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DISSERTAÇÃO DE MESTRADO

IMPORTÂNCIA DAS RESOLVINAS NA INFECÇÃO POR *LEISHMANIA AMAZONENSIS*

HAYNA MALTA SANTOS

Salvador - Bahia
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Orientadora: Dra. Valéria de Matos Borges
Co-orientadora: Dra. Jaqueline França Costa

Dissertação apresentada ao Curso de Pós-Graduação em Patologia Humana para a obtenção do grau de Mestre

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"IMPORTÂNCIA DAS RESOLVINAS NA INFECÇÃO POR LEISHMANIA AMAZONENSIS"

HAYNA MALTA SANTOS

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COMISSÃO EXAMINADORA

Dra. Cristiana Santos Macedo
Pesquisadora
Fiocruz/RJ

Dr. Edgar Marcelino Carvalho Filho
Pesquisador
IGM / Fiocruz

Dra. Valeria de Matos Borges
Pesquisadora Titular
IGM / Fiocruz

Dedico esse trabalho a
A Deus, dono de toda ciência, sabedoria e poder
Matheus, meu amado companheiro, amigo e parceiro
Minha família, minha base e porto seguro.

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RESUMO

INTRODUÇÃO: A Leishmaniose Cutânea Difusa (LCD) é uma manifestação clínica rara causada pela *Leishmania amazonensis* que é caracterizada por uma resposta celular parasitária ineficiente e macrófagos intensamente parasitados nas lesões cutâneas. Mediadores lipídicos e seus precursores desempenham um papel crucial durante a infecção por *Leishmania*. Estudos prévios demonstram que pacientes com leishmaniose tegumentar, exibem um distinto balanço de eicosanoides *in situ* e sistêmico. Recentemente, demonstrou-se que mediadores lipídicos especializados na pró-resolução desempenham um papel crítico na redução de processos inflamatórios patológicos induzindo a restauração da homeostasia em diferentes modelos experimentais. Entre esses mediadores, as resolvinas da série D exibem potente atividade anti-inflamatória e imuno-regulatória que inclui a inibição da quimiotaxia leucocitária e bloqueio na produção de citocinas pró-inflamatórias. No entanto, ainda é desconhecido se as resolvinas desempenham um papel significativo no estabelecimento e persistência da infecção por *Leishmania*. **OBJETIVO**: Nesse estudo, avaliamos os níveis circulantes de Resolvina D1 (RvD1) em pacientes com leishmaniose tegumentar apresentando a forma clínica cutânea localizada (LCL) ou difusa. **RESULTADOS:** Nossos resultados demonstram que pacientes com LCD apresentam maiores níveis plasmáticos de RvD1 quando comparados a LCL ou controles endêmicos. Além disso, os níveis séricos de RvD1 em pacientes com LCD se correlacionam positivamente com a Arginase I e TGF-β, enquanto que inversamente com os níveis sistêmicos de TNF-α. Experimentos adicionais *in vitro* utilizando macrófagos humanos revelaram que a RvD1 promove a replicação intracelular da *L. amazonensis* por um mecanismo associado a indução da enzima heme oxigenase-1. **CONCLUSÃO:** Os resultados sugerem que a via de produção da RvD1 pode servir como uma potencial estratégia terapêutica para os pacientes com LCD.

Palavras chave: Leishmaniose Tegumentar, *L. amazonensis*, Resolvina D1.

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ABSTRACT

INTRODUCTION: Diffuse Cutaneous Leishmaniasis (DCL) is a rare clinical manifestation caused by *Leishmania amazonensis* that is characterized by an inefficient parasite-specific cellular responses and heavily parasitized macrophages in skin lesions. Lipid mediators and their precursors play a crucial role during *Leishmania* infection. Previous works have shown that patients with cutaneous leishmaniasis exhibit a distinct *in situ* and systemic balance of this eicosanoids. Recently, pro-resolution lipid mediators have been shown to play critical role in dampening pathological inflammatory processes to reestablish homeostasis in a diverse range of experimental settings. Among these mediators, resolvins from D series have been described to exhibit potent anti-inflammatory and immune-regulatory activities that include inhibition of leukocyte chemotaxis and blockage on the production of proinflammatory cytokines. However, whether resolvins play significant roles in establishment and persistence of *Leishmania* infection is currently unknown. **AIM**: We addressed this question by assessing circulating levels of resolvin D1 (RvD1) in tegumentary leishmaniasis patients presenting localized cutaneous leishmaniasis (LCL) or diffuse disease. **RESULTS**: We found that DCL patients have higher plasma levels of RvD1 when compared with LCL patients or endemic controls. In addition, the levels of this mediator were positively correlated with arginase-I and TGF-β and were negatively correlated with TNF-α levels. Additional *in vitro* experiments using primary human macrophages revealed that resolvin D1 promotes the intracellular *L. amazonensis* replication for a mechanism dependent on induction of heme oxygenase-1 enzyme. **CONCLUSION**: These results indicate that targeting RvD1 could serve as potential strategy for DCL patients.

Keys word: Cutaneous leishmaniasis, *L. amazonensis,* Resolvin D1.

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LISTA DE ABREVIATURAS

DNA	Ácido Desoxirribonucléico
IDRM	Reação Intradérmica de Montenegro
IFN-γ	Interferon gama
IL	Interleucina
LCD	Leishmaniose Cutânea Difusa
LCL	Leishmaniose Cutânea Localizada
LCM	Leishmaniose Cutânea Mucosa
LT	Leishmaniose Tegumentar
LV	Leishmaniose Visceral
PBMC	Células mononucleares de sangue periférico
PGE	Prostaglandina E
LTB	Leucotrieno B
TGF- β	Fator transformante de crescimento β, do inglês *"Transforming growth factor β"*
Th	Célula T auxiliadora, do inglês "*T helper*"
TNF-α	Fator de necrose tumoral
COX	Ciclooxigenase
LO	Lipoxigenase
SPM	Mediadores especializados na pró-resolução, do inglês "Specialized pro-resolving mediators"
DHA	Ácido docosahexanóico
EPA	Ácido eicosapentanóico
RvD1	Resolvina D1
RvD2	Resolvina D2
iNOS	Óxido Nítrico Sintase induzida
NO	Óxido Nítrico
SOD	Super Óxido Desmutase
AA	Ácido araquidônico
PUFAs	Ácidos graxos poli-insaturados
LPG	Lipofosfoglicano

SUMÁRIO

1. INTRODUÇÃO

1.1 CICLO BIOLÓGICO DA *LEISHMANIA SP*.

As Leishmanioses são doenças causadas por parasitas protozoários intracelulares pertencentes à ordem Kinetoplastidae, família Trypanosomatidae e ao gênero *Leishmania*, que acometem ao homem e diferentes espécies de animais silvestres e domésticos (WHO, 2010). Esses parasitos caracterizam-se pela presença de um cinetoplasto, estrutura formada por ácido desoxirribonucleico (DNA) altamente compactado dentro da mitocôndria, estando relacionada ao fornecimento de energia para o batimento flagelar e consequente locomoção do parasito (MACNEILL, 2014).

Leishmanias são parasitos que apresentam um ciclo de vida digenético, ou seja, apresentam duas formas evolutivas: promastigotas flagelados presentes no trato digestivo do hospedeiro invertebrado e os amastigotas, estado não móvel encontrados em macrófagos de hospedeiros vertebrados (HENARD *et al.*, 2014).

A transmissão natural da doença ocorre quando as formas promastigotas são inoculadas no tecido subcutâneo, por fêmeas de insetos hematófagos da subfamília Phlebotominae (Díptera, Psychodidae), denominados genericamente de flebótomos. A infecção do inseto ocorre no momento da alimentação, quando ele suga o sangue contendo amastigotas ou macrófagos infectados. No trato digestivo do vetor, ocorre rompimento da membrana dos macrófagos e as amastigotas liberadas diferenciam-se na forma promastigota. Estas formas são afiladas, apresentam flagelo externalizado e são capazes de se multiplicar no intestino do vetor (GOSSAGE *et al.*, 2003).

Ainda no vetor, as formas promastigotas passam por um processo conhecido como metaciclogênese onde sofrem uma série de modificações bioquímicas em sua superfície, perdem a capacidade de adesão ao epitélio do intestino do vetor, tornando-se

móveis e altamente infectantes, chamadas de metacíclicas (AKROPYANTS *et al.*, 2004). As formas metacíclicas migram para a probóscide onde serão transmitidas para o hospedeiro vertebrado no momento da picada, durante o repasto sanguíneo do vetor (PETERSEN; GREENLEE, 2011). No local da picada, as formas metacíclicas são fagocitadas por células do sistema fagocítico mononuclear presentes na derme (Figura 1). Os parasitos internalizados ficam localizados dentro de vacúolos parasitóforos, também conhecidos como fagolisossomas. Essa organela é formada a partir da fusão do fagossoma, gerado durante a fagocitose do parasito, com os lisossomos e são ricas em hidrolases e ácidos (LODGE; DESCOTEAUX, 2005) (PESSOA *et al.*, 2016). Apesar do ambiente altamente inóspito que é o interior do vacúolo parasitóforo, as *Leishmanias* desenvolveram mecanismos que são capazes de escapar da atividade microbicida das células. Nesse sentido, as promastigotas conseguem sobreviver e se diferenciar em amastigotas (COURRET *et al.*, 2002), sendo essa a forma evolutiva responsável pelo estabelecimento da doença e disseminação da infecção pela sua replicação no interior da célula hospedeira (HENARD *et al.*, 2014). Após diversas multiplicações, os macrófagos ficam intensamente infectados e rompem-se, liberando as amastigotas que são rapidamente fagocitadas por novos macrófagos.

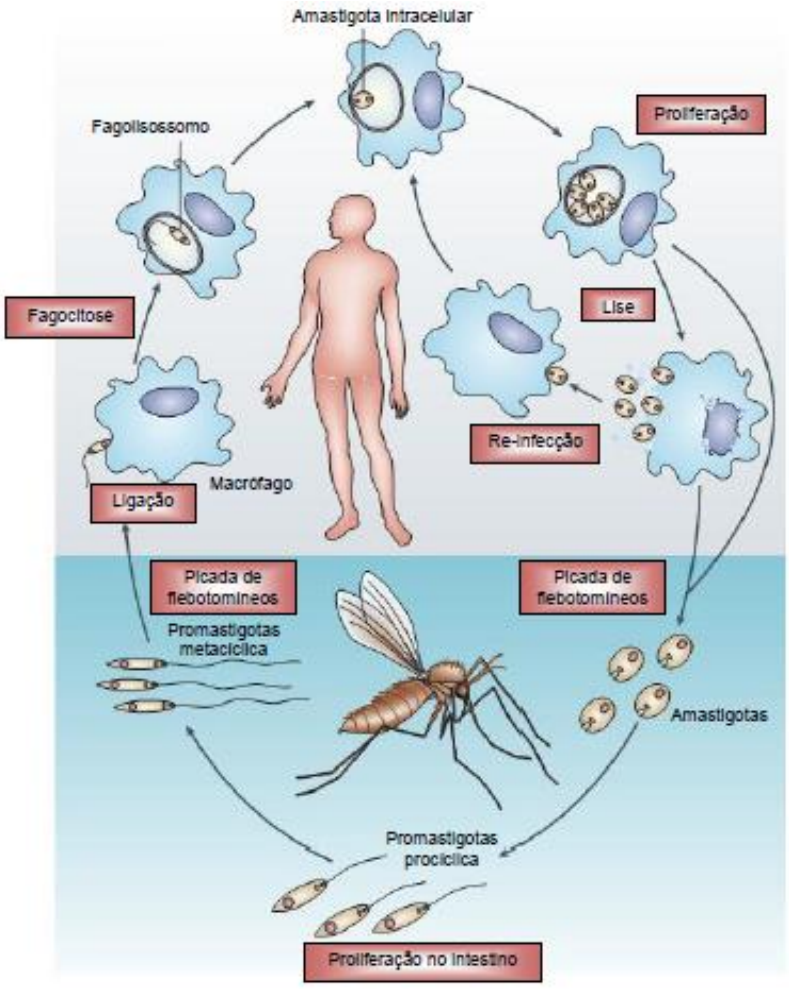


Figura 1. **Ciclo de vida da *Leishmania sp.*** (Modificado de CHAPPUIS *et al.*, 2007).

1.2 CARACTERÍSTICAS GERAIS E FORMAS CLÍNICAS DA LEISHMANIOSE

As leishmanioses são endêmicas em mais de 98 países, distribuídos pela África, Ásia, Europa e América Latina, estimando-se até 1,3 milhões de novos casos e de 20 a 30 mil mortes por ano (WHO, 2010). As leishmanioses têm causa multifatorial e representam um complexo de doenças com grande diversidade epidemiológica e espectros clínicos (ORYAN; AKBARI, 2016). Caracteriza-se pela diversidade da resposta no hospedeiro, agentes etiológicos e vetores, já que pode ser causada por mais de 20 espécies de *Leishmania* e transmitida por diferentes espécies de flebotomíneos vetores (ORYAN; AKBARI, 2016).

No país já foram identificadas sete espécies de *Leishmanias* responsáveis pela doença, sendo agrupadas em dois grandes subgêneros: *Leishmania* e *Viannia* (REITHINGER; DUJARDIN; LOUZIR, 2007). Além disso, as espécies também podem ser classificadas de acordo com a distribuição geográfica das áreas endêmicas como espécies do Velho Mundo (Europa e Ásia) e do Novo Mundo (Américas) (BRASIL, 2010). Vetores do gênero *Lutzomyia* estão associados à transmissão de espécies de Leishmania do Novo Mundo, enquanto que o gênero *Phlebotomus*, transmite espécies do Velho Mundo (GONZÁLEZ *et al.*, 2015).

As leishmanioses se apresentam sob diversas formas clínicas, podendo ser divididas em: Leishmaniose Tegumentar (LT) e Leishmaniose Visceral (LV) ou calazar (WHO, 2016). Segundo a Organização Mundial da Saúde a LV é uma infecção crônica que apresenta altas taxas de mortalidade sendo considerada a forma mais grave da doença. A LV caracteriza-se por afetar principalmente os órgãos internos como baço e fígado, além de estar associado à pancitopenia nos pacientes (WHO, 2010).

A LT, forma cutânea da doença, é a mais comum. Caracteriza-se por causar úlceras espalhadas pelo corpo que embora, na maioria dos casos curem espontaneamente, podem deixar cicatrizes graves e desfigurantes (WHO, 2010). A Leishmaniose Tegumentar pode se manifestar em quatro formas clínicas principais: Leishmaniose Cutânea Localizada (LCL), Leishmaniose Mucocutânea (LCM), Leishmaniose Disseminada (LD) e a Leishmaniose Cutânea Difusa (LCD).

1.3 DADOS EPIDEMIOLÓGICOS E CLÍNICOS DA LEISHMANIOSE TEGUMENTAR

A LT é uma antropozoonose considerada uma das doenças mais negligenciadas do mundo e constitui-se um grave problema de saúde pública, atingindo 88 países, sendo que 75% dos casos se concentram apenas em 10: Afeganistão, Argélia, Colômbia, Brasil, Irã, Etiópia, Peru, Costa Rica, Arábia Saudita e Síria (ALVAR *et al.*, 2012).

A LT apresenta ampla distribuição mundial, sendo notificados casos em países do Velho e Novo Mundo (WHO, 2010). Na Europa, a maioria dos casos notificados se concentra na Espanha e Portugal, enquanto que no Novo Mundo, os casos relatados se espalham desde o extremo dos Estados Unidos até a Argentina, na América do Sul (BRASIL, 2010) (ALVAR *et al.*, 2012) (Figura 2). No Brasil, a distribuição da LT é alta, com registro de casos em todas as regiões brasileiras. Em 2004, foram registrados 28.569 casos, distribuídos em 1.926 municípios brasileiros (BRASIL, 2010).

O crescente número de casos na última década vem sendo atribuído ao melhor diagnóstico e com o aumento de notificações. Além disso, acredita-se que esse cenário pode ser resultado de uma série de fatores de risco como o aumento do desmatamento, urbanização, controle inadequado do vetor (ORYAN; AKBARI, 2016).

A importância da LT reside não somente na sua alta distribuição geográfica e incidência, mas também na possibilidade de assumir manifestações clínicas distintas. Formas graves da doença podem apresentar lesões dermatológicas incapacitantes e desfigurantes, afetando o paciente psicologicamente, com reflexos no campo social e econômico, com graves repercussões na vida do indivíduo (GONTIJO; CARVALHO, 2003).

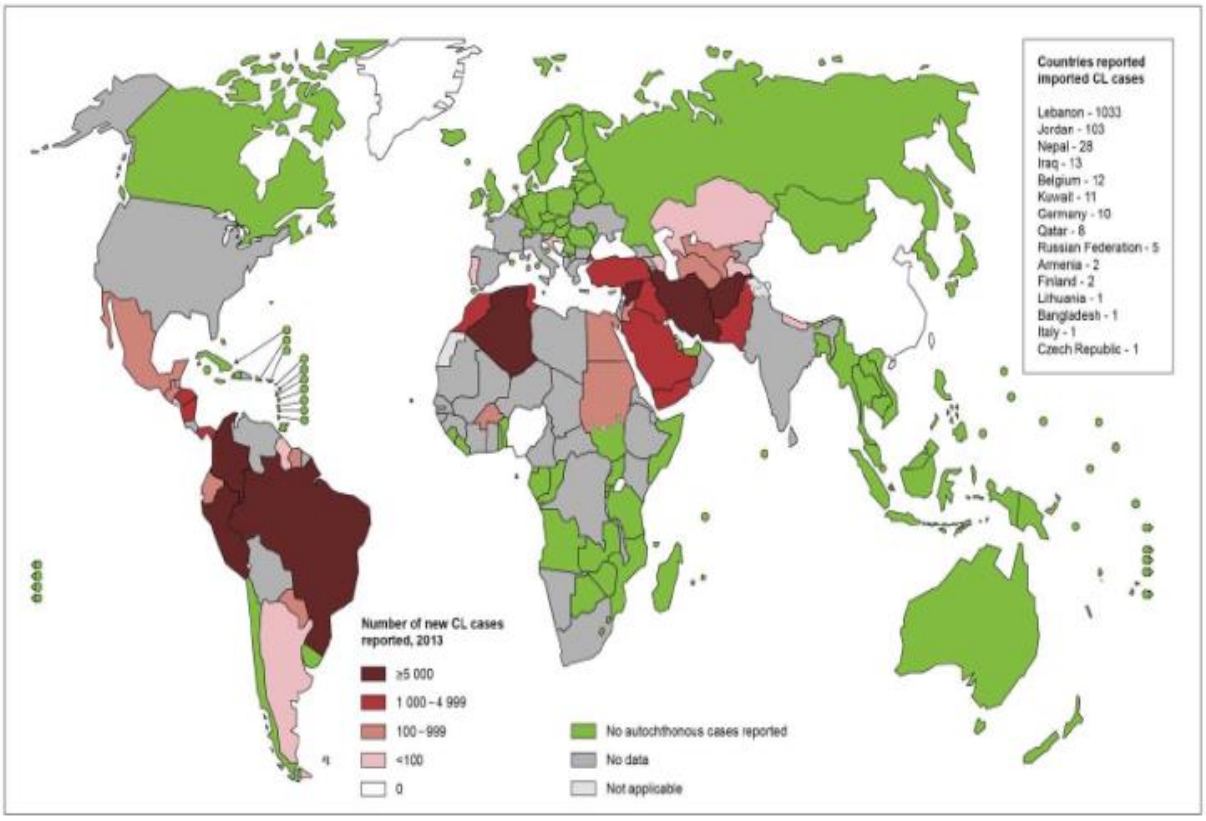


Figura 2. **Distribuição endêmica da Leishmaniose Tegumentar** (WHO, 2013)

No espectro intermediário de manifestações clínicas da LT, a forma mais frequente é a LCL que pode ser causada no Novo Mundo pelas espécies: *L. braziliensis*, *L. amazonensis* e *L. guyanensis*. As lesões de pacientes com LCL são cutâneas, bem definidas por bordas elevadas, suportadas por uma eficiente resposta de células T, que geralmente favorece uma boa resposta à terapia antimonial tradicional, tendendo à cicatrização (BRASIL, 2010). Os cortes histológicos revelam um infiltrado inflamatório tecidual misto com poucos parasitos na lesão estabelecida.

A LCM e LCD são as formas mais graves da doença encontrando-se em lados opostos no espectro imunológico (SCOTT; NOVAIS, 2016) (Figura 3). No polo hiperérgico, a LCM caracteriza-se pela exacerbada reação imune fortemente associada com elevada produção de IFN-ϒ e TNF-α que levam a necrose do tecido mucoso da nasofaringe e consequente escassez de parasitos na lesão (SILVEIRA, F. T. *et al.*,

2009). No polo anérgico, encontra-se a LCD, com grande quantidade de parasitas nas lesões, devido aos baixos níveis de citocinas Th1 e altos títulos de anticorpos circulantes. Além disso, pacientes com LCD são fortemente marcados pela elevada produção de IL-4 e IL-10, enquanto os níveis dessas citocinas são encontradas em baixas concentrações nos pacientes com LCM (SILVEIRA *et al.*, 2009) (SCOTT; NOVAIS, 2016).

