



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



Programa de Pós-Graduação em Patologia Humana e Experimental

TESE DE DOUTORADO

**ESTUDO DA MODULAÇÃO DA MIGRAÇÃO DE CÉLULAS HOSPEDEIRAS
INDUZIDA POR *LEISHMANIA* E SEU IMPACTO NA DISSEMINAÇÃO DO
PARASITO NO HOSPEDEIRO**

AMANDA REBOUÇAS PAIXÃO

Salvador - Bahia

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AMANDA REBOUÇAS PAIXÃO

Tese apresentada ao Programa de Pós-Graduação em
Patologia Humana do Instituto Gonçalo Muniz para a
obtenção do grau de Doutora.

Orientadora: Profa. Dra. Juliana P. B. de Menezes
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Amanda Rebouças Paixão

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Dra. Juliana Perrone Bezerra de Menezes Fullam
Pesquisadora
IGM/FIOCRUZ

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RESUMO

Leishmania spp. infectam diversos hospedeiros vertebrados, incluindo o homem. As doenças causadas por estes protozoários, as leishmanioses, podem apresentar diferentes tipos de manifestações clínicas, desde lesões localizadas na pele que cicatrizam espontaneamente, lesões de pele, que se espalham de forma disseminada, a forma mucocutânea da doença, além da leishmaniose visceral, forma mais grave que podem levar à morte se não tratada. A disseminação e *homing* de células infectadas contendo antígenos de *Leishmania* são fundamentais para a sobrevivência deste parasito no hospedeiro e para o estabelecimento da lesão. Ainda hoje, pouco se sabe sobre os mecanismos envolvidos na adesão da célula hospedeira e sua migração na infecção por este protozoário. Assim, este trabalho teve como objetivo avaliar a migração de monócitos, macrófagos e células dendríticas humanas na infecção por *Leishmania* e os mecanismos envolvidos nesse processo. Células hospedeiras humanas oriundas de doadores saudáveis foram obtidas, infectadas por diferentes espécies de *Leishmania* e submetidas a migração direcional e randômica por sistema transwell e em tempo real, respectivamente. Avaliamos ainda a formação de complexos de adesão pela imunomarcagem de p-FAK e p-paxilina. Adicionalmente, investigamos a dinâmica de actina por meio de análise da expressão de Rac1, Rho-A, Cdc42 e marcação por faloidina nestas células, bem como a formação de podossomos por meio da imunomarcagem de vinculina. Por fim, investigamos o papel da via de sinalização associada a AKT/PI3K na migração de células hospedeiras infectadas por estes parasitos, através da análise da expressão desta proteína por *western blot*, além do efeito da inibição dessa via na migração de células hospedeiras infectadas. Nossos dados mostram uma redução na migração bidimensional de monócitos e macrófagos humanos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*, associado a uma redução na formação de complexos de adesão, podossomos e dinâmica de actina nestas células. Entretanto, observamos um aumento na migração de células dendríticas infectadas por *L. infantum* ou isolados de *Leishmania* oriundos de pacientes com a forma disseminada ou difusa da doença. Adicionalmente, observamos que o aumento da migração nessas células está associado a um aumento na formação de complexos de adesão, podossomos, dinâmica de actina e expressão de CCR7. Por fim, foi observado que a infecção por *Leishmania* induz a ativação da via de AKT/PI3K. Em conjunto, nossos dados apontam para um papel de células dendríticas, mas não de macrófagos e monócitos na disseminação de *Leishmania* no hospedeiro vertebrado.

Palavras-chave: Célula hospedeira. *Leishmania*. Migração celular.

PAIXÃO, Amanda Rebouças. **Study of the modulation of host cell migration induced by *Leishmania* and its impact on parasite dissemination in the host.** 2024. 177f. Tese (Doutorado em Patologia Humana e Experimental) – Universidade Federal da Bahia, Faculdade de Medicina. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2024.

ABSTRACT

Leishmania spp. infect several vertebrate hosts, including humans. The diseases caused by this protozoan, leishmaniasis, can present different clinical manifestations, from localized skin lesions that heal spontaneously, to disseminated skin lesions, mucocutaneous form of the disease, and visceral leishmaniasis, the most severe form that can lead to death if left untreated. The dissemination and homing of infected cells containing *Leishmania* are crucial for the parasite's survival in the host and the establishment of the lesions. However, the understanding of the mechanisms underlying host cell adhesion and migration during infection by this protozoan remains limited. Therefore, this study aimed to evaluate the migration of human monocytes, macrophages, and dendritic cells in *Leishmania* infection, and the mechanisms involved in this process. Human host cells from healthy donors were obtained, infected with different isolates of *Leishmania*, and subjected to directional and random migration using a transwell system and real-time tracking, respectively. We also assessed the formation of adhesion complexes by immunostaining p-FAK and p-paxillin. Additionally, we investigated actin dynamics by analyzing the expression of Rac1, Rho-A, Cdc42, and phalloidin staining in these cells, as well as the formation of podosomes by immunostaining vinculin. Finally, we investigated the role of the AKT/PI3K signaling pathway in the migration of host cells infected by these parasites, through the analysis of protein expression by western blot, and the effect of inhibition of this pathway on the migration of infected host cells. Our results showed a reduction in the two-dimensional migration of human monocytes and macrophages infected with *L. amazonensis*, *L. braziliensis*, or *L. infantum*, associated with a decrease in the formation of adhesion complexes, podosomes, and actin dynamics in these cells. However, we observed an increase in the migration of dendritic cells infected by *L. infantum* or *Leishmania* isolates from patients with the disseminated or diffuse form of the disease. Additionally, we observed that the increase in migration in these cells is associated with an increase in the formation of adhesion complexes, podosomes, actin dynamics, and CCR7 expression. Finally, we observed that *Leishmania* infection induces activation of the AKT/PI3K pathway. Taken together, our data point to a role of dendritic cells, but not macrophages and monocytes, in the dissemination of *Leishmania* parasites in the vertebrate host.

Keywords: Host cell. *Leishmania*. Cell migration.

