UNIVERSIDADE FEDERAL DA BAHIA
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FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISA GONÇALO MONIZ

Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

ATIVAÇÃO DA HEME OXIGENASE-1 E VIA DA NECROPTOSE COMO MECANISMOS IMUNOPATOGÊNICOS NA INFECÇÃODE MACRÓFAGOS POR *Leishmania infantum*

NÍVEA FARIAS LUZ

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A meus pais Domingos e Sônia, minhas asas e raízes

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RESUMO

A Leishmaniose visceral (LV) apresenta ampla distribuição geográfica e é fatal caso não seja tratada. As manifestações hematológicas são constantes na LV e em casos não tratados os pacientes evoluem à óbito por sangramento maciço ou anemia grave. Neste cenário, mecanismos ligados à morte celular, hemólise, metabolismo do heme e atividade da enzima heme oxigenase podem estar envolvidos na imunopatogênese da LV. A heme oxigenase (HO) tem importantes propriedades regulatórias e está envolvida em processos fisiológicos e patofisiológicos como citoproteção e inflamação. Nesse projeto testamos a hipótese de que a ativação da enzima heme oxigenase-1 (HO-1) favorece a infecção por *Leishmania infantum chagasi*, principal agente etiológico da LV humana no Brasil e de que mecanismos de morte celular inflamatória induzida por heme estão associados com a resistência ao parasita. Nossas observações nesse trabalho indicam que a enzima HO-1 é induzida em macrófagos durante a infecção por *L. chagasi* e que a indução farmacológica da HO-1, pela CoPP aumenta a carga parasitária de macrófagos infectados por *L. chagasi* e reduz a produção de mediadores pró-inflamatórios. Além disso, a HO-1 favorece um ambiente anti-inflamatório onde prevalece a presença de IL-10 sobre a de TNF. Macrófagos derivados de medula óssea de camundongos deficientes no gene HO-1 têm menor carga parasitária, quando infectados por *L. chagasi* em comparação aos macrófagos de camundongos selvagens. Além disso, pacientes com LV apresentam maiores níveis de heme-oxigenase 1 e de heme no soro. Nossas observações indicam que heme é capaz de induzir necroptose em macrófagos humanos, e de que moléculas da via da necroptose estão associadas com a resistência na infecção por *Leishmania*. A molécula RIPK1 controla a replicação de *Leishmania* por um mecanismo independente da produção de IL-1β, enquanto que a molécula PGAM5 depende de IL-1βpara controlar o crescimento do parasita. Por fim, encontramos que essas proteínas participam do controle da replicação por *Leishmania* em um modelo experimental de Leishmaniose cutânea. Esses achados indicam um potencial deletério para a HO-1 na infecção por *L. chagasi*, e um papel protetor da necroptose na infecção por*Leishmania.*

Palavras-chave: Macrófago, *Leishmania,* Necroptose, Heme-Oxigenase, Heme

LUZ, Nivea Farias. Activation of heme oxygenase-1 and necroptosis pathway as immunopathogenic mechanisms in macrophages infection with *Leishmania Infantum.* 139 f. il. Tese (Doutorado) – Universidade Federal da Bahia. Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

ABSTRACT

Visceral leishmaniasis (VL) is a widespread disease and is fatal if left untreated. Hematological manifestations are common in VL and untreated patients evolve to death from massive bleeding and severe anemia. In this scenario, mechanisms related to cell death pathways, hemolysis, heme metabolism and enzymatic activity of heme oxygenase may be involved in the immunopathogenesis of the disease. Heme oxygenase (HO) has important regulatory properties and is involved in patho-physiological processes such as cytoprotection and inflammation. This project tested the hypothesis that heme oxygenase- 1 (HO-1) activation favors *Leishmania infantum chagasi* infection, the main etiologic agent of human VL in Brazil, we also tested whether heme induced inflammatory cell death pathways are involved in resistance to *Leishmania* infection. Our observations indicate that HO-1 is induced in macrophages infected with *L. infantum chagasi* and pharmacological induction for HO-1 by CoPP increases parasite load of infected macrophages and reduces production on inflammatory mediators. In addition, HO-1 contributes to the anti inflammatory pathway that favors *L. chagasi* replication through a higher IL-10/TNF-α ratio in macrophages. We also observed that bone marrow derived macrophages knockout to HO-1 gene have a significant lower parasite load when infected by *L. infantum chagasi* than their wild type counterparts. Beyond this, we found that patients with VL presented higher systemic concentrations of HO-1 and heme than healthy individuals. We found that heme is able to induce programmed necrosis "*necroptosis*" in human cells and that molecular players from necroptosis pathway contribute to resistance to *Leishmania* infection. RIPK1 controls *Leishmania r*eplication through a mechanism independent of IL-1β production, while PGAM5 requires IL-1β to control *Leishmania* replication. Finally, we found that RIPK1 and PGAM5 play an important role in controlling *Leishmania* replication in a cultaneous leishmaniasis experimental model. Our findings argue that HO-1 has a critical role in *L. chagasi* replication and necroptosis pathway is involved in resistance against *Leishmania* infection.

Key-words: Macrophage, *Leishmania*, Necroptosis, Heme-Oxygenase, Heme

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BACH 1 *basic leucine zipper transcription factor*

HBV Vírus da HepatiteB

IFN Interferon

IL-10 Interleucina 10

IL-1β Interleucina 1 β

IL-6 Interleucina6

iNOS óxido nítrico sintetase induzível

LPS Lipopolissacarídeo

MCP Proteína Quimiotática de Monócitos do inglês "*Monocyte chemoattractant protein*"

Myd88 *Myeloid Differentiation Primary Response88*

NADPH Nicotinamida adenina dinucleotídeo P

NLRP3 *pyrin domain containing3.*

NRF2 fator receptor nuclear "*nuclear receptor factor*"

PGE2 Prostaglandina E2

PKC Proteína quinase C, do inglês *protein kinase*C

PPAR-γ receptor ativado da proliferação de peroxissomos, do inglês "*peroxisome proliferator-activatedreceptors*"

ROS Espécies reativas de oxigênio, do inglês "*reactive oxygenspecies*"

TGF-β Fator transformante de crescimento β "*Transforming growth factor β*" Th célula T auxiliadora, "*Thelper*"

TLR *Toll-like receptors*

TNF fator de necrose tumoral

UV Ultravioleta

ZnPP Zinco protoporfirina "Zinc protophorfirynIX"

SUMÁRIO

1. INTRODUÇÃO

1.1 ASPECTOS GERAIS DA LEISHMANIOSE E CICLO DO PARASITA

As Leishmanioses são endêmicas em mais de 80 países, distribuídos pela África, Ásia, Europa e América Latina. Esta doença apresenta uma prevalência estimada de 12 milhões e uma incidência de 2 milhões de casos por ano em todo o mundo. Além disso, estima-se que 350 milhões de pessoas estão em risco de contrair Leishmaniose (WHO, 2010).

As Leishmanioses são enfermidades causadas por protozoários intracelulares obrigatórios da ordem Kinetoplastida, família Trypanosomatidae, gênero *Leishmania*, que acometem o homem e diferentes espécies de animais silvestres e domésticos em vários países da América Latina, África, Índia, parte da Ásia ocidental e central e alguns países europeus próximos ao Mediterrâneo (WHO, 2010). A Leishmaniose é uma doença multifatorial, caracterizada pela diversidade e complexidade da resposta do hospedeiro, agentes etiológicos e vetores, uma vez que pode ser causada por mais de 20 espécies de *Leishmania* e transmitida para os humanos por aproximadamente 30 espécies de flebotomíneos vetores (PEARSON; SOUSA, 1996).

A Leishmania trata-se de um parasito digenético encontrado na forma promastigota (flagelada) no trato digestivo do hospedeiro invertebrado (vetores flebotomíneos) e na forma amastigota nos hospedeiros vertebrados. A transmissão natural da *Leishmania* ocorre pela picada das fêmeas infectadas durante a alimentação pelo repasto sanguíneo, momento em que esses vetores infectados injetam a saliva juntamente com os parasitos na forma promastigota no hospedeiro vertebrado (DE ALMEIDA et al., 2003). Essas formas flageladas são fagocitadas por macrófagos do hospedeiro e transformam-se em formas amastigotas que se replicam no interior desta célula. O ciclo é completado quando o hospedeiro vertebrado torna- se uma nova fonte de parasitas para os vetores, quando estes, ao se alimentarem, ingerem sangue e células infectadas, revisto por (ANDRADE et al., 2007) (**Figura 1**).

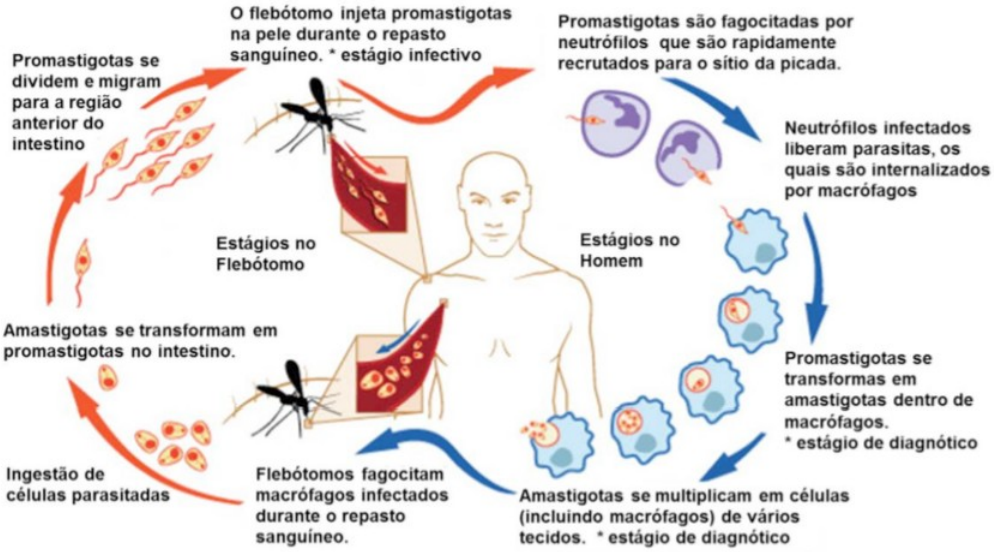

Figura 1. Ciclo biológico da *Leishmania* (traduzido e adaptado de http://www.niaid.nih.gov/topics/leishmaniasis/pages/lifecycle).

Embora parasitas do gênero *Leishmania* sejam capazes de interagir e infectar uma vasta variedade de células do hospedeiro, o macrófago é considerado uma das células mais importantes na determinação do curso de infecção por este parasito. Tanto o controle quanto o estabelecimento dos parasitas é determinado pelo grau de ativação dessas células (PODINOVSKAIA; DESCOTEAUX, 2015). Após internalização do parasita pelos macrófagos, a *Leishmania* sobrevive e multiplica em ambiente ácido conhecido como vacúolo parasitóforo.

Os macrófagos são as principais células efetoras responsáveis pela destruição dos parasitas. Essas células podem produzir óxido nítrico e espécies reativas de oxigênio, as quais são moléculas tóxicas para o parasita (ARANGO DUQUE; DESCOTEAUX, 2015; GANTT et al., 2009). A infecção por *L. amazonensis* em macrófagos deflagra ainda a ativação de NF-kB (fator nuclear kappa B) o qual desempenha um papel importante na regulação da resposta imune por regular a expressão de quimiocinas, citocinas e enzimas associadas com o favorecimento ou inibição da replicação do parasita (CALEGARI-SILVA et al., 2009, 2015).

Entender como os parasitas do gênero *Leishmania* são capazes de sobreviver e manipular a célula hospedeira favorecendo sua sobrevivência e transmissão é extremamente

importante para o desenvolvimento de novas drogas ou terapias estratégicas contra a doença causada por esse parasita.

1.1.1 Formas Clínicas e Epidemiologia da Leishmaniose

As Leishmanioses apresentam-se sob diversas características clínicas e epidemiológicas, podendo ser agrupadas em: Leishmaniose tegumentar e Leishmaniose visceral ou calazar (CHAPPUIS et al., 2007).

A Leishmaniose tegumentar pode manifestar-se em três formas principais de expressão clínica: Leishmaniose cutânea localizada (LCL), Leishmaniose mucocutânea (LCM) e Leishmaniose cutânea difusa (LCD). A Leishmaniose tegumentar está entre as seis doenças infecto-parasitárias de maior importância segundo a Organização Mundial da Saúde (OMS). A Leishmaniose tegumentar é prevalente em 82 países e sua incidência é estimada em 1,5 milhões de casos por ano. Mais de 90% dos casos ocorrem na África (principalmente em Marrocos, Etiópia e Tunísia), Oriente Médio (principalmente no Afeganistão, Paquistão, Irã, Iraque, Síria e Arábia Saudita) e América Latina (principalmente no Brasil, a Bolívia, Colômbia, Equador, Peru e Venezuela) (WHO, 2010). Na Bahia a Leishmaniose tegumentar tem maior impacto epidemiológico na região Sul e Sudoeste do Estado, onde os municípios de Jequié, Vitória da Conquista, Itagí, Itagiba, Apuarema e Contendas do Sincorá são os mais afetados. Em 2000 o povoado de São Gonçalo, localizado no município de Contendas do Sincorá, foi acometido por um surto epidêmico que atingiu 22,5% da população (BACELLAR, 2005; COSTA, 2005; DIAS et al., 2007).

A LCL é a forma mais freqüente da Leishmaniose tegumentar e pode ser causada por *L. braziliensis*, *L. amazonensis* e *L. guyanensis*. Esta forma caracteriza-se por uma ou mais lesões cutâneas ulceradas, mantidas por uma eficiente resposta de células T, que geralmente favorece uma boa reação à terapia antimonial tradicional e tendem à cicatrização (BARRAL- NETTO et al., 1986; SALMAN; RUBEIZ; KIBBI, 1999). No Brasil, a LCD é causada pela *L. (L.) amazonensis*, sendo a maioria dos casos relatados no Maranhão (40%), Pará (20%), Bahia (12,5%), Mato Grosso (10%) e em menor intensidade em estados do Norte do país (COSTA et al., 2005). Já a LCM caracteriza-se por escassez de parasitos na lesão e reação imune exacerbada com intensa destruição tecidual. Essa forma da doença acomete as mucosas oral, nasal, faríngea e laríngea, levando à lesões desfigurantes (STRAZZULLA et al., 2013). Estima-se que 3% dos casos de Leishmaniose tegumentar causados por *L. braziliensis* desenvolvam lesão cutânea mucosa (BOAVENTURA et al., 2009). Por fim, a Leishmaniose visceral visceral (LV) é considerada a forma clínica mais

grave entre as leishmanioses, consistindo de infecção crônica que apresenta altas taxas de morbidade e mortalidade em muitos países em desenvolvimento. A maioria das espécies que causam Leishmaniose visceral (LV) pertencem ao complexo *L. infantum* (LUKES et al., 2007).

1.1.2 Aspectos Gerais da LV

Existem aproximadamente 500.000 casos de LV e mais de 50.000 mortes dessa doença por ano no mundo, um número que só é ultrapassado entre as doenças parasitárias pela malária (WHO, 2010). A maioria dos casos (>90%) ocorre em apenas seis países: Bangladesh, Índia, Nepal, Sudão, Etiópia e Brasil (**Figura 2),** afetando principalmente comunidades pobres, geralmente em áreas rurais remotas (CHAPPUIS et al., 2007). Os sintomas mais prevalentes da LV 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). Altos níveis de mortalidade estão normalmente associados com uma co-infecção com HIV (OKWOR; UZONNA, 2013) e/ou bactérias e hemorragia (ABDELMOULA et al., 2003). 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 (SAMPAIO et al., 2010). A gravidade em casos pediátricos de LV está associada com altos níveis de citocinas pro-inflamatórias séricas (COSTA et al., 2013).

Figura 2- Distribuição da leishmaniose visceral nomundo
Fonte- Chappuis, *et al.,*2007

Os sinais e sintomas da LV são apresentados após um período de incubação de 2 a 6 semanas de infecção, consistindo de inflamação sistêmica persistente e invasão parasitária do sangue e do sistema retículo endotelial, assim como aumento de linfonodos, baço e fígado (CHAPPUIS et al., 2007). As manifestações hematológicas são constantes na LV. As anormalidades mais frequentes relatadas envolvem pancitopenia, anemia megaloblástica e ferropriva, síndrome hemolítica e alterações do sistema de coagulação sanguínea. Diversos mecanismos patogênicos estão envolvidos neste processo, incluindo hiperesplenismo, mecanismos imunológicos, auto-imunidade e disfunção da medula-óssea (BACELLAR; CARVALHO, 2008). A fadiga e fraqueza são agravadas pela anemia, que é causada pelo estado de inflamação persistente, hiperesplenismo (a destruição periférica dos eritrócitos no baço) e algumas vezes pelo sangramento (AL-JURAYYAN et al., 1995).

Mecanismos ligados à hemólise, metabolismo do heme e ativação da enzima heme oxigenase podem estar envolvidos no sangramento, na fase da doença que envolve coagulação e anemia grave e, especialmente na interação de células do sistema imune com o parasito. Entretanto, o eixo heme-heme oxigenase na doença permanece por ser explorado. Assim, na primeira parte desse trabalho buscamos investigar o papel da enzima heme oxigenase-1 (HO-1) na resposta à infecção por *L. infantum chagasi*.

1.2 O HEME E SEUS EFEITOS

O Heme é um composto metálico hidrofóbico que contém um átomo de ferro no seu anel protoforfirínico. O heme ferroso (Fe^{2+}) têm uma carga química neutra, enquanto que o heme férrico (Fe^{3+}) tem uma carga positiva, e pode se ligar a ânios (MARTINEZ; HAUSINGER,2015).

A síntese do heme ocorre em diversos organismos metazoários via oito etapas enzimáticas que utilizam glicina, succinil-CoA e ferro como substrato. A primeira e as três últimas etapas da síntese de heme ocorrem na mitocôndria, enquanto que as outras etapas ocorrem no citosol (AJIOKA; PHILLIPS; KUSHNER, 2006; LANE et al., 2015). A formação de heme envolve a participação sequencial de oito enzimas: aminolevulanato sintase (ALA), aminolevulanato dehidratase (ALA-D), porfobilinogênio deaminase (PBGD), uroporfirinogênio III sintase (URO-S), uroporfirinogênio III descarboxilase (URO-D), coproporfirinogênio III oxidase (CPO), protoporfirinogênio III oxidase (PPO) e ferroquelatase (FC). O heme recém sintetizado pode então ser incorporado em heme proteínas ou pode ser degradado (SCHULTZ et al., 2010).

As heme proteínas têm um diverso espectro de funções biológicas: transporte e armazenamento de gases oxigênio, óxido nítrico e monóxido de carbono; modulação transcricional de gene; transferência de elétrons; regulação de ritmo circadiano; regulação transducional; regulação de canais iônicos; processamento de micro-RNA e metabolismo de drogas (HENTZE et al., 2010; LARSEN et al., 2012). A função do heme é atribuída pela proteína a qual está ligado. Em proteínas como hemoglobina e mioglobulina, o heme age como carreador de gases, já em proteínas como citocromo C, citocromo C oxidase e redutase, o heme age como transportador de elétrons. (GOZZELINO et al., 2010).

A taxa mais rápida de síntese de heme ocorre entre eritrócitos na medula óssea (75% do total de heme no corpo) e nos hepatócitos (SASS; BARIKBIN; TIEGS, 2012). No corpo humano, 65-75% do ferro total está presente como heme nas células vermelhas do sangue (WAGENER et al., 2001). Após uma meia vida de 120 dias, eritrócitos senescentes são fagocitados por macrófagos do sistema fagocítico mononuclear retículo-endotelial e removidos da circulação. Uma vez que os eritrócitos sejam lisados nos macrófagos, o anel de heme é liberado no lúmen do fagolisossomo e é degradado pela enzima heme oxigenase 1 (HO-1). O ferro gerado nessa degradação é estocado na ferritina ou exportado pela ferroportina (FPN1) (DONOVAN et al., 2005; HENTZE et al., 2010). O heme e a

hemoglobina (Hb) são liberados no plasma durante a destruição de eritrócitos senescentes e enucleação dos eritroblastos. A haptoglobina (Hp) e hemopexina (Hpx) são proteínas plasmáticas responsáveis pela reciclagem do heme. A haptoglobina forma complexos solúveis com os dímeros de hemoglobina e o complexo Hp-Hb liga-se ao receptor CD163 na superfície de macrófagos sendo endocitado (KRISTIANSEN et al., 2001). Já a Hpx liga-se ao heme livre com alta afinidade e os complexos Hpx-Heme são internalizados pelo receptor CD91 principalmente em hepatócitos (HVIDBERG et al., 2005; SMITH; MCCULLOH, 2015). Embora o heme seja importante para diversos processos fisiológicos, o excesso desse composto pode ser deletério. O heme livre provoca dano celular e tecidual por amplificar a formação de espécies reativas de oxigênio (ROS), resultando em estresse oxidativo (JENEY et al., 2002; VERCELLOTTI et al., 1994). Por conta do seu baixo peso molecular, o heme é lipofílico e intercala-se nas membranas, desestabilizando a bicamada lipídica e as organelas (RYTER; TYRRELL, 2000). Supostamente, qualquer forma de heme livre ou heme proteína pode agir na reação de Fenton e catalisar a geração de $OH^{\cdot}$ (BRESGEN; ECKL, 2015; RYTER; TYRRELL, 2000).

Em concentrações acima de 5 mM, fora de células vermelhas, os tetrâmeros de hemoglobina dissociam-se espontaneamente em dímeros (ROTHER et al., 2005). Na presença de ROS ou espécies reativas de nitrogênio (RNS), os dímeros de hemoglobina são prontamente oxidados em metahemoglobina (metHb), liberando seu grupamento heme (BALLA et al., 2000). Dessa forma, o heme livre pode ser altamente citotóxico para células endoteliais, um evento patogênico que expõe a matriz protrombótica endotelial para a cascata de coagulação, levando a formação de trombos microvasculares com concomitante vaso-oclusão (BALLA et al., 2000, 2007).

Muitas doenças autoimunes e hereditárias (como anemia falciforme) são complicadas por hemólise, e os tecidos podem ser subsequentemente expostos a grandes quantidades de heme livre ou heme proteínas (LARSEN et al., 2012). Sob condições patofisiológicas o heme pode ser liberado de heme proteínas as quais está ligado covalentemente, isso produz o “heme livre”, o qual é um potente pró-oxidante devido ao átomo de ferro contido no seu anel protoporfirínico (GOZZELINO; SOARES, 2011; SEIXAS et al., 2009).

O heme livre pode interferir no recrutamento de leucócitos para os tecidos por diferentes mecanismos: *(a)* induzindo a expressão de moléculas de adesão em células endoteliais; *(b)* aumentando a permeabilidade vascular; *(c)* aumentando a expressão e

secreção de quimiocinas e *(d)* induzindo a migração de leucócitos, especialmente neutrófilos (GRAÇA-SOUZA et al., 2002; WAGENER et al.,2003).

O heme funciona como molécula quimiotática por induzir a produção de leucotrieno B4 em macrófagos (MONTEIRO et al., 2011) e estimular a migração de neutrófilos; nessas células acredita-se que a geração de ROS se dá através da ativação de um receptor acoplado à proteína Gα em neutrófilos (PORTO et al., 2007), amplificando assim a resposta inflamatória. Autores do mesmo grupo demonstraram que o heme aumenta a letalidade e secreção de citocinas induzida pelo LPS, assim como outros agonistas para receptores do sistema imune inato (FERNANDEZ et al., 2010). Ainda em macrófagos foi descrito que o heme induz necrose programa, denominada necroptose, através da produção autocrina de TNF e ROS (FORTES et al., 2012). Mais recentemente, o mesmo grupo demonstrou que heme induz a ativação da plataforma do inflamasoma NLRP3, assim como clivagem de caspase-1 e processamento e liberação de IL-1β (DUTRA; BOZZA, 2014; DUTRA et al., 2014).

1.2.1 SISTEMA HEME-HEME OXIGENASE: CARACTERIZAÇÃO E FUNÇÃO

O único mecanismo fisiológico de degradação enzimática do grupamento heme é a enzima heme oxigenase (HO). A HO-1 desempenha fundamentalmente dois processos fisiológicos importantes: *(1)* reciclagem da molécula de ferro dos eritrócitos e *(2)* manutenção da homeostasia celular em condições de estresse (RYTER; ALAM; CHOI, 2006; WILKS; HEINZL, 2014). O entendimento atual do sistema heme-heme oxigenase é que os efeitos biológicos da HO-1 refletem os efeitos dos produtos da degradação do heme. Esse conceito é corroborado pelos achados que formas clivadas de HO-1 que perderam a atividade enzimática podem exercer atividades biológicas, que incluem citoproteção (LIN et al., 2007) e transcrição de genes associados à mecanismo anti-oxidativos como glutationa peroxidase e catalase (COLLINSON et al., 2011; LINARES et al., 2013). De todo modo, o catabolismo do heme *per se* é um componente crítico mediando os efeitos biológicos atribuídos à HO-1 e em particular a sua atividade citoprotetora (GOZZELINO; JENEY; SOARES, 2010).

A HO foi originalmente descrita por Tenhune e cols. em 1968. As isoformas de HO podem metabolizar o heme (protoporfirina IX), liberando ferro, monóxido de carbono e biliverdina que é convertida em bilirubina pela biliverdina redutase (TENHUNEN; MARVER; SCHMID, 1968) e são altamente conservada entre as espécies **(Figura3).**

As HOs estão ancoradas no retículo endoplasmático por uma sequência de aminoácidos na extremidade carboxi-terminal da proteína. A HO é expressa virtualmente em todas todas as formas de vida, bactérias, fungos, plantas e humanos, regulando uma variedade de processos celulares. Em condições fisiológicas normais, a maioria das células expressam baixas quantidades de HO-1 (32 kDa), enquanto que HO-2 (36 kDa) é constitutivamente expressa. A expressão da HO-3 ainda não é bem caracterizada. Embora tida como constitutiva, a transcrição de HO-2 pode ser induzida por alguns agentes como opióides e glicocorticóides adrenais (LI; DAVID CLARK, 2000; LIU et al., 2000). De toda forma, a enzima HO-2 confere certo grau de proteção contra o heme livre e tem um papel secundário na detecção de oxigênio e hipoxia celular (MUÑOZ-SÁNCHEZ; CHÁNEZ-CÁRDENAS, 2014).

A expressão de HO-1 é induzida por vários estímulos e envolve várias vias de sinalização celular. O gene que codifica a HO-1, o *Hmox1*, é fortemente induzido por agentes ou condições que aumentam o estresse oxidativo (DENNERY, 2014). A expressão de HO-1 é regulada no nível transcricional e pode ser induzida por estímulos que incluem: metais pesados, lipossacarídeos de bactérias, hipoxia, hiperoxia, isquemia, radiação UV, H_2O_2, citocinas, óxido nítrico e o seu próprio substrato, o heme (GOZZELINO; JENEY; SOARES, 2010; PRAWAN; KUNDU; SURH, 2005).

Bach 1 é o repressor transcricional que está ligado a vários elementos responsivos a estresse (STREs) e inibe a transcrição do gene *Hmox1* constitutivamente. A ligação Bach1 ao ligação ao heme resulta em mudanças conformacionais que levam ao desligamento do repressor Bach 1 da região reguladora do gene *Hmox1* o que resulta na transcrição do gene *Hmox1* (HIRA et al., 2007). De fato, camundongos *knockout* para a proteína Bach1 expressam constitutivamente altas quantidades de RNA mensageiro para a proteína HO-1 (SUN et al., 2002).

O estresse oxidativo também pode suprimir a atividade da Bach 1 e impedir sua ligação aos STREs. De fato, o desligamento da Bach 1 da região reguladora do gene *Hmox1* permite que o fator nuclear responsivo de transcrição (Nrf2) acesse os STREs e induza a transcrição do gene *Hmox1* (CEDERBAUM, 2013; ITOH et al., 2015). Sabe-se que, por conta do seu papel pró-oxidante, o heme produzido induz a expressão de HO-1, tanto por promover desligamento do repressor Bach 1 quanto por promover o translocação do Nrf2. Além disso, várias vias de sinalização independentes do balanço redox estão envolvidos na regulação do gene *Hmox1*, incluindo quinases, proteína quinase C (PKC), proteína quinase

A e fosfatases (ALAM; COOK, 2007). Dentre as vias de sinalização, o PPAR-γ se destaca, por ser um receptor nuclear que ativa a transcrição de diversos genes associados ao estresse oxidativo, dentre eles o gene da HO-1. A sinalização via PPAR-γ também já foi implicada no controle da proliferação celular e funções inflamatórias de macrófagos (TONTONOZ; SPIEGELMAN, 2008). Acredita-se que a indução de HO-1 e por conseqüência liberação de CO e bilirrubina seja o mecanismo principal pelo qual PPAR-γ exerce um papel anti-inflamatório (KRONKE et al., 2007; NDISANG, 2014).

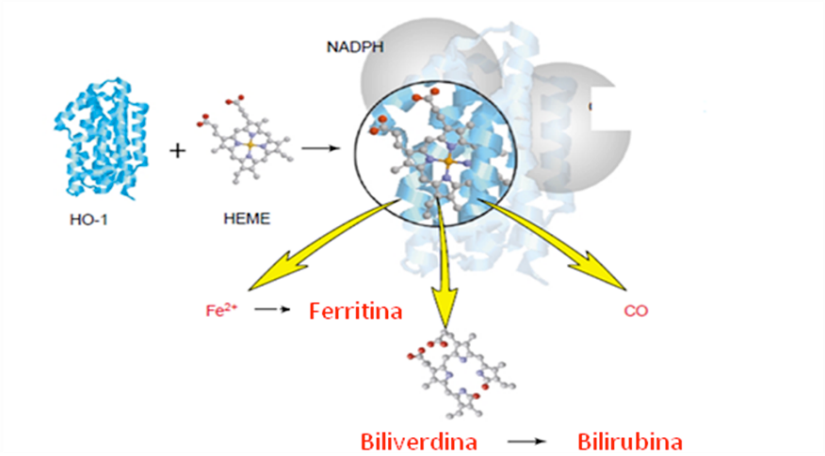

Figura 3- Degradação do heme pelaHO-1
Adaptado de Otterbein & Soares,2003

Além do seu papel anti-inflamatório, a HO tem um papel importante na reciclagem de ferro feita por macrófagos, principalmente do sistema retículo endotelial (HULL; AGARWAL; GEORGE, 2014). Apenas 10% do ferro necessário é adquirido na absorção intestinal, o resto é fornecido por macrófagos que reciclam o ferro do heme internamente (HENTZE et al., 2010). Macrófagos fagocitam eritrócitos senescentes e catabolizam heme utilizando a HO-1, enquanto o ferro resultante dessa degradação é exportado para o meio extracelular (EVSTATIEV; GASCHE, 2012). A HO-1 é expressa no fígado, baço, pâncreas, intestino, rim, coração, retina, próstata, pulmão, pele, cérebro e células endoteliais (SASS; BARIKBIN; TIEGS, 2012; WAGENER et al.,2003).

Muitos estudos têm associado a HO-1 a propriedades anti-inflamatórias. Essa descrição tem sido corroborada pelo fato de que camundongos deficientes no gene *Hmox1* desenvolvem um estado crônico de inflamação (POSS; TONEGAWA, 1997). Há um relato

de caso de um ser humano com deficiência na atividade da enzima HO-1, esse indivíduo tinha anemia hemolítica, altas quantidades de heme e morreu de síndrome inflamatória (YACHIE et al.,1999).

No entanto, humanos diferem consideravelmente na sua habilidade de montar uma resposta de aumento de expressão de HO-1. Dois polimorfismos na região promotora do gene codificante para HO-1 já foram relatadas na modulação da quantidade de HO-1 em resposta a um estímulo dado (YAMADA et al., 2000). Muitos estudos têm demonstrado que a capacidade dos pacientes, com certos genótipos, de responder fortemente em termos de aumentar a expressão de HO-1 pode ser um importante fator protetor ou preditor de gravidade (HIRAI et al., 2003; MENDONÇA et al., 2012; ONO; MANNAMI; IWAI,2003).

1.2.2 PAPEL DA HO-1 E DO HEME EM DOENÇAS INFECCIOSAS NÃO PARASITÁRIAS

A participação da enzima HO-1 já foi descrita em diversas doenças infecciosas e seu efeito está essencialmente relacionado no balanço entre os mediadores pró e anti-inflamatórios durante a infecção (GOZZELINO; JENEY; SOARES, 2010). Em um modelo de endotoxemia, camundongos *knockout* para o gene *Hmox1* têm maior mortalidade, estresse oxidativo e dano tecidual quando expostos a bactérias Gram-negativas (WIESEL et al., 2000). Em contraste a esse efeito, a administração de sub-produtos da ação da HO-1 como CO ou biliverdina-bilirubina reduz a inflamação e atenua o dano tecidual em modelo experimental de colite causado por *Escherichia coli* (MAHARSHAK et al.,2015).

Em relação à outras infecções bacterianas, camundongos *knockout* para o gene *Hmox1* têm menor taxa de sobrevivência quando submetidos à um modelo de sepse que consiste na perfuração e ligação do ceco (CLP) (CHUNG; HALL; PERRELLA, 2009). **Larsen e cols. (2010) demonstraram que os efeitos protetores da HO-1 na sepse por CLP são proporcionados pela capacidade da HO-1 de prevenir o dano tecidual provocado pelo heme liberado da hemoglobina durante a infecção.** Além disso, os autores exploraram o papel da hemopexina (Hpx) na proteção contra os efeitos tóxicos do heme na sepse, demonstrando então que a administração de Hpx protege do dano tecidual e da letalidade. Por fim, os autores descreveram que pacientes que não sobreviveram à sepse tinham menores quantidades de Hpx no soro (LARSEN et al., 2010; SMITH; MCCULLOH, 2015).

A *Mycobacterium tuberculosis* (MTB) destaca-se por provocar um dos maiores

problemas de saúde pública, a tuberculose (WHO, 2010). A MTB expressa uma série de genes conhecidos como reguladores da dormência. Foi demonstrado que a HO-1é induzida em macrófagos infectados com MTB *ex vivo* e *in vivo* e o CO é bem tolerado pela MTB (KUMAR et al., 2008). Mais relevante ainda, os autores demonstraram que o CO induz dormência em MTB, sendo que na ausência de HO-1, os genes de dormência não são suprimidos. Esses resultados levaram à proposição da hipótese de que quando a MTB estabelece sua infecção latente, o organismo induz uma resposta imune que resulta na indução de HO-1 e formação de granuloma. Consequentemente, a MTB é exposta a HO-1 e CO e menores quantidades de oxigênio no granuloma, o que ativa a dormência e permite sobrevivência do organismo (ZACHARIA; SHILOH, 2012).