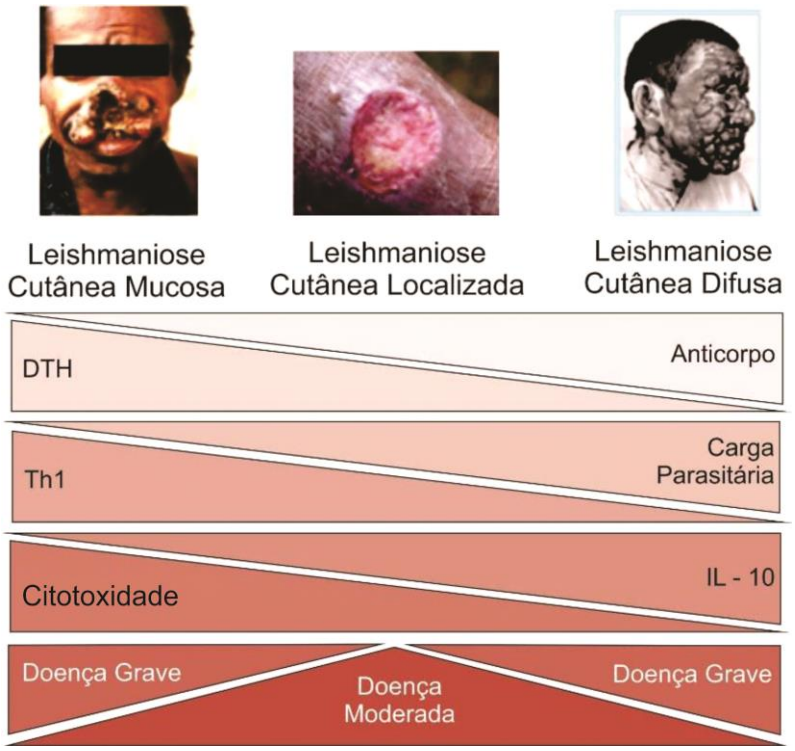


Figura 3. **Aspectos imunológicos observados na Leishmaniose Tegumentar.** (Adaptado de: Manual de Vigilância Tegumentar Americana, Ministério da Saúde, 2009; e SCOTT; NOVAIS, 2016).

1.4 LEISHMANIOSE CUTÂNEA DIFUSA

No Brasil a espécie *Leishmania amazonensis* é o agente etiológico responsável pela LCD, sendo a maioria dos casos relatados no Maranhão, Pará, Bahia e Mato Grosso (REIS *et al.*, 2008). A LCD representa uma forma rara da LT. Entretanto, por

causa das consequências estigmatizantes para o paciente, é reconhecida como um importante problema de saúde pública (DESJEUX, 2004).

Constitui uma forma clínica grave, caracterizando-se por um maciço comprometimento dérmico de natureza crônica que progride lentamente e pode persistir por décadas (PEARSON *et al.*, 1996). A maioria dos pacientes apresenta recidiva ao tratamento. Após vários esquemas terapêuticos anti-Leishmania observa-se melhoras clínicas caracterizadas por redução significativa de lesões que voltam a aumentar após a falha terapêutica (COSTA *et al.*, 1995).

A doença geralmente inicia-se com uma lesão primária e única que em seguida dissemina-se envolvendo outras partes do corpo (GREVELINK; LERNER, 1996). As lesões observadas na LCD evoluem de forma lenta com formação de eritemas, pápulas, tubérculos, nódulos ou infiltrações difusas de distribuição simétrica na face, tronco e nos membros, podendo disseminar-se para todo o corpo após meses ou anos da infecção (COSTA *et al.*, 2009). As lesões em geral não cicatrizam de forma espontânea e são classicamente resistentes ao tratamento medicamentoso (GONTIJO; CARVALHO, 2003). Além disso, seu aspecto nodular e com infiltrações cutâneas pronunciadas simulam quadro de hanseníase virchowiana (PEARSON *et al.*, 1996).

Histologicamente, as lesões ativas da LCD caracterizam-se por um denso infiltrado dérmico que é constituído principalmente por macrófagos vacuolados intensamente parasitados, obscurecendo estruturas da derme e da hipoderme (CONVIT *et al.*, 1972) (BARRAL *et al.*, 1995). Nas lesões que involuem espontaneamente ou por ação de tratamento quimioterápico, vêm-se grande infiltrado linfoplasmocitário e áreas de fibrose e necrose (BITTENCOURT, 2009). No entanto, as recidivas podem ser atribuídas a reinfecção ou alterações no estado imunológico do paciente (BARRAL *et al.*, 1995).

Do ponto de vista imunológico, o teste de Reação Intradérmica de Montenegro (IDRM) são negativos, uma vez que as culturas de células mononucleares do sangue periférico após serem estimuladas com antígeno de *Leishmania* não respondem aos testes de proliferação celular e produção de IFN-γ. No entanto, mesmo diante da ausência de repostas celulares específicas os níveis circulantes de anticorpos anti-*Leishmania* nesses pacientes é alta (BARRAL *et al.,* 1995). Essa ausência de resposta celular específica para antígenos de *Leishmania* caracteriza a LCD e está associada a grande proliferação do parasito bem como a disseminação da doença (BRASIL, 2010).

Alguns trabalhos têm mostrado que células do PBMC de pacientes com LCD apresentam um perfil de citocinas da resposta imune predominante do tipo Th2, com redução nos níveis de IFN-γ e TNF-α ao mesmo tempo em que apresenta altos níveis de IL-10 e IL-4 (BOMFIM *et al.*, 1996) (Figura 3). Além disso, o processo inflamatório é desorganizado, não sendo capaz de controlar a infeção.

O perfil de citocinas pode variar durante os diferentes estágios da doença. Em lesões cutâneas de pacientes com LCD quase não foi detectado a expressão de IFN-γ em contraste com elevada expressão de mRNA de IL-4 e IL-10 nas mesmas amostras (SILVEIRA *et al.,* 2004). O mesmo foi observado quando analisado a expressão de mRNA desses mediadores no PBMC de pacientes com LCD (BOMFIM *et al.*, 1996). Entretanto, observa-se que durante cura transitória pós-tratamento, ocorre uma alta expressão de IFN-γ e baixa expressão de IL-10 (BOMFIM *et al.*, 1996). Esse quadro foi revertido nos pacientes que apresentaram recidiva.

Outras citocinas também estão envolvidas no estado anérgico da LCD. O TNF-α, um importante potencializador do IFN-γ, encontra-se reduzido no soro de pacientes com LCD quando comparados com pacientes LCM (CASTES *et al.,* 1993). Estes dados demonstraram que a inabilidade em produzir IFN-γ pode estar associada à

imunossupressão observada na LCD e que a IL-10 pode ser importante nessa modulação negativa (Bonfim *et al*., 1996). Além disso, nosso grupo mostrou um balanço nos níveis plasmáticos entre marcadores anti-inflamatórios e pró-inflamatórios em pacientes com LCD. Enquanto que os níveis de IL-12, MCP-1, TNF-α e CXCL 10 foram reduzidos no plasma de LCD comparados com o plasma de LCL, observamos um aumento nos níveis circulantes de arginase-1 e TGF-β (FRANCA-COSTA *et al.*, 2015).

1.5 IMPORTÂNCIA DA VIA DE METABOLISMO DA *L*-ARGININA E ANTI-OXIDATIVO NA RESPOSTA DE MACRÓFAGOS A INFECÇÃO POR *LEISHMANIA*.

Sabe-se que as *Leishmanias* utilizam de vários mecanismos para impedir as respostas antimicrobianas do hospedeiro. Dentre os mecanismos de resposta à infecção mais importantes, encontram-se a indução de citocinas (SACKS e SHER, 2002), antioxidantes (LUZ *et al.*, 2012) (KHOURI *et al.*, 2009) e espécies reativas de oxigênio (ROS) e hidrogênio (RATNA; ARORA, 2016).

A infecção por Leishmania pode modular a secreção de citocinas alterando o estado de ativação macrofágico (WANDERLEY *et al.*, 2012). Nesse sentido, a ativação da via clássica caracteriza-se pelo processamento da *L*-arginina pela enzima Óxido Nítrico Sintase induzida (iNOS). A ativação dessa via é induzida por citocinas do tipo Th1 como o IFN-γ e o TNF-α, que oxidam a *L*-arginina em Óxido Nítrico (NO). O aumento da síntese da iNOS induz a maior produção de NO ativando mecanismos microbicidas da célula hospedeira. A via de ativação alternativa, por sua vez, é induzida por citocinas do perfil Th2 como o IL-4 e IL-10 (MORI; GOTOH, 2000). Essas

citocinas induzem a expressão da Arginase-1, que ao degradar a L-arginina leva à produção de poliaminas que são cruciais para o sucesso da infecção.

Nesse sentido, trabalhos *in vitro* vêm demonstrando que a ativação da via alternativa é importante para a sobrevivência de várias espécies de *Leishmania*. Formas amastigotas de *L. major* e *L. infantum* proliferam mais em macrófagos incubados com citocinas do tipo Th2, IL-4, IL-10 e TGF-β, que induzem o aumento da atividade da arginase 1 (INIESTA *et al*., 2001). Castellano e colaboradores demonstraram que a infecção por *Leishmania* é capaz de induzir a diferenciação de células T para uma resposta do tipo Th2 caracterizada pela persistência da infecção, mediada pela produção de IL-4 e IL-10 (CASTELLANO *et al.*, 2009). Além disso, Calegari-Silva e colaboradores demonstraram que a *L. amazonensis* é capaz de reduzir a regulação da iNOS, inibindo a produção do NO e favorecendo a manutenção da infecção (CALEGARI-SILVA *et al.*, 2015).

Trabalhos anteriores do grupo demonstram que a inibição da via da arginase é capaz de conter a carga parasitária em macrófagos humanos infectados por *L. amazonensis*. O tratamento com nor-NOHA, inibidor da arginase, resultou na alteração do perfil de citocinas produzidas, com redução dos níveis TGF-β e PGE_2 e aumento nos níveis de TNF-α e IL-12 no sobrenadante de culturas de macrófagos humanos infectados (FRANCA-COSTA *et al.*, 2015). Além disso, o trabalho mostrou que os níveis de arginase-1 correlacionava-se positivamente com o TGF-β e PGE_2 no plasma de pacientes com LCD, podendo estar contribuindo com a ineficiente resposta imune observada nesses pacientes (FRANCA-COSTA *et al.*, 2015).

A produção de ROS, incluindo peróxidos de hidrogênio e superóxido, pelos macrófagos é o principal mecanismo de defesa contra patógenos em células humanas (DULTRA; GOLLOB, 2008). Nesse contexto, Khouri e colaboradores demonstraram

que o aumento da Superóxido Dismutase (SOD), induzida pelo IFN-β, é capaz de aumentar a carga parasitaria de macrófagos humanos infectados devido à inibição de ROS (KHOURI *et al.*, 2009). Além disso, a atividade dessa enzima foi maior no plasma de pacientes com leishmaniose cutânea quando comparado com os controles endêmicos, sugerindo que os níveis plasmáticos da SOD podem refletir o estado clínico da doença, podendo ser utilizado como biomarcador (KHOURI *et al.*, 2014).

Outro importante mecanismo anti-oxidante com impacto na infecção por Leishmania é a indução da heme oxigenasse 1 (HO-1). Essa enzima também vem sendo associada à manutenção da infecção em diversas doenças infecciosas e seu efeito vem sendo associado ao balanço entre citocinas pró e anti-inflamatórios. Luz e colaboradores demonstraram que a infecção por *L. infantum* induz a expressão de HO-1 em macrófagos murinos e humano e essa indução também esta associada à indução da SOD-1 (LUZ *et al.*, 2012). A importância dessa enzima para a persistência da infecção foi evidenciada quando a infecção de macrófagos de camundongos $Hmox1^{-/-}$ foi capaz de reduzir a carga parasitária em relação aos camundongos *wild type*. Além disso, observou-se que a HO-1 foi capaz de modular a produção de citocinas pró-inflamatórias, reduzindo os níveis de TNF-α (LUZ *et al.*, 2012).

Além da importância das citocinas na ativação de macrófagos, seja ela a alternativa ou a clássica, vem sendo descrito que os mediadores lipídicos também são capazes de induzir essa ativação. Nesse contexto, destacam-se as prostaglandinas onde, a depender do modelo experimental, podem desempenhar papeis antagônicos durante a ativação macrofágica. Seta e colaboradores demonstraram que durante inflamações pulmonares o aumento na expressão de COX-2 e consequente aumento na produção de PGE_2 está altamente relacionado à exacerbação expressão da HO-1 (SETA *et al.*, 2011).

Por sua vez, durante a sepse a secreção de prostaglandinas é capaz de induzir a arginase-1, juntamente com as citocinas do tipo Th2 (POPOVIC *et al.*, 2007).

2 MEDIADORES LIPÍDICOS

2.1 MEDIADORES LIPÍDICOS NA RESPOSTA INFLAMATÓRIA: SÍNTESE E FUNÇÃO.

Mediadores lipídicos são moléculas orgânicas de curta duração que são liberadas durante a resposta inflamatória e estão fortemente associadas com uma série de processos fisiológicos e patológicos incluindo câncer, cicatrização de lesões e doenças inflamatórias e infecciosas (TUNCER; BENERJEE, 2015). Os eicosanoides, mediadores lipídicos mais estudados, são moléculas sinalizadoras derivadas da oxidação do ácido araquidônico (AA), molécula bio-ativa de 20 carbonos (SANAK, 2016).

A biossíntese dos mediadores lipídicos ocorre em três etapas: inicialmente ocorre a conversão dos ácidos graxos poli-insaturados (PUFAs) derivados dos fosfolipídios em AA e os PUFAs derivados do ω-3 em EPA (ácido eicosapentanóico e o DHA (ácido docosahexanóico) (SZEFEL *et al.*, 2015). A disponibilidade desses ácidos, depende da capacidade das enzimas de retira-los dos fosfolipídios de membrana, isso caracteriza a segunda etapa de biossíntese, onde a fosfolipase A_2 (PLA_2) catalisa a liberação de ácidos graxos livres de fosfolipídios (BERRY *et al.*, 2016). A enzima PLA_2 possui três famílias: a secretória e a citosólica, que são dependentes de cálcio, e a $iPLA_2$, independente de cálcio. A PLA_2 citosólica está envolvida no processo de síntese dos eicosanóides e pode ser ativada por trauma, citocinas específicas, complexos imunes, hormônios ou certos microrganismos (FUNK *et al.*, 2001) (BROCK; PETERS-GOLDEN, 2007). Por fim, na última etapa de síntese, o AA liberado pela PLA_2 pode ser

então metabolizado pelas enzimas ciclooxigenase (COX) ou pela lipoxigenase (LO) em eicosanoides biologicamente ativos (Figura 4). (PETERS-GOLDEN *et al.*, 2007).

Os eicosanoides sintetizados pelas vias da COX ou LO incluem uma série de prostanóides, leucotrienos (LTs), epoxinas e lipoxinas que são diferencialmente expressas em diferentes tipos celulares e interações cooperativas entre enzimas (BOZZA *et al.*, 2011). O AA metabolizado pela via da COX formam as prostaglandinas (PGs) e tromboxano A_2. A COX-1 tem expressão constitutiva, sendo a enzima responsável pela síntese dos prostanóides, enquanto a COX-2 é uma enzima induzível e por isso é importante em vários processos inflamatórios (SANAK, 2016). Ainda existe a COX-3, que é produto de um *splicing* alternativo da COX-1 e seu estado de expressão ainda é questionado (SANAK, 2016).

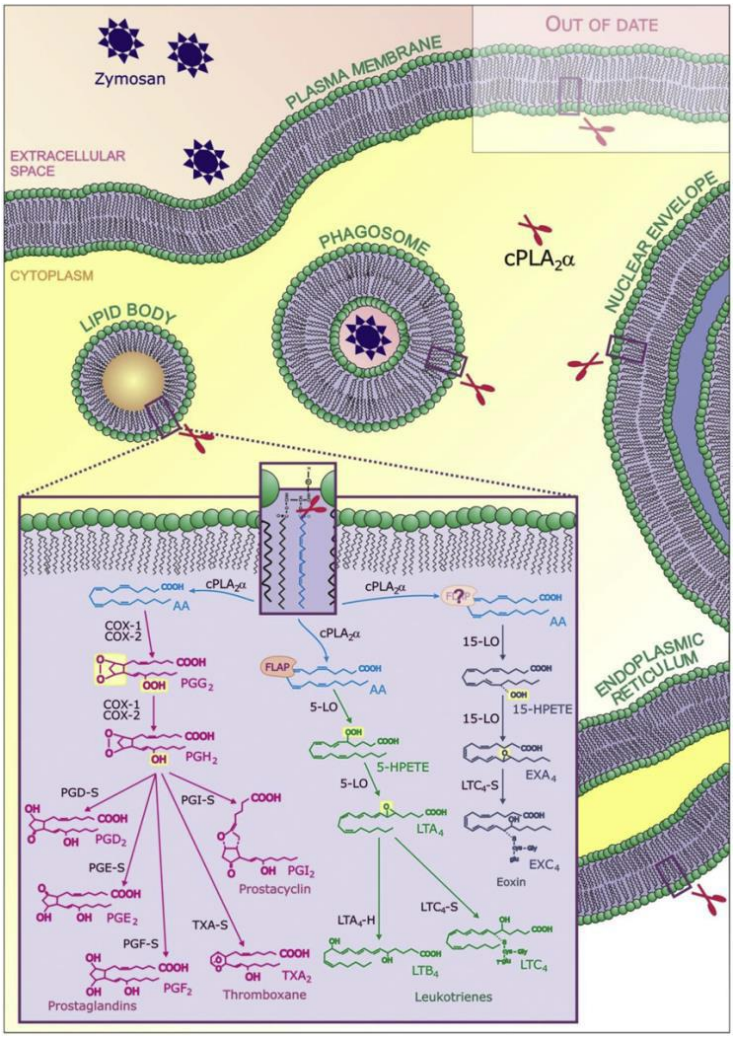


Figura 4. **Representação da via de produção dos eicosanoides.** (Adaptado de BOZZA *et al.*, 2011).

A diversidade de efeitos fisiológicos apresentados por esses mediadores pode estar associada a diferentes receptores celulares. A prostaglandina E_2, por exemplo, pode desempenhar diferente papeis, a depender do receptor envolvido na ativação da célula hospedeira. Esse eicosanoide apresenta 4 receptores acoplados a proteínas G expressos diferencialmente em macrófagos: o EP1 e EP3, associados com a resposta pró-inflamatória, e o EP2 e EP4, envolvidos com a resposta anti-inflamatória (KAUL *et al.*, 2012).

A via da LO, outra enzima que metaboliza o AA, leva a formação dos LTs e lipoxinas, produzidos pela 5-LO, e os mais recentes descobertos mediadores especializados na pró-resolução da inflamação (SPMs), sintetizados pela 15-LO. A expressão da 5-LO está associada aos eventos iniciais da resposta inflamatória. Quando o AA é metabolizado pela 5-LO, resulta na produção de LTA_4 que é transformado em LTB_4 pela LTA_4 hidrolase (HARIZI; CORCUFF; GUALDE, 2008). O LTB_4, está associado com a produção de citocinas pró-inflamatórias e radicais de oxigênio em resposta a agentes infecciosos, e os chamados cistenil-leucotrienos LTC_4, LTD_4 e LTE_4, envolvidos na resposta alérgica (PETERS-GOLDEN *et al.*, 2007) (BERRY *et al.*, 2016). Por outro lado, o LTA_4 pode dar origem as LXAs via 12 ou 15-LO (HARIZI; CORCUFF; GUALDE, 2008). A ativação da 15-LO e seus produtos são importantes durante a fase de resolução da inflamação (HORN *et al.*, 2013).

Os eicosanoides são conhecidos como sinalizadores lipídicos envolvidos na modulação do sistema imune (HARRIS *et al.*, 2002) (SAKA; VALDIVIA, 2012), além de serem importantes em processos patológicos (BOZZA *et al.*, 2011). Esses mediadores também desempenham um importante papel em todos os estágios da

resposta inflamatória, desde os eventos iniciais até a resolução da inflamação. Após lesão tecidual ou invasão de organismo, inicia-se uma sequência de eventos que caracterizam a resposta inflamatória aguda, que tem como objetivo o reparo tecidual ou eliminação dos organismos invasores (SERHAN *et al.*, 2015).

A produção de mediadores pró-inflamatórios como as prostaglandinas (PGs) e leucotrienos (LTs) gerados durante a fase aguda da resposta inflamatória, são responsáveis pela vasodilatação, aumento da permeabilidade vascular e recrutamento celular de neutrófilos (LAWRENCE; WILLOUGHBY; GILROY, 2002) (SERHAN *et al.*, 2015). Essa fase inicial está associada à produção de diferentes mediadores lipídicos inflamatórios a depender do tipo de célula e substratos presentes (SUGIMOTO *et al.*, 2016). Já nos estágios tardios, a fagocitose de neutrófilos apoptóticos pelos macrófagos recrutados para o sítio da lesão, induz uma reprogramação no exsudato com produção de mediadores especializados na pró-resolução que promovem a pausa na produção dos mediadores pró-inflamatórios e redução do influxo de células ao sítio inflamatório, ocasionando a resolução da inflamação (SUGIMOTO *et al.*, 2016).