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LISTA DE ABREVIATURAS

p-FAK	(phospho focal adhesion kinase)
PI3K	(phosphoinositide 3- kinase)
LC	(Leishmaniose cutanea)
LV	(Leishmaniose visceral)
LCL	(Leishmaniose cutanea localizada)
LCD	(Leishmaniose cutanea disseminada)
LD	(Leishmaniose difusa)
LCM	(Leishmaniose muco-cutanea)
ROS	(Especies reativas de oxigenio)
LTB4	(Leucotrieno B4)
TLR	(Toll like receptor)
CR1	(Complement receptor 1)
CR3	(Complement receptor 3)
GP63	(Glicoproteina 63)
LPG	(Lipofosfoglicano)
iNOS	(nitric oxide synthase)
TNF-alfa	(Tumor necrosis fator)
CXCL18	(Chemokine ligand 18)
CCL2	(Chemokine ligand 2)
IL-12	(Interleucina 12)
MEC	(Matriz extracelular)
AF	(Adesao focal)
ATP	(Adenosina trifosfato)
MMPs	MMPs (Metaloproteinases)
PtdIns3P	(Fosfatidilinositol)
JAK	(Janus kinase)
STAT	(Signal transducer activator of transcription)
ROCK	(Rho-associated protein kinase)
LAMP	(Lysosomal-associated membrane protein)
LPS	(Lipopolissacarideo)
M-CSF	(Macrophage colony-stimulating factor)

IFNγ	(Interferon gama)
HCMV	(Human cytomegalovirus)
CCR7	(C-Chemokine receptor 7)
CCR2	C-Chemokine receptor 2)
CCL2	(C-ligand 2)
CCL3	C-ligand 3)
MCP1	(Monocyte chemoattractant protein-1)
MHC II	(Major histocompatibility complex II)
HIV	(Human immunodeficiency virus)
dLND	(draining lymphonode)
PBS	(Phosphate buffer saline)
TBS	(Tris-buffered saline)
BSA	(Bovine serum albumin)
CD86	(Cluster of differentiation 86)
CD80	(Cluster of differentiation 80)
CD205	(Cluster of differentiation 205)
iNOS	(Inducible nitric oxide synthase)
NO	(Nitric oxyde)
TGF-β	(Transforming growth factor-beta)
IL-10	(Interleukin 10)
TLR2	(Toll like receptor 2)
TLR4	(Toll like receptor 4)
TRL9	(Toll like receptor 9)
ROI	(Reactive oxygen intermediates)
CXCL8	(C-X-C motif chemokine ligand 8)
ROS	(Reactive oxygen species)
CXCL2	(C-X-C motif chemokine ligand 2)
IL-4	(Interleukin 4)
MAPK	(Mitogen-activated protein kinase)
PAMP	(Pathogen-associated molecular pattern)
DAMP	(Damage-associated molecular pattern)
TH1	(Type 1 T helper)

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1 INTRODUÇÃO

1.1 Leishmanioses

As leishmanioses são doenças com elevada incidência e ampla distribuição geográfica, sendo endêmica em 98 países, com uma estimativa de 700 mil a 1 milhão de novos casos anualmente. A presença da leishmaniose está diretamente associada a fatores sociais, ambientais e climáticos que influenciam diretamente a epidemiologia da doença (WHO, 2023). No velho mundo, os agentes etiológicos incluem *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica*, bem como *Leishmania infantum* ou *Leishmania donovani*. Dentre as principais espécies no novo mundo estão o complexo *Leishmania mexicana* (*Leishmania mexicana*, *Leishmania amazonensis*), ou o subgênero *Viannia* (*Leishmania guyanensis*, *Leishmania panamensis*, *Leishmania infantum*). Nos últimos anos, mais de 1 milhão de casos de leishmaniose cutânea (LC) e cerca de 70.000 novos casos de leishmaniose visceral (LV) foram registrados nas Américas. Estima-se que na LC cerca de 95% dos casos ocorrem nas Américas, Oriente Médio e Asia Central, com uma incidência de 200 mil novos casos reportados anualmente (WHO, 2023).

No Brasil e região andina, foram notificados cerca de 800.000 casos entre 2001 a 2021, correspondendo a 37,60% e 40,79% dos casos de LC, respectivamente (OPAS, 2022). Clinicamente, a LC é dividida em cutânea localizada (LCL), cutânea disseminada (LCD), cutânea difusa (LD) e muco-cutânea (LCM) (Desjeux, 2004). Na LCL, podem surgir uma ou mais lesões de pele que tendem a cicatrizar, com úlceras geralmente indolores de formato arredondado, base eritematosa e fundo necrótico, podendo ser causada principalmente por parasitos das seguintes espécies no Brasil: *Leishmania braziliensis*, *Leishmania amazonensis*, *Leishmania guyanensis*, *Leishmania panamensis* (Steverding, 2017). A LCD é caracterizada pela presença de múltiplas lesões papulares polimórficas e frequente envolvimento da mucosa nasal, sendo causada no Brasil principalmente por *L. braziliensis* (Machado et al., 2019). A LMC, por sua vez, apresenta lesões destrutivas em vias aéreas superiores, como boca, nariz e garganta, sendo causada primariamente por *L. braziliensis* ou *L. amazonensis*. Já a LD (forma anérgica), causada no Brasil por *L. amazonensis*, apresenta nódulos infiltrados com ausência de úlceras ou envolvimento de mucosas (Machado et al., 2019; Salgado et al., 2016).

Com relação à forma visceral da doença, nos últimos vinte anos, houve cerca de 70.000 novos casos nas Américas, e, apesar de 13 países dessa região registrarem casos de LV, cerca de 93% dos casos ocorrem no Brasil (OPAS, 2022). A LV, causada por *L. infantum* no Brasil,

é caracterizada por lesões que acometem fígado, baço, medula óssea e linfonodos, podendo levar a um quadro de hepatoesplenomegalia, que pode levar à morte se não tratadas (OPAS, 2022).

1.2 Ciclo biológico do parasito

O ciclo biológico do parasito *Leishmania* envolve duas fases principais, uma no vetor transmissor e outra no hospedeiro vertebrado. Durante o repasto sanguíneo, promastigotas metacíclicas, formas flageladas e infectivas, são transmitidas através da picada de flebotomíneos fêmeas do gênero *Phlebotomus* ou *Lutzomia* (Sacks, 1989). Após o reconhecimento e internalização de formas promastigotas por células hospedeiras, os parasitos sofrem diversas alterações morfológicas e moleculares, transformando-se em amastigotas, que sobrevivem e se multiplicam no interior de compartimentos fagolisossomais (Mcconville et al., 1992; Veras et al., 1992) (Figura 1). Alguns estudos mostram que, no interior dessas células, o parasito dissemina-se pelos diferentes tecidos do hospedeiro causando lesões (Fiorini et al., 2002; Leon et al., 2007; Moll et al., 1993). Durante um novo repasto sanguíneo, os fagócitos infectados são ingeridos pelos vetores, onde ocorrerá a transformação das formas amastigotas em promastigotas no intestino de flebótomos, completando o ciclo de transmissão (Kaye e Scott, 2011).