A importância da HO-1 na tuberculose humana também já foi investigada. **Andrade e cols. (2013) demonstraram que maiores níveis de HO-1 são encontrados no soro de indivíduos com forma ativa e extra pulmonar de tuberculose.** Os autores também demonstraram que os níveis séricos de HO-1 são reduzidos após o tratamento farmacológico contra tuberculose (ANDRADE et al., 2013). Recentemente, o mesmo grupo de autores investigou diferentes coortes de pacientes com a forma ativa de tuberculose. Eles encontraram que a expressão de HO-1 em pacientes e em culturas de macrófagos *in vitro* está negativamente relacionada a expressão de MMP-1, mostrando que HO-1 pode participar do remodelamento tecidual na tuberculose (ANDRADE et al.,2015).

Os fungos tem o potencial de serem patogênicos. Por exemplo, a *Candida albicans,* em situações de baixa da resposta imune, pode se tornar um patógeno invasivo que coloniza o hospedeiro, levando à doença disseminada, que está associada com alta mortalidade (PENDRAK; YAN; ROBERTS, 2004). No referido artigo, os autores demonstraram que quando *C. albicans* é exposta à hemoglobina há aumento dos receptores de fibronectina, levando a adesão à células vasculares do hospedeiro vertebrado ou tecidos específicos do hospedeiro. Dessa forma, os autores propuseram a hipótese de que a hemoglobina pode ser um fator do hospedeiro que leva à infecção disseminada por *C. albicans*. **Pendrak e cols. (2004) ainda discutiram que como a biliverdina protege dos efeitos fungicidas do macrófago como o estresse oxidativo, e o CO e a HO-1 têm propriedades anti-inflamatórias, a HO-1 pode trazer outras vantagens para a *C. albicans* como patógeno oportunista no hospedeiro (CHUNG; HALL; PERRELLA,2009).**

Em relação às infecções virais, as hepatites representam uma das causas mais comuns de doença hepática. Por conta do seu papel na resolução da inflamação, a HO-1 pode proteger o fígado das conseqüências deletérias da resposta imune do hospedeiro contra

o vírus da hepatite B (HBV) (SASS; BARIKBIN; TIEGS, 2012). Em um modelo murino de infecção aguda por HBV, Protzer e cols. **(2007) demonstraram que a indução de HO-1 protege o fígado do do dano tecidual decorrente da infecção por HBV por uma atividade antiviral do hospedeiro**. Os autores ainda evidenciaram que a indução de HO-1 pela Cobalto Protoporfirina (CoPP) ou por um adenovírus resultou em uma atividade anti-viral, agindo na replicação do HBV, além dos efeitos anti-inflamatórios (PROTZER et al., 2007). Esses resultados foram corroborados mais recentemente por outro grupo de autores. **Utilizando uma linhagem de hepatócitos humanos, Shen e cols. (2015) demonstraram que a indução de HO-1 reduz a carga do vírus HBV nessas células (SHEN et al.,2015).**

Adicionalmente, Devadas & Dhawan (2006) investigaram as conseqüências biológicas da indução farmacológica de HO-1, utilizando heme como mecanismo de defesa do hospedeiro contra a infecção por HIV. Em monócitos e células T humanas, a indução de HO- 1 resulta em menor infectividade e supressão da replicação do HIV (DEVADAS; DHAWAN, 2006; TAKEDA et al., 2015). Além disso, uma das drogas utilizadas no tratamento contra AIDS, o inibidor de protease Rotinavir, parece ter seu efeito mediado parcialmente pela indução de HO-1 (MÜHL et al.,2004).

1.2.3 PAPEL DA HO-1 EM DOENÇAS INFECCIOSAS PARASITÁRIAS

Em se tratando de doenças parasitárias, um modelo bastante estudado é o da malária, no qual a expressão de HO-1 interfere na resolução da doença (FERREIRA et al., 2008). A infecção pelo *Plasmodium*, tanto em humanos quanto em camundongos, está associada com a indução de altas quantidades de HO-1. Há um aumento de expressão de HO-1 em lesões cerebrais de pacientes com malária, assim como na micróglia próxima às áreas de hemorragia (FERREIRA et al., 2008). Além disso, a infecção experimental de camundongos por diferentes espécies de *Plasmodium* aumenta a expressão de HO-1 no cérebro e fígado desses animais (EPIPHANIO et al., 2008; SEIXAS et al., 2009). Camundongos Balb/c expressam maiores quantidades de HO-1 quando comparados à linhagem C57BL/6. A diferença na expressão de HO-1 contribui para a susceptibilidade da linhagem C57BL/6 ao desenvolvimento da malária cerebral experimental quando infectados pelo *P. berghei* (PAMPLONA et al.,2007a).

No estudo do papel da HO-1 em um modelo murino experimental de malária cerebral, Pamplona e cols demonstraram em 2007 que a HO-1 e seu subproduto monóxido de carbono evitam o rompimento da barreira hemato-encefálica e a consequente congestão

microvascular, hemorragia e neuroinflamação visto nos estágios eritrocíticos e sanguíneos da infecção por *Plasmodium*. O efeito protetor da HO-1 ou CO tem sido atribuído à ligação com a hemoglobina livre, suprimindo assim a acumulação de heme na circulação e no cérebro o que é deletério ao hospedeiro infectado (FERREIRA et al., 2011; PAMPLONA et al., 2007b).

Epiphanio e cols. (2008) demonstraram que a infecção por *Plasmodium* no fígado induz a expressão de HO-1, que serve para proteger hepatócitos infectados, promovendo assim a fase hepática da infecção (EPIPHANIO et al., 2008). Os mesmos autores do referido artigo explanaram que a indução de HO-1 por um adenovírus aumenta a carga parasitária de hepatócitos infectados com *P. berghei*. Da mesma forma, a inibição de HO-1 *in vivo* reduz a replicação do parasito circulante no sangue, bem como no fígado. Em contraste aos efeitos benéficos para o hospedeiro no modelo experimental de malária cerebral, a indução de HO-1 serve como um estágio obrigatório no estabelecimento da fase hepática, por controlar a resposta imune do hospedeiro e proteger hepatócitos infectados. Dessa forma, existe uma significante diferença no papel da HO-1 dependendo do estágio da doença (ZHENG; TAN; XU,2014).

A HO-1 é protetora também na forma não cerebral da malária (DEY et al., 2014). Quando infectados por *P. chabaudi*, camundongos apresentam altas concentrações de TNF e heme livre no plasma, desenvolvendo uma forma letal de falência hepática associada com morte celular disseminada. A neutralização de TNF (com anticorpo) ou do heme (pela indução de HO-1) protege hepatócitos de sofrer morte celular e suprime a falência hepática em um modelo experimental de malária (SEIXAS et al.,2009).

Em se tratando de malária humana, trabalhos do nosso grupo demonstraram que os níveis de heme estão aumentados em pacientes com forma sintomática de malária, causada por *P. vivax* e está diretamente relacionado com a patogênese da doença (ANDRADE et al., 2010). Corroborando os dados encontrados em modelos experimentais de malária, nosso grupo também demonstrou que o polimorfismo para o gene *Hmox1* contribui para o desenvolvimento da doença, uma vez que indivíduos que expressam o "polimorfismo curto", que implica na expressão reduzida de HO-1, desenvolvem a forma sintomática da doença (MENDONÇA et al., 2012,2015).

No contexto de doenças causadas por tripanossomatídeos Paiva e cols. **2012 demonstraram que a HO-1 atribui resistência a infecção por *Trypanosoma cruzi* em macrófagos e em modelo experimental da doença de chagas (PAIVA et al.,2012).**

Nessa tese exploramos o papel da HO-1 nas funções do macrófagos no contexto da

infecção por *L. infantum chagasi*, além de investigar o papel da HO-1 na patogenênese e como potencial marcador da LV humana.

1.3 MECANISMOS DE MORTE CELULAR: NECROPTOSE, SISTEMA IMUNE E INFECÇÃO

A morte celular é parte integrante da vida do organismo, sendo imprescindível para a manutenção de tecidos e órgãos. Morfologicamente três tipos distintos de morte celular foram reconhecidos: apoptose, autofagia, e necrose (GALLUZZI et al., 2014). A apoptose é um tipo de morte celular programada que preserva a integridade da membrana plasmática, resulta na retração do citoplasma e núcleo da célula, com diminuição de seu volume, condensação e agregação da cromatina, originando núcleos picnóticos, assim como a sua fragmentação a partir da clivagem oligonucleossomal do DNA por ação de endonucleases. Mais tardiamente, a célula pode se fragmentar, dando origem aos corpos apoptóticos que são englobados por fagócitos, o que inibe a produção de mediadores pró-inflamatórios e induz a produção de mediadores anti-inflamatórios como PGE_2 e TGF-β(FADOK et al., 1998; SAVILL; FADOK, 2000). Diferentes vias que levam à apoptose, em quase sua totalidade, levam a ativação de cisteína proteases chamadascaspases.

A apoptose é mediada pelos receptores de morte TNF, Fas e TRAIL os quais quando ligados aos seus ligantes sofrem oligomerização e iniciam a cascata de sinalização que pode levar a apoptose. Uma vez ativados os receptores de morte disparam a poli ubiquitinação de RIP1 (do inglês *Fas-interacting serine/threonine kinase receptor interacting protein-1*) por moléculas inibidoras da apoptose(IAPs).

O estado de ubiquitinação de RIP1 determina se a molécula vai desempenhar função "scaffold" ou quinase que é a que promove morte celular (MCQUADE; CHO; CHAN, 2013; MOQUIN; MCQUADE; CHAN, 2013). A ativação de RIP1 no resíduo de lisina (K63) promove a ativação "downstream" de MAPK e NFkB, o que governa a expressão de genes de sobrevivência. As proteínas deubiquitinantes CYLD e A20, por sua vez, podem remover as cadeias de ubiquitina de RIP1 o que elimina o papel de RIP1 na ativação de NFkB e promove a ligação a caspase 8 (caspase iniciadora) que cliva as caspase 3 e 7 (caspases executoras) o que leva a execução da apoptose (SILKE; RICKARD; GERLIC, 2015). Normalmente, a ativação de caspase-8 inibe a fosforilação de RIP3 e ativa apoptose, mas caso essa caspase esteja ausente ou bloqueada, RIP1 e RIP3 se tornam fosforilados o que leva ao evento denominado necroptose, também chamado de necrose programada (GÜNTHER et al., 2011; VANDENABEELE et al., 2010). Uma vez ativada e fosforilada,

RIP3 fosforila as moléculas *downstream* efetoras da via da necroptose: *(i)* a quinase MLKL (do inglês *mixed lineage kinase domain-like*) que promove a oligomerização e desestabilização da membrana plasmática,influxodesódioecálcioelisedamembranacelular (DONDELINGERetal.,2014; WU et al., 2013); (*ii*) e a fosfatase mitocondrial PGAM5 (do inglês *phosphatase phosphoglycerate mutase family member 5*) que promove a fissão mitocondrial e produção de ROS (MORIWAKI; CHAN, 2013; WANG et al., 2012).

A necroptose é o mais recente tipo de morte celular descrito, tendo sido denominada como 'necrose programada' por Chan e cols. **(2003) revisado em (CHAN; LUZ; MORIWAKI, 2015); uma vez que as células que morrem por essa via apresentam as características morfológicas da necrose classicamente descrita.** Chan e cols. demonstraram que células Jukart infectadas com o vírus da vaccínia morriam por 'necrose programada' em reposta ao TNF e a deficiência de RIP protegia tais células da citotoxicidade induzida pelo TNF (CHAN et al., 2003). Entretanto, o termo *necroptose* foi originalmente descrito por Degterev e cols. **(2005), ao demonstrarem que o tratamento de células com TNF em presença de inibidores de caspases (zVAD-fmk), ou em presença de mutação de caspase-8, levava à necrose ao invés de apoptose, como normalmente ocorre na ausência das condições acima descritas (DEGTEREV; YUAN, 2008; DEGTEREV et al., 2005).** Morfologicamente, as células que sofrem necroptose tornam-se intumescidas, assim como suas organelas, havendo ruptura da membrana plasmática e perda de conteúdo intracelular (CHAN; LUZ; MORIWAKI, 2015).

Considerando caracteres moleculares/bioquímicos, a necrose programada pode ser iniciada por diferentes estímulos incluindo estresse oxidativo, dano no DNA da célula, infecções e ativação de receptores de reconhecimento padrão (PRR). **Fortes e cols. (2012) demonstraram que a molécula heme é capaz de induzir necroptose em macrófagos murinos através da produção de autócrina de TNF e ROS (FORTES et al., 2012).** As implicações clínicas desses achados podem ser relacionadas às diversas doenças ou infecções que causam hemólise, com consequente dano tecidual einflamação.

A piroptose é outra forma importante de morte celular não apoptótica com características morfológicas de necrose. Esse tipo de morte celular é desencadeada pela formação do inflamassoma, que é um complexo multiproteico intracelular que atua na ativação de enzimas da família cisteína-aspartato proteases (caspases) como uma estrutura essencial para a regulação da imunidade em condições fisiológicas e no reconhecimento de sinais de perigo a diferentes componentes. Estes sinais podem ser produtos microbianos, adjuvantes ou alterações no ambiente iônico intra e extracelular. A participação do

inflamassoma está confirmada na patogênese de várias doenças inflamatórias, cuja atuação é moldada pelo tipo de ativação e influenciada pelo microambiente, criando um perfil patogênico diferente para cada doença (GUO; CALLAWAY; TING, 2015). Essa plataforma multiproteica denominada inflamassoma ativa a caspase-1, que é responsável pela produção das formas ativas de duas importantes citocinas inflamatórias: a IL-1β e a IL-18 (KEYEL, 2014; SOLLBERGER et al., 2014).

No contexto da infecção por *Leishmania*, Lima-Junior e cols. **(2013) demonstraram que a ativação do inflamassoma de NLRP3 é crucial para a resistência do hospedeiro à parasita.** Esse controle é mediado por mecanismos dependentes da produção de IL-1β e IFN-γ, os quais induzem a geração de óxido nítrico que contribui para a restrição da replicação do parasito no interior de macrófagos (LIMA-JUNIOR et al., 2013; ZAMBONI; LIMA- JUNIOR, 2015).

A autofagia, por sua vez, é responsável pela degradação do conteúdo citoplasmático, tais como proteínas e organelas e pela formação de autofagolisossomos. Ao contrário dos demais tipos de morte celular, a autofagia é essencialmente um mecanismo de sobrevivência deflagrado em resposta ao estresse (hipóxia, anoxia, disfunção mitocondrial e infecção) e que promove a sobrevivência dos tecidos. Entretanto, a autofagia pode ocorrer descontroladamente ocasionando degradação celular excessiva e condições patológicas (DERETIC; LEVINE, 2009; FITZWALTER; THORBURN, 2015).

O sistema imune responde às infecções de forma variada, abrangendo a ativação de sinais de sobrevivência celular ou sinais que levam a programas de morte da célula. Por outro lado, sabe-se que muitos patógenos deflagram programas específicos de morte celular nos hospedeiros e que estas são estratégias de sobrevivência do próprio patógeno, sendo assim a necroptose uma morte pró-inflamatória, ao contrário da apoptose. (RILEY et al., 2015; SILKE; RICKARD; GERLIC, 2015).

Nesse estudo pretendemos explorar o papel das moléculas da via da necroptose na infecção por *Leishmania*, bem como os mecanismos inflamatórios deflagrados por essa via que podem contribuir para a resistência a infecção *in vitro* e em modelo experimental de Leishmaniose.

2. JUSTIFICATIVA

A LV apresenta uma série de alterações hematológicas cujos mecanismos imunopatogênicos ainda são pouco esclarecidos. Um estudo demonstrou, utilizando ferramentas de *microarray,* que o gene *Hmox1* é um dos genes induzidos no contexto da infecção de macrófagos humanos e células THP-1 por *L. donovani* (EL FADILI et al., 2008). Nesse sentido, a enzima heme oxigenase-1 pode ter um envolvimento nas infecções por *Leishmania* do complexo *L. donovani*, inclusive a *L. infantum* (responsável pelos casos da forma visceral no Brasil). Por se tratar de uma parasitemia sistêmica visceralizante associada à hemólise e consequente liberação de heme, a enzima HO-1 pode ter participação na modulação da carga parasitária bem como na proteção aos efeitos tóxicos doheme.

Sabe-se que a infecção de macrófagos com promastigotas e amastigotas de *Leishmania* resulta na produção de superóxido (GANTT et al., 2009; PODINOVSKAIA; DESCOTEAUX, 2015). **Pham e cols. (2005) demonstraram que amastigotas de *Leishmania* possuem estratégias para evitar a produção de radicais de oxigênio, e que esse fenômeno ocorre na dependência da ativação da enzima HO-1.** Amastigotas de *Leishmania pifanoi* inviabilizam a formação do complexo NADPH (uma enzima que contém grupamento heme) no vacúolo parasitóforo, via ativação da enzima HO-1, o que resulta na degradação do heme e impede a formação do polipeptídio intermediário que compõe a enzima NADPH oxidase, molécula de intensa atividade leishmanicida (PHAM; MOURIZ; KIMA, 2005). No entanto, o referido estudo não explora quais as conseqüências da ativação da HO-1 na replicação intracelular da *Leishmania* e quais mecanismos imunoregulatórios são deflagrados na célula hospedeira.

O significado biológico da necroptose na saúde e na doença, bem como dos mecanismos que levam a essa morte celular também tem sido cada vez mais explorado. É fato que a necroptose está associada a várias doenças como isquemia cerebral, doenças neurodegenerativas, síndrome da resposta inflamatória sistêmica letal e infecções virais (SMITH; YELLON, 2011a, 2011b). Por outro lado, até o presente momento não existem trabalhos que avaliem o efeito deste tipo de morte celular em infecções parasitárias, muito menos no contexto da infecção por*Leishmania*.

Sabendo-se que a indução da morte da célula hospedeira tem sido demonstrada em casos de infecções por bactérias, vírus e parasitos, com importantes consequência na

imunopatogênese, investigamos nesse trabalho o papel da necroptose na resistência a infecção por *Leishmania.*

Tendo em vista que este trabalho tem duas abordagens, uma em relação aos aspectos da *Leishmania* em termos de subversão da resposta leishmanicida e outra do aspecto de resistência a infecção por *Leishmania*, achamos conveniente dividi-lo em duas partes.

3. PARTE I
3.1 HIPÓTESE

A ativação da enzima heme oxigenase-1 favorece a infecção por *Leishmania chagasi* em macrófagos e está associada com a LV humana.

3.2 OBJETIVOS

OBJETIVO GERAL

Avaliar o papel da heme oxigenase-1 (HO-1) na infecção por *L. chagasi*.

OBJETIVOS ESPECÍFICOS

- Avaliar o efeito da infecção por *L. chagasi* na expressao da proteína HO-1 em macrófagos;
- Investigar o papel da HO-1 na infecção por *L. chagasi*, utilizando drogas ativadoras da expressão e atividade da enzima;
- Averiguar a relevância do gene *Hmox1* da célula hospedeira na infecção por *L. chagasi*, utilizando macrófagos *knockout* para o gene;
- Avaliar a produção por macrófagos murinos de mediadores pró e anti-inflamatórios na infecção por *L. chagasi*, bem como frente à estimulação por LPS e tratamento com a droga indutora da HO-1;
- Investigar o papel da HO-1 na infecção por *L. chagasi* em macrófagos humanos;
- Verificar os níveis plasmáticos de HO-1 em indivíduos sintomáticos com LV humana em comparação aos controles endêmicos e após o tratamento leishmanicida;
- Correlacionar os níveis plasmáticos de HO-1 em indivíduos sintomáticos com os níveis de citocinas inflamatórias clássicas associadas à LV humana.

3.3 MANUSCRITO 1

HEME OXYGENASE-1 PROMOTES THE PERSISTENCE OF *LEISHMANIA CHAGASI* INFECTION.

Esse trabalho investiga a participação da HO-1 na infecção de macrófagos por *L. chagasi*, bem como sua relevância na LV humana.

Resumo:
Macrófagos murinos e humanos foram infectados com promastigotas de *L. chagasi* ou tratados com seu LPG. Observamos a indução da enzima HO-1 nesses macrófagos até 72 horas após a infecção ou após incubação com o LPG. A indução farmacológica da HO-1, pela CoPP aumenta a carga parasitária de macrófagos infectados por *L. chagasi* e reduz a produção de mediadores pró-inflamatórios frente à estimulação por LPS, tais como ROS, TNF, NO, MCP-1 e IL-6. Além disso, a HO-1 favorece um ambiente anti-inflamatório onde prevalece a presença de IL-10 sobre a de TNF e incremento da expressão de Cu/Zn Superóxido Dismutase. Macrófagos derivados de medula óssea de camundongos deficientes no gene *Hmox1* têm menor carga parasitária, quando infectados por *L. chagasi* em comparação aos macrófagos de camundongos selvagens. Ainda demonstramos que pacientes com LV apresentam altos níveis séricos de HO-1, e que esses níveis são reduzidos após o tratamento anti-leishmanial, sugerindo que HO-1 está associada com a susceptibilidade a doença. Esses achados indicam um potencial deletério para a HO-1 na infecção por *L. chagasi*, bem como sugerem possíveis mecanismos envolvidos na imunopatogênese da LV.

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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.

metabolizes heme and releases free iron, carbon monoxide, and biliverdin, which rapidly undergoes conversion to bilirubin (18). Recently, the HO-1 isoform encoded by the *Hmox1* gene has emerged as a key regulator of inflammation by its anti-inflammatory, cytoprotective, antiapoptotic, and antiproliferative effects. Interestingly, HO-1 seems also to modulate innate as well as adaptive immunity (19). Studies have emphasized the participation of HO-1 in host-tolerance mechanisms facing infections by means of its heme detoxifying activity (20). Therefore, HO-1 can overcome the pathogenesis of a variety of immune system-mediated inflammatory conditions, such as malaria (20, 21), ischemia/reperfusion injury (22), intrauterine fetal growth restriction (23), sepsis (24, 25), graft rejection (26, 27), and sickle hemoglobin (28). Intriguingly, the immunomodulatory effects of HO-1 can drive both beneficial and detrimental consequences in the host immunity against infections agents (reviewed in Ref. 29). In fact, HO-1 protects *Plasmodium*-infected hepatocytes, thereby promoting the establishment of those parasites (30). In contrast, HO-1 enhances bacterial clearance during polymicrobial sepsis caused by cecal ligation and puncture (24), arguing that this antioxidant enzyme plays an important role in the antimicrobial process without inhibiting the inflammatory response (i.e., resistance to infection).

Despite the recognition of the importance of HO-1 in immunoregulatory mechanisms, the direct role of this enzyme in the host cell–*Leishmania* interplay has not been addressed. Pham and colleagues (31) reported that during infection of macrophages with *L. pifanoi*, HO-1 is involved in the suppression of superoxide production by inducing heme degradation, which hampers the maturation of $gp91^{phox}$, a subunit of NADPH oxidase enzyme complex. However, it was not clear whether this event had any impact in parasite survival or cytokine production.

In the current study, we report that both *L. chagasi* and LPG isolated from promastigotes induce HO-1 expression in murine macrophages. Interestingly, stimulation of macrophages with cobalt protophorphyrin IX (CoPP), a pharmacologic inductor of HO-1, resulted in a significant increase of the parasite burden. Upon *L. chagasi* infection, bone marrow-derived macrophages (BMMs) from $Hmox1^{-/-}$ mice showed lower parasite loads then macrophages from wild-type (WT) mice. Finally, we found that HO-1 is strongly associated with human VL in a cohort of patients from a highly endemic area in Brazil. These results represent the first evidence, to our knowledge, for the importance of HO-1 in regulating host immune responses to *L. chagasi* infection. Our study opens up new perspectives suggesting that HO-1 might be a therapeutic target for human VL.

Materials and Methods

Reagents

CoPP (Frontier Scientific, Logan, UT) was dissolved in 0.1 N NaOH and RPMI 1640 medium (Invitrogen, Carslbad, CA) and adjusted to concentrations of 50 μM for in vitro assays. Rosiglitazone and GW9662 were obtained from Cayman Chemical (St. Louis, MO) and dissolved in DMSO from ACROS Organics (New Jersey, NJ). RPMI 1640 medium, L-glutamine, penicillin streptomycin, and dihydroethidium (DHE) were from Invitrogen. The following primary Abs were used: anti-mouse HO-2 from R&D Systems (Minneapolis, MN) and anti-mouse β-actin from Cell Signaling Technology (Ann Arbor, MI). Schneider's insect medium, *Escherichia coli* LPS (serotype 0127:b8), IFN-γ, and pentoxifylline (PTX) were purchased from Sigma-Aldrich (St. Louis, MO). Human HO-1 ELISA kit was from Assay Designs (Ann Arbor, MI), and mouse HO-1 ELISA kit was from Takara Bio (Madison, WI). The Cu/Zn superoxide dismutase (SOD-1) Protein ELISA kit was purchased from Calbiochem (San Diego, CA). Cell Proliferation Kit II XTT was from Roche Applied Science (Indianapolis, IN), and Cytometric Bead Array mouse inflammation kit was from BD Biosciences (San Jose, CA).

Parasites and LPG

L. infantum chagasi (MCAN/BR/89/Ba262) (referred throughout the text as *L. chagasi*) promastigotes at stationary phase were cultured at 24°C in Schneider's insect medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. LPG was isolated and purified from Ba262 *L. chagasi* strain following previously described protocol (13).

Mice

Inbred male or female C57BL/6 mice aged 8–10 wk were obtained from the animal facility of the Centro de Pesquisas Gonçalo Moniz/Fundação Oswaldo Cruz, Salvador, Brazil. All experimental procedures were approved and conducted according to the Animal Care Committee of the Centro de Pesquisas Gonçalo Moniz (L-IGM-024/2009). $Hmox1^{-/-}$ mice bone marrow, a generous gift from Dr. Miguel Soares (Instituto Gulbenkian de Ciência, Oeiras, Portugal), was isolated by crushing femur bones from 10–15-wk-old WT or $Hmox1^{-/-}$ from SCID or BALB/c mice.

Mouse and human macrophages

C57BL/6 mice were injected i.p. with 3% thioglycolate solution. Four days after injection, peritoneal lavage was performed using 8 ml RPMI 1640 medium supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. BMMs from $Hmox1^{-/-}$ and $Hmox1^{+/+}$ mice from both BALB/c and SCID strains were cultured in RPMI 1640 medium containing 20% FBS, gentamicin, HEPES, and 20% L929-conditioned medium for 7 d. Human monocytes were isolated from peripheral blood of healthy donors through Ficoll gradient centrifugation and plastic adherence; cells were cultivated in RPMI 1640 medium supplemented with 10% FBS for 7 d to obtain differentiated macrophages in vitro. In some experiments, total PBMCs from normal blood donors were infected with *L. chagasi* in the presence of 1 mM PTX, an inhibitor of TNF-α production, for 12 h.

Infection assays

Macrophages (3×10^5) were seeded onto glass coverslips in 24-well plates. Cells were allowed to adhere for 2 h at 37°C and 5% CO_2; nonadherent cells were removed by washing each well with sterile saline. *L. chagasi* promastigotes in early stationary phase were added to macrophage cultures at a macrophage/parasite ratio of 1:10 in RPMI 1640 medium supplemented with 10% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Macrophages (1×10^6) were seeded on 48-well plates in the experiments in which the cell lysate or supernatants were used for HO-1 or cytokine measurements. The pharmacological modulation of HO-1 during the *L. chagasi* infection was performed in presence of CoPP, an inductor of HO-1 production (32). After 4 h, infected cells were washed to remove extracellular parasites, fresh medium was replaced with the same stimuli, and plates were returned to the incubator until the desired time. After 4, 24, 48, and 72 h, supernatants were harvested and cleared by centrifugation and stored at −20°C. Cells on glass coverslips were fixed with methanol and stained by Diff-Quick (American Scientific Products, McGraw Park, IL). Intracellular amastigotes were counted under light microscopy in 200 macrophages per slide in a blind fashion manner. Results are shown as amastigote number per 100 macrophages and percentage of infected macrophages in relation to control (group infected only with *L. chagasi*). Alternatively, intracellular load of *L. chagasi* was estimated by production of viable promastigotes in Schneider medium as described previously (33). Briefly, after 72 h of infection, RPMI 1640 medium was replaced by Schneider medium supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and the plate was then kept at 24°C. Intracellular survival of *L. chagasi* was quantified by counting proliferating extracellular motile promastigotes in a Neubauer hemocytometer until the seventh day after the medium replacement. To rule out a possible toxicity induced in cells or parasites treated with porphyrins, we performed a cell viability assay using XTT (Roche Applied Science). In some experiments, macrophages were incubated with LPS (100 ng/ml) or pretreated overnight with an antagonist (5 μM GW9662) or an agonist (10 μM rosiglitazone) of the peroxisome proliferator-activated receptor γ (PPARγ).

HO-1 measurement by ELISA

Murine and human macrophages were infected with *L. chagasi* or treated with LPG and HO-1 was measured in supernatants or cell lysates obtained through the use of lysis buffer available in murine and human HO-1 ELISA kit, following the manufacturer's instructions.

Measurements of the inflammatory mediators

TNF-α, IL-6, MCP-1, and IL-10 were measured in cell supernatants using a cytometric bead array mouse inflammation kit. NO production was measured in the supernatants of *L. chagasi*-infected macrophages pretreated with 100U/ml IFN-γ by the Griess method, as described elsewhere (34, 35). Intracellular reactive oxygen species (ROS) levels were measured by staining with the oxidative fluorescent dye probe DHE 5 μM (Invitrogen/Molecular Probes, Grand Island-NY) for 30 min at 37°C and then analyzed using flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) using the FL2 emission filter. Data were displayed as histograms, and the geometric median fluorescence intensity was evaluated using FlowJo software (Tree Star, Ashland, OR).

HO-1 measurements in VL serum samples

Serum samples were obtained from patients with VL (n = 52) and sex-matched endemic healthy controls (HC) (n = 42) from an endemic area in the Northeast of Brazil. The baseline characteristics of the study participants are shown in the Supplemental Table I. The patients with VL were followed up and HO-1 was measured before the antileishmanial therapy and after the treatment was finished due to clinical cure. HO-1 was measured using a human ELISA kit following the manufacturer's instructions. This study was approved by Institutional Review Board of Federal University of Sergipe, Brazil, where the field study was performed. All clinical investigations were conduced according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians.

Statistical analysis

Each experiment was performed at least three times, and at least five mice were used in each experimental group. Data are reported as mean ± SD of representative experiments and were analyzed using GraphPad Prism Software 5.0 (GraphPad, San Diego, CA). After performing a normality test, Kruskal–Wallis nonparametric test followed by Dunn's posttest or linear trend analysis were used to evaluate statistical significance among the groups. In some assays, comparisons between two groups were explored using the Mann–Whitney U test. Correlations among HO-1 and IL-10, TNF-α, and IL-6 were performed using the Spearman test. The Wilcoxon matched pairs test was performed to estimate statistical significance before and after the antileishmanial treatment. Receiver-operator characteristic (ROC) curves with C-statistics were used to establish the threshold value of HO-1 able to discriminate between VL and HC. A logistic regression model adjusted for age was also applied to check the strength of the association between HO-1 systemic concentrations and the occurrence of VL. A p value <0.05 was considered statistically significant.

Results

L. chagasi *infection enhances HO-1 expression by mouse macrophages*

Crescent concentrations of HO-1 protein were detected in mouse peritoneal macrophages infected with *L. chagasi* compared with uninfected cells (Fig. 1A, 1B), with significant trend to increase over time postinfection (linear trend $p < 0.0001$) in either cell-culture supernatants (Fig. 1A) or cell lysates (Fig. 1B). In addition, infected macrophages treated with 50 μM CoPP, an inductor of HO-1 (32), amplified the production of HO-1 (Fig. 1C, 1D), when compared with *L. chagasi*-infected macrophage alone. Because HO-1 is a microsomal enzyme, and we were able to detect HO-1 protein in the culture supernatants, cell death could be occurring during in vitro infection. To rule out an important toxic effect of CoPP in cell culture, we tested whether treatment with this porphyrin could affect cell viability. The stimulus was not significantly toxic for either infected macrophages (Supplemental Fig. 1A) or *Leishmania* parasites (Supplemental Fig. 1B). As expected, HO-2, the constitutive form of HO, remained unchanged in both infected macrophages or with CoPP treatment (data not shown). Therefore, we conclude that mouse macrophages display consistently high amounts of HO-1 upon infection with *L. chagasi*.

HO-1 promotes L. chagasi *infection in macrophages*

Once inside macrophages, *Leishmania* parasites may circumvent various host defense mechanisms to survive. Because HO-1 induction is associated with the persistence of *Plasmodium* in the liver (30) and also favors *Mycobacterium tuberculosis* inside macrophages during a latent infection via induction of the dormancy-associated genes (36), we hypothesized that the elevated concentrations of HO-1 detected in infected macrophages treated with CoPP could bring key benefits for the *Leishmania*. To address this question, we infected murine macrophages in vitro in presence of CoPP and evaluated the parasite load. Our experiments revealed that induction of HO-1 led to an increased in the percentage of infected macrophages (Fig. 2A), number of intracellular amastigotes (Fig. 2B), and viability of *L. chagasi* inside macrophages (Fig. 2C), whereas inhibition of HO-1 activity by Tin protoporphyrin had no impact on the parasite burden (data not shown). Interestingly, BMMs from *Hmox1*$^{-/-}$ mice from either BALB/c (Fig. 2D) or SCID (Fig. 2E) mouse strains presented significantly reduced parasite burden compared with the strain-matched WT. Infected BMMs from *Hmox1*$^{-/-}$ mice from both genetic backgrounds incubated or not with CoPP displayed reduced *Leishmania* viability at different time points postinfection, confirming that the primary effect of CoPP on parasite survival within macrophages was due to the induction of HO-1 (Fig. 2D, 2E). These results are consistent with the idea that induction of HO-1 by infected macrophages is a key event promoting *Leishmania* survival.

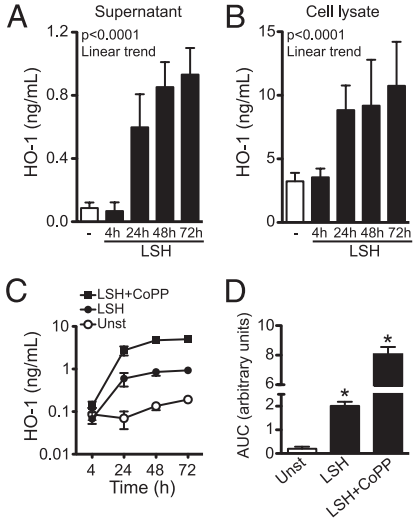

FIGURE 1. *Leishmania* infection induces HO-1 by macrophages. C57BL/6 peritoneal macrophages were infected with *L. chagasi* (LSH) at a multiplicity of infection of 10. HO-1 production was analyzed in cell-culture supernatant (**A**) and cell lysate (**B**) at 4, 24, 48, and 72 h postinfection. Data were compared using one-way ANOVA with linear trend posttest. Macrophages infected with *L. chagasi* promastigotes in the presence of 50 μM CoPP were tested for HO-1 release in cell-culture supernatants at different time points poststimulation (**C**). HO-1 release was induced by CoPP in response to *L. chagasi* infection (**D**), as showed by the area under the curve (AUC); Mann–Whitney U test was used for the pairwise comparisons. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. Bars represent mean ± SD. *$p < 0.05$.

