishmania infantum chagasi* Infection Through Modulation of Eicosanoids**

A Saliva de *Lutzomyia longipalpis* Favorece a Infecção por *Leishmania infantum chagasi* Através da Modulação de Eicosanoides

Este trabalho avalia o efeito da saliva no modelo peritoneal murino de macrófagos quanto à formação de corpúsculos lipídicos e a produção de eicosanoides associada a essas organelas, bem como vias de sinalização envolvidas neste processo.

Resumo dos resultados: Neste trabalho, nós avaliamos o efeito da saliva de *Lutzomyia longipalpis* sobre a produção de eicosanoides durante os momentos iniciais da infecção por *L. i. chagasi* no modelo peritoneal murino. Nós observamos que a saliva aumentou a viabilidade intracelular de *L. i. chagasi* tanto em monócitos como em neutrófilos recrutados para a cavidade peritoneal. As células recrutadas para cavidade peritoneal apresentaram maiores níveis da relação PGE_2/LTB_4 e o pré-tratamento com NS-398, o inibidor de COX-2, reverteu o efeito da saliva sobre a viabilidade intracelular dos parasitas.

Este artigo será submetido ao periódico internacional Parasites & Vectors (Fator de impacto JCR 2011 = 2.937).

1 ***Lutzomyia longipalpis* Saliva Favors *Leishmania infantum chagasi* Infection Through**
2 **Modulation of Eicosanoids**

3

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21

22 Running Title
23 *Leishmania* escape by saliva-driven eicosanoid

24

25

26 **Abstract**

27 Monocytes and neutrophils are considered the first line of host defense against infections, and
28 seem to be implicated in the immunopathogenesis of leishmaniasis. Here, we evaluated the
29 effect of *Lutzomyia longipalpis* salivary gland sonicate (SGS) on neutrophil and monocyte
30 recruitment and activation of eicosanoid production in a murine model of inflammation.
31 Intraperitoneal injection of *L. longipalpis* SGS together with *L. i. chagasi* induced an early
32 increased parasite viability inside monocytes and neutrophils. *L. longipalpis* SGS increased
33 PGE_2 but reduced LTB_4 production *ex vivo* in peritoneal leukocytes. In addition, the
34 pharmacological inhibition of COX-2 with NS-398 decreased parasite viability during
35 *Leishmania* infection in the presence of *L. Longipalpis* SGS indicating that PGE_2 is important
36 to prevent parasite killing. These findings point out *L. longipalpis* SGS as a critical factor
37 driving immune evasion of *Leishmania* through modulation of eicosanoids, which may
38 represent an important mechanism on establishment of the infection.

39

40 **Introduction**

41

42 Despite of efforts towards the development of an antileishmanial vaccine and effective
43 antiparasite agents, visceral leishmaniasis (VL) continues to cause high morbidity and
44 considerable mortality worldwide (WHO, 2002). In America VL is transmitted by the bite of
45 *Lutzomyia longipalpis* sand flies. Transmission of *Leishmania* sp. by hematophagous sand fly
46 vectors occurs during blood feeding, when salivary content is inoculated alongside
47 *Leishmania* into host skin. Sand fly saliva enhances *Leishmania* infection on several
48 experimental models [1–3] through its modulatory effects on the host immune system [4,5]. A
49 successful blood feeding depends on the formation of a blood hemorrhagic pool [6]. In such
50 microenvironment there are many inflammatory cells [4], and *L. longipalpis* saliva has been

51 shown to enhance recruitment of immune cells, including monocytes and neutrophils [7–9].
52 Macrophage recruitment induced by *L. longipalpis* saliva has been previously described by
53 our group using the air pouch model. However, restrictions related to this model make it
54 impossible to point out a more detailed effect of saliva in the leukocytes recruited. The
55 peritoneal cavity is a self-contained and delineated compartment [9], and for this reason,
56 several studies have described the use of the peritoneal model to investigate the leukocyte
57 migration induced by sand fly salivary gland extracts [9–11] as well as by *Leishmania*
58 [9,12,13]. We have previously shown that *L. longipalpis* salivary gland sonicate (SGS) is able
59 to modulate eicosanoid release in monocytes and neutrophils recruited to peritoneal cavity
60 [14]. In neutrophils, SGS benefits *L. i. chagasi* infection stimulating production of PGE_2 in
61 vitro [15].
62 In the present study, we explore the effect of *L. longipalpis* SGS on the eicosanoids
63 production in the context of *L. i. chagasi* infection *in vivo* using the peritoneal model in mice.
64 In addition, we demonstrate that eicosanoids can be important in modulation of immune
65 response elicited by SGS allowing increase in parasite viability as well as burden during early
66 moments of *L. i. chagasi* infection.
67
68 **Methods**
69
70 **Antibodies and Reagents**
71 Schneider's insect medium, N-(1-naphthyl)-ethylenediamine and p-Aminobenzene-
72 sulfanilamide were purchased from SIGMA (St. Louis, MO). RPMI 1640 medium and L-
73 glutamine, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA).
74 Nutridoma-SP was from Roche (Indianapolis, In, USA). A23187 calcium ionophore was from
75 Calbiochem Novabiochem Corp. (La Jolla, CA). NS-398, PGE_2 and LTB_4 enzyme-linked

76 immunoassay (EIA) Kits were from Cayman Chemical (Ann Arbor, MI). Dimethylsulfoxide
77 (DMSO) was purchased from ACROS Organics (New Jersey, NJ).
78
79 **Animals**
80 Inbred male C57BL/6 mice, age 6–8 weeks, were obtained from the animal facility of Centro
81 de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (CPqGM-FIOCRUZ, Bahia, Brazil).
82 The animals were kept at a temperature of 24 °C, with free access to food and water and light
83 and dark cycles of 12 hours each.
84
85 **Ethics Statement**
86 All experiments were performed in strict accordance with the recommendations of the
87 Brazilian National Council for the Control of Animal Experimentation (CONCEA). The
88 Ethics Committee on the use of experimental animals (CEUA) of the Centro de Pesquisas
89 Gonçalo Moniz, Fundação Oswaldo Cruz – (Permit Number: 27/2008) approved all protocols.
90
91 **Parasite**
92 *L. i. chagasi* (MCAN/BR/89/BA262) promastigotes were cultured at 25°C in Schneider's
93 insect medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml
94 penicillin, and 100 µg/ml streptomycin.
95
96 **Sand flies and preparation of salivary glands**
97 Adult *Lutzomyia longipalpis* captured in Cavunge (Bahia, Brazil) were reared at the
98 Laboratório de Imunoparasitologia/CPqGM/FIOCRUZ (Bahia, Brazil) as described
99 previously [8]. Salivary glands were dissected from 5- to 7-day-old *L. longipalpis* females
100 under a stereoscopic microscope (Stemi 2000, Carls Zeiss, Jena, Germany) and stored in

101 groups of 10 pairs in 10 µl endotoxin-free PBS at -70°C. Immediately before use, glands were
102 sonicated (Sonifier 450, Brason, Danbury, CT) and centrifuged at 10,000 x g for 4 minutes.
103 The supernatants of salivary gland sonicate (SGS) were used for the experiments. The level of
104 LPS contamination of SGS preparations was determined using a commercially available LAL
105 Chromogenic Kit (QCL-1000, Lonza Bioscience) resulting in negligible levels of endotoxin
106 in the salivary gland supernatant. All experimental procedures used SGS equivalent to 0.5
107 pair of salivary gland per group which possesses approximately 0.7 micrograms of proteins
108 [16].
109
110 **Mice infection**
111 C57BL/6 mice were submitted to intra-peritoneal (i.p.) injection of with 0.1 ml of SGS (0.5
112 pair/cavity), 0.1 ml of *L. i. chagasi* ($3x10^6$/cavity), 0.1 ml of endotoxin-free saline per cavity
113 (negative control) or 0.1 ml of LPS (20µg/ml; positive control). One hour post stimulus the
114 total leukocytes that migrated to the peritoneal cavity was harvested by peritoneal lavage with
115 injection of 10 ml endotoxin-free saline. Alternatively, C57BL/6 mice were previously treated
116 with an i.p. injection of NS398 2 mg/kg or DMSO as a vehicle control. Total counts were
117 performed on a Neubauer hemocytometer after staining with Turk's solution. Differential cell
118 counts (200 cells total) of infected cells were carried out microscopically on cytospin
119 preparations stained with Diff-Quick.
120
121 **Assessment of intracellular load of *L. i. chagasi***
122 Intracellular load of *L. i. chagasi* was estimated by production of proliferating extracellular
123 motile promastigotes in Schneider medium [17]. Briefly, after 1h of infection, peritoneal cells
124 were centrifuged, supernatants containing non-internalized promastigotes were removed and
125 medium was replaced by 250 µl of Schneider medium supplemented with 20% inactive FBS,

126 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Infected cells were
127 cultured at 25°C for additional 3 days. In the third day promastigotes in the cultures were
128 counted in a Neubauer hemocytometer.
129
130 **Transmission Electron Microscopy**
131 Peritoneal cells from mice infected with *L. i. chagasi* were centrifuged (1500 rpm, 10 min)
132 and the pellets were resuspended and fixed in a mixture of freshly prepared aldehydes (1%
133 paraformaldehyde and 1 % glutaraldehyde) in 0.1 M phosphate buffer, pH7.4 overnight at
134 4°C. The cells were washed in the same buffer and embedded in molten 2% agar (Merk).
135 Agar pellets containing the cells were post-fixed in a mixture of 1% phosphate-buffered
136 osmium tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h and
137 processed for resin embedding (PolyBed 812, Polysciences, Warrington, PA). The section
138 were mounted on uncoated 200-mesh copper grids and viewed with a transmission electron
139 microscope (EM 109; Zeiss, Germany). Electron micrographs were randomly taken at the
140 magnifications of 7 000 – 30 000X to study the entire cell profile.
141
142 **PGE_2 and LTB_4 measurements**
143 PGE_2 and LTB_4 levels were measured *ex vivo* from leukocytes harvested by peritoneal cavity
144 washing with Ca^{2+}-Mg^{2+}-free HBSS. After, recovered cells ($1x10^6$ cells/ml) were
145 ressuspended in HBSS contained Ca^{2+}-Mg^{2+} and then stimulated with A23187 (0.5 µM) for
146 15 min. Reactions were stopped on ice, and samples were centrifuged at 500 x g for 10 min at
147 4°C. Supernatants were collected to measure PGE_2 and LTB_4 by enzyme-linked immunoassay
148 (EIA), according to manufacturer's instructions.
149
150 **Statistical analysis**

151 Each experiment was repeated at least three times. The data are presented as the mean and
152 SEM (standard error) of representative experiments and were analyzed using the GraphPad
153 Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The comparisons between
154 two groups were analyzed using Mann-Whitney test. The differences were considered
155 statistically significant when $p \leq 0.05$.
156

157 **Results**

158 ***L. longipalpis* SGS enhances parasite viability during *L. i. chagasi* infection *in vivo***

159 We have previously shown that the main cell types recruited to the inoculation site by *L.*
160 *longipalpis* saliva are neutrophils [8] and macrophages [7,14]. Here we observed this event
161 early (Supporting Information fig. S1) and curiously, when inoculated together *L. i. chagasi*,
162 SGS does not alter the number of infected neutrophils (fig. 1A) or monocytes (fig. 1B)
163 recovered from the peritoneum of mice injected with *L. i. chagasi* plus SGS after 1 hour.
164 However, significant increase in the number of viable parasites were obtained from cultures
165 of peritoneum recovered cells after the same time (fig. 1C). We have also evaluated the
166 presence of parasites inside peritoneal neutrophils and monocytes by electron microscopy
167 (fig. 2A-D). Cells recovered from mice injected with *L. i. chagasi* alone frequently presented
168 degenerated parasite inside vacuoles from neutrophils (fig. 2A) and monocyte (fig. 2B). In
169 contrast, when *L. longipalpis* SGS was inoculated together with *Leishmania*, viable parasites
170 were recurrently observed in both neutrophils (fig. 2C) and monocytes (fig. 2D), although the
171 relative number of these leukocytes were not enhanced by SGS plus *L. i. chagasi* inoculum
172 (fig. S1).
173

174 **Effect of *L. longipalpis* SGS on eicosanoids production and *L. i. chagasi* infection *ex vivo***

175 It has been well demonstrated that different classes of eicosanoids promote both cellular
176 recruitment and safe removal of inflammatory cells coordinating the initial events of

177 inflammation [18]. In addition, LTB_4 is important in host responses to infection because it
178 enhances leukocyte accumulation and phagocytic capacity [19]. We have previously
179 demonstrated that *L. longipalpis* is able to recruit neutrophils [20] and monocytes [14] to
180 peritoneal cavity and increases PGE_2 but not LTB_4 in these cells [14]. Considering these
181 findings, our further interest was to investigate whether *L. longipalpis* saliva is involved in
182 augmenting of PGE_2 and LTB_4 production by *L. i. chagasi*-infected mice. We measured PGE_2
183 and LTB_4 levels on supernatants of peritoneal cells recovered 1 hour after injection with *L. i.*
184 *chagasi* in the presence or absence of *L. longipalpis* SGS. Addition of SGS to *Leishmania* did
185 not alter PGE_2 levels by peritoneal cells (fig. 3A) while LTB_4 production was dramatically
186 reduced (fig. 3B). Concerning the fact that PGE_2 and LTB_4 present antagonistic effects on
187 inflammation and *Leishmania* infection [21,22], we addressed the inflammatory status of this
188 dynamic process by plotting the PGE_2/LTB_4 ratio (fig. 3C). *L. longipalpis* salivary
189 components triggered high PGE_2/LTB_4 ratio 1 h after stimulation (fig. 3C). Based on these
190 observations we hypothesized that *L. longipalpis* SGS favors *L. i. chagasi* infection by
191 inhibiting LTB_4 production and favoring PGE_2 formation. To assess if the manipulation of the
192 eicosanoid balance driven by SGS is important to *L. i. chagasi* infection *in vivo*, we inhibited
193 pharmacologically the PGE_2 production by using a cicloxigenase-2 (COX-2) selective
194 inhibitor NS398. The inhibition of COX-2 decreased number of viable parasites in peritoneal
195 cells after *L. i. chagasi* infection in the presence of SGS (fig. 4).
196
197 **Discussion**
198 Sand fly saliva displays an important role in the first steps of *Leishmania* infection. In this
199 regard, saliva induces cellular recruitment to inflammatory site, inhibits proinflammatory
200 cytokines and deactivates dendritic cells to mobilize regulatory T cells [5]. Previous studies
201 have shown the participation of eicosanoid in the inflammatory response triggered by sand fly
202 saliva [9,14,23]. Herein, we showed for the first time that saliva can modulate eicosanoid

203 profile with a balance skewed towards COX-2 driven PGE_2 over LTB_4 during early time post
204 *L. i. chagasi* inoculation, benefiting infection.
205 PGE_2 production supports establishment of several pathogen infections [24]. In rats and mice,
206 *Trypanossoma cruzi* infection induces PGE_2 by macrophages [25–27]. During *Mycobacterium*
207 *bovis* infection, the increase of PGE_2 and TGF-β1 production by macrophages that phagocyte
208 apoptotic neutrophils in the inflammatory site increases infection [28]. In addition, the
209 interaction between human apoptotic neutrophils and macrophages also increases *L.*
210 *amazonensis* infection via PGE_2 and TGF-β1 production [29]. On the other branch of the
211 inflammatory response is LTB_4. The production of LTB_4 is associated to increase of pathogen
212 killing [19,30]. In the context of *Leishmania* infection, LTB_4 is involved in nitric oxide
213 production and reduced parasite burden in susceptible and resistant mice to *L. amazonensis*
214 [21]. We have previously shown that *L. longipalpis* saliva promptly activates macrophages to
215 produce PGE2 but not LTB4 [14] in vitro and *ex vivo*. In addition, SGS increases PGE_2
216 production by neutrophils during *L. i. chagasi* infection [15]. Here we demonstrate that *L.*
217 *longipalpis* SGS reduce the early LTB_4 production during *L. i. chagasi*, infection whereas
218 orchestrates an anti-inflammatory response by increment of PGE_2 production. In addition, the
219 inoculation of *L. longipalpis* SGS plus *L. i. chagasi* increased parasite viability inside
220 peritoneal cells. The pharmacological inhibition of COX-2 reversed the effect of SGS on
221 enhancing parasite viability. These data suggest that the presence of sand fly SGS favors an
222 inflammatory balance which could facilitate the parasite transmissibility and infection since
223 eicoisanoid can be released faster than other mediators as cytokines and chemokines, which in
224 general need to be expressed after stimuli. In set our data show that eicosanoid profile induced
225 by sand fly saliva displays an important role in the inflammatory modulation during early
226 stages of *L. i. chagasi* infection and point out potential implications of the eicosanoid balance
227 in the immunopathogenesis of visceral leishmaniasis.

228

229 **Acknowledgements**

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231 Lanfredi for electronic microscopy support.

232

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331 **Figure legends**

332

333 **Figure 1. *L. longiplapis* SGS favors *L. i. chagasi* survival inside neutrophils and**
334 **monocytes.** C57BL/6 mice were inoculated with *L. i. chagasi* and/or SGS according to
335 methods. Percentage of infected (A) neutrophils and (B) monocytes were estimated on Diff-
336 Quick-stained cytospin preparations. (C) The figure shows viable parasite counting recovered
337 by total infected peritoneal cells. Bars represent the mean ± SEM, n = 3. *p* values are shown
338 on graphs.

339

340 **Figure 2. *L. longiplapis* SGS favors viability of *L. i. chagasi* inside neutrophils and**
341 **monocytes.** C57BL/6 mice were inoculated with *L. i. chagasi* and/or SGS according to
342 methods. Transmission electron microscopic images of peritonial cells after 1h infection with
343 *L. i. chagasi* are shown. Disrupted *L. i. chagasi* inside neutrophils (A) and monocytes (B) are
344 showed. Viable parasites were observed in neutrophils (C) and monocytes (D) those animals
345 infected in the presence of *L. longipalpis* SGS. Insets indicated by white arrowheads shows
346 details of parasite inside parasitophorous vacuoles (PV) outlined in white (50k-fold
347 increase). P-parasite.

348

349 **Figure 3. Eicosanoid production in response to *L. longipalpis* SGS during *L. i. chagasi***
350 **infection.** C57BL/6 mice were injected i.p. with saline (control), *L. i. chagasi* and/or SGS
351 according to methods. One hour after stimulation, peritoneal cavities were washed and cells
352 were harvested. The cells were then incubated with A23187 (0.5 mM) for 15 min at 37ºC to
353 evaluate LTB_4 and PGE_2 production. The concentrations of PGE_2 (A) and LTB_4 (B) in the
354 supernatant were measured by ELISA. (C) The figure shows the PGE_2/LTB_4 ratios. The data
355 are the means and SEM from an experiment representative of three independent experiments.
356 *p* values are showed on graphs.
357

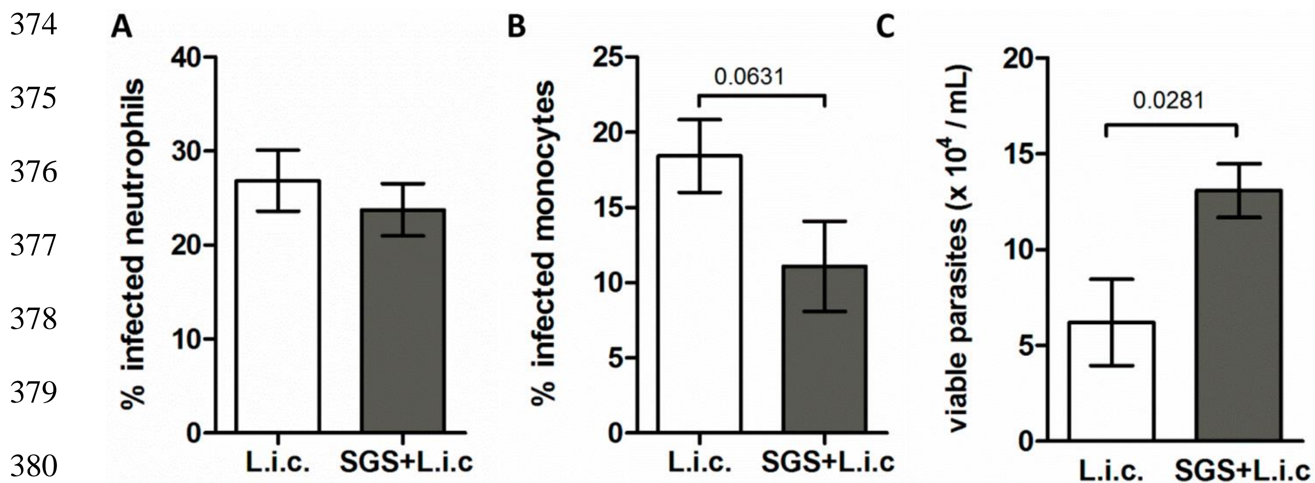
358 **Figure 4. Eicosanoid inhibition affects the parasite viability *in vivo* during *L. i. chagasi***
359 **infection in the presence of SGS.** C57BL/6 mice were treated with DMSO (vehicle – Veh),
360 NS398 2 mg/kg. After 1h of treatment, mice were injected i.p. with *L. i. chagasi* and SGS
361 according to methods. Graph shows viable parasite counting recovered by total infected
362 peritoneal cells. The data are the means and SEM from an experiment representative of three
363 independent experiments.
364