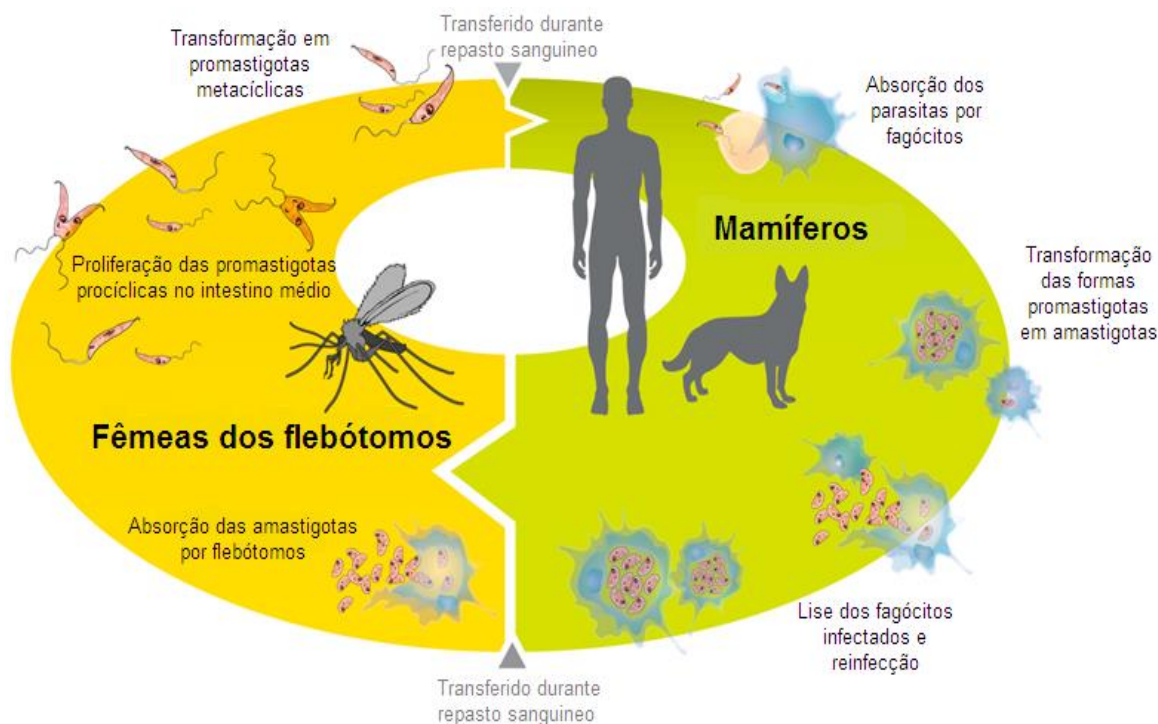


Figura 1 - Ciclo biológico da *Leishmania*. O ciclo de vida da *Leishmania* consiste em dois estágios alternados conhecidos como amastigota e promastigota. Fêmeas flebotomíneas infectadas com as formas promastigotas inoculam parasitos no hospedeiro vertebrado durante o repasto sanguíneo. No hospedeiro vertebrado, esses parasitos são fagocitados por células hospedeiras, se transformam em amastigotas, podendo sobreviver e se multiplicar no interior de vacúolos parasitóforos. A multiplicação de parasitos pode levar ainda à lise de células hospedeiras, permitindo que os mesmos sejam internalizados por flebótomos em um novo repasto sanguíneo.

Fonte: Adaptado de (Veras; Menezes, 2016).

1.3 Células hospedeiras na infecção por *leishmania*

Leishmania sp. interage com células hospedeiras do sistema imune, tais como neutrófilos, macrófagos, monócitos e células dendríticas. Esta interação inicial do parasito com as diferentes células do sistema imune é fundamental para a determinação do curso da infecção e desfecho da doença (Liu; Uzonna, 2012; Desjeux, 2004). A interação inicial de fagócitos com o parasito ocorre via diferentes receptores, que reconhecem ligantes padrões na superfície celular do parasito (Rotureau et al., 2009; Gough; Gordon, 2000; Forestier et al., 2011). Durante a fagocitose, ocorre a ligação específica receptor-ligante, através de receptores como fucose-manose, *toll-like* (TLR), fibronectina, CR1 e CR3, que reconhecem moléculas de superfície do parasito, como a glicoproteína 63 Kda (GP63), que apresenta atividades proteolíticas, ou o lipofosfoglicano (LPG), que é associado a proteção do parasito da lise pelo complemento e a destruição decorrente da ação de espécies reativas de oxigênio como produto da fagocitose (Kelleher et al., 1995; Gough; Gordon, 2000). Estas moléculas são ancoradas por glicofosfatidilinositol (GPI) na superfície da *Leishmania* e favorecem a entrada do parasito na

célula hospedeira (Turco; Descouteaux, 1992). Trabalho anterior demonstrou que a baixa expressão de GP63 em promastigotas de *L. amazonensis* levou a uma menor taxa de sobrevivência intracelular (Chen et al., 2000). O LPG, por sua vez, também é um importante fator de virulência, associado a sobrevivência do parasito tanto no hospedeiro, quanto no vetor flebotomíneo (Descouteaux; Turco, 1999; Sacks; Kamhawi, 2001). Adicionalmente, já foi demonstrado que o LPG de *Leishmania* auxilia na subversão da lise pelo sistema complemento da célula hospedeira ao inibir a montagem de moléculas desse sistema (Puentes et al., 1988). Após o reconhecimento inicial, promastigotas do parasito são internalizadas pelas células hospedeiras onde sinais intracelulares desencadeiam alterações na membrana plasmática e no citoesqueleto da célula, levando a formação do fagossomo (Roy et al., 2014; Desjardins; Griffiths, 2003).

Uma vez internalizados, os parasitos sobrevivem e se multiplicam no interior de compartimentos com características fagolisossomais, conhecidos como vacúolos parasitóforos (Young; Kima, 2019). No interior destes compartimentos, a *Leishmania* é capaz de absorver nutrientes necessários para sua sobrevivência e replicação, visto que o vacúolo parasitóforo traz produtos da via endocítica que fornecem fontes de carbono, açúcares, aminoácidos e lipídios (Saunders et al, 2018; Naderer et al, 2015). Devido à sua associação com a via endocítica, a biogênese do vacúolo parasitóforo envolve ainda a aquisição de diversas moléculas, incluindo as proteínas de membrana associadas ao lisossomo 1 e 2 (LAMP 1 e LAMP 2), componentes moleculares de fusão como as Rab GTPases e a bomba vacuolar de H⁺ (Courret et al, 2002; Antoine et al, 1998). Adicionalmente, a fagocitose de *Leishmania* leva à produção de moléculas microbidas como os radicais intermediários de oxigênio (ROI) e o óxido nítrico (NO), que favorecem a destruição do parasito (Stafford et al, 2002; Panaro et al, 2001).