HO-1 regulates the production of proinflammatory mediators by L. chagasi*-infected macrophages*

It is well established that the essential mechanisms of protection against *Leishmania* involves activation of macrophages and production of proinflammatory cytokines, ROS (37, 38), and NO (39). In this study, we addressed whether the role of HO-1 in promoting *Leishmania* persistence within macrophages involves regulation of proinflammatory cytokines and/or oxidative stress. Induction of HO-1 by CoPP decreased production of TNF-α (Fig. 3A), but did

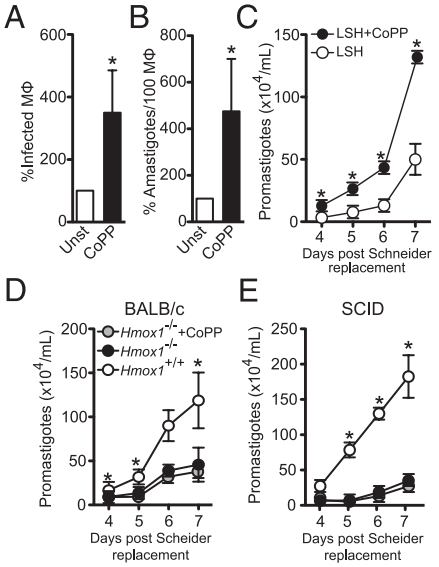

FIGURE 2. HO-1 promotes *Leishmania* persistence in infected macrophages. Macrophages were infected in vitro with *L. chagasi* (multiplicity of infection 10) in the absence or presence of 50 μM of CoPP. The parasite load was measured by optical microscopy at 72 h postinfection as described in *Materials and Methods*. The percentage of *L. chagasi* (LSH)-infected macrophages (MΦ) (**A**) and the number of amastigotes per 100 macrophages (**B**) are displayed as percentage of control (group infected only with *L. chagasi*). (**C**) After 72 h of infection, RPMI 1640 medium was replaced by Schneider (*Leishmania* medium), and extracellular *L. chagasi* promastigotes were counted following 4, 5, 6, and 7 d. Data were analyzed using the Kruskal–Wallis test. Bars represent mean ± SD. BMMs from *Hmox1*$^{-/-}$ mice from both BALB/c (**D**) and SCID (**E**) genetic backgrounds or from strain-matched WT mice were infected with *L. chagasi* and CoPP. Parasite load was measured by the Schneider method; after 72 h of infection, the RPMI 1640 medium was replaced by Schneider medium, and extracellular *L. chagasi* promastigote number was measured 4, 5, 6, and 7 d postreplacement. Data were evaluated using Kruskal–Wallis test. Points and error lines represent mean ± SD. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. *$p < 0.05$.

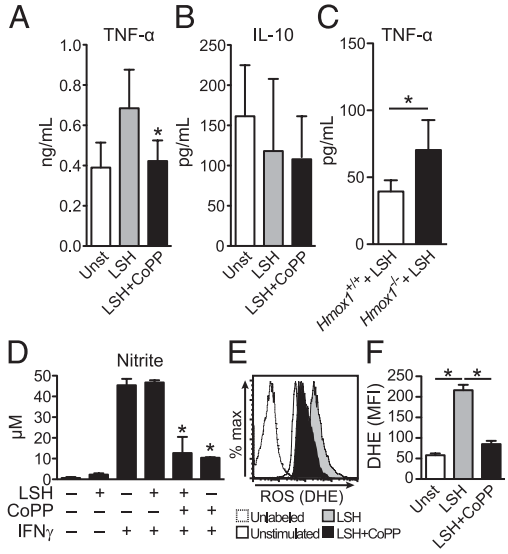

FIGURE 3. HO-1 regulates production of proinflammatory mediators by macrophages infected with *Leishmania*. Quantification of TNF-α (**A**) and IL-10 (**B**) in the supernatants from macrophages infected in vitro with *L. chagasi* in the presence or absence of 50 μM CoPP. (**C**) TNF-α production by BMMs from WT or *Hmox1*$^{-/-}$ mice infected in vitro with *L. chagasi*. (**D**) Murine peritoneal macrophages were stimulated with IFN-γ (100 U/ml) during in vitro infection in the presence or absence of 50 μM CoPP, and supernatants were harvested after 48 h; nitrite was measured using Griess reaction. Representative histograms (**E**) and median fluorescence intensity (MFI) (**F**) for ROS in macrophages infected with *L. chagasi* in the presence or absence of CoPP evaluated by flow cytometry using the probe DHE. (F) MFI of DHE-stained macrophages. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. Bars represent mean ± SD. Kruskal–Wallis test was used to compare experimental groups. *$p < 0.05$.

not change the amounts of IL-10 (Fig. 3B) at 24 h post-*L. chagasi* infection. BMMs from *Hmox1*$^{-/-}$ (SCID genetic background) produced higher amounts of TNF-α than WT cells upon infection (Fig. 3C), reinforcing the participation of HO-1 in the modulation of TNF-α production. Moreover, macrophages primed with IFN-γ presented significantly diminished NO production when treated with CoPP (Fig. 3D). Additionally, we tested if the modulation of the proinflammatory mediators induced by HO-1 could be robust enough to revert the inflammatory profile of infected macrophages primed with LPS. Indeed, induction of HO-1 by CoPP consistently increased parasite burden (Supplemental Fig. 2A) and reduced production of TNF-α (Supplemental Fig. 2B), NO (Supplemental Fig. 2E), IL-6 (Supplemental Fig. 2F), and MCP-1 (Supplemental Fig. 2G), but not IL-10 (Supplemental Fig. 2C) by these cells. Thus, a higher IL-10/TNF-α ratio was found in infected macrophages primed with LPS upon induction of HO-1 (Supplemental Fig. 2D). In addition, CoPP treatment reduced *L. chagasi*-induced ROS production by macrophages (Fig. 3E, 3F). These data suggest that HO-1 promotes *Leishmania* survival within macrophages by precluding inflammation and oxidative stress.

L. chagasi *promastigotes and LPG induce HO-1 independently of PPARγ activation*

LPG from the cell surface of *Leishmania* promastigotes has been described as a major virulence factor (40), involved in the ability to induce lesions in mice (41) and activation of immune cells (15). To test if this virulence factor from the parasite surface could be able to induce HO-1, we incubated macrophages with LPG isolated from the *L. chagasi* strain Ba262. Indeed, LPG was capable of inducing HO-1 production by mouse macrophages (Fig. 4B). HO-1 expression is transcriptionally regulated by PPARγ (42). Because PPARγ is induced by *L. donovani* and could exacerbate the disease in a chronic experimental model of VL (43), we determined whether PPARγ was involved in the induction of HO-1 by *L. chagasi* and its LPG. Interestingly, induction of HO-1 by macrophages stimulated with *L. chagasi* (Fig. 4A) or LPG (Fig. 4B) persisted in the presence of a PPARγ antagonist (GW 9662) or an agonist (rosiglitazone). Thus, the induction of HO-1 does not seem to be regulated by PPARγ in the context of *L. chagasi* infection.

HO-1 promotes Leishmania *persistence in human macrophages*

To verify if our results were reproducible in human macrophages, we performed in vitro infections with *L. chagasi* in the presence of CoPP. Induction of HO-1 favored increased percentage of infected macrophages (Fig. 5A), number of intracellular amastigotes (Fig. 5B), and the viability of *L. chagasi* inside macrophages (Fig. 5C). With an attempt to verify a potential mechanism by which the induction of HO-1 favors *L. chagasi* persistence inside the human macrophages, we tested surrogates of the oxidative stress. Treatment of the cells with CoPP led to reduction in the production of superoxide radicals triggered by *L. chagasi* infection (Fig. 5D). Interestingly, some antioxidant mechanisms of HO-1 require interplay with another class of antioxidant enzyme, the superoxide

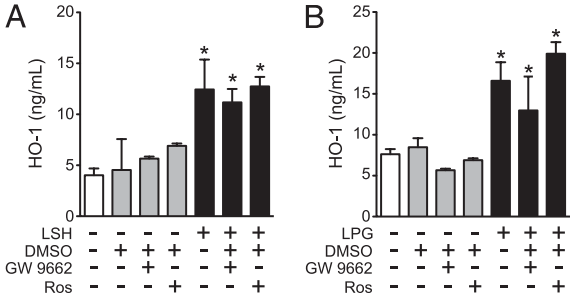

FIGURE 4. LPG from *Leishmania* induces HO-1 in macrophages. Murine peritoneal macrophages were pretreated with PPARγ agonist (5 μM rosiglitazone) or antagonist (10 μM GW9662) following interaction with *L. chagasi* promastigotes (LSH) (**A**) or LPG (**B**). HO-1 was measured in cell lysates at 48 h poststimulation. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. Bars represent mean ± SD. Kruskal–Wallis test was used to compare the experimental groups. *$p < 0.05$ when compared with untreated macrophages.

dismutase (44, 45). Therefore, we investigated the association between the induction of HO-1 and SOD-1. Notably, SOD-1 has been shown to favor *Leishmania* growth in human macrophages (35). We found hereby that the induction of HO-1 by CoPP upon *L. chagasi* infection is also associated with induction of SOD-1 protein (Fig. 5E) in human macrophages. These findings argue that induction of HO-1 favors *Leishmania* survival by its antioxidant activities per se and also by the induction of superoxide dismutases, which are potent scavengers of superoxide anions generated during infection.

HO-1 is strongly associated with human visceral leishmaniasis

The ultimate goal of our current investigation was to investigate whether HO-1 was associated with the human disease caused by *L. chagasi* infection. We then evaluated serum samples obtained from patients with VL and sex-matched endemic controls from a highly endemic area in the northeast of Brazil. The baseline characteristics of the study participants are shown in Supplemental Table I. Patients with VL presented higher serum concentrations of HO-1 compared with healthy individuals ($p < 0.0001$; Fig. 6A). A logistic regression adjusted for age revealed that HO-1 is indeed strongly associated with VL (odds ratio: 30.28; 95% confidence interval: 12.72–45.01; $p < 0.0001$). The systemic concentrations of HO-1 were significantly reduced 15–30 d after the antileishmanial treatment ($p < 0.01$; Fig. 6B). An analysis using ROC curves revealed that HO-1 could be used to discriminate patients with VL from those uninfected in the cohort studied (Fig. 6C). In individuals with VL, HO-1 presented positive correlation with IL-10 ($r = 0.56$; $p < 0.0001$; Fig. 6F), a major factor involved in the clinical severity of VL (46, 47). HO-1 was also correlated with diverse markers of systemic inflammation, such as TNF-α ($r = 0.23$; $p = 0.0117$; Fig. 6D), IL-6 ($r = 0.58$; $p < 0.0001$; Fig. 6G), and IL-8 ($r = 0.44$; $p < 0.0001$; Fig. 6H). These results reinforce the notion that HO-1 is associated with *L. chagasi* in humans and that HO-1 may be linked to susceptibility to human VL. Given that HO-1 is a stress-responsive gene, this finding is consistent with the notion that the disease is associated with increased oxidative stress, thereby explaining the increased level of expression of this enzyme. As expected, after the antileishmanial treatment, systemic levels of TNF-α were reduced ($p < 0.001$; Fig. 6E) and no longer correlated with HO-1 ($r = 0.4920$; $p = 0.0625$). The next step was to test whether it is possible to interfere with HO-1 expression by inhibiting proinflammatory cytokines in human cells. We found that treatment of human PBMC with PTX, a pharmacological TNF-α inhibitor with wide clinical use, resulted in reduction of HO-1 production upon infection with *L. chagasi* (Supplemental Fig. 3A, 3B).

Discussion

Studies have emphasized that the cytoprotective enzyme HO-1 plays a pivotal role in maintaining cellular homeostasis during inflammation (48), and its expression is increased in a variety of pathological conditions (29). The protective actions of HO-1 during infection are usually associated with the reduction in immunopathology caused by the oxidative stress (49, 50). Indeed, a higher expression of HO-1 results in diminished damage of cells and tissues even at the relatively high infection burden, what is called tolerance to infection (20, 51). In this study, we show that HO-1 seems to affect host effector molecules that drive resistance to infection, such as inflammatory cytokines and free radicals, which favors *Leishmania* persistence. Our results also suggest that HO-1 may play an essential dual role during *Leishmania* infection. On one hand, HO-1 can protect host tissues against injury by damping excess of inflammation. On the other hand, induction of HO-1 protects the parasite against the host defense. Similarly, upon *Plasmodium* infection, induction of HO-1 seems to be an

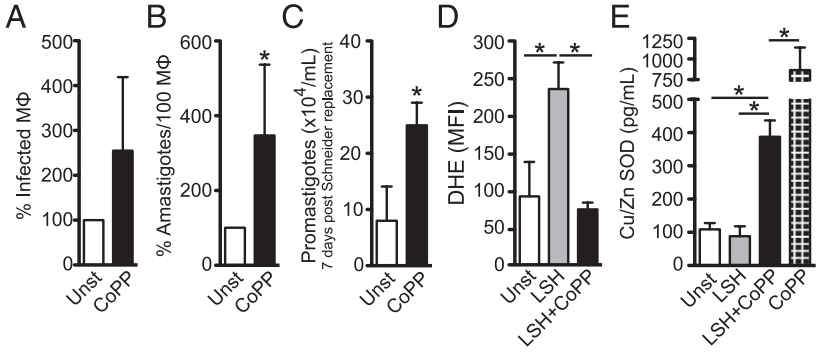

FIGURE 5. HO-1 promotes *Leishmania* persistence in infected human macrophages. Monocyte-derived human macrophages infected with *L. chagasi* were cultured with or without 50 μM CoPP for 72 h, and the percentage of infected macrophages (**A**) and number of intracellular amastigotes (**B**) were quantified. Results shown in (A) and (B) are displayed as percentage of control (group infected only with *L. chagasi*). (**C**) Intracellular survival of *L. chagasi* amastigotes was quantified by transformation of proliferating extracellular motile promastigotes in Schneider's medium. Each bar represents the mean ± SD of six to eight donors. Differences were estimated using Mann–Whitney *U* test. (**D**) Median fluorescence intensity (MFI) for ROS in macrophages infected with *L. chagasi* in the presence or absence of CoPP, evaluated by flow cytometry using the probe DHE. (**E**) Cu/Zn SOD expression of protein in cell-culture supernatant of those cells was quantified by ELISA. Each bar represents the mean ± SD. Kruskal–Wallis nonparametric test followed by Dunn's posttest was used to evaluate statistical significance. *$p < 0.05$.

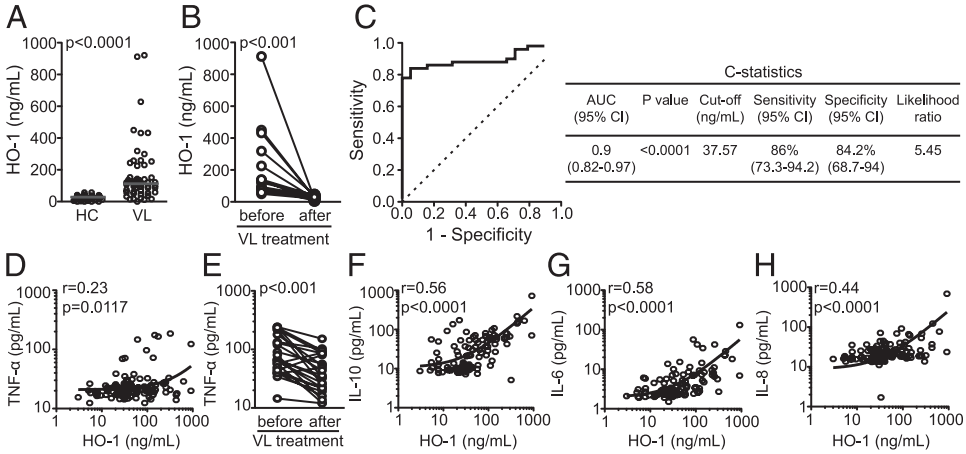

FIGURE 6. HO-1 is strongly associated with human visceral leishmaniasis. Serum samples were obtained from individuals at an endemic area in the Northeast of Brazil. Serum levels of HO-1 were measured in healthy endemic controls (HC; $n = 42$) and in patients with VL ($n = 50$) at admission to a reference hospital (**A**) and after 15 d of the antileishmanial treatment (**B**). (**C**) An ROC curve was used to evaluate the power of serum HO-1 to discriminate VL patients from the healthy endemic controls. Concordance (C)-statistics are illustrated in the table (*right panel*) and were used to verify the validation of the ROC curves and the discrimination power of HO-1. Mann–Whitney *U* test was used to verify differences between HC and VL. Wilcoxon matched-pairs signed rank test was performed to calculate the statistical significance in HO-1 and TNF-α (**E**) serum levels before and after treatment. Correlations between systemic concentrations of HO-1 and TNF-α (**D**), IL-10 (**F**), IL-6 (**G**), and IL-8 (**H**) were calculated using the Spearman test. The values of p and r are illustrated in each graph. AUC, Area under the curve.

obligatory step in the liver stage by controlling host innate inflammatory responses and protecting infected hepatocytes from cell death (21, 30). To date, little is known about the role of HO-1 in *L. chagasi* pathogen burden, and our data demonstrate that HO-1 enhances *L. chagasi* survival within host macrophages and that HO-1 is a highly associated with human VL. We propose that HO-1 plays two major roles following *L. chagasi* infection: 1) prevents damage to host cells; and 2) decreases the host ability to limit the intracellular growth of the parasite.

Hmox1 gene expression involves multiple pathways, including redox-dependent and -independent signaling molecules and immune mediators (52–54). Most of the studies that tried to address the role of HO-1 in infectious diseases have been focused on the anti-inflammatory effects, especially during malaria infection. Indeed, Pamplona and colleagues (20) have demonstrated that infection of BALB/c mice with *Plasmodium berghei* causes upregulation of HO-1 in the brain. It has been also suggested that *P. berghei* infection upregulates HO-1 in hepatocytes in vivo and peritoneal macrophages in vitro (30). In the current study, we observed that *L. chagasi* infection induced HO-1 in mouse macrophages, whereas concentrations of HO-2, the constitutive HO isoform, remained unchanged upon infection or treatment with CoPP. The obvious further step was to try to identify what molecule from the parasite would be able to trigger HO-1. A natural candidate would be LPG, which is the major *Leishmania* parasite surface molecule and has been implicated in *Leishmania* survival within mammalian macrophages in vitro (41, 55). Interestingly, we found that LPG was able to strongly induce HO-1 in mouse macrophages. Although tempted, we cannot assume that LPG is the most important trigger for HO-1 induction, because we did not rule out other *Leishmania* surface proteins. The absence of available mutants for *L. chagasi* still underlines the need for further experiments. After the identification of a possible trigger, we tested whether PPARγ, a known transcription factor of upstream *Hmox1* gene activation (42), would be involved in *L. chagasi*-induced HO-1 expression. PPARγ has been implicated in alternative activation of macrophages upon infection with *L. major*, which favors parasite survival (56) and interferes with adaptive immunity to exacerbate the pathogenesis of experimental VL (43). Our experiments performed using macrophages cocultured with an agonist or an antagonist of PPARγ showed no alteration in HO-1 induction by either *L. chagasi* or LPG, suggesting that PPARγ does not seem to be the major orchestrator of *Hmox1* induction in this experimental model. Thus, the specific transcription factors upstream of HO-1 that are induced by *Leishmania* or LPG remain to be determined.

Our results suggest that the HO-1 expression in macrophages is an important subversion mechanism by which *Leishmania* parasites can escape from the oxidative burst. Similarly, Pham and colleagues (31) have shown that *L. pifanoi* avoids elicitation of superoxide production during their internalization, and this phenomenon is dependent on HO-1 production. However, the authors did not address whether this escape via HO-1 has any impact on infection burden and *Leishmania* survival. Our study expands the current knowledge, as we demonstrate that induction by HO-1 by CoPP markedly increases parasite burden within infected macrophages. We then tested whether the genetic deficiency of HO-1 expression would be associated with modulation of parasite burden. Indeed, we found that lack of HO-1 expression leads to a significant reduction in parasite load and that this effect was correlated with a higher TNF-α production in response to infection, indicating that expression of *Hmox1* gene promotes *Leishmania* survival. In fact, infection burdens in BMMs from *Hmox1*$^{-/-}$ mice treated with CoPP were unaffected, indicating that CoPP acts through HO-1 to favor parasite persistence. We propose that in *L. chagasi* infection, HO-1 plays a similar role as in malaria, in which overexpression of HO-1 increases *P. berghei* liver infection, and that HO-1 is required to protection of infected hepatocytes by controlling the inflammatory responses (30). TNF-α is a central inflammatory cytokine in the induction of macrophage antimicrobial activities (57, 58) and has been associated with disease severity in patients with VL (59, 60). In fact, we found that induction of HO-1 reduced production of TNF-α and ROS upon *L. chagasi* infection of macrophages, consistent with mechanisms of cell protection by HO-1 (48). Furthermore, induction of HO-1 caused reduction of proinflammatory mediators such as NO, MCP-1, and IL-6 by macrophages primed with IFN-γ or LPS. A recent study suggested that LPS might contribute to the cytokine

storm and cellular activation in patients with VL (61). We propose that HO-1 is probably increasing the tolerance to *L. chagasi* infection by reducing the inflammatory status of activated macrophages and that this is probably critical in human VL, which is frequently associated with bacterial coinfection.

Finally, our findings on human macrophages confirm that HO-1 induction increases parasite load and indicate that this mechanism may be important for the pathogenesis of human disease. Patients with VL had higher serum levels of HO-1 than those not infected, and the systemic concentrations of HO-1 were significantly reduced at 15 d post-antileishmanial treatment. HO-1 is primarily thought to be an intracellular enzyme (62), and the increased serum levels of HO-1 suggest that some degree of cell death is occurring during VL. Indeed, knowing the source of extracellular HO-1 is still needed. Our data show that high concentrations of serum HO-1 are associated with a higher chance to have VL in our cohort of patients. Consistent with our results, increased concentrations of HO-1 have already been associated with other diseases such as vasculitis in Henoch-Schonlein purpura (63), hemophagocytic syndromes from hematological conditions (64, 65), type 2 diabetes (66), and prostate cancer (67). Thus, although showing strong associations between HO-1 and VL, our study expands the list of the diseases in which HO-1 potentially plays a fundamental role. In the current study, we also show that systemic concentrations of HO-1 are positively correlated to diverse cytokines, such as TNF-α, IL-6, IL-8, and IL-10. In this context, HO-1 could play a role similar to the one previously described for IL-10, which is anti-inflammatory but strongly associated with inflammatory conditions and also in human VL (2, 46). We found positive correlation between serum HO-1 and TNF-α. Of note, there is increased production of several cytokines and chemokines in VL patients, and much of the response appears to be proinflammatory, as indicated by the elevated plasma protein levels of IL-1, IL-6, IL-8, and TNF-α (reviewed in Ref. 58). Indeed, elevated serum levels of TNF-α have been associated with VL (60). We speculate that the systemic amounts of the proinflammatory cytokines could be even higher in the absence of HO-1. The high levels of TNF-α and cytokine storm, features of VL, could be inducing higher levels of HO-1 as a counterregulatory response. Similar to our results, patients with hemophagocytic syndrome also present a positive correlation between HO-1 expression and serum TNF-α (65), suggesting that this pattern may be common in inflammatory conditions in vivo. Our results argue that it is possible that the susceptibility to infection would be worse in case of the lack or absence of HO-1 in human VL. Whether the systemic concentrations of the cytokines and HO-1 evaluated in this study represent their respective amounts in the tissues in which the infected cells are localized deserves further investigation.

In summary, the current study shows that HO-1 drives *L. chagasi* infection within macrophages by means of its anti-inflammatory properties, reinforcing that *Hmox1* gene is required for *Leishmania* survival and persistence. Moreover, we show that HO-1 is strongly associated with human VL. Therefore, HO-1 may represent an important escape mechanism required for the control of *Leishmania* replication by immune cells and could be used as a therapeutic target to reduce VL severity. Protection against excessive inflammatory response may preclude deleterious effects of VL.

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Disclosures

The authors have no financial conflicts of interest.

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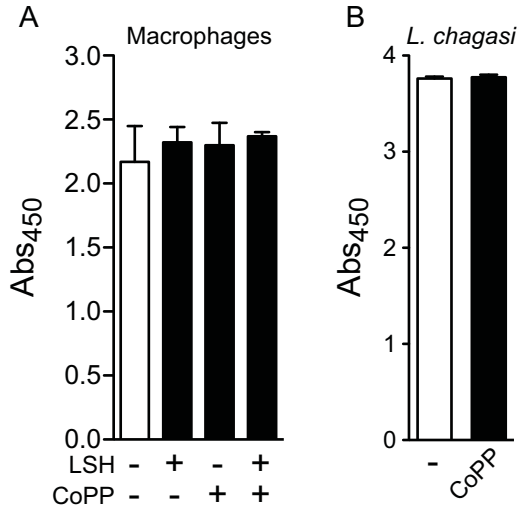
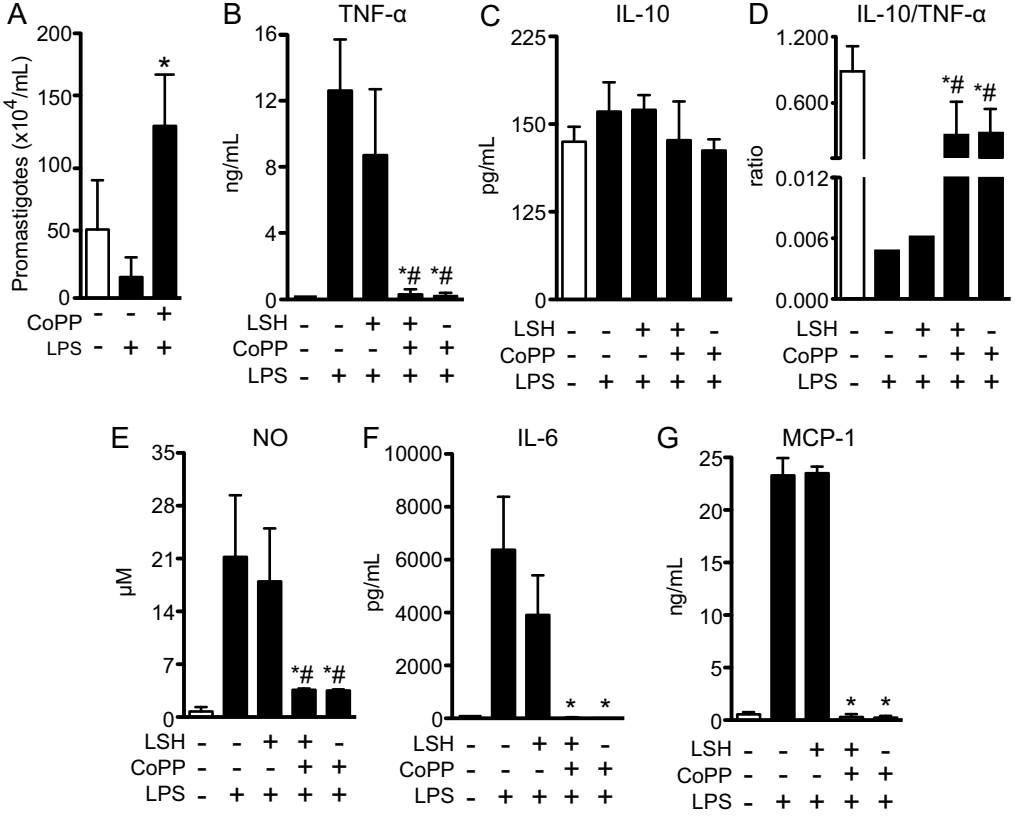
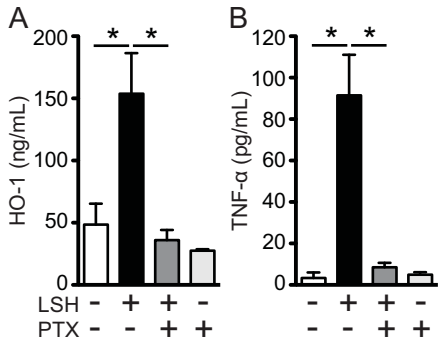


Supplementary figure 1: Viability of murine macrophages and *Leishmania* promastigotes treated with CoPP. (A) Cytotoxicity of 50μM CoPP towards murine peritoneal macrophages infected with *L. chagasi* or towards axenic promastigotes cultures (B) was determined by the XTT assay. Data are from one representative experiment performed with at least 4 mice per experimental group. Data represent mean ± SD and were evaluated using Kruskal-Wallis test.



Supplementary figure 2: HO-1 modulates inflammatory responses in infected macrophages primed with LPS. Murine peritoneal macrophages were infected in vitro with *L. chagasi* in the presence or absence of 50 μM CoPP and/or 100 ng/mL LPS. (A) Intracellular survival of *L. chagasi* amastigotes was quantified by transformation of proliferating extracellular motile promastigotes in Schneider's medium at 7 days post medium replacement. (B) TNF-α, (C) IL-10, (D) IL-10/TNF-α ratio, (E) NO (F) IL-6 and (G) MCP-1 were measured in cell culture supernatants as describe in methods. Data represent mean ± SD from one representative experiment performed with at least 4 mice per experimental group. Kruskal-Wallis test with Dunn's multiple comparisons post test was used to compare the groups. * $p < 0.05$, when compared to LPS treated macrophages; # $p < 0.05$, when compared to *L. chagasi* infected macrophages treated with LPS.



Supplementary figure 3: The role of *L. chagasi* infection on the levels of HO-1 and TNF-α. PBMCs (10^6/well) from six healthy volunteers were cultured in the presence of 1mM Pentoxifylline (PTX) and/or *L. chagasi* promastigotes as described in Materials and Methods. HO-1 (A) was measured in cell extracts and TNF-α (B) was measured in the supernatants, 24h and 12h after treatment, respectively. Bars and lines represent means and SD. Kruskal-Wallis tests with Dunn's multiple comparisons or selected pairs were used to evaluate statistical significance. *p<0.05.

Supplementary Table 1:

Baseline characteristics of the study participants

Variable	Healthy endemic controls (HC)	Individuals with Visceral Leishmaniasis (VL)	P value
	n=42	n=50	
Age (years) - mean and SD	22.51±13.64	12.88±11.86	0.0002
Male (%)	40.47	60	ns
Hemoglobin (g/dL)	13.21±1.05	8.82±1.5	<0.05
Platelets (no./L)	241.5±46.42	170.5±63.14	<0.05
Neutrophils (% of total leucocytes)	47.19±11.79	33.83±16.18	<0.0001

Data represented by percentages were evaluated using Fisher's exact test whereas Mann-Whitney test was used to verify differences between HC and VL, represented by mean and SD. ns, non significant

4. PARTE II

4.1 HIPÓTESE

A indução de necroptose contribui para a inflamação e controle da carga parasitária em macrófagos infectados por *Leishmania.*

4.2 OBJETIVOS

OBJETIVO GERAL

Avaliar o papel da via da necroptose na infecção por *Leishmania*.

OBJETIVOS ESPECÍFICOS

1) Analisar o papel do heme na morte celular necrótica em células humana;
2) Analisar o papel dos inibidores de RIPK1, RIPK3 e MLKL na morte celular induzida por heme;
3) Investigar a relevância da RIP1, RIP3 e MLKL na replicação de *Leishmania* em macrófagos tratados com heme;
4) Investigar a participação de moléculas da via da necroptose na replicação de *Leishmania* em macrófagos;
5) Analisar a participação de moléculas da via da necroptose em um modelo experimental de Leishmaniose.
6) Investigar os mediadores inflamatórios associados a via da necroptose e importantes para a resistência na infecção por *Leishmania.*

4.3 MANUSCRITO 2

PROGRAMMED NECROSIS IN THE CROSS TALK OF CELL DEATH AND INFLAMMATION.

Nessa revisão nós discutimos o papel da necrose programada em diversos processos biológicos.

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Programmed Necrosis in the Cross Talk of Cell Death and Inflammation

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Keywords

necroptosis, RIPK1, RIPK3, MLKL, TNF, inflammation, TRIF, DAI, MCMV, vaccinia virus

Abstract

Cell proliferation and cell death are integral elements in maintaining homeostatic balance in metazoans. Disease pathologies ensue when these processes are disturbed. A plethora of evidence indicates that malfunction of cell death can lead to inflammation, autoimmunity, or immunodeficiency. Programmed necrosis or necroptosis is a form of nonapoptotic cell death driven by the receptor interacting protein kinase 3 (RIPK3) and its substrate, mixed lineage kinase domain-like (MLKL). RIPK3 partners with its upstream adaptors RIPK1, TRIF, or DAI to signal for necroptosis in response to death receptor or Toll-like receptor stimulation, pathogen infection, or sterile cell injury. Necroptosis promotes inflammation through leakage of cellular contents from damaged plasma membranes. Intriguingly, many of the signal adaptors of necroptosis have dual functions in innate immune signaling. This unique signature illustrates the cooperative nature of necroptosis and innate inflammatory signaling pathways in managing cell and organismal stresses from pathogen infection and sterile tissue injury.

INTRODUCTION

Cell death is an important biological process that sculpts the development of multicellular organisms. In the immune system, cell death plays critical roles in immune cell development and pathogen defense. Apoptosis is an orderly form of cell death marked by chromatin condensation, DNA fragmentation, and membrane blebbing into apoptotic bodies. Apoptosis can be triggered by receptors in the tumor necrosis factor (TNF) superfamily (extrinsic pathway) or through direct activation of mitochondrial effectors (intrinsic pathway). Caspases are cysteine proteases that drive apoptosis. Effector caspases cleave and inactivate the flippase adenosine triphosphatase type 11C and scramblase X Kell blood group precursor related family member 8 (Xkr8) (1, 2). This results in exposure of phosphatidyl serine (PS) on the cell surface, which flags the dying cell for uptake and clearance by professional phagocytes such as macrophages (3). The rapid clearance of apoptotic cells ensures minimal risk of detrimental inflammation. This explains why apoptosis is the preferred and dominant pathway by which multicellular organisms eliminate unwanted cells during development. In contrast, necrosis is marked by rapid loss of plasma membrane integrity. Plasma membrane leakage in necrosis is widely thought to occur prior to or concomitant with exposure of PS and other eat-me signals. This early rupture of the plasma membrane releases endogenous danger signals or danger-associated molecular patterns (DAMPs), which are potent stimulants of inflammation (4). As such, necrosis is often detected in infections and inflammatory diseases. This association has led to the popular view that necrosis represents pathological cell death, whereas apoptosis is more central for development.