365 **Supporting Information**

366 **Figure S1. Leukocyte recruitment in response to *L. longipalpis* SGS during *L. i. chagasi***
367 **infection.** C57BL/6 mice were injected i.p. with saline (control), *L. i. chagasi* and/or SGS
368 according to methods. One hour after stimulation, peritoneal cavities were washed and cells
369 were harvested. (A) Total leucocytes, (B) monocytes and (C) neutrophil were estimated on
370 Diff-Quick-stained cytospin preparations. The data are the means and SEM from an
371 experiment representative of three independent experiments. *p* values are showed on graphs.
372

373 Figure 1

374–380



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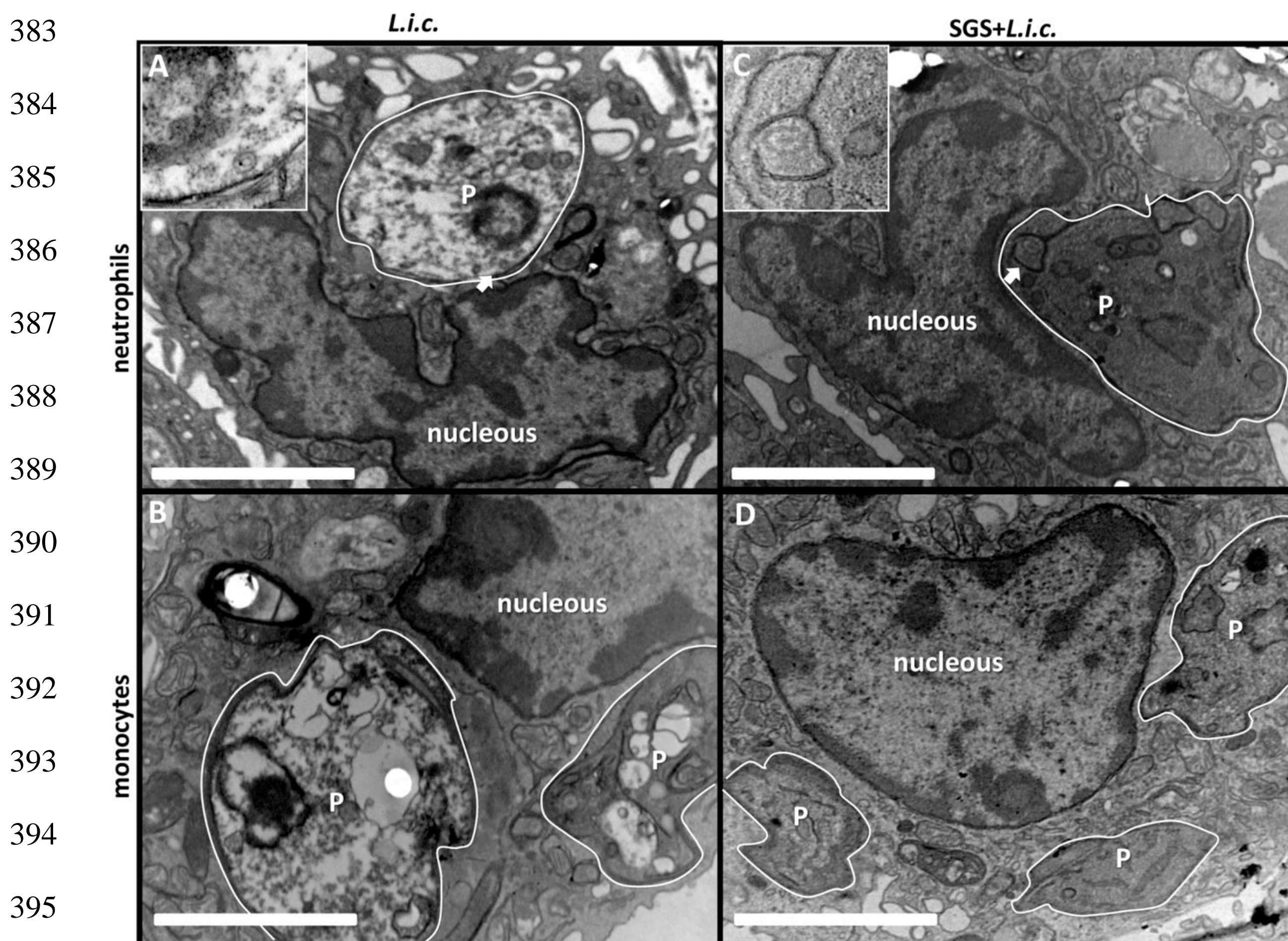

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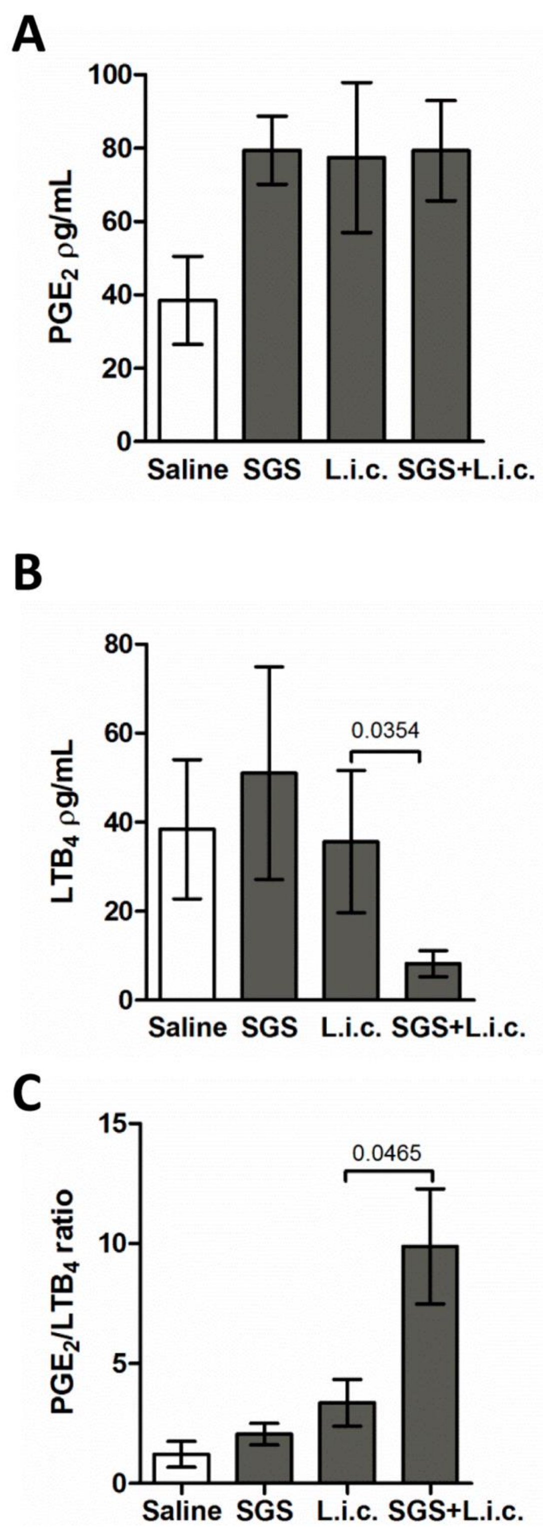

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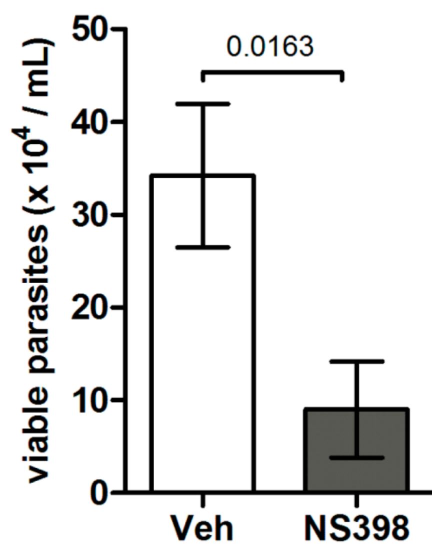

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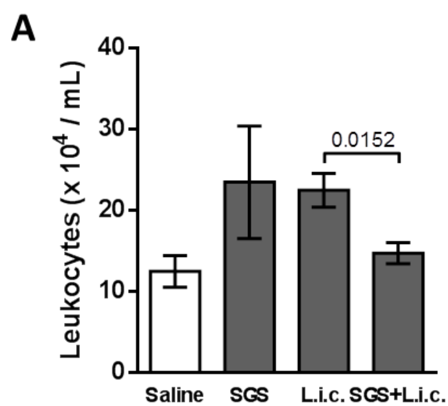

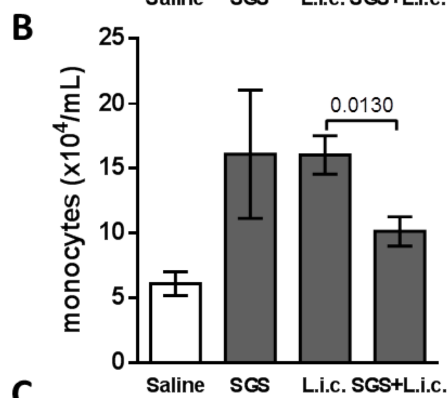

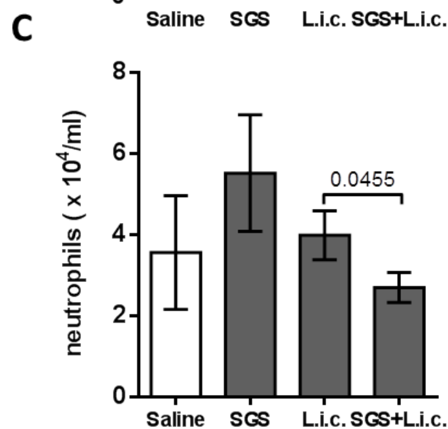

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4.4. MANUSCRITO IV

Prostaglandin $F_{2\alpha}$ Production in Lipid Bodies from *Leishmania infantum chagasi* is a Critical Virulence Factor

A Produção Prostaglandina $F_{2\alpha}$ em Corpúsculos Lipídicos de *Leishmania infantum chagasi* é um crítico fator de virulência

Durante os estudos anteriores, ao tentarmos avaliar a formação de CLs em células infectadas observamos a presença de CLs restrita ao parasito *Leishmania.* Neste trabalho, nós caracterizamos a dinâmica de formação dos CLs de *L. i. chagasi*. Além disso, verificamos o papel dessa organela na produção de prostaglandina $F_{2\alpha}$ pelo parasita e a importância deste eicosanoide durante a infecção de macrófagos.

Resumo dos resultados: Neste estudo nós descrevemos a dinâmica de formação e a distribuição celular dos CLs nas distintas formas evolutivas de *L. i. chagasi* utilizando técnicas de microscopia ótica convencional, confocal e microscopia eletrônica de transmissão. Aqui, nós verificamos que a quantidade de CLs é aumentada durante a metaciclogênese. Além disso, a expressão de $PGF_{2\alpha}$ sintase (PGFS) foi maior nas formas metacíclicas quando comparada às outras formas e a enzima foi localizada nos CLs. A adição de ácido araquidônico AA à cultura de *Leishmania* aumentou a quantidade de CLs por parasita, bem como os níveis de $PGF_{2\alpha}$ nos sobrenadantes de cultura. A infecção com as diferentes formas de *L. i. chagasi* não foi capaz de estimular a formação de CLs na célula hospedeira. Entretanto, os parasitas intracelulares apresentaram maiores quantidades de CLs. A infecção estimulou uma rápida expressão de COX-2, mas não foi detectado aumento na produção de $PGF_{2\alpha}$ nos sobrenadantes de

células infectadas. Por fim, nós verificamos a presença do receptor de $PGF_{2\alpha}$ (FP) nos vacúolos parasitóforos e o pré-tratamento das células com um antagonista do receptor FP inibiu os índices de infecção de forma dose-dependente.

Este artigo foi submetido ao periódico internacional PLoS Neglected Tropical Diseases (Fator de impacto JCR 2011 = 4.716) e encontra-se em segunda fase de revisão por pares.

Prostaglandin $F_{2\alpha}$ Production in Lipid Bodies from *Leishmania infantum chagasi* is a Critical Virulence Factor

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21 **Footnote Page**

22 The authors declare they have no commercial association that might pose a conflict of
23 interest.

24

25 **Running Title**

26 Lipid bodies from *L. i. chagasi* produce $PGF_{2\alpha}$

27

28 **Abstract**

29 Lipid bodies (LB) are cytoplasmic organelles involved in eicosanoid production in
30 leukocytes. Eicosanoids such as prostaglandins (PG) have been implicated in the
31 immune response control. Parasites such as *Leishmania* are also capable of producing
32 PGs, but the role of parasite LBs in biosynthesis of PGs has not yet been investigated.
33 In this work, we studied the dynamics of LB formation and PG release from *Leishmania*
34 *infantum chagasi*. Using light and electron microscopy techniques, we described here
35 the cellular arrangement and abundance of LBs during development of the protozoan *L.*
36 *i. chagasi*. In this regard, a virulent metacyclic state of *Leishmania* displayed more LBs
37 as well as expressed high levels of $PGF_{2\alpha}$ synthase (PGFS) compared to others
38 developmental stages. Moreover, PGFS was localized in the parasite LBs and the
39 addition of exogenous arachdonic acid to procyclic *Leishmania* cultures increased
40 parasite LBs formation and $PGF_{2\alpha}$ release. During macrophage infection with *L. i.*
41 *chagasi*, LBs were restricted to parasites inside the parasitophorous vacuoles (PV).
42 Notwithstanding, *Leishmania* infection upregulated COX-2 expression but this was not
43 followed by $PGF_{2\alpha}$ release by macrophages. We detected $PGF_{2\alpha}$ receptor (FP) on the
44 *Leishmania* PV surface by immunogold electron and fluorescence microscopy. The
45 blockage of FP receptor with AL8810, a selective antagonist, dramatically hampered

46 *Leishmania* infection suggesting that $PGF_{2\alpha}$ should be important to parasite infectivity.
47 Overall these results suggest that $PGF_{2\alpha}$ production in LBs is a virulence factor to
48 metacyclic forms of *L. i. chagasi*. The data demonstrate novel functions for LBs and
49 $PGF_{2\alpha}$ in the cellular biology of *Leishmania*, with possible implications for interactions
50 with the surrounding host microenvironment.
51

52 **Author Summary**

53 *Leishmania* parasites contain the enzymes to synthesize prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). It is
54 unknown whether $PGF_{2\alpha}$ associates with lipid body (LB) formation in parasites, and
55 whether LB from the parasite and/or the host macrophage contribute to parasite
56 infectivity. We report here that LBs increased in abundance during development of the
57 protozoan *L. i. chagasi* to a virulent metacyclic state, as did the expression of $PGF_{2\alpha}$
58 synthase (PGFS). The abundance of parasite LBs, and of PGFS and $PGF_{2\alpha}$ were
59 modulated by exogenous arachdonic acid, a substrate of PGFS. Infected macrophages
60 rapidly upregulate COX-2 expression but this was not followed by $PGF_{2\alpha}$ release,
61 suggesting that the macrophage metabolites were used by parasites inside the
62 parasitophorous vacuole. Moreover, inhibition of the host $PGF_{2\alpha}$ receptor dramatically
63 hampered *Leishmania* infection, suggesting that this prostaglandin may facilitate
64 parasite infectivity. The data demonstrate novel functions for prostaglandin $F_{2\alpha}$
65 production in LBs and for the $PGF_{2\alpha}$ receptor (FP) in the cellular biology of *Leishmania*
66 with critical implications for the host-parasite interactions.
67

68 **Introduction**

69 Lipid bodies (also called lipid droplets) (LBs) are cytoplasmic organelles involved in
70 the storage and processing of lipids and are present in all cell types [1]. In leukocytes

71 and endothelial cells, LBs are critically involved in eicosanoid production because they
72 contain the necessary enzymatic machinery and substrates [2]. Several intracellular
73 pathogens take advantage of the LB formation in the host cells. The increase in the
74 number of host cell LBs and their recruitment to parasitophorous vacuoles have been
75 demonstrated in infections with *Trypanossoma cruzi* [3], *Toxoplasma gondii* [4],
76 *Plasmodium falciparum* [5], *Chlamydia trachomatis* [6] and *Mycobacterium leprae* [7]
77 The location of LBs close to phagolysosomes suggests that LBs could be used as a
78 source of nutrients by pathogens. In addition, an increase in the LB number in the
79 cytoplasm of macrophages is associated with release of PGE_2 and enhancement of
80 *M.bovis* [8,9] and *T. cruzi* [3]. All together, these findings argue that induction of LB
81 formation by intracellular pathogens promotes their survival [10].
82 Notwithstanding the morphological similarity between the LBs in leukocytes and
83 parasites, the function of parasite LBs and the eicosanoid production by its LBs have
84 not been demonstrated. Eicosanoids, such as prostaglandins (PG), are bioactive
85 molecules produced from arachidonic acid (AA) metabolism by specific enzymes, such
86 as cyclooxyganase (COX) and prostaglandin synthases. Prostaglandins have been
87 implicated in the control of immune responses [11,12]. Despite the absence of COX
88 genes and homologous proteins in the Order *Trypasomatidae* protozoa, parasites such
89 as *Leishmania* are capable of producing PGs [13]. These parasites contain the
90 prostaglandin $F_{2\alpha}$ synthase (PGFS) responsible for $PGF_{2\alpha}$ production [14]. $PGF_{2\alpha}$ acts
91 directly on the $PGF_{2\alpha}$ receptor (FP) and triggers the activation of the COX pathway
92 [15]. However, the question of whether PG biosynthesis localizes in parasite has not
93 been investigated. Beside is unknown what the role of $PGF_{2\alpha}$ and your FP receptor in
94 the *Leishmania*-host interplay.

95 In this study, we investigated the dynamics of LB formation and $PGF_{2\alpha}$ release in
96 *Leishmania infantum chagasi* (*L. i. chagasi*). In addition, we investigated the role of the
97 FP receptor in macrophages during *L. i. chagasi* infection. Our findings demonstrated
98 an increase in the expression of PGFS during *L. i. chagasi* metacyclogenesis and
99 showed that parasite-derived $PGF_{2\alpha}$ plays a critical role in macrophage infection.
100

101 Materials and Methods

102

103 Antibodies and Reagents

104 The L-glutamine, penicillin, streptomycin, RPMI 1640 medium, Ca^{2+} Mg^{2+}-free $HBSS^{-/-}$
105 and $HBSS^{+/+}$ with Ca^{2+} and Mg^{2+} were purchased from Gibco (Carlsbad, CA).
106 Dimethylsulfoxide (DMSO) was purchased from ACROS Organics (New Jersey, NJ).
107 The rabbit anti-FP receptor antibody, $PGF_{2\alpha}$ enzyme-linked immunoassay (EIA) Kit and
108 AA were from Cayman Chemical (Ann Arbor, MI). The 4,4-difluoro-1,3,5,7,8-
109 pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503) was obtained from
110 Molecular Probes (Eugene, OR) and osmium tetroxide (OsO_4) was from Electron
111 Microscopy Science (Fort Washington, PA). Aqua-polymount was from Polysciences
112 (Warrington, PA). Thiocarbo-hydrazide and N-ethyl-N'- (3-dimethylaminopropyl)
113 carbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich (St. Louis,
114 MO). Rat 1D4B anti-LAMP antibody was from the University of Iowa (Iowa City, IA).
115 The Texas Red-conjugated with goat anti-rabbit IgG and Vectashield H-1000 and 1200
116 medium were purchased from Vector Labs (Burlingame, CA). Alexa Fluor 647 and
117 488-conjugated with goat anti-rat IgG were purchased from Molecular Probes
118 (Carlsbad, CA).

119

120 **Animals**

121 Inbred male BALB/c mice, age 3–5 weeks, were obtained from the animal facility of

122 Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (CPqGM-FIOCRUZ,

123 Bahia, Brazil). The animals were kept at a temperature of 24 °C, with free access to

124 food and water and light and dark cycles of 12 hours each.

125

126 **Ethics Statement**

127 All experiments were performed in strict accordance with the recommendations of the

128 Brazilian National Council for the Control of Animal Experimentation (CONCEA). The

129 Ethics Committee on the use of experimental animals (CEUA) of the Centro de

130 Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz – (Permit Number: 27/2008)

131 approved all protocols.

132

133 **Wild-type Parasites**

134 The *Leishmania chagasi* promastigotes (MHOM/BR/00/1669) were serially passed

135 through Syrian hamsters and isolated from spleens. Parasites were cultured in

136 hemoflagellate-modified minimal essential medium (HOMEM) containing 10% HI-FCS

137 for 7–9 days until the culture reached stationary phase. To obtain a pure population of

138 logarithmic-phase promastigotes, the cultures were re-diluted every 2 days for at least 3

139 consecutive cycles [16]. Metacyclic promastigotes were isolated from the stationary

140 cultures using the Ficoll-Hypaque (Sigma St. Louis, MO) density gradient separation

141 method described previously [17]. Amastigotes were isolated from the spleens of the

142 infected male Syrian hamsters and were incubated overnight h in amastigote growth

143 medium containing 20% FCS at 37ºC and 5% CO_2, pH 5.5 [18].