Ainda durante os estágios iniciais da infecção, os monócitos, fagócitos mononucleares, contribuem significativamente para a indução de respostas inflamatórias (Mildner et al., 2016). A interação do monócito com a *Leishmania* depende de receptores do tipo toll e do sistema complemento como o CD11b (Rosenthal et al, 1996). Uma vez internalizados por monócitos inflamatórios, os parasitos têm sua proliferação limitada por meio da produção de ROS e de óxido nítrico sintase induzível (iNOS), como demonstrado em trabalho anterior (Heyde et al., 2018; Romano et al., 2017; Glennie et al., 2017). Essas células também desempenham um papel essencial na resposta imune inata contra *Leishmania* e contribuem para a resposta imune adaptativa, participando da resposta inflamatória inicial ao liberar TNF- α e quimiocinas, como CXCL8 e CCL2 (Gordon; Taylor, 2005; Serbina et al., 2008). Adicionalmente, essas células têm sido associadas à morte de parasitos *L. major* e ao dano tecidual de lesões cutâneas por *L.*

braziliensis (Badolato et al., 1996; Ritter e Moll, 2000; Vargas-Inchaustegui et al., 2010). Além disso, já foi demonstrado que a infecção por *L. braziliensis* favorece o recrutamento de monócitos através de gradientes quimiotáticos, além de estarem associados à produção de CXCL10 durante a infecção ativa (Vargas-Inchaustegui et al., 2010).

A interação de fagócitos e sua ativação decorrente do contato com *Leishmania* são cruciais para o desfecho da infecção. A complexa interação macrófago-*Leishmania* se inicia com o reconhecimento de moléculas do parasito por diferentes receptores na célula hospedeira, como receptores de complemento (CRs), receptores de manose e receptores de fibronectina (Mosser; Rosenthal, 1993; Mosser; Miles, 2007). Nesse contexto, os macrófagos desempenham papel fundamental na fagocitose de *Leishmania* e na sua sobrevivência no hospedeiro vertebrado, sendo as principais células hospedeiras deste parasito (Liu; Uzonna, 2012). Estudos mostram ainda que o controle da infecção por *Leishmania* é dependente da capacidade de destruição do parasito por macrófagos, por mecanismos muitas vezes dependentes de óxido nítrico e superóxido (Green et al., 1990; Liew et al., 1990; Behin et al., 1979; Nacy et al., 1983; Assreuy et al., 1994; Mukbel et al., 2007). Nesse sentido, já foi mostrado que macrófagos oriundos de camundongos deficientes para iNOS, tornam-se susceptíveis à infecção por *L. major*, tendo sua capacidade de eliminação do parasito prejudicada (Wei et al., 1995; Murray & Nathan, 1999). Adicionalmente, diferentes espécies de *Leishmania* são capazes induzir a produção de TGF- β e IL-10, responsáveis por inibir a eliminação de parasitos intracelulares pela redução da síntese de óxido nítrico (Kupani et al., 2021). Por outro lado, a sobrevivência do parasito está associada à indução da arginase e secreção de IL-4 e IL-13, que favorecem a proliferação intracelular de *Leishmania* (Liu; Uzonna, 2012).

Células dendríticas são células apresentadoras de antígeno profissionais originárias da medula óssea, que participam do controle de infecções após capturarem e processarem antígenos, podendo ainda migrar para os linfonodos, onde apresentam os antígenos aos linfócitos T. Estas células são classificadas em diferentes subtipos de acordo com sua origem, marcadores de superfície e localização (Barragan et al., 2012; Tibúrcio et al., 2019). Durante esse trajeto e após a detecção de padrões moleculares associados a patógenos (PAMPs), as células dendríticas passam por um processo de maturação através da expressão molecular de MHC-classe I e II, CD11c, CD86 e CD80 (Caux et al., 1994). Durante o processo migratório, essas células expressam ainda níveis elevados do receptor de quimiocina CCR7, que é crucial para a interação entre as células dendríticas e os linfócitos durante a apresentação de antígenos (Foster et al., 1999; Ohl et al., 2004).

Dentre os receptores que medeiam a interação célula dendrítica-*Leishmania* estão os receptores tipo toll (TLRs: TLR2, TLR4, TLR9) além de receptores de lectina (CLRs). Adicionalmente, foi demonstrado que TLR1 e TLR2 participam do reconhecimento do lipofosfoglicano (LPG) de *Leishmania* (Tibúrcio et al., 2019; Lima et al., 2017). Estudos anteriores mostraram que a neutralização desses receptores leva à deficiência na apresentação de antígeno durante a infecção por *L. major*. (Becker et al., 2003; Argueta et al., 2016). Diversos estudos destacam ainda o papel de células dendríticas frente a infecção por *Leishmania* na organização da resposta imune, evidenciando que o desfecho da infecção por este parasito em camundongos resistentes e suscetíveis pode estar relacionado a diferenças funcionais nessas células durante o processo de diferenciação de células T auxiliares CD4⁺ em células efetoras (Suzue et al., 2008; Gorak et al., 1998; Von Stebut et al., 1998; Leon et al., 2007). Trabalho anterior mostrou que células dendríticas infectadas por *L. major* derivadas de tecido de camundongos BALB/c e C57BL/6 regulam positivamente suas moléculas co-estimulatórias e produzem altos níveis de citocinas pró-inflamatórias, como IL-12 (Von Stebut et al., 1998). Adicionalmente, foi mostrado que camundongos BALB/c infectados com *L. major* têm um número aumentado dessas células em seus linfonodos drenantes, associado a um maior recrutamento de células dendríticas logo após a infecção, em comparação com camundongos C57BL/6 infectados (Baldwin et al., 2004). Dados da literatura mostram ainda que, em camundongos Balb/c, a infecção por *L. amazonensis* prejudica a função das células dendríticas, como a diferenciação e maturação, bem como a produção de citocinas (Favali et al., 2007).

1.4 Migração celular

A migração celular é um processo cíclico e fisiológico que envolve diferentes eventos moleculares e estruturais integrados e é fundamental no estabelecimento e manutenção da organização de organismos multicelulares (Trepatt et al., 2012). A migração de fagócitos é essencial para a resposta imune, reparo de lesões e homeostase do tecido, além disso, uma migração não-organizada pode levar a diversas patologias como câncer por exemplo (Fireld et al., 2008).

Para migrar, as células adquirem uma modulação estrutural que permite transformar as forças geradas intracelularmente em translocação do seu corpo celular, como a projeção de uma morfologia polarizada, ou seja, uma distinção entre a parte anterior e traseira da célula (Wynn et al., 2013). Tais processos são mediados por integrinas, que são receptores de superfície celular para a matriz extracelular (MEC). Diferentes tipos celulares, incluindo macrófagos,

monócitos e células dendríticas, atuam na defesa imunológica e, nesse cenário, a migração é uma função fundamental para uma gama de processos fisiológicos e patológicos, que vão desde o desenvolvimento, homeostase do tecido, metabolismo, resposta a lesões e regeneração, até a condições patológicas como câncer (Wynn et al., 2013; Friedl et al., 2008). De forma geral, processos patológicos ou inflamatórios dependem do recrutamento celular, conhecido por envolver a adesão ao endotélio e subsequente transmigração, seguida por migração direcionada para o local da inflamação e fagocitose. Adicionalmente, defeitos na execução dessas funções resultam em complicações patológicas (Zaidel-Bar et al., 2007).