Pathologists have historically relied on morphology to distinguish between apoptosis and necrosis. Apoptosis is marked by cell shrinking, the appearance of membrane blebs called apoptotic bodies, and condensation of chromatin. In contrast, necrosis is associated with cell and organelle swelling and limited chromatin condensation. Biologists have long considered necrosis as the consequence of trauma or accidental injury. This view has now been revised with recent advances showing the existence of dedicated molecular pathways controlling necrotic cell death. Notably, the classical markers that define apoptosis can sometimes be detected in necrosis. For example, Annexin V staining is a commonly used method to detect exposure of PS on the outer leaflet of the plasma membrane in early apoptotic cells. In some necrotic cells, PS exposure can be detected without significant plasma membrane leakage (5). PS exposure is supposed to mark apoptotic cells for clearance by phagocytes. However, researchers have also described scavenger receptors that recognize necrotic cells (6, 7). Moreover, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, which detects DNA strand breaks in apoptosis, also detects such breaks in necrotic cells (8). These observations suggest that the morphological definition of apoptosis and necrosis is insufficient to distinguish between these two cell death modules. Instead, we favor a molecular definition based on genetic pathways. Terms such as programmed necrosis, regulated necrosis, and necroptosis are now used to describe necrosis induced by the receptor interacting protein kinases (RIPKs) (9). In addition, certain forms of regulated necrosis can occur without the RIPKs (10, 11).

Here, we focus our discussion on RIPK-driven necrosis. The term necroptosis is used throughout to distinguish RIPK-dependent necrosis from other forms of regulated necrosis. As is discussed below, pathways that control necroptosis and inflammation often use overlapping signaling adaptors. The sharing of common signal adaptors establishes an intimate link between inflammation and necroptosis that goes beyond their association in disease pathologies. Hence, necroptosis and inflammation can be mutually reinforcing processes that govern not only inflammatory diseases but also immune and organismal homeostasis.

THE MOLECULAR MACHINERY OF PROGRAMMED NECROSIS

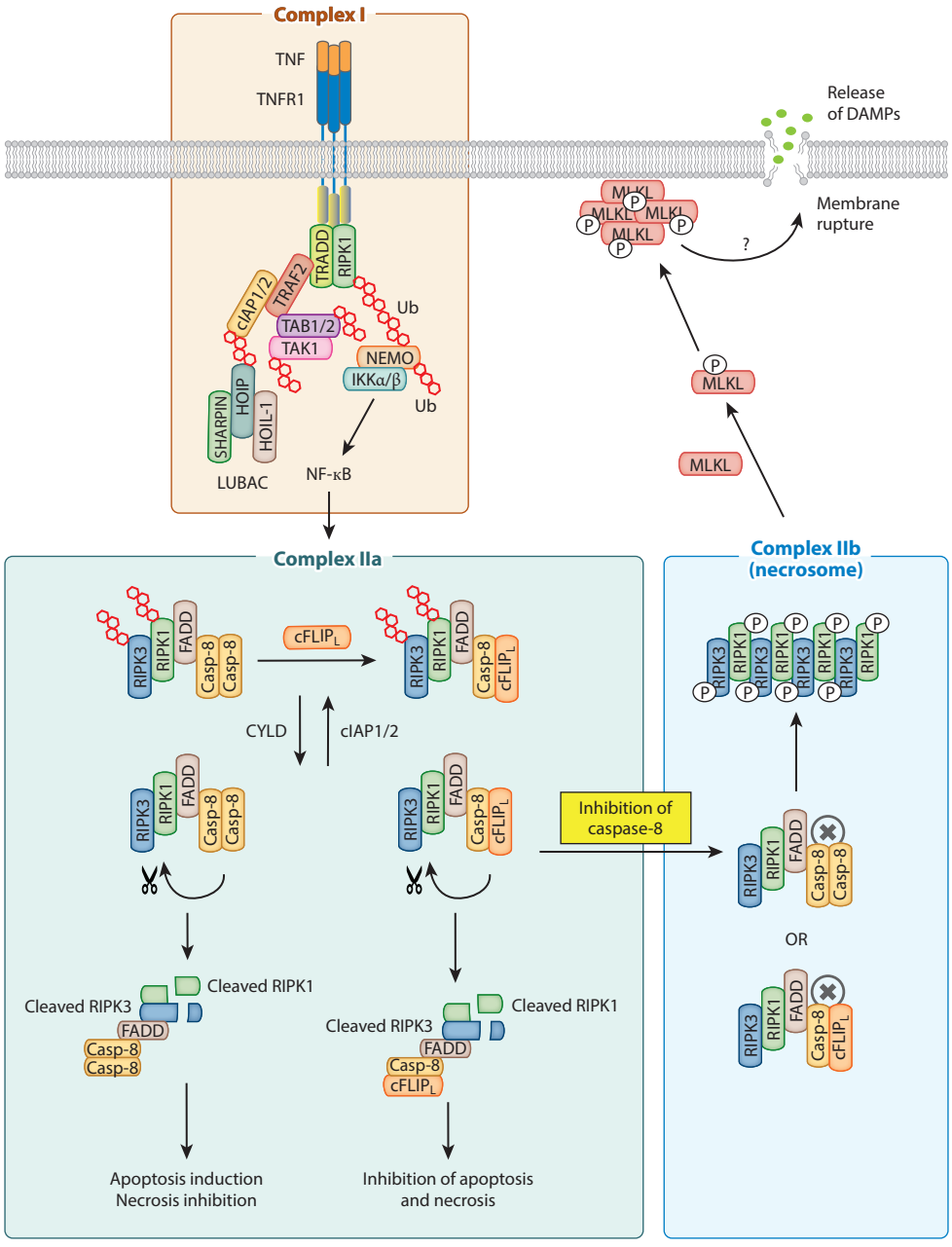
Necroptosis can be activated by death receptors in the TNF superfamily, Toll-like receptor 3 (TLR3) and TLR4, and the interferon receptors (12). The signaling pathway for necroptosis is best characterized for TNF receptor 1 (TNFR1). TNFR1 is the prototypic member of a subfamily within the TNF receptor superfamily that contains an essential protein interaction domain called the death domain (DD). DD-containing death receptors include CD95/FAS/APO-1, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 and 2, death receptor 3 (DR3), DR6, and ectodysplasin A receptor. However, cell death is not the only signaling outcome for the death receptors. In fact, nuclear factor-κB (NF-κB) activation is often the dominant response emanating from these receptors. TNFR1 is a prime example of such a receptor. Ligation of the preassembled TNFR1 trimer (13) with TNF causes a conformation change that promotes formation of a short-lived membrane-signaling complex termed Complex I by Micheau & Tschopp (14). This membrane complex is composed of the adaptors TNF receptor–associated death domain (TRADD), TNF receptor–associated factor 2 (TRAF2), RIPK1, cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, and the linear ubiquitin chain assembly complex (LUBAC), which is made up of the subunits RanBP-type and C3HC4-type zinc finger containing 1 (aka HOIL-1), ring finger protein 31 (aka HOIP), and Shank-associated RH domain interacting protein (SHARPIN) (15). The E3 ligases cIAP1, cIAP2, and HOIL-1 within LUBAC critically control ubiquitination of many of the adaptors within Complex I. Ubiquitin linkages of different types have been found with various adaptors in Complex I. This ubiquitin network within Complex I is essential for recruitment and activation of the inhibitor of κB kinase (IKK) complex. The activated IKK phosphorylates IκBα, leading to IκBα degradation by the proteasome and nuclear translocation of NF-κB dimers.

NF-κB is a key transcription factor for many proinflammatory and survival genes. A proper NF-κB response is crucial for cell survival and to counteract the cytotoxic effects of TNF. The prosurvival effect of NF-κB is mediated in part by its transcriptional targets cIAP1, cIAP2, and the long form of cellular FLICE-like inhibitor protein ($cFLIP_L$) (16, 17). As we discuss below, $cFLIP_L$ and the cIAPs critically regulate cellular sensitivity to apoptosis and necroptosis. Hence, Complex I is a critical checkpoint for cell death versus cell survival signaling (**Figure 1**). In addition, Complex I adaptors appear to have NF-κB-independent survival functions. For example, mice lacking both cIAP1 and cIAP2 die at an earlier stage in embryonic development than RelA/p65-deficient mice (18). The adaptor TRAF2 stabilizes cIAP1 expression by preventing its autoubiquitination and proteasomal degradation (19). Consistent with this association, *Traf2*$^{-/-}$ mice also exhibit embryonic lethality (20). In contrast, *cpdm* mice that lack the LUBAC subunit SHARPIN show defective NF-κB activation but are nonetheless viable (21–23). These results demonstrate that the cytoprotective effects of TRAF2, cIAP1, cIAP2, and X-linked IAP (XIAP) are mediated through NF-κB-dependent and -independent functions.

Unlike conventional death receptors such as Fas or TRAIL receptors, Fas-associated via death domain (FADD) and caspase-8 are not recruited to the TNFR1-associated Complex I (14, 24). Instead, rapid receptor internalization is important for docking of the adaptor FADD and the initiator caspase, caspase-8, to the complex. Although evidence suggests that FADD and caspase-8 can be recruited to the TNFR1 complex (25), standard biochemical pull-down supports a model in which TNFR1 dissociation occurs prior to docking of FADD and caspase-8 (14). The cytosolic complex that contains FADD and caspase-8 is often referred to as Complex IIa (**Figure 1**). Normally, apoptosis is prevented by dimerization between caspase-8 and $cFLIP_L$, an enzyme-inactive homolog of caspase-8. The caspase-8/$cFLIP_L$ heterodimer inhibits full activation of caspase-8 and apoptosis but retains cleavage of essential necrosis regulators such as RIPK1, RIPK3, and

cylindromatosis (CYLD) (26–30). Hence, NF-κB-dependent induction of $cFLIP_L$ inhibits apoptosis as well as necroptosis.

Active caspase-8 in Complex IIa not only initiates the caspase cascade and the apoptotic program but also cleaves and inactivates essential necroptosis mediators such as RIPK1, RIPK3, and CYLD. Hence, inhibition of caspase-8 or its upstream adaptor FADD primes cells for necroptosis by preserving the integrity of RIPK1 and RIPK3. Stabilization of RIPK1 and recruitment of RIPK3 convert Complex IIa to Complex IIb or the necrosome (**Figure 1**). The



cytosolic complexes that contain the RIPKs have also been referred to as the ripoptosome (31, 32), although this term does not make the distinction between apoptosis and necroptosis. RIPK1 and RIPK3 interact via the RIP homotypic interaction motif (RHIM) to form an amyloid-like complex that is essential for recruitment and activation of the downstream RIPK3 substrate mixed lineage kinase domain-like (MLKL) (33–35). RIPK3 phosphorylates MLKL at Thr357 and Ser358 to stimulate its oligomerization and translocation to intracellular and plasma membranes (34, 36–39) (**Figure 1**). The precise mechanism by which MLKL induces membrane rupture is controversial, with some reports implicating disruption of calcium or sodium ion channels (36, 37) and others showing direct binding to membrane phospholipids and disruption of membrane integrity (38, 39). In contrast to MLKL, another reported substrate of RIPK3, the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) (40), may not be crucial, as small hairpin RNA–mediated knockdown of *Pgam5* did not consistently confer protection against TNF-induced necroptosis (41, 42). In agreement with the notion that PGAM5 is not a core component of the necroptosis machinery, widespread depletion of mitochondria did not impair necroptosis (43). The differential requirement for mitochondrial signaling further distinguishes necroptosis from apoptosis.

Because excessive necrosis in FADD- or caspase-8-deficient mice was rescued by inactivation of RIPK1 or RIPK3 (28, 44, 45), FADD and caspase-8 are paradoxically prosurvival factors during development. This yin-yang relationship between the RIPKs and FADD/caspase-8 also plays out in the skin keratinocytes, intestinal epithelium, and T cells (46–49). Genetic evidence also provides a mechanistic explanation for the biochemical interaction between FADD, caspase-8, and the RIPKs. Intriguingly, although caspase-8-deficient Jurkat T cells are sensitized to necroptosis induced by TNF, Fas ligand (FasL), and TRAIL, FADD-deficient Jurkat cells are only sensitized to TNF-induced necroptosis (50). The molecular basis for the resistance of these cells to FasL- and TRAIL-induced necrosis is unknown. One possibility is that because FADD is the apical adaptor recruited to Fas and TRAIL receptors, downstream signaling will be completely blunted in its absence. Although this is certainly the case for FADD-mediated apoptosis, it is insufficient to explain RIPK3 signaling in T cells. *Fadd*$^{-/-}$ T cells undergo RIPK1- and RIPK3-dependent necroptosis in response to T cell receptor (TCR) stimulation (45, 48, 49). Because *Fadd*$^{-/-}$*Ripk3*$^{-/-}$ mice develop lymphoproliferation resembling that caused by the Fas mutant in *lpr* mice (51), one can argue that Fas triggers necroptosis of *Fadd*$^{-/-}$ T cells through RIPK3. Alternatively, necroptosis of *Fadd*$^{-/-}$ T cells could be the consequence of direct TCR signaling. As we explore further below, RIPK3 is also capable of signaling for necroptosis in the absence of RIPK1 under certain conditions. These perplexing results highlight the fact that the traditional model of Complex I to Complex II transition may not be adequate to account for signaling in necroptosis.

Figure 1

TNF-induced signaling complexes. The membrane-associated Complex I is chiefly responsible for NF-κB activation. The ubiquitin chains are represented by red hexagons. Induction of $cFLIP_L$ expression by NF-κB inhibits apoptosis and necroptosis. Active caspase-8 in Complex IIa promotes apoptosis and inhibits necroptosis by cleavage of RIPK1, RIPK3, and CYLD (*scissors*). When caspase-8 is inactive (*circled X*), RIPK1 and RIPK3 initiate Complex IIb assembly, amyloid conversion, and recruitment of MLKL. Both Complex IIa and Complex IIb are also regulated by protein ubiquitination. CYLD acts as the deubiquitinase that promotes Complex II activity by removing ubiquitin chains on RIPK1 and RIPK3. (Abbreviations: cIAP, cellular inhibitor of apoptosis 1; CYLD, cylindromatosis; DAMP, damage-associated molecular pattern; FADD, Fas-associated via death domain; IKK, inhibitor of κB kinase; LUBAC, linear ubiquitin chain assembly complex; MLKL, mixed lineage kinase domain-like; NF, nuclear factor; P, phosphorylation; RIPK, receptor interacting protein kinase; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; Ub, ubiquitin.)

UBIQUITINATION: A CRITICAL CHECKPOINT FOR NECROPTOSIS

RIPK1 and other Complex I adaptors are key substrates of the E3 ubiquitin ligases cIAP1 and cIAP2. As discussed above, the ubiquitin network within Complex I functions to recruit the IKK complex and to promote survival through NF-κB-dependent and -independent mechanisms (52–56). Bivalent IAP antagonists or Smac mimetics (SMs) are often used to deplete cIAP1, cIAP2, and XIAP. SMs are small-peptide mimetics of second mitochondrial-derived activator of caspases (Smac) that trigger autoubiquitination and degradation of the IAPs. Because RIPK1 ubiquitination does not occur in the absence of the IAPs, SM tips the balance of TNF signaling toward cell death. Moreover, because the NF-κB-inducing kinase (NIK) is constitutively targeted for ubiquitination and degradation by the IAPs (57–60), SM can additionally stabilize NIK, leading to noncanonical NF-κB activation and autocrine TNF production. Thus, SM primes cells for cell death through two mutually reinforcing mechanisms: elimination of a cytoprotective ubiquitin network and induction of TNF. Given that many tumors overexpress cIAPs and are resistant to traditional chemotherapies, SM can provide a powerful one-two punch to trigger cancer cell death through either apoptosis or necroptosis (61, 62). Physiologically, IAP depletion occurs in response to stimulation of TNFR2, the TNF receptor whose expression is highly inducible (63). Although TNFR2 does not contain a cytoplasmic DD, it recruits TRAF2 and the cIAPs and triggers their proteasomal degradation. Hence, similar to the action of SM, TNFR2 also skews TNF signaling toward cell death (64–67). Hence, the IAPs and ubiquitination play important roles in fending off the cytotoxic effects of TNF.

The importance of cIAPs and the ubiquitin machinery in regulating RIPK activities and necroptosis is illustrated by the partial rescue of embryonic lethality of *ciap1*$^{-/-}$*xiap*$^{-/-}$ or *ciap1*$^{-/-}$*ciap2*$^{-/-}$ embryos by loss of *Ripk3* or a single *Ripk1* allele (18). Systemic autoinflammatory disease of mice with myeloid-specific deletion of cIAP1, cIAP2, and XIAP was also corrected by inactivation of RIPK1 or RIPK3 (68). These results provide strong evidence that the IAPs are crucial guardians that keep RIPK1 and RIPK3 in check to prevent deleterious cell injury and inflammation. Further evidence that the ubiquitin network within Complex I serves critical functions in limiting cell death and inflammation comes from mice lacking the LUBAC components SHARPIN or HOIL-1. Cells lacking SHARPIN or HOIL-1 are sensitized to apoptosis as well as necroptosis (21, 69), and mice lacking these components develop systemic autoinflammatory diseases (21–23, 70). Interestingly, the severe skin and multiorgan inflammation in SHARPIN-deficient *cpdm* mice was corrected by crosses to knock-in mice expressing kinase-inactive RIPK1 (*Ripk1-K45A*) (71). Fibroblasts and macrophages from *Ripk1-K45A* knock-in mice exhibit normal mitogen-activated protein kinase and NF-κB responses but are resistant to TNF-induced necroptosis (71, 72). Hence, excessive cell death appears to be the major driver for RIPK1-dependent inflammation in *cpdm* mice. However, because the kinase activity of RIPK1 is required for apoptosis under certain conditions (73), it remains to be determined if RIPK1-induced inflammation in *cpdm* mice is driven by apoptosis or necroptosis.

In addition to mouse models, human patients with mutations in the E3 ligase subunit of LUBAC HOIL-1 exhibit chronic inflammation, increased cytokine expression in response to IL-1β, cardiomyopathy, and susceptibility to pyogenic bacteria due to impaired NF-κB activation (74, 75). Consistent with the key role of the LUBAC complex for recruitment of the IKK complex, mutations in the IKK regulatory subunit NF-κB essential modulator (NEMO)/IKKγ cause incontinentia pigmenti (IP), a disease marked by skin lesions and multiorgan inflammation. Because *Nemo* is an X-linked gene, male mice lacking NEMO are embryonic lethal (76–78), and male patients of IP are rarely found. In the few rare cases of male patients harboring mild mutations in NEMO, patients develop a variant form of the disease called hypohidrotic ectodermal

dysplasia, which is marked by abnormalities in the teeth, hair, and eccrine sweat glands (79). The chronic inflammatory phenotypes caused by mutations in the cIAPs, LUBAC, and NEMO seem to contradict the fact that cells lacking these adaptors are impaired in cytokine-induced NF-κB responses. Because cells lacking these components in the ubiquitin network are also highly sensitive to death signals, heightened cell death is likely the driver of the chronic inflammation. It will be interesting to determine if the kinase function of RIPK1 is similarly responsible for driving the lethal inflammatory disease of *Nemo*$^{-/-}$ mice as in *cpdm* mice (71).

The NF-κB transcriptional target A20 and the tumor suppressor CYLD are believed to facilitate Complex I transition to Complex II by promoting deubiquitination of RIPK1. Although both A20 and CYLD are recruited to Complex I, small interfering RNA (siRNA) knockdown of CYLD, but not A20, protects cells against TNF-induced necroptosis (69, 80, 81). Surprisingly, RIPK1 ubiquitination in Complex I was not altered in *Cyld*$^{-/-}$ cells. Rather, RIPK1 and RIPK3 ubiquitination within the necrosome was greatly elevated in *Cyld*$^{-/-}$ cells (81). Hence, rather than regulating RIPK1 ubiquitination in Complex I, CYLD acts within the necrosome to deubiquitinate RIPK1 and RIPK3. Moreover, these results suggest that in addition to regulating NF-κB activation within Complex I, the E3 ligases cIAP1 and cIAP2 may also control necrosome activation through ubiquitination of RIPK1 and RIPK3.

THE JANUS NATURE OF RIPK1

Besides acting as the upstream activator of TNF-induced, RIPK3-mediated necroptosis, RIPK1 is also required for SM-primed, TNF-induced apoptosis (73). Paradoxically, RIPK1 also functions as an inhibitor of RIPK3- and caspase-8-mediated cell injury and inflammation. *Ripk1*$^{-/-}$ mice suffer from perinatal lethality that was originally believed to be caused by defective NF-κB-mediated induction of survival genes (82). However, *Ripk1*$^{-/-}$ mice are born alive, whereas *RelA*$^{-/-}$ mice die in utero at E15.5. In addition, a recent report argues that RIPK1 does not play a significant role in NF-κB activation (83). These results suggest that defective NF-κB activity may not fully account for the perinatal lethality of *Ripk1*$^{-/-}$ mice. Mice that lack multiple cIAPs, such as *ciap1*$^{-/-}$*Xiap*$^{-/-}$ mice, suffer from embryonic lethality at E10.5. This lethality is eerily similar in mice lacking *Fadd*, *Casp8*, or *cFlip*. Strikingly, hemizygous *Ripk1* deficiency significantly prolonged survival of *ciap1*$^{-/-}$*Xiap*$^{-/-}$ mice until weaning age (18). Thus, researchers have proposed an alternative model in which RIPK1 ubiquitination sterically hinders recruitment of downstream cell death effectors (55, 56).

Recently, the molecular basis that underlies the perinatal lethality of *Ripk1*$^{-/-}$ mice was examined in further detail. *Ripk1*$^{-/-}$ mice exhibit extensive cleaved caspase-3 in multiple tissues and a systemic increase in inflammatory cytokines. The increase in caspase-3 activation and apoptosis appears to be partly due to a failure to upregulate $cFLIP_L$ expression (84). Cleaved caspase-3 and apoptosis were significantly reduced in *Ripk1*$^{-/-}$*Casp8*$^{-/-}$ mice. However, these mice still succumbed to perinatal lethality (85), indicating that apoptosis is not the only driver for the lethal phenotype. Deletion of *Ripk3* also had minimal effect on the survival of *Ripk1*$^{-/-}$ mice (84). However, *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice survived until adulthood and developed an *lpr*-like autoimmune disease that is also observed in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ mice (84–86). Tissue-specific deletion of *Ripk1* and bone marrow reconstitution experiments show that RIPK1 is essential for the survival of hematopoietic stem cells, skin keratinocytes, and intestinal epithelial cells (85, 87, 88). Inactivation of TNFR1 and the IFN receptor significantly increased survival of *Ripk1*$^{-/-}$ mice (84), suggesting that RIPK1 inhibits TNFR1- and IFN-induced cell death in multiple cell types (**Figure 2**). Surprisingly, knock-in mice expressing kinase-inactive RIPK1 are viable and do not exhibit the abnormalities found in *Ripk1*$^{-/-}$ mice (71, 72). Hence, although

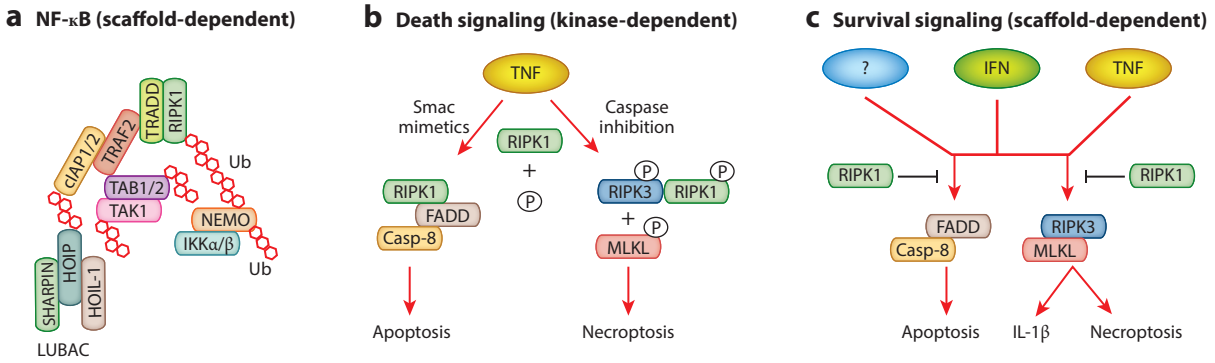


Figure 2

RIPK1 mediates cell survival and cell death through distinct mechanisms. (*a*) RIPK1 facilitates assembly of the ubiquitin scaffold that stimulates NF-κB activation. This function does not require the kinase activity of RIPK1. (*b*) The kinase activity of RIPK1 promotes apoptosis and necroptosis. (*c*) The scaffolding function of RIPK1 promotes survival and suppresses inflammation by inhibiting FADD–caspase-8 and RIPK3-MLKL activation. This RIPK1 function is required to neutralize deleterious signals from the interferon receptor, the TNF receptor, and other yet-to-be-identified receptors. The kinase activity of RIPK1 is dispensable for this survival function. (Abbreviations: cIAP, cellular inhibitor of apoptosis; FADD, Fas-associated via death domain; HOIL-1, heme-oxidized IRP2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; IFN, interferon; IKK, inhibitor of κB kinase; LUBAC, linear ubiquitin chain assembly complex; MLKL, mixed lineage kinase domain-like; NEMO, NF-κB essential modulator; NF, nuclear factor; P, phosphorylation; RIPK, receptor interacting protein kinase; SHARPIN, SHANK-associated RH domain interacting protein (SHARPIN); Smac, second mitochondrial-derived activator of caspases; TAB, TAK1-binding protein; TAK, TGF-β-activated kinase; TNF, tumor necrosis factor; TRADD, TNF receptor–associated death domain; TRAF, TNF receptor–associated factor 2; Ub, ubiquitin.)

its kinase activity promotes cell death through apoptosis and necroptosis, RIPK1 has a separate scaffolding function that curbs the death signals emanating from multiple innate immune and death receptors (**Figure 2**).

RHIM-MEDIATED AMYLOID CONVERSION IN NECROPTOSIS

In addition to needing to avoid caspase-8-mediated cleavage and deubiquitination by CYLD, induction of necroptosis also requires a RHIM-mediated interaction between RIPK1 and RIPK3 (89, 90). The RHIM is defined by a highly conserved tetrapeptide core flanked by hydrophobic residues that predominantly form β-sheets (**Figure 3**). RHIM-like adaptors are found in viruses and in *Drosophila*, arguing for a critical role for RHIM-mediated interaction immunity throughout evolution (see below). Strikingly, RHIM-containing adaptors exhibit a strong propensity to adopt an amyloid-like conformation either alone or in complex with another RHIM-containing adaptor. This unique structural scaffold is important for signaling. In the case of RIPK1 and RIPK3, disruption of this amyloid scaffold severely impairs autophosphorylation and activation of RIPK1 and RIPK3, as well as downstream execution of necroptosis (33).

Although RHIM-mediated interaction is essential for RIPK1- and RIPK3-dependent necroptosis downstream of TNFR1, not all RHIM-mediated interactions lead to cell death. For example, Toll/Interleukin-1 receptor (TIR) domain–containing adaptor-inducing interferon-β (TRIF) and RIPK1 interact via their respective RHIMs to mediate NF-κB activation downstream of TLR3 or TLR4 (91, 92). By contrast, RIPK3 inhibits this response, apparently through disruption of RHIM-RHIM interaction between RIPK1 and TRIF. Similarly, the murine cytomegalovirus (MCMV) necrosis inhibitor M45/viral inhibitor of RIPK activation (vIRA) inhibits premature

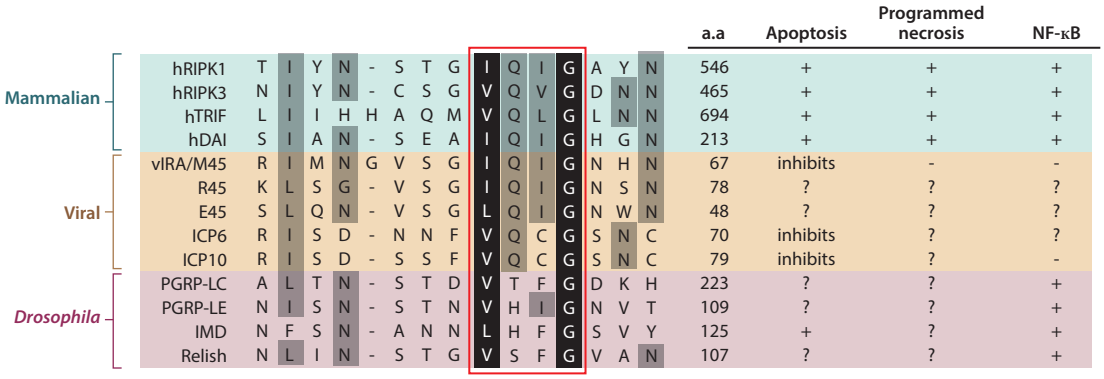


Figure 3

The RHIM is a conserved signaling motif in innate immune and death signaling adaptors. Sequence alignment of mammalian (human), viral, and *Drosophila* RHIM-containing adaptors is shown. R45 and E45 are the M45 homologs in the Maastricht and English isolates of rat CMV, respectively. ICP6 and ICP10 are the M45 homologs of HSV-1 and HSV-2, respectively. Although all the viral RHIM adaptors encode a ribonucleotide reductase domain, not all are active enzymes (149). The *Drosophila* RHIM-like adaptors and receptors are included for comparison, although there is currently no evidence to indicate that they function like the mammalian RHIMs. In addition to the PGRPs, the *Drosophila* IMD and Relish also contain RHIM-like motifs (A. Kleino & N. Silverman, personal communication). The red box indicates the tetrapeptide core of the RHIM. The black and gray shading represents highly and moderately conserved residues, respectively, as defined by functional side chains. (Abbreviations: +, positive inducer of the indicated response; –, unable to induce the indicated response; ?, undefined function for the indicated response; a.a., amino acid position of the last residue shown in the sequence alignment; CMV, cytomegalovirus; HSV, herpes simplex virus; IMD, immune deficiency; NF, nuclear factor; PGRP, peptidoglycan recognition protein; RHIM, receptor interacting protein homotypic interaction motif.)

necroptosis in infected cells by binding to RIPK3 and preventing it from interacting with its partner, DNA activator of interferon (DAI) (93, 94) (see below). Because all these RHIM-containing adaptors have the propensity to form amyloid fibrils in vitro (33), it will be vital to determine whether amyloid conversion also occurs in situations that do not result in cell death.

Amyloid complexes are widely perceived to be the etiological agents for age-related dementias such as Alzheimer's disease (AD) and Parkinson's disease. The neurological pathologies in these diseases are often marked by necrosis and inflammation. Recent evidence indicates that signaling through the nucleotide-binding oligomerization domain-like receptor, pyrin domain containing 3 (NLRP3) inflammasome drives the disease pathology in AD (95, 96). Interestingly, the NLRP3 inflammasome is activated by agonists that are aggregate in nature, including silica, uric acid crystals, and amyloid β (Aβ) peptides. This raises the tantalizing possibility that the RIPK1-RIPK3 amyloid fibrils may stimulate neuronal injury and inflammation by activating the NLRP3 inflammasome. Alternatively, released RIPK1-RIPK3 amyloid fibrils from dying neurons may seed the amyloid conversion of pathogenic amyloid proteins such as Aβ, α-synuclein, or tau. RIPK1 has been implicated in several models of ischemia-reperfusion-induced brain injury (97, 98). It will be interesting to determine if there is any interaction between the RIPK1-RIPK3 complex and any of the neurotoxic, amyloid-like peptides and whether the RIPKs contribute to age-related neurodegeneration.

NONCANONICAL NECROSOMES

As we alluded to in the above section, the RHIM is also found in the TLR3/4 adaptors TRIF and DAI. A common link for the mammalian RHIM-containing adaptors is that they share functions in innate immune signaling, cell death signaling, or both (**Figure 3**).

In the presence of caspase inhibition, TLR3 and TLR4 stimulation causes necroptosis mediated by TRIF and RIPK3 (99, 100). Similar to RIPK1 and RIPK3, TRIF is a cleavage substrate of caspase-8. TRIF cleavage by caspase-8 inhibits its ability to stimulate NF-κB-dependent cytokine gene expression (101). However, researchers do not know whether caspase-8 cleavage of TRIF also inhibits necroptosis. TRIF-dependent necroptosis requires binding to RIPK3 via the RHIM. In contrast to that of TRIF and RIPK3, the role of RIPK1 in TLR3- and TLR4-induced necroptosis is enigmatic. The RIPK1 inhibitor necrostatin-1 (Nec-1) inhibited TLR3- and TLR4-induced necroptosis in primary bone marrow–derived macrophages, the macrophage cell line J774, and, to a lesser extent, the endothelial cell line SVEC4-10 (99, 100). However, *Ripk1*$^{-/-}$ fibroblasts or siRNA knockdown of RIPK1 in 3T3 fibroblasts and SVEC4-10 did not rescue TLR3-induced necroptosis. Because Nec-1 was able to enhance survival of TLR3- and TLR4-induced necroptosis in J774 macrophages with silenced expression of RIPK1 (100), the protection conferred by Nec-1 might be due to off-target effects (102, 103).

Unlike RIPK1, TRIF does not possess kinase activity. This implies that the mechanism by which TRIF activates RIPK3 is different from that used by RIPK1. We therefore propose the term noncanonical necrosome to distinguish pronecrotic RIPK3 complexes that do not contain RIPK1. In addition to TRIF, RIPK3 can also partner with DAI to induce necroptosis during MCMV infection (see below). Canonical necrosome activation requires RIPK1-dependent phosphorylation of RIPK3 at specific sites including Ser199, Ser357, and Ser358 (34, 104). Will these modifications also be required for noncanonical necrosome activation? If they are, what kinases mediate these events in the absence of RIPK1? These and other questions will need to be addressed in the future.

NECROPTOSIS IS CONTROLLED BY PHOSPHORYLATION

Both RIPK1 and RIPK3 are heavily phosphorylated in the necrosome. Mass spectrometry analyses have identified multiple phosphorylation sites on RIPK1 and RIPK3 (34, 105), with the majority of these phosphorylation sites localized within the N-terminal kinase domains. Interestingly, expression of truncated RIPK1 or RIPK3 lacking the kinase domain, but not full-length proteins, results in spontaneous formation of amyloid fibrils. Because alanine substitutions of individual serine or threonine residues on RIPK1 have little effect on RIPK1 kinase activity and TNF-induced necroptosis (104), these results are most consistent with a model in which the kinase domain inhibits RIPK activation by masking the RHIM. In this model, phosphorylation of RIPK1 in the kinase domain alters the conformation of the kinase, perhaps through charge repulsion, to allow RHIM-mediated interaction with downstream signal adaptors (**Figure 4*a***).