LcJ Parasites

The LcJ parasite line, derived from wild-type *L. i. chagasi*, converts between promastigote and amastigote forms in axenic culture. LcJ promastigotes were maintained in HOMEM, and amastigotes were maintained in a low pH medium with fetal calf serum, as reported [19]. Parasites were switched from one stage to the other every 3 weeks. To ensure that the LcJ promastigotes or amastigotes were fully converted, the experiments were performed using parasites that were passaged three times under conditions specific for each stage[18].

Cloning, Expression and Purification of Prostaglandin F2α Synthase from *L. i. chagasi*

The prostaglandin f2-alpha synthase/D-arabinose dehydrogenase (PGFS) coding region (genedb code: LinJ31_V3.2210) was amplified from *L. i. chagasi* total DNA using the polymerase chain reaction (PCR) and was cloned into the *Bam*HI site of pBluescript vector using the primers 5'-CGGGATCCATGGCTGACGTTGGTAAGGC-3' and 5'-CCAAGCTTTAGAACTGCGCCTCATCGGG-3' (the restriction sites are underlined). Amplification of the correct gene sequence was confirmed by DNA sequence analysis (CPqGM – FIOCRUZ facility) and the coding region was subcloned in frame with an N-terminal His_6 tab in the pQE30 expression vector (Qiagen, Germany).

Expression of the recombinant protein was induced in *E. coli* cultures by the addition of 2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37ºC. The bacterial lysates were loaded onto nitrilotriacetic acid (NTA) chromatographic columns, and the

167 protein purification was performed in accordance with the manufacturer's instructions
168 (Qiagen, Germany).

169 **Production of Antiserum Against *L. i. chagasi* Prostaglandin F2α Synthase**

170 C57BL/6 mice were immunized intraperitoneally with 50μg of *L. i. chagasi* PGFS
171 recombinant protein in the presence of a 50% solution of Freud's incomplete adjuvant
172 (Sigma-Aldrich) three times with a 15-day interval between each immunization. After
173 each immunization, mouse serum was collected and evaluated the anti-PGFS antibody
174 production using ELISA on plates coated with the PGFS recombinant protein (see
175 Figure S1A). The specificity of the antiserum to the *L. i. chagasi* PGFS protein was
176 evaluated by Western blot analysis of the *L. i. chagasi* total protein (see Figure S1B) as
177 described below.

178

179 **Western Blotting**

180 *Leishmania* parasites ($2x10^8$/mL) at different stages were lysed using LyseM solution
181 (Roche Mannheim, Germany). Sample protein concentrations were measured using the
182 BCA protein assay (Pierce, Rockford, IL). Total proteins (30μg) were separated by 10%
183 SDS–PAGE and were transferred to nitrocellulose membranes. The membranes were
184 blocked in Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TT) plus 5%
185 dry milk for 1 h before incubation overnight in murine anti-PGFS (1:1,000) antibodies.
186 After the removal of the primary antibody, the membranes were washed five times in
187 TT and were incubated in the peroxidase-conjugated secondary antibody (1:5,000) for
188 1h. The membranes were washed and developed using the ECL chemiluminscence kit
189 (Amersham, UK). The membranes were stripped in accordance with the manufacturer's
190 instructions (Amersham, UK) and reprobed with primary anti-α tubulin (1:1,000)

191 antibody as a loading control. The protein bands were detected using the ImageQuant
192 LAS 4000 system (GE, Piscataway, NJ).

193 **Culture and Infection of Bone Marrow Macrophages**

194 Bone marrow cells were harvested from BALB/c mouse femurs and cultured at 37ºC
195 and 5% CO_2 in RPMI-1640 medium supplemented with 10% HI-FCS, 2 mM L-
196 glutamine, 100 U/ml penicillin, and 50 mg/ml streptomycin (RP-10), and 20% L929 cell
197 culture supernatant (American Tissue Type Collection, Manassas, VA) as a source of
198 macrophage colony-stimulating factor. After 7–9 days, differentiated adherent bone
199 marrow derived macrophages (BMMs) were detached from the plate using 2.5 mg/ml
200 trypsin plus 1 mM EDTA (Gibco) [18]. Bone marrow macrophages ($3x10^5$/well) were
201 plated on coverslips in 24-well plates and cultured at 37ºC, 5% CO_2 in RP-10 for 24
202 hours.

203 BMMs were either treated with 50, 10 and 1 µM of AL 8810 isopropyl ester or with
204 ethanol as the vehicle control. Treated macrophages were infected with non-opsonized
205 metacyclics promastigotes at a multiplicity of infection (MOI) of 10:1, LcJ
206 promastigotes at a MOI of 20:1 or LcJ amastigotes at a MOI of 3:1. Macrophage
207 binding was synchronized by centrifugation of BMMs and parasites for 3 min at 1,200
208 rpm and 4ºC, followed by placement at 37ºC, 5% CO2 at time = 0.

209 After 30 min extracellular parasites were removed by rinsing twice with HBSS without
210 Ca^{++} or Mg^{++} ($HBSS^{-/-}$) followed by the addition of fresh RP-10. After specified times,
211 some coverslips were fixed, and stained with Diff Quik (Wright-Giemsa). Intracellular
212 parasites were counted under light microscopy. Other coverslips were harvested after 1,
213 4, 8, 24, 48 or 72 h, fixed in 2% paraformaldehyde and analyzed by confocal
214 microscopy as described below.

215 **Measurement of PGF_{α} production**

216 Supernatants from *Leishmania* cultures medium or infected macrophages were collected
217 for measurement of $PGF_{2\alpha}$ by enzyme-linked immunoassay (EIA) according to the
218 manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

219 **COX-2 Expression**

220 Total RNA was extracted from infected BMMs using RNeasy Protect Mini Kit (Qiagen,
221 USA) 1, 4 and 24 hours after infection. First-strand cDNA synthesis was performed
222 with 1µg of RNA in a total volume of 25 µL by using SuperScript II (Gibco, USA).
223 Oligonucleotide primers used were: GAPDH 5'-CTGACATGCCGCCCTGGAG-3' and
224 3´-TCAGTGTAGCCCAGGATGCC-5'; COX-2 5'-
225 GCTCAGGTGTTGCACGTAGTCTT-3' and 3'-TTCGGGAGCACAACAGAGTG-5'.
226 All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).
227 RT-PCRs were performed by using µ10 L Fast SYBR Green Master Mix in a total
228 volume of 20µL including cDNA samples and primers. The results were expressed by
229 ΔCt.

230

231 **Confocal microscopy Analysis**

232 Parasites were washed by centrifugation in $HBSS^{-/-}$ and subjected to cytospin onto glass
233 slides, fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 10 min,
234 and rinsed with $HBSS^{-/-}$. The parasites were incubated overnight in anti-PGFS
235 antiserum, and non-immune mouse serum as the negative control.

236 Infected macrophages were fixed in 2% paraformaldehyde and permeabilized with 0.1%
237 Triton X-100 in PBS for 15 min and blocked with 5% dry milk for 1 hour. To stain

238 parasitophorous vacuoles, BMMs were incubated with rat 1D4B anti-LAMP-1 (1:100)
239 in 5% milk/PBS overnight at 4°C, washed and incubated with secondary antibodies
240 (1:200) Alexa Fluor 647 or 488-conjugated with goat anti- rat IgG for 1h at room
241 temperature.

242 Both parasites and infected BMMs were stained for lipid bodies and nuclei. Cells were
243 first incubated in BODIPY® 493/503 (10 μM) at room temperature for 1h to stain the
244 lipid bodies. Cells were washed and then stained with 5ηg/mL ethidium bromide to
245 stain the nuclei. Images were analyzed by confocal microscopy using a Zeiss 510
246 microscope equipped with ZEN2009 software (Carl Zeiss, Inc., Thornwood, NY).

247 In addition, uninfected and infected macrophages were stained with anti-FP receptor
248 antibody (1:20) overnight at 4ºC, washed and incubated with Texas Red-conjugated
249 with goat anti-rabbit IgG for 1h at room temperature. The FP receptor staining was
250 colocalized with anti-LAMPI and DAPI staining (Vector Laboratories, Burlingame,
251 CA). Samples were observed by AX-70 Olympus microscopy and images were
252 acquired using the software Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

253

254 **Transmission Electron Microscopy**

255 Metacyclic *L. i. chagasi* or infected BMMs were centrifuged, and the pellets were
256 resuspended and fixed in a mixture of freshly prepared aldehydes (1%
257 paraformaldehyde plus 1% glutaraldehyde) in 0.1 M phosphate buffer (pH 7.4)
258 overnight at 4°C. A subset of metacyclic parasites were fixed using an imidazole-based
259 technique to stain the neutral lipids [20] prior to fixation. All cells were washed using
260 the 0.1 M phosphate buffer (pH 7.4) and embedded in molten 2% agar (Merck). Agar
261 pellets containing the cells were post-fixed in a mixture of 1% phosphate-buffered

262 osmium tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h and
263 processed for resin embedding (PolyBed 812, Polysciences, Warrington, PA). The
264 sections were mounted on uncoated 200-mesh copper grids and were viewed using a
265 transmission electron microscope (JEOL JEM-1230, Tachikawa, Tokyo). Grids were
266 examined at 50–120,000X magnification.
267

268 **Immunogold Electron Microscopy**

269 The infected macrophages and metacyclic *L. i. chagasi* were processed for immunogold
270 staining. Cells were fixed in 4% paraformaldehyde, 1% glutaraldehyde (Sigma, grade I),
271 and 0.02% picric acid in 0.1 M cacodilate buffer (pH 7.2) at 4 °C. Free aldehyde groups
272 were quenched in a 0.1-M glycine solution for 60 min. Cells were then dehydrated in a
273 methanol series and embedded at progressively lowered temperatures in Lowicryl K4M.
274 Thin sections containing the parasites were stained with mouse anti-PGFS antibody
275 (1:20), and the thin sections containing the infected macrophages were stained with
276 rabbit anti-FP receptor antibody (1:20) overnight at 4℃. After incubation the sections
277 were washed with HBSS$^{-/-}$ and incubated with 10 ηm colloidal gold-AffiniPure-
278 conjugated anti-mouse or anti-rabbit IgG (H + L) for 1h at room temperature. The
279 samples were examined at 120,000X magnification using a transmission electron
280 microscope (JEOL JEM-1230, Tachikawa, Tokyo).
281

282 **Statistical Analyses**

283 Each experiment was repeated at least three times. The data are presented as the mean
284 plus SEM (standard error) of representative experiments and were analyzed using the
285 GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The dose-
286 response experiments were analyzed using one-way ANOVA with post-test to linear

287 trend, and comparisons between the two groups were analyzed using Student´s t-test.
288 The differences were considered statistically significant when $p \leq 0.05$.
289

290 **Results**

291 **Lipid Body Arrangement During *L. i. chagasi* Metacyclogenesis**

292 Lipid bodies can be visualized using techniques to stain neutral lipids, such as osmium
293 impregnation or BODIPY (a fluorescent probe) [21]. Both light and confocal
294 microscopic analyses were used to visualize and enumerate the LBs content in the
295 different developmental forms of *L. i. chagasi* (Figure 1A-F). We used the *LcJ L. i.*
296 *chagasi* parasite cell line that converts between promastigote and amastigote forms in
297 axenic culture [18]. Graphical representation of the numbers of lipid bodies per parasite
298 showed that LcJ amastigotes contained more LBs per cell than LcJ promastigotes in
299 logarithmic stage growth (Figure 1G). Similarly, wild-type (wt) *L. i. chagasi*
300 amastigotes isolated from spleens of infected hamsters contained more LBs than
301 promastigotes (Figure 1G). Remarkably, the LB content increased during
302 metacyclogenesis, with the lowest numbers in logarithmic, higher in unpurified
303 stationary and highest content in isolated metacyclic forms. The amount of LBs per
304 metacyclic promastigote cell did not differ statistically from LB number per amastigote
305 (Figure 1G). Next we investigated the ultrastructural arrangement of LBs in the
306 metacyclic forms of the *L. i. chagasi*, because this is the infective stage of the parasite.
307 Confocal microscopy showed that the LBs were arranged in a linear sequence near to
308 the cell nucleus (Figure 2A-B). In addition, we confirmed that the observed structures
309 were LBs using osmium imidazole-based (Figure 2C) and conventional Transmission
310 Electron Microscopy (TEM) (Figure 2D). The TEM analysis clearly showed the

311 location of the LBs close to the mitochondrion and cell nucleus in the metacyclic forms
312 (Figure 2D).
313

314 ### *L. i. chagasi* Lipid Bodies are Intracellular Sites for the Production of $PGF_{2\alpha}$

315 In *Trypasomatidae*, the only two enzymes in the eicosanoid synthesis pathway that have
316 been described are phospholipase A_2 and PGFS [14]. To address whether PGFS is
317 associated with LBs and whether this association correlates with virulence of parasite
318 forms, we generated an anti-PGFS mouse antiserum against *L. chagasi* PGFS
319 recombinant protein (Supporting Information Figure S1A-B). The LcJ promastigotes
320 expressed higher levels of PGFS than amastigotes (Figure 3A). Strikingly, the PGFS
321 expression in *L. i. chagasi* metcyclic forms was increased compared to wt amastigotes
322 and procyclic forms (Figure 3A).
323 Lipid bodies are intracellular sites of eicosanoid synthesis in mammalian cells [22]. We
324 tested if this is the case for *L. i. chagasi* by investigating the subcellular localization of
325 PGFS in the metacyclic forms of the parasite. We verified that staining for PGFS was
326 strictly localized in the LBs (Figure 3B-D). Furthermore, we incubated wt *L. i. chagasi*
327 procyclic forms with different doses of arachdonic acid (AA) (3.75 – 30 μM), a major
328 eicosanoid precursor. AA induced both LB formation and a dose-dependent release of
329 $PGF_{2\alpha}$ by *Leishmania* (Figure 4B-C). However, there was no detectable effect on the
330 cellular content of PGFS in the AA-stimulated *L. i. chagasi* procyclic forms (Figure
331 4A). These results suggest that: (i) *L. i. chagasi* LBs are the intracellular sites for $PGF_{2\alpha}$
332 production, (ii) the promastigote production of $PGF_{2\alpha}$ increases in response to AA and
333 (iii) this prostaglandin is released from the parasite to the extracellular environment.
334 Because compartmentalization is an important component of eicosanoid synthesis, a

335 failure to induce the total cellular abundance of the PGFS biosynthetic enzyme does not
336 signify a failure to increase its activity.
337

338 ***Leishmania*-driven $PGF_{2\alpha}$ promotes *L. i. chagasi* infection of macrophages**

339 Several intracellular pathogens induce LB formation and recruitment to parasitophorous
340 vacuoles [22]. Intriguingly, our data suggest that the different developmental stages of
341 *L. i. chagasi* forms did not induce host cell LB formation during infection of bone
342 marrow-derived macrophages (BMMs). In contrast, we observed that the LB staining
343 was restricted to the *L. i. chagasi* cell itself within the infected BMM (Figure 5A-B;
344 Figure 7A-D and Video S1). Because we have documented $PGF_{2\alpha}$ release from *L. i.*
345 *chagasi*, we decided to assess the role of this eicosanoid in BMM infection. $PGF_{2\alpha}$ acts
346 directly on the FP receptor and triggers the activation of the COX pathway [15]. The
347 distribution of FP receptor was observed by confocal immunofluorescence in uninfected
348 and *L.i. chagasi* -infected BMMs for 1h (Figure 6). The FP receptor staining in
349 uninfected cell present diffuse in the cytoplasm while in infected cell it was punctual
350 and near to the early phagocytic vacuoles and to parasitophorous vacuoles containing
351 parasites (Figure 6). Using immunogold TEM to investigate BMMs infected for 1 hr
352 with *L.i. chagasi*, we observed that the FP receptor, which recognizes $PGF_{2\alpha}$, was
353 localized near to the parasitophorous vacuoles (Figure 7E-F).
354 In addition, BMMs triggered a rapid expression of COX-2 mRNA after 1-4 hours of
355 infection (Figure 8A). Surprisingly, the infected BMMs did not release $PGF_{2\alpha}$ at the
356 early time points (Figure 8B). These results suggest that the COX-2 products of AA
357 metabolism could be being internalized and used by the parasites. Accordingly,
358 pretreatment of BMMs with AL8810, a specific inhibitor of the FP receptor, resulted in
359 a dose-dependent decrease in *L. i. chagasi* infection (Figure 9A-C). Furthermore,

360 inhibition of the FP receptor decreased the infection index levels in BMMs infected
361 with all forms of parasites examined, i.e., amastigotes, procyclics and metacyclics
362 (Figure 9A-C). Taken together, these results indicate that $PGF_{2\alpha}$ plays an important role
363 in *L. i. chagasi* infection.
364

365 **Discussion**

366 Lipid bodies can play important roles as nutritional sources and in eicosanoid
367 production during host-pathogens interactions [23,24]. Eicosanoids released by
368 macrophage LBs have the potential to modulate immune response [10,22]. Despite of
369 this, the role of eicosanoids produced by parasites and the cellular mechanism involved
370 in their production have not been previously addressed. In the present study, we
371 demonstrate that the LBs in *L. i. chagasi* are intracellular sites of prostaglandin
372 production. Because LBs increase during both metacyclogenesis and in the intracellular
373 amastigote form, we hypothesize that they could act as virulence factors. In addition, the
374 LBs in *L .i. chagasi* are responsible for the production of $PGF_{2\alpha}$, which we also
375 demonstrate here that is important for the modulation of macrophage infection.
376 LBs have been associated with other infectious agents, such as *T. gondii* and *P.*
377 *falciparum* [10]. The increase in the number of LBs in these parasites was demonstrated
378 in *in vitro* cultures and is associated with the acquisition of lipids, such as
379 triacylglycerol (TAG), from the host cell during infection [25]. Herein, we demonstrate
380 that *L. i. chagasi* increases the lipid storage in the LBs and amplifies the expression of
381 PGFS during metacyclogenesis, demonstrating that the parasites can mobilize the
382 eicosanoid machinery in the infective forms of the parasite.
383 The biology of LBs in mammalian cells is relatively well understood. In leukocytes, LB
384 formation is a coordinated process involving the activation of receptors and kinase

385 proteins [2]. Similarly, recent studies in leukocytes have shown that *T. brucei* modulate
386 the LB number via the activation of a specific parasite kinase named lipid droplet kinase
387 LDK [26]. In the current study, we found that AA, a substrate of parasite PGFS,
388 increases both the number of LBs and the release of $PGF_{2\alpha}$ by *L. i. chagasi*. Previous
389 studies have shown that AA induce parasites to release prostaglandins, such as PGE_2,
390 PGD_2 and $PGF_{2\alpha}$ [13,14,27,28]. Here we extend these observations and show an
391 association of these mediators with parasite infectivity. Our data suggest that *L. i.*
392 *chagasi*-derived $PGF_{2\alpha}$ may be important for parasite virulence because the expression
393 of PGFS in the parasite increase during metacyclogenesis. In addition, the PGFS is
394 expressed predominantly in LBs, indicating that LBs are the major intracellular site for
395 the production of prostaglandins in *L. i. chagasi* (Figure 10).
396 It has been reported that the host cell LBs are an important source of TAG and
397 cholesterol for pathogens [23]. Indeed, pathogens can recruit host cell LBs to their
398 parasitophorous vacuoles during infection [3,6]. A recent study suggested that
399 *Leishmania* may use a similar mechanism to acquire lipids and to induce foam cell
400 formation [29]. However, our data demonstrated that the LBs formed during the *L. i.*
401 *chagasi* infection are exclusively from the parasites because the LBs are located inside
402 the parasites within the parasitophorous vacuoles in the infected macrophages. Further
403 studies will be necessary to elucidate how *Leishmania* acquires lipids from the host cells
404 for its metabolism.
405 The role of $PGF_{2\alpha}$ in the immune response is not well understood. Macrophages can
406 produce $PGF_{2\alpha}$ during inflammation [30] or during *L. donovani* infection [27]. $PGF_{2\alpha}$
407 ligates and activates the FP receptor to enhance COX-2 expression in the 3T3-L1 cell
408 line, and the autocrine signaling of this mediator increases PGE_2 and $PGF_{2\alpha}$ levels [15].
409 Herein, we demonstrate that the FP receptor is localized in the early phagocytic

410 vacuoles and surface parasitophorous vacuoles during macrophages infection with
411 metacyclic forms of *L. i. chagasi* (Figure 10). In addition, the *L. i. chagasi*-infected
412 macrophages rapidly express COX-2 but do not release $PGF_{2\alpha}$. These results are
413 consistent with previous studies showing that *Leishmania* infections trigger COX-2
414 expression [29,31–33]. We hypothesize that the COX-2 expression observed in the *L. i.*
415 chagasi-infected macrophages is induced by the $PGF_{2\alpha}$ released from parasites, and that
416 the metabolites from COX-2 enzyme, such as prostaglandin H_2 (PGH_2), in the
417 macrophages could be harvested by the *L. i. chagasi* inside the parasitophorous
418 vacuoles. We further reinforce this idea by showing that the inhibition of the FP
419 receptor in the macrophages diminishes the *L. i. chagasi* parasite load 72h after
420 infection.
421 Our findings demonstrate that LBs and PGFS from *L. i. chagasi* are upregulated in the
422 metaclyclic forms of the parasites and the role of $PGF_{2\alpha}$ and your FP receptor in the
423 *Leishmania*-host interplay. They also suggest that parasite derived eicosanoids may
424 enhance the survival of the parasite inside macrophages. Further studies will be
425 necessary to elucidate how intracellular *Leishmania* could acquire lipids from the host
426 cells and if and how they in turn release eicosanoid precursors into the infected
427 macrophage cytoplasm. Ultimately this could reveal a major mechanism through which
428 the parasite controls the inflammatory microbicidal state of the infected host cell.
429
430