2.2 MEDIADORES LIPÍDICOS ESPECIALIZADOS NA PRÓ-RESOLUÇÃO DA INFLAMAÇÃO

Além do envolvimento dos eicosanoides em diferentes modelos de inflamação e doenças infecciosas, uma nova classe de mediadores lipídicos derivados enzimaticamente de ácidos graxos poli-insaturados derivados do ω - 3 (ω - 3 PUFAs), como o EPA (que após sua biossíntese produzem as resolvinas da série E (RvE1)); e o DHA (que produzem as resolvinas da série D (RvD1), vem sendo estudado. Eles funcionam como SPMs (mediadores especializados na pró-resolução), marcando a transição da fase aguda para a fase de resolução durante a resposta inflamatória

(SERHAN *et al.*, 2015). Esses SPMs incluem lipoxinas, resolvinas, protectinas e maresinas, e compreendem a nova família de autacóides com potente ação anti-inflamatória e de pró-resolução (SERHAN *et al.*, 2008) (Figura 5).

As resolvinas, assim como os outros SPMs estão associadas com a resolução da inflamação aguda e com a restauração do tecido à homeostasia (SERHAN *et al.*, 2015). A maioria dos estudos tem focado nas resolvinas da série D, representada principalmente pela resolvina D1 (RvD1) e D2 (RvD2) (SERHAN *et al.*, 2015).

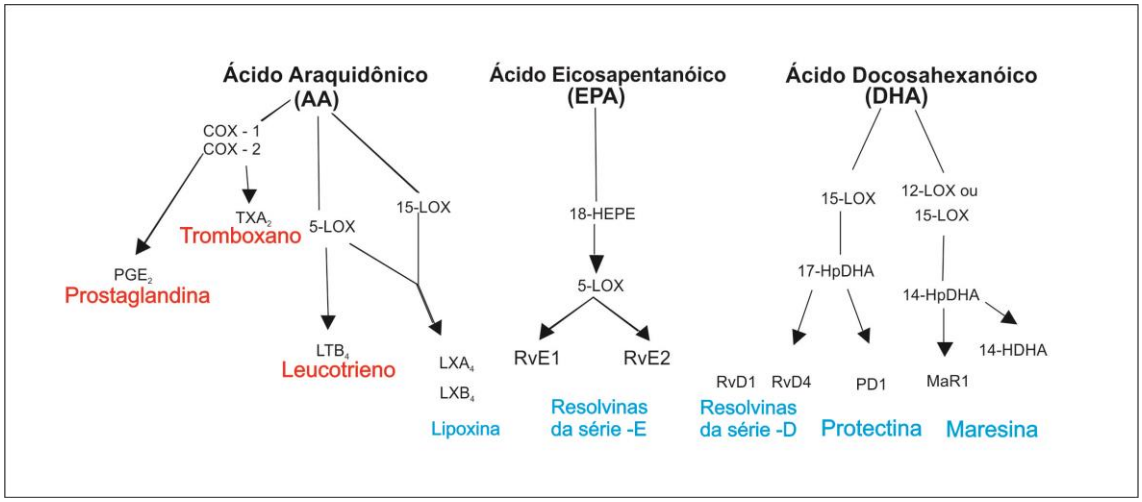


Figura 5. **Via bioquímica de produção de mediadores especializados na pró-resolução** (Adaptado de FREDMAN, G.; SERHAN, C. 2011)**.**

Os efeitos biológicos atribuídos a esses mediadores estão altamente associados com suas propriedades anti-inflamatórias e imuno-modulatória, que incluem a inibição da quimiotaxia dos leucócitos, bloqueio da produção de TNF-α and IL-6, ao mesmo tempo em que induz o aumento da expressão de IL-10 (WANG *et al.*, 2011).

A atividade anti-inflamatória das resolvinas vem sendo descrita em vários modelos de doenças inflamatórias, incluindo doenças cardiovasculares, câncer, inflamação renal aguda e lesões pulmonares (SERHAN *et al.*, 2015). Esther Titos e

colaboradores demonstraram que tanto a RvD1, como seu precursor (DHA) foram capazes de induzir fortemente a expressão da arginase-1 enquanto reduzia a produção de IFN-γ e citocinas do perfil Th1 durante a inflamação crônica no tecido adiposo (TITOS *et al.*, 2013).

O papel das resolvinas também já foi descrito no contexto de infecções virais e bacterianas (SERHAN *et al.*, 2015). Durante infecções virais por herpes simplex resolvinas da série E foram capazes de reduzir o influxo de células T CD4, reduzindo os níveis de INF-γ (NAJASAGI *et al.*, 2011). Resultados similares foram observados após o tratamento com RvD1em modelos de infecções bacterianas, onde a RvD1 foi capaz de regular a enzimas e citocinas inflamatórias após a infecção (CROASDELL *et al.*, 2016). Mais recentemente, foi demonstrado o papel anti-inflamatório da RvD1 em pacientes com Doença de Chagas. Células mononucleares do sangue periférico (PBMCs) de pacientes, depois de tratadas com altas doses de RvD1 foram capazes de reduzir a produção de INF-γ e a proliferação celular induzida pelos antígenos de *T. cruzi*, sugerindo o efeito imuno-modulatório desse mediador nesse modelo de doença infecciosa parasitária (OGATA *et al.*, 2016).

Embora trabalhos anteriores do nosso grupo tenham demonstrado que os níveis de diferentes mediadores inflamatórios e eicosanoides no plasma de pacientes com leishmaniose tegumentar são importantes da patogênese da doença (FRANÇA-COSTA *et al.*, 2015) (FRANÇA-COSTA *et al.*, 2016), ainda não é conhecido como as resolvinas interferem no estabelecimento e manutenção da infecção por *Leishmania*.

2.3 IMPORTÂNCIA DOS MEDIADORES LIPÍDICOS NA LEISHMANIOSE.

Durante infecções por organismos intracelulares, foi demonstrado que a produção de PGE_2 pode estar envolvida com a imunossupressão do hospedeiro, bem como com a progressão da doença. Isso ocorre, pois esse mediador suprime a resposta imune Th1 inibindo a produção de IFN-γ, TNF-α e IL-12 (KALINSKI, 2011) ao mesmo tempo em que é capaz de induzir a produção citocinas do perfil Th2, bem como IL-10, IL-4 e TGF- β (BARATELLI *et al.*, 2010). Os produtos da via da 5-LO como o LTB_4, por sua vez, induzem o aumento da produção de mediadores pró-inflamatórios e óxido nítrico, importante no controle de infecções em diversos modelos (PETERS-GOLDEN, 2007).

No contexto de infecção por *Leishmania* a dicotomia na expressão dos mediadores produzidos está implicada no sucesso ou controle da infecção. A secreção PGE_2 vem sendo associada ao favorecimento da sobrevivência do parasito, enquanto que o aumento dos níveis de LTB_4 controla a carga parasitária em células hospedeiras infectadas (MORATO *et al.*, 2014). Serezani e colaboradores mostraram que a liberação de PGE_2 durante a infecção de macrófagos por *L. amazonensis* está associada com a sobrevivência de parasitos nos macrófagos, enquanto a secreção de LTB_4 com a eliminação do parasito e controle da infecção (SEREZANI *et al.*, 2006).

O efeito do LTB_4 em contribuir com a ação microbicida do macrófago vem sendo associado à sua capacidade de indução de espécies reativas de oxigênio (RODRIGUES *et al.*, 2015). Trabalhos do grupo também mostram a participação do LTB_4 em conter a infecção por *L. amazonensis* em neutrófilos pela indução da produção de ROS e a ativação do NFκB levando a produção de LTB_4 via TLR2 (TAVARES *et al.*, 2014). A inibição da 5-LO, via biosintética de produção do LTB_4, com o zileuton foi capaz de aumentar a carga parasitária de neutrófilos infectados, confirmando a importância desse mediador em controlar a infecção (TAVARES *et al.*, 2014).

Embora vários trabalhos venham demonstrando a importância das prostaglandinas para a persistência da infecção acredita-se que a *Leishmania* pode induzir distintas respostas, a depender da espécie envolvida e dos receptores envolvidos.

Segundo Kaul e colaboradores, a sinalização do PGE_2 através do EP2 promove uma resposta imune do tipo Th2 que induz a supressão da atividade microbicida dos macrófagos favorecendo seu crescimento e proliferação (LAUNOIS *et al.*, 1999) (KAUL *et al.*, 2012). Trabalhos do grupo mostraram que os níveis de expressão de RNAm do receptor EP2 é maior em lesões de pacientes com LCD quando comparado com os pacientes com LCL bem como de outras enzimas envolvidas na síntese de PGE_2 (FRANÇA-COSTA *et al.*, 2015).

O perfil de eicosanoides difere entre pacientes com diferentes formas clínicas da leishmaniose. Pacientes com LCL apresentam significativamente maiores níveis plasmáticos de PGE_2 quando comparados com pacientes com LCM, por outro lado apresentam menores níveis de $PGF_{2\alpha}$ e RvD1 quando comparado com os controles endêmicos (FRANCA-COSTA *et al.*, 2016).

O balanço entre os níveis de PGE_2 e LTB_4 tem sido utilizado para determinar o desfecho clínico em outras doenças (MAYER-BARBER; ANDRADE; OLAND, 2014). Mais recentemente, foi demonstrado que o balanço dos níveis plasmáticos desses mediadores pode estar associado com os diferentes estados inflamatórios observado na LCM e LCL, e que esses mediadores podem ser utilizados como potenciais biomarcadores na LT (FRANCA-COSTA *et al.*, 2015).

Embora trabalhos anteriores do nosso grupo tenham ampliado o conhecimento a respeito do papel dos eicosanoides na modulação da resposta imune e controle da infecção por Leishmania, ainda não é conhecido o papel dos mediadores lipídicos de resolução dentro desse contexto. Nesse estudo, exploramos o papel da RvD1 e seus

efeitos anti-inflamatórios no contexto da infecção por *L. amazonensis* e na patogênese de pacientes com LCD.

3 JUSTIFICATIVA

A progressão da LCD é atribuída à falta de imunidade mediada por células específica para antígeno do parasito com presença de inúmeros macrófagos vacuolizados e intensamente parasitados.

Mais recentemente trabalhos do grupo demonstraram que a imunossupressão observada nos pacientes com LCD podem estar associada com altos níveis plasmáticos de PGE_2, arginase-I e TGF-β. Embora haja evidências na literatura da importância desses mediadores na LCD, se os altos níveis plasmáticos de RvD1 estão associados com esses mediadores e com a desativação da resposta imune da LCD, bem como se o aumento da replicação do parasito na LCD está associado com o aumento da produção de resolvinas ainda não foi demonstrado.

Devido à associação da produção de resolvinas com respostas supressoras e antioxidantes, nesse estudo iremos buscar entender o papel das resolvinas na LCD e se ela está envolvida no favorecimento da proliferação da *L. amazonensis*, principal agente etiológico da forma difusa da doença. Com esse estudo iremos ampliar o conhecimento da imunopatogênese da LCD e possibilitar uma possível alternativa de via terapêutica.

4 HIPÓTESE

Resolvina D1 favorece a infecção por *L. amazonensis.*

5 OBJETIVOS

5.1 OBJETIVO GERAL

Avaliar o papel da RvD1 na infecção por *L. amazonensis.*

5.2 OBJETIVOS ESPECÍFICOS

1. Comparar a produção de resolvina no plasma de pacientes com LCL, LCD e voluntários não infectados da região endêmica;

2. Avaliar se a infecção por *L. amazonensis* induz a produção de RvD1 em macrófagos humanos;

3. Testar se a suplementação de RvD1 em culturas axênicas de promastigotas de *L. amazonensis* interfere no crescimento do parasito;

4. Quantificar a carga parasitária e mediadores produzidos no sobrenadante de macrófagos humanos infectados por *L. amazonensis* na presença de RvD1;

5. Testar se a inibição da via de produção das resolvinas interfere na carga parasitária da infecção por *L. amazonensis.*

6 MANUSCRITO

6.1 MANUSCRITO 1

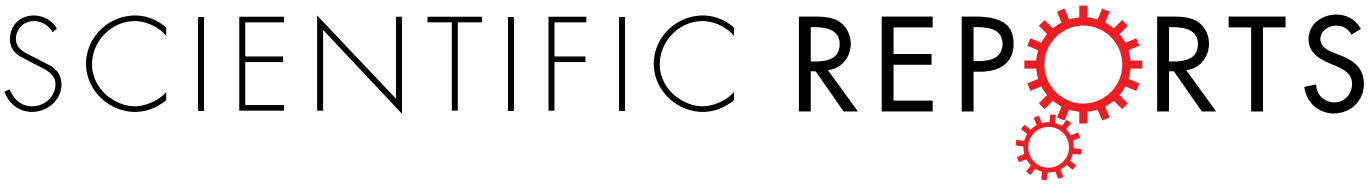
Título: Resolvin D1 drives establishment of *Leishmania amazonensis* infection.

Esse trabalho investiga a participação da RvD1 na infecção de macrófagos por *L. amazonensis*, bem como sua relevância na LCD.

Situação: Aceito para publicação

Resumo:

Nesse trabalho demonstramos que os pacientes com LCD apresentam altos níveis plasmáticos de RvD1 em comparação a pacientes com LCL, e que esses níveis se correlacionam com importantes mediadores envolvidos na patogênese da doença. Além disso, nós observamos que a infecção por *Leishmania* foi capaz de induzir a produção de RvD1 no sobrenadante de macrófagos nas horas iniciais de infecção. O tratamento desses macrófagos com crescentes doses de resolvina sintética foi capaz de aumentar a carga parasitária bem como a viabilidade do parasito através de um mecanismo dependente de HO-1. Esses dados sugerem que as resolvinas podem estar envolvidas no estabelecimento de um ambiente que favorece a replicação da *L. amazonensis*, bem como a progressão da doença.



OPEN

Resolvin D1 drives establishment of *Leishmania amazonensis* infection

Hayna Malta-Santos[1,2], Bruno B. Andrade[1,3], Dalila L. Zanette[1], Jackson M. Costa[1], Patrícia T. Bozza[4], Christianne Bandeira-Melo[5], Aldina Barral[1,2], Jaqueline França-Costa[1,2,*] & Valéria M. Borges[1,2,*]

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Previous studies have indicated that the balance between different eicosanoids reflect the intensity of the inflammatory profile in patients with tegumentary leishmaniasis. More recently, pro-resolution lipid mediators have been shown to play critical roles in dampening pathological inflammatory processes to reestablish homeostasis in a diverse range of experimental settings. Among these lipid mediator, resolvins from D series have been described as potent anti-inflammatory and immunomodulatory mediators, and its activities include inhibition of leukocyte chemotaxis and blockage production of proinflammatory cytokines, while increasing the expression of regulatory mediators. Whether resolvins play significant roles in establishment and persistence of *Leishmania* infection is currently unknown. We addressed this question in the current study by assessing circulating levels of D-series resolvins in tegumentary leishmaniasis patients presenting with localized or diffuse disease. We found heightened expression of resolvin D1 in diffuse cutaneous leishmaniasis which was correlated with expression profile of biomarkers associated with disease pathogenesis. Additional *in vitro* experiments using primary human macrophages indicated that resolvin D1 may promote intracellular *Leishmania amazonensis* replication through a mechanism associated with induction of heme oxygenase-1. These results suggest that targeting resolvin D1 could serve as potential strategy for host directed therapy in diffuse cutaneous leishmaniasis.

Resolvins are oxygenated lipid mediators derived from ω-3 polyunsaturated fatty acids that have been associated with resolution of acute inflammation and restoration of tissue homeostasis[1]. The majority of the studies involving resolvins has focused on those from the D series, represented mainly by resolvin D1 (RvD1) and D2 (RvD2)[1]. The biological effects attributed to resolvins have been linked to its anti-inflammatory and immunomodulatory properties, which include inhibition of leukocyte chemotaxis, blocking production of pro-inflammatory cytokines, while increasing the expression of anti-inflammatory mediators such as heme oxygenase 1 (HO-1)[2,3]. The anti-inflammatory activity of resolvins has been extensively described in several inflammatory disease models, including cardiovascular diseases, cancer, acute kidney and lung injuries and metabolic inflammation in adipose tissue[1,3]. More recently, resolvins have also been implicated in host protective responses during viral and bacterial infections[1]. Although lipid mediator levels in plasma have been reported in patients with tegumentary leishmaniasis[4], it is still unknown whether resolvins associate with disease pathogenesis driven by *Leishmania* infection.

Tegumentary leishmaniasis is a disease caused by *Leishmania* parasites and exhibits a spectrum of clinical manifestations associated with the balance between parasite replication and immune-mediated inflammatory destruction of the skin tissue[5]. In the most common clinical form, named localized cutaneous leishmaniasis (LCL), single self-healing skin ulcers are usually observed. In this setting, a modest infiltration of macrophages is detected, with very few parasites, due moderate inflammation and cell-mediated immune responses[5]. A rare clinical form, diffuse cutaneous leishmaniasis (DCL), is characterized by the numerous nonulcerated nodular lesions with heavily parasitized macrophages[6]. Patients with DCL lack protective cell-mediated immunity and are reported to present

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil. [2]Universidade Federal da Bahia, Salvador, Brazil. [3]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Fundação José Silveira, Salvador, Brazil. [4]Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. [5]Instituto de Biofísica Carlos Chagas Filho (IBCCF), Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to J.F.-C. (email: jaquefcosta@ufba.br) or V.M.B. (email: vborges@bahia.fiocruz.br)

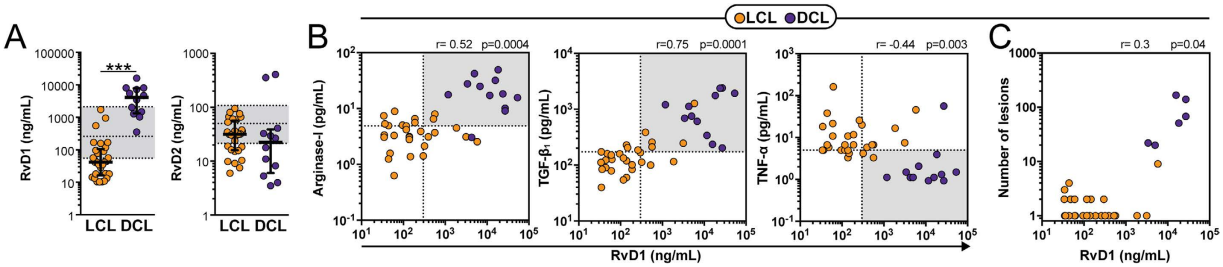

Figure 1. Differential plasma concentrations of RvD1 and RvD2 in patients with tegumentary leishmaniasis. (**A**) Serum levels of RvD1 and RvD2 in patients with localized cutaneous leishmaniasis (LCL; n = 29) or diffuse cutaneous leishmaniasis (DCL; n = 12). Shaded area represent median and interquartile range values obtained in 42 healthy endemic controls to serve as reference. Data were compared using the Mann-Whitney *U* test (***$P < 0.0001$). (**B**) Correlations between plasma RvD1 levels and arginase-I, transforming growth factor-β1 (TGF-β) and tumor necrosis factor-α (TNF-α) in active LCL and DCL patients. Dotted lines on the X-axis represent the median value of RvD1 within the entire study population whereas dotted lines on each Y-axis indicate median values for each mediator. Gray areas designate the quadrants that include the individuals simultaneously displaying values of RvD1 and madiators above the medians or in the case of TNF-α below the median. (**C**) Correlation between plasma RvD1 levels and number of lesions in the study population. Correlations were tested using Spearman ranks.

elevated expression of anti-inflammatory mediators such as TGF-β and arginase-I while exhibiting reduced circulating levels of TNF-α and IL-12p70[7], highlighting a relative state of immunosuppression. Probably for this reason, DCL disease continues to develop for decades and exhibits treatment resistance and relapse[6].

In vitro experiments have demonstrated that during *Leishmania* infection, there is increased production of arginase-I, transforming growth factor β (TGF-β) and HO-1 by infected macrophages, which has been associated with augmented intracellular parasite proliferation[7–10]. Moreover, the role of these three important mediators in *Leishmania* infection has been further established in experiments using macrophages from mice genetically lacking HO-1[11], and pharmacological inhibition of arginase-I[7] or antibody-mediated blockage of TGF-β[12] in infected human macrophages. In all these experimental settings, reduction of HO-1, arginase-I and TGF-β resulted in substantial increase in anti-parasite effector mechanisms and production of inflammatory cytokines such as TNF-α, resulting in better control of parasite loads *in vitro*[7,11,12]. Whether resolvins play any role in these phenomena in the context of *L. amazonensis in vitro* or *in vivo* is unknown.

Here, to characterize the role of RvD1 and RvD2 in *Leishmania* infection, we assessed circulating levels of these lipid mediators in patients with LCL or DCL from an endemic area in Brazil. We observed that RvD1 concentrations in plasma samples from DCL patients were substantially increased compared to those with LCL and that its levels were positively correlated with arginase-I and TGF-β, while being negatively correlated with TNF-α levels. In addition, we performed *in vitro* assays with primary monocyte-derived human macrophages infected with *L. amazonensis*. Notably, *L. amazonensis* infection of macrophages primed RvD1 production and its supplementation to cultures amplified intracellular parasite replication, arguing that RvD1 may promote parasite persistence. The findings presented here, which still need further validation in different patient populations and epidemiological settings, point to the idea that interfering with the RvD1 pathway could potentially serve as an adjunctive therapy for DCL.