Although it is widely accepted that RIPK1 is the upstream kinase that activates RIPK3, evidence suggests that RIPK3 can signal for necroptosis independently of RIPK1. For example, inducible dimerization of RIPK3 drives RHIM- and MLKL-dependent necroptosis independently of RIPK1 (106–108). These results argue that the major function of RIPK1 is to initiate the nucleation event for RIPK3 oligomerization. Indeed, the phosphomimetic mouse RIPK3 mutant S204D (S199D in human RIPK3) restored TNF-induced necroptosis in *Ripk3*$^{-/-}$ fibroblasts that was no longer sensitive to inhibition by the RIPK1 kinase inhibitor Nec-1 or siRNA knockdown of RIPK1 (104). Moreover, overexpression of RIPK3 also leads to TNF-induced necroptosis that is independent of RIPK1 (109). Because RIPK3 expression is highly inducible by different activation signals (9, 90), the result from RIPK3 overexpression suggests that TNF-induced necroptosis can indeed proceed without RIPK1 under certain physiological conditions. The current model predicates that RIPK1 is essential for recruitment and activation of RIPK3; thus, these results do raise questions about

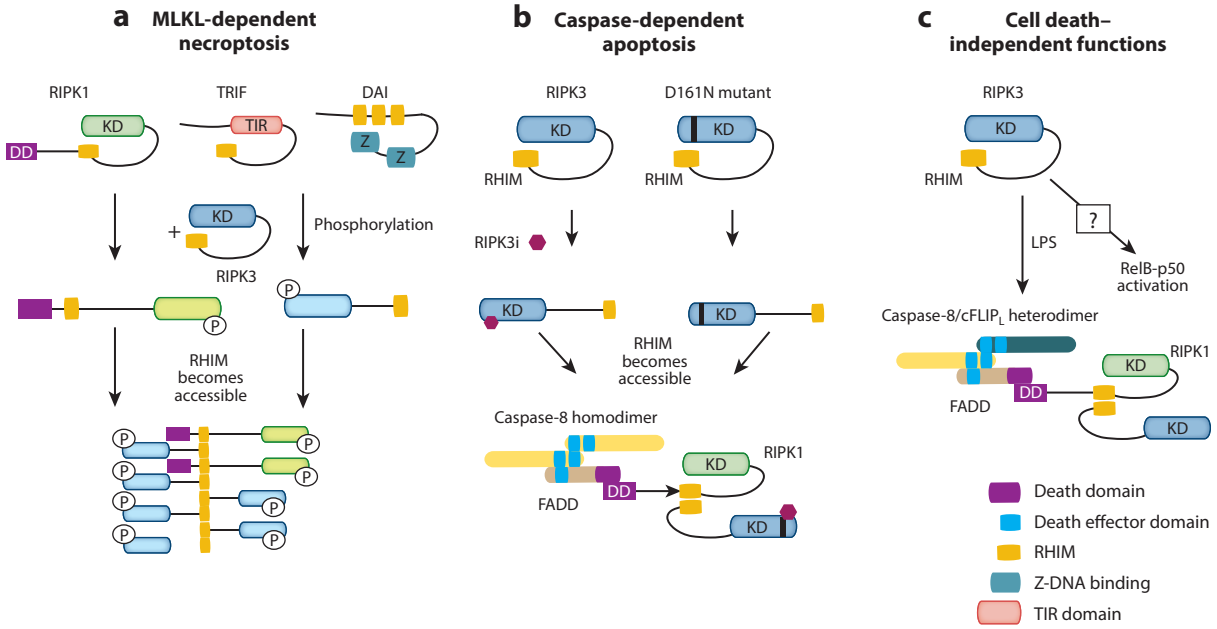


Figure 4

RIPK3 signals for cell death and inflammation through diverse mechanisms. (*a*) RIPK3 mediates necroptosis by binding to RIPK1 or other RHIM-containing adaptors. This causes amyloid conversion of RIPK3, which serves as a platform for docking and recruitment of the RIPK3 substrate MLKL. (*b*) Binding of the RIPK3 kinase inhibitor or introduction of the D161N mutation causes a conformational change that promotes a different form of RHIM-mediated interaction between RIPK1 and RIPK3 that leads to FADD and caspase-8 binding and apoptosis. (*c*) Although the mechanisms have yet to be defined, RIPK3 can also induce pro-IL-1β processing through caspase-1 and caspase-8. In overexpression studies, RIPK3 has also been shown to either enhance or inhibit NF-κB signaling. (Abbreviations: DAI, DNA activator of interferon; DD, death domain; FADD, Fas-associated death domain protein; KD, kinase domain; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like; NF, nuclear factor; P, phosphorylation; RHIM, RIP homotypic interaction motif; RIPK, receptor interacting protein kinase; RIPK3i, RIPK3 inhibitor; TIR, Toll/interleukin-1 receptor; TRIF, TIR domain–containing adaptor-inducing interferon-β.)

the veracity of the traditional Complex I to Complex II transition model downstream of TNFR1 signaling.

In addition to Ser204, phosphorylation of RIPK3 at Ser227 was reported to mediate MLKL binding (34). Although alanine substitution at this residue abrogated RIPK3 function (34), a phosphomimetic mutant was unable to restore TNF-induced necroptosis (104). Therefore, the negative charge that results from phosphorylation of RIPK3 at Ser227 is not crucial for MLKL recruitment. Rather, Ser227 phosphorylation may convert RIPK3 into a permissive conformation to interact with MLKL. This type of conformation-sensitive interaction involving RIPK3 is also found in the kinase-inactive RIPK3 mutant D161N. Mice and cells that express RIPK3-D161N undergo apoptosis following assembly of an alternative caspase-8-activating complex that contains RIPK1, RIPK3-D161N, FADD, and caspase-8 (72). However, not all kinase-inactive mutants of RIPK3 drive assembly of this apoptosis-inducing complex. For example, expression of RIPK3-K51A and RIPK3-D143N are not toxic to cells. Surprisingly, high doses of RIPK3-specific kinase inhibitors can drive assembly of this apoptosis complex in cells that express wild-type RIPK3 or the kinase-inactive RIPK3 mutants K51A or D143N (110) (**Figure 4*b***). Because an intact RHIM is also required to drive assembly of this caspase-8-activating complex, RHIM-mediated interaction alone is not sufficient to determine the cell death mode. Additional factors such as differences

in conformation or recruitment of distinct adaptors are likely important in determining the cell death module being activated.

RIPKs, NF-κB ACTIVATION, AND IL-1β

The receptors that induce necroptosis are also potent inducers of the proinflammatory transcription factor NF-κB. NF-κB induces expression of prosurvival factors such as cFLIP and the cIAP genes and hence is generally considered to be a mutually exclusive signaling outcome from apoptosis or necroptosis. However, this is not always the case. For example, activated T cells upregulate expression of TNFR2 and are highly sensitive to cell death signals (111, 112). The sensitization to cell death can be recapitulated in Jurkat T cell leukemia by expression of TNFR2. Under these conditions, enhanced TNF-induced apoptosis or necroptosis is accompanied by strong NF-κB activation (64). In addition, SM, which sensitizes cells to death cytokines, also causes noncanonical NF-κB activation (57, 58, 113). Hence, NF-κB and necroptosis can synergize with each other to maximize the inflammatory response to stress signals.

To further highlight the cross talk between necroptosis and inflammation signaling, both RIPK1 and RIPK3 can promote NF-κB activation. As discussed above, RIPK1 facilitates NF-κB downstream of TNFR1 and other innate immune receptors such as TLR3 and TLR4 (114, 115). Because of its homology to RIPK1, early studies on RIPK3 also focused on its ability to modulate NF-κB signaling. Overexpression of RIPK3 either stimulates or inhibits NF-κB activation in a context-dependent manner (92, 116–119). However, embryonic fibroblasts and macrophages from *Ripk3*$^{-/-}$ mice were normal for TNF- and TLR4-induced IκBα phosphorylation and degradation, and cytokine expression was unaffected (90, 120). Although these results suggest that RIPK3 is not a core component of the NF-κB pathway, we found that RIPK3 can indeed modulate NF-κB signaling, especially that of RelB and p50, in certain dendritic cell subsets (121) (**Figure 4*c***). Taken together, these results indicate that RIPK1 and RIPK3 can promote inflammation in vivo through necrosis-dependent and -independent mechanisms.

Besides its role in necroptosis and NF-κB activation, RIPK3 has also been implicated in facilitating pro-IL-1β processing in macrophages and dendritic cells. IL-1β is an innate inflammatory cytokine that requires NF-κB-dependent de novo synthesis as well as cleavage and maturation by caspase-1. Caspase-1 cleavage of pro-IL-1β happens as a result of activation of a macromolecular complex termed the inflammasome, which consists of a sensor such as absent in melanoma 2 (AIM2) or NLRP3, the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1. This basic signaling scheme is eerily similar to that used by death receptors, suggesting that the apoptosis and inflammasome signaling pathways share common evolutionary ancestry. Although the necroptosis signaling pathway does not use a similar signaling scheme, the necrosome and inflammasome both require the assembly of a higher-order filamentous complex for activation. For the AIM2 and NLRP3 inflammasomes, cryoelectron microscopy revealed that activation of the inflammasome sensor causes a nucleation reaction driven by the pyrin domain of the adaptor ASC, leading to multimerization of caspase-1 and formation of an elongated, filamentous complex (122). This prion-like property again highlights the potential link between cell death, inflammation, and neurodegeneration. This multimerization model of caspase-1 activation contrasts with that of the widely accepted model of proximity-induced dimerization ofapoptosis-inducing caspases (123). However, more recent work reveals that oligomerization is also important for caspase-8 activation by TNFR-like death receptors (124, 125). Interestingly, although the filamentous inflammasome complex is not amyloid in nature, it can apparently seed further polymerization reactions in neighboring cells as it is released from cells undergoing pyroptosis (126, 127). Higher-order oligomerization appears to be an emerging

theme in innate and cell death signaling, as other intracellular pattern-recognition receptors and sensors including retinoic acid inducible gene-I and mitochondrial antiviral signaling protein are also activated by similar polymerization mechanisms (128–130).

In addition to processing by the caspase-1-associated inflammasome, pro-IL-1β can also be processed by caspase-8 in certain situations (131–136). For example, the chemotherapeutic agent doxorubicin exclusively induces caspase-8-mediated pro-IL-1β processing in bone marrow–derived dendritic cells (133). Moreover, in lipopolysaccharide (LPS)-primed macrophages that lack cIAP1, cIAP2, and XIAP, pro-IL-1β processing is mediated through caspase-1 and caspase-8 in a RIPK3-dependent manner (137). The mechanism by which RIPK3 promotes IL-1β processing is unclear at present. As we discussed above, RIPK3 inhibitors and the kinase-inactive RIPK3 mutant D161N can drive formation of an alternative caspase-8-activating complex. Could a similar complex be involved in caspase-8-mediated pro-IL-1β processing? In the case of the D161N mutant, this complex promotes apoptosis. However, if caspase-8 is paired with its inhibitor $cFLIP_L$, this complex may no longer promote apoptosis but instead facilitate pro-IL-1β processing. This model is consistent with published reports that the caspase-8/$cFLIP_L$ heterodimer exhibits altered substrate specificity compared with the caspase-8 homodimer (29). Because these effects are manifested when the cIAPs are depleted, the IAPs are crucial gatekeepers of RIPK3 activity in cell death and inflammation.

In addition to promoting caspase-8, RIPK3 can also promote caspase-1-mediated pro-IL-1β processing. *Fadd*$^{-/-}$ and *Casp8*$^{-/-}$ macrophages and dendritic cells produced greatly elevated levels of IL-1β that was reversed by deletion of *Ripk3* (138, 139). However, researchers disagree on whether the enhanced IL-1β production was due to increased necrosis-associated release of DAMPs or direct effects of RIPK3 on caspase-1 activation. Regardless of the mechanism, RIPK3 can clearly promote caspase-1- and caspase-8-mediated pro-IL-1β processing via distinct mechanisms.

The necrosis-independent effects of RIPK1 and RIPK3 on NF-κB and pro-IL-1β processing illustrate an important principle: The RIPKs facilitate inflammation through multiple means. They also reinforce the notion that death-signaling adaptors often have important functions beyond cell death. The multifaceted nature of death-inducing adaptors is not a novel concept. FADD, for example, has been implicated in regulating cell cycle entry (140–142), and caspases have important functions in cell differentiation, wound repair, and pruning of neuronal dendrites (143–145). The diverse functions of RIPK3 remind us that inhibition of necroptosis is not the only possible explanation for why *Ripk3*$^{-/-}$ mice often show protection in many inflammatory disease models.

NECROPTOSIS IN VIRAL INFECTIONS

Because the release of DAMPs can stimulate pattern-recognition receptors such as TLRs, necroptosis is widely recognized to be beneficial in innate immune responses against pathogens. However, studies also show that necrosis-dependent inflammation can lead to detrimental pathology in sterile injury–induced diseases (**Table 1**). Given the fact that caspase inhibition is a priming signal for necroptosis, perhaps it is not surprising that viruses that encode caspase inhibitors are susceptible to host cell necroptosis (146). Poxviruses are master evaders of the host cell death machinery. In the case of vaccinia virus, the viral serpin Spi2/B13R is a potent inhibitor of caspase-1 and caspase-8. As in the case of most pathogens, vaccinia virus infection causes an early wave of TNF expression, which triggers RIPK1/RIPK3-dependent necroptosis in different infected tissues (65, 90). In vitro experiments confirmed that wild-type cells infected with vaccinia virus were sensitized to TNF-induced cytotoxicity, but infected *Ripk1*$^{-/-}$ and *Ripk3*$^{-/-}$ cells were highly resistant to TNF-induced necroptosis (65, 90). *Ripk3*$^{-/-}$ mice had reduced necrosis and inflammation in

Table 1 Necroptosis-related diseases

Cause of cell injury	Disease	Model	Reference(s)
Viral infection	Vaccinia virus	*Ripk3*$^{-/-}$ and *Ripk1-D138N* mice	65, 90, 147
	Murine cytomegalovirus	*Ripk3*$^{-/-}$ and *Dai*$^{-/-}$ mice	93, 94
Bacterial infection	*Mycobacterium tuberculosis*	Zebrafish	158
	Salmonella enterica serovar Typhimurium	*Ripk3*$^{-/-}$ mice and macrophages; necrostatin-1	157
Sterile injury–induced inflammation	Psoriasis	Keratinocyte-specific deletion of *Fadd* or *Casp8*; *cpdm* mice	47, 71, 187
	Inflammatory bowel disease	Intestinal epithelium–specific deletion of *Fadd* or *Casp8*	8, 46
	Myocardial infarction	Necrostatin-1	170
	Hypoxia-ischemia-induced brain injury	Necrostatin-1	97
	Ischemia-reperfusion kidney injury	Necrostatin-1; *Ripk3*$^{-/-}$ mice	161, 188
	Retinal degeneration	Retinal detachment; interphotoreceptor retinoid-binding protein–deficient mice; poly(I:C)-induced retinal injury; rd10 mice; pde6c mutant zebrafish	175–178
	Pancreatitis	Cerulein-induced pancreatitis in *Ripk3*$^{-/-}$mice	153, 168, 189
	Atherosclerosis	*Ripk3* deletion in apolipoprotein E or low-density lipoprotein cholesterol receptor–deficient mice	174
	Gaucher's disease	Conduritol B epoxide inhibition of glucocerebrosidase in *Ripk3*$^{-/-}$ mice	169

infected tissues and ultimately succumbed to the infection because of failure to control viral replication. In agreement with these results, mice expressing a kinase-inactive RIPK1 (D138N) were also partially impaired in clearance of vaccinia virus (147). These results established necroptosis as an important antiviral response against certain viral pathogens. This innate immune defense mechanism may be important to tamp down viral replication before robust, virus-specific T cell responses are mobilized (**Figure 5*a***).

The results from vaccinia virus are surprising because they seem to indicate that by blocking caspase activation, viruses set themselves up for destruction by the host. Because ablation of necroptosis leads to rapid death of the host, blocking necroptosis may actually deprive the virus of the opportunity to disseminate and infect another host. From this perspective, one can argue that necroptosis is beneficial not only to the host but also to the invading virus (**Figure 5*a***). Moreover, when compared to other naturally occurring poxviruses, vaccinia virus contains large deleted gene segments (148). Thus, a tantalizing possibility is that the gene that inhibits necroptosis is lost in vaccinia virus because of these gene deletions. In this scenario, triggering of necroptosis may be an exception rather than a rule for vaccinia virus. To this end, it will be important to determine if other poxviruses also cause infected cells to undergo necroptosis.

Viral inhibition of apoptosis is widely perceived to give the virus an edge in its struggle with the host. Because of its antiviral effects, it stands to reason that some viruses may have also developed strategies to counteract necroptosis. Herpesviruses are highly adept at countering the

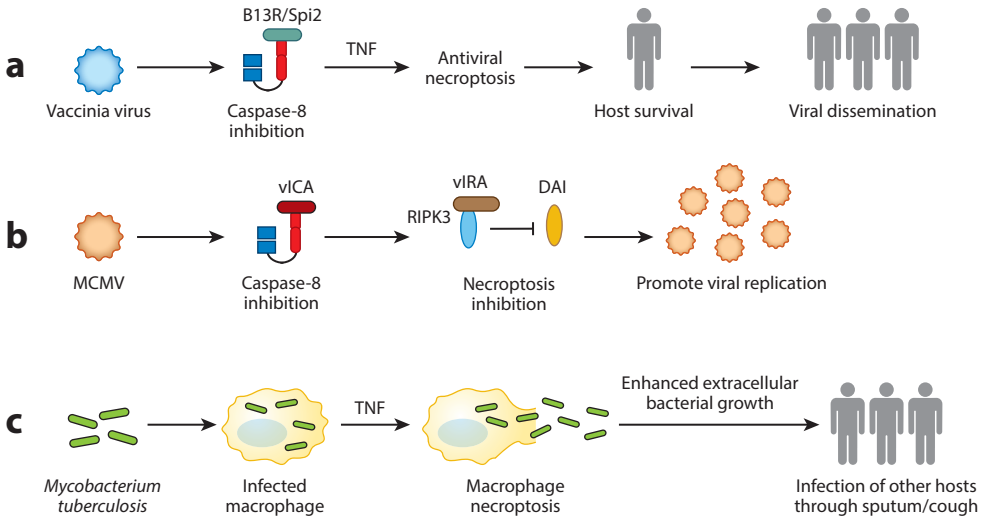


Figure 5

Necroptosis in host-pathogen interactions. (*a*) Vaccinia virus inhibits caspase-8 via the viral inhibitor B13R/Spi2. This primes the cells toward necroptosis. Although necroptosis and the ensuing inflammation have antiviral effects, they may in fact promote viral dissemination to another host by avoiding premature death of the infected host. (*b*) MCMV inhibits caspase-8 and necroptosis via vICA and vIRA. Genetic experiments show that vIRA is essential to prevent premature death of the infected cells. Hence, vIRA-mediated necroptosis inhibition is important for the virus to complete its replication cycle and to generate more viral progeny. (*c*) *Mycobacterium tuberculosis* uses RIPK3-dependent necroptosis to release the bacteria into a growth-permissive environment, which in turn enhances spread of the pathogen to uninfected hosts via the sputum. (Abbreviations: DAI, DNA activator of interferon; MCMV, murine cytomegalovirus; RIPK, receptor interacting protein kinase; TNF, tumor necrosis factor; vICA, viral inhibitor of caspase-8-induced apoptosis; vIRA, viral inhibitor of RIPK activation).

host cell death machinery. MCMV encodes several viral cell death inhibitors, one of which is the viral inhibitor of caspase-8-induced apoptosis (vICA). Because inhibition of caspase-8 is a priming signal for necroptosis, one would expect that cells infected with MCMV would become susceptible to necroptosis. However, MCMV-infected cells are spared from necroptosis because the virus also encodes vIRA, the product of the *M45* gene. M45/vIRA is a RHIM-containing viral cell death inhibitor that binds to RIPK3 to prevent virus-induced necroptosis (94, 146). Recombinant MCMV expressing a tetra-alanine-substitution RHIM mutant of vIRA fails to inhibit RIPK3 and succumbs to rapid necrosis. Because of this premature cell death, the mutant virus fails to establish a productive infection in cells and mice. Productive infection was reestablished with the mutant virus in *Ripk3*$^{-/-}$ cells and *Ripk3*$^{-/-}$ mice (93).

In contrast to RIPK3, TNF signaling and RIPK1 are both dispensable for mutant MCMV-induced necroptosis. Instead, RIPK3 interacts with DAI, another RHIM-containing adaptor, to form a noncanonical necrosome that drives virus-induced necrosis (**Figure 5*b***). *M45* encodes a ribonucleotide reductase (RNR) with no enzymatic activity. Interestingly, many RNRs from other herpesviruses also encode a RHIM (**Figure 3**) (149). This suggests that viral inhibitors that target the RIPKs via the RHIM represent a common viral immune evasion strategy for herpesviruses. The results from vaccinia virus and MCMV highlight the importance of necroptosis in acute viral infections. Yet questions still remain on whether necroptosis can influence the quality and magnitude of adaptive immune responses, the generation of immunological memory, and viral latency.

BACTERIAL AND PARASITIC INFECTIONS

TNF is a major driver of bacterial sepsis, a life-threatening condition marked by systemic cytokine storm and multiorgan failure. In agreement with the idea that RIPK-dependent necroptosis promotes damaging inflammation, *Ripk3*$^{-/-}$ mice are resistant to TNF-induced systemic inflammatory syndrome (SIRS) (150, 151). In contrast to RIPK3, the role of RIPK1 in TNF-induced SIRS is more controversial. Although several reports show that mice expressing kinase-inactive RIPK1 and wild-type mice treated with RIPK1 kinase inhibitors are protected from TNF-induced SIRS (71, 151, 152), another study found that Nec-1 exacerbates the disease (150). Furthermore, the response of *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice against cecal ligation and puncture–induced sepsis is also variable (151, 153). Because *Ripk3*$^{-/-}$ mice and *Ripk3*$^{-/-}$ macrophages exhibit a normal response to LPS (120, 121), RIPK1 and RIPK3 likely play minor roles in acute bacterial sepsis.

Although the role of the RIPKs in LPS-induced responses is ambiguous, they are nonetheless crucial in controlling certain bacterial pathogens. *Yersinia pestis*, the etiological agent of the black death pandemic, causes rapid RIPK1- and caspase-8-dependent macrophage apoptosis. As in TNF- and SM-induced apoptosis, *Y. pestis*–induced macrophage apoptosis requires intact RIPK1 kinase activity. In addition, RIPK1 is required for inflammatory cytokine production in response to *Y. pestis* infection (154, 155). However, RIPK3 appears to play a minimal role in *Y. pestis* infection. *Salmonella enterica*, a flagellated, gram-negative bacterium, is also a potent inducer of macrophage cell death. Although it is widely accepted that macrophage cell death induced by *Salmonella* is caused by inflammasome activation and caspase-1-mediated pyroptosis (156), a recent report argues that RIPK3-dependent necroptosis is also involved (157). These discrepant conclusions could be reconciled by the fact that RIPK3 can also modulate inflammasome and caspase-1 activation (137, 139).

Host control of *Mycobacterium tuberculosis* (Mtb) critically requires TNF. One of the major protective functions of TNF is to promote granuloma formation, which is thought to be crucial in containment of the bacteria. Using zebrafish as a model, Roca & Ramakrishnan (158) show that RIPK1 and RIPK3 are both required to trigger TNF-induced reactive oxygen species (ROS) production and necroptosis in response to tuberculosis infection. Although necroptosis of infected macrophages initially inhibits bacterial growth, it later enhances growth as bacteria are released into the growth-permissive extracellular environment. Hence, unlike the situation with vaccinia virus and MCMV, one can view Mtb as a pathogen that hijacks the host necroptosis machinery to promote its own growth and dissemination (**Figure 5*c***). Consistent with this thesis, necrosis is often associated with severe Mtb infection (159).

Mechanistically, TNF-induced necroptosis in Mtb-infected macrophages requires mitochondrial cyclophilin D (CypD) and acid sphingomyelinase–induced ceramide production. Ceramide has long been implicated in death receptor–induced apoptosis. However, its role in mammalian cell necroptosis has yet to be thoroughly tested. CypD is an inner mitochondrial protein and an important component of the mitochondrial permeability transition pore. It is required for certain forms of necrosis, such as that induced by calcium and ROS (160). CypD and RIPK3 act in synergy to mediate acute kidney injury in an ischemia-reperfusion model (161). However, CypD deficiency did not rescue excessive necroptosis of *Casp8*$^{-/-}$ T cells (48). Moreover, widespread elimination of mitochondria through induced mitophagy did not alter the cellular response to TNF-induced necroptosis (43). Hence, rather than being a core component of the necroptosis machinery, the CypD pathway appears to be uniquely involved in Mtb-induced necroptosis. It will be interesting to determine whether similar mechanisms involving RIPK1, RIPK3, CypD, and ceramide are involved in immune defense against Mtb infections in mammals.

Parasitic diseases such as malaria and leishmaniasis target red blood cells, leading to anemia, hemolysis, and bleeding in some cases. These symptoms are caused by red blood cell lysis, which releases cell-free hemoglobin into the circulation. Oxidation of hemoglobin releases heme to trigger the Fenton reaction and generation of highly reactive oxygen radicals. The oxidative stress drives lipid and protein peroxidation, DNA damage, and other insults to the cell (162). Free heme greatly sensitizes hepatocytes to TNF-induced apoptosis in response to infection with *Plasmodium*, the etiological agent for malaria (163). In addition to hepatocytes, macrophages are also highly susceptible to heme-induced cytotoxicity. Through a poorly defined mechanism, heme directly activates TLR4, leading to autocrine TNF and ROS production, which synergize with each other to induce RIPK1- and RIPK3-dependent necroptosis (164). By inducing macrophage necroptosis, RIPK1 and RIPK3 may restrict the niche within which parasites can replicate. In vivo infections will be required to validate the biological role of necroptosis in parasitic infections.

NECROPTOSIS IN STERILE INFLAMMATION

Besides its role in pathogen infections, necrosis is also a hallmark of acute and chronic sterile inflammation. In agreement with induced expression of RIPK3 in response to acute and chronic exposure to alcohol, *Ripk3*$^{-/-}$ mice were protected from alcoholic liver disease (165). Moreover, *Ripk3*$^{-/-}$ mice were protected from acetaminophen-induced liver injury (166), and elevated phospho-MLKL signals were detected in drug-induced liver diseases (38). Repeated doses of cerulein led to a biphasic cell death reaction in the acinar cells that was partially dependent on TNF, RIPK3, and MLKL (167). As such, *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice are partially protected from cerulein-induced acute pancreatitis (153, 168). RIPK3 deficiency also improves the neurological manifestation of Gaucher's disease, a lysosomal storage disease caused by mutations in glucocerebrosidase (169).

Necroptosis appears to be an important mechanism of cell injury in ischemia-reperfusion-induced tissue injury. As discussed above, RIPK3-dependent necroptosis is partially responsible for ischemia-reperfusion-induced kidney injury (161). The RIPK1 kinase inhibitor Nec-1 is effective in alleviating hypoxia-ischemia-induced oxidative brain injury and inflammation in neonatal mice (97). Nec-1 also reduced mouse and rat models of ischemia-reperfusion-induced myocardial cell death and infarct formation (170). However, in a model of permanent left anterior descending coronary artery ligation, the resulting inflammation and tissue remodeling were impaired in *Ripk3*$^{-/-}$ mice (171). Because Nec-1 has been shown to exhibit off-target effects (102, 172), these results need to be interpreted with caution. As mice expressing kinase-inactive RIPK1 have recently been generated (71, 72), they will be useful in further dissecting the kinase-dependent necroptotic signaling versus scaffold-dependent non-necroptotic signaling in these disease models.

In addition to drug- and trauma-induced tissue injury and inflammation, RIPK3-dependent necroptosis also contributes to chronic inflammatory diseases such as atherosclerosis. Macrophage necrosis is widely viewed as a key factor in atherosclerotic plaque formation (173). Mice deficient in apolipoprotein E or low-density lipoprotein receptor (LDL-R) that are fed a high-fat diet developed atherosclerosis marked by macrophage necrosis in the atherosclerotic plaques. Strikingly, RIPK3 deletion ameliorates macrophage necrosis in the plaques and atherosclerosis in *ApoE*$^{-/-}$ and *Ldl-r*$^{-/-}$ mice (174). Given that *Ripk3*$^{-/-}$ macrophages are resistant to oxidized LDL–induced necroptosis, these results strongly suggest that RIPK3-dependent macrophage necroptosis is a direct driver of atherosclerotic plaque formation. Finally, investigators have also shown RIPK3-dependent necroptosis to be causative of mouse models of retinal injury (175–178). These examples point to the emerging role of the RIPKs in diverse inflammatory diseases. However, researchers

need to consider both necroptosis-dependent and -independent effects of the RIPKs when interpreting these results.

EVOLUTIONARY PERSPECTIVES

Studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have contributed greatly to our knowledge of apoptosis signaling mechanisms. The conservation of apoptosis machinery through evolution illustrates its importance in the maintenance of organismal homeostasis. Is the mammalian necroptosis pathway also conserved in *C. elegans* and *Drosophila*? Interestingly, RHIM-like adaptors are found in *Drosophila* (**Figure 3**). In response to gram-negative bacteria, the innate immune receptors peptidoglycan recognition protein (PGRP)-LC and PGRP-LE stimulate antimicrobial peptide expression through immune deficiency (IMD), a RIPK1-like adaptor, and Relish, a *Drosophila* NF-κB (179). The tetrapeptide core sequences of *Drosophila* RHIM-containing adaptors differ from those in the mammalian RHIMs (**Figure 3**). Mutations of the RHIM-like motif in PGRP-LC and PGRP-LE compromise antimicrobial peptide expression in response to peptidoglycan stimulation (180), indicating that these variant RHIMs are functional. The structural similarity between mammalian and *Drosophila* RHIM adaptors argues that they may have evolved from a common primordial pathway. Although the PGRP-IMD-Relish pathway is generally not known to promote cell death, overexpression of IMD has been shown to result in cell death. Interestingly, IMD-induced cell death was only partially rescued by the caspase inhibitor p35 (181), suggesting the possibility that nonapoptotic cell death may be involved.

In addition to the IMD pathway, transgenic overexpression of the *Drosophila* TNF ortholog *Eiger* in the developing eye primordium leads to JNK-dependent necrosis-like cell death (182). Interestingly, suppression of genes involved in glycolysis and mitochondrial respiration inhibits *Eiger*-induced cell death (182). The apoptosis protease activating factor 1 (Apaf1) interacts with caspase-9 and cytochrome *c* to form the apoptosome, a macromolecular structure essential for mitochondria-mediated apoptosis. Surprisingly, an Apaf1 hypomorph mutant also exhibits progressive wing cell necrosis, which triggers a systemic inflammatory response, wasting, and expression of antimicrobial peptides (183). In both Eiger- and Apaf1-mediated necrosis, the cell death phenotype is associated with changes in energy metabolism. This is in contrast to mammalian necroptosis, which does not require JNK or mitochondria (43, 102). Other pathways of nonapoptotic cell death have recently been described in *Drosophila* nurse cells (184) and developing neuroblasts (185). It will be interesting to determine if similar principles that govern mammalian necroptosis are conserved in these situations.

CLOSING THOUGHTS

In considering the molecular machinery that controls necroptosis and its roles in different diseases, perhaps it will be helpful to step back and ponder why evolution has preserved this unique cell death module. Phylogenetic analysis indicates that modern-day RIPKs evolved through a series of gene duplication events. The relatively short branch between RIPK1 and the RIPK progenitor suggests that RIPK1 is probably the most ancient RIPK (**Figure 6**). This evolutionary model is appealing, as RIPK1 is the only RIPK that is crucial for embryonic survival and beyond. Although the *Drosophila* IMD was once thought to be a RIPK1 ortholog (181), it shares homology only in the DD and lacks the essential kinase domain. The absence of RIP-like kinases in lower organisms such as *Drosophila* or *C. elegans* argues that these kinases are relatively novel products of evolution. The earliest example of RIPK1-like kinases is found in bony fish. How are we supposed to make sense of this? One possible explanation is that necroptosis is the product of coevolution with certain

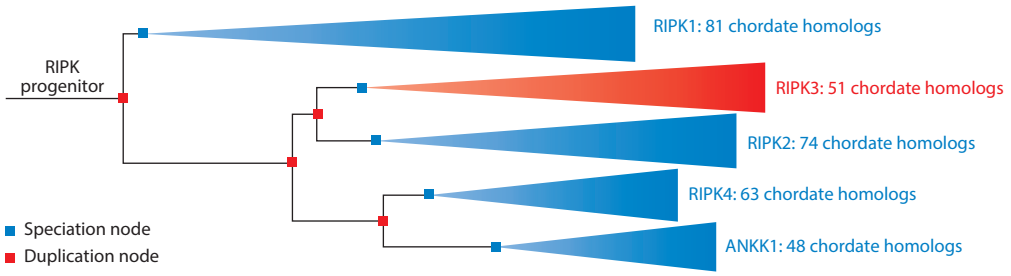


Figure 6

The RIPK gene family evolved through a series of gene duplication events. This reconstructed phylogeny was generated by the Ensembl genome browser (186). Internal nodes correspond to key speciation (*purple*) and gene duplication (*red*) events. Branch lengths correspond to rates of evolutionary change. ANKK1 does not function as a RIPK but is closely related to RIPK4 and other RIPKs. (Abbreviations: ANKK1, ankyrin repeat and protein kinase domain–containing protein 1; RIPK, receptor interacting protein kinase.)

viruses that target vertebrates. The strongest support for this argument comes from the examples of vaccinia virus and MCMV. According to this model, sterile injury–induced necroptosis is the price we pay in this evolutionary struggle with viruses. This is appealing because necroptosis tends to associate with detrimental pathologies in sterile inflammation. Studies of more viruses will be crucial to validating this hypothesis.