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543 ## Figure Legends

544 **Figure 1. LB number during *in vitro* differentiation of *L. i. chagasi*.** LcJ axenic
545 parasite strain, which converts between amastigote and promastigote forms *in vitro* and
546 wild-type (wt) metacyclic parasites were stained with (A-C) osmium tetroxide. (D-F)
547 show merged images of confocal microscopy of the parasites to LBs stained with
548 BODIPY (green), DNA stained with ethidium bromide (red), and cell contours (DIC).
549 (G) shows the number of LBs in the different stages of *Leishmania* including the
550 amastigote and promastigote LcJ axenic parasites strain and wt parasites. Ama:
551 Amastigote; Pro: Procyclic; Log: Logarithmic; Sta: Stationary: Meta: Metacyclic. Bars
552 represent the mean ± SEM from LB per parasite; n = 3; ***, $p<0.001$ between groups
553 (One-way ANOVA).
554

555 **Figure 2. Cellular characterization of LBs in metacyclic promastigotes of *L. i.*
556 *chagasi*.** (A) Schematic representation of the arrangement of the LBs in most
557 metacyclic forms, also shown microscopically in (B) by merge between LBs (green),
558 DNA (red), and cell contours (DIC). Neutral lipids were detected using osmium
559 imidazole-based (C) or conventional TEM (D). Lipid bodies are indicated with white
560 arrowheads. C-D, Left panels show details of indicated LBs. m – mitochondrion; k –
561 kinetoplast.
562

563 **Figure 3. *Leishmania* LBs are intracellular sites for the production of $PGF_{2\alpha}$.** (A)
564 Immunoblot comparing the abundance of PGFS at different stages in the wild-type (wt)
565 or LcJ strain of *L. i. chagasi*, as described in the methods section. Blots were incubated
566 with polyclonal antiserum to recombinant PGFS (see Figure S1A- B). B, left panel
567 shows merged image of metacyclic promastigotes visualized by confocal microscopy

568 with anti-PGFS (blue), LBs stained with BODIPY (green), DNA stained with ethidium
569 bromide(red), and cell contours (DIC). B, right panels show the left white box area as
570 individual stains and a merged image to visualize PGFS co-localization with LBs. C and
571 D show PGFS localized close to the LBs in the metacyclic forms of two different
572 parasites by post-embedding immunogold staining (120k-fold increase). Black
573 arrowheads indicate the immunogold staining of PGFS.
574
575 **Figure 4. LBs formation and $PGF_{2\alpha}$ release are modulated by arachdonic acid.** (A)
576 Immunoblot documents the abundance of PGFS in the procyclic forms of *L. i. chagasi*
577 stimulated with arachdonic acid (AA), vehicle (veh) or buffer (CTR) for 12 h. (B)
578 Parasites were incubated with different doses of AA (3.75 – 30 µM) for 72 h, and then
579 stained with osmium tetroxide to enumerate the LBs. (C) Supernatants from
580 promastigotes in panel B were harvested and $PGF_{2\alpha}$ levels were measured. Significance
581 was tested by one-way ANOVA with post-test linear trend. Bars represent the mean ±
582 SEM, n = 3.
583
584 **Figure 5. LBs are restricted to parasites during macrophage infection.** (A) shows
585 images of BMMs infected with LcJ amastigotes and promastigotes for 24 h. Nuclei
586 were stained with ethidium bromide (red), parasitophorous vacuole (PV) membranes
587 were stained with anti-Lamp1 (blue), and LBs were stained with BODIPY (green). (B)
588 shows a z-section sequence of images through an infected BMM. White arrowheads
589 indicate the LBs inside PVs after 1 hour of LcJ amastigote infection (see Video S1).
590
591 **Figure 6. Localization of FP receptor during early macrophage infection.** BMMs
592 were infected or not with metacyclic *L. i. chagasi* for 1h and FP receptor localization

593 was shown in the uninfected (left panel) and infected cells (right panels). Nuclei were
594 stained with DAPI (red), parasitophorous vacuole (PV) membranes were stained with
595 anti-Lamp1 (blue), and $PGF_{2\alpha}$ receptors (FP) were stained using anti-FP receptor or IgG
596 control (green). Merge of fluorescence and differential interference contrast (DIC)
597 microscopy shows images from uninfected and infected.
598

599 **Figure 7. LBs and FP receptor arrangement in the *Leishmania*-infected**
600 **macrophage.** Transmission electron microscopic images of BMMs after 1h infection
601 with metacyclic *L. chagasi* are shown. (A) shows an infected BMM with
602 parasitophorous vacuoles (PV) outlined in white. Panels B (80k-fold increase), C, and
603 D (120k-fold increase) show details of LBs inside the parasites. (E) shows post-
604 embedding immunogold staining for FP receptor (50k-fold increase). (F) shows details
605 of FP receptor arrangement close to the PVs in the black box region from the panel B
606 (120k-fold increase). FP receptor staining is indicated by black arrowheads. P –
607 parasite.
608 **Figure 8. COX-2 expression and $PGF_{2\alpha}$ release during macrophage infection.** (A)
609 shows the COX-2 transcript levels measured using qPCR in BMMs infected with LcJ
610 amastigotes, promastigotes and wt metacyclics for 1, 4 and 24 hours and processed
611 immediately. * $p<0.05$ (Student's t-test). (B) shows the kinetic of $PGF_{2\alpha}$ levels released
612 by BMMs infected with *L. i. chagasi* metacyclic forms for 1-48 hours. * $p<0.05$
613 (Student's t-test).
614

615 **Figure 9. Inhibition of the FP receptor hampers *L. i. chagasi* infection.** BMMs were
616 pretreated for 1 h with AL8810 (50-1 μM), a FP receptor antagonist, and infected with
617 (A) LcJ amastigotes, (B) promastigotes or (C) wt metacyclic forms of the parasite for 72

618 h. Infection index is illustrated (One-way ANOVA with post-test´s linear trend). Bars
619 represent the mean ± SEM, n = 3.
620
621 **Figure 10. Schematic view of LB formation and $PGF_{2α}$ release in *L. i. chagasi*
622 during macrophage infection.** (i) LBs are intracellular sites of $PGF_{2α}$ in *L. i. chagasi*.
623 PGFS is localized in the LBS and increase during metacyclogenesis. In addition, LBs
624 and $PGF_{2α}$ can be up regulated by AA in promastigote forms. (ii) FP receptor is
625 mobilized to macrophages PVs, and there it is activated by $PGF_{2α}$ increasing parasite
626 infectivity.
627
628 **Supporting Information**
629
630 **Figure S1.** Specificity of antiserum against prostaglandin F synthase from *L. i. chagasi*.
631 (A) C57BL/6 mice were immunized intraperitoneally with three doses of PGFs
632 recombinant protein (30 µg) plus incomplete Freud´s adjuvant (IFA), and the serum
633 conversion was measured using ELISA using plates coated with recombinant PGFS. (B)
634 Immunoblot showing the specific binding of the PGFS antiserum in to membranes
635 containing *L. chagasi* total promastigote lysate.
636
637 **Video S1.** LBs are restricted to parasitophorous vacuoles during macrophage infection.
638 BMMs were infected with LcJ amastigotes at an MOI of 3 parasites:1 macrophage for 1
639 h. Nuclei were stained with ethidium bromide (red), parasitophorous vacuole (PV)
640 membranes were stained with anti-Lamp1 (blue), and LBs were stained with BODIPY
641 (green). The movie shows the z-section sequence of images.
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643 **Figure 1**

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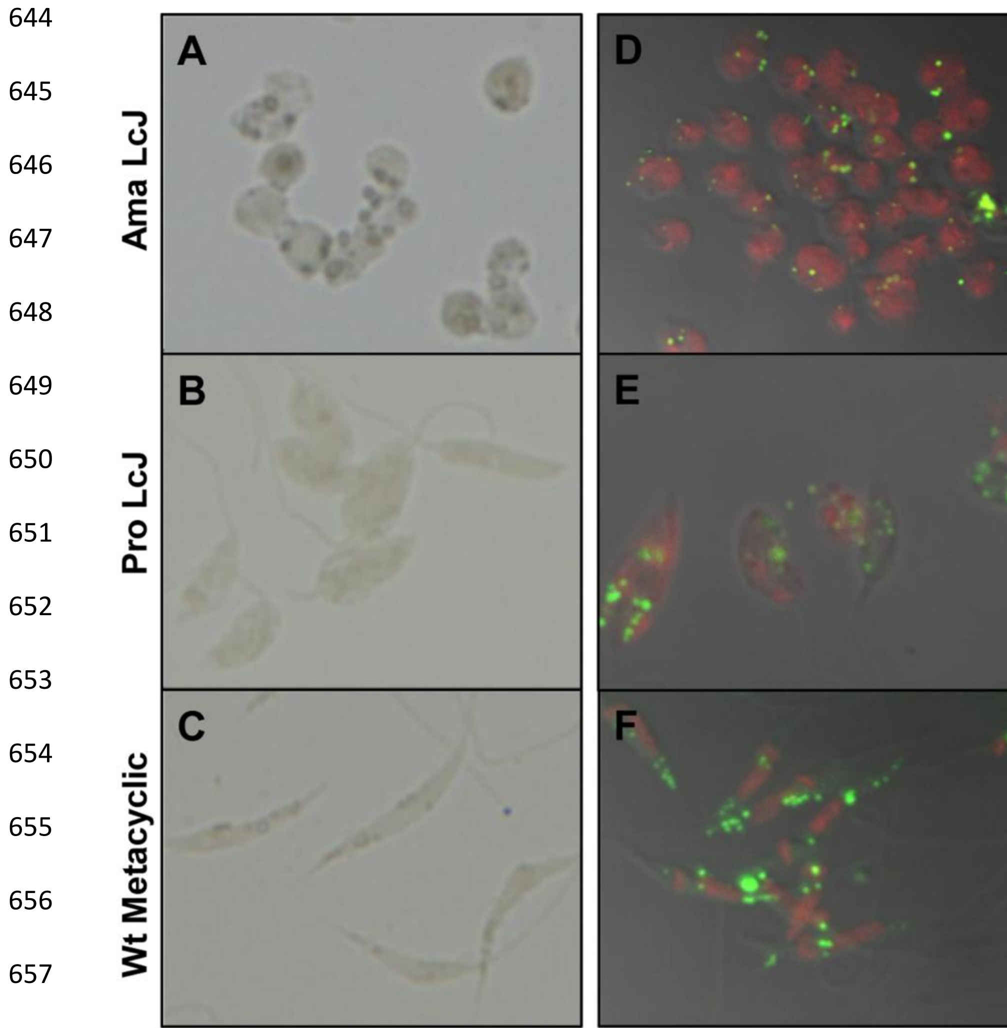

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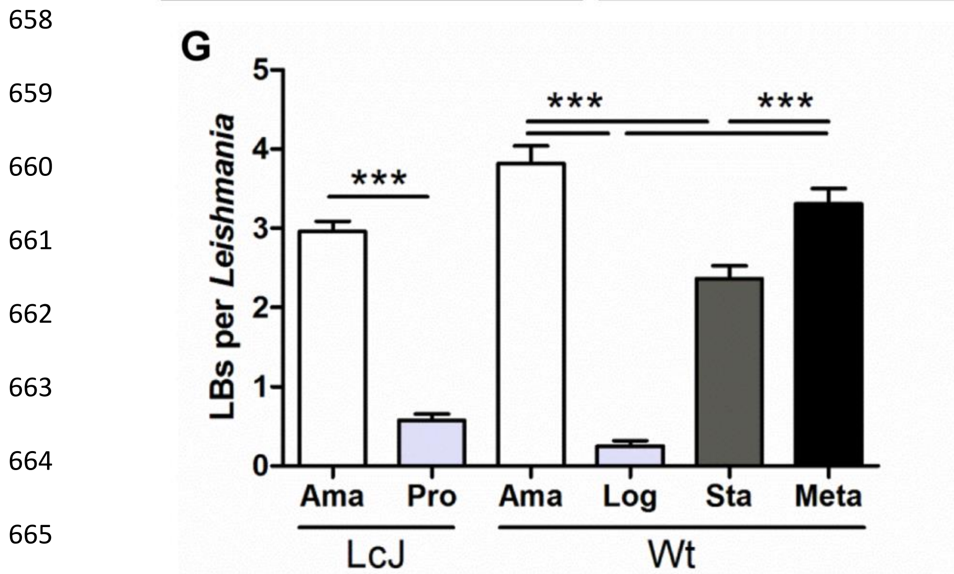

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668 **Figure 2**

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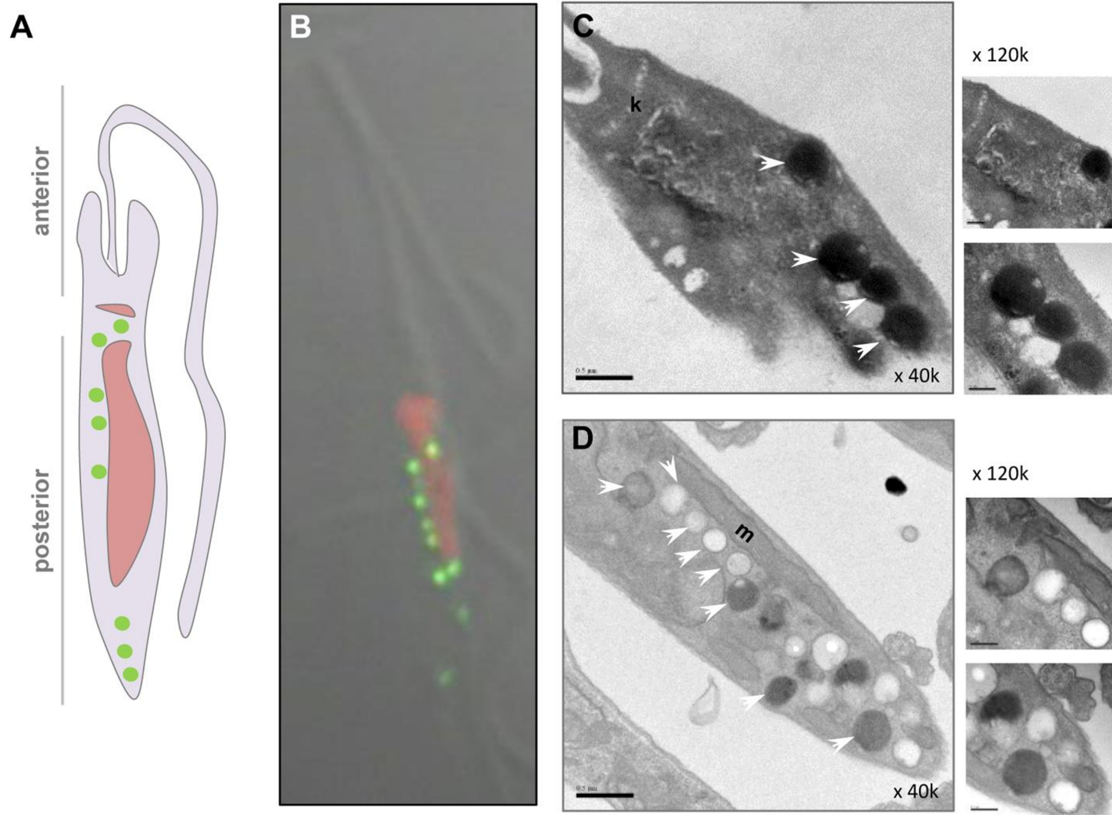

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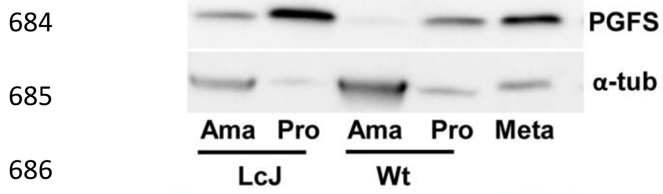

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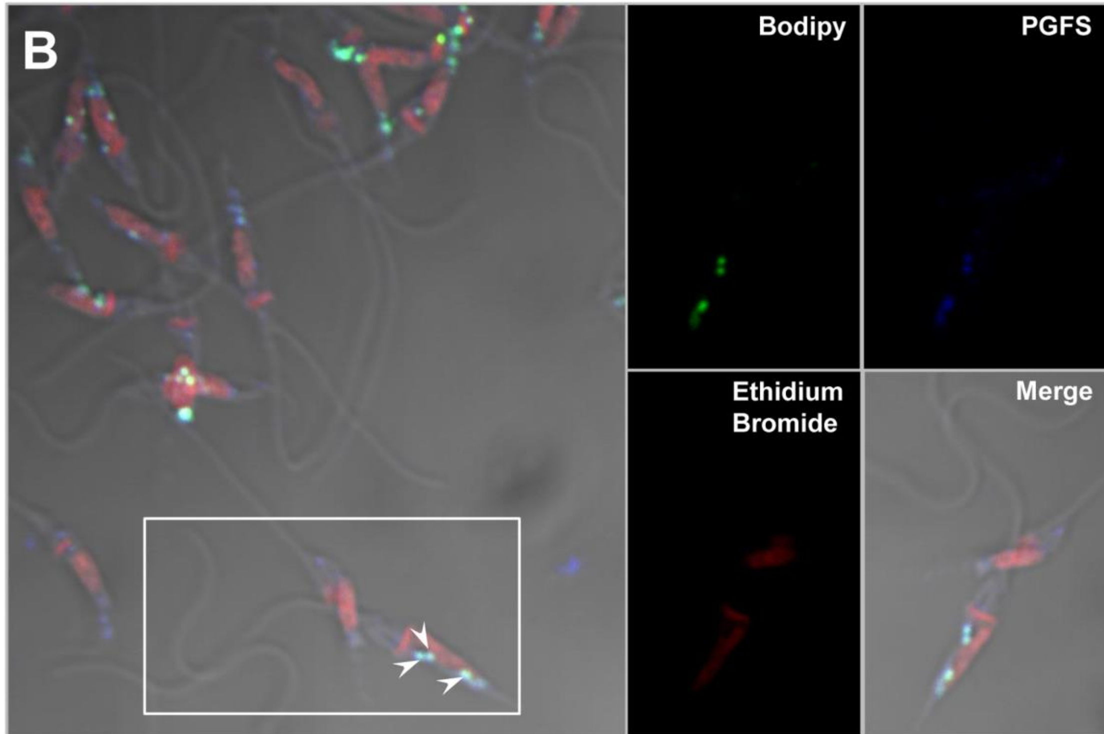

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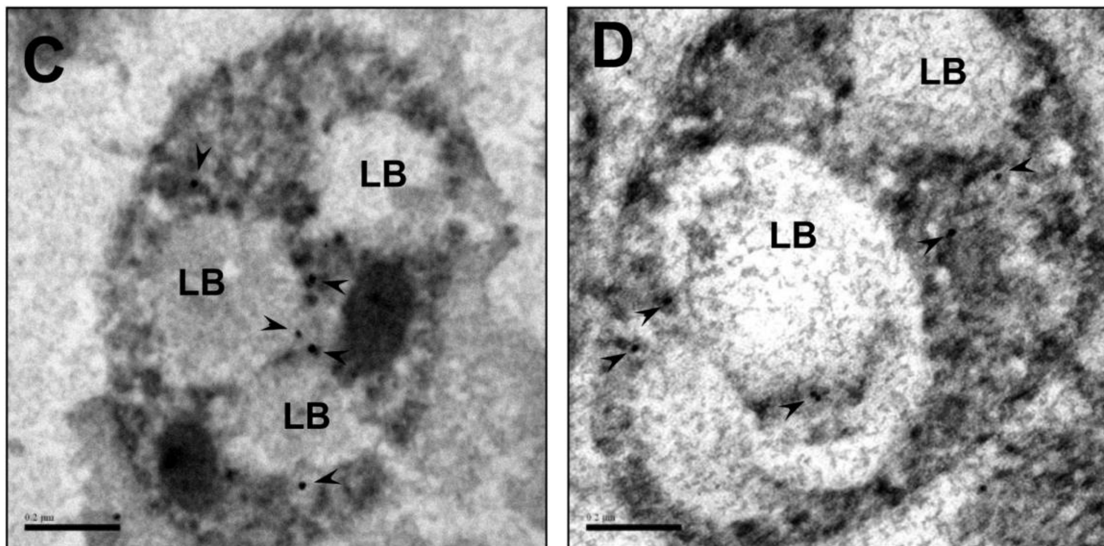