Results

RvD1 plasma levels are associated with an immune profile that distinguishes patients with DCL from those with LCL. Patients with DCL displayed at least 100-fold higher levels of RvD1 in plasma compared with those with LCL (Fig. 1A). Concentrations of RvD2 were not different between these clinical groups (Fig. 1A). These findings made us hypothesize that RvD1 levels could be associated with an immunological environment that favors the development of DCL. A recent study reported that DCL patients present heightened levels of arginase-I and TGF-β whereas those with LCL display increased plasma concentrations of TNF-α[7]. We next tested if RvD1 levels are associated with this immune profile. RvD1 levels in plasma exhibited strong positive correlations with concentrations of arginase-I and TGF-β and were negatively correlated with TNF-α levels (Fig. 1B). To test if the high plasma levels of RvD1 is associated with increased *Leishmania* replication *in vivo*, we performed additional analyses, which revealed that RvD1 levels were positively correlated with the number of lesions in the study population (Fig. 1C). These observations suggested that RvD1 but not RvD2 may be differentially implicated in the pathogenesis of LCL and DCL and that RvD1 could be associated with the dampened inflammation observed in DCL, which favors *Leishmania* replication.

RvD1 enhances *L. amazonensis* infection burden in human macrophages. To better explore the direct association between RvD1 and *Leishmania* infection, we employed an *in vitro* system using primary human monocyte-derived macrophages. In cultures of macrophages infected with *L. amazonensis*, we observed a >1.5-fold induction of RvD1 concentrations in supernatants at 4 h post-infection compared to that in uninfected cell cultures (Fig. 2). This infection-driven induction in RvD1 production was transient as the detected

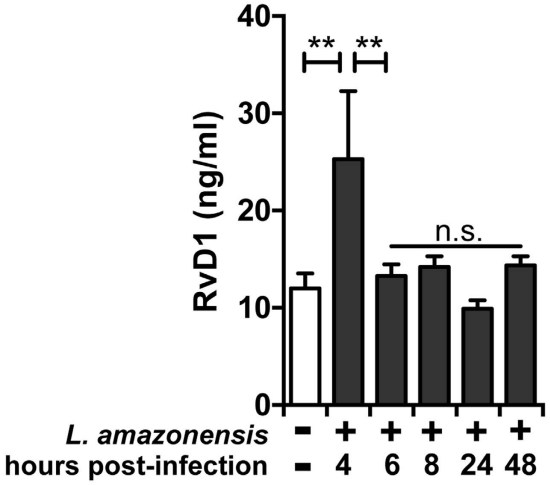

Figure 2. ***L. amazonensis*** **infection induces the RvD1 production by macrophages.** Monocyte-derived human macrophages (n = 6) were infected with *L. amazonensis* (MOI 6:1). RvD1 levels were measured in culture supernatants at indicated timepoints post-infection. Data shown are mean and SE of one representative out two independent experiments. *P < 0.05, **P < 0.01.

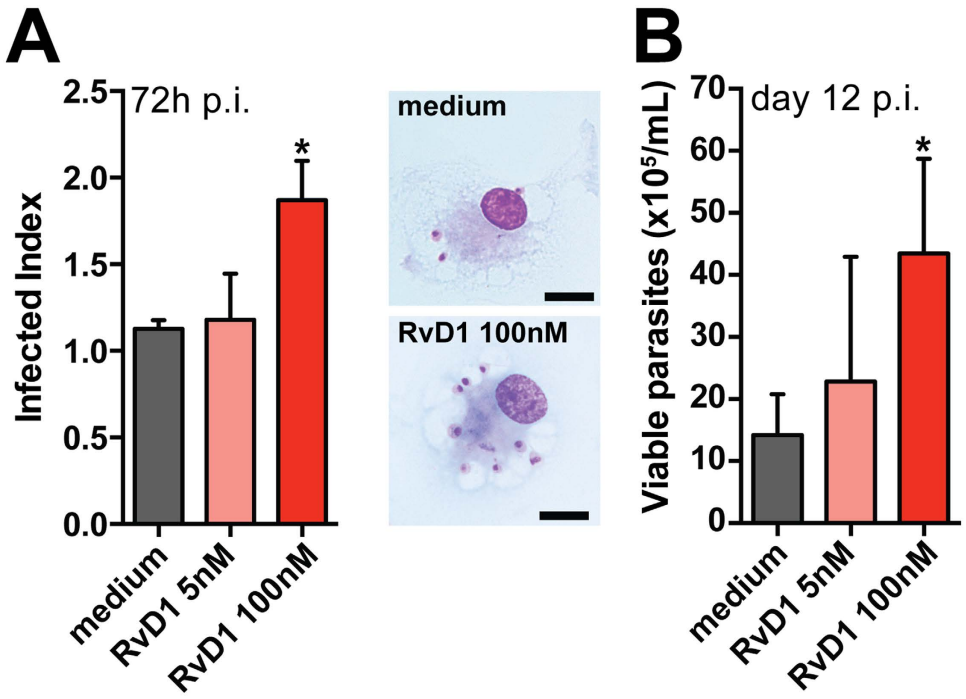

Figure 3. Effectiveness of Resolvin D1 supplementation in cultures of ***L. amazonensis*****- infected human macrophages.** Monolayers of infected macrophages were cultured with medium alone or with indicated doses of synthetic RvD1. (**A**) Intracellular infection burden was assessed by light microscopy; micrographs from *L. amazonensis*-infected human macrophages unstimulated or treated with RvD1 for 72 h. Original magnification x 1000. (**B**) Counting of viable parasites was performed as described in Methods. Data represent mean and SE of one representative out two independent experiments. *P < 0.05, **P < 0.01.

concentrations significantly dropped at as soon as 6 h post infection and persisted for up to 48 h at low levels similar to pre-infection status (Fig. 2).

To mimic an environment with high RvD1 levels such as that observed in DCL patients, we supplemented cultures of macrophages infected with *L. amazonensis* with increasing doses of this lipid mediator. We found an increased infection index as well as augmented number of viable replicating parasites inside macrophages treated with 100 nM RvD1 (Fig. 3A). Photomicrographs illustrated that treatment with RvD1 was able to increase intracellular parasite burden more efficiently than cells in the untreated group (Fig. 3A).

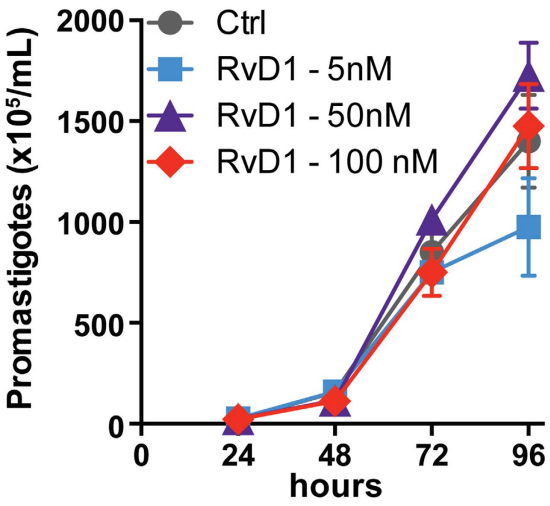

Figure 4. Effect of Resolvin D1 (RvD1) in axenic *L. amazonensis* cultures. Parasites were incubated for 4 days with medium alone (Ctrl) or with indicated doses of RvD1. The number of viable parasites was evaluated by direct counting. Each point represents mean and SE. Data are representative of at least 3 independent assays and were collected in triplicate for each condition.

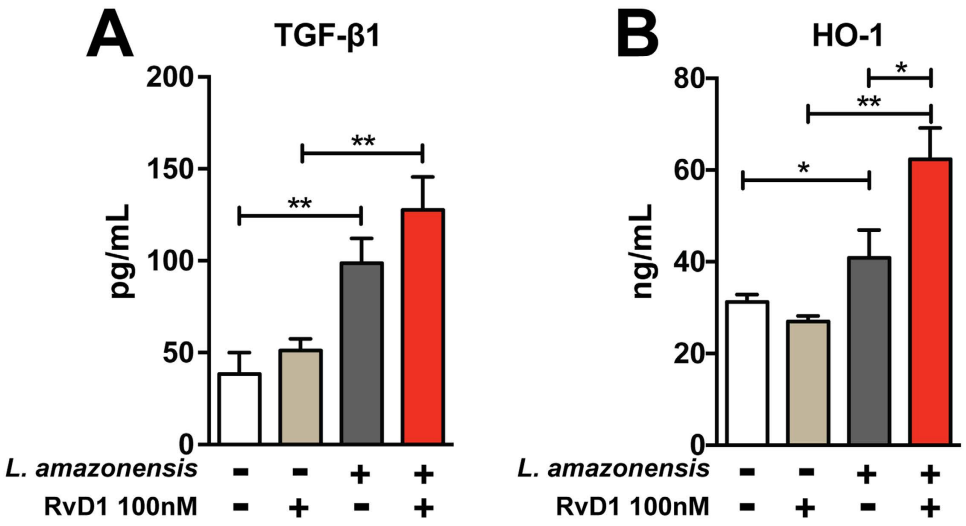

Figure 5. RvD1 induces the HO-1 production in *L. amazonensis*-infected human macrophages. Supernatants from infected macrophages were collected after 24 h post infection and evaluated for the levels of transforming growth factor β (TGF-β; **A**) whereas hemoxygenase-1 (HO-1; **B**) protein expression was assessed in cell extracts as described in Methods. Data shown are mean and SE of one representative out two independent experiments. *$P < 0.05$, **$P < 0.01$.

To assess if RvD1 could be exerting an effect in the parasite growth or survival *in vitro* we treated axenic *L. amazonensis* promastigote cultures with increasing doses of RvD1. We found that RvD1 supplementation in parasite cultures did not affect the growth curves (Fig. 4) These results are consistent with the idea that the parasite triggers an early and transient production of RvD1 by macrophages which should be important for the *Leishmania* intracellular proliferation.

RvD1 supplementation amplifies *L. amazonensis* driven HO-1 production in infected macrophages. It is well established that induction of cytokines is a critical evasion mechanism used by *Leishmania* parasites[10,13]. Here, we confirmed that *L. amazonensis* infection induces TGF-β production by infected macrophages, as previously reported[14] (Fig. 5A). Interestingly, treatment of cultures with RvD1 was not able to further increase TGF-β levels in this *in vitro* system (Fig. 5A). Furthermore, we found no differential induction of arginase activity and TNF-α expression in cultures treated with RvD1 (data not shown).

An important mechanism used by *Leishmania* parasites to subvert host antimicrobial responses is the induction of antioxidants[11,15]. In the present study, *L. amazonensis* infection of macrophages induced HO-1 expression similarly to a previous observation in *L. chagasi* infected cells[11]. Notably, RvD1 supplementation induced

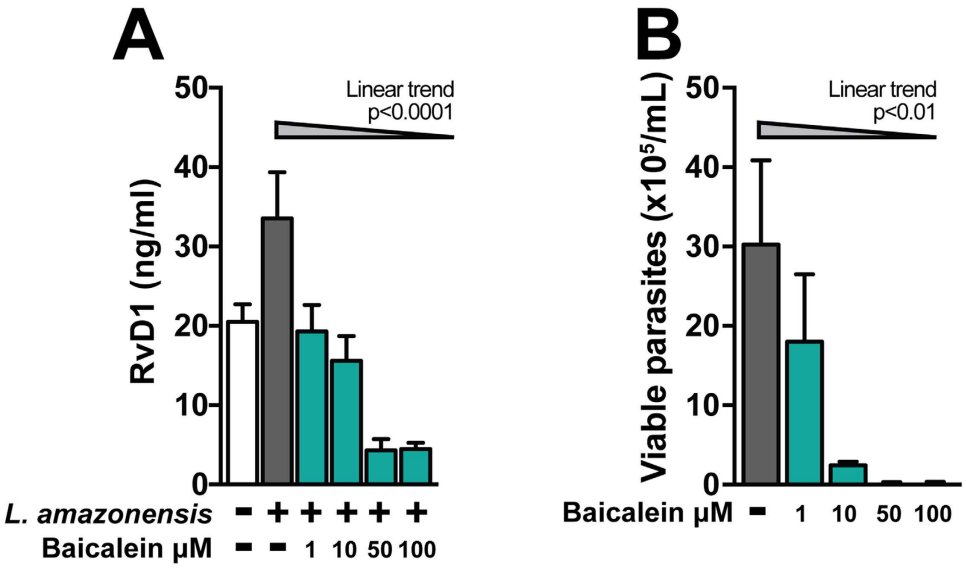

Figure 6. Baicalein inhibits the *L. amazonensis* proliferation. The effect of 15-lipoxygenase in *L. amazonensis* intracellular growth was examined by treating infected macrophage cultures with indicated doses of Baicalein. (**A**) RvD1 levels were measured in culture supernatants 4 h post infection. (**B**) Counting viable parasites was assessed in cells treated with increasing doses of baicalein. Data shown are mean and SE of one representative out two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

substantially higher expression of HO-1 in cell lysates compared to untreated cultures (Fig. 5B). Despite these differences observed *in vitro*, we found that plasma levels of HO-1 were undistinguishable between LCL and DCL patients (data not shown).

Pharmacological treatment of macrophage cultures with Baicalein reduces intracellular *L. amazonensis* infection. To further delineate the direct link between intrinsic RvD1 induction and *L. amazonensis* replication, we treated infected macrophages with Baicalein, an inhibitor of 15-lipoxygenase[16], which is a key enzyme involved in the production of RvD1[17]. Treatment with Baicalein resulted in a dose dependent reduction of RvD1 production at 4 h post-infection (Fig. 6A). This reduction was associated with a significant decrease in intracellular parasite viability (Fig. 6B). These findings strongly argue that blocking RvD1 production may serve as a strategy to reduce intracellular *L. amazonensis* infection burden.

Discussion

Resolvins are derived from ω-3 fatty acid and represent a new class of lipid mediator[1]. Studies have demonstrated that resolvins play a pivotal role during the resolution phase of acute inflammation and restoration of tissue homeostasis[18,19]. Its biological effects have been described in several inflammatory diseases[17] as well as in viral or bacterial infections[1]. However, the role of resolvins in leishmaniasis is largely unexplored.

In the present study, we show that plasma levels of RvD1, but not of RvD2, were substantially higher in DCL patients compared with that detected in LCL patients. These results suggest that distinct resolvins from the D series may be differentially implicated in the pathogenesis of LCL and DCL. Corroborating with the idea that RvD1 has been shown to modulate inflammatory responses[1], our correlation analyses revealed that circulating levels of RvD1 exhibited strong positive associations with concentrations of arginase-I and TGF-β, while negatively correlating with TNF-α levels. Previous data from our group in plasma samples from the same patient population have shown high levels of arginase-I and TGF-β, while reduced TNF-α concentrations in DCL patients compared to uninfected controls or individuals with LCL[7]. This study has further demonstrated that *in situ* arginase-I expression was increased in lesions from DCL vs. those from LCL patients. The association between high levels of RvD1 and arginase-I and TGF-β could be ultimately contributing to suppression of host immune responses observed in DCL patients[7,20] and can potentially be critical to disease progression.

In active disease, DCL patients present with disseminated nodular lesions and high intracellular parasite burden in tissue macrophages[21]. Our *in vitro* experiments revealed that RvD1 is associated with increased parasite replication in macrophages. In addition, we found that DCL patients presenting with the highest numbers of cutaneous lesions were the same individuals exhibiting the highest plasma levels of RvD1. These results argue that high RvD1 expression is associated with the anti-inflammatory profile observed in DCL patients, which in turn reflects parasite replication and disease progression. Additional prospective studies are warranted to answer whether RvD1 levels directly affects disease activity in DCL.

It has been demonstrated that resolvins are produced rapidly during the inflammatory response, but have a prolonged effect in different experimental models[18,22]. Our study expands the current knowledge in the context of parasite infection, as we demonstrate that RvD1 is transiently induced in macrophages during the first 4 hours upon exposure to *L. amazonensis* but its biological effect in promoting intracellular parasite replication persists up to 72 h post-infection. Our observations suggest that initial RvD1 production by infected macrophages may

be crucial to promote *L. amazonensis* intracellular survival and proliferation. Although we demonstrate that *Leishmania* is capable of inducing the RvD1 production in our experimental *in vitro* system, the specific parasite component triggering activation of signaling pathways that drive RvD1 production are yet to be defined.

To our knowledge, no previous study has described the role of RvD1 in *L. amazonensis* proliferation. Our data clearly demonstrated treatment with heightened doses of this lipid mediator was able to increase intracellular parasite loads. Interestingly, treatment of axenic cultures of synthetic RvD1 did not alter the growth curves of the parasite, indicating that the biological effect of this lipid mediator in our experimental system relies on macrophage function rather than on the parasite itself. We then hypothesized that the mechanisms linking macrophage exposure to RvD1 and enhanced intracellular parasite replication could involve subversion of anti-parasite macrophage effector functions. During the *Leishmania* infection, it is well established that parasite can present a series of escape mechanisms, such the deactivation of macrophages[23], induction of antioxidants[11,15] and anti-inflammatory cytokines, such TGB-β[14]. In other experimental models, resolvins have been shown to induce high levels of TGF-β, increasing phagocytosis of cells by macrophages and inducing an anti-inflammatory phenotype[24]. Herein, we found that *Leishmania* infection triggered the production of TGF-β by macrophages, but RvD1 supplementation in cultures failed to amplify such effect. This result argues that RvD1 may affect another mechanism aside from TGF-β production by macrophages, which may result in increased parasite replication. Notably, TGF-β can be produced by many other cell types[25,26] and thus it is possible that the positive correlation between RvD1 and TGF-β plasma levels observed in our patient population could result from an effect in cells other than macrophages. Future studies investigating the role of RvD1 on other cell types in the context of *Leishmania* infection are needed to better understand these discrepancies between the *in vivo* and *in vitro* systems.

In addition to its anti-inflammatory and pro-resolution effects[1], RvD1 has been reported to potently reduce oxidative stress damage through induction of HO-1[2,27]. In the context of *Leishmania* infection, we have demonstrated that parasite-driven HO-1 production represents an important escape mechanism, which works through modulation of reactive oxygen species production by infected human and mouse macrophages[11]. Here we showed that RvD1 supplementation in macrophage cultures resulted in robust induction of HO-1 protein expression at 24 h post *L. amazonensis* infection. We suggest that aside from the induction of HO-1, there may be additional mechanisms driving the effects of RvD1 on parasite replication. HO-1 is an intracellular enzyme and thus its circulating levels may not directly represent *in situ* expression. Indeed, we found no associations between plasma levels of RvD1 and of HO-1 in tegumentary leishmaniasis patients. We are currently performing studies in additional patients to examine *in situ* expression of HO-1 pathway in DCL patients. Our results are associative rather than definitive and thus investigations depicting other potential mechanisms explaining the effects of RvD1 on intracellular *Leishmania* infection are necessary to improve our knowledge about the modulation of macrophage effector functions by this pro-resolvin lipid mediator.

The role of TNF-α and its relationship with oxidative responses during parasite infections has been described in different models[28,29]. Previous work from our group has shown that downregulation of TNF-α production is a key event linking HO-1 driven increased survival of *L. infantum chagasi* in infected macrophages[11]. More recently, a study using peripheral blood mononuclear cells (PBMCs) from patients with Chagas Disease-associated cardiomyopathy demonstrated that RvD1 supplementation in cell cultures interfered with cellular survival without affecting TNF-α production[30], suggesting that the biological action of RvD1 is not directly linked to TNF-α production. The results presented here, showing no link between RvD1-associated HO-1 induction and changes in TNF-α production, reinforce the idea that RvD1 may act independent of TNF- α in our infection model. The effects of RvD1 and HO-1 on TNF-α production may differ according to the infection model or parasite species, and additional studies with other *Leishmania* species are necessary to confirm this hypothesis.

The inhibition of enzymes from the lipid mediators pathways has been widely used to efficiently control inflammatory responses[31] as well as *Leishmania* infection burden[32]. In our experimental model, we found that treatment with an inhibitor of 15-lypoxigenase resulted in a reduction of RvD1 production, which mirrored a decrease in intracellular parasite viability. These data suggest that pharmacological inhibition of RvD1 production may serve as a strategy to reduce *L. amazonensis* infection burden.

Our study has some limitations. Quantifying resolvin levels in plasma of patients may not accurately represent *in situ* responses. In addition, our clinical study was cross-sectional and we believe that prospectively assessing RvD1 concentrations at different time points upon initiation of leishmanicidal treatment may give important insights on whether the levels of RvD1 reflect diseases activity. Larger cohort studies from our group are underway to address this question. We had no access to skin biopsy specimens from the study population, and for this reason we could not examine the expression of enzymes related to RvD1 production *in situ* or perform correlations with parasite loads in the skin. Regardless, the positive correlation between RvD1 levels in plasma and number of lesions in the context of DCL (which is indirectly linked to parasite burden *in vivo*) argues that RvD1 production is closed associated with increased parasite replication. Although our *in vitro* experiments clearly demonstrated a relevant biological effect on infected human macrophages, future investigation is necessary to clarify the role of RvD1 in the parasite persistence.

Heightened circulating levels of RvD1 detected in patients with DCL together with the observed strong relationships with biomarkers closely related to an anti-inflammatory environment strongly indicate that this lipid mediator participates in DCL pathogenesis. Furthermore, our *in vitro* results suggest that RvD1 may induce anti-oxidative mechanisms in *L. amazonensis*-infected macrophages, which cause these cells to become more permissive to parasite infection and proliferation.