SUMMARY POINTS

1. The receptors that stimulate programmed necrosis or necroptosis are also apoptosis inducers.
2. Necroptosis is regulated by RIPK3 and MLKL.
3. Protein phosphorylation, ubiquitination, and FADD/caspase-8-mediated proteolytic processing are the three major post-translational mechanisms that control the induction of necroptosis.
4. RIPK3 promotes inflammation through necroptosis, NF-κB activation, and caspase-1 and 8-mediated pro-IL-1β maturation.
5. RIPK-dependent necroptosis contributes to pathogen-induced and sterile inflammation.
6. Necroptosis can promote or suppress antiviral immune responses in a pathogen-specific manner.
7. The existence of viral inhibitors of necroptosis argues for an important role for necroptosis in host-pathogen interactions.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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4.4 MANUSCRITO 3

RIPK1 AND PGAM5 CONTROL *LEISHMANIA* REPLICATION THROUGH DISTINCT MECHANISMS

Este artigo se encontra em fase de preparação e será submetido ao *The Journal of Immunology*.

Esse trabalho investiga a participação de moléculas da via da necroptose no controle da infecção por *Leishmania*

Resumo:

Macrófagos humanos foram tratados com heme e com inibidores específicos de RIPK1, RIPK3 e MLKL. Observamos que o heme induz necroptose em células humanas. Macrófagos infectados com *L. chagasi* e tratados com heme têm menor carga parasitária, enquanto que apenas o tratamento com inibidores de RIPK1 é capaz de reverter o efeito de redução da carga parasitária. Além disso, PGAM5, outra molécula da via da necroptose também participa na resistência na infecção por *Leishmania*. Quando infectado com diferentes espécies de *Leishmani*a, macrófagos *knockout* para o gene *Pgam5* e com RIPK1 deficiente, apresentam maior carga parasitária. Esse mecanismo parece ser dependente da redução da produção de IL-1β em *Pgam5*$^{-/-}$ e independente de IL-1β em RIP1K deficiente. Por fim, em um modelo experimental de leishmaniose cultânea, encontramos que RIPK1 e PGAM5 participam no controle da lesão de camundongos infectados com *L. amazonensis*. Esses achados indicam que moléculas da via da necroptose podem participar do controle da infecção por *Leishmania* sem necessariamente ativar morte celular necroptótica.

RIPK1 and PGAM5 Control Leishmania Replication through Distinct Mechanisms

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Abstract

Leishmaniasis is an important parasitic disease found in the tropics and sub-tropics. Human infection is mediated by phlebotomic bit from sand flies. The two major forms of the disease affect the skin and visceral organs and are referred to as cutaneous and visceral leishmaniasis respectively. Cutaneous and visceral leishmaniasis affects an estimated 1.5 million people worldwide. Despite its human health relevance, relatively little is known about the host immune mechanisms that control Leishmania replication. Necroptosis is a recently identified form of cell death with potent anti-viral effects. RIPK1 is a critical kinase that mediates necroptosis downstream of death receptors and toll-like receptors. Heme, a product of catabolism of hemoglobin during certain intracellular pathogen infections, is also a potent inducer of macrophage necroptosis. In this study, we examined the impact of heme and necroptosis on Leishmania replication. We found that although heme potently inhibited Leishmania replication in bone marrow derived macrophages (BMDMs), Leishmania did not induce extensive necroptosis. Surprisingly, inhibition of RIPK1 kinase activity dramatically enhanced parasite replication in BMDMs and in mice. We further found that the putative necroptosis downstream effector phosphoglycerate mutase family member 5 (PGAM5) was also required for inhibition of Leishmania replication. In mouse infection, both PGAM5 and RIPK1 kinase function are required for IL-1β expression in response to Leishmania. However, PGAM5, but not RIPK1 kinase activity, was directly responsible for IL-1β secretion in BMDMs challenged with Leishmania. Collectively, these results revealed RIPK1 and PGAM5 as two novel host factors that exert optimal control of Leishmania replication through distinct mechanisms.

Introduction

Leishmaniasis is a vector-borne parasitic disease caused by protozoan of the genus *Leishmania*. *Leishmania* parasites exist as extracellular flagellated promastigotes in sandflies. Upon entry into mammalian hosts, they exist as intracellular amastigotes [1]. In human, disease manifestation varies depending on the *Leishmania* species and can range from asymptomatic infections, self-healing cutaneous lesions, to life-threatening infections involving visceral organs. For instance, *Leishmania* (L.) *amazonensis* and *L. major,* are both known to cause cutaneous ~~and mucosal~~ infections, while *L. infantum chagasi* causes visceral leishmaniasis (VL). VL can be extremely debilitating, with significant morbidity and mortality, if not promptly treated. In addition, disease progression is also influenced by host immune response [2]. Macrophages are widely believed to play critical roles in the control of Leishmania infection [3]. Interestingly, they also serve as major reservoirs in which Leishmania amastigotes replicate.

The host immune response that controls Leishmaniasis is not fully understood. The inflammatory cytokine IL-1β is critical for innate immune defense against many different pathogens. Production of mature IL-1β requires two signals: NF-κB-driven *de novo* synthesis of pro-IL-1β and inflammasome-mediated cleavage of pro-IL-1β into the mature cytokine. Mice that are deficient in the essential inflammasome sensor NLRP3, the adaptor ASC, or the IL-1β cleavage protease caspase 1 exhibited impaired control of Leishmania replication [4], suggesting that IL-1β is a critical cytokine in innate

immune control of Leishmania replication. IL-1β confers protection against Leishmania in part through induction of nitric oxide and by promoting Th1 responses [5].

In contrast to IL-1β, the stress-induced enzyme heme oxygenase-1 (HO-1) has been shown to facilitate Leishmania replication by limiting inflammatory cytokine expression [6]. A key function of HO-1 is to cleave heme into biliverdin, which is subsequently converted to bilirubin by biliverdin reductase; free iron and carbon monoxide. Heme release is found in infections with intracellular pathogens such as *Plasmodium falciparum* and Plasmodium vivax, the etiological agents of malaria [7]. Severe visceral leishmaniasis is also characterized by hematological alterations and spontaneous bleeding associated with marked inflammatory imbalance.

Interestingly, free heme has been shown to induce murine macrophage necroptosis [8], a recently described form of inflammatory cell death that has important protective functions in certain viral infections [9-12]. Two serine/threonine kinases, the receptor interacting protein kinase 1 (RIPK1) and RIPK3, are critical adaptors for necroptosis [13]. RIPK1 and RIPK3 activation leads to recruitment and activation of two major necroptosis effectors, the pseudokinase mixed lineage kinase domain-like (MLKL) and the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) [14-16]. Hence, HO-1 may facilitate Leishmania replication by limiting heme-induced necroptosis, thereby preserving the parasite replication reservoir. However, the roles of heme, RIP kinases and necroptosis have not been investigated in Leishmania infection.

In this study, we report that RIPK1 and RIPK3 kinase activities, but not PGAM5, are required for heme-induced necroptosis in macrophages. Heme reduced *L. chagasi*

replication in macrophages, which was restored in the presence of RIPK1 kinase inhibitors. By contrast, RIPK3 kinase inhibitors and MLKL inhibitor did not restore *L. chagasi* replication in heme-treated macrophages, suggesting that RIPK1 does not regulate *L. chagasi* replication through necroptosis. Indeed, *L. chagasi* alone did not cause extensive cell death in infected macrophages. Similar to the effect of RIPK1 kinase inhibitors, macrophages and mice expressing kinase inactive RIPK1 also exhibited elevated *Leishmania* replication. In addition to RIPK1, the downstream effector PGAM5 was also required for optimal control of *Leishmania* replication. We found that PGAM5 limits *Leishmania* replication by facilitating IL-1β secretion. By contrast, RIPK1 kinase activity suppresses *Leishmania* replication independent of IL-1β. Collectively, our results identified RIPK1 and PGAM5 as two novel host factors that control *Leishmania* replication through distinct mechanisms.

Results

Heme induced necroptosis in human macrophages

Previous work shows that heme causes RIPK1- and RIPK3-dependent necroptosis in mouse macrophages [8]. To test whether heme-induced necroptosis is conserved in human cells, we treated human peripheral blood mononuclear cells (PBMCs) from different healthy donors with different doses of heme. We found that human PBMCs underwent cell death in response to 30 μM heme, but were otherwise resistant to heme at lower doses (Fig. 1a). By contrast, human monocyte-derived macrophages were more sensitive to heme than PBMCs, with detectable cell death at as little as 10 μM heme (Fig. 1b). The human monocytic cell line THP-1 differentiates into macrophages when stimulated with the phorbol ester PMA. Similar to primary PBMCs and macrophages, PMA-treated THP-1 cells also underwent cell death with increasing doses of heme (Fig. 1c). Heme-induced cell death of THP-1 cells was inhibited by the RIPK1 kinase inhibitor necrostatin-1 (Nec-1), the RIPK3 kinase inhibitors GSK840 and GSK843 [17], and the MLKL inhibitor necrosulfonamide (NSA) (Fig. 1d-e). Since RIPK1, RIPK3 and MLKL are key adaptors for necroptosis [12], we conclude that heme induced classical necroptosis in human macrophages.

Leishmania replication is inhibited by heme and RIPK1 kinase activity

Heme release is a pathological consequence of hemolytic intracellular pathogen infection such as malaria [18]. *Leishmania* promastigotes have been shown to degrade hemoglobin, which could lead to release of heme [19]. Since heme is catabolized by

HO-1 and HO-1 has been shown to promote *Leishmania* replication and that a key function of HO-1 is to catabolize heme [6], we asked whether heme might inhibit *Leishmania* replication in macrophages. Indeed, we found that *L. chagasi* replication in PMA-treated THP-1 cells was severely impaired by heme (Fig. 2a). Moreover, the RIPK1 kinase inhibitors 7-Cl-O-Nec-1, GSK963, but not the inactive enantiomer GSK962, reversed the heme-induced inhibition of *Leishmania* replication in THP-1 cells (Fig. 2b). Surprisingly, the RIPK3 inhibitors GSK840 and GSK843, and the MLKL inhibitor NSA had no effects on heme-induced inhibition of Leishmania replication (Fig. 2b). Since necroptosis critically depends on the kinase activity of RIPK3 and MLKL, these results suggest that RIPK1 kinase activity might regulate *Leishmania* replication independent of necroptosis. Indeed, the RIPK1 inhibitors 7-Cl-O-Nec-1 and GSK963 similarly enhanced *L. chagasi* replication in the absence of heme in THP-1 cells while the RIPK3 inhibitor GSK 843 had no effect (Fig. 2c). These results therefore show that RIPK1 kinase activity inhibits *L. chagasi* replication independent of heme- induced necroptosis.

The putative RIPK1 downstream effector PGAM5 regulates replication of different strains of Leishmania

The effect of the RIPK1 kinase inhibitors on *Leishmania* replication prompted us to examine the underlying mechanism. Since pharmacological inhibitors can have off-target effects, we first confirmed the role of RIPK1 kinase activity in *Leishmania* replication using bone marrow derived macrophages (BMDMs) from knock-in mice expressing a kinase inactive RIPK1 (*Ripk1*$^{kd/kd}$) [20]. Similar to THP-1 cells treated with

RIPK1 inhibitors, the number of *L. chagasi* amastigotes in infected *Ripk1*$^{kd/kd}$ BMDMs was far greater than that of wild type BMDMs (Fig. 3a-b). Similar increase in amastigotes in infected *Ripk1*$^{kd/kd}$ BMDMs was observed with two other strains of Leishmania, *L. amazonensis* (Fig. 3c-d) and *L. major* (Fig. 3e-f). These results indicate that RIPK1 kinase activity inhibits *Leishmania* replication in macrophages.

The mitochondrial phosphatase PGAM5 has been reported to act downstream of RIPK1 and RIPK3 in necroptosis [14]. Although subsequent studies challenges the role of PGAM5 in necroptosis [21], it appears to facilitate RIPK1/RIPK3-mediated IL-1β maturation in caspase 8$^{-/-}$ dendritic cells [22]. We therefore explored the possibility that RIPK1 functions through PGAM5 to inhibit *Leishmania* replication. Indeed, we found that the number of amastigotes in *Pgam5*$^{-/-}$ BMDMs infected with *L. chagasi* (Fig. 3a-b), *L. amazonensis* (Fig. 3c-d) and *L. major* (Fig. 3e-g) were also increased compared to wild type BMDMs. Similar increase in *Leishmania* strains replication was observed in *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ BMDMs when viable promastigotes were counted (Fig. 3g-h).

RIPK1 and PGAM5 are required for in vivo control of Leishmania infection

We next examined if RIPK1 and PGAM5 are required for control of *Leishmania* infection *in vivo*. Because *L. chagasi* does not cause productive infection in mice, we used *L. amazonensis* for *in vivo* infection instead. After 10 weeks of infection, we counted the number of parasite in the infected footpad. Consistent with the results in BMDMs, parasite count was higher in both *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 4a). Consistent with the increased parasite load, the lesion size in the infected footpad was significantly increased in *Ripk1*$^{kd/kd}$ mice at 10 weeks post-infection (Fig. 4b). However,

192 lesion size was not significantly increased in *Pgam5*$^{-/-}$ mice (Fig. 4b-c). These results
193 indicate that RIPK1 has a more prominent role than PGAM5 in the control of
194 *Leishmania* replication *in vivo*.
195
196 *RIPK1 and PGAM5 controls Leishmania replication through different mechanisms*
197 The differential phenotypes of the *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice suggest that they
198 might control *Leishmania* replication through distinct mechanisms. Previous studies by
199 several groups show that NLRP3 inflammasome-induced IL-1β secretion plays a crucial
200 role in the control of *Leishmania* replication [4,5]. Since both RIPK1 and PGAM5 have
201 been implicated to regulate IL-1β cleavage and secretion under certain conditions
202 [22,23], we examined whether IL-1β expression was affected in the mutant mice.
203 Indeed, reduced IL-1β was detected in the footpad of infected *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$
204 mice at 4 weeks post-infection (Fig. 5a). Importantly, TNF expression was normal in
205 *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 5a). By 10 weeks post-infection, the difference in IL-
206 1β was no longer detected in *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 5b). These results
207 strongly imply that the RIPK1 and PGAM5 regulate early IL-1β release during
208 *Leishmania* infection.
209 We next asked whether PGAM5 and RIPK1 kinase activity directly regulate IL-1β
210 secretion by macrophages. We first examined IL-1β secretion by LPS-primed
211 macrophages stimulated with the NLRP3 inflammasome agonist nigericin. Surprisingly,
212 reduced IL-1β secretion was only observed in *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$ BMDMs (Fig.
213 5c). In fact, *Ripk1*$^{kd/kd}$ BMDMs secreted increased amount of IL-1β compared to wild
214 type BMDMs. These results suggest that RIPK1 does not directly control IL-1β

215 expression in response to Leishmania infection. To further test this hypothesis, we
216 stimulated splenocytes from infected mice with *L. amazonensis* and found that *Pgam5*$^{-/-}$,
217 but not *Ripk1*$^{kd/kd}$ splenocytes, secreted reduced level of IL-1β ex vivo (Fig. 5d). IL-1β
218 confers protection against Leishmania in part by induction of nitric oxide (NO) [4].
219 Consistent with a defect in IL-1β secretion, Leishmania-induced NO was significantly
220 reduced in *Pgam5*$^{-/-}$ BMDMs (Fig. 5e). In contrast, the reduced NO production by
221 *Ripk1*$^{-/-}$ BMDMs was not statistically significant. The defect in IL-1β secretion by
222 *Pgam5*$^{-/-}$ BMDMs was not due to survival defects, since *Pgam5*$^{-/-}$ BMDMs were similarly
223 sensitive to heme and other cell death inducers (Fig. 5f and data not shown). This is
224 also consistent with the fact that *Leishmania* infection in BMDMs did not elicit
225 substantial cell death (Fig. 5g). Collectively, these results indicate that PGAM5 and
226 RIPK1 controls *Leishmania* replication through distinct and cell death-independent
227 mechanisms.
228

Discussion

Leishmaniasis is endemic in the tropics and sub-tropics and a major public health concern in the affected regions. Despite its importance, the host immune defense mechanism against *Leishmania* infection is poorly understood. Certain intracellular pathogens such as *Plasmodium falciparum* and Plasmodium vivax can elicit release of heme, which can cause oxidative damage, inflammation and necroptosis [8,18]. Free heme is catabolized by HO-1, an enzyme that promotes Leishmania replication [6]. Although previous work demonstrated that heme is one of the essential nutrients that Leishmania must acquire from host cells to survive the accumulation of heme might inhibit Leishmania replication. To our surprise, although RIPK1 kinase inhibitors did suppress Leishmania replication, RIPK3 kinase inhibitors or NSA, which inhibit the downstream necroptosis effector MLKL [15], had no effects in the presence of heme. Hence, although RIPK1 kinase activity is known to mediate cell death, it has a cell death-independent function in host response against the intracellular parasite *Leishmania*.

Recent studies suggest that the inflammatory cytokine IL-1β has a key role in the control of *Leishmania* infection. Mature IL-1β secretion requires processing of pro-IL-1β by the inflammasome. Macrophages and mice lacking inflammasome components such as NLRP3, ASC or caspase 1 failed to control Leishmania replication in vitro and in vivo. IL-1β and other inflammatory cytokines such as TNF and INFγ have been shown to enhance NO production by macrophages [4,24], which is a hallmark of microbicidal function and can limit parasite replication by inducing macrophage cell death [25]. In addition, deficiency in the NLRP3 inflammasome led to an ineffective Th2-skewed

immune response against *Leishmania* [5]. However, we found that *Leishmania* did not induce significant cell death in wild type, *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ BMDMs. Hence, although RIPK1 and PGAM5 were originally identified as cell death signal adaptors, they regulate Leishmania replication independent of cell death.

Although several recent reports have identified the NLRP3 inflammasome and IL-1β as key innate immune regulators of Leishmania infection, the underlying mechanism by which Leishmania stimulates the NLRP3 inflammasome was unknown. Our results identified the mitochondrial phosphatase PGAM5 and RIPK1 as novel regulators for *Leishmania*-induced inflammasome activation and IL-1β secretion *in vivo*. Interestingly, *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$ splenocytes, exhibited defective IL-1β secretion in response *L. amazonensis*. The differential requirement for PGAM5 for IL-1β secretion was also observed in response to AIM2 inflammasome agonists, RNA viruses and other NLRP3 agonists (unpublished observation). Since PGAM5 is a key regulator of multiple inflammasomes, it is unlikely to be a specific sensor for Leishmania.

Although *Ripk1*$^{kd/kd}$ mice exhibited reduced IL-1β secretion and elevated Leishmania replication, RIPK1 kinase activity does not directly regulate inflammasome activation and IL-1β secretion. *Ripk1*$^{kd/kd}$ splenocytes and BMDMs produced normal level of IL-1β in response to Leishmania antigen and other inflammasome agonists. This is consistent with our recent observation that RIPK1 kinase activity is dispensable for inflammasome activation and IL-1β secretion [23]. However, it is noteworthy that an intact RIPK1 is required for optimal inflammasome activation and pro-IL-1β processing under certain conditions [23,26,27]. Collectively, our results indicate that RIPK1 indirectly controls IL-1β secretion in vivo during Leishmania infection. Previous studies

show that RIPK1 kinase activity is essential for death receptor-induced necroptosis and certain forms of apoptosis [10,28], but dispensable for NF-κB and MAP kinase activation [29-31]. However, *Leishmania* infection did not result in aberrant cell death of *Ripk1*$^{kd/kd}$ BMDMs. Moreover, activation of NF-κB and the MAP kinase p38 was also normal in *Ripk1*$^{kd/kd}$ BMDMs (unpublished observation). More work is needed to fully elucidate the mechanism by which RIPK1 kinase activity promotes control of Leishmania replication.

Inflammatory cell death of macrophages as mediated by RIPK1 and PGAM5 could represent a general signal that alerts the immune system during Leishmania infection. Manipulation of necroptosis pathways could be helpful for designing therapeutic intervention to reduce disease severity associated with intense oxidative burst and unspecific tissue damage.

290

291 **Figure legends**

292

293 Figure 1: Heme induces RIPK1-RIPK3-MLKL dependent necroptosis in human
294 macrophages.
295 (a-c) Heme induced dose-dependent cell death in (a) human PBMCs, (b) primary
296 human monocyte-derived macrophages and (c) PMA-differentiated THP-1 cells. (d-e)
297 THP-1 cells were pre-treated with 10 μM necrostatin-1, 5 μM necrosulfonamide (NSA),
298 or the RIPK3 kinase inhibitors GSK840 and GSK843 (2 μM) for 1 hour prior to treatment
299 with 30 mM heme. Results shown are mean ± SEM.

300

301 Figure 2: RIPK1 kinase activity regulates Leishmania replication independent heme-
302 induced necroptosis.
303 (a) Heme inhibited *Leishmania chagasi* replication in THP-1 cells. Promastigotes were
304 counted as described in materials and methods. (b) RIPK1 kinase inhibitors, but not
305 RIPK3 or MLKL inhibitors, reversed heme-induced inhibition of *Leishmania chagasi*
306 replication in THP-1 cells. PMA-differentiated THP-1 cells were pretreated with the
307 indicated inhibitors for 1 hour, followed by infection with *Leishmania chagasi* for 4 hours
308 prior to stimulation with heme. Parasite load was determined 24 hours post-infection.
309 (c) RIPK1 kinase activity regulates *Leishmania chagasi* replication in THP-1 cells
310 independent of heme-induced necroptosis. PMA-differentiated THP-1 cells were pre-
311 treated with the indicated inhibitors for 1 hour prior to infection with *Leishmania chagasi.*

Parasite load was determined 24 hours post-infection. Results shown are mean ± SEM.

Figure 3: RIPK1 and PGAM5 are required for control of Leishmania replication in mouse BMDMs.

(a) Wild type (WT), *Pgam5*$^{-/-}$ and *Ripk1*$^{kd/kd}$ BMDMs were infected with (a, b, g) *Leishmania chagasi,* (c, d, h) *Leishmania amazonensis* or (e, f, i) *Leishmania major*. Amastigotes were counted 72 hours post-infection. (b, d, f) Representative images of amastigotes in infected BMDMs were shown. (g, h, i) Extracellular Promastigote counts show similar results to that of amastigote counts. Results shown are mean ± SEM.

Figure 4: RIPK1 kinase activity and PGAM5 are required for *in vivo* control of Leishmania replication.

Wild type, *Ripk1*$^{kd/kd}$ or *Pgam5*$^{-/-}$ mice were infected with *Leishmania amazonensis* in the footpad. (a) Parasite load in the footpad was determined 10 weeks after infection. (b-c) Lesion size on the footpad were monitored weekly.

Figure 5: RIPK1 and PGAM5 control Leishmania through distinct mechanisms.

(a-b) Production of IL-1β, but not TNF, was reduced in Leishmania-infected *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$ mice. (a) Splenocytes from mice infected with *Leishmania amazonensis* for 4 weeks were stimulated with Leishmania particular antigen for 48 hours. (b) Tissue extracts from mice infected with *Leishmania amazonensis* for 10

weeks were examined for IL-1β and TNF expression by ELISA. (c) *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$ BMDMs are defective for IL-1β secretion. BMDMs were primed with LPS for 3 hours followed by 3 hours of stimulation with nigericin. IL-1β secretion was determined by ELISA. (d) Splenocytes from mice infected with *Leishmania amazonensis* were stimulated with Leishmania particular antigen. IL-1β secretion was measured 48 hours after stimulation. (e) BMDMs of the indicated genotypes were left untreated or infected with *L. amazonensis* (L.A.), *L. chagasi* (L.C.) or *L. major* (L.A.) for 4 hours and NO was determined. (f) PGAM5 deficiency does not affect heme-induced necroptosis. BMDMs were stimulated with 30 μM heme and cell death was determined as described in materials and method. (g) BMDMs were infected with the indicated Leishmania strains. Cell death was determined 24 hours post-infection. Results shown are mean ± SEM.

Material and Methods

Mouse infection

All experimental procedures were approved and conducted according to the Institutional Animal Care and Use Committee of the University of the University of Massachusetts Medical School. *Ripk1*$^{kd/kd}$ (*Ripk1*$^{K45A/K45A}$) have been described before [20]. Characterization of the *Pgam5*$^{-/-}$ mice will be published in a different manuscript. For *in vivo* infection with Leishmania, mice were infected with 4 x 10^6 *L. amazonensis* per footpad in a volume of 20 μl. Swelling of the footpad was measured weekly by digital calipers (Fisher Scientific) and compared to the uninfected footpad. For quantification of *L. amazonensis* parasite load footpads from infected mice were harvested 10 weeks post-infection, homogenized, and serially diluted (1:2) in 96-well plates in complete Schneider media. After 7 days of incubation at 26°C, the number of viable parasites was calculated from the highest dilution at which parasites were observed. For assessment of cytokine production, footpads from mice were homogenized in 1 ml PBS supplemented with protease inhibitors. The footpad extracts were analyzed for TNF and IL-1β by ELISA (BD Biosciences). For *ex vivo* analysis of IL-1β and TNF production, single-cell suspensions were prepared from the spleen of mice infected after 10 weeks. After red blood cell lysis, 1 x 10^6 cells/ml in 1 ml were plated in 24-well plates. Cells were stimulated with 50 μg/ml of *L. amazonensis* particulate antigens (L.A.). The supernatants were harvested after 48 hours and analyzed by ELISA. The Griess method was used to measure nitric oxide (NO) production via its stable reaction product, nitrite (NO_2^-).

372 *Mouse and human cell culture*

373 Bone marrow derived macrophages (BMDMs) were generated from femoral bone
374 marrow cells by culture for seven days in DMEM medium supplemented with 20% L929
375 conditioned medium as described before [32]. Human primary monocyte-derived
376 macrophages were generated from peripheral blood mononuclear cells (PBMCs) from
377 healthy volunteers. After Ficoll Paque (GE healthcare) density centrifugation, PBMCs
378 were plated at 2 x 10^6 cells/well on 96-well plate. After 1 hour, non-adherent cells were
379 removed, and adherent cells were cultured in RPMI 1640 supplemented with 2 mM L-
380 glutamine and 10% FBS for 7 days. THP1 cells were stimulated with 200 nM phorbol
381 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 3 days, followed by culture in medium
382 without PMA for another 2 days.
383
384 *Cell death assay*
385 BMDMs (10^5 cells/well), PBMCs (2 x 10^5 cells/well), monocyte-derived macrophages (2
386 x 10^5 cells/well) and pro-monocytic THP-1 cells (2 x 10^5 cells/well) were cultured with 3
387 μM, 10 μM or 30 μM of heme. Twelve hours later, release of lactate dehydrogenase
388 (LDH) was measured in cell-free culture supernatant by the cytotoxicity detection kit
389 (Roche Applied Science, Germany). Where it is indicated, THP-1 cells were pre-treated
390 with RIPK1 or RIPK3 inhibitors for 1 hour before treatment with heme. BMDMs cell
391 viability was assessed by CytoTox96 Non-Radioactive Cytotoxicity Assay. In vitro IL-1β
392 production by BMDMs was measured by ELISA after stimulation with 200 ng/ml
393 ultrapure LPS (InvivoGen) for 3 hours, followed by 10 μM nigericin for another 3 hours.
394

Leishmania infection in BMDMs

L. amazonensis (strain IFLA/BR/67/PH8), *L. major* (strain MHOM/IL/81/Friedlin) and *L. chagasi* (MCAN/BR/89/Ba262) promastigotes were grown in Schneider medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. Stationary-phase promastigotes were used in all experiments. BMDMs and THP-1 cells were plated on coverslips one day before infection. Cells were infected with parasites at early stationary phase (MOI=5 for *L. amazonensis* and *L. major*, MOI=10 for *L. chagasi*). After 4 hours, cells were washed and further cultured for 72 hours. In some experiments, 30 μM heme was added 4 hours after infection. Cells were fixed with methanol and stained with Diff-Quick (Thermo Scientific). The number of amastigotes per 100 macrophages was enumerated by counting on a light microscope. In addition, parasite load was determined by light microscopy and the production of viable promastigotes in Schneider medium. Briefly, 72 hours post-infection, cell culture medium was replaced by Schneider medium and the plates were then kept at 24^{o}C. Seven days later, proliferating extracellular motile promastigotes were counted in a Neubauer hemocytometer. For THP-1 cells *in vitro* infection, PMA-differentiated cells were treated for 1 hour with GSK RIPK1 and RIPK3 inhibitors before infection with promastigotes.

Statistical analyses

All results shown are mean ± SEM. Statistical significance was determined by Student's t test.

Figure 1: Heme induced necroptosis in human macrophages

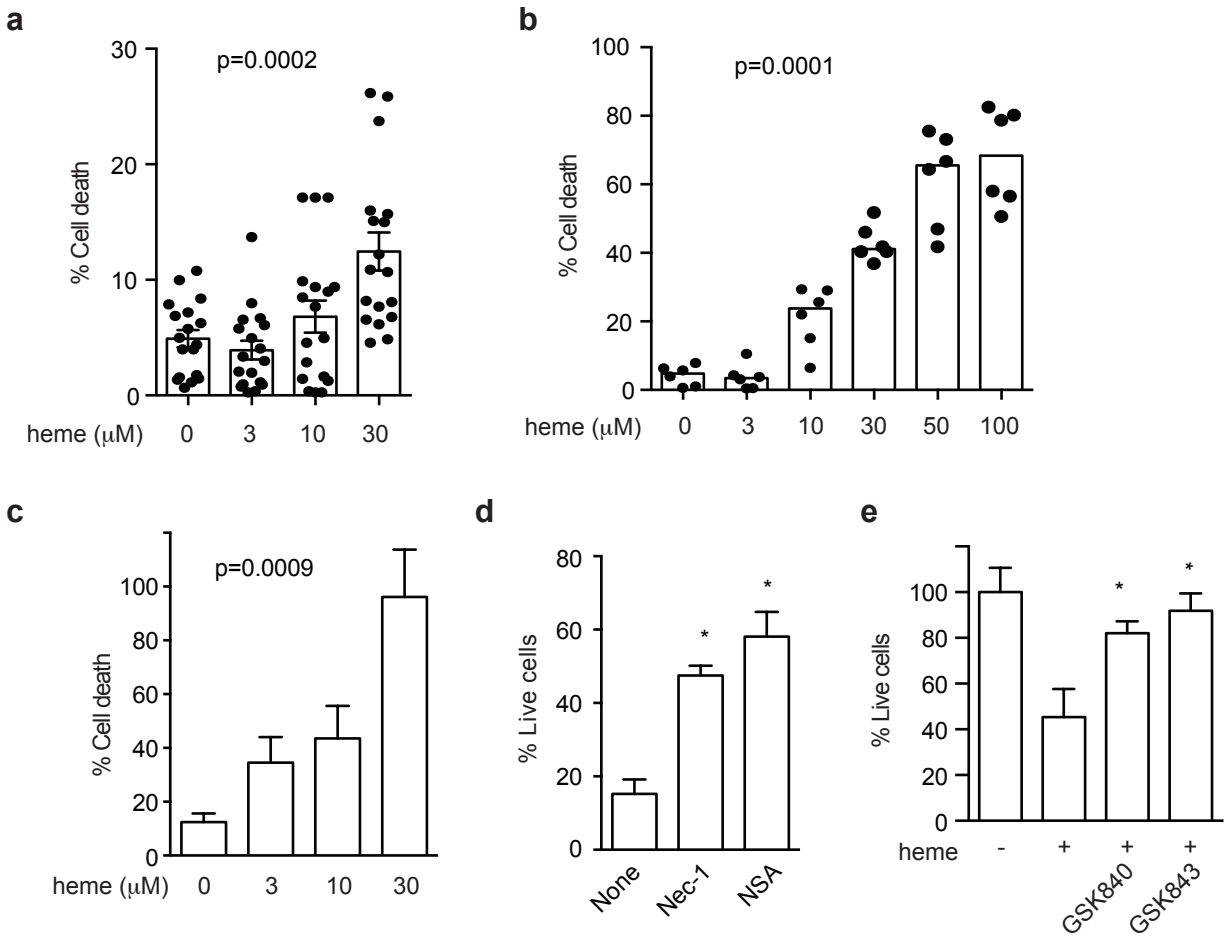


Figure 2: RIPK1 kinase activity is required for control of Leishmania replication