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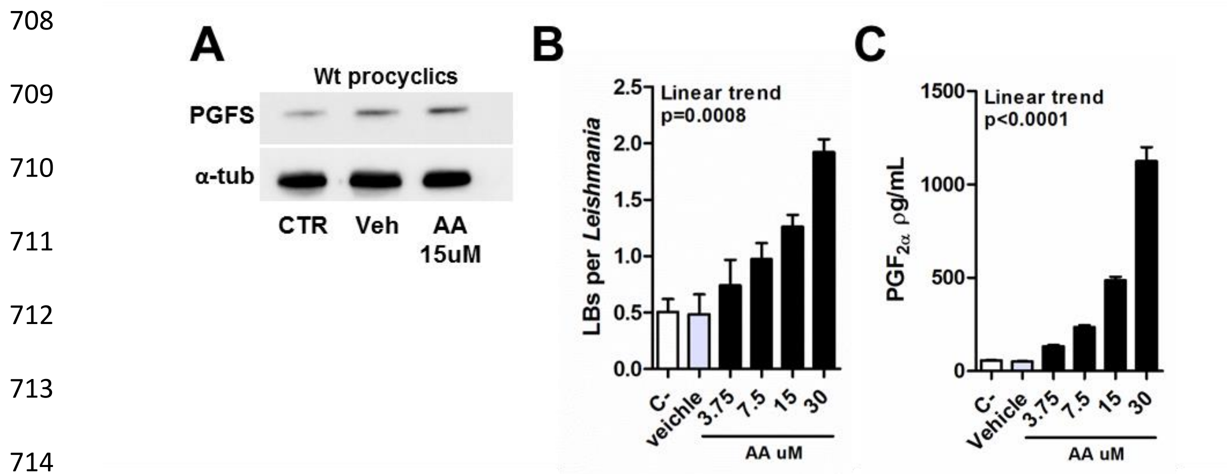
Figure 4



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732 **Figure 6**

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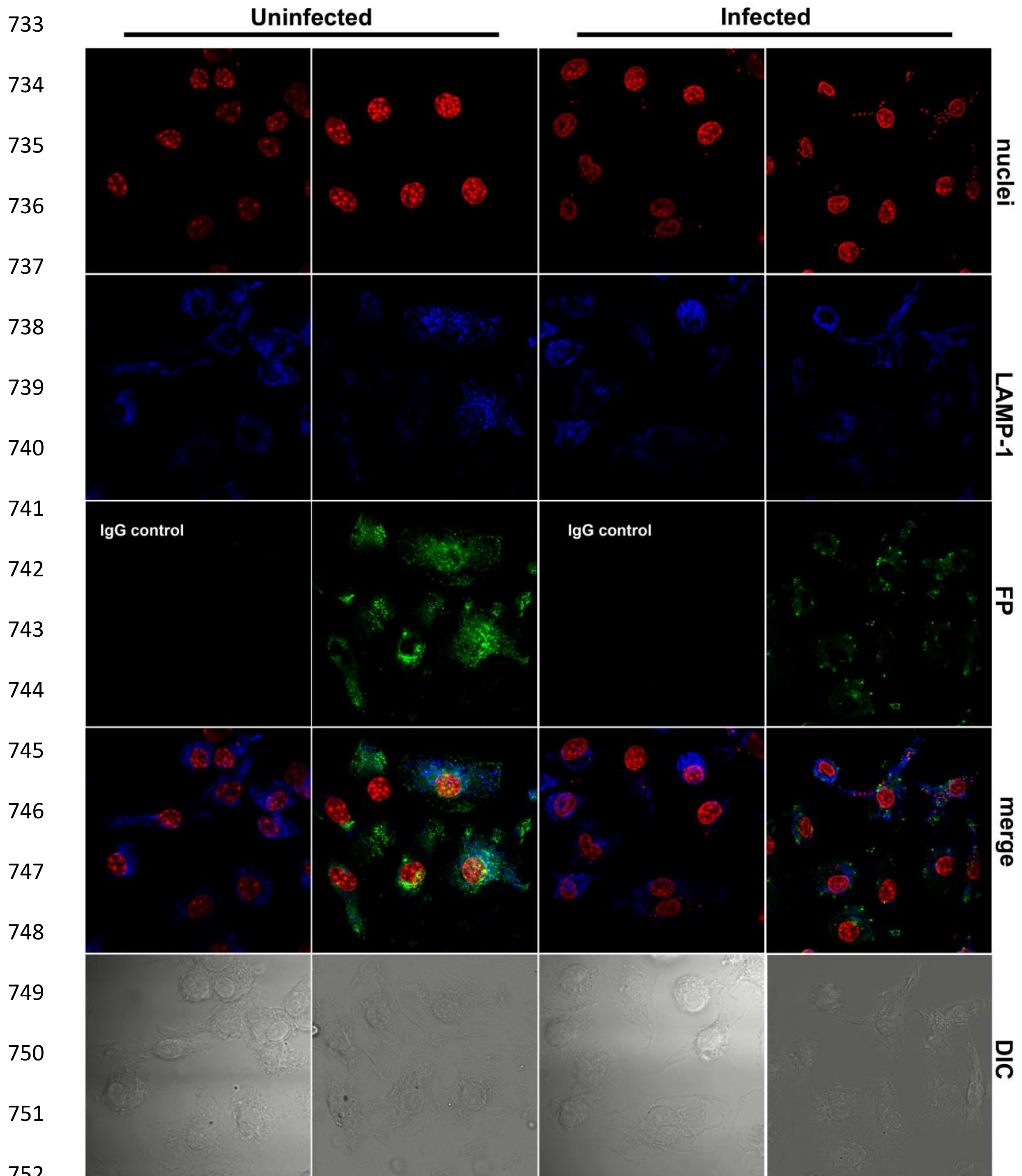

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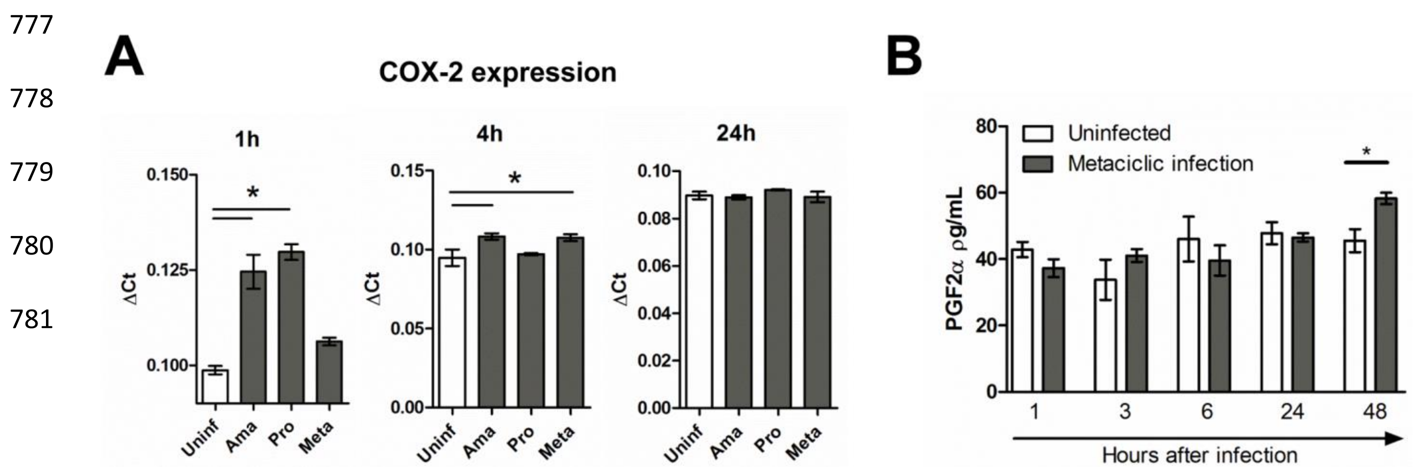

782 **Figure 9**

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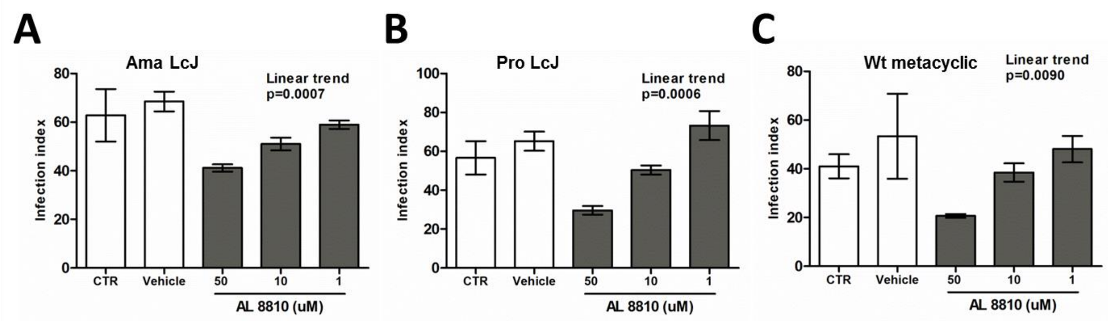

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790 **Figure 10**

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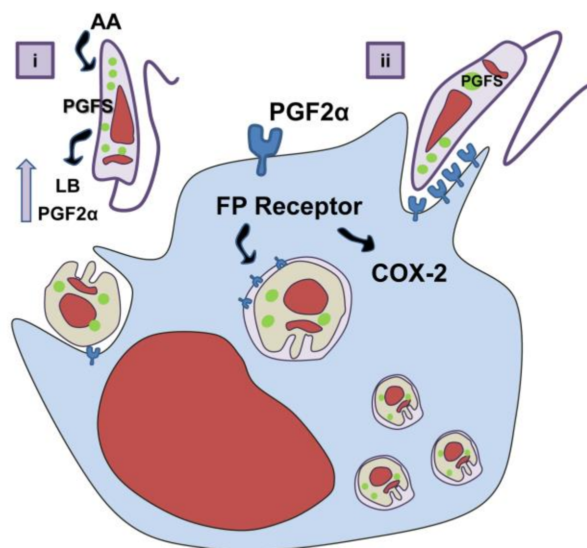

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799 **Figure S1**

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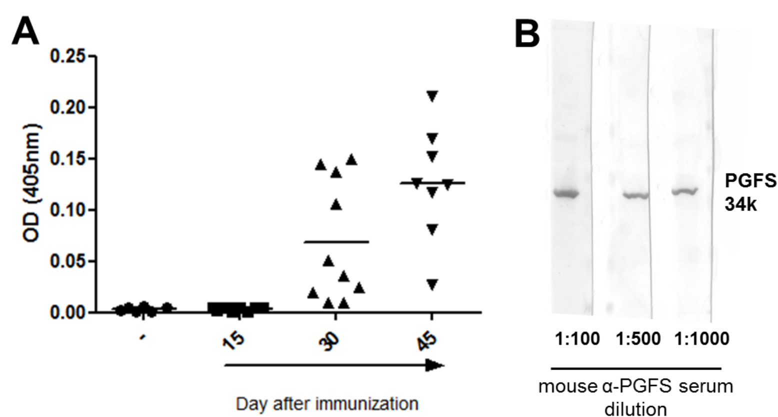

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5. DISCUSSÃO

Sob condições inflamatórias, eicosanoides são prioritariamente produzidos em organelas citoplasmáticas denominadas corpúsculos lipídicos, os quais são formados em leucócitos e outras células envolvidas na resposta inflamatória às infecções e diversos outros estímulos (BOZZA et al., 2009). Os eicosanoides exercem um importante papel na infecção por *Leishmania*. Nessa tese foram abordadas as participações de eicosanoides e corpúsculos lipídicos na interface da interação parasita-vetor-célula hospedeira. Nós verificamos que: (1) a saliva de *L. longipalpis* é capaz de modular a biogênese dos corpúsculos lipídicos e a produção de eicosanoides; (2) o perfil de mediadores lipídicos favorece o estabelecimento da infecção e possivelmente a transmissão do parasito e, além disso, (3) nós demonstramos os mecanismos pelo qual a *L. i. chagasi* produz eicosanoides e que estes também são importantes para a infectividade da forma metacíclica, a forma envolvida na fase inicial de transmissão do parasita do flebótomo para o hospedeiro vertebrado.

A saliva de flebotomíneos induz uma resposta inflamatória caracterizada pelo influxo celular seguido por um mecanismo de supressão da resposta imunológica e hemostática do hospedeiro (ANDRADE et al., 2005). Nosso grupo de pesquisa e outros tem demonstrado o papel da saliva como marcador epidemiológico e como modulador da resposta imune do hospedeiro (CHARMOY et al., 2010; PETERS; SACKS, 2009) (MANUSCRITO II). Entretanto, a participação da saliva na indução de eicosanoides, bem como sua associação com a biogênese de corpúsculos lipídicos ainda não haviam sido investigadas até o presente estudo. Aqui, nós mostramos que a saliva de *L. longipalpis* induz a formação de corpúsculos lipídicos e produção de PGE_2 em macrófagos peritoneais *ex vivo* e *in vitro* via a fosforilação de quinases e ativação de COX-2 (MANUSCRITO I).

Estudos anteriores demonstraram em vários modelos experimentais que a saliva de flebótomo é capaz de induzir o recrutamento celular (CARREGARO et al., 2008; MONTEIRO et al., 2007; SILVA et al., 2005; TEIXEIRA et al., 2005). Peters e cols. (2008) mostraram um perfil semelhante de recrutamento durante a picada de flebótomo usando um sistema de aquisição de imagem intravital. Aqui, nós confirmamos os relatos anteriores de que a saliva de *L. longipalpis* induz um infiltrado inflamatório composto principalmente de macrófagos e neutrófilos. Além disso, mostramos que o recrutamento celular induzido pela saliva ocorre concomitante com a produção de PGE_2 e LTB_4 (MANUSCRITOS I e III). Neste cenário, os eicosanoides poderiam estar deflagrando o recrutamento celular. A produção de LTB_4 por macrófagos residentes é responsável por induzir a migração de neutrófilos (OLIVEIRA et al., 2008). Além disso, outros estímulos inflamatórios como o LPS induzem a migração de macrófagos através da produção de PGD_2 e PGE_2 (TAJIMA et al., 2008).

A PGE_2 é o eicosanoide mais comumente produzido por células inflamatórias, e que é conhecido por exercer efeitos anti-inflamatórios e vasodilatadores. Esses efeitos são úteis para a manutenção da hematofagia de alguns insetos. A saliva do carrapato *Ixodes scapularis*, por exemplo, contém níveis farmacológicos de PGE_2, o qual está implicado na atividade imunomoduladora da saliva na ativação de células dendríticas e macrófagos (SÁ-NUNES et al., 2007). Estudos anteriores utilizando a saliva de *Phlebotomus* sugerem que as propriedades anti-inflamatórias da saliva podem ser atribuídas à produção PGE_2 e IL-10 por células dendríticas (CARREGARO et al., 2008; MONTEIRO et al., 2005). Nestes estudos, o recrutamento celular induzido pela estimulação OVA foi inibido em presença da saliva, o qual foi associado com um perfil anti-inflamatório dependente da produção de IL-10, IL-4 (MONTEIRO et al., 2005) e PGE2 (CARREGARO et al., 2008). Já a saliva de *L. longipalpis* contém o maxadilan,

um peptídeo vasodilatador com atividades imunomoduladoras que é capaz de induzir em macrófagos ativados com LPS a produção de PGE_2 via ativação de COX-1 (SOARES et al., 1998). Aqui, nós demonstramos que a saliva de *L. longipalpis* induz a produção de PGE_2 em macrófagos residentes pela ativação da COX-2, uma vez que a inibição farmacológica com NS-398 reverteu esse efeito da saliva (MANUSCRITO I). Além disso, nós investigamos a presença de PGE_2 na saliva de *L. longipalpis*, mas não encontramos níveis detectáveis deste eicosanoide (dado não mostrado).

Corpúsculos lipídicos de células inflamatórias podem conter enzimas relacionadas com o metabolismo de eicosanoides tais como a COX e 5-LO (BOZZA et al., 2009). Estudos anteriores têm mostrado que vários estímulos inflamatórios e infecciosos são capazes de induzir a formação de CLs em macrófagos (BOZZA; MELO; BANDEIRA-MELO, 2007; BOZZA et al., 2009). Nós verificamos que a saliva *L. longipalpis* induz a formação de CLs em macrófagos *in vivo* e *in vitro*, sugerindo que a saliva atua diretamente sobre estas células. Além disso, os CLs induzidos em macrófagos pela saliva de *L. longipalpis* parecem estar comprometidos com a produção de PGE2, uma vez que nós observamos a co-localização das enzimas COX-2 e PGE-sintase nestas organelas (MANUSCRITO I).

Dados referentes ao efeito direto dos componentes da saliva de *L. longipalpis* sobre vias de sinalização nas células hospedeiras são escassos. MAP quinases como ERKs e proteína quinase C (PKC), estão entre as principais enzimas envolvidas na sinalização nas respostas celulares, incluindo a produção de eicosanoides. As quinases ERK1 e ERK2 induzem a ativação de $cPLA_2$, uma enzima que hidrolisa fosfolipídios de membrana liberando o AA, o qual é metabolizado em prostaglandina H_2 pelas COXs (BOZZA et al., 2009). Estudos anteriores demonstraram a compartimentalização em CLs de MAP quinases e $cPLA_2$ (MOREIRA et al., 2009; YU et al., 1998), bem como de

COX-2 e PGE-sintase (ACCIOLY et al., 2008; D'AVILA et al., 2006; PACHECO et al., 2002). Aqui, nós verificamos que a saliva de *L. longipalpis* ativa a fosforilação de ERK-1/2 e PKC-α em macrófagos (MANUNSCRITO I).

A ativação de COX-2 e a produção de PGE_2 em macrófagos estimulados com LPS são dependentes da fosforilação de quinases tais como PKC-α (GIROUX; DESCOTEAUX, 2000) e ERK-1/2 (WEST et al., 2000). Nós mostramos que a produção de PGE_2 induzida pela saliva de *L. longipalpis* é dependente da atividade de ERK-1/2 e PKC-α (MANUSCRITO I). Esta associação entre a ativação de quinases e o metabolismo de eicosanoides dentro de CLs pode servir para aumentar a rápida produção de eicosanoides em resposta a estímulos extracelulares tais como a saliva. Além do seu papel na regulação da resposta do hospedeiro à infecção pela modulação da produção de eicosanoides, os CLs também podem servir como fontes ricas de nutrientes para os patógenos intracelulares, favorecendo assim a replicação intracelular patógeno (BOZZA et al., 2009; D'AVILA; MAYA-MONTEIRO; BOZZA, 2008).

Apesar de grande parte dos estudos realizados sobre eicosanoides na infecção por *Leishmania* envolver espécies que acometem o sistema tegumentar, parece claro que existe uma dicotomia na resposta imune, em que a produção de produção de PGE_2 beneficia a viabilidade do parasita (AFONSO et al., 2008; LONARDONI et al., 1994; PINHEIRO et al., 2008), enquanto que a produção de LTB_4 favorece a resolução da infecção (SEREZANI et al., 2006). Por outro lado, Ansted e cols. (2001) demonstraram de forma elegante que a produção de PGE_2 facilitava a visceralização de *L. donovani* em animais submetidos a uma dieta com restrição de Cu e Zn, mas não afetava a parasitemia dos animais infectados (Ansted et al.; 2001), sugerindo que em outras espécies de *Leishmania* o efeito da PGE_2 poderia estar associado a disseminação do parasita. A maioria dos estudos envolvendo eicosanoides negligencia em quais etapas

da infecção os eicosanoides poderiam estar envolvidos. Aqui, nós mostramos que a saliva modula o perfil de eicosanoides de maneira que a ativação de COX-2 coordena a produção de PGE_2 em detrimento da produção de LTB_4 nos momentos iniciais da infecção por *L. i. chagasi* (MANUSCRITO III).

A importância da produção de PGE_2 para o estabelecimento da infecção foi demonstrada para alguns patógenos (D'AVILA; MAYA-MONTEIRO; BOZZA, 2008). Em ratos e camundongos, a infecção com *Trypanosoma cruzi* induz produção de PGE_2 por macrófagos (D'AVILA et al., 2011; FREIRE-DE-LIMA et al., 2000; MELO et al., 2003). Um dos fatores responsáveis pela indução da produção de PGE_2 por macrófagos é o reconhecimento de células apoptóticas (FREIRE-DE-LIMA et al., 2000). A interação entre neutrófilos apoptóticos e macrófagos aumenta a infecção por *Mycobacterium bovis* via o aumento dos níveis de PGE_2 e TGF-β1 (D'AVILA et al., 2006). Um mecanismo similar foi demonstrado para infecção por *L. amazonensis*, onde a interação entre neutrófilos apoptóticos e macrófagos humanos aumentou a infecção com a participação de PGE_2 e TGF-β1 (AFONSO et al., 2008).

A saliva de *L. longipalpis* aumenta a apoptose de neutrófilos ao mesmo tempo em que aumenta a produção de PGE_2 durante a infecção por *L. i. chagasi in vitro* (PRATES et al., 2011). *In vivo*, é possível notar a interação entre macrófagos e neutrófilos infectados, após poucas horas da infecção por *L. i. chagasi* (dado não mostrado). Aqui, nós observamos que a saliva de *L. longipalpis* reduz a produção de LTB_4 nos momentos iniciais da infecção por *L. i. chagasi*, ao mesmo tempo que estimula uma resposta anti-inflamatória pelo aumento da produção de PGE_2 (MANUSCRITO III). Este ambiente induzido pela saliva em que prevalece a produção de PGE_2 sobre LTB_4 aumenta a viabilidade dos parasitas dentro das células peritoneais. Neste sentido, nós verificamos que a inibição farmacológica de COX-2 reverteu o efeito

da saliva de *L. longipalpis* sobre a viabilidade dos parasitas (MANUSCRITO III), sugerindo que a presença da saliva favorece um balanço inflamatório que poderia facilitar a transmissibilidade e infecção de *L. i. chagasi* , uma vez que eicosanoides podem ser produzidos mais rápido do que outros mediadores tais como citocinas e quimiocinas, os quais precisam ser expressos *de novo*.