Methods

Ethics Statement. Written informed consent was obtained from all participants or their legally responsible guardians, and all clinical investigations were performed according to the principles expressed in the Declaration of Helsinki. The clinical protocols from which the plasma samples were used in the present study were approved by Institutional Review Board of Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Brazil (license number 136/2007).

Patients. The present study used cryopreserved EDTA plasma samples from age- and sex- matched patients with LCL (n = 29, male to female ratio, 1.9; mean age [±standard deviation, SD], 34 ± 15 years) and those with DCL (n = 12, male to female ratio, 1.4; mean age [±SD], 23 ± 17 years). Patients with LCL were recruited at our reference clinic in Jiquiriçá, Bahia, Brazil. Patients with DCL were from Maranhão State. LCL patients were recruited at the time of disease presentation, were treatment naïve and had no previous diagnosis of tegumentary leishmaniasis. Clinical and epidemiological characterization, as well as the diagnostic approaches, have been reported previously[7]. To serve as a reference for the plasma assays, we have included plasma samples from 42 age and gender matched healthy endemic controls, who were family members of the patients with DCL, exhibiting no cutaneous lesions or prior CL history and negative DTH response.

Immunoassays. Concentrations of RvD1 and RvD2 were measured using an enzyme-linked immunoassay (Cayman Chemical, Ann Harbor, MI) according to the manufacturer's instruction. TGF-β, TNF-α (R&D Systems, Minneapolis, Minnesota) and RvD1 (Cayman Chemical) expression was measured in supernatants cells according to the manufacturer's protocols. The expression of heme oxygenase-1 (HO-1) in cell lysates were measured an ELISA kit (Enzo Life Sciences, NY) by the protocols.

Cell Culture. Column purified $CD14^+$ monocytes were obtained from buffy coats from healthy blood donors (from the Hematology and Hemotherapy Foundation of Bahia, HEMOBA), and plated at 2×10^6 cell/well in 24-well plates containing RPMI with 10% fetal bovine serum and treated for 7 d with 50 ng/mL MCSF (Peprotech, Rock Hill, NJ) to differentiate in human macrophages as previously described[33]. Macrophages were infected (multiplicity of infection 6:1) with stationary phase *L. amazonensis* promastigotes (MHOM/BR/87/BA336) isolated from a patient with DCL. After 4 h incubation at 37 °C, free parasites were removed by extensive washing with phosphate-buffered saline. Then, indicated concentrations of synthetic RvD1 (Cayman Chemical), with or without Baicalein (a 15-LO inhibitor, TOCRIS, Bristol, UK) were added to cultures. Culture supernatants were collected after 24 h post infection for measurement inflammatory mediators as described above. The intracellular parasite loads were quantified by microscopy and viable promastigotes in Schneider medium. The infectivity index (percentage of infected macrophages x average number of amastigote per macrophage) was determined by randomly counting at least 200 macrophages per slide in light microscope, using the immersion objective (100x).

***In vitro* treatments.** Axenic cultures of *L. amazonensis* promastigotes were incubated at 26 °C in Schneider's complete medium (Invitrogen) supplemented with 10% inactive Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Invitrogen). For assessment of a potential direct biological effect of resolvins on parasite cultures, promastigotes in stationary phase (2×10^5/mL) were cultured with supplemented Schneider medium alone or in combination with indicated concentrations of RvD1 or RvD2 (5, 50 and 100 nM). Cultures were incubated for 4 days at 24 °C and the effect of these drugs on parasite growth was evaluated by directing counting daily live motile parasites using a Neubauer chamber.

Statistical analysis. Median and interquartile ranges were used as measures of central tendency for the *ex vivo* analyses. Mean and standard errors were used to display data from the *in vitro* experiments. Differences between groups were calculated using the Mann–Whitney *U* test (2-groups) or the Kruskal-Wallis test with the Dunn multiple comparisons or linear trend analysis post tests (more than 2 groups). Correlations were tested using Spearman ranks. Differences with p-values < 0.05 were considered statistically significant.

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Author Contributions

H.M.-S., J.F.-C. performed the experiments; H.M.-S., J.F.-C., D.L.Z., C.B.M., P.T.B., B.B.A. and V.M.B. designed the experiments; J.M.C., A.B., C.B.M., P.T.B., B.B.A. and V.M.B. provided materials and infrastructural support; D.L.Z., J.F.-C., B.B.A. and V.M.B. mentored the work; H.M.-S., J.F.-C., B.B.A. and V.M.B. wrote the manuscript. The authors declare that they do not have a commercial association that might pose a conflict of interest.

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7 DISCUSSÃO

Resolvinas são docosanoides derivados de ácidos graxos polinsaturados ω-3 e representam uma nova classe de mediadores lipídicos (SERHAN *et al.,* 2015). Estudos tem demonstrado que as resolvinas desempenham um papel fundamental durante a resolução da fase aguda da inflamação e na restauração da homeostasia tecidual (SPITE *et al.,* 2014) (WANG *et al.,* 2011). O efeito biológico desse mediator vem sendo descrito em várias doenças inflamatórias (XU *et al.,* 2013) bem como em infecções virais e bacterianas (SERHAN *et al.,* 2015). No entanto, o papel das resolvinas nas leishmanioses ainda permanece pouco explorado.

Nesse estudo, nós demonstramos que os níveis plasmáticos de RvD1, mas não RvD2, estavam aumentados nos pacientes de LCD quando comparado com os valores detectados nos pacientes com LCL. Esses resultados sugerem que diferentes resolvinas da série D podem agir diferencialmente na patogênese da LCL e da LCD. Nossas análises de correlação revelaram que os níveis circulantes de RvD1 exibiram fortes correlações positivas com os níveis plasmáticos de arginase-I e TGF-β, enquanto exibiram uma correlação negativa com os níveis de TNF-α, em pacientes com LCD. Trabalhos anteriores do grupo realizados com amostras de plasma dos mesmos pacientes utilizados neste estudo demonstraram altos níveis de arginase-1 e TGF-β, enquanto os níveis de TNF-α estavam reduzidos em pacientes com LCD quando comparados com controles não infectados ou indivíduos com LCL (FRANCA-COSTA *et al.,* 2015). Esse estudo também mostrou que a expressão da arginase-1 estava aumentada *in situ* nas lesões de pacientes com LCD comparado com LCL. A associação os altos níveis de RvD1 com arginase-1 e TGF-β, pode estar contribuindo com a supressão da resposta imune do hospedeiro observada nos pacientes de LCD e pode ser

potencialmente importante para a progressão da doença (FRANCA-COSTA *et al.*, 2015). Nossos dados corroboram a ideia de que a RvD1 pode estar contribuindo com a supressão da resposta inume observada pacientes com LCD.

A expressão de enzimas envolvidas na via de produção de mediadores lipídicos foi identificada em lesões de pacientes com distintas formas de Leishmaniose Tegumentar (FRANCA-COSTA *et al.*, 2015) (FRANCA-COSTA *et al.*, 2016). Entretanto, permanece em aberto se a expressão da enzima 15-LO, responsável pela via de produção da RvD1, está aumentada *in situ* nas lesões de pacientes LCD em comparação a outras formas clínicas da leishmaniose.

Durante a fase ativa da doença, os pacientes com LCD apresentam lesões nodulares disseminadas com carga parasitária intracelular elevada nos macrófagos teciduais (SILVEIRA *et al.*, 2004). Em nosso estudo, testamos se havia uma correlação entre os níveis plasmáticos da RvD1 e a aumentada carga parasitária observada em macrófagos infectados em lesões de pacientes com LCD. Corroborando nossa hipótese, verificou-se que os pacientes com LCD que apresentavam o maior número de lesões cutâneas eram os mesmos indivíduos que exibiam os maiores níveis plasmáticos de RvD1.

Juntos os resultados citados acima, argumentam que a expressão elevada de RvD1 pode estar associada com perfil imunossuprimido observado nos pacientes com LCD, o que por sua vez, pode conduzir a replicação do parasito e a progressão da doença. Apesar de termos encontrado uma correlação positiva entre os níveis plasmáticos da RvD1 com o aumento do número de lesões, sabemos que esses mediadores são produzidos dinamicamente durante a resposta inflamatória. Por essa razão, pretendemos ampliar nossas observações iniciais desse estudo exploratório de corte transversal para estudos prospectivos adicionais. Isto se faz necessário para

responder se os níveis de RvD1 refletem a atividade da doença na LCD em comparação ao estado recidivo da doença. Além disso, será importante testar se os níveis de RvD1, bem como outros mediadores lipídicos envolvidos na fase de resolução da doença, variam em resposta ao tratamento anti-leishmanicida em outras formas clínicas da LT.

Trabalhos recentes têm demonstrado que as resolvinas são produzidas rapidamente durante a resposta inflamatória e tem seu efeito prolongado em diferentes modelos experimentais (SPITE *et al.*, 2014) (FREDMAN; VAN DYKE; SERHAN, 2011). Nosso estudo expande o conhecimento atual no contexto de infecção parasitária. Aqui nós demonstramos que a produção de RvD1 é induzida em macrófagos durante as primeiras 4 horas após a infecção por *L. amazonensis*, mas seu efeito biológico persiste até 48 horas após a infecção. Embora a produção de RvD1 induzida pela *Leishmania* tenha sido transitória, nossas observações indicam que a produção inicial desse mediador lipídico por macrófagos infectados pode ser crucial para promover a sobrevivência e proliferação intracelular da *L. amazonensis.* Apesar de demonstrar que a *Leishmania* é capaz de induzir a produção de RvD1 em nosso modelo experimental *in vitro*, o componente específico do parasito que desencadeia a ativação das vias de sinalização e impulsionam a produção de RvD1 ainda precisa ser definido.

Dentre as moléculas envolvidas, o lipofosfoglicano (LPG) destaca-se devido ao seu envolvimento em uma grande variedade de funções imunes. O LPG, glicoconjugado majoritário expresso na superfície de *Leishmania*, é reconhecido durante as interações parasito-hospedeiro pelo receptor Toll Like 2 (TLR2) ativando vias de sinalização complexas (BECKER *et al.*, 2003) (OLIVIER; GREGORY; FORGET, 2005) inibição da maturação fagossomal, modulação da produção de NO, IL-12 e da via dos eicosanoides (AVILA *et al.*, 2006). Foi demonstrado que o LPG de *L. major*, após ser reconhecido pelo TLR2, induz a ativação de células NK e macrófagos (BEKER *et al.*,

2003). Além disso, Nogueira e colaboradores demonstraram que o LPG de cepas de *L. amazonensis*, foi capaz de ativar a resposta imune via TLR4 (NOGUEIRA *et al.,* 2016). Além da ativação celular, esses receptores são importantes por controlar a infecção por *Leishmania* em macrófagos humanos através da secreção de TNF-α e INF-γ (GALLEGO *et al.*, 2011).

Sabe-se que os SPMs são biosintétizados a partir de ácidos graxos polinsaturados e desempenham um papel crítico durante a resolução da resposta inflamatória. Recentemente, foi descrito em um modelo de inflamação das vias respiratórias, que TLR7 pode mobilizar e ativar a via biosintética de produção do DHA, em macrófagos murinos e humano, levando a produção de protectinas e resolvinas da série D (KOLTSIDA *et al.*, 2013). A indução de RvD1 mediada pelo TLR7 levou à resolução da inflamação das vias aéreas. No entanto, esse efeito foi perdido quando utilizado animais *Alox15*$^{-/-}$ (KOLTSIDA *et al.*, 2013). Embora a ativação de TLR7 induza a biossíntese de resolvinas da série D, ainda é desconhecido se esses receptores são capazes de reconhecer o LPG ou outras moléculas presentes na superfície da *Leishmania*, assim como, é desconhecido se a ativação de TLRs é capaz de interferir na biossíntese de RvD1 induzida pela infecção.

O tratamento com resolvinas vem sendo utilizado em uma série de modelos inflamatórios onde sua administração exógena induz a resolução da inflamação (WANG *et al.*, 2011). Inicialmente o receptor de lipoxina A4 (ALX4) foi identificado como o sendo o mesmo receptor das RvD1 em leucócitos humanos (KRISHNAMOORTHY *et al.*, 2010). A inibição desse receptor com seu agonista resultou na inibição do efeito protetor induzido pelas resolvinas em lesões pulmonares induzidas pelo LPS (WANG *et al.*, 2011). Mais recentemente um receptor específico para RvD1 foi descrito. O receptor acoplado a proteínas G, GPR32 é expresso em leucócitos humanos, tecido adiposo e

macrófagos humanos (SCHMID *et al.,* 2016). Interessante que o GPR32 não é expresso em camundongos, sugerindo que em camundongos o efeito da RvD1 seja mediado via ALX 4 (SCHMID *et al.*, 2016).

No contexto da infecção por *L. amazonensis*, bem como para outras espécies de *Leishmania*, não sabemos se o receptor para RvD1 é regulado positivamente ou como ocorre sua interação com a resolvina induzida na célula hospedeira. Estudos futuros são necessários para avaliar se os efeitos mediados pelas resolvinas estão sendo via GRP32 e se a infecção induz sua expressão na célula hospedeira. Curiosamente, o tratamento de culturas axênicas de promastigotas com RvD1 sintética não foi capaz de alterar a curva de crescimento do parasito. Nesse contexto, nossos dados sugerem que o efeito biológico das resolvinas em nosso modelo experimental depende da função dos macrófagos e não da forma promastigota do parasito, podendo essa estar exercendo algum efeito sobre a amastigota, forma que mantém a infecção no hospedeiro.

Confirmando nossa hipótese em experimentos *in vitro*, demonstramos claramente que o tratamento com a RvD1 sintética foi hábil em aumentar a carga parasitária intracelular em macrófagos humanos.

Diante disso, avaliamos se os mecanismos ativados durante a exposição de RvD1 e o aumento da replicação intracelular do parasito estariam relacionados com a subversão das funções efetoras dos macrófagos. Está bem descrito na literatura que durante a infecção por *Leishmania,* os parasitas podem apresentar uma série de mecanismos de escape como a desativação de macrófagos (PROBST *et al.*, 2012), indução de antioxidantes (LUZ *et al.*, 2013) (KHOURI *et al.*, 2009), e citocinas anti-inflamatórias, como o TGF-β (BARRAL *et al.,* 1995). Aqui verificou-se que a infecção por *Leishmania* desencadeou a produção de TGF-β pelos macrófagos, mas a suplementação com RvD1 sintética nas culturas falhou em amplificar esse efeito. Esse

resultado sugere que a RvD1 pode afetar outro mecanismo, além da produção de TGF-β pelos macrófagos, o que pode resultar no aumento da suscetibilidade da replicação do parasita. Estudos futuros são necessários para avaliar se as resolvinas são capazes de aumentar os níveis de outras citocinas relacionadas ao favorecimento do crescimento do parasito como o IL-4 e IL-10.

Embora tenhamos observado que os pacientes com LCD que apresentavam altos níveis plasmáticos de RvD1 eram os mesmos indivíduos que exibiam os maiores níveis de TGF-β, a produção *in vitro* desse mediador não foi induzida pela RvD1. Sabe-se que o TGF-β pode ser produzido por muitos outros tipos de células como as plaquetas (MALONEY *et al.*, 2016)(SAVILL; FADOK, 2000). Assim é possível que a correlação positiva entre os níveis plasmáticos de RvD1 e TGF-β observado em nossa população de pacientes possa ser resultado de um efeito da RvD1 em outras células que não os macrófagos, já que a produção sistêmica desses mediadores envolve vários tipos celulares, diferente das culturas de macrófagos purificados utilizadas nos nossos ensaios *in vitro.*

No contexto da infecção por Leishmania, Gonçalves e colaboradores demonstraram que as plaquetas podem ser ativadas por *L. major* e que essa ativação é importante no recrutamento de leucócitos para o sítio da infecção, podendo contribuir com a defesa do hospedeiro (GONCALVES *et al.*, 2011).

As resolvinas da série E também são capazes de regular e ativar plaquetas humanas (FREDMAN, VAN DYKE & SERHAN, 2010). Essa ativação é mediada via o receptor de RvE1 presente nas plaquetas, o ChemR23 (SERHAN *et al.*, 2011). Mais recentemente, Lannan e colaboradores demonstraram que plaquetas humanas expressam outros receptores para os SPMs, dentre eles o GPR32 e o ALX (LANNAN *et al.*, 2016), além de demonstrar que a RvD1 e a lipoxina A4 também são capazes de ativar

plaquetas via esses receptores, respectivamente (LANNAN *et al.,* 2016). Embora seja conhecida a importância das plaquetas no contexto da leishmaniose e que as resolvinas são capazes de induzir e ativar essas células, para melhor compreender as discrepâncias observadas em nosso sistema *in vivo* e *in vitro*, são necessários estudos que investiguem o papel da RvD1 em plaquetas que se encontram em andamento.

Além dos efeitos anti-inflamatórios e de pró-resolução (SERHAN *et al.,* 2015), as resolvinas vem sendo descritas com um importante potencial em reduzir o estresse oxidativo através da indução da HO-1 (WANG *et al.*, 2014) (CHIANG *et al.*, 2013). No contexto de infecção por *Leishmania*, o grupo demonstrou que a indução de HO-1 pelo parasito representa um importante mecanismo de escape que funciona através da modulação da produção de espécies reativas de oxigênio por macrófagos humanos e murinos (LUZ *et al.*, 2013). Aqui nós mostramos que a suplementação com RvD1 nas culturas de macrófagos resultou na indução robusta da expressão da HO-1. A HO-1 é uma enzima intracelular e seus níveis circulantes podem não representar diretamente a expressão *in situ*. De fato, não encontramos associação entre os níveis plasmáticos de RvD1 com a HO-1 nos pacientes com leishmaniose tegumentar. Além de analisar a expressão da via dessa enzima *in situ*, investigações que descrevam outros mecanismos potenciais que expliquem o efeito da RvD1 na infecção intracelular de *Leishmania* são necessários para ampliar nosso conhecimento sobre a modulação das funções efetoras de macrófagos por esse mediador lipídico. Um possível candidato seria a indução da SOD pela RvD1, uma vez que já foi demonstrado que esse mediador é capaz de favorecer a infecção por *L. amazonensis* e *L. braziliensis* (KHOURI *et al.,* 2009).

O TNF-α tem um papel crítico na resposta oxidativa durante infecções parasitárias (MAGEZ *et al.*, 2007) (DAULOUEDE *et al.*, 2001). Trabalhos recentes do grupo mostram que a regulação negativa da produção de TNF-α induzida pela HO-1 é o

evento chave que dirige o aumento da sobrevivência da *L. infantum chagasi* em macrófagos humanos e murinos infectados (LUZ *et al.*, 2012). Mais recentemente, um estudo utilizando PBMCs de pacientes com cardiomiopatia associada à Doença de Chagas demonstrou que a suplementação com RvD1 na cultura de células interferiu na sobrevivência celular sem afetar a produção de TNF-α e IL-10, por outro lado a RvD1 foi capaz de reduzir os níveis de INF-ϒ (OGATA *et al.*, 2016). Nossos dados estão de acordo com os encontrados na literatura, onde o efeito biológico da RvD1 não altera a produção TNF-α. Além disso, nossos resultados não mostram qualquer associação entre a indução da HO-1 associada a RvD1 e alterações na produção de TNF-α, sugerindo que essa relação depende do modelo estudado. Isso reforça a ideia que a RvD1 pode atuar independentemente do TNF-α no nosso modelo. Além disso, é importante avaliar se a RvD1 é capaz de reduzir outras citocinas pró-inflamatórias como o INF-ϒ e IL-12, durante a infecção por *L. amazonensis*.

Estudos adicionais com outras espécies de *Leishmania* são necessários para avaliar o efeito da RvD1 e HO-1 sobre a produção de TNF-α, já que se sabe que sua produção pode ser alterada a depender do modelo ou espécie de parasita envolvida. Dados preliminares indicam que a adição de RvD1 sintética também favorece o aumento da carga parasitária em macrófagos humanos infectados com *L. braziliensis* (dados não mostrados).

A inibição de enzimas da via dos eicosanoides tem sido amplamente utilizada para controlar respostas inflamatórias (MEDEIROS *et al.*, 2012), bem como a carga parasitária de infecções por *Leishmania* (AFONSO *et al.*, 2008) (ARAÚJO-SANTOS *et al.*, 2015). Em nosso modelo experimental *in vitro*, nós demonstramos que o tratamento com a baicaleina, inibidor da 15-LO (lipoxigenase 15), resultou na redução da produção de RvD1 que refletiu a diminuição da viabilidade intracelular do parasita.

Embora a baicaleina tenha sido capaz de inibir a produção de RvD1, ela não é um inibidor específico da 15-LO, podendo inibir também outras lipoxigenases. Logo, estudos futuros utilizando inibidores específicos da 15-LO são necessários afim de utilizar a inibição farmacológica da produção de RvD1 como uma possível estratégia terapêutica para reduzir a carga da infecção por *L. amazonensis.* A recente descoberta de receptores específicos para RvD1 abre novas perspectivas como estratégia terapêutica, uma vez que a utilização de inibidores seletivos ou agonistas desses receptores poderiam reduzir os efeitos induzidos pela RvD1 dentro da célula, como o aumento da carga parasitária. No entanto, estudos adicionais são necessários para avaliar esses aspectos, bem como o efeito desse mediador em outras espécies de *Leishmania*.