1.4.1 Diferentes etapas e modos de migração celular

Para migrar de forma eficaz, as células devem polarizar e reorganizar seu citoesqueleto e esta polarização é caracterizada por dividir a célula em duas regiões, uma frontal e outra traseira. Uma vez polarizada, o movimento celular ocorre em diferentes etapas: protrusão, na qual a membrana plasmática é empurrada para frente na face anterior da célula; ligação, na qual o citoesqueleto de actina se liga ao substrato; e, posteriormente, a tração, no qual a célula desprende sua parte posterior e todo seu corpo é tracionado para a frente (Figura 2) (Wiesner et al., 2014; Ridley et al 2003; Bianco et al, 2007).

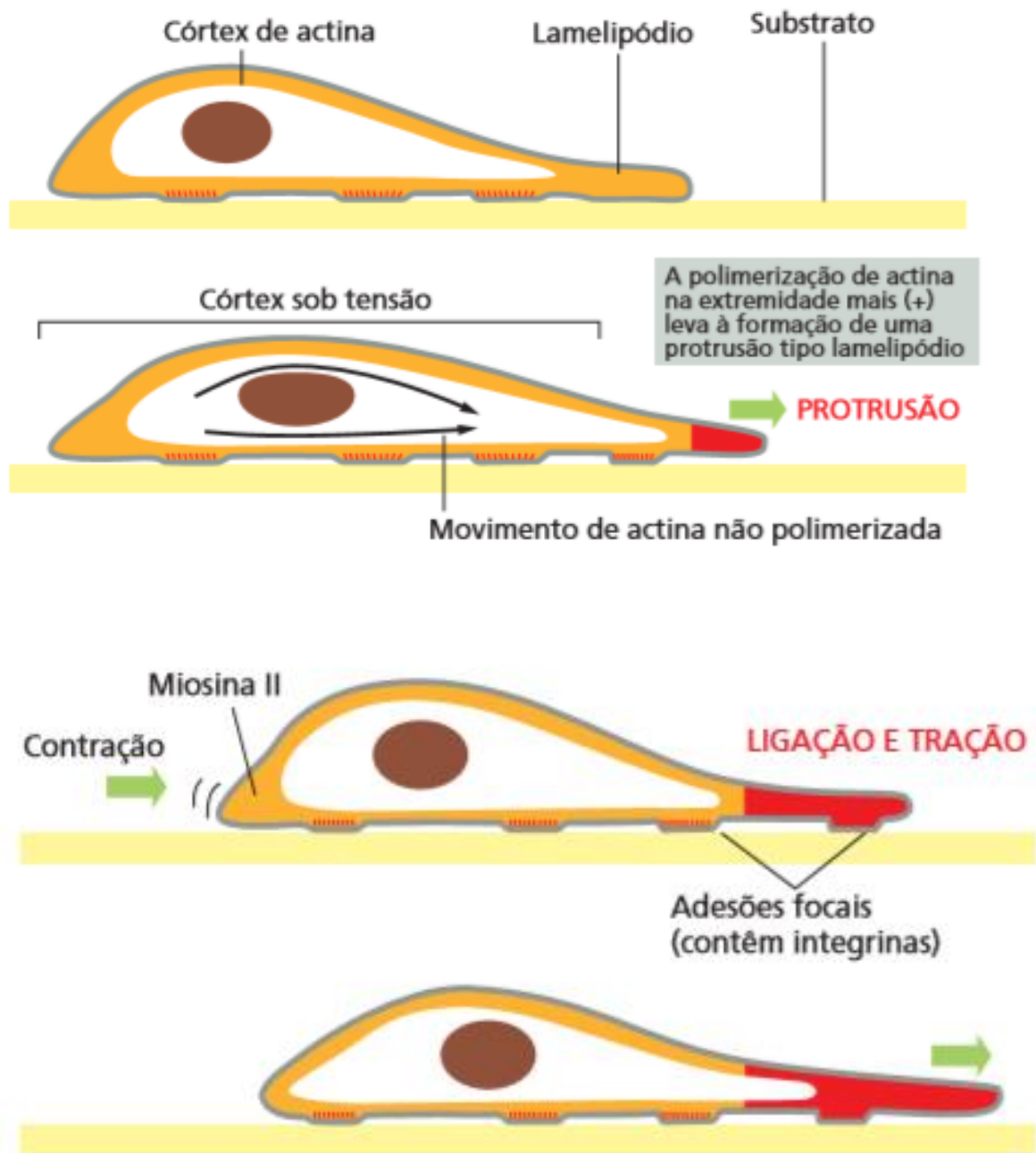


Figura 2 - Etapas da migração celular. O movimento celular é dividido em etapas: formação da protrusão de membrana; adesão ao substrato; contração e desprendimento da parte posterior.
Fonte: (Alberts, 2015).

Embora a migração celular tenha sido estudada extensivamente em condições de cultura de células essencialmente 2D, cada vez mais atenção tem sido dada ao movimento das células em ambientes 3D. A matriz 3D atua como uma estrutura que produz suporte físico para células que podem afetar a morfologia celular e induzir o crescimento ou migração celular (Yamada et

al, 2005; Verollet et al, 2011). Adicionalmente, o ambiente 3D mimetiza a migração de células em tecidos do hospedeiro vertebrado, além da matriz poder induzir variação nas cascatas de sinalização nas células por meio de aderências e forças de tração (Amatangelo et al, 2005).

A maioria dos modos de migração já estudados anteriormente em ambientes 2D também ocorrem em ambientes 3D. No entanto, a morfologia celular de leucócitos e fibroblastos é bastante diferente nos dois ambientes (Even-Ram; Yamada, 2005). Na cultura de células 2D, fibroblastos têm grandes lamelipódios e filopódios ao passo que, em géis de colágeno 3D exibem lamelipódios e filopódios menores, adquirindo uma conformação mais cilíndrica (Frield; Gilmor, 2009). Em uma matriz 3D, a migração celular pode ocorrer de dois modos: amebóide, característico da migração em matriz frouxa, e independente da secreção de proteases; e mesenquimal, presente em matriz densa, na qual a célula apresenta um corpo celular mais extenso e dependente da secreção de proteases e degradação de matriz extracelular para migração. De forma geral, diversos tipos celulares, como macrófagos, células dendríticas, células cancerosas e fibroblastos, podem mudar seu modo de migração a depender do ambiente em que se encontram (Figura 3) (Frield, et al 2009; Cougole et al 2012).