A despeito da produção de eicosanoides pela célula hospedeira, parasitas também são capazes de produzir eicosanoides (KUBATA et al., 2007). Entretanto, o mecanismo celular envolvido nesta produção, bem como a importância dos eicosanoides produzidos pelo parasito para a infecção permanece por ser esclarecida. Nós demonstramos que os CLs de *L. i. chagasi* são sítios intracelulares de produção de prostaglandina (MANUSCRITO IV). Uma vez que os CLs de *L. i. chagasi* aumentam em número durante a metaciclogênese nós acreditamos que os CLs e as PGs proveniente destes CLs sejam fatores de virulência em *L, i. chagasi* (MANUSCRITO IV).

Os corpúsculos lipídicos têm sido associados com a virulência de diversos patógenos, tais como *T. gondii* e *P. falciparum* (SAKA; VALDIVIA, 2012). O aumento no número de CLs nos parasitas foi demonstrado em culturas *in vitro* e está associado com a aquisição de lipídeos como o triacilglicerol (TAG) da célula hospedeira durante a infecção por *Toxoplasama* (NISHIKAWA et al., 2005). Aqui, nós demonstramos que *L. i. chagasi* aumenta o estoque de lipídios em CLs durante a metaciclogênese (MANUSCRITO IV), sugerindo que os parasitas podem mobilizar o metabolismo lipídico em suas formas infectivas.

A biologia dos CLs de leucócitos e outras células de mamíferos é relativamente bem conhecida. Em leucócitos, a formação de CLs é um processo controlado e que envolve a ativação de receptores de membrana, a fosforilação de proteínas quinase e a

produção de eicosanoides (BOZZA; MAGALHÃES; WELLER, 2009). Similarmente, um estudo recente mostrou que a formação de CLs em *T. brucei* depende da ativação de uma quinase específica do parasita denominada proteína quinase de corpúsculo lipídico (LDK) (FLASPOHLER et al., 2010). Entretanto a associação dos CLs de outras células eucarióticas que não as mamíferas, ainda não haviam sido associadas à produção de eicosanoides até o presente estudo. *Leishmania* não possui PLA_2 descrita em seu genoma e não apresenta proteínas análogas às COXs para o metabolismo de AA à eicosanoides. Kabutu e cols. (2003) descreveram a presença de uma PGFS em *L. donovani* capaz de metabolizar AA à $PGF_{2\alpha}$ (KABUTUTU et al., 2003). Aqui, nós verificamos que a expressão da PGFS de *L. i. chagasi* aumenta durante a metaciclogênese. Além disso, a PGFS foi localizada predominantemente em CLs, indicando que CLs são os principais sítios intracelulares para a produção de prostaglandinas em *L. i. chagasi* (MANUSCRITO IV), sugerindo que este pode ser um fator de virulência.

A quantidade de CLs e a produção de eicosanoides podem ser moduladas pela presença de AA (BOZZA et al., 2002; MOREIRA et al., 2009; WELLER; DVORAK, 1985). Estudos anteriores mostraram que o tratamento com AA induz *L. donovani* a produzir as prostaglandinas PGE_2, PGD_2 e $PGF_{2\alpha}$ (KABUTUTU et al., 2003; KUBATA et al., 2000, 2007). Nós estendemos esses achados e demonstramos que a incubação de *L. i. chagasi* com AA aumenta tanto a quantidade de CLs, quanto a produção de $PGF_{2\alpha}$, embora a expressão da PGFS permaneça quase inalterada (MANUSCRITO IV).

Corpúsculos lipídicos das células hospedeiras são importantes fontes de TAG e colesterol para os patógenos (MURPHY, 2012). Além disso, patógenos podem recrutar CLs das células hospedeiras para o vacúolo parasitóforo durante a infecção (COCCHIARO et al., 2008; D’AVILA et al., 2011). Um estudo recente sugeriu que

Leishmania pode utilizar um mecanismo similar para aquisição de lipídios do hospedeiro (RABHI et al., 2012). Entretanto, nossos dados sugerem que os CLs formados durante a infecção são exclusivamente do parasito intracelular, uma vez que os CLs estão restritos aos parasitas dentro dos vacúolos parasitóforos dos macrófagos infectados (MANUSCRITO IV). Estudos posteriores serão essenciais para elucidar como *Leishmania* adquire lipídios da célula hospedeira para o seu metabolismo.

O papel do $PGF_{2\alpha}$ na resposta imune ainda não havia sido elucidado até o presente estudo. Macrófagos produzem $PGF_{2\alpha}$ durante a inflamação (LEE et al., 2012) ou durante a infecção por *L. donovani* (REINER; MALEMUD, 1985). $PGF_{2\alpha}$ se liga, ativa o receptor FP e induz a expressão de COX-2 em células de linhagem 3T3-L1, e a sinalização autócrina deste mediador aumenta a produção de PGE_2 e $PGF_{2\alpha}$ (UENO; FUJIMORI, 2011). Aqui, nós verificamos que o receptor FP está localizado na superfície dos vacúolos parasitóforos de *L. i. chagasi* nos momentos iniciais da infecção. Além disso, macrófagos infectados com *L. i. chagasi* expressaram rapidamente COX-2 mas não liberaram $PGF_{2\alpha}$ (MANUSCRITO IV). Nossos resultados são consistentes com estudos anteriores que mostraram que a infecção com *Leishmania* ativa a expressão de COX-2 (GIROUX; DESCOTEAUX, 2000; GREGORY et al., 2008; MATTE et al., 2001). Nós hipotetizamos que a expressão de COX-2 observada em macrófagos infectados é induzida pelo $PGF_{2\alpha}$ produzido pelos parasitas e que os metabólitos da enzima COX-2, tais como a prostaglandina H_2 (PGH_2) poderiam ser captados pela *L. i. chagasi* nos vacúolos parasitóforos (MANUSCRITO IV). Essa idéia é reforçada pela evidência encontrada durante a inibição do FP receptor em macrófagos, a qual reduziu a carga parasitária nos macrófagos infectados (MANUSCRITO IV). Esses dados sugerem que a $PGF_{2\alpha}$ atua beneficiando a *L. i. chagasi* durante a infecção.

Em conjunto, os nossos dados sugerem que tanto o balanço de eicosanoides modulado pela saliva, quanto à prostaglandinas produzidas pela *L. i. chagasi* desempenham um papel importante nos momentos iniciais da infecção. Embora não tenha sido o foco desse estudo, nós nos perguntamos quais seriam as implicações dos nossos achados na LV crônica. Não existem dados experimentais ou clínicos sobre o status de produção dos eicosanoides durante a LV. Em uma análise preliminar nós verificamos que os níveis de PGE_2 no soro de pacientes adultos com LV não alteram com a infecção, enquanto que os níveis de $PGF_{2\alpha}$ estiveram aumentados em relação aos grupos de indivíduos assintomáticos (ver Anexo). Esses dados sugerem que $PGF_{2\alpha}$ pode ser importante para a infecção por *L. i. chagasi* mesmo durante a fase crônica da doença. Estudos posteriores serão necessários para avaliar o papel das prostaglandinas durante a doença estabelecida e serão importantes para estabelecer novos perfis de tratamento em pacientes com LV.

6. CONCLUSÕES

- A saliva de *L. longipalpis* induz a formação de CLs em macrófagos associada a produção de PGE_2 via fosforilação de PKC-α e ERK-1/2 e ativação de COX-2;
- A produção de PGE_2 induzida pela saliva de *L. longipalpis* favorece a viabilidade intracelular de *L. i. chagasi* in vivo em neutrófilos e macrófagos;
- Corpúsculos lipídicos são sítios intracelulares de produção de PGs em *L. i. chagasi*;
- Prostaglandina F sintase é localizada em CLs e aumenta durante a metaciclogênese de *L. i. chagasi*;
- A formação de CLs e a produção de $PGF_{2\alpha}$ pode ser modulada pela presença de AA em formas procíclicas de *L. i. chagasi*;
- A infeção por *L. i. chagasi* não induz a formação de CLs em macrófagos;
- O receptor FP é mobilizado para o VP de macrófagos e é importante para infectividade de *L. i. chagasi.*

7. REFERÊNCIAS BIBLIOGRÁFICAS

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8. ANEXO

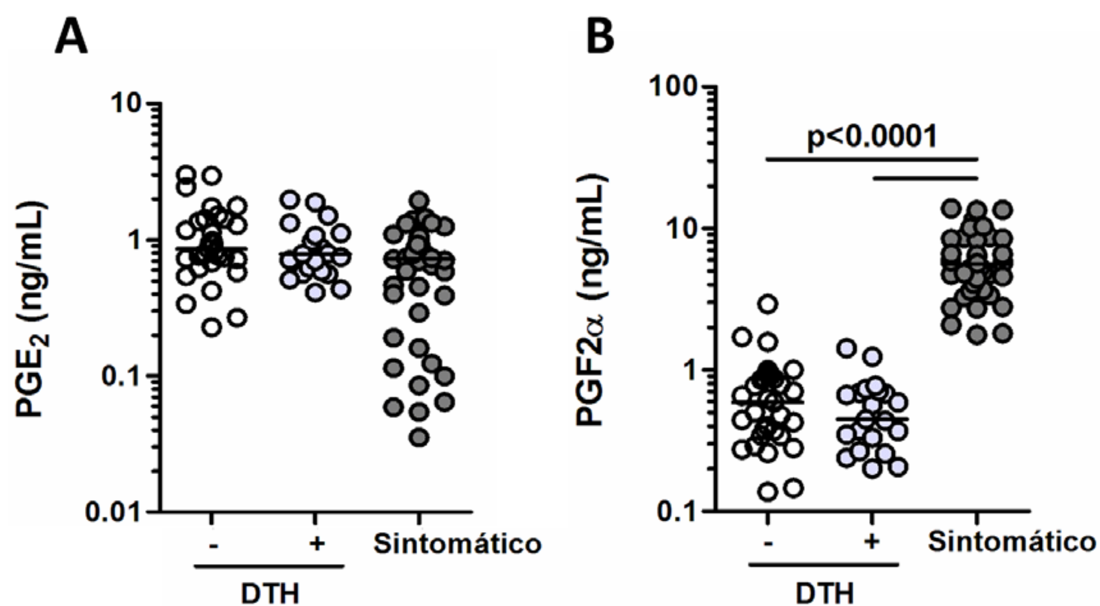

Figura suplementar 1. Níveis séricos de PGE_2 e $PGF_{2\alpha}$ em pacientes com LV. O soro de indivíduos com LV ativa (N = 54) de Aracaju/SE ou familiares classificados com DTH - (n = 31) e DTH + (n=21) foram coletados e os níveis de PGE_2 (A) e (B) foram quantificados por EIA. As diferenças entre os grupos foram avaliadas pelo teste de Kruskal-Walli com pós-teste de Dunn e os valores de significância estatística são mostrados sobre os gráficos.

9. APÊNDICE

Artigos produzidos em colaboração durante o período do doutorado e que não entraram no corpo da tese.

ANDRADE, B. B.; **ARAÚJO-SANTOS, T.**; LUZ, N. F.; KHOURI, R.; BOZZA, M. T.; CAMARGO, L. M. A.; BARRAL, A.; BORGES, V. M.; BARRAL-NETTO, M. Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria. **Journal of immunology (Baltimore, Md. : 1950)**, v. 185, n. 2, p. 1196-204, 15 jul. 2010.

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Article

Lutzomyia longipalpis saliva drives apoptosis and enhances parasite burden in neutrophils

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ABSTRACT

Neutrophils are considered the host's first line of defense against infections and have been implicated in the immunopathogenesis of Leishmaniasis. *Leishmania* parasites are inoculated alongside vectors' saliva, which is a rich source of pharmacologically active substances that interfere with host immune response. In the present study, we tested the hypothesis that salivary components from *Lutzomyia longipalpis,* an important vector of visceral Leishmaniasis, enhance neutrophil apoptosis. Murine inflammatory peritoneal neutrophils cultured in the presence of SGS presented increased surface expression of FasL and underwent caspase-dependent and FasL-mediated apoptosis. This proapoptosis effect of SGS on neutrophils was abrogated by pretreatment with protease as well as preincubation with antisaliva antibodies. Furthermore, in the presence of *Leishmania chagasi*, SGS also increased apoptosis on neutrophils and increased PGE_2 release and decreased ROS production by neutrophils, while enhancing parasite viability inside these cells. The increased parasite burden was abrogated by treatment with z-VAD, a pan caspase inhibitor, and NS-398, a COX-2 inhibitor. In the presence of SGS, *Leishmania*-infected neutrophils produced higher levels of MCP-1 and attracted a high number of macrophages by chemotaxis in vitro assays. Both of these events were abrogated by pretreatment of neutrophils with bindarit, an inhibitor of CCL2/MCP-1 expression. Taken together, our data support the hypothesis that vector salivary proteins trigger caspase-dependent and FasL-mediated apoptosis, thereby favoring *Leishmania* survival inside neutrophils, which may represent an important mechanism for the establishment of *Leishmania* infection. *J. Leukoc. Biol.* 90: 575–582; 2011.

Introduction

Neutrophils play complex roles in infection. They provide an important link between innate and adaptive immunity during parasitic infections [1, 2] but also undergo apoptosis and are ingested by macrophages, thereby triggering secretion of anti-inflammatory mediators [1, 3, 4]. At the onset of *Leishmania* infection, neutrophils establish a cross-talk with other cells in the development of an immune response [5], but the ultimate outcome is controversial, as protective [6–8] and deleterious [9–12] effects to the host have been shown.

Leishmania is transmitted by bites from sandflies looking for a blood meal. Tissue damage caused by sandfly probing [10] and sandfly saliva [13] is a potent stimulus for neutrophil recruitment, which results in a rapid migration and accumulation of neutrophils at the site of the vector's bite [10, 12, 14]. Pharmacological properties of the saliva from sandflies are diverse [15, 16], and we have shown recently that saliva from *Lutzomyia longipalpis*, the main vector of *Leishmania chagasi* in Brazil, triggers important events of the innate immune response [17]. Despite the recognition of the importance of phlebotomine saliva and neutrophils in the initial steps of leishmanial infection, the direct role of saliva on the parasite-neutrophil interplay has not been addressed.

Recent studies demonstrated the presence of *Leishmania*-infected apoptotic neutrophils at the sandfly bite site [10];

Abbreviations: bindarit=2 methyl-2-1-(phenylmethyl)-1H-indazol-3yl[methoxy] propanoic acid, CNPq=Conselho Nacional de Desenvolvimento Científico e Tecnológico, CPqGM-FIOCRUZ=Centro de Pesquisa Gonçalo Moniz-Fundação Oswaldo Cruz, H_2DCFDA=dihydrodichlorofluorescein diacetate, L=ligand, PS=phosphatidylserine, SGS=salivary gland sonicate

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however, a possible role of the sandfly saliva in this phenomenon remains unclear. Herein, we show an important FasL- and caspase-dependent apoptosis effect of *Lu. longipalpis* SGS upon neutrophils. In addition, the SGS-induced apoptosis favors *L. chagasi* survival inside neutrophils. These results represent the first evidence of direct effects of *Lu. longipalpis* SGS on host neutrophils and bring implications for the innate immune response to *Leishmania* infection.

MATERIALS AND METHODS

Mice and parasites

Inbred male C57BL/6 mice, aged 6–8 weeks, were obtained from the animal facility of CPqGM-FIOCRUZ (Bahia, Brazil). This study was carried out in strict accordance with the recommendations of the International Guiding Principles for Biomedical Research Involving Animals. All experimental procedures were approved and conducted according to the Brazilian Committee on the Ethics of Animal Experiments of the FIOCRUZ (Permit Number: 027/2008). *L. chagasi* (MCAN/BR/89/BA262) promastigotes were cultured at 25°C in Schneider's insect medium, supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Sandflies and preparation of salivary glands

Adult phlebotomines from a *Lu. longipalpis* colony from Cavunge (Bahia, Brazil) were reared at the Laboratório de Imunoparasitologia/CPqGM/FIOCRUZ, as described previously [16]. Salivary glands were dissected from 5- to 7-day-old *Lu. longipalpis* females under a stereoscopic microscope (Stemi 2000; Carl Zeiss, Jena, Germany) and stored in groups of 10 pairs in 10 μl endotoxin-free PBS at –70°C. Immediately before use, glands were sonicated (Sonifier 450; Brason, Danbury, CT, USA) and centrifuged at 10,000 *g* for 4 min. Supernatants of SGS were used for experiments. The level of LPS contamination of SGS preparations was determined using a commercially available *Limulus* amoebocyte lysate chromogenic kit (QCL-1000, Lonza Bioscience, Walkersville, MD, USA); negligible levels of endotoxin were found in the salivary gland supernatant. All experimental procedures used SGS in an amount equivalent to 0.5 pair of salivary glands/group, representing ~0.7 μg protein [18].

Reagents

Anti-Gr-1-FITC, anti-mouse CD178L-PE (FasL; CD95L), PE hamster IgG κ isotype control (anti-TNP), CBA mouse inflammation kit, neutralizing antibody anti-mouse FasL, and hamster IgG κ isotype control were purchased from BD Biosciences (San Jose, CA, USA). Anti-mouse Ly-6G Alexa Fluor 647 was from BioLegend (San Diego, CA, USA). Annexin-V, PI (apoptosis detection kit), and z-VAD-FMK were from R&D Systems (Minneapolis, MN, USA). NS-398 and DMSO were from Cayman Chemical (Ann Arbor, MI, USA). Proteinase K was from Gibco, Invitrogen (Grand Island, NY, USA). RPMI-1640 medium and L-glutamine, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA). Schneider's insect medium and etoposide (VP-16) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nutridoma-SP was from Roche (Indianapolis, In, USA), and thioglycolate was from Difco (Detroit, MI, USA). Bindarit was from Angelini Farmaceutici (Santa Palomba-Pomezia, Rome, Italy).

Inflammatory neutrophils

Peritoneal exudate neutrophils were obtained as described previously [19]. Briefly, C57BL/6 mice were i.p.-injected with aged 3% thioglycolate solution. Seven hours after injection, peritoneal lavage was performed using 10 ml RPMI-1640 medium supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To remove adherent cells, exudate cells were incubated at 37°C in 5% CO_2 for 1 h in 250-ml flasks (Costar, Cambridge, MA, USA); cells on supernatants were then recovered and quantified in a hemocytometer by microscopy. Cell viability was >95%, as determined by trypan blue exclusion (data not shown). Nonadherent cells were stained with anti-Gr-1 and Ly-6G to assess purity and were subsequently analyzed by flow cytometry using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA). Gr-1$^+$ Ly-6G$^+$ cells were routinely >95% pure.

Neutrophil apoptosis assay

For cell cultures, neutrophils (5×10^5/well) were cultured in 200 μl RPMI-1640 medium, supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well plates (Nunc, Denmark) in the presence of different doses of *Lu. longipalpis* SGS (0.5, 1.0, and 2.0 pairs/well). In some experiments, etoposide (20 μM) or LPS (100 ng/well) was used as a positive control. Three hours and 20 h after stimuli, neutrophil apoptosis was assessed by PS, exposed in the outer membrane leaflet through labeling with annexin-V-FITC by FACS analyses in combination with PI nuclear dye [19]. Annexin-V specificity was tested using Ca^{2+}-free buffer; binding was not observed in this case. Morphological criteria for apoptosis, such as separation of nuclear lobes and darkly stained pyknotic nuclei, were also applied for quantification purposes using cytospin preparations stained by Diff-Quick under light microscopy [19]. Neutrophils were graded as apoptotic or nonapoptotic after examination of at least 200 cells/slide. To FasL-blocked assays, neutrophils were pretreated with a neutralizing antibody specific for FasL (10 μg/mL) or an IgG isotype control (10 μg/mL) for 30 min before use. In some experiments, SGS was preincubated with sandfly antisaliva serum (0.5 salivary gland pair plus 50 μl serum preincubated for 1 h at 37°C) [20] or with proteinase K (10 mg/ml) at 65°C for 2 h and then for 5 min at 95°C for enzyme inactivation before use.

Anti-sandfly saliva serum

Hamster-derivated serum was obtained as described previously [20]. Briefly, hamsters (*Mesocricetus auratus*) were exposed to bites from 5- to 7-day-old female *Lu. longipalpis*. Animals were exposed three times to 50 sandflies every 15 days. Fifteen days after the last exposure, serum was collected and tested for IgG antisaliva detection by ELISA.

Human neutrophil assay

Human blood from healthy donors was obtained from Hemocentro do Estado da Bahia (Salvador, Brazil) after donors had given written, informed consent. This study was approved by the Research Ethics Committee of FIOCRUZ-Bahia. Human neutrophils were isolated by centrifugation using PMN medium, according to the manufacturer's instructions (Robbins Scientific, Sunnyvale, CA, USA). Briefly, blood was centrifuged for 30 min at 300 *g* at room temperature. Neutrophils were collected and washed three times at room temperature by centrifugation at 200 *g*. Cells/well (10^6) were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated FBS (Hyclone, Ogden, UT, USA), 2 mM/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen) for 3, 6, and 20 h at 37°C, 5% CO_2, in the presence or absence of *Lu. longipalpis* SGS (0.5 pair/well) or etoposide (20 μM). Cells were then cytocentrifuged and stained with Diff-Quick, and pyknotic nuclei were analyzed by light microscopy.