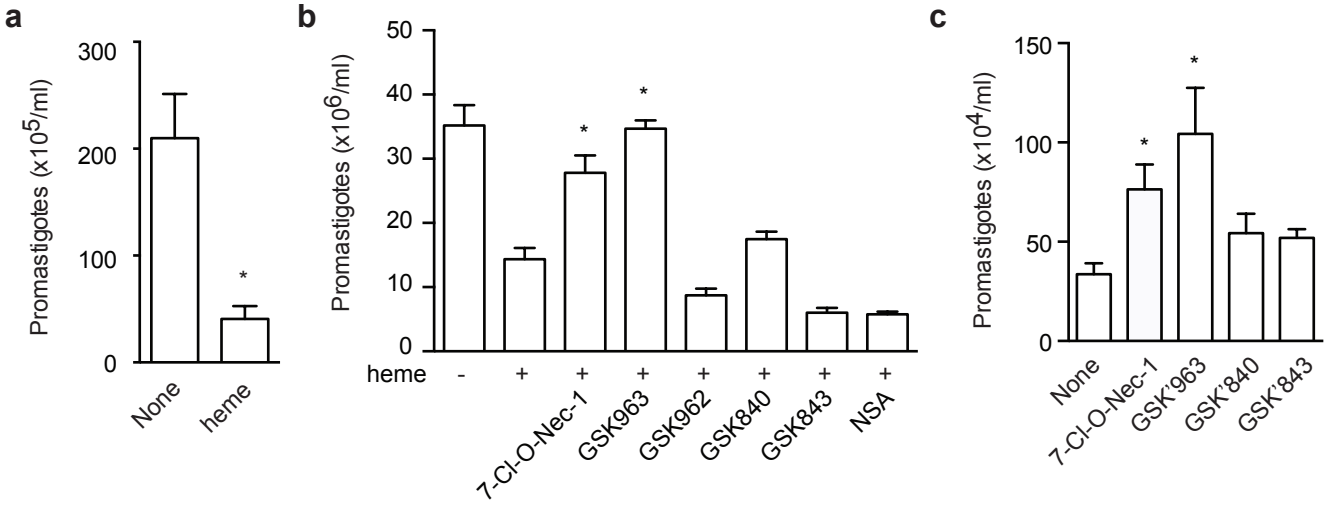


Figure 3

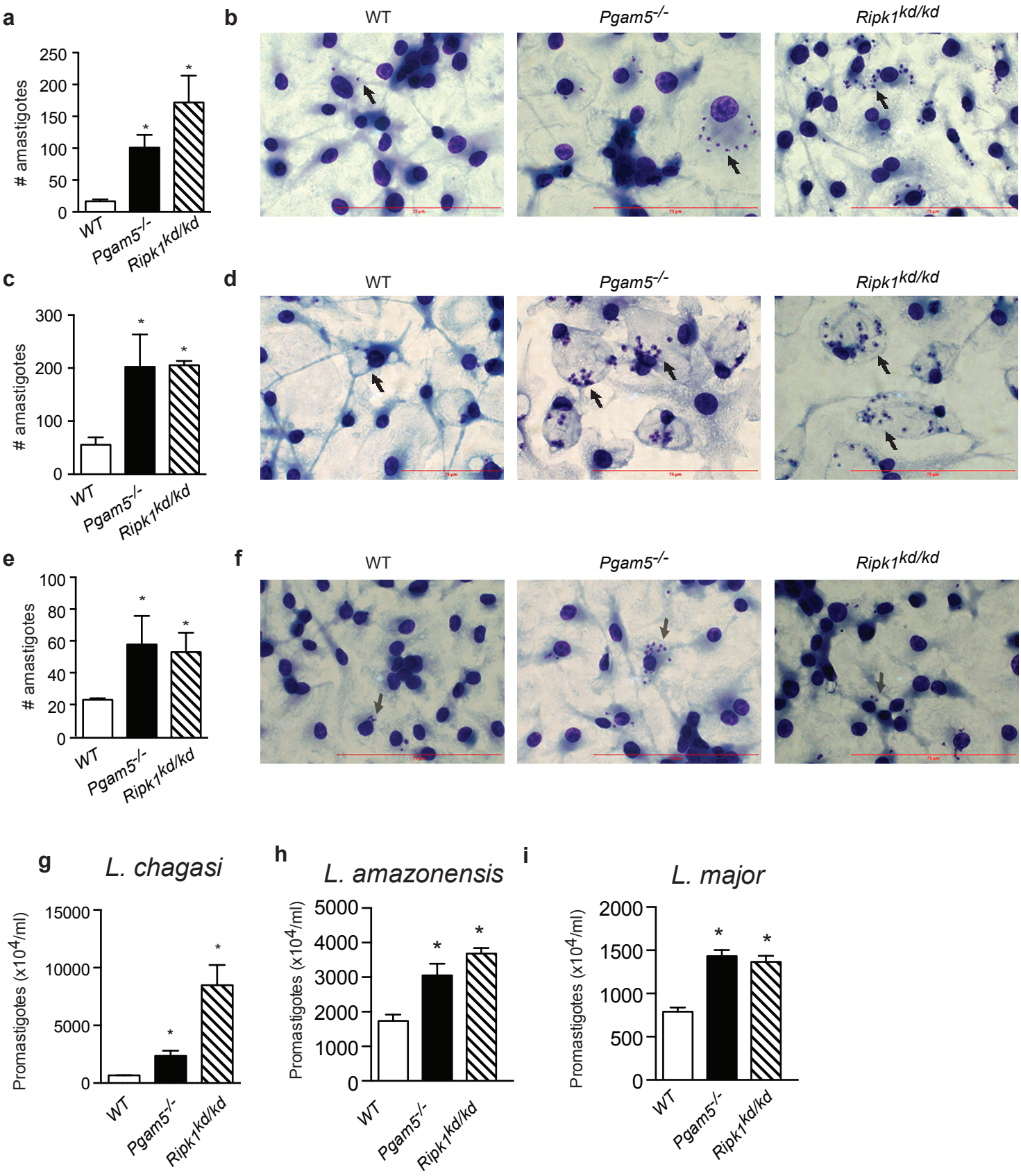

a
amastigotes
250
200
150
100
50
0
WT
Pgam5-/-
Ripk1kd/kd
b
WT
Pgam5-/-
Ripk1kd/kd
c
amastigotes
300
200
100
0
WT
Pgam5-/-
Ripk1kd/kd
d
WT
Pgam5-/-
Ripk1kd/kd
e
amastigotes
100
80
60
40
20
0
WT
Pgam5-/-
Ripk1kd/kd
f
WT
Pgam5-/-
Ripk1kd/kd
g
L. chagasi
Promastigotes (x10^4/ml)
15000
10000
5000
0
WT
Pgam5-/-
Ripk1kd/kd
h
L. amazonensis
Promastigotes (x10^4/ml)
5000
4000
3000
2000
1000
0
WT
Pgam5-/-
Ripk1kd/kd
i
L. major
Promastigotes (x10^4/ml)
2000
1500
1000
500
0
WT
Pgam5-/-
Ripk1kd/kd

Figure 4: RIPK1 kinase activity and PGAM5 are also required for in vivo control of LSH replication

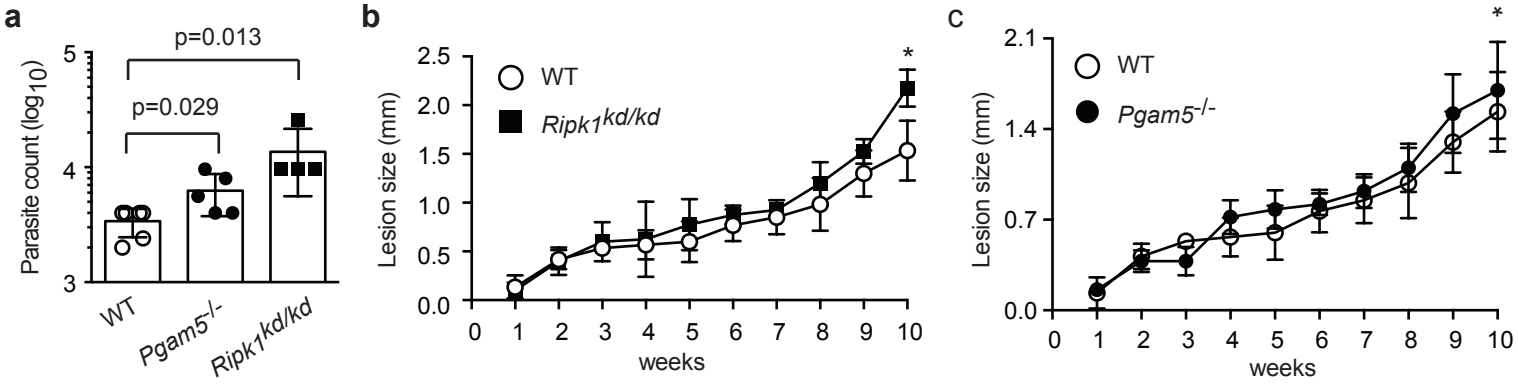
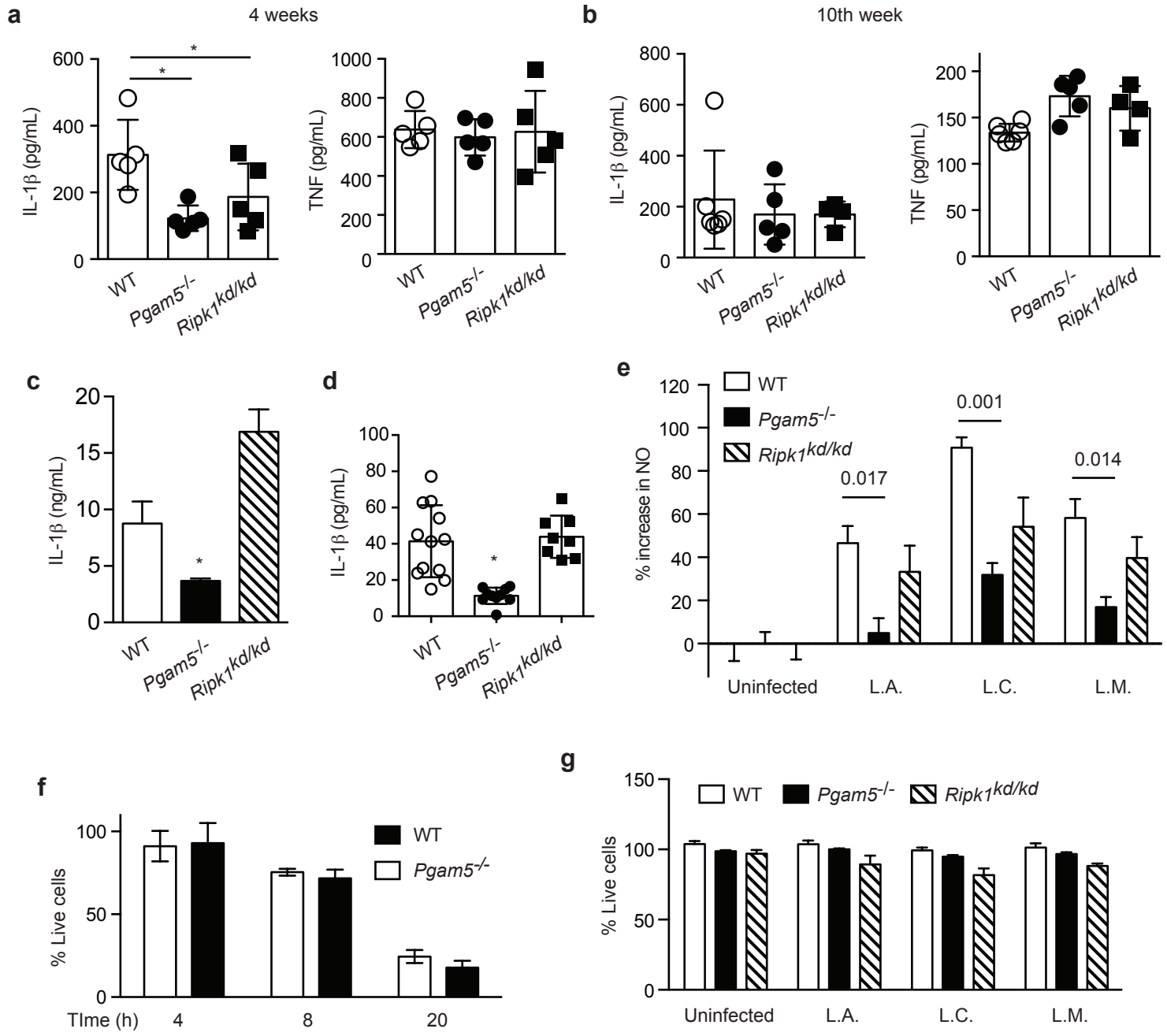


Figure 5: RIPK1 and PGAM5 control LSH replication through distinct mechanisms



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5. DISCUSSÃO

Conhecer a biologia da interação patógeno-célula hospedeira durante a infecção por *Leishmania* é essencial para entender os mecanismos regulatórios imune em resposta ao patógeno. Parasitas do gênero *Leishmania* têm um pronunciado tropismo por macrófagos, embora tenham capacidade de infectar diversos tipos celulares. Amastigotas de *Leishmania* proliferam no fagolisossomo de macrófagos e adquirem nutrientes necessários para seu metabolismo (HUYNH; ANDREWS, 2008). No entanto, esse processo não é passivo, pois a célula hospedeira é capaz de limitar a disponibilidade de nutrientes para o parasito no vacúolo parasitóforo, bem como induzir a produção de moléculas efetoras no controle da infecção (PODINOVSKAIA; DESCOTEAUX, 2015). Para a *Leishmania*, desenvolver mecanismos de resistência e subversão da resposta microbicida na célula hospedeira é crucial para o sucesso da infecção e persistência do parasito no hospedeiro. Nesse contexto, a enzima heme oxigenase 1 (HO-1) foi escolhida como alvo desse estudo para buscar compreender seu papel durante a infecção de macrófagos por *L. infantum chagasi*, principal agente etiológico dos casos da forma visceral da Leishmaniose noBrasil.

As manifestações hematológicas são constantes na LV (VARMA; NASEEM, 2010). Mecanismos ligados à hemólise, metabolismo do heme e atividade da enzima heme oxigenase podem estar envolvidos nas alterações hematológicas, especialmente na fase da doença que envolve coagulação e anemia grave. Por se tratar de uma parasitemia visceralizante associada à hemólise e consequente liberação de heme, a enzima HO-1 pode ter participação na modulação da carga parasitária bem como na proteção aos efeitos tóxicos do heme. Aqui nós investigamos o papel da HO-1 na infecção por *L. chagasi* e a modulação das funções pró e anti-inflamatórias *in vitro* de macrófagos, exploramos também a associação da enzima HO-1 no contexto da LV humana.

Nossos dados mostraram que promastigotas de *L. chagasi* são capazes de induzir a liberação de HO-1 no sobrenadante de macrófagos infectados, bem como no lisado dessas células. O perfil de indução da HO-1 deflagrado pela infecção por promastigotas de *L. chagasi* em macrófagos mostrou-se sustentado após 24 horas de interação com o parasita. O mesmo aconteceu para a interação com lipofosfoglicano (LPG), que é abundante na superfície do parasita *Leishmania*, e relacionado com a subversão da resposta leishmanicida do macrófago e importante para o sucesso da infecção (FORESTIER; GAO; BOONS, 2014). É importante ressaltar que outras moléculas de superfície do parasita podem também estar envolvidas na indução do gene da HO-1.

Atualmente, as vias de indução de HO-1 durante a infecção por *Leishmania* não são completamente esclarecidas. Sabe-se que o gene *Hmox 1* é induzido por vários estímulos e envolve múltiplos padrões de sinalização, dentre esses estímulos o gene *Hmox 1* é fortemente induzido por condições de estresse oxidativo (WAGENER et al., 2003). Em nossos experimentos encontramos que a infecção por promastigotas de *L. chagasi* resulta na indução de ROS em macrófagos, e que a indução de HO-1 reduz a produção deste importante mecanismo microbicida. Entretanto, não podemos descartar que o parasita também induza HO-1 por vias independentes do balanço redox associadas à ativação do gene *Hmox*, como quinases, MAPK, Proteína quinase C (PKC) e fosfatases (ALAM; COOK, 2007; FERNANDEZ-BUSTAMANTE et al., 2015). O fator nuclear PPAR-γconhecidamente regula a expressão do gene *Hmox1* (NDISANG, 2014), no entanto em nossos experimentos a pré- incubação com antagonistas de PPAR-γno contexto da infecção, bem como o tratamento de macrófagos com LPG não encontramos qualquer alteração nos níveis de HO-1 produzidos, indicando que outros fatores nucleares podem estar envolvidos na indução de HO-1 por *L. chagasi*. Nrf2 (*Nuclear factor-erythroid 2-related factor 2*) o fator nuclear que induz a expressão do gene *Hmox1* está aumentado em soro de pacientes com LV (DAS et al., 2013), indicando que Nrf2 pode ser um dos fatores nucleares pelos quais Leishmania induz HO-1.

Pamplona e col. (2007) demonstraram com um modelo experimental de malária cerebral, que na infecção de camundongos Balb/c por *Plasmodium berghei* os níveis da proteína HO-1 são aumentados (PAMPLONA et al., 2007a). Epiphanio e cols. (2009) demonstraram que os níveis de mRNA e da proteína HO-1 são aumentados em hepatócitos na infecção *in vivo* com *P. berghei* e em macrófagos peritoneais na infecção por *P. berghei* e *P.yoelli* (EPIPHANIO et al., 2008). Posteriormente, autores do mesmo grupo demonstraram que HO-1 é requerida para indução de Ferritina H em resposta a infecção por *P. chabaudi*, bem como para os efeitos protetores da Ferritina (GOZZELINO et al., 2012). Nossas observações reforçam a idéia de que a HO-1 é induzida em diversas doenças parasitárias e está relacionada à proteção ou susceptibilidade do hospedeiro.

Outros modelos de infecções têm demonstrado que a HO-1 participa no contexto da interação de macrófago com patógeno. **Recentemente, Gobert e cols. (2014) demonstraram que macrófagos infectados por *Helicobacter pylori* têm maior expressão de HO-1.** A inibição de HO-1 em camundongos infectados por *H. pylori* polariza os macrófagos para um perfil M1 o que atribui a esses macrófagos menor carga bacteriana e predomínio de citocinas inflamatórias (GOBERT et al., 2014).

No contexto da infecção por *T. cruzi* Paiva e cols. **(2012) encontraram que a indução de HO-1 por CoPP reduz a carga parasitária de macrófagos infectados, assim como a parasitemia e mortalidade em camundongos infectados.** Os resultados dos autores indicam que a infecção por *T. cruzi* é favorecida pelo estresse oxidativo, uma vez que a administração de pró-oxidantes aumenta a parasitemia de camundongos infectados (PAIVA et al., 2012). Nesse contexto, a HO-1 reduziria então a carga parasitária por reduzir o estresse oxidativo necessário para a replicação do parasita (PAIVA; BOZZA, 2014). Não podemos descartar a possibilidade de encontrar resultados diferentes caso outras espécies de *Leishmania* sejam utilizadas.

Camundongos deficientes em $gp91^{phox}$ e infectados por *L. donovani* apresentam maior carga parasitária e retardo do controle do crescimento do parasita (MURRAY; NATHAN, 1999), *L. pifanoi* inviabiliza a formação do complexo NADPH oxidase na membrana do vacúolo parasitóforo (PHAM; MOURIZ; KIMA, 2005), esses achados indicam que diferentes espécies de *Leishmania* têm diferentes estratégias de lidar com o ROS. Além disso, a expressão de HO-1 em condições como malária, tuberculose e infecções virais têm sido relacionada a um papel protetor do hospedeiro na resposta desencadeada pelas células da imunidade inata e adaptativa à infecção (CHUNG; HALL; PERRELLA, 2009; RYTER; CHOI, 2015). Interessantemente, um trabalho publicado recentemente demonstrou que em um modelo experimental de Leishmaniose cutânea, a expressão de HO-1 está aumentada em lesões de camundongos C57BL/6 e BALB/c infectados por *L. amazonensis*, reforçando que HO-1 é uma proteína de estresse fortemente associada a infecção por *Leishmania* (ARAUJO; GIORGIO,2015).

Um dos aspectos mais estudados da HO-1 em doenças infecciosas têm sido o seu papel anti-inflamatório e quais as conseqüências para a proliferação do patógeno na célula hospedeira. Apesar do estudo conduzido por Pham e colaboradores, 2005, demonstrar que amastigotas de *L. pifanoi* possuem estratégias para evitar a produção de radicais de oxigênio e que esse mecanismo é dependente da ativação da enzima HO-1, os autores não exploram qual o impacto desses achados no controle da carga parasitária durante a infecção por *Leishmania*. Nesse sentido, nosso trabalho mostra pela primeira vez quais as implicações da ativação da HO-1 na sobrevivência de *L. chagasi* em macrófagos infectados.

Para melhor explorar o papel da HO-1 na infecção de macrófagos por *L. chagasi* nós utilizamos o indutor farmacológico da HO-1. A Cobalto protoporfirina (CoPP) é um potente indutor da HO-1 via Bach1 e Nrf2 (fatores nucleares associados à regulação do gene

Hmox1) (SHAN et al., 2006). Observamos que em macrófagos peritoneais de camundongos C57BL/6 e em macrófagos humanos derivados de monócitos a indução de HO-1 pela CoPP corresponde à um considerável aumento do percentual de macrófagos infectados e quantidade de amastigotas por macrófago. A indução de HO-1 polariza macrófagos derivados de monócitos humanos para um fenótipo anti-inflamatório (WEIS et al., 2009). Em nosso modelo o aumento do percentual de macrófagos infectados indica que a HO-1 torna os macrófagos susceptíveis à infecção por *L. chagasi*, enquanto que o aumento do número de amastigotas por macrófago é um forte indicador de que a HO-1 é requerida para exercer seu papel anti-inflamatório que auxiliaria na desativação de mecanismos leishmanicidas do macrófago. De forma similar, o aumento da expressão de HO-1 utilizando um adenovírus em camundongos Balb/C leva ao aumento da replicação de *P. berghei* no fígado quando comparado aos animais controle transfectados apenas com adenovírus não codificante, demonstrando dessa forma que a HO-1 é requerida também para o favorecimento da replicação de *P. berghei in vivo* (EPIPHANIO et al., 2008).

No caso da infecção de macrófagos humanos, encontramos que a indução de HO-1 implica na redução de ROS e aumento da expressão de Cu/Zn Superóxido Dismutase (SOD- 1). Esses dados são consistentes com trabalhos publicados por Khouri e cols. **(2009 e 2010) demonstrando que SOD-1 favorece a replicação e imunossupressão em macrófagos humanos infectados por *L. amazonensis* (KHOURI et al., 2009) e contribui para o sucesso da infecção por *Leishmania* em um modelo experimental de Leishmaniose cutânea (KHOURI et al., 2010).**

Para descartar possíveis efeitos pleiotrópicos da manipulação de HO-1 farmacologicamente e esclarecer de forma definitiva o efeito da ausência de HO-1 na infecção L. *chagasi* nós utilizamos macrófagos derivados de precursores mononucleares de medula óssea de camundongos Balb/c e Scid selvagens ou deficientes no gene *Hmox1 (knockout)*. Observamos que os macrófagos de camundongos *knockout* para o gene *Hmox1* têm uma menor carga parasitária quando comparado aos macrófagos selvagens. Esse efeito foi observado em ambas as linhagens de camundongos. Corroborando os nossos achados, Epiphanio e cols., 2009 encontraram que na infecção por *P. berghei* e *P. yoelli*, os camundongos *knockout* para o gene *Hmox1* têm menor carga parasitária no sangue e no fígado quando comparado aos camundongos selvagens. Além disso, o tratamento de camundongos Balb/c com o RNA de interferência para o gene da HO-1induz uma menor carga parasitária em animais infectados. Coletivamente, nossos achados demonstram que a indução de HO-1 é requerida no estabelecimento da infecção por *L. chagasi*.

Já foi demonstrado que a CoPP induza expressão da HO-1 (MARKS,1994). De forma similar, nossos resultados mostraram que a CoPP foi capaz de induzir aumento na liberação de HO-1 nos macrófagos peritoneais de camundongos C57BL/6, tanto na infecção por *L. chagasi* quanto apenas no tratamento com CoPP. Como esperado, a CoPP aumenta significativamente a liberação da proteína HO-1 no sobrenadante dessas culturas. Esse resultado nos permite inferir que os efeitos da CoPP sobre carga parasitária na infecção de macrófagos devem-se de fato à proteína HO-1.

A partir das observações de que a infecção por *L. chagasi* induz HO-1 e de que a CoPP induz aumento da replicação do parasita nós hipotetizamos que a HO-1 estaria reduzindo potenciais mecanismos leishmanicidas do macrófagos, dentre eles o TNF. De fato na LV humana, sintomas como anemia e fraqueza, podem estar associados a níveis aumentados de TNF. Esse mediador foi detectado em pacientes com LV e sua presença tem sido associada à atividade da doença (BARRAL-NETTO et al., 1991; PERUHYPE-MAGALHÃES et al., 2006). Além disso, o TNF desempenha um importante papel na defesa do hospedeiro contra patógenos intracelulares e na regulação de padrões inflamatórios (LOCKSLEY; KILLEEN; LENARDO, 2001). Nesse conjunto, as observações de que o tratamento com CoPP reduz a produção de TNF induzida pela *Leishmania*, corroboram o efeito anti-inflamatório da HO-1 e explica um dos mecanismos pelos quais a *Leishmania* se beneficia da indução de HO-1 em macrófagos murinos. Esse achado ainda nos sugere que a indução de HO-1 provocada somente pela infecção é uma estratégia do parasita para desativar mecanismos leishmanicidas domacrófagos.

Uma possível explicação de como a redução da produção de TNF deve favorecer a replicação do parasita é que os mecanismos leishmanicidas do TNF são mediados principalmente por indução de estresse oxidativo (ENGWERDA; ATO; KAYE, 2004; SHEEL; ENGWERDA, 2012). A indução de HO-1 corresponde também a ativação de genes associados à proteção do estresse oxidativos (COLLINSON et al., 2011). Logo, a indução de HO-1 parece ser um mecanismo inicial da infecção que permite um ambiente menos hostil para o parasita. Nesse sentido, Pham. e colaboradores, demonstraram que durante a interação de amastigotas e promastigotas de *Leishmania pifanoi* com macrófagos, ambas as formas evolutivas são capazes de induzir HO-1. Segundo esses autores, a enzima HO-1 degrada o heme do complexo NADPH oxidase, impedindo assim a formação viável das subunidades do complexo capaz de gerar superóxido no vacúolo parasitóforo como parte do escape da ativação dos mecanismos leishmanicidas do macrófago (PHAM; MOURIZ; KIMA,2005).

Alguns mediadores pró e anti-inflamatórios são produzidos frente a um primeiro estímulo (*priming*), o LPS é um agonista de TLR4 que polariza e ativa macrófagos para a produção de mediadores pró-inflamatórios (LATZ et al., 2002; LIEN et al., 2000). Para melhor elucidar o efeito da indução de HO-1 pela CoPP nós ativamos as células com LPS e avaliamos alguns mediadores que poderiam estar envolvidos na resposta leishmanicida do macrófagos. Curiosamente, observamos que a indução de HO-1 pela CoPP aumenta a carga parasitária de macrófagos infectados por *L. chagasi* independente da presença de LPS. Esses achados nos permitem afirmar que embora a infecção por *L. chagasi* tenha efeito na polarização e modulação do macrófago, alguns efeitos na produção de mediadores independem da infecção e parecem ser exercidos primariamente pela HO-1 induzida pela CoPP

Nós também investigamos a produção de componentes antiinflamatórios que poderiam estar envolvidos no favorecimento da replicação do parasita. Vários estudos experimentais murinos têm demonstrado que um dos principais fatores para a progressão da Leishmaniose é a IL-10. O bloqueio do receptor IL-10 em camundongos infectados com *L. donovani* reduz drasticamente a infecção (MURRAY et al., 2002) e camundongos IL-$10^{-/-}$ são altamente resistentes à LV (MURPHY et al., 2001).

Nós achamos que no tratamento com CoPP, prevalece a produção de IL-10 quando comparado aos níveis de TNF, ou seja, a indução de HO-1 deve favorecer a proliferação da *L. chagasi* porque contribui para a prevalência de um ambiente anti-inflamatório.

As respostas associadas a IL-10 são geradas para balancear a excessiva resposta Th1e prevenir a imunopatologia. A alta produção de IL-10 está associada a redução da resposta inflamatória levando à susceptibilidade à infecção por *Leishmania* (BELKAID et al., 2001; ANDERSON et al., 2005; STAGER et al., 2010). Os efeitos anti-inflamatórios da IL-10 são exercidos via indução da HO-1 (LEE e CHAU, 2002) o que polariza a diferenciação de macrófagos (SIERRA-FILARDI et al., 2010) e células dendríticas (CHAUVEAU et al., 2005) para um perfil de resposta anti-inflamatória. Dessa forma, podemos especular que na infecção por *L. chagasi* a indução de HO-1 pela CoPP concomitante com a prevalência de IL-10 pode significar a persistência de mais de um estímulo capaz de favorecer a replicação do parasita, induzindo assim uma resposta anti-inflamatória na célula hospedeira. Além disso encontramos em amostras de pacientes com LV uma correlação positiva entre a presença de IL-10 e HO-1 no soro, corroborando relatos de que a persistência da LV humana está associada também com altos níveis de IL-10 no

soro dos pacientes (GHALIB et al., 1993; PERUHYPE- MAGALHÃES et al., 2006).

Em modelos murinos *in vitro* e *in vivo* de infecção por *Leishmania* é bem estabelecido que a ativação de macrófagos por citocinas como IFNγ e TNF leva à produção de espécies reativas de oxigênio e nitrogênio, que são responsáveis pela atividade leishmanicida (DEY et al., 2007). A produção de NO é o principal mecanismo microbicida em macrófagos murinos (DIAS COSTA et al., 2007; GANTT et al., 2001). Em nosso estudo o tratamento com CoPP foi capaz de reduzir a produção de nitrito à níveis basais ao de uma célula não estimulada com LPS, esse efeito pode ser especificamente atribuído à indução de HO-1, uma vez que as células tratadas com LPS e CoPP apresentaram a mesma redução dos níveis de nitrito, independente da infecção por *L. chagasi*. Esse achado nos permite deduzir que a indução de HO-1 pela CoPP desativa o macrófago frente à estimulação com LPS e que esse mecanismo reduz a produção de um importante mecanismo efetor no controle da infecção por *Leishmania*.

Os antígenos de *Leishmania* são capazes de induzir a produção de mediadores associados à ativação da célula infectada. Dentre esses mediadores a interleucina-6 (IL-6) destaca-se por estar envolvida na regulação do balanço da resposta Th1-Th2 associada à infecção por *Leishmania* (DE LIMA et al., 2007). Deste modo, resolvemos avaliar a influência do tratamento com CoPP em macrófagos tratados com LPS e infectados com *L. chagasi*. A indução de HO-1 pela CoPP reduziu os níveis de IL-6 frente à estimulação por LPS. Esse dado nos remete à importância do papel supressor da HO-1 na produção de mediadores inflamatórios, uma vez que a redução dos níveis de IL-6 em pacientes com LV está associado com sucesso no tratamento e, além disso, altos níveis de IL-6 em pacientes com LV é considerado um marcador da forma ativa da doença (PERUHYPE-MAGALHAES et al., 2006). O papel supressor da HO-1 sobre a produção de IL-6 já foi demonstrado em modelo agudo de agressão hepática (KAMIMOTO et al., 2009), assim como em um modelo de inflamação sistêmica em camundongos *knockout* no gene *Hmox1* (KAPTURCZAK et al., 2004).

Não podemos descartar a possibilidade de que os efeitos protetores e anti-inflamatórios da HO-1 estejam de fato sendo desempenhado pelos produtos da degradação do heme, que são o CO, Ferro e Biliverdina. Os mecanismos pelos quais a HO-1 exerce seu efeito protetor são parcialmente descritos e muito se sabe à respeito do efeito cito-protetor do monóxido de carbono (CO) (OTTERBEIN; CHOI, 2008; RYTER; CHOI, 2015). O papel do monóxido de carbono já foi demonstrado em diversos modelos de dano celular. A HO-1 protege células endoteliais da apoptose mediada por TNF e agonistas pró-apoptóticos

(SOARES et al., 2002). Da mesma forma, esse efeito ocorre quando as células são tratadas com CO, levando à idéia de que os efeitos protetores da HO-1 são fortemente mediados pela produção de CO.

Ao contrário do átomo de Fe contido no anel de protoporfirina IX de heme, o Fe liberado do heme pode ser neutralizado por uma variedade de mecanismos associados ao metabolismo do ferro, incluindo a Ferritina (FtH), que é induzida pelo próprio Fe e tem um papel cito-protetor (GOZZELINO; AROSIO, 2015; GOZZELINO; SOARES, 2014). A expressão de FtH protege células da apoptose mediada por TNF por prevenir o efeito pró-oxidante do Fe livre na ativação constante da c-Junk (quinase), que leva à ativação de caspases efetoras (GOZZELINO et al., 2012). O Fe é um co-fator para muitas enzimas e perfis metabólicos e a sua aquisição é fortemente regulada pelas células (GOZZELINO et al., 2012). Algo ainda interessante em relação ao efeito do Fe é que além do papel cito-protetor, esse componente já foi demonstrado como imprescindível suporte nutricional para tripanossomatídeos como *Trypanossoma* e *Leishmania* (FLANNERY; RENBERG; ANDREWS, 2013; HUYNH; ANDREWS, 2008).

L. chagasi expressa uma NADPH redutase dependente de Fe, que é capaz de converter o ferro oxidado (Fe^{3+}) em ferro reduzido (Fe^{2+}) que é mais solúvel (WILSON et al., 2002). A adição de holotransferrina é capaz de alcançar o compartimento intracelular dos parasitas e promover o crescimento de *Leishmania* em macrófagos murinos (BORGES; VANNIER-SANTOS; DE SOUZA, 1998). O transpotador de ferro, LIT1, está na membrana plasmática de *L. amazonensis*, e é essencial para a replicação intracelular e axênica e virulência do parasita (RENBERG et al., 2015). Conjuntamente esses relatos demonstram a importância nutricional do ferro para *Leishmania* e que o parasita tem capacidade de sequestrar esse nutriente. Essa observação sugere que em nosso modelo experimental um dos mecanismos pelos quais a indução da HO-1 pela CoPP pode aumentar a carga parasitária de macrófagos infectados poderia estar associado ao aumento da disponibilidade de ferro como produto da degradação do heme.

O efeito do próprio heme sobre a *Leishmania* não pode ser descartado. Existe apenas um artigo indicando a possível existência de uma enzima análoga à HO-1 e algumas enzimas relacionadas à síntese do heme em promastigotas de *L. donovani.* Srivastava e cols., 1997 explanaram sobre a presença de enzimas como δ-aminolevulinato sintase e ferroquelatase em promastigotas de *L. donovani* assim como heme oxigenase e biliverdina redutase (SRIVASTAVA et al., 1997). Embora inovador, esse estudo não é conclusivo

porque os autores investigaram apenas a atividade dessas enzimas, não havendo qualquer análise protéica ou genômica dessas proteínas em *Leishmania*. De qualquer forma, o estudo aponta para a capacidade de síntese de heme como suporte nutricional e indica que a *Leishmania* possui estratégias de detoxicação dos efeitos tóxicos do heme. Portanto, não podemos descartar a participação dos produtos da degradação do heme e do próprio heme no favorecimento da replicação da *L. chagasi* em macrófagos. Pelo contrário, nossos achados indicam que outras protoforfirinas, além da CoPP, ou mecanismos moleculares associados à indução da HO-1 podem favorecer a replicação do parasita. O papel protetor *in vivo* das proteínas ligadoras de heme, como a hemoglobina, hemopexina, haptoglobina e albumina que estão associadas à mecanismos de detoxicação do heme extracelular (KRISTIANSEN et al., 2001; QUAYE, 2008, 2015) necessita ser melhor explorado.

Por fim, investigamos o papel da HO-1 na LV humana. Encontramos que a HO-1 está aumentada no soro de pacientes com LV quando comparados aos níveis de indivíduos da mesma área endêmica. Encontramos também que os níveis de HO-1 estão positivamente relacionados com os níveis de IL-10, IL-8 e IL-6. Acredita-se que a HO-1 exerça sua atividade enzimática de degradação do heme no meio intracelular (YOSHINAGA; SASSA; KAPPAS, 1982)., dessa forma o aumento da HO-1 no soro parece está relacionada com o dano tecidual característico da LV, isso parece ser o caso, uma vez que os mesmos indivíduos apresentaram redução dos níveis de HO-1 no soro quinze dias após o tratamento leishmanial.

Nossos achados são a primeira evidência de que a infecção por *L. chagasi* é capaz de induzir a secreção da proteína HO-1 e que a indução da mesma, favorece a replicação do parasita por reduzir mecanismos leishmanicidas da célula hospedeira está associada com a LV humana. Dessa forma, esse estudo levanta uma série de novas perspectivas de investigação do papel da HO-1 e proteínas relacionadas à detoxicação do heme no esclarecimento de mecanismos imunopatogênicos associados à patogênese da LV humana e uma possível forma de intervenção terapêutica para forma letal da Leishmaniose.