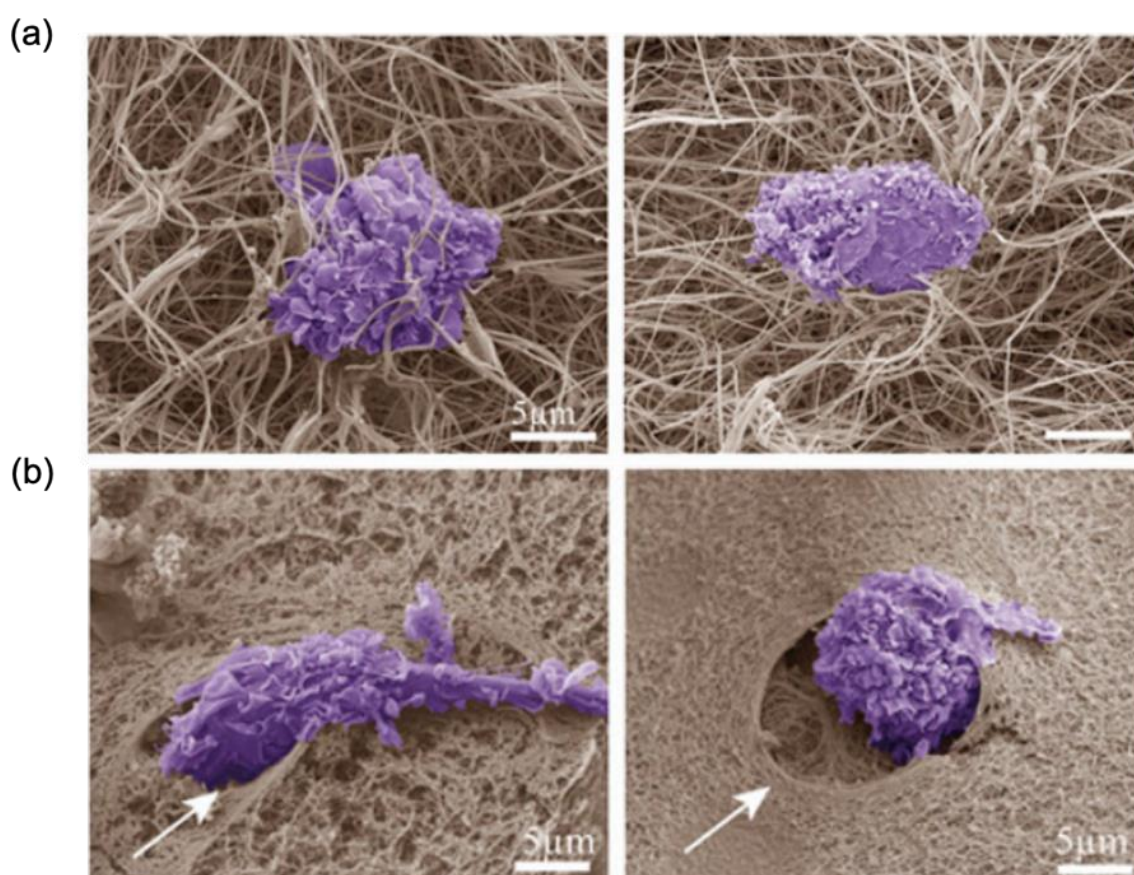


Figura 3 - Diferentes arquiteturas da matriz extracelular. (a) Matriz de colágeno I frouxo com macrófago imerso. (b) Matrigel, matriz densa com macrófago imerso.

Fonte: (Van Goethem, et al, 2010).

Três fatores importantes regulam a migração de células em ambientes 3D: adesões à matriz extracelular, a família Rho de pequenas GTPases e proteases. Na cultura 2D, as integrinas são principalmente responsáveis pelas adesões celulares à MEC na forma de adesões focais (AFs), contatos focais e podossomos. No entanto, na cultura de células 3D, ocorre uma redução no número de AFs e suas integrinas componentes. Assim, por exemplo, a integrina $\alpha V\beta 3$, que é altamente expressa em cultura de células 2D, não foi detectada nas adesões de matriz 3D de fibroblastos, e o nível de fosforilação de FAK foi amplamente reduzido (Kole et al, 2005). Adicionalmente, em superfícies bidimensionais (2D), foi demonstrado que a adesão mediada por integrinas é necessária para a migração eficaz de leucócitos, como células dendríticas e macrófagos, uma vez que a capacidade de adesão de células com receptores de integrinas depletados foi prejudicada em ensaio *in vitro* em ambiente 2D (Lammerman et al, 2008).

1.4.2 Formação de complexos de adesão

Adesões focais são estruturas dinâmicas que passam por ciclos de montagem e desmontagem e sua regulação é parte fundamental da migração celular (Treat et al, 2012). A regulação da ligação entre F-actina e integrinas é indispensável no controle da protusão e retração celular (Choi et al, 2008; Gupton et al, 2006). Nesse contexto, FAK (do inglês focal adhesion kinase) é uma cinase citoplasmática chave que funciona como um fator de regulação da montagem e desmontagem da adesão focal durante o movimento celular. No entanto, os mecanismos de regulação envolvidos nesse processo ainda são pouco compreendidos (Zaidel-Bar, et, al, 2007).

Os domínios citoplasmáticos da integrina recrutam uma variedade de proteínas para os focos de adesão para que esses focos sirvam como condutores de força necessária para a migração celular e para a sinalização entre o interior da célula e a matriz extracelular (Deakin & Turner, 2008). Inicialmente, a composição de proteínas associadas a adesão focal era limitada a proteínas estruturais, como talina e vinculina, que juntas participam da ancoragem do domínio citoplasmático da integrina ao citoesqueleto de actina (Figura 4) (Burridge et al., 1988). Atualmente, se compreende melhor a complexidade molecular das adesões focais que são caracterizadas por possuir mais de 125 proteínas individuais (Turner, 2000; Zaidel-Bar et al., 2007).