In vitro neutrophil infection

Peritoneal neutrophils were infected in vitro with *L. chagasi* promastigotes stationary-phase at a ratio of 1:2 (neutrophil:parasites) in the presence or absence of SGS (0.5 pair/well) in RPMI-1640-supplemented medium. In some experiments, neutrophil infection was performed in the presence of etoposide (20 μM). For inhibitory assays, neutrophils were pretreated for 30 min with z-VAD-FMK (100 μM) to block caspase activation or preincubated for 1 h with NS-398 (1 μM), a COX-2 inhibitor. DMSO (vehicle) 0.4% was used as control. After 20 h, infected neutrophils were centri-

fuged, supernatants containing noninternalized promastigotes were collected, and medium was replaced by 250 μl Schneider medium, supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Infected neutrophils were cultured at 25°C for an additional 3 days. Intracellular load of *L. chagasi* was estimated by production of proliferating extracellular motile promastigotes in Schneider medium [21].

Quantification of ROS production

Intracellular ROS detection in neutrophils cultured at 5×10^5 cells/well was performed using H_2DCFDA fluorescent probe following analyses by FACS, according to the manufacturer's instructions. For investigation of ROS production, the purified neutrophil population was analyzed by forward- and side-scatter parameters following application of the H_2DCFDA-FITC probe.

Measurement of PGE_2 production

Supernatants from neutrophil cultures were collected 20 h after incubation with *L. chagasi* or *L. chagasi* plus SGS and cleared by centrifugation. PGE_2 was measured by the EIA kit from Cayman Chemical. All measurements were performed according to the manufacturer's instructions.

MCP-1/CCL2 measurement

Supernatants from neutrophil cultures were collected 20 h after incubation with RPMI medium, SGS, *L. chagasi*, or *L. chagasi* plus SGS and cleared by centrifugation. MCP-1 (CCL2) chemokine was measured using the CBA mouse inflammation kit (BD Biosciences), according to the manufacturer's instructions.

Chemotaxis assays

Neutrophils were pretreated or not with bindarit propanoic acid (Angelini Farmaceutici; 100 μM) for 30 min before incubation with medium, SGS, *L. chagasi*, or *L. chagasi* plus SGS, and supernatants were harvested. The culture supernatants were added to the bottom wells of a 96-well chemotaxis microplate ChemoTx system (Neuro Probe, Gaithersburg, MD, USA). Macrophages were obtained 4 days after i.p. injection of 1 ml 3% thioglycolate solution on C57BL/6 mice and ressuspended in RPMI-1640 medium before being added to the top wells (10^5 cells/well) and incubated for 1.5 h at 37°C under 5% CO_2. Following incubation, cells that migrated to the bottom wells were counted on a hemocytometer. Macrophage migration toward RPMI-1640 medium alone (radom chemotaxis) was used as a negative control and toward LPS as a positive control. The chemotaxis indexes were calculated as the ratio of the number of migrated cells toward supernatants taken from *L. chagasi*-infected or not infected neutrophils cultured in the presence or absence of SGS to the number of cells that migrated to RPMI-1640 medium alone.

Statistical analysis

The in vitro systems were performed using at least five mice/group. Each experiment was repeated at least three times. Data are reported as mean and SE of representative experiments and were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Data distribution from different groups was compared using the Kruskal-Wallis test with Dunn's multiple comparisons, and comparisons between two groups were explored using the Mann-Whitney test. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

Lu. longipalpis SGS induces neutrophil apoptosis

Different doses of *Lu. longipalpis* SGS (0.5–2.0 pairs/well) were capable of inducing apoptosis of neutrophils from C57BL/6 mice (**Fig. 1A** and **C**). Such effect was significantly higher than that observed in untreated controls (Fig. 1A and B). The occurrence of apoptosis was similar between the conditions containing diverse doses of SGS (Fig. 1A). We then decided to keep the lowest dose of SGS with biological effect in our model (0.5 pair of salivary gland/well) for further experiments.

Neutrophils exhibited markers of apoptosis up to 20 h upon incubation with SGS, such as PS exposure (Fig. 1D) and the pyknotic nuclei (Fig. 1E). At 3 h after stimulus with SGS, indicators had levels similar to those observed in unstimulated cells. Etoposide was used as a positive control to induce neutrophil apoptosis, and its effect was evident at 3 h by annexin-V detection (Fig. 1D) and 20 h by pyknotic nuclei analyses (Fig. 1E). These results confirm the proapoptotic effect of *Lu. longipalpis* SGS upon murine neutrophils.

Our further interest was to explore whether *Lu. longipalpis* SGS displays a proapoptotic effect on human neutrophils. To address this question, neutrophils obtained from healthy donors were incubated in the presence or absence of SGS or etoposide (Fig. 1F). Strikingly, 3 h after incubation, SGS induced human neutrophil apoptosis (Fig. 1F). At further times (6 and 20 h), this proapoptotic effect was no longer evident by comparison with negative control.

Neutrophil apoptosis induced by SGS is caspase-dependent and mediated by FasL

To evaluate the mechanisms triggered by *Lu. longipalpis* saliva to induce neutrophil apoptosis, we incubated C57BL/6 murine neutrophils with z-VAD, a pan-caspase inhibitor, for 30 min before addition of *Lu. longipalpis* SGS (**Fig. 2A**). Treatment of neutrophils with z-VAD prevented apoptosis induced by SGS, in contrast to treatment with the vehicle (DMSO) alone (Fig. 2A). Caspase activation can be induced by FasL, a molecule whose expression relates to susceptibility in *Leishmania* infection [22]. We then assessed FasL expression in neutrophils exposed to *Lu. longipalpis* SGS, which induced increased expression of FasL in neutrophils concerning intensity/cell (Fig. 2B) and also the percentage of neutrophils expressing FasL (Fig. 2C). Moreover, blockade of FasL prevented neutrophil apoptosis induced by *Lu. longiplapis* SGS (Fig. 2D). These results indicate that *Lu. longipalpis* SGS induces neutrophil apoptosis by a mechanism that involves activation of caspases and expression of FasL.

Lu. longipalpis SGS proteins induce neutrophil apoptosis

To depict initially the composition of the *Lu. longipalpis* salivary components responsible for the proapoptosis effect on neutrophils, we preincubated SGS with proteinase K before in vitro neutrophil stimulation. We observed a reduction of proapoptotic activity of SGS by incubation with proteinase K (**Fig. 3A**). This result suggests that apoptosis of neutrophils induced by *Lu. longipalpis* SGS is mediated by one or more proteic components.

Furthermore, as many evidences point out the immunogenicity of sandfly salivary proteins [13, 23, 24], we hypothesized that the

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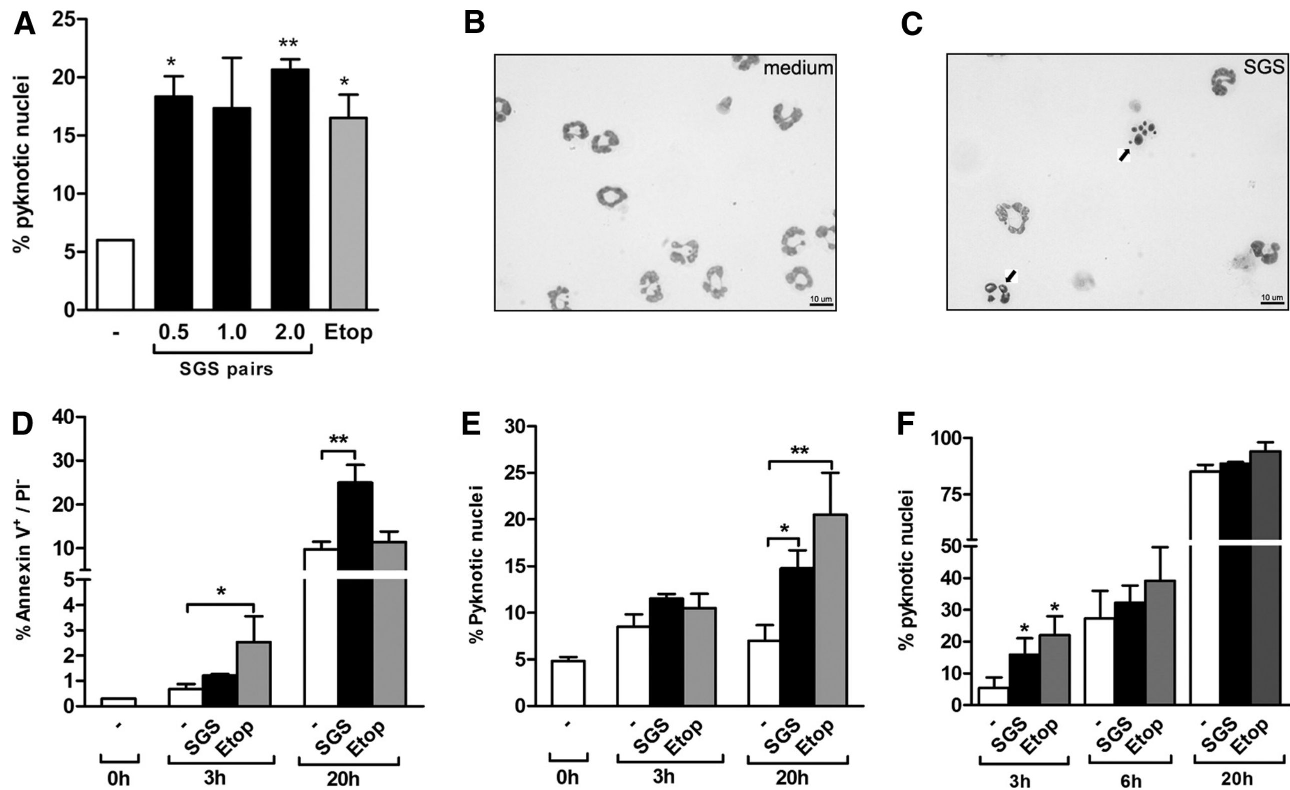

Figure 1. Effect of *Lu. longipalpis* SGS on neutrophil apoptosis. (A–E) Neutrophils from C57BL/6 mice were kept unstimulated (–) or stimulated with SGS or etoposide (Etop) 20 μM (positive control). (A) Neutrophil apoptosis induced by SGS in different doses was assessed by counting cells with pyknotic nuclei 20 h after stimulation. (B and C) Representative image of inflammatory neutrophils, unstimulated (B) or stimulated with *Lu. longipalpis* SGS (0.5 pair/well; original magnification, ×1000; C). Arrows point to neutrophil pyknotic nuclei. (D and E) Kinetic of neutrophil apoptosis in response to *Lu. longipalpis* SGS. Three hours and 20 h after stimulation, apoptosis was assessed by flow cytometry after annexin-V staining (D) and by counting cells with pyknotic nuclei (E) on Diff-Quick-stained cytospin preparations. (F) Human neutrophil apoptosis induced by SGS (0.5 pair/well). Data shown are from a single experiment that is representative of three independent experiments. $^*P \leq 0.05$; $^{**}P \leq 0.01$, compared with the unstimulated cells.

proteic component of the *Lu. longipalpis* saliva could be targets for the host's antibodies. To test this possibility, we preincubated the SGS with polled sera from hamsters pre-exposed to *Lu. longipalpis* bites. Strikingly, preincubation of SGS with specific antiserum completely abrogated induction of neutrophil apoptosis after 20 h in culture (Fig. 3B), reinforcing that components present in *Lu. longipalpis* saliva with proapoptotic activity are proteins and can be neutralized by antibodies.

Effect of *Lu. longipalpis* SGS in apoptosis and parasite burden of infected neutrophils

After determining the proapoptotic effect of *Lu. longipalpis* SGS, we evaluated whether *L. chagasi*, the parasite transmitted by this sandfly, can modify this effect in vitro. Analysis of PS exposure on inflammatory neutrophils demonstrated that *L. chagasi* was also able to induce neutrophil apoptosis (**Fig. 4A**). Moreover, this effect was exacerbated when neutrophils were coincubated with parasite and saliva (*L. chagasi* vs. *L. chagasi* plus SGS: 29.19% vs. 46.39%; Fig. 4A).

Neutrophils can act as important host cells for *Leishmania* [10, 25, 26]. As sandfly saliva exacerbates *Leishmania* infection [27], we investigated the infection of inflammatory neutrophils with *L. chagasi* in the presence of *Lu. longipalpis* SGS in vitro. Saliva increased the viability of *L. chagasi* inside neutrophils (Fig. 4B). Infection in the presence of etoposide did not enhance parasite burden in neutrophils compared with the control cultures infected with *L. chagasi* alone (Fig. 4B). Apoptotic neutrophils displayed a high number of parasites (Fig. 4C). To investigate whether neutrophil apoptosis induced by *Lu. longipalpis* saliva affects this increase of parasite burden in vitro, we pretreated the cultures with z-VAD (Fig. 4D), which abolished the increase in *L. chagasi* replication induced by SGS (Fig. 4D). COX activation is associated with an increase of *Leishmania* infection [28]. Herein, we evaluated the role of COX-2, an inflammatory form of COX, in the increase of parasite burden triggered by SGS. NS-398, a COX-2 inhibitor, led to an inhibition of viable parasite number (Fig. 4D) when added to the neutrophil culture before infection. Moreover, PGE_2, a product of COX-2, favors intracellular pathogen growth, a phenomenon that could be reverted by treatment with COX-2 inhibitors [29, 30]. Indeed, our experiments show that SGS increased production of PGE_2 by *Leishmania*-infected neutrophils (Fig. 4E).

As ROS production is a primarily important microbicidal mechanism from neutrophils, we evaluated the effect of SGS on

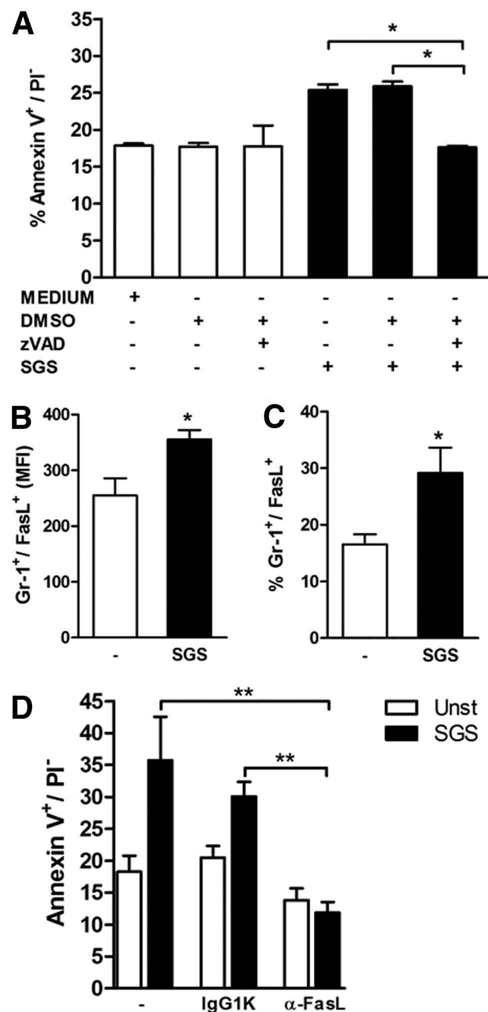

Figure 2. FasL expression and inhibition of neutrophil apoptosis by z-VAD and anti-FasL. (A) Neutrophils from C57BL/6 mice were pretreated with the pan-caspase inhibitor z-VAD (100 μM) or with vehicle (DMSO) before incubation with SGS. Twenty hours after incubation, apoptosis was assessed by annexin-V staining. (B and C) FasL expression induced by SGS on neutrophils was analyzed by flow cytometry 20 h after incubation. Results are expressed as the mean fluorescence intensity (MFI) (B) and percentage of FasL-expressing neutrophils on the Gr-1 population (C). (D) Mouse neutrophils were pretreated with neutralizing antibody specific for FasL (α-FasL; 10 μg/ml) or with IgGk1 (10 μg/ml). Apoptosis was assessed by annexin-V staining after 20 h. Data shown are from a single experiment representative of three independent experiments. –, Unstimulated (Unst) cells. $*P \leq 0.05$; $**P \leq 0.01$.

ROS production by these cells (Fig. 4E). Addition of SGS on the neutrophil cultures induced a partial reduction on ROS production 1 h after infection with *L. chagasi* (Fig. 4E). In summary, these results suggest that neutrophil apoptosis induced by *Lu. longipalpis* SGS favors *L. chagasi* infection by COX-2 activation and PGE_2 production, while reducing ROS generation.

CCL2/MCP-1 released by *L. chagasi*-infected neutrophils induces macrophage recruitment

We next examined whether supernatans from neutrophils incubated with *L. chagasi* and SGS are able to induce macrophage recruitment in vitro. We found that supernatants obtained from neutrophil cultures in the presence of *L. chagasi* could attract macrophages (**Fig. 5A**) and that *Lu. longipalpis* saliva induced a synergistic effect (Fig. 5A). Analyses of the MCP-1 (CCL2) revealed that neutrophils incubated with *L. chagasi* plus SGS produced significantly higher amounts of this chemokine (Fig. 5B). To investigate whether the macrophage recruitment was a result of production of CCL2/MCP-1 induced by *L. chagasi* plus SGS, we previously treated the neutrophils with bindarit, an inhibitor of CCL2/MCP-1 synthesis, before incubation with SGS, *L. chagasi*, or both. Treatment with bindarit resulted in total reduction of macrophage chemotaxis (Fig. 5B).Taken together, these results indicate that SGS synergizes with *L. chagasi* to enhance neutrophil apoptosis, CCL2/MCP-1 production, and macrophage recruitment.

DISCUSSION

The present study provides the first evidence that salivary components from a *Leishmania* vector play a relevant and direct role on neutrophils, which in turn, influence the *L. chagasi* parasite burden. We found that *Lu. longipalpis* salivary components induced neutrophil FasL-mediated and caspase-dependent apoptosis, and this event was associated with *Leishmania* survival inside these cells.

Neutrophils are now generally considered an initial target of *Leishmania* parasites [10, 31]. Significant numbers of neutrophils are present at the parasite inoculation site, as well as in lesions and draining LNs in *Leishmania* experimentally infected mice [11, 32–35]. Moreover, *Lu. longipalpis* SGS induces accumulation of neutrophils on an air-pouch model [20]. These experimental data are reinforced by the the fact that massive dermal neutrophilic infiltrates are noted in *Lu. longipalpis* [13] and *Phlebotomus duboscqi* bite sites [10], suggesting that accumulation of this cell type may be orchestrated, at least in part, by sandfly saliva constituents. Besides neutrophil recruitment, there are no previous reports about the

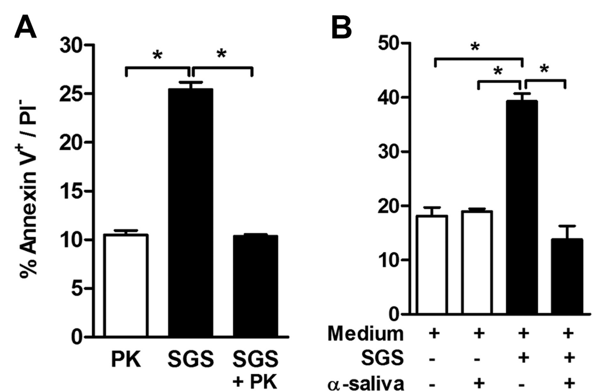

Figure 3. Inhibition of neutrophil apoptosis after *Lu. longipalpis* SGS treatment with proteinase K and α-saliva serum. Annexin-V staining from C57BL/6 mice neutrophils incubated for 20 h with SGS pretreated with proteinase K (PK; A) or with SGS preincubated for 1 h with anti-*Lu. longipalpis* saliva serum (B). Data shown are from a single experiment representative of three independent experiments. $*P \leq 0.05$.