8 CONCLUSÃO

A elevação dos níveis circulantes de RvD1 detectados nos pacientes com LCD, juntamente com as fortes relações com os biomarcadores estreitamente relacionados com o ambiente anti-inflamatório desses pacientes, indicam que esse mediador lipídico participa da patogênese da LCD. Além disso, nosso resultados *in vitro* sugerem que a RvD1 pode induzir um mecanismo antioxidativo via ativação da enzima heme-oxigenase 1 nos macrófagos infectados por *L. amazonensis* o que faz com que essas células se tornem mais permissivas à infecção e proliferação do parasito.

9 REFERÊNCIAS

Aqui estão listadas as referências utilizadas na introdução e discussão geral da dissertação. As referências citadas apenas no manuscrito não estão listadas nesta seção.

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10. ANEXOS

10.1 Anexo 1

MAJOR ARTICLE

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

Jaqueline França-Costa,[1] Johan Van Weyenbergh,[1] Viviane S. Boaventura,[1,2] Nívea F. Luz,[1,2] Hayna Malta-Santos,[1,2] Murilo Cezar Souza Oliveira,[1,2] Daniela Conceição Santos de Campos,[1,2] Ana Cristina Saldanha,[3] Washington L. C. dos-Santos,[1] Patrícia T. Bozza,[4] Manoel Barral-Netto,[1,2,5] Aldina Barral,[1,2,5] Jackson M. Costa,[1] and Valeria M. Borges[1,2,5]

[1]Centro de Pesquisas Gonçalo Moniz/FIOCRUZ-BA and [2]Faculdade de Medicina, Universidade Federal da Bahia, Salvador, [3]Universidade Federal do Maranhão, UFMA, [4]Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, and [5]Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia, São Paulo, Brazil

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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Correspondence: Valéria Borges, PhD, Rua Waldemar Falcão, 121, Candeal, CEP 40295-001, Salvador, Bahia, Brazil (vborges@bahia.fiocruz.br).

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and is highly expressed in lesions from patients with LCL [5]. This enzyme metabolizes L-arginine into urea and L-ornithine, the substrate used for ornithine decarboxylase (ODC) to produce polyamines that are crucial for parasite replication. Macrophages infected with *Leishmania major* or *Leishmania infantum* and treated with LOHA (L-hydroxyl arginine), an arginase inhibitor, exhibit significant decrease in parasite load [6]. Moreover, DFMO, a potent ODC inhibitor, effectively inhibits *Leishmania donovani* promastigote growth in culture [7].

Leishmania amazonensis, the sole agent implicated in DCL in Brazil [8], increases arginase I, transforming growth factor β (TGF-β), and prostaglandin E_2 (PGE_2) expression, contributing to intra-macrophage parasite proliferation [9–11]. Nevertheless, the relevance of these mediators in DCL pathogenesis remains unknown. In the present study, we investigated the systemic levels and in situ expression of inflammatory mediators in samples from patients with DCL, patients with LCL, and healthy controls, who were identified among family members of patients with DCL. High levels of arginase I, ODC, PGE_2, and TGF-β were observed in plasma specimens from patients with DCL. In addition, in situ transcriptomic analyses reinforced the association between arginase I expression and the enzymes involved in the pathways of prostaglandin and polyamine biosynthesis at the messenger RNA (mRNA) level. Moreover, arginase and ODC inhibitors prevented parasite replication and modulated inflammatory mediators in human monocyte-derived macrophages infected with *L. amazonensis*. Our study highlights that arginase I, ODC, and COX-2 biosynthetic enzymes can be used as potential drug targets for *Leishmania* infection.

MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized. The project was approved by the institutional review board of Centro de Pesquisas Gonçalo Moniz, FIOCRUZ–BA (license number 136/2007), and comply with the guidelines of the Declaration of Helsinki.

Patient Characteristics

All patients with DCL (n = 12) were followed by one of the authors (J. M. C.), and their characteristics have been reported previously [3]. Their diagnoses were established as described elsewhere [12]. Patients with DCL exhibited a chronic evolution of the disease with several remissions, multiple nodular and highly parasitized lesions throughout the skin, and a negative DTH response. Patients with LCL (n = 29) with a single or few ulcerated lesions present for up to 2 months and a positive DTH response were followed by one of the authors (A. B.) [13]. The clinical and epidemiological data from patients with DCL and those with LCL are summarized in Table 1. Controls were identified among 49 family members of the patients with DCL and underwent careful physical examinations, exhibiting no cutaneous lesions or prior CL history and a negative DTH response. Individual blood samples were collected after examination. Skin biopsy specimens (3 or 4 mm) were obtained from 4 patients with DCL and 7 patients with LCL, embedded in a cryopreservation resin, snap frozen, and stored in liquid nitrogen. We did not have lesion biopsy specimens available from all patients during the period of this study, and since the procedure is too invasive not all patients have allowed collection of a new lesion biopsy specimen.

Table 1. Epidemiological and Clinical Parameters for Patients With Diffuse Cutaneous Leishmaniasis (DCL) and Those With Localized Cutaneous Leishmaniasis (LCL)

Characteristic	LCL Group (n = 29)	DCL Group (n = 12)	*P* value
Sex, no.			.09
Male	19	7	
Female	10	5	
Age, y			.06
Mean ± SD	34 ± 15	17 ± 23	
Range	13–65	4–41	
Active lesions, no.			.001
Mean	1	138	
Range	1–9	22–500	
DTH positivity, %	100	0	.0001
Disease duration, mo			.0001
Mean	3	142	
Range	1–6	36–276	

Abbreviations: DTH, delayed hypersensitivity skin-test response; SD, standard deviation.

Inflammatory Mediator Measurements

Plasma levels of arginase I (Hycult Biotech, Uden, the Netherlands), ODC (Wuhan EIAAB Science, Wuhan, China) and TGF-β (R&D Systems, Minneapolis, Minnesota) were measured using enzyme-linked immunosorbent assay ELISA according to the manufacturer's instructions. The total TGF-β level was measured in plasma after acidification, according to the manufacturer's instructions. The plasma levels of interleukin 10 (IL-10), interleukin 12 (IL-12), interferon γ (IFN-γ), monocyte chemotactic protein 1 (MCP-1), CXCL-10, and tumor necrosis factor α (TNF-α) were measured using a Cytometric Bead Array (CBA) Human Inflammatory kit (BD Biosciences Pharmingen, San Diego, California) according to the manufacturer's protocol. The flow cytometry assay was performed and analyzed by a single operator. PGE_2 production was measured using a specific enzyme immunoassay (the PGE_2 EIA kit, Cayman Chemical, Ann Arbor, Michigan) according to the manufacturer's instructions. TGF-β, PGE_2, TNF-α, and IL-12 expression was measured in supernatants by the same protocols.

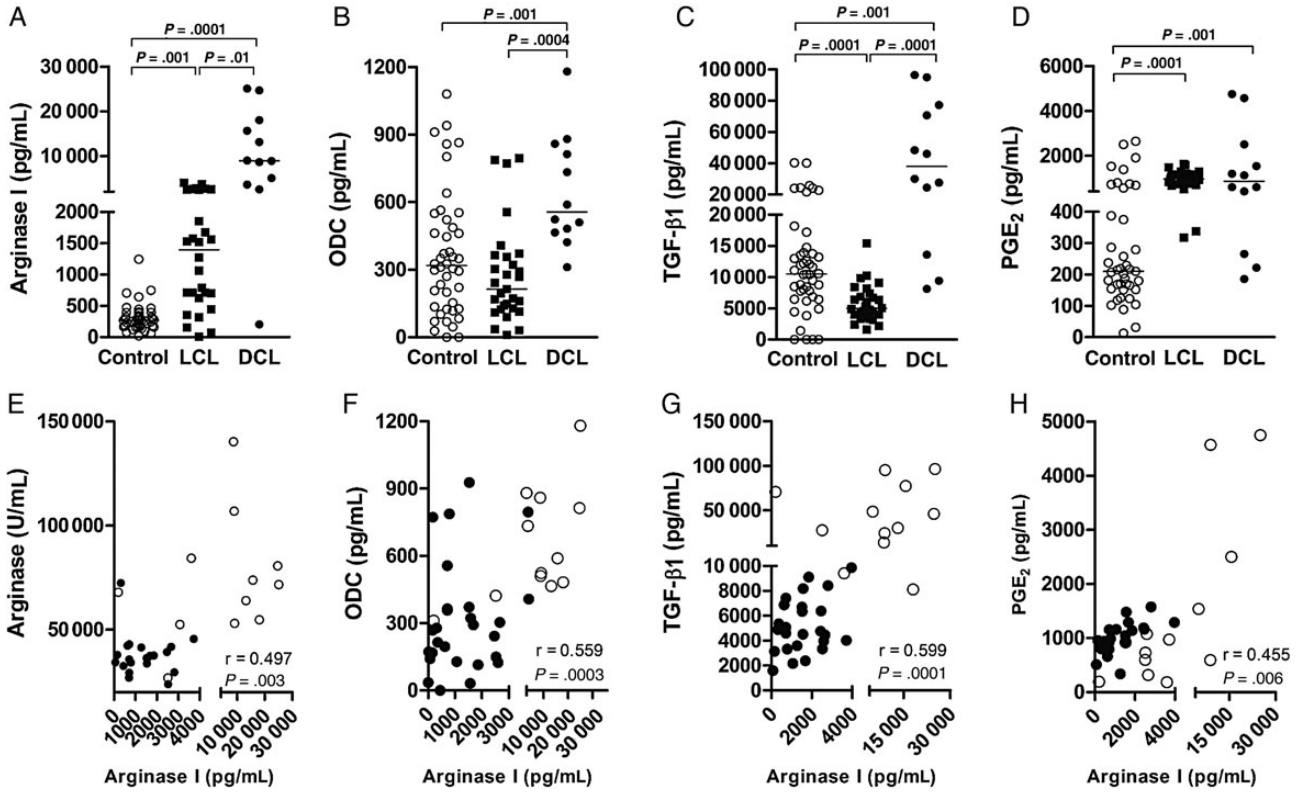

Figure 1. Arginase I, ornithine decarboxylase (ODC), and antiinflammatory mediators in plasma specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Plasma protein levels of arginase I (*A*), ODC (*B*), transforming growth factor β (TGF-β; *C*), and prostaglandin E_2 (PGE_2; *D*) were measured by an enzyme-linked immunosorbent assay in healthy controls (n = 35), patients with active DCL (n = 12), and patients with localized cutaneous leishmaniasis (LCL; n = 30). Correlations between arginase I and arginase activity (*E*), ODC (*F*), TGF-β (*G*), and PGE_2 (*H*) were calculated. Each point represents a different donor, and each bar represents the median. Open circles represent patients with DCL, and closed circles represent patients with LCL. Differences between groups were tested by the Kruskal–Wallis test with the Dunn multiple comparisons post hoc test. The Spearman test was used to verify the significance of correlations between parameters. Data are representative of at least 2 independent assays performed in duplicate for each patient or control.

Measurement of Arginase Enzymatic Activity

Arginase activity was measured by a colorimetric assay for the detection of urea in plasma samples, as previously described [14].

Immunohistochemistry

Immunohistochemistry was performed as previously reported by us [15], using primary antibodies against human arginase I (2 μg/mL; 1:100; Santa Cruz Biotechnology, Dallas, Texas), ODC (4 μg/mL; 1:100; Cayman Chemical, Ann Arbor, Michigan), and COX-2 (2 μg/mL; 1:50; Cayman Chemical, Ann Arbor, Michigan). Digital images were obtained from fields of 400× original magnification and captured using a Nikon E600 microscope and an Olympus Q-Color 1 digital camera with the Image-Pro Plus program.

nCounter Analysis

Total RNA was extracted from lesion biopsy specimens from 4 patients with DCL and 7 patients with LCL, using TRIzol, according to the manufacturer's protocol, with an additional purification step performed using RNeasy columns (Qiagen Benelux, Venlo, the Netherlands). nCounter (NanoString Technologies, Seattle, Washington) analysis was performed at the VIB MicroArray Facility (Leuven, Belgium) based on direct molecular bar coding of target RNA transcripts and digital detection [16]. Through the use of color-coded probe pairs and direct hybridization and without the use of reverse transcriptase or amplification, the following host-specific cellular genes were quantified: *ARGI*, which encodes arginase I; *ODC*, which encodes ornithine decarboxylase; *EP1*, which encodes prostaglandin E receptor 1; *EP2*, which encodes prostaglandin E receptor 2; *EP3*, which encodes prostaglandin E receptor 3; *EP4*, which encodes prostaglandin E receptor 4; *PLA2G4A*, which encodes phospholipase A2, group IVA (cytosolic, calcium-dependent); *PTGS1*, which encodes prostaglandin-endoperoxide synthase 1; *PTGS2*, which encodes prostaglandin-endoperoxide synthase 2; *PTGES*, which encodes prostaglandin E synthase; *SMS*, which encodes spermine synthase; and *SRM*, which encodes spermidine synthase. The following housekeeping genes were also quantified for normalization at the femtomolar range: *GUSB*,

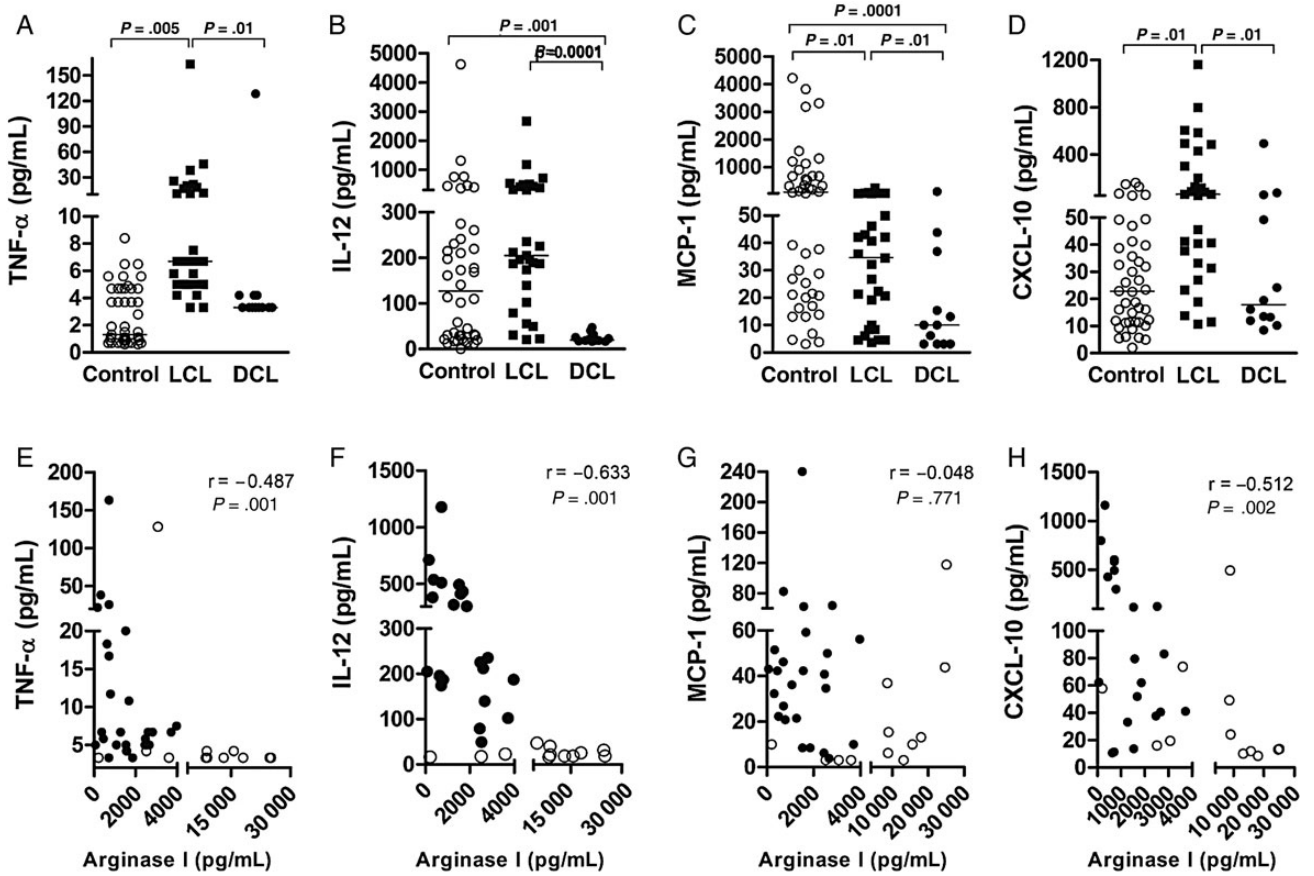

Figure 2. Proinflammatory cytokines and chemokines in plasma specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Plasma protein levels of tumor necrosis factor α (TNF-α; *A*), interleukin 12 (IL-12; *B*), monocyte chemotactic protein 1 (MCP-1; *C*), and CXCL-10 (*D*) were measured by the Cytometric Bead Array kit in healthy controls (n = 35), patients with active DCL (n = 12), and patients with LCL (n = 30). Correlations between arginase I and TNF-α (*E*), IL-12 (*F*), MCP-1 (*G*), and CXCL-10 (*H*) levels were measured. Each point represents a different individual, and each bar represents the median. Open circles represent patients with DCL, and closed circles represent LCL patients. Differences between groups were tested by the Kruskal–Wallis test with the Dunn multiple comparisons post hoc test. The Spearman test was used to verify the significance of correlations between parameters. Data are representative from at least 2 independent assays performed in duplicate for each patient or control.

which encodes β-glucuronidase; *G6PD*, which encodes glucose-6-phosphate dehydrogenase; *GAPDH*, which encodes glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, which encodes hypoxanthine phosphoribosyltransferase; and *CD45*, which encodes the pan-leukocyte marker CD45.

Cell Culture

PBMCs and monocytes were isolated and cultured as previously described [17]. The promastigotes used in these experiments came from an *L. amazonensis* strain (MHOM/BR/87/BA336) isolated from a patient with DCL. The methods of parasite culture and macrophage infection have been previously reported [18]. Macrophages were infected with early stationary phase *L. amazonensis* at a parasite to cell ratio of 6:1. After 4 hours of incubation at 34°C, free parasites were removed by extensive washing with phosphate-buffered saline, and 10 μg/mL nor-NOHA or 0.5 mM DFMO (arginase and ODC inhibitors) was added to the cultures (both from Sigma-Aldrich, St. Louis, Missouri). The intracellular parasite load was estimated at 24 and 72 hours after infection by light microscopy and the production of viable promastigotes in Schneider medium, as described previously [18].

Statistical Analysis

For ordinal variables, differences between groups were calculated using the nonparametric Kruskal–Wallis test with the 2-tailed Dunn multiple comparisons post hoc test and the Mann–Whitney unpaired *t* test for 2-group comparisons. The χ^2 test was used to compare differences between categorical variables. The Spearman test was used to verify the significance of correlations between arginase I and the plasma levels of arginase activity, ODC, TGF-β, PGE_2, TNF-α, IL-12, MCP-1, IFN-γ, and CXCL-10 (nonparametric data). For transcriptomic

(nCounter) analysis, in situ mRNA levels were normalized to CD45 mRNA levels, followed by log transformation, allowing linear regression analysis of the interactions between inflammatory mediators, as previously described [19,20]. The values in the text represent medians ± standard deviations. Differences were considered statistically significant at P values of ≤ .05. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software).

RESULTS

Inflammatory Imbalance Mediators in Plasma Specimens From Patients With DCL

Plasma specimens from patients with DCL exhibited the highest arginase I levels, by at least 5-fold, compared with patients with LCL or controls (Figure 1*A*). Patients with DCL also exhibited higher arginase activity (69.850 ± 29.017 U/mL) than patients with LCL (36.602 ± 9.659 U/mL) and controls (28.971 ± 11.037 U/mL). L-Ornithine, the arginase product, can be used by ODC, resulting in polyamine production [14]. ODC was augmented in DCL plasma when compared with the control and LCL groups (Figure 1*B*).

We next investigated the mediators known to modulate the L-arginine metabolic pathways. TGF-β displayed higher levels in plasma from patients with DCL than patients with LCL (Figure 1*C*). Similarly, PGE_2 levels were higher in patients with DCL than in controls, but no difference was detected between patients with DCL and patients with LCL (Figure 1*D*). Arginase I exhibited a positive correlation with antiinflammatory mediators, including arginase activity, ODC, TGF-β, and PGE_2 (Figure 1*E*–*H*). However, the proinflammatory cytokines and chemokines TNF-α (Figure 2*A*), IL-12 (Figure 2*B*), MCP-1 (Figure 2*C*), and CXCL-10 (Figure 2*D*) were reduced in plasma specimens from the DCL group, compared with levels in plasma from the LCL and control groups. Arginase I exhibited a negative correlation with TNF-α, IL-12, and CXCL-10 (Figure 2*E*–*H*) but not with MCP-1 (Figure 2*G*).

To determine whether arginase I, ODC, and COX-2 were also expressed in situ, we performed a comprehensive analysis of genes implicated in this inflammatory pathways. The mRNA levels of the selected genes were quantified and normalized to the mRNA level of the pan-leukocyte marker CD45 to correct varying levels of leukocyte infiltration between patient lesion biopsy specimens. Cells in lesion biopsy specimens from the DCL group exhibited significantly increased arginase I mRNA levels, compared with those from the LCL group (Table 2), corroborating the increased levels of this enzyme found in plasma specimens from patients with DCL. However, the ODC mRNA levels did not significantly differ between the DCL and LCL groups. The mRNA levels of IL-4 and IL-10 were significantly increased, although the TNF-α mRNA level was decreased in lesions from the DCL group in situ (Table 2). PGE_2 binds to 4 cellular receptors, of which EP2 mRNA was significantly overexpressed in lesion biopsy specimens from the DCL group, compared with those from the LCL group (Table 2). Present upstream of PGE_2, phospholipase A2 is the crucial enzyme for arachidonic acid production, and levels of this enzyme were significantly higher in DCL lesions, compared with LCL lesions (Table 2).