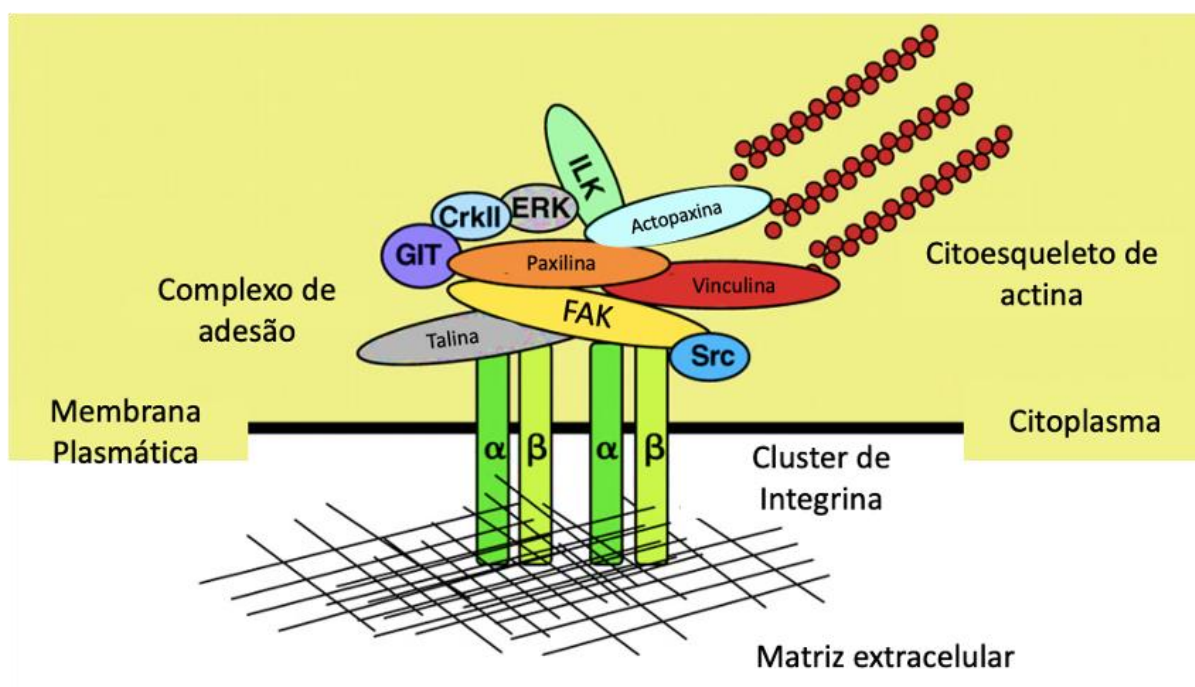


Figura 4 - Complexo de adesão. Proteínas como FAK e paxilina formam o complexo de adesão, onde juntas, ancoram filamentos de actina à integrinas transmembrana.

Fonte: (Turner; Deakin, 2008).

Estudos mostram FAK como um mediador central da sinalização de integrina, bem como um importante componente da sinalização por outros receptores de superfície celular em células que contribuem para a patogênese do câncer e outras doenças (McLean et al., 2005). Por ser uma proteína-tirosina cinase intracelular recrutada e ativada nos complexos de adesão, FAK contribui na sinalização de fatores de crescimento e componentes da matriz extracelular. Esta proteína recruta c-Src em adesões focais para formar um complexo de sinalização FAK-Src, que por sua vez fazem a fosforilação umas das outras em tirosinas específicas, criando um sítio de encaixe para os membros da família Src de tirosinas-cinase citoplasmáticas, ativando assim diversas vias de sinalização na regulação da migração celular (Parsons, 2003). Dados da literatura reforçam a importância de FAK na migração celular. Foi demonstrado que, em neutrófilos humanos, a depleção da expressão de FAK ocasionou uma redução na transmigração dessas células, e em macrófagos deficientes para esta proteína, foi observado uma motilidade prejudicada (Parsons et al, 2010; Owen et al., 2007).

Paxilina é outra proteína importante recrutada na formação dos complexos de adesão da célula (Deakin; Turner, 2008). Além de seu papel na montagem de adesões focais, a paxilina também regula a migração celular ao contribuir para a desmontagem dessas adesões, conforme mostrado pela estabilização de adesões em células desprovidas de paxilina (Webb et al., 2004). Em macrófagos, já foi demonstrado que a fosforilação de paxilina auxilia na migração, bem

como sua inibição leva a uma redução da capacidade migratória desses fagócitos (Nayal et al., 2006; Delorme-walker et al., 2011; Menezes et al., 2017). Para monócitos com deficiência em paxilina foi observado uma redução significativa na motilidade (Nogalski et al., 2011).

1.4.3 Dinâmica de actina

O processo de migração celular depende da montagem e desmontagem dinâmica dos filamentos de actina (Schacks et al., 2019). Esses filamentos se organizam em subdomínios onde protrusões da membrana plasmática como lamelipódios e filopódios, e são altamente complementares às estruturas de adesão celular, como complexos focais ou contatos focais, estruturas que servem como focos de ancoragem para actina, conhecida como fibra de estresse, além de podossomos e invadosomos (Skruber et al., 2018; Schacks et al., 2019).

A actina é um componente importante do citoesqueleto, que impulsiona a migração celular (Schacks et al., 2019). Ele se reúne em filamentos lineares conhecidas como F-actina, com duas extremidades. Essa polarização estrutural dos filamentos de actina é induzida por motores moleculares que transformam a energia liberada durante a hidrólise do trifosfato de adenosina (ATP) em energia mecânica (Mogilner; Oster, 1996).

A ancoragem e desmontagem da F-actina são reguladas por vários cofatores, como por exemplo, forminas e o complexo Arp2/3 (Faix; Rottner, 2006; Gupton; Gertler, 2007, Pollard; Borisy, 2003). A adição de monômeros de actina aos filamentos gera uma força, que impulsiona a membrana para frente e seguem uma regulação dinâmica (Forscher & Smith, 1988; Lin & Forscher, 1995; Pollard; Borisy 2003; Ponti et al., 2004). Inicialmente, há a transmissão da polimerização do filamento na borda da célula, seguidos da transmissão de forças de tração impulsionadas pela miosina, e finalmente, a protusão associada em tração contra a matriz extracelular, que puxa o corpo celular para frente (Figura 5) (Harris, 1973; Jurado et al., 2005; Suter; Forscher, 2000). Trabalhos anteriores evidenciaram a importância da polimerização de actina para células hospedeiras. Newman e colaboradores inicialmente mostraram que a inibição da estabilização de actina leva a redução do processo fagocítico e internalização de grandes moléculas (Newman et al., 1991). Trabalhos mais recentes mostram que alterações na nucleação da actina modulam a motilidade de queratinócitos por meio da regulação de genes associados ao citoesqueleto (Sharili et al., 2016). Além disso, o tratamento de macrófagos com citocalasina favoreceu a redução da migração das células (Pergola et al., 2017). Adicionalmente, a inibição da organização de actina em células dendríticas derivadas de medula óssea induziu a redução da velocidade dessas células (Paterson et al., 2022; Ricart et al., 2011).