Diversas doenças infecciosas estão associadas com manifestações hematológicas como a hemólise, a liberação e acúmulo de heme livre no soro. Nessas doenças altos níveis séricos de heme estão associados com a gravidade, indicando que a homeostase dos níveis de heme no soro está intimamente relacionado com a patogênese dessas doenças (LARSEN et al., 2012). Em modelos de malaria cerebral experimental altos níveis de heme são encontrados na circulação e estão associados com o rompimento da barreira hematoencefálica (PAMPLONA et al., 2007a). De forma similar, na malaria humana nosso

grupo encontrou que o heme está aumentado em pacientes que apresentam a forma sintomática da doença e que os níveis de heme servem como preditor de gravidade (ANDRADE et al., 2010). Levando em consideração que a LV apresenta diversas manifestações hematológicas, decidimos investigar os níveis de heme nos mesmos pacientes e indivíduos da área endêmica onde determinamos os níveis HO-1. Encontramos que os níveis de heme estão significativamente aumentados em pacientes com LV quando comparado a indivíduos controles, e que esses níveis se encontram positivamente correlacionado com os níveis de HO-1 (Anexo 1), indicando que o heme pode participar na patogênese da LV.

Fortes e cols. (2012) demonstraram que em macrófagos murinos o heme induz uma necrose programada, que é também denominada de necroptose. Os autores demonstraram que o tratamento de macrófagos com heme induz perda da integridade da membrana plasmática e morte celular com aspecto morfológico necrótico. Essa morte celular parece ser dependente da produção de TNF através de TNFR1 e Myd88. Nesse modelo a inibição de RIPK1 reverte o efeito de dano celular induzido pelo heme, e há indução de RIPK1 e RIPK3 após o tratamento com heme (FORTES et al., 2012). Em nossos experimentos utilizando PBMCs, macrófagos derivados de monócitos humanos e macrófagos derivado de células THP-1 também encontramos que o heme é capaz induzir dano membranar em células humanas. Da mesma forma a inibição de RIPK1, RIPK3 e MLKL, moléculas acessórias e efetoras na via da necroptose, reverteu o dano celular induzido pelo heme, indicando dessa forma que heme é capaz de induzir necroptose clássica também em célulashumanas.

Diversos trabalhos indicam a importância da necroptose na resistência a infecções. Na infecção por vírus *vaccinia* a replicação do vírus é controlada pelo hospedeiro pela produção de TNF através de RIPK1 e RIPK3 (FORTES et al., 2012). Da mesma forma na infecção por citomegalovírus murino a necroptose é relevante para a restrição da replicação viral. Esse vírus apresenta um mecanismo de subversão da resposta do hospedeiro que consiste em codificar a proteína viral vIRA que inibe o domínio quinase de RIPK3, o que por sua vez inibe a necroptose (UPTON; CHAN, 2014; UPTON; KAISER; MOCARSKI, 2010).

O papel de moléculas da via da necroptose também tem sido relevante no controle da infecção por *Mycobacterium tuberculosis* (MTB), uma vez que a inibição de necrose programada, através da inibição de RIPK1, RIPK3, MLKL e PGAM5, resulta inicialmente em uma redução da atividade bactericida inicial e redução da morte necrótica de

macrófagos. Esse mecanismo resulta posteriormente em aumento da carga bacteriana extracelular uma vez que bactérias são liberadas em um ambiente permissivo para sua replicação, com níveis reduzidos de TNF e ROS que são essenciais para a formação do granuloma e atividade bactericida (ROCA; RAMAKRISHNAN, 2013; SRIDHARAN; UPTON,2014).

Considerando os diversos trabalhos que demonstram a importância da necroptose, esperávamos encontrar em nosso modelo que a necroptose induzida pelo heme no contexto da LV contribuiria para a atividade leishmanicida de macrófagos. De fato encontramos que heme reduz a carga parasitárias de macrófagos infectados *in vitro* por *L. infantum chagasi*. No entanto, a inibição da necroptose através da inibição farmacológica de RIPK1, RIPK3 e MLKL demonstrou que apenas a inibição de RIPK1 reverte o efeito de heme de redução da carga parasitária, o que nos indica que RIPK1 pode regular a replicação do parasita por um mecanismo independente de necroptose. Para descartar o efeito do heme de indução direta de necroptose, avaliamos a carga parasitária de macrófagos infectados com *L. chagasi* e não tratados com heme, o que confirmou nosso resultado anterior de que apenas a inibição de RIPK1 contribui para um fenótipo de favorecimento do crescimento do parasita. Uma vez que a inibição de RIPK3 não se mostrou relevante para o crescimento do parasita, é possível que em nosso modelo a RIPK1 controle a replicação de *Leishmania* por um mecanismo independente da ativação de necroptose. De fato, além da participação no processo de necroptose a molécula RIPK1 participa também da ativação de NFkB (CHAN; LUZ; MORIWAKI, 2015). Uma vez ativada a RIPK1 promove a fosforilação de IkBα por IKK, levando a degradação de IkBα pelo proteossoma e translocação nuclear dos dímero de NFkB, o que por sua vez regula a expressão gênica que promove inflamação (HUMPHRIES et al., 2014).

A ativação de NFKB já se mostrou importante no controle da replicação de *Leishmania* (REINHARD et al., 2012; TAVARES et al., 2014). Na infecção de células dendríticas por *L. infantum,* o parasita é capaz de clivar a subunidade NFkB RelA, causando a desativação dessas células (NEVES et al., 2010). A infecção de macrófagos por *L. mexicana* revelou que esse parasita tem capacidade de clivar subunidade RelA e gerar uma subunidade p35 inativa, o que bloqueia o efeito do IFN-γ indução de óxido nítrico (NO) (NEVES et al., 2010). No contexto da infecção de macrófagos por *L. major* o parasita é capaz de inibir a ativação de RelA e ERK e induzir a formação do complexo p50-c-Rel o que participa na regulação de citocinas inflamatórias, especialmente na redução de TNF (GUIZANI-TABBANE et al., 2004; RICARDO-CARTER et al., 2013). Macrófagos

infectados por *L. amazonensis* ativam a formação do complexo repressor (p50/p50) que leva a inativação de NFkB e expressão de iNOS o que reduz a produção de NO e favorece o crescimento do parasita (CALEGARI-SILVA et al., 2009, 2015). Dessa forma, nossos dados podem indicar que no contexto da infecção de macrófagos por *L. chagasi* a RIPK1 promove a resistência a infecção por um mecanismos dependente de NFkB e independente de necroptose. De fato, experimentos adicionais são necessários para definir quais as vias relacionadas a NFkB e RIPK1 que podem estar implicadas no controle da infecção por *L. chagasi*.

Como o efeito de redução da carga parasitária por RIP quinases parece ser independente do efeito necroptótico do heme testamos o efeito da RIP quinase no controle da replicação de outras espécies de *Leishmania* que são agentes etiológicas de forma clínicas da doença não associadas com manifestações hematológicas como a LV. Uma vez que estávamos interessados nos efeitos de RIPK1, utilizamos macrófagos com o domínio quinase de RIP1 inativo, o que nos permitiu investigar o papel de RIPK1 sem os efeitos pleiotrópicos da inibição farmacológica (BERGER; BERTIN; GOUGH, 2015; BERGER et al., 2014). Nossos achados confirmaram que a RIPK1 participa do controle na infecção por *Leishmania*, uma vez que a infecção com *L. major*, *L. amazonensis* e *L. chagasi* revelaram que macrófagos que possuem o domínio quinase de RIP1 inativo são incapazes de controlar a infecção. Esse achado indica que no contexto de ausência ou RIP1 funções leishmanicidas do macrófago podem estar ausentes por conta da menor ativação deNFkB.

A geração de ROS parece ser essencial no desenvolvimento do processo de necroptose. Nesse contexto a proteína mitocondrial PGAM5, participa da mitofagia, fissão mitocodrial e geração de ROS após a formação do complexo RIP1-RIP3-MLKL (WANG et al., 2012). Nossos dados indicaram que a proteína PGAM5 desempenha um importante papel no controle da infecção por *Leishmania*. Estudos posteriores desafiaram o papel de PGAM5 como proteína essencial no processo de necroptose, uma vez que o silenciamento dessa proteína com RNA de interferência não impede a desenvolvimento da necroptose em diversos tipos celulares (MARSHALL; BAINES, 2014; REMIJSEN et al., 2014). Uma vez que a produção de IL-1β é um importante mecanismo leishmanicida de macrófagos (ZAMBONI; LIMA-JUNIOR, 2015) e a proteína PGAM5 é essencial para a ativação do inflamassoma NLRP3 com concomitante secreção de IL-1β em células dendríticas (WANG et al., 2012) e em macrófagos (MORIWAKI et al., dados não publicados); sugerimos que em nosso modelo PGAM5 pode participar do controle da replicação do parasita por um mecanismo independente da necroptose e dependente da produção de IL-1β.

IL-1 é uma citocina pró-inflamatória, considerada como "citocina de alarme" que é secretada por macrófagos. A família da citocina IL-1 inicia e propaga a inflamação por induzir a expressão de moléculas de adesão em células endoteliais e leucócitos (STUTZ; GOLENBOCK; LATZ, 2009; VORONOV et al., 2010). IL-1 tem sido relatada como uma citocina que afeta a patogênese da Leishmaniose por gerar uma resposta inflamatória e por modular a resposta adaptativa que age para conter a disseminação do parasita (KOSTKA et al., 2006; VON STEBUT et al., 2003). Voronov e cols., 2010 demonstraram que animais deficientes no gene da IL-1β ou no receptor de IL-1β são mais susceptíveis à infecção por *L. major* do que os camundongos selvagens (VORONOV et al., 2010). De fato, utilizando um modelo experimental de leishamaniose cutânea encontramos que RIPK1 e PGAM5 têm um papel relevante no controle da lesão de camundongos infectados por *L.amazonensis*. Esse papel parece ser dependente da indução inicial (4 semanas após a infecção) da produção de IL-1β na lesão, já que o estágio tardio (10 semanas após a infecção) da lesão não revelou diferença alguma nos níveis de IL-1β na lesão e no baço cultivado *ex vivo*.

Nossos dados corroboram os dados de Lima-Junior e cols. (2012) que demonstraram a relevância da produção inicial de IL-1β para o sucesso do controle da infecção por *L. amazonensis* (LIMA-JUNIOR et al., 2013), da mesma forma Gurung e cols. (2015) demonstraram que na infecção por *L. major* IL-1β é produzida inicialmente na lesão e essa produção é reduzida em camundongos caspase-1, NLRP3 e ASC *knockout*. (GURUNG et al., 2015). Muito embora a produção *in vivo* e *ex vivo* de IL-1β tenha sido reduzida em ambos camundongos PGAM5 e RIP1 quinase inativo, nosso dados de estimulação de macrófagos *in vitro* também indicam que a RIPK1 não é requerida para a ativação do inflamassoma NLRP3, o que nos indica que PGAM5 e RIPK1 podem controlar a infecção por *Leishmania* através de diferentes mecanismos além do inflamassoma. Novamente, o papel de RIPK1 na ativação de NFkB pode revelar um papel importante dessa quinase no controle da infecção por *Leishmania* através da ativação diferencial ou inativação da resposta inflamatória de NFkB.

A investigação de possíveis mecanismos leishmanicidas efetores do macrófago no nosso modelo experimental revelou que a produção de NO está reduzida em macrófagos *Pgam5*$^{-/-}$ infectados com diferentes espécies de *Leishmania*, mas não em RIP1 quinase inativo. Esse resultado reforça a tese de que RIPK1 e PGAM5 controlam a replicação de *Leishmania* por diferentes mecanismos. De fato, Lima-Junior e cols. (2013) demonstraram que no contexto da infecção por *L. amazonensis* IL-1β confere proteção através da

produção de NO.

Dessa forma, nossos dados demonstram que moléculas da via da necroptose estão envolvidas no controle da infecção por *Leishmania*, sem necessariamente ativação da morte celular necroptótica. Experimentos e investigações adicionais são necessários para elucidar os mecanismos leishmanicidas deflagrados por essa via e como esses podem ser explorados como novos alvos para a intervenção terapêutica no tratamento da Leishmaniose.

6. PRINCIPAIS ACHADOS DA TESE

1. A infecção por *L. chagasi* é capaz de subverter mecanismos leishmanicidas do macrófago por aumentar a expressão dos níveis de HO-1;
2. A proteína HO-1 do hospedeiro participa na sobrevivência e replicação da *L.chagasi*;
3. A indução farmacológica da HO-1 pelo CoPP favorece a replicação do parasito caracterizada pela redução nos níveis de importantes mediadores leishmanicidas;
4. Macrófagos tratados com LPS em presença de CoPP induzem um ambiente anti-inflamatório marcado pelo aumento da razão IL-10 em relação às quantidades de TNF;
5. A HO-1 favorece o crescimento de *L. chagasi* por favorecer a expressão de genes que reduzem o estresse oxidativo, assim como a SOD-1;
6. A HO-1 está associada com a LV humana e serve como marcador do estágio ativo da doença e de associação com citocinas inflamatórias;
7. O promove necroptose em células humanas;
8. As moléculas RIPK1 e PGAM5 são requeridas para o controle da infecção por *Leishmania*;
9. A produção ineficiente de IL-1β em camundongos RIP quinase inativo e *Pgam5*$^{-/-}$ participa do controle na infecção por *L.amazonensis*.

7. CONCLUSÃO GERAL

Com base nos resultados aqui apresentados podemos concluir que a enzima HO-1 está associada à patogênese da LV humana e aos mecanismos de interação da célula hospedeira favorecendo a persistência do parasito e reduzindo mecanismos leishmanicidas do macrófago, enquanto que moléculas da via de morte por necroptose contribuem para a resistência à infecção por *Leishmania*.

8. REFERÊNCIAS

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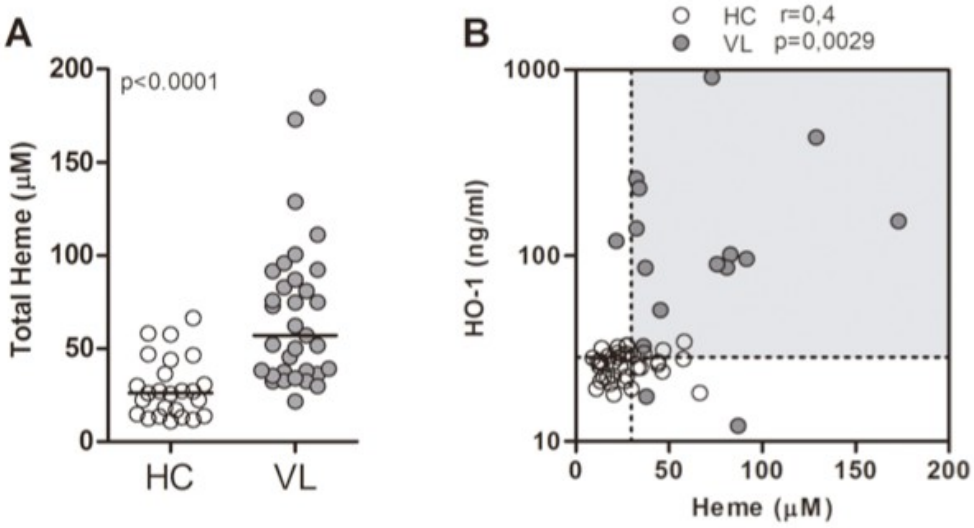
9. ANEXO 1
ASSOCIAÇÃO ENTRE HEME E HO-1 NA LV HUMANA



Heme is strongly associated with HO-1in human visceral leishmaniasis. Plasma level of heme was compared in healthy endemic controls and in patients with VL from a reference hospital (A). Each point on the graph represents a donor. Mann–Whitney test was used to verify differences among the groups. Correlations between plasma levels of heme and HO-1 (B) was assessed using the Spearman test.

10. ANEXOS
Artigos produzidos em colaboração durante o período do doutorado

10.1 Hepatitis B Infection Is Associated with Asymptomatic Malaria in the Brazilian Amazon

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PLoS one

Hepatitis B Infection Is Associated with Asymptomatic Malaria in the Brazilian Amazon

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Abstract

Background: Areas that are endemic for malaria are also highly endemic for hepatitis B virus (HBV) infection. Nevertheless, it is unknown whether HBV infection modifies the clinical presentation of malaria. This study aimed to address this question.

Methodology and Findings: An observational study of 636 individuals was performed in Rondônia, western Amazon, Brazil between 2006 and 2007. Active and passive case detections identified *Plasmodium* infection by field microscopy and nested Polymerase Chain Reaction (PCR). HBV infections were identified by serology and confirmed by real-time PCR. Epidemiological information and plasma cytokine profiles were studied. The data were analyzed using adjusted multinomial logistic regression. *Plasmodium*-infected individuals with active HBV infection were more likely to be asymptomatic (OR: 120.13, $P<0.0001$), present with lower levels of parasitemia and demonstrate a decreased inflammatory cytokine profile. Nevertheless, co-infected individuals presented higher HBV viremia. *Plasmodium* parasitemia inversely correlated with plasma HBV DNA levels ($r=-0.6$; $P=0.0003$).

Conclusion: HBV infection diminishes the intensity of malaria infection in individuals from this endemic area. This effect seems related to cytokine balance and control of inflammatory responses. These findings add important insights to the understanding of the factors affecting the clinical outcomes of malaria in endemic regions.

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Introduction

Malaria continues to be a major health threat worldwide. Most regions highly endemic for malaria are also endemic for other infectious diseases, which may affect the malaria infection [1]. In this context, hepatitis B virus (HBV) infections are common in many of the malaria endemic areas. HBV induces a robust pro-inflammatory Type 1 immune response (Th1), which is important for *Plasmodium* clearance, but is also implicated in disease severity [2]. Whilst intriguing, little is known of the effects of HBV on the clinical presentation of malaria. Intrahepatic HBV replication is inhibited by *P. yoelii* infection in mice [3], and there is enhanced interferon (IFN)-γ and IFN-α/β production in the liver. In humans, results from a small investigation suggest that acute falciparum malaria modulates HBV viremia in patients with chronic HBV infection [4]. Moreover, a study performed in a Vietnamese hospital showed that patients with cerebral malaria had a slightly greater risk of registering positive serology for the HBV surface antigen (HBSAg) [5]; however, this study did not show a significant association between the overall risk of death caused by severe falciparum malaria and positivity for HBSAg [5]. There is no clear evidence that the clinical status of underlying hepatitis B-related liver disease is affected during malaria infection. In addition, the impact of HBV infection on malaria symptoms has not been adequately addressed. Here, we report a study aimed at comparing co-infected individuals to individuals with single infections of HBV or *P. falciparum* and/or *P. vivax* to evaluate how HBV infection influences the malaria burden in a region from the Brazilian Amazon.

Methods

Ethics statement

Written informed consent was obtained from all participants or their legally responsible guardians, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The project was approved by the institutional review board

10.2 *Lutzomyia longipalpis* saliva drives apoptosis and enhances parasite burden in neutrophils

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JLB

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: 000–000; 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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10.3 Association between the Haptoglobin and Heme Oxygenase 1 Genetic Profiles and Soluble CD163 in Susceptibility to and Severity of Human Malaria

IAI

Association between the Haptoglobin and Heme Oxygenase 1 Genetic Profiles and Soluble CD163 in Susceptibility to and Severity of Human Malaria

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Intravascular hemolysis is a hallmark event in the immunopathology of malaria that results in increased systemic concentrations of free hemoglobin (Hb). The oxidation of Hb by free radicals causes the release of heme, which amplifies inflammation. To circumvent the detrimental effects of free heme, hosts have developed several homeostatic mechanisms, including the enzyme haptoglobin (Hp), which scavenges cell-free Hb, the monocyte receptor CD163, which binds to Hb-Hp complexes, and heme oxygenase-1 (HO-1), which degrades intracellular free heme. We tested the association between these three main components of the host response to hemolysis and susceptibility to malaria in a Brazilian population. The genetic profiles of the *HMOX1* and *Hp* genes and the plasma levels of a serum inflammatory marker, the soluble form of the CD163 receptor (sCD163), were studied in 264 subjects, including 78 individuals with symptomatic malaria, 106 individuals with asymptomatic malaria, and 80 uninfected individuals. We found that long (GT)*n* repeats in the microsatellite polymorphism region of the *HMOX1* gene, the *Hp2* allele, and the *Hp2.2* genotype were associated with symptomatic malaria. Moreover, increased plasma concentrations of heme, Hp, HO-1, and sCD163 were associated with susceptibility to malaria. The validation of these results could support the development of targeted therapies and aid in reducing the severity of malaria.

Malaria infection has high morbidity and mortality rates worldwide. During the blood stage of malarial infection, hemoglobin (Hb) is released from red blood cells that have ruptured due to *Plasmodium* multiplication (27). This unique characteristic of the *Plasmodium* life cycle leads to increased concentrations of cell-free Hb in the circulation because of intravascular hemolysis and the possible release of the heme prosthetic group from hemoglobin (25). Free heme is highly harmful to cells and tissues, as it can induce oxidative stress, cytotoxicity and inflammation (25), and cell death (30). Patients with severe malaria may exhibit high circulating levels of free heme, which impairs regulatory responses and can cause inflammatory imbalances (1). Under homeostatic conditions, haptoglobin (Hp) can rapidly scavenge cell-free Hb by forming the stable Hb-Hp complex, which is recognized and internalized by the CD163 receptor expressed by monocytes and macrophages in the red pulp of the spleen. Once internalized, the heme is usually degraded by the antioxidant enzyme heme oxygenase-1 (HO-1) (39). A thorough understanding of the factors and pathways that control the accumulation of free heme and the determinants of the unfavorable events that are triggered by this molecule can drive the development of novel therapeutic approaches to treat malaria and other hemolytic diseases.

Haptoglobin is a tetrameric protein ($\alpha 2\beta 2$) that is characterized by α-chain heterogeneity due to an intragenic duplication that resulted in two different alleles, *Hp2* and *Hp1* (including two subvariants, *Hp1F* and *Hp1S*). The diversity in the *Hp* phenotypes causes different binding affinities for cell-free Hb (*Hp1.1* > *Hp1.2* > *Hp2.2*) and CD163 (*Hp2.2* > *Hp1.2* > *Hp1.1*) (39). Additionally, polymorphisms in the *Hp* gene have been associated with different functional capabilities and organic responses, including alterations in immune regulation, oxidative stress, and iron delocalization within monocytes (8, 9, 31, 42–44, 54). Thus, it is necessary to consider the strategies used to study the mechanisms associated with heme regulation by HO-1, Hp, and the Hp receptor, CD163, and their contribution to the susceptibility to malaria.

The haptoglobin receptor CD163 is a member of a group of B cysteine-rich scavenger membrane receptors that is expressed on monocytes and macrophages and has been linked to inflammation. The soluble form of the CD163 receptor (sCD163) is a surrogate for its cellular expression, and sCD163 levels are elevated in many inflammatory processes (18, 29, 33, 45, 46, 50–52, 60, 66). Only one study has shown that sCD163 levels are more elevated in uncomplicated falciparum malaria than in severe malarial anemia and cerebral malaria, and all malaria patients have higher levels of sCD163 than uninfected individuals (41).

In experimental models of malaria, the induction of HO-1 is mostly associated with increased tolerance to *Plasmodium* infection (26, 53) as a result of the ability of HO-1 to control nonspe-

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10.4 Metabolic Adaptation to Tissue Iron Overload Confers Tolerance to Malaria

Cell Host & Microbe
Article

Metabolic Adaptation to Tissue Iron Overload Confers Tolerance to Malaria

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SUMMARY

Disease tolerance is a defense strategy that limits the fitness costs of infection irrespectively of pathogen burden. While restricting iron (Fe) availability to pathogens is perceived as a host defense strategy, the resulting tissue Fe overload can be cytotoxic and promote tissue damage to exacerbate disease severity. Examining this interplay during malaria, the disease caused by *Plasmodium* infection, we find that expression of the Fe sequestering protein ferritin H chain (FtH) in mice, and ferritin in humans, is associated with reduced tissue damage irrespectively of pathogen burden. FtH protection relies on its ferroxidase activity, which prevents labile Fe from sustaining proapoptotic c-Jun N-terminal kinase (JNK) activation. FtH expression is inhibited by JNK activation, promoting tissue Fe overload, tissue damage, and malaria severity. Mimicking FtH's antioxidant effect or inhibiting JNK activation pharmacologically confers therapeutic tolerance to malaria in mice. Thus, FtH provides metabolic adaptation to tissue Fe overload, conferring tolerance to malaria.

INTRODUCTION

There are two evolutionarily conserved defense strategies against infection that limit host disease severity. One relies on the capacity of the host's innate and adaptive immune system to reduce pathogen burden, i.e., resistance to infection. The salutary effects of this defense strategy are illustrated by the protective effect of vaccination against a wide range of infectious diseases. There is, however, another host defense strategy that limits disease severity irrespectively of pathogen burden, i.e., disease tolerance (Ayres and Schneider, 2012; Medzhitov et al., 2012; Schneider and Ayres, 2008). Revealed originally in plants and thereafter in flies, disease tolerance also operates in mammals, as demonstrated for *Plasmodium* (Råberg et al., 2007; Seixas et al., 2009) and polymicrobial (Larsen et al., 2010) infection in mice.

Once infected, mammals restrict Fe availability to pathogens, reducing Fe acquisition via gut epithelial cells while inhibiting cellular Fe export and sequestering intracellular Fe within different tissues (Ganz, 2009; Weiss, 2002). A major "trade-off" of this host defense strategy is tissue Fe overload, which can act in a pro-oxidant and cytotoxic manner to promote tissue damage and exacerbate disease severity. Presumably, this pathologic outcome is countered by a series of mechanisms providing host metabolic adaptation to tissue Fe overload. We hypothesized that these mechanisms involve the expression of ferritin H chain (*Fth*), a stress-responsive gene (Torti et al., 1988) that regulates Fe metabolism (Harrison and Arosio, 1996; Hentze and Kühn, 1996; Vanoaica et al., 2010).

FtH is an evolutionarily conserved Fe sequestering protein that acts in a cytoprotective manner (Balla et al., 1992; Berberat et al., 2003; Cozzi et al., 2003; Pham et al., 2004). This cytoprotective effect relies on the ferroxidase activity of FtH, which converts Fe(II) into Fe(III), hence limiting the participation of Fe(II) in the production of free radicals via the Fenton chemistry (Pham et al., 2004). The antioxidant effect of FtH inhibits c-Jun N-terminal kinase (JNK) activation in vitro through a mechanism targeting redox-sensitive phosphates and conferring cytoprotection (Chang et al., 2006; Kamata et al., 2005; Pham et al., 2004). We now demonstrate that inhibition of JNK activation by FtH confers tolerance to malaria, the disease caused by *Plasmodium* infection.

RESULTS

FtH Confers Tolerance to Malaria in Mice

Plasmodium chabaudi chabaudi (*Pcc*) infection, i.e., administration of *Pcc*-infected red blood cells (RBCs), was associated with the induction of FtH expression in the liver of C57BL/6 mice (Figure 1A), namely in hepatocytes (Figures 1A and S1A). FtH protein expression (Figure S1B) was associated with concomitant, albeit less pronounced, *Fth* mRNA expression (Figure S1C). Ferritin L chain (FtL) protein (Figure S1B) and mRNA (data not shown) expression were not induced in the liver of *Pcc*-infected

10.5 PLGA nanoparticles loaded with KMP-11 stimulate innate immunity and induce the killing of *Leishmania*

POTENTIAL CLINICAL RELEVANCE

ELSEVIER

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Research Article

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PLGA nanoparticles loaded with KMP-11 stimulate innate immunity and induce the killing of *Leishmania*

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Abstract

We recently demonstrated that immunization with polyester poly(lactide-co-glycolide acid) (PLGA) nanoparticles loaded with the 11-kDa *Leishmania* vaccine candidate kinetoplastid membrane protein 11 (KMP-11) significantly reduced parasite load in vivo. Presently, we explored the ability of the recombinant PLGA nanoparticles to stimulate innate responses in macrophages and the outcome of infection with *Leishmania braziliensis* in vitro. Incubation of macrophages with KMP-11-loaded PLGA nanoparticles significantly decreased parasite load. In parallel, we observed the augmented production of nitric oxide, superoxide, TNF-α and IL-6. An increased release of CCL2/MCP-1 and CXCL1/KC was also observed, resulting in macrophage and neutrophil recruitment in vitro. Lastly, the incubation of macrophages with KMP-11-loaded PLGA nanoparticles triggered the activation of caspase-1 and the secretion of IL-1β and IL-18, suggesting inflammasome participation. Inhibition of caspase-1 significantly increased the parasite load. We conclude that KMP-11-loaded PLGA nanoparticles promote the killing of intracellular *Leishmania* parasites through the induction of potent innate responses.

From the Clinical Editor: In this novel study, KMP-11-loaded PLGA nanoparticles are demonstrated to promote the killing of intracellular *Leishmania* parasites through enhanced innate immune responses by multiple mechanisms. Future clinical applications would have a major effect on our efforts to address parasitic infections.

Key words: *Leishmania*; PLGA; Nanoparticle; Macrophage; Innate response

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Conflict of interest statement if applicable: The authors declare that they have no conflicts of interest.

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10.6 Prostaglandin E2/leukotriene B4 balance induced by *Lutzomyia longipalpis* saliva favors *Leishmania infantum* infection.

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Parasites & Vectors

RESEARCH Open Access

Prostaglandin E_2/Leukotriene B_4 balance induced by *Lutzomyia longipalpis* saliva favors *Leishmania infantum* infection

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Abstract

Background: Eicosanoids and sand fly saliva have a critical role in the *Leishmania* infection. Here, we evaluated the effect of *Lutzomyia longipalpis* salivary gland sonicate (SGS) on neutrophil and monocyte recruitment and activation of eicosanoid production in a murine model of inflammation.

Methods: C57BL/6 mice were inoculated intraperitonealy with *Lutzomyia longipalpis* SGS or *Leishmania infantum* or both, followed by analyses of cell recruitment, parasite load and eicosanoid production.

Results: Intraperitoneal injection of *Lutzomyia longipalpis* SGS together with *Leishmania infantum* induced an early increased parasite viability in monocytes and neutrophils. *L. longipalpis* SGS increased prostaglandin E_2 (PGE_2), but reduced leukotriene B_4 (LTB_4) production *ex vivo* in peritoneal leukocytes. In addition, the pharmacological inhibition of cyclooxygenase 2 (COX-2) with NS-398 decreased parasite viability inside macrophages during *Leishmania* infection in the presence of *L. longipalpis* SGS arguing that PGE_2 production is associated with diminished parasite killing.

Conclusions: These findings indicate that *L. longipalpis* SGS is a critical factor driving immune evasion of *Leishmania* through modulation of PGE_2/LTB_4 axis, which may represent an important mechanism on establishment of the infection.

Keywords: *Lutzomyia longipalpis*, *Leishmania infantum*, Saliva, Prostaglandina E_2, Leukotriene B_4

Background

Leishmania infantum in America is transmitted by the bite of infected *Lutzomyia longipalpis* sand flies. Transmission of *Leishmania* sp. by hematophagous sand fly vectors occurs during blood feeding, when salivary content is inoculated with regurgitated *Leishmania* into host skin. Sand fly saliva enhances *Leishmania* infection on several experimental models [1-3] through its modulatory effects on the host immune system [4,5]. A successful blood feeding depends on the formation of a blood hemorrhagic pool [6]. In such a microenvironment there are many inflammatory cells [4], and *L. longipalpis* saliva has been shown to enhance recruitment of different cells, including monocytes and neutrophils [7-10].

Eicosanoids display an important role during *Leishmania* infection [11-16]. In this context, there are results showing that Prostaglandin E_2 (PGE_2) production benefits parasite survival [15-18] while Leukotriene B_4 (LTB_4) is related with parasite killing by host cells [12,14,19]. In addition, sand fly saliva seems to modulate the eicosanoid production by host cells in a polarized way towards PGE_2 [10,11,15,20]. Maxadilan, a vasodilatory peptide present in *L. longipalpis* salivary glands, is shown to increase production of PGE_2 by macrophages [21]. *L. longipalpis* salivary gland sonicate (SGS) is able to modulate PGE_2 and LTB_4 release in monocytes and neutrophils recruited to the peritoneal cavity [20]. In neutrophils, SGS increases *L. infantum* infection-driven production of PGE_2 *in vitro* [15]. However, it remains to be addressed whether sand fly saliva

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10.7 Arginase I, polyamine, and prostaglandin E2 pathways suppress the inflammatory response and contribute to diffuse cutaneous leishmaniasis.

MAJOR ARTICLE

Arginase I, Polyamine, and Prostaglandin E_2 Pathways Suppress the Inflammatory Response and Contribute to Diffuse Cutaneous Leishmaniasis

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Diffuse cutaneous leishmaniasis (DCL) is a rare clinical manifestation of tegumentary leishmaniasis. The molecular mechanisms underlying DCL pathogenesis remain unclear, and there is no efficient treatment available. This study investigated the systemic and in situ expression of the inflammatory response that might contribute to suppression in DCL. The plasma levels of arginase I, ornithine decarboxylase (ODC), transforming growth factor β (TGF-β), and prostaglandin E_2 (PGE_2) were higher in patients with DCL, compared with patients with localized cutaneous leishmaniasis (LCL) or with controls from an area of endemicity. In situ transcriptomic analyses reinforced the association between arginase I expression and enzymes involved in prostaglandin and polyamine synthesis. Immunohistochemistry confirmed that arginase I, ODC, and cyclooxygenase2 expression was higher in lesion biopsy specimens from patients with DCL than in those from patients with LCL. Inhibition of arginase I or ODC abrogates *L. amazonensis* replication in infected human macrophages. Our data implicate arginase I, ODC, PGE_2, and TGF-β in the failure to mount an efficient immune response and suggest perspectives in the development of new strategies for therapeutic intervention for patients with DCL.

Keywords. *Leishmania amazonensis*; diffuse cutaneous leishmaniasis; arginase I; ornithine decarboxylase; prostaglandin E_2; TGF-β.

Cutaneous leishmaniasis exhibits a wide spectrum of clinical manifestations varying from self-healing localized cutaneous leishmaniasis (LCL) with a moderate cell-mediated immune response to diffuse cutaneous leishmaniasis (DCL) [1]. DCL is distinct from disseminated cutaneous leishmaniasis [2] and is characterized by the presence of several nonulcerated nodular skin lesions, the predominance of highly parasitized macrophages in the lesions, an absent or modest in vitro antileishmanial antigen cellular immune response, a negative delayed-type hypersensitivity (DTH) response, and resistance to antiparasite therapy [3]. The molecular mechanisms underlying DCL pathogenesis remain unclear, and there is no efficient treatment available.

In patients with DCL, antiinflammatory cytokines are abundant in lesions and in restimulated peripheral blood mononuclear cells (PBMCs), whereas proinflammatory cytokines and chemokines are absent or present at low levels [1]. However, the mechanisms responsible for this imbalance are not yet understood.

The arginase I pathway is emerging as a critical mechanism of immune regulation in *Leishmania* infection [4]

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