Table 2. Messenger RNA (mRNA) Levels of Inflammatory Mediators in Lesion Biopsy Specimens From Patients With Diffuse Cutaneous Leishmaniasis (DCL) and Those With Localized Cutaneous Leishmaniasis (LCL)

Mediator	LCL Group (n = 7)	DCL Group (n = 4)	*P* value
Arginase I	0.02	1.02	<.04
ODC	−0.63	−0.88	<.64
EP-2	−0.96	−0.53	<.006
PLA2G4A	−1.28	−1.02	<.09
Interleukin 4	−3.02	−1.72	<.02
Interleukin 10	−2.47	1.66	<.01
TNF-α	−0.35	−0.68	<.01

Values were obtained by nCounter digital quantification of mRNA levels and normalized to the CD45 mRNA level, followed by log transformation. Negative values thus reflect a ratio of mediator mRNA level to CD45 mRNA level of <1.

Abbreviations: EP-2, prostaglandin E receptor 2; ODC, ornithine decarboxylase; PLA2G4A, phospholipase A2, group IVA; TNF-α, tumor necrosis factor α.

As shown in Figure. 3, arginase I mRNA levels were positively correlated with PLA2G4A, PTGES, and PTGS2 mRNA levels (Figure 3*A*–*C*), all of which are enzymes involved in prostaglandin synthesis. Arginase I expression was also positively correlated with spermine synthase and spermidine synthase expression (Figure 3*D* and 3*E*), which are enzymes responsible for polyamine synthesis.

Arginase I, ODC, and COX-2 Expression in Lesion Biopsy Specimens From Patients With DCL

Arginase I staining was more widespread in lesion biopsy specimens from the DCL group (n = 3) than in those from the LCL group (n = 3), as shown in Figure 4. The expression of ODC and COX-2, which are involved in polyamine and PGE_2 synthesis, respectively, exhibited an increase in DCL lesions when compared with LCL lesions. No reactivity was detected using an isotype control antibody (data not shown).

Arginase and ODC Inhibition Control *L. amazonensis* Infection in Human Macrophages

Macrophages infected with *Leishmania* organisms exhibited $10.9 \times 10^4 \pm 2.1 \times 10^4$ viable parasites at 24 hours after infection. The groups treated with nor-NOHA or DFMO presented no difference in parasite load at the same time (Figure 5*A*). However, 72 hours after infection, the unstimulated cells group showed parasite proliferation ($6.2 \times 10^7 \pm 1.75 \times 10^7$), and the groups treated with nor-NOHA and DFMO exhibited $3.6 \times 10^4 \pm 6.05 \times 10^4$ and $1 \times 10^5 \pm 1.40 \times 10^4$ viable parasites, respectively,

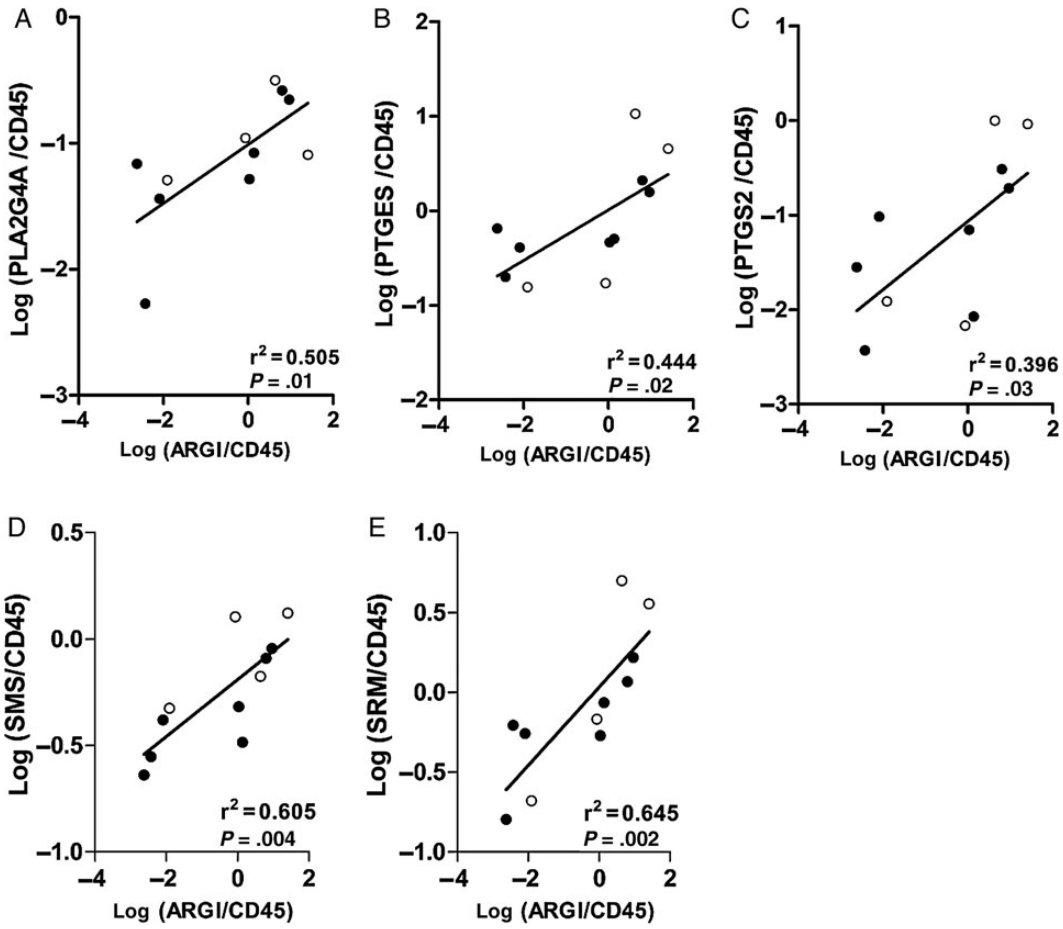

Figure 3. Arginase I in situ is related to polyamine and PG pathways in lesion biopsy specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Total RNA was extracted from lesion biopsy specimens for patients with LCL (n = 7) and those with DCL (n = 4), and the messenger RNA transcripts of host-specific cellular genes were quantified by nCounter (Nanostring), including that of a housekeeping gene encoding CD45, for normalization. Linear regression between ARG1 (which encodes arginase I) and PLA2G4A (which encodes phospholipase A2; (*A*), PTGES (which encodes prostaglandin E synthase; (*B*), PTGS2 (which encodes prostaglandin-endoperoxide synthase [known as cyclooxygenase 2]; (*C*), and the polyamine biosynthetic pathways for SMS (which encodes spermine synthase; (*D*) and SMR (which encodes spermidine synthase; (*E*). Open circles represent patients with DCL, and closed circles represent patients with LCL. The statistical significance of differences was determined by linear regression. The data were collected in triplicate for each donor.

which were both significantly lower than the level in unstimulated cells (Figure 5*A*). In addition, at 72 hours after infection, photomicrographs confirmed that cells treated with nor-NOHA and DFMO were able to control the parasite burden more efficiently than cells in the untreated group (Figure 5*B*).

Next, we measured essential mediators of *L. amazonensis* survival and proliferation in human macrophage culture supernatants at 24 hours. First, we observed that nor-NOHA or DFMO treatment decreased TGF-β (Figure 5*C*) and PGE_2 (Figure 5*D*) production by at least 50%, compared with the control group. Subsequently, we measured the levels of key cytokines that control *Leishmania* infection. nor-NOHA–treated cells exhibited a 3-fold increase in both TNF-α (Figure 5*E*) and IL-12 (Figure 5*F*) levels, whereas DFMO-treated cells did not exhibit altered cytokine levels, compared with untreated cells.

DISCUSSION

DCL is characterized by an inefficient parasite-specific cellular response and heavily parasitized macrophages. However, the immunopathogenic mechanisms underlying this disease remain unclear. The findings reported here lead us to propose a straightforward pathway by which arginase I, ODC, PGE_2, and TGF-β contribute to DCL lesions, thus resulting in a permissive microenvironment for *Leishmania* proliferation and progression to chronic disease.

Plasma from patients with DCL exhibited higher levels of arginase I expression and activity, compared with plasma from patients with LCL or controls. High levels of arginase activity in plasma and saliva have been reported in several other human pathologic conditions as a useful biological biomarker,

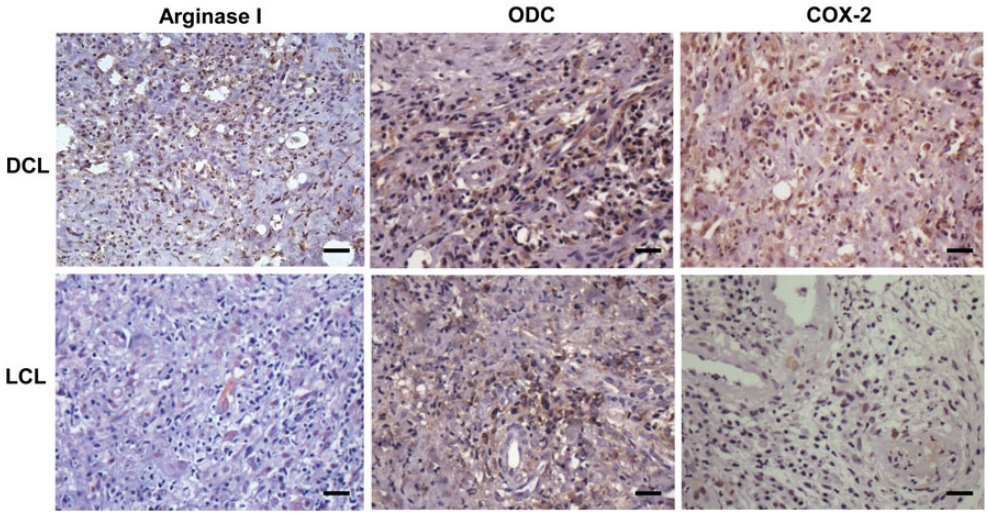

Figure 4. Arginase I, ornithine decarboxylase (ODC), and COX-2 expression in lesions biopsy specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Immunohistochemistry was performed on paraffin-embedded sections of lesion biopsy specimens from patients with DCL and those with LCL, using primary antibodies against arginase I (2 μg/mL; 1:100), ODC (4 μg/mL; 1:100), and COX-2 (2 μg/mL; 1:50). The labeling was revealed with diaminobenzidine (DAB), followed by counterstaining with Harris hematoxylin. Data are representative of at least 3 independent assays and were collected in duplicate for each patient. Digital images (400× original magnification) were captured using a Nikon E600 microscope and an Olympus Q-Color 1 digital camera with the Image Pro Plus program. Bars represent 10 μm.

in some cases representing an indicator of disease progression [21, 22], but the mechanism by which arginase I is released remains unclear. Recently, increased arginase I activity was demonstrated in situ in LCL lesions from Ethiopian patients [5], as well as in plasma specimens from patients with visceral leishmaniasis [14]. The difference in arginase I expression in plasma and lesions could be due to parasite-related factors or could be a consequence of polymorphisms in host genes, as indicated for other pathologic conditions [23, 24]. Regardless of arginase I source, the data presented here suggest that arginase I levels in plasma are unbalanced in patients with DCL and those with LCL, compared with healthy controls. Furthermore, the relationship between arginase I levels and clinical presentations suggests that arginase I may represent a biomarker for cutaneous leishmaniasis severity.

The arginase I pathway is preferentially driven by the presence of antiinflammatory mediators, such as the lipid mediator PGE_2 [25] and TGF-β [26]. Although PGE_2 production in plasma specimens from patients with DCL and those with LCL did not differ, both patient subgroups exhibited higher values, compared with values for controls, indicating that PGE_2 might be relevant to cutaneous leishmaniasis. PGE_2 can use 4 G-protein–coupled E-prostanoid receptors (prostaglandin E receptors 1–4) [27]. Interestingly, differences between DCL and LCL lesions were only detected in EP2 mRNA levels. PGE_2 signaling through EP2 promotes T-helper type 2 (Th2) immune responses [28] and the suppression of the microbicidal activity of alveolar macrophages through prostaglandin E receptors 2/4, increasing cAMP levels and inhibiting the assembly and activation of p47phox [29].

Indeed, the potent immunomodulatory effects exhibited by PGE_2 are ambiguous, depending on the profile of in situ mediators. PGE_2 has been shown to synergize with TNF-α to induce high levels of IL-12 production by dendritic cells [30], whereas TGF-β synergizes with PGE_2 to block IFN-γ and TNF-α secretion [31]. These effects are associated with suppression of the host immune response and a switch from a Th1 to a Th2 response [32]. Therefore, systemic PGE_2 in LCL, in concert with TNF-α and IL-12, appears to support effective activation of the immune system, whereas in patients with DCL, PGE_2 and TGF-β have the opposite immunosuppressive effect. Indeed, arginase I plasma levels are upregulated by antiinflammatory mediators and downregulated by proinflammatory mediators, as shown by correlation analysis. This setting appears to be decisive in the clinical outcome of cutaneous leishmaniasis—localized versus metastatic, diffuse disease. The dispersion of mediators found in the plasma suggests that there are subgroups of patients with DCL with possible differences in the pattern of inflammatory response. Since the present study has a limited number of patients with DCL and therefore subgroup analyzes would not have statistical significance, further studies are necessary to clarify this question.

In addition to arginase I, ODC, the subsequent downstream enzyme required for polyamine synthesis, was also enhanced in plasma specimens from patients with DCL, compared with those from patients with LCL. In addition to ODC, L-ornithine

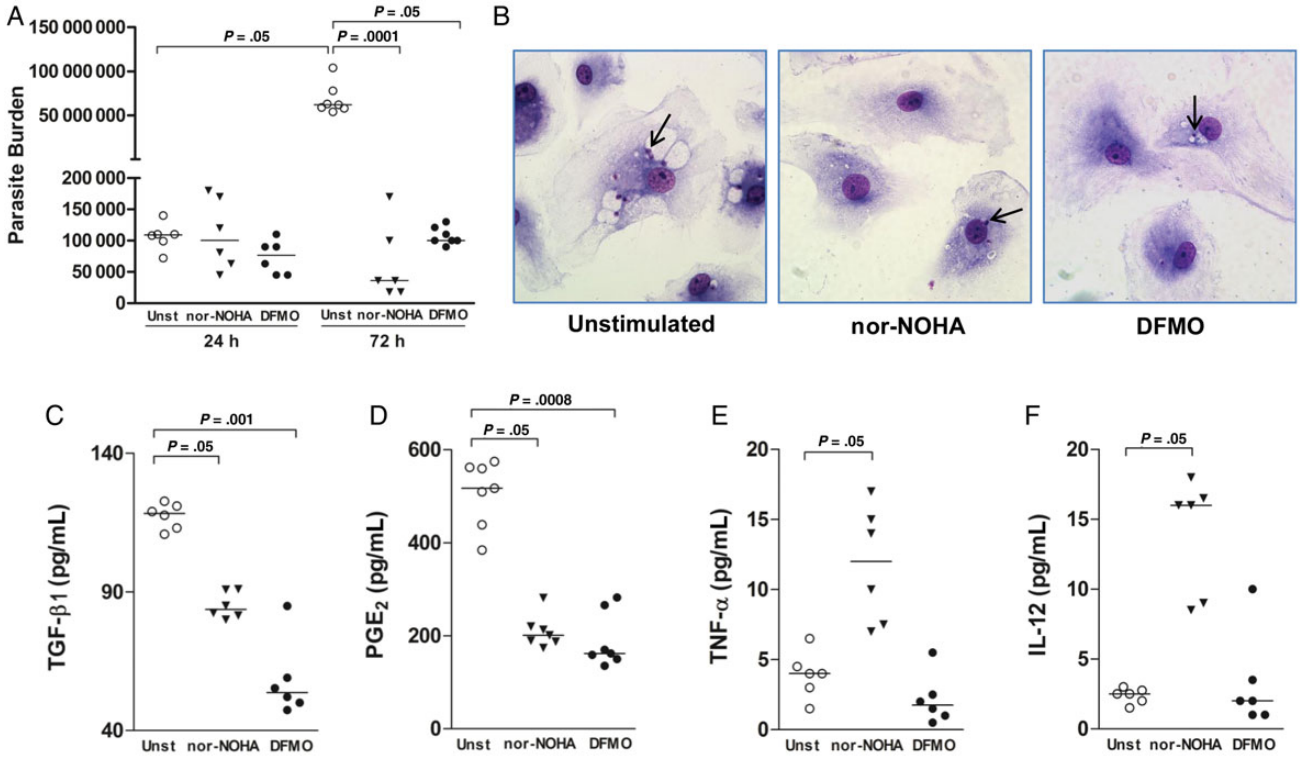

Figure 5. Inhibition of arginase I and ornithine decarboxylase abrogate *Leishmania* replication in infected human macrophages. Infected macrophage monolayers were cultured with medium alone (○), NOHA (▾), or DFMO (•) (*A*). Parasite burden 24 and 72 hours after infection. Each point represents a different donor, and each bar represents the median. *B*, Micrographs from *Leishmania amazonensis*–infected human macrophages unstimulated or in the presence of 100 mM nor-NOHA or DFMO for 72 hours. Arrows point to amastigotes inside a parasitophorous vacuole. Original magnification × 1000. Bars represent 10 μm. Differences were evaluated using the Kruskal–Wallis test with the Dunn multiple comparisons post hoc test. Supernatants were collected after 24 hours and assayed for the presence of transforming growth factor β (TGF-β; *C*) and prostaglandin E_2 (PGE_2; *D*), by enzyme-linked immunosorbent assay, and the presence of tumor necrosis factor α (TNF-α; *E*) and interleukin 12 (IL-12; *F*), by the Cytometric Bead Array kit. Statistical significance was determined by a 2-tailed Mann–Whitney test. Data are representative of at least 3 independent assays and were collected in triplicate for each condition. Abbreviation: Unst, unstimulated.

can also be metabolized to proline by ornithine aminotransferase, which is related to collagen production. The increased expression of ODC indicates that the arginase pathway is being deviated toward the first step of polyamine biosynthesis. In addition, both arginase I and ODC exhibit increased expression in DCL lesions, compared with LCL lesions, implying increased polyamine production in situ. Likewise, we observed positive correlations between arginase I mRNA levels and the levels of spermine and spermidine synthase mRNA, the 2 enzymes responsible for polyamine synthesis. Histological analysis of DCL lesions revealed heavily parasitized cells, whereas in LCL lesions, parasites were scarce [13]. Therefore, the high expression of polyamine biosynthetic enzymes may possibly contribute to the intense *Leishmania* proliferation in macrophages in patients with DCL. However, whether the level of the final product polyamine is actually increased in patients with DCL remains unclear and needs to be further investigated.

Arginase I was also related to phospholipase A2, COX-1, COX-2, and prostaglandin E2 synthase, which are all enzymes involved in PGE_2 syntheses. The relationship between arginase I and the prostaglandin biosynthetic pathway is reinforced by the difference in in situ COX-2 expression between DCL and LCL lesions. COX-2 expression is induced by stimuli such as growth factors and cytokines. TGF-β has been associated with COX-2/PGE_2 expression in peripheral blood lymphocytes [33], where it suppresses the degradation of COX-2 mRNA [34]. In addition, TGF-β enhances arginase activity in macrophages and hence increases polyamine release [35]. In agreement with the literature, our data demonstrate that both TGF-β and COX-2 are increased in patients with DCL and are positively correlated with arginase I, suggesting their involvement in its production.

The inhibition of polyamine pathway enzymes is efficient for experimental *Toxoplasma* and trypanosome infections [36] and has been used as a treatment in patients with African sleeping sickness [37]. Arginase and ODC inhibition directly affects the production of polyamines, which are critical for the survival and replication of *Leishmania* parasites [6, 38]. Hence, in our model, control of *L. amazonensis* replication in macrophages

treated with nor-NOHA (arginase inhibitor) and DFMO (ODC inhibitor) could be mediated by a decrease in polyamine levels. Furthermore, nor-NOHA and DFMO appear to modulate the inflammatory response. Arginase and ODC inhibition decreased levels of TGF-β and PGE_2, which are crucial to parasite survival and proliferation [39]. Moreover, treatment with nor-NOHA increased levels of TNF-α and IL-12, which can activate macrophages to produce nitric oxide [40], a potent leishmanicidal molecule. Accordingly, treatment with the arginase inhibitor nor-NOHA altered the cytokine profile, suggesting that the functional status of macrophages was changed from classical activation to alternative activation. Interesting, DFMO treatment did not increase proinflammatory cytokine production, although DFMO treatment was as efficient as nor-NOHA in controlling parasite load. These data suggest that polyamines are sufficient to support *L. amazonensis* survival and multiplication and that the inhibition of polyamines is decisive for the outcome of infection.