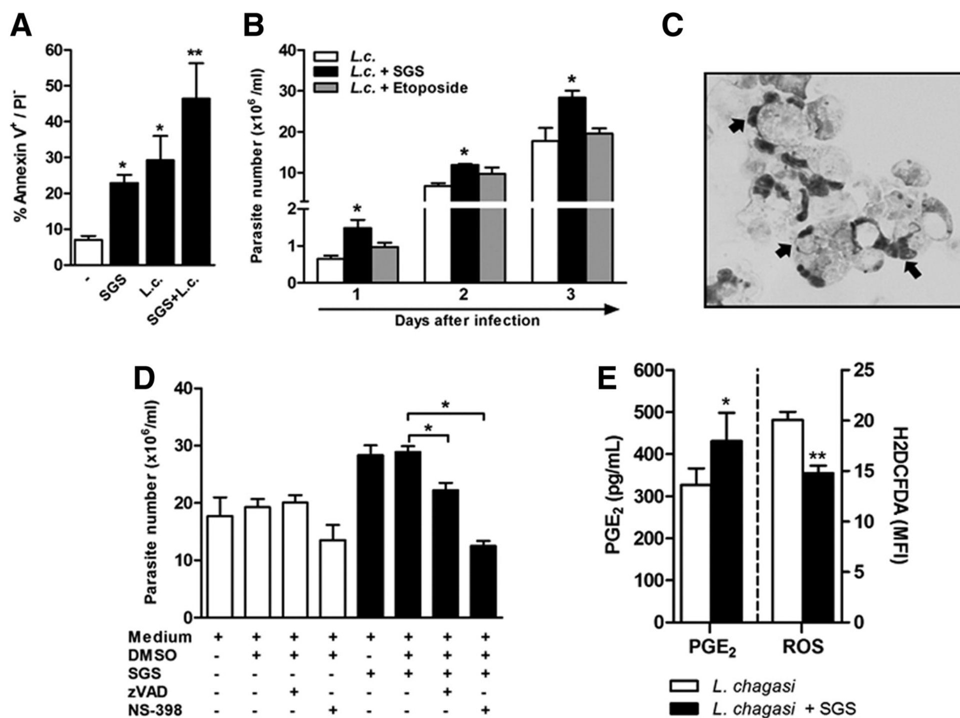

Figure 4. Effect of *Lu. longipalpis* SGS on neutrophil apoptosis and infection. (A) Inflammatory neutrophils from C57BL/6 mice were kept unstimulated (–) or stimulated with SGS (0.5 pair/well), *L. chagasi* (*L.c.*; 2:1) or SGS + *L. chagasi*. After 20 h, apoptosis was assessed by annexin-V staining. (B) In vitro neutrophil infection in the presence of SGS or etoposide (20 μM), followed by cultivation at 26°C and viable promastigote counts after 1, 2, and 3 days. (C) Representative image of *L. chagasi*-infected apoptotic neutrophils stimulated with *Lu. longipalpis* SGS (0.5 pair/well; original magnification, ×1000). Arrows point to infected apoptotic neutrophils. (D) Prior treatment of neutrophils with z-VAD (100 μM) and NS-398 (1 μM), followed by infection in the presence or absence of SGS. Viable promastigote counts were performed after 3 days. (E) PGE_2 levels of supernatants from neutrophils incubated for 20 h with *L. chagasi* and/or SGS (left side). ROS production by neutrophils cultured with *L. chagasi* for 1 h in the presence or absence of SGS (right side). Neutrophils were incubated with H_2DCFDA, and ROS production was evaluated by flow cytometry. Data shown are from a single experiment representative of three independent experiments. $^*P \leq 0.05$; $^{**}P \leq 0.01$.

further effects of sandfly saliva on neutrophils. Interestingly, studies performed with tick saliva reveal that the inhibition of critical functions of neutrophils favors the initial survival of spirochetes [36–38].

Our findings on human neutrophils confirm apoptosis induction by SGS and interestingly, indicate that mice and human neutrophils have a different kinetic of spontaneous and saliva-induced apoptosis. Notably, the apoptosis of human neutrophils induced by *Lu. longipalpis* SGS also indicates that this mechanism may be important for the pathogenesis of human disease. Indeed, phagocytosis of apoptotic human neutrophils increases parasite burden in macrophages infected with *Leishmania amazonensis* [28].

It is likely that proteins from SGS trigger neutrophil apoptosis, as reincubation of *Lu. longipalpis* SGS with proteinase K abrogated its proapoptosis effect. Additionally, antisaliva serum was able of block neutrophil apoptosis. This is particularly interesting, as it reinforces the idea of a host protection mediated by the immune response against sandfly saliva, allowing for the development of an immune response against *Leishmania*. Interestingly, SGS-induced neutrophil apoptosis was associated with caspases and FasL expression. Previous studies have implicated FasL in neutrophil apoptosis [39]. Likewise, turnover of neutrophils mediated by FasL drives *Leishmania major* infection [22]. Further studies are necessary to deeply address this observation.

Our results demonstrate that SGS increases the neutrophil leishmanial burden by inducing neutrophil apoptosis, as inhibition of apoptosis by z-VAD reduced the viable parasite numbers in vitro. Indeed, treatment with z-VAD blocks lymphocyte apoptosis and increases in vitro and in vivo resistance to *Trypanosoma cruzi* infection [30, 40]. van Zandbergen and colleagues [12] have proposed that infected apoptotic neutrophils can serve as "Trojan horses" for *Leishmania*. Alternatively, uptake of parasites egressing from dying neutrophils in an anti-inflammatory environment created by the phagocytosis of these cells, per se, could favor the infection ("Trojan rabbit" strategy) [41]. Our findings that *Lu. longipalpis* SGS could favor neutrophil apoptosis and infection by *L. chagasi* seem to give support to either of these two proposed hypotheses.

We found that neutrophil infection in the presence of SGS induced PGE_2 release, but was decreased in the presence of COX-2 inhibitor NS-398, indicating the participation of COX-2 products in parasite survival. Indeed, PGE_2, a major product from COX-2, facilitates *Leishmania* infection by deactivating macrophage microbicidal functions [19, 28–30]. Moreover, addition of exogenous PGE_2 to macrophage cultures induces a marked enhancement of *Leishmania* infection [19, 42]. Exposure of neutrophils to SGS caused a marked reduction of ROS production, which is a primarily important microbicidal mechanism of neutrophils. In this regard, *Lu. longipalpis* salivary proteins could be contributing to deactivation of the neutrophil inflammatory response, favoring the early steps of *Leishmania* infection. Taken together, our data suggest that the presence of sandfly SGS drives an anti-inflammatory response in *L. chagasi*-infected neutrophils by initially reducing ROS production, favoring the parasite survival. Furthermore, SGS could be triggering neutrophil deactivation through induction of apoptosis, activation of COX-2, and PGE_2 production by these cells. *L. major* promastigotes drive a selective fusion of azuro-

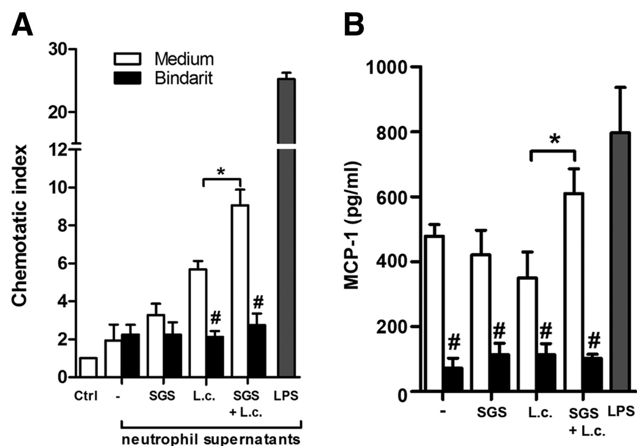

Figure 5. Macrophage recruitment and CCL2/MCP-1 release by *L. chagasi*-infected neutrophils in the presence of *Lu. longipalpis* SGS. (A) Macrophages were allowed to migrate toward supernatants from neutrophils infected or not with *L. chagasi* in the presence or absence of SGS (white bars), as described in Materials and Methods. Migration toward supernatants from bindarit-pretreated neutrophils (black bars). Following incubation, the migrated macrophages were counted, and the chemotatic index was calculated. Crtl, Negative control of radom chemotaxis. (B) CCL2/MCP-1 production (white bars) in the supernatants of neutrophil cultures after 20 h and its inhibition by bindarit pretreatment (black bars). Data are representative of two independent experiments performed in triplicate for each sample. *$P \leq 0.05$; #$P \leq 0.05$, compared with no bindarit-treated neutrophils.

philic granules into parasite-containing phagosomes in human neutrophils [43]. It remains to be elucidated whether, in the present system, SGS modulates neutrophil granule mobilization and contributes to early *L. chagasi* survival.

Macrophages are the preferential host cells for *Leishmania*, and the recruitment of these cells could provide safe havens for the parasite [31]. Neutrophils infected by *L. major* produce chemokines such as MIP-1β [12, 44], and sandfly SGS leads to increased expression of the macrophage chemokine MCP-1 at the site of injection [20], leading to macrophage recruitment. We have shown here that neutrophils infected with *L. chagasi* in the presence of SGS displayed higher MCP-1 production, corroborating with macrophage recruitment. This result was reinforced with the use of bindarit, an original indazolic derivative that has been shown the ability to inhibit CCL2/MCP-1 synthesis [45]. As a matter of fact, *L. chagasi*-infected neutrophil supernatants are able to recruit mouse macrophages, even though they did not induce significant MCP-1 production, which suggests that other chemotatic factors could be implicated in this event. A direct chemotatic activity of sandfly saliva has been described with several experimental models [13, 20, 46]. Herein, we also report an indirect chemotactic effect of SGS by inducing chemokine production by neutrophils.

In summary, our data demonstrate that *Lu. longipalpis* saliva orchestrates FasL- and caspase-dependent apoptosis of neutrophils. At the same time, saliva proapoptosis activity is of benefit to the parasite and may represent an important mechanism to facilitate *Leishmania* infection. These results contribute to a better understanding of the interactions between vector saliva and neutrophils in innate immunity to *Leishmania* infection.

AUTHORSHIP

D.B.P., T.A-S., B.B.A., M.B-N., V.M.B., and A.B. conceived of and designed the experiments. D.B.P., T.A-S., N.F.L., J.C., B.B.A., L.A., and J.F-C. performed the experiments. D.B.P., T.A-S., J.C., L.A., M.B-N., V.M.B., and A.B. analyzed the data. J.C.M., M.B-N., V.M.B., and A.B. contributed reagents/materials/analysis tools. D.B.P., T.A-S., B.B.A., M.B-N., and V.M.B. wrote the paper. D.B.P., T.A-S., B.B.A., P.T.B., G.A.D., C.B., M.B-N., V.M.B., and A.B. participated in critical discussion of the manuscript.

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KEY WORDS:
Leishmania chagasi · sand fly · cell death · FasL · chemotaxis

Heme Impairs Prostaglandin E_2 and TGF-β Production by Human Mononuclear Cells via Cu/Zn Superoxide Dismutase: Insight into the Pathogenesis of Severe Malaria

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In many hemolytic disorders, such as malaria, the release of free heme has been involved in the triggering of oxidative stress and tissue damage. Patients presenting with severe forms of malaria commonly have impaired regulatory responses. Although intriguing, there is scarce data about the involvement of heme on the regulation of immune responses. In this study, we investigated the relation of free heme and the suppression of anti-inflammatory mediators such as PGE_2 and TGF-β in human vivax malaria. Patients with severe disease presented higher hemolysis and higher plasma concentrations of Cu/Zn superoxide dismutase (SOD-1) and lower concentrations of PGE_2 and TGF-β than those with mild disease. In addition, there was a positive correlation between SOD-1 concentrations and plasma levels of TNF-α. During antimalaria treatment, the concentrations of plasma SOD-1 reduced whereas PGE_2 and TGF-β increased in the individuals severely ill. Using an in vitro model with human mononuclear cells, we demonstrated that the heme effect on the impairment of the production of PGE_2 and TGF-β partially involves heme binding to CD14 and depends on the production of SOD-1. Aside from furthering the current knowledge about the pathogenesis of vivax malaria, the present results may represent a general mechanism for hemolytic diseases and could be useful for future studies of therapeutic approaches. ***The Journal of Immunology*, 2010, 185: 1196–1204.**

Severe malaria is a highly lethal condition and a major health threat in many tropical countries. Multiple factors have been implicated in the pathogenesis of the severe complications of this condition, such as uncontrolled cytokine production (1, 2), hemolysis (3), and erythropoiesis suppression (4). Severe malaria was firstly described as originating from *Plasmodium falciparum* infection (5), but severe cases, including those with lethal outcomes, have also been observed from *Plasmodium vivax* infections (6–8). One of the major factors thought to be involved in sustaining systemic inflammation is the release of free heme, as a consequence of hemolysis inherent to the life cycle of *Plasmodium* within RBCs (9). Recently, heme has been implicated in the pathogenesis of severe forms of malaria in mice (10, 11). Under homeostasis, the heme released from hemoproteins such as cell-free hemoglobin (Hb) is scavenged by plasma proteins such as hemopexin or albumin as well as by lipoproteins (12). However, these proteins can be depleted during severe hemolytic conditions, such as associated with *Plasmodium* infection (13). This leads to the accumulation of free Hb tetramers in the plasma (14), which dissociate spontaneously into dimers. In the presence of reactive oxygen species (ROS) or other free radicals, cell-free Hb dimers are readily oxidized into methemoglobin, releasing their heme prosthetic groups (12). As a consequence, in malaria and other hemolytic disorders, the concentrations of heme can reach levels of up to 50 μM in the bloodstream (15), which can trigger an intense oxidative burst and unspecific tissue damage (11). Moreover, a crystal form of heme molecules produced by *Plasmodium sp.*, and referred to as hemozoin, also acts as a proinflammatory agonist and thus could be associated with the development of severe forms of malaria (16–18). Hemozoin inhibits PGE_2 production in both mice (19) and humans (20, 21), and there is an inverse relationship between PGE_2 and blood mononuclear cell cyclooxygenase-2 with disease severity in children with *P. falciparum* malaria (22). Until now there is no clear description of the effect of free heme on the PGE_2 production.

During malaria infection, superoxide anions are thought to be the main form of ROS produced (23). In this context, the antioxidant enzyme Cu/Zn superoxide dismutase (SOD-1) is activated and may display an important role in the pathological oxidative injury. Notwithstanding, SOD-1 has been linked to an increased inflammatory activity by amplifying TNF-α production on macrophages (24). In addition, overexpression of SOD-1 increases NF-κB–related rapid responses, such as immune response and antiapoptosis factors (25). Therefore, studies have correlated SOD-1 activity with

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Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; A, asymptomatic; ALT, alanine aminotransferase; CoPPIX, cobalt protoporphyrin IX; CRP, C-reactive protein; DETC, diethyldithiocarbamate; Hb, hemoglobin; HO-1, heme oxygenase-1; M, mild; NAC, *N*-acetyl-L-cysteine; NI, noninfected individual; PPIX, protoporphyrin IX; ROS, reactive oxygen species; S, severe; siRNA, small interfering RNA; SnPPIX, Tin protoporphyrin IX; SOD-1, Cu/Zn superoxide dismutase.

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Heme Oxygenase-1 Promotes the Persistence of *Leishmania chagasi* Infection

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Visceral leishmaniasis (VL) remains a major public health problem worldwide. This disease is highly associated with chronic inflammation and a lack of the cellular immune responses against *Leishmania*. It is important to identify major factors driving the successful establishment of the *Leishmania* infection to develop better tools for the disease control. Heme oxygenase-1 (HO-1) is a key enzyme triggered by cellular stress, and its role in VL has not been investigated. In this study, we evaluated the role of HO-1 in the infection by *Leishmania infantum chagasi*, the causative agent of VL cases in Brazil. We found that *L. chagasi* infection or lipophosphoglycan isolated from promastigotes triggered HO-1 production by murine macrophages. Interestingly, cobalt protoporphyrin IX, an HO-1 inductor, increased the parasite burden in both mouse and human-derived macrophages. Upon *L. chagasi* infection, macrophages from *Hmox1* knockout mice presented significantly lower parasite loads when compared with those from wild-type mice. Furthermore, upregulation of HO-1 by cobalt protoporphyrin IX diminished the production of TNF-α and reactive oxygen species by infected murine macrophages and increased Cu/Zn superoxide dismutase expression in human monocytes. Finally, patients with VL presented higher systemic concentrations of HO-1 than healthy individuals, and this increase of HO-1 was reduced after antileishmanial treatment, suggesting that HO-1 is associated with disease susceptibility. Our data argue that HO-1 has a critical role in the *L. chagasi* infection and is strongly associated with the inflammatory imbalance during VL. Manipulation of HO-1 pathways during VL could serve as an adjunctive therapeutic approach. *The Journal of Immunology*, 2012, 188: 000–000.

Visceral leishmaniasis (VL) continues to be a major health threat worldwide and is classified as one of the most neglected diseases by the World Health Organization. VL is a chronic infection clinically characterized by progressive fever, weight loss, splenomegaly, hepatomegaly, anemia, and spontaneous bleeding associated with marked inflammatory imbalance (1). The hallmark of this disease is thought to be a lack of cellular immune responses against the parasite and high systemic levels of IFN-γ and IL-10 (2). The New World *Leishmania infantum chagasi* is the major species implicated in the VL in Brazil. *Leishmania* parasites are obligate intracellular protozoa that replicate preferentially inside macrophages (3). It is well known that *L. chagasi* is able to evade pro-oxidative responses and other macrophage effectors mechanisms (4), possibly hampering the activation of adaptive immune responses against infection (5). During parasite–host interactions, complex signaling pathways are triggered by the recognition of key molecules from parasite (4). In this context, lipophosphoglycan (LPG), a glycoconjugate expressed on the surface of *Leishmania* parasites and TLR2 agonist (6, 7), has been implicated in the modulation of a wide range of innate immune functions. Those may include resistance to complement, attachment and entry into macrophages, protection against proteolytic damage within acidic vacuoles (8), inhibition of phagosomal maturation (9), modulation of NO and IL-12 production (10–13), inhibition of protein kinase C (14), induction of neutrophil extracellular traps (15), and induction of protein kinase R (16). However, specific aspects of how the parasites regulate some protective responses are still unknown. Moreover, it is not fully understood whether LPG from *Leishmania* is the major regulator of the effectors pathways associated with the protective responses against this protozoan.

Excess of heme is very hazardous for the cells, and we have previously shown that heme suppresses some anti-inflammatory mediators in human malaria caused by *Plasmodium vivax* (17). Heme oxygenase-1 (HO-1) is a stress-responsive enzyme that

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Abbreviations used in this article: BMM, bone marrow-derived macrophage; CoPP, cobalt protoporphyrin IX; DHE, dihydroethidium; HC, healthy control; HO-1, heme oxygenase-1; LPG, lipophosphoglycan; PPARγ, peroxisome proliferator-activated receptor γ; PTX, pentoxifylline; ROC, receiver-operator characteristic; ROS, reactive oxygen species; SOD-1, Cu/Zn superoxide dismutase; VL, visceral leishmaniasis; WT, wild-type.

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Control of *Mycobacterium fortuitum* and *Mycobacterium intracellulare* infections with respect to distinct granuloma formations in livers of BALB/c mice

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Mycobacterium fortuitum *is a rapidly growing nontuberculous* Mycobacterium *that can cause a range of diseases in humans. Complications from* M. fortuitum *infection have been associated with numerous surgical procedures. A protective immune response against pathogenic mycobacterial infections is dependent on the granuloma formation. Within the granuloma, the macrophage effector response can inhibit bacterial replication and mediate the intracellular killing of bacteria. The granulomatous responses of BALB/c mice to rapidly and slowly growing mycobacteria were assessed in vivo and the bacterial loads in spleens and livers from* M. fortuitum *and* Mycobacterium intracellulare*-infected mice, as well as the number and size of granulomas in liver sections, were quantified. Bacterial loads were found to be approximately two times lower in* M. fortuitum*-infected mice than in* M. intracellulare*-infected mice and* M. fortuitum*-infected mice presented fewer granulomas compared to* M. intracellulare*-infected mice. These granulomas were characterized by the presence of Mac-1$^+$ and CD4$^+$ cells. Additionally, IFN-γ mRNA expression was higher in the livers of* M. fortuitum*-infected mice than in those of* M. intracellulare*-infected mice. These data clearly show that mice are more capable of controlling an infection with* M. fortuitum *than* M. intracellulare. *This capacity is likely related to distinct granuloma formations in mice infected with* M. fortuitum *but not with* M. intracellulare.

Key words: *Mycobacterium fortuitum* - *Mycobacterium intracellulare* - granuloma - liver - control of infection

Nontuberculous mycobacteria (NTM) include different species of the genus *Mycobacterium* that do not belong to the *Mycobacterium tuberculosis* complex. These include both slowly growing [e.g., *Mycobacterium avium-intracellulare* (MAI)] and rapidly growing (e.g., *Mycobacterium fortuitum* and *Mycobacterium abscessus*) species (Runyon 1959). NTM are human opportunistic pathogens and are predominantly acquired from the environment. A large number of NTM species have been recovered from soil, household dust, water, dairy products, cold-blooded animals, vegetation and human faeces (Ho et al. 2006). These species can also colonize surgical equipment and materials, such as endoscopes and solutions (Brown-Elliott & Wallace 2005).

In humans, NTM are organisms that belong to a heterogeneous group in which each species of bacteria should be studied separately (Alvarez-Uria 2010). These pathogens can cause a range of diseases affecting a variety of tissues, including the lungs, lymph nodes, skin and soft and skeletal tissue. These diseases can also affect the genitourinary systems and cause disseminated infections (Ho et al. 2006, Griffith et al. 2007, Jarzembowski & Young 2008). MAI is primarily a pulmonary pathogen and is the NTM species most commonly associated with human disease (Griffith et al. 2007). Inhalation of this bacterium may cause pulmonary disease, whereas the ingestion of contaminated water may cause a disseminated disease. A cutaneous manifestation can be attributed to direct inoculation, direct contact or disseminated disease (Weitzul et al. 2000). Infections caused by rapidly growing NTM including *M. fortuitum* can appear after surgical procedures, such as liposuction, silicone injection and breast implantation, or after intravenous catheter insertion, exposure to prosthetic material and pacemaker placement (Sungkanuparph et al. 2003, Palwade et al. 2006, Uslan et al. 2006). There is still no defined optimal treatment for NTM infections because these organisms are resistant to the standard antituberculous agents. In addition, susceptibility to anti-mycobacterial agents varies across different NTM species (ATS 1997).

A protective immune response against pathogenic mycobacterial infections depends on the ability of individuals to form organ granulomas. During infection, mycobacteria induce the formation of these organized immune complexes of differentiated macrophages, lymphocytes and other cells, which are critical for the maintenance of the granuloma architecture and for the restriction of the infection. In the centre of the granuloma, macrophages produce a response that can effectively prevent the replication of bacteria and/or mediate the killing of the intracellular pathogen. On the other hand, compromised granuloma formation is accompanied by dissemination. In addition, the course of the infection in individuals that

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