Taken together, our data implicate the local and systemic release of arginase I, prostaglandins, and polyamines in the inability of patients with DCL to mount an efficient immune response against *L. amazonensis* infection, providing a favorable environment for parasite replication and the dissemination of the disease. Considering that DCL is still without an effective treatment, the discovery of novel targets for chemotherapy is extremely important. Our study highlights arginase I, ODC, and COX-2 as promising targets for DCL treatment, and inhibitors of these enzymes are already used successfully in the control of other pathologic conditions. Drugs interfering with these metabolic pathways are commercially available and could be used in future clinical trials, reestablishing a balanced inflammatory response to control parasite replication in DCL.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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10.1 Anexo 2

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BRIEF REPORT

Differential Expression of the Eicosanoid Pathway in Patients With Localized or Mucosal Cutaneous Leishmaniasis

Jaqueline França-Costa,[1] Bruno B. Andrade,[1,2] Ricardo Khouri,[1] Johan Van Weyenbergh,[1,6] Hayna Malta-Santos,[1,3] Claire da Silva Santos,[1] Cláudia I. Brodyskn,[1,3,4] Jackson M. Costa,[1] Aldina Barral,[1,3,4] Patrícia T. Bozza,[5] Viviane Boaventura,[1,3] and Valeria M. Borges[1,3]

[1]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research Initiative, Fundação José Silveira, and [3]Universidade Federal da Bahia, Salvador, [4]Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia, São Paulo, and [5]Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil; and [6]Department of Microbiology and Immunology, Rega Institute for Medical Research, University of Leuven, Belgium

Unfettered inflammation is thought to play critical role in the development of different clinical forms of tegumentary leishmaniasis. Eicosanoids are potent mediators of inflammation and tightly associated with modulation of immune responses. In this cross-sectional exploratory study, we addressed whether targets from the eicosanoid biosynthetic pathway, assessed by multiplexed expression assays in lesion biopsy and plasma specimens, could highlight a distinct biosignature in patients with mucocutaneous leishmaniasis (MCL) or localized cutaneous leishmaniasis (LCL). Differences in immunopathogenesis between MCL and LCL may result from an imbalance between prostaglandins and leukotrienes, which may serve as targets for future host-directed therapies.

Keywords. tegumentary leishmaniasis; inflammation; eicosanoids; prostaglandin; leukotrienes; biomarkers.

Tegumentary leishmaniasis is a vector-borne disease caused by *Leishmania* parasites and exhibits a wide spectrum of clinical presentations. The most common clinical form of the disease caused by *Leishmania braziliensis* is localized cutaneous leishmaniasis (LCL), characterized by ulcerated dermal lesions, which usually heal spontaneously [1]. A more severe form of this disease, mucocutaneous leishmaniasis (MCL), is observed in 3% of individuals with LCL [2]. Patients with MCL usually present with severe and progressive destruction of nasopharyngeal and/or laryngeal structures [2]. MCL lesions exhibit intense inflammation and tissue damage and, counterintuitively, very few parasites. Necrosis of mucosal tissue is associated with a strong T-cell–mediated response, reflected by an exacerbated delayed-type hypersensitivity (DTH) reaction to *Leishmania* antigens [3]. Possible mechanisms linked to increased disease severity in MCL are still unknown, but the lack of immune modulation leading to uncontrolled inflammation seems to be critically involved [3].

Eicosanoids have been described to regulate key aspects of the host immune responses during *Leishmania* infection [4]. Prostaglandin E_2 (PGE_2) has been shown to enhance parasite survival, whereas increased leukotriene B_4 (LTB_4) production leads to enhanced intracellular parasite killing by infected host cells [5, 6]. These findings suggest that the balance between prostanoids and leukotrienes may directly affect the capacity of the host to control *Leishmania* infection. However, whether this dichotomy in the expression of eicosanoids is relevant in the context of MCL remains unknown.

We performed a cross-sectional exploratory study in patients with MCL and those with LCL from an area of endemicity in Brazil, assessing circulating levels, as well as in situ RNA expression of mediators from the eicosanoid pathway. We identified a distinct biosignature of MCL, with a hallmark of decreased expression of enzymes and receptors of prostaglandins, compared with LCL. Moreover, plasma levels of PGE_2 and LTB_4 indicated that patients with MCL are prone to skew the eicosanoid balance toward leukotrienes, whereas individuals with LCL exhibit an enriched prostanoid signature. These distinct expression profiles have potential implications for the understanding of tegumentary leishmaniasis pathogenesis, which can lead to development of new host-directed therapies targeting the eicosanoid pathway.

PATIENTS AND METHODS

This study was approved by the institutional review board from Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (number 136/2007). All clinical investigations were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized.

The present study assessed age- and sex-matched patients with MCL (n = 13; male to female ratio, 1.4; mean age [± standard deviation {SD}], 59 ± 17 years) and those with LCL (n = 29; male to female ratio, 1.9; mean age [±SD], 34 ± 15 years) recruited at our reference clinic in Jiquiriçá, Brazil. The 2 groups were not significantly different with respect to age ($P = .894$) or sex distribution ($P = .921$). Individuals included in the present study were required to have no previous diagnosis of tegumentary leishmaniasis and to be treatment naive. For plasma analyses, we included samples from 43 healthy controls

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Correspondence: V. M. Borges, Centro de Pesquisas Gonçalo Moniz, Rua Waldemar Falcão, 121, Candeal, Salvador, BA 40295-001, Brazil (vborges@bahia.fiocruz.br).

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(matched by age and sex to the LCL and MCL groups) from a region of endemicity who had negative results of an anti-*Leishmania* DTH test. LCL and MCL diagnoses were confirmed by the presence of an ulcerated skin lesion or granulomatous mucosal lesion, respectively, in addition to at least 1 of the following: positive results of an anti-*Leishmania* DTH test, detection of anti-*Leishmania* antibody, or detection of *Leishmania* parasites in biopsy tissue specimens by either immunohistochemistry or qualitative polymerase chain reaction (PCR) assays. Patients with LCL exhibited a single or few ulcerated lesions for up to 2 months, while patients with MCL had symptoms for a prolonged period (mean disease duration [±SD], 10 ± 14 years) with mucosal lesions involving the nasal cavity (100%), pharynx (35%), and/or larynx (11%). Tissue samples from which we had high-quality messenger RNA (mRNA) were obtained from a subset of 4 patients with MCL and 7 patients with LCL. These patients were similar to their respective groups with regard to age and sex (data not shown). Nasal mucosal samples were obtained from turbinoplasty nasal surgery and performed under local anesthesia. All tissue specimens were obtained before treatment.

Total RNA was extracted from cryopreserved lesion biopsy specimens, using Trizol reagent (Invitrogen, Carlsbad, California), with an additional purification step using RNeasy columns (Qiagen, Venlo, Netherlands) as previously described [7]. nCounter analysis (NanoString Technologies, Seattle, Washington) was performed at the VIB MicroArray Facility (Leuven, Belgium), based on direct molecular bar coding of target RNA transcripts and digital detection [7]. The chosen targets were as follows: *PGES (*PGE synthase*)*, *PGDS* (PGD synthase), *PGD2R* (PGD 2 receptor), *PTGFR* (PGF receptor), *PTGS1* (COX-1), *PTGS2* (COX-2), *PLAS2G4A* (phospholipase S2G4A), *PLA2G6* (phospholipase 2G6), *LXA4R* (lipoxin A4 receptor), *PTGER1* (E-prostanoid receptor 1 [EP1]), *PTGR2* (EP2), *PTGER3 (*EP3*)*, *PTGER4* (EP4), *ALOX15* (arachidonate 15-lipoxygenase), *ALOX12* (arachidonate 12-lipoxygenase), and *ALOX5* (arachidonate 5-lipoxygenase). To account for differences in leukocyte infiltration between patient lesions, data were normalized for *CD45*, which encodes the pan-leukocyte marker CD45, detectable at the femtomolar range as previously reported [7].

Concentrations of PGE_2, PGD_2, $PGF_{2\alpha}$, LTB_4, and resolvin D1 (RvD1) were measured in cryopreserved ethylenediaminetetraacetic acid (EDTA)–treated plasma samples from all patients, using an enzyme-linked immunoassay (Cayman Chemical, Ann Harbor, Michigan).

Median values with interquartile ranges (IQRs) were used as measures of central tendency. For expression assays, the Mann–Whitney test was used to compare the variables. Plasma values were compared using the Kruskal–Wallis test with the Dunn multiple comparisons ad hoc test. Unsupervised 2-way hierarchical cluster analyses (Ward's method) with bootstrap were used to test whether patients with MCL and those with LCL can be grouped separately on the basis of simultaneous assessment of plasma eicosanoids. Two models of principal component analysis were used to test the contribution of PGE_2 levels to the power of the combined assessment of several eicosanoids to distinguish MCL from LCL cases. A P value of $<.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To characterize the eicosanoid signaling pathways expressed in situ during MCL and LCL, we performed a comprehensive analysis of targeted RNA transcripts isolated from mucosal versus skin biopsy specimens. The target transcripts were represented within the context of an eicosanoid signaling pathway, using ingenuity pathway analysis (Figure 1*A*). Remarkably, patients with MCL exhibited substantial downmodulation in several genes from the prostaglandin pathway, compared with those with LCL (Figure 1*B*). Among all the genes examined, we found that *PGES*, *PTGER3*, *PGDS*, *PTGFR*, *PTGS1*, and *ALOX5* expression were significantly lower, whereas LXA4R expression was higher in MCL cases than in individuals with LCL (Figure 1*C*).

Interestingly, differences found in expression of constitutively expressed targets, such as *PTGS1* gene/COX-1, and for the inducible isoforms, such as *PTGS2* gene/COX-2, indicate that LCL and MCL activate distinct prostaglandins synthase pathways. PGE_2 acts through 4 distinct G protein-coupled receptors, EP1, EP2, EP3, and EP4 [8]. Once PGE_2 binds to different receptors, it can activate different signaling pathways inducing multiple, and sometime paradoxical, effector functions. Notably, it has been reported that *Leishmania major* infection upregulates EP1 and EP3 expression while downregulating EP2 and EP4 in vitro [9]. Our analyses suggested that differential expression of EP3 might be an important parameter related to the pathogenesis of MCL and LCL. Our exploratory findings warrant the design of additional studies that assess the role of EP receptor signaling in leishmaniasis.

We next tested whether the differences in gene expression observed in situ could be reflected in a distinct profile of plasma concentrations in patients with MCL and those with LCL. By quantifying plasma levels of PGE_2, PGD_2, and $PGF_{2\alpha}$, as well as LTB_4 and RvD1, we found that patients with LCL exhibited a distinct expression profile from those with MCL (Figure 2*A*). We observed that PGE_2 levels were significantly higher in LCL cases, compared with individuals with MCL (Figure 2*B*). Whether the augmented circulating levels of these prostanoids are directly related to the increased COX1/*PTGS1* expression in skin lesions observed in patients with LCL deserves future clarification. Importantly, compared with values detected in healthy controls, PGD_2, $PGF_{2\alpha}$, LTB_4, and RvD1 levels were significantly lower in patients with LCL (Figure 2*B*). Conversely, concentrations of all eicosanoids except $PGF_{2\alpha}$ were undistinguishable

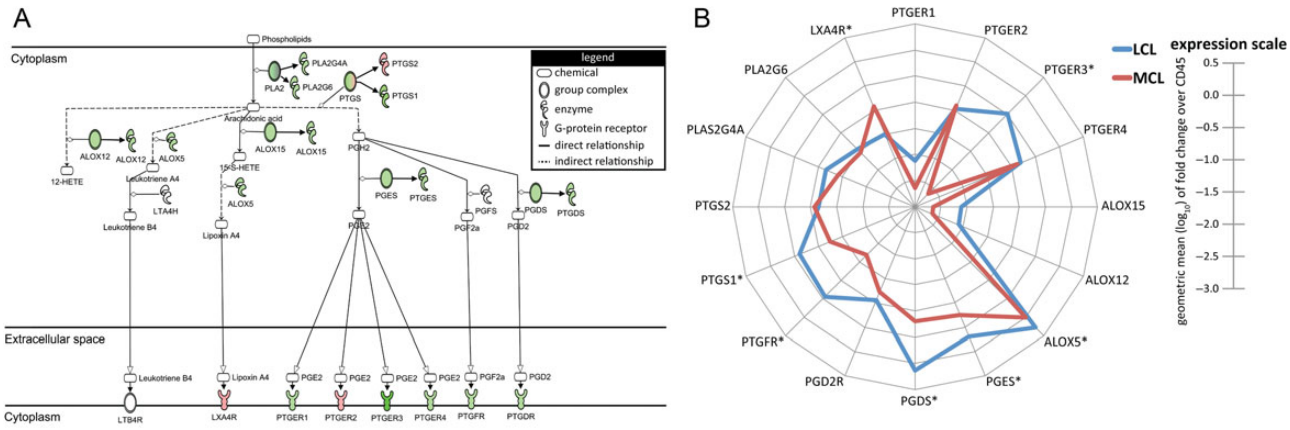

Figure 1. Differential expression of selected genes of eicosanoid pathways in skin or mucosal lesions from patients with tegumentary leishmaniasis. Total RNA was extracted from lesion biopsy specimens obtained from 7 patients with localized cutaneous leishmaniasis (LCL) and 4 with mucocutaneous leishmaniasis (MCL). Indicated messenger RNA transcripts of host-specific cellular genes were quantified by nCounter (Nanostring), including the pan-leukocyte gene CD45, for normalization of immune infiltration into tissues. *A*, The targeted genes were represented within the context of an eicosanoid signaling pathway, using Ingenuity Pathway Analysis. Red and green colors infer higher or lower gene expression in patients with MCL, relative to that in patients with LCL, respectively. *B*, A representative profile of geometric mean values (log_{10} transformed) for indicated genes is displayed for each clinical group. *C*, Scatterplots of gene expression relative to CD45 are shown. Lines represent median values and interquartile ranges. Data were compared using the Mann–Whitney test. $*P < .05$.

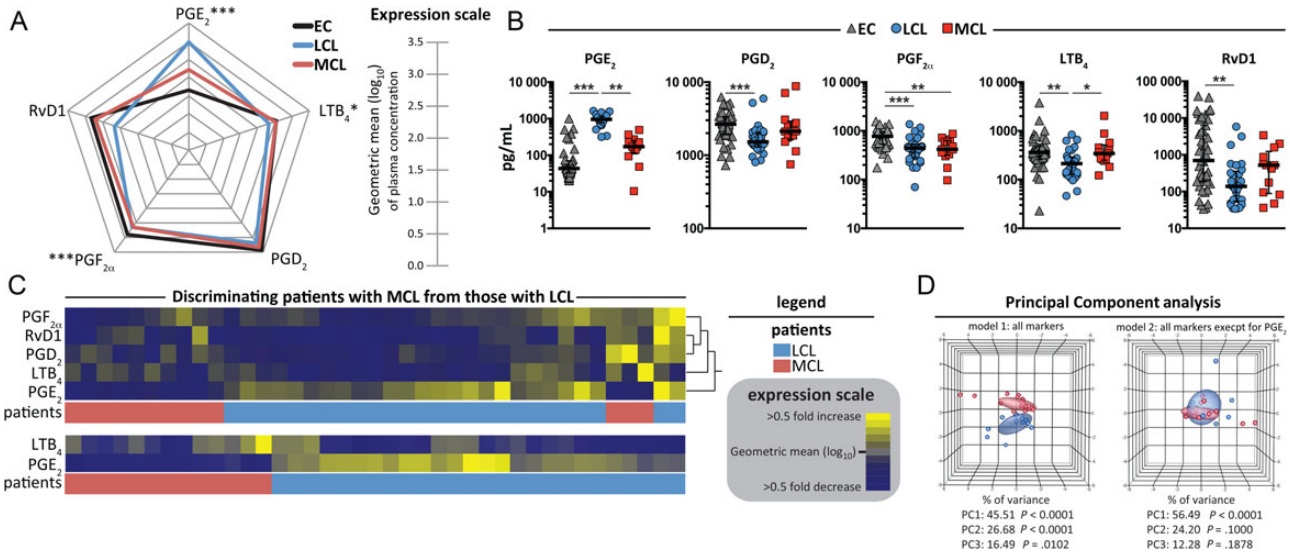

Figure 2. Plasma concentrations of eicosanoids in patients with localized cutaneous leishmaniasis (LCL) or mucosal cutaneous leishmaniasis (MCL). *A*, Plasma levels of COX-2–derived prostanoids prostaglandin E_2 (PGE_2), PGD_2, and $PGF_{2\alpha}$, as well as 5-LO–derived lipid mediators leukotriene B_4 (LTB_4) and resolvin D1 (RvD1), were compared between 29 patients with LCL or 13 with MCL, as well as 43 healthy controls from an area of endemicity (EC). Data were compared using the Kruskal–Wallis test with the Dunn multiple comparisons ad hoc test. Lines represent median values and interquartile ranges. $*P < .05$, $**P < .01$, and $***P < .001$. *B*, Univariate analyzes with scatterplots of the comparisons are shown. *C*, A hierarchical clustering analysis (Ward's method) was used to test whether the overall expression profile of all the lipid mediators (upper panel) or just PGE_2 and LTB_4 (lower panel) in plasma could distinguish MCL from LCL cases. *D*, Two principal component (PC) analysis models were used to examine the contribution of PGE_2 in explaining the differences observed between the LCL and MCL study groups.

between patients with MCL and controls (Figure 2*B*). Noteworthy, levels of LTB_4 were >1 log higher in patients with MCL than in LCL cases (Figure 2*B*). Leukotrienes are highly bioactive, and minor differences in plasma measurements could reflect major differences in inflammation, as observed in other disease models [10]. These results indicate that a downmodulatory effect may be more relevant in LCL than in MCL, compared with healthy donors. Thus, systemic mediators observed in LCL and MCL may be useful as biomarkers of active disease.

Together, these observations led us to hypothesize that a balance in the circulating levels of lipid mediators is associated with the differential inflammatory status observed in MCL or LCL. In this scenario, prostaglandins derived from cyclooxygenases would prevail over lipoxygenase-derived products in patients with LCL, compared with those with MCL. To test this hypothesis, we performed an unsupervised hierarchical cluster analysis in which plasma values of all the eicosanoids measured in patients with MCL or LCL were inputted. We confirmed that simultaneous assessment of key prostaglandins, LTB_4 and RvD1, could successfully segregate the different clinical groups evaluated (Figure 2*C*).

RvD1, an important specialized proresolving mediator, is endogenously generated during the spontaneous resolution phase in many models of acute and chronic inflammation diseases [11]. Counterintuitively at a first glance, although our data reveal that there is no statistically significant difference for RvD1 plasma levels between patients with LCL and those with MCL, we noticed a trend of RvD1 median levels to be approximately 1.2 log decreased during LCL, compared with MCL (Figure 2*B*). Whether RvD1 participates in the control and resolution of inflammation or promotion of parasite survival in patients with tegumentary leishmaniasis needs to be further investigated.

Notably, to our knowledge, this is the first study to demonstrate increased levels of prostanoids in plasma specimens from patients with tegumentary leishmaniasis. Interestingly, we found that $PGF_{2\alpha}$ was in general reduced in patients with tegumentary leishmaniasis, compared with controls (Figure 2*B*). Recent studies from our group reported that $PGF_{2\alpha}$ is uniquely involved in the cellular metabolism of *Leishmania* species and its immune evasion capacity in murine models [12, 13]. Modulation of $PGF_{2\alpha}$ production could be a potential mechanism by which the host restricts a key mediator for promotion of parasite growth.

Strikingly, additional hierarchical clustering analyses confirmed that patients with MCL and those with LCL could be better separated when data on only 2 eicosanoids, PGE_2 and LTB_4, were considered (Figure 2*C*). The balance between these eicosanoids has been described to determine clinical outcomes in other diseases [10]. Two models of principal component analysis supported the idea that differential expression of PGE_2 in plasma is probably the most important parameter leading to distinction between patients with MCL and those with LCL (Figure 2*D*). Importantly, the PGE_2/LTB_4 ratio was >8-fold higher in patients with LCL than in those with MCL (median values, 5.3 [IQR, 3.5–6.4] vs 0.6 [IQR, 0.1–0.8]; $P < .0001$). Thus, circulating levels of PGE_2 and LTB_4 could be tested as potential biomarkers of mucosal involvement in tegumentary leishmaniasis. Considering that MCL cases exhibit longer periods with disease prior to diagnosis and that this disease form may progress from localized lesions, it is possible that our results may be affected by illness duration. Prospective studies focused on early detection of MCL may help clarify this issue. In addition, differences in the expression profile of these biomarkers may reflect distinctions in the infiltration of leukocytes in the lesions. Although MCL and LCL exhibit, in general, cellular infiltrates enriched for mononuclear cells, we have previously shown an important role for neutrophils contributing to inflammation in MCL [14]. Cellular analyses using flow cytometry could be performed to extensively phenotype cellular subsets recruited to tegumentary lesions, thus elucidating associations between the leukocyte infiltrate and the differential eicosanoid expression described here. A limitation of the present study was the lack of access to skin/mucosal biopsy specimens from healthy controls for comparison with those from patients with leishmaniasis. In addition, we could not test correlations between eicosanoid expression and parasite burden in the lesions, because the parasite quantification was very low and the sensitivity of the histological technique was insufficient to provide reliable quantitative values in such a small sample set of individuals from whom we had in situ data. Regardless, our data indicate that eicosanoids pathways, and PGE_2 in particular, may be explored as novel targets for therapeutic interventions of tegumentary leishmaniasis.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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