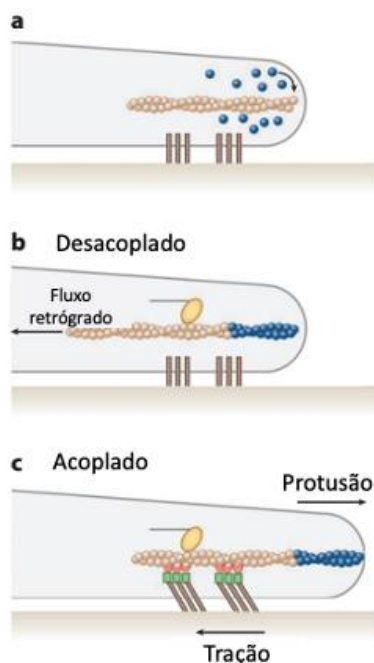


Figura 5 - Diagrama esquemático da montagem de actina de uma célula em migração. (a) Monômeros de actina montados na extremidade do filamento de actina na borda celular associadas a integrinas. (b) Actina recém-montada impulsionando o filamento para trás. (c) Interações indiretas entre as moléculas de adesão focal de ligação à actina e moléculas de adesão focal de ligação à integrina para imobilizar o filamento.

Fonte: (Wiesner et al, 2014).

A organização dos filamentos de actina é altamente regulada por proteínas da família Rho gtpases (Lin; Forscher, 1995). A maioria das Rho GTPases atuam nas membranas e afetam o movimento dessas membranas, alterando o citoesqueleto de actina associado à elas. Os membros da família mais bem estudados são RhoA, Rac1 e Cdc42 e, em células de mamíferos, Rac1 e Cdc42 são conhecidas por induzir protrusões da membrana plasmática como lamelipódios e filopódios, estimulando a polimerização da actina (Figura 6). RhoA atua na borda traseira de células, induzindo o desprendimento dessa região, fornecendo contratilidade à base de actomiosina necessária para a contração do corpo celular e retração da cauda, contribuindo assim para a motilidade celular. Adicionalmente, RhoA é fundamental na indução da polimerização de actina na borda dianteira da célula, na forma de lamelipódios de membrana, facilitando o impulsionamento da célula para a frente, além de gerar a força motriz para a motilidade celular (Jaffe; hall, 2005; Machacek et al., 2009; Heasman et al., 2010). Já Cdc42, induz polimerização de actina em invadopódios, que estão envolvidos em degradar a matriz extracelular, permitindo que as células migrem através de um ambiente tridimensional,

além de ser importante para a migração direcional e quimiotaxia em alguns tipos celulares, predominantemente por polarizar o citoesqueleto de microtúbulos (Pollard; Borisy., 2003).

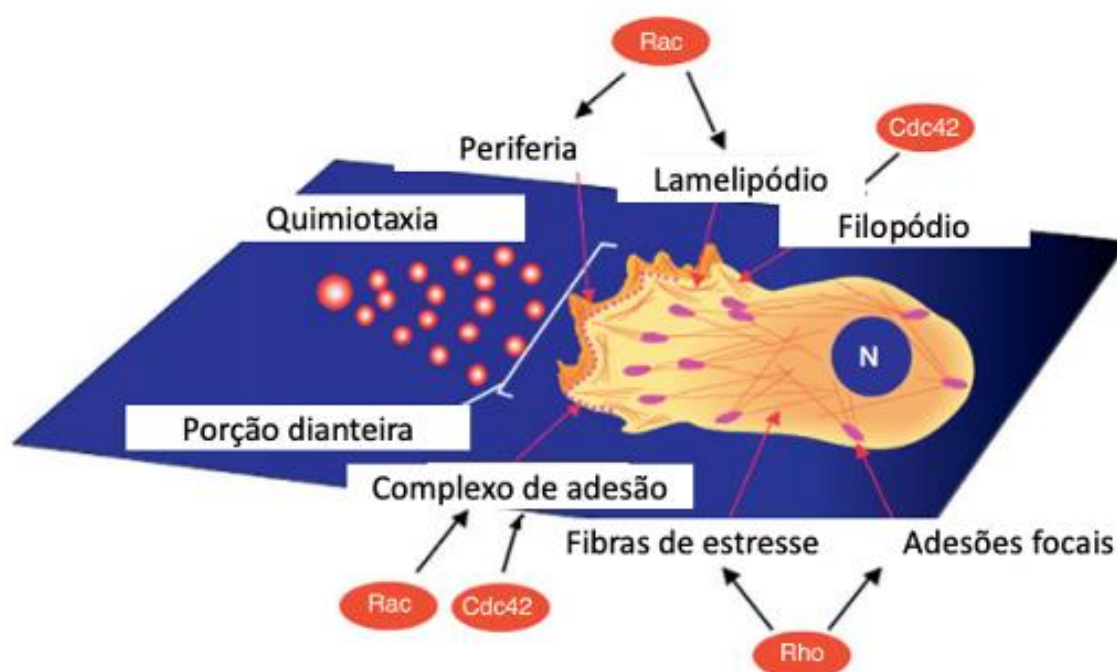


Figura 6 - Rho GTPases na migração celular. A família das Rho GTPases regulam a polimerização de actina. Rac1 regula a formação de lamelipódios e complexos focais; Cdc42 regula a formação de filopódios e complexos focais e Rho regula formação de fibras de estresse e adesões focais na parte posterior da célula.

Fonte: (Rikitake et al., 2011).

As Rho GTPases afetam a dinâmica dos microtúbulos, o que também é importante para a migração celular. Estudos sugerem que células dendríticas com RhoA inibidas tem sua capacidade migratória suprimida em comparação com as células não tratadas com o inibidor (Riol-blanc et al., 2005). Além disso, a depleção transitória de Rac1, RhoA e Cdc42 em fibroblastos resultou em uma diminuição da motilidade (Monypenny et al., 2009) e leucócitos tratados com inibidores específicos tiveram sua motilidade reduzida em comparação aos grupos não tratados (Lammerman et al., 2008; Babier et al., 2019). Embora as funções principais de Rho GTPases na migração celular já foram estabelecidas, ainda não é totalmente compreendido como essas proteínas e seus efetores são regulados, e como sua dinâmica interfere exatamente na migração celular (Villalonga; Ridley, 2006).

1.4.4 Podossomos e proteases

Podossomos são organelas altamente dinâmicas, que passam por constantes rearranjos. Tais organelas pertencem ao grupo de invadosomos que exibem a capacidade de degradação focal da matriz extracelular por proteases de matriz (Moreau et al., 2003). Podossomos são formados constitutivamente em células como macrófagos e células dendríticas, mas também podem ser encontrada ou induzida em uma variedade de outras células, como células endoteliais e musculares (Sabeh et al, 2009; Guet et al, 2011). Essas estruturas consistem de um núcleo rico em F-actina em forma de anel, que também contém proteínas associadas à actina como por exemplo o complexo Arp2/3, cortactina, gelsolina, talina e vinculina (Figura 7) (Linder et al., 2003; Linder et al., 2005). Além de estarem envolvidas na degradação da matriz extracelular, os podossomos são estruturas associadas a adesão, mecanossensores e transdutores, além de serem relacionados à capacidade de degradação da matriz extracelular (Linder et al., 2007; Linder et al., 2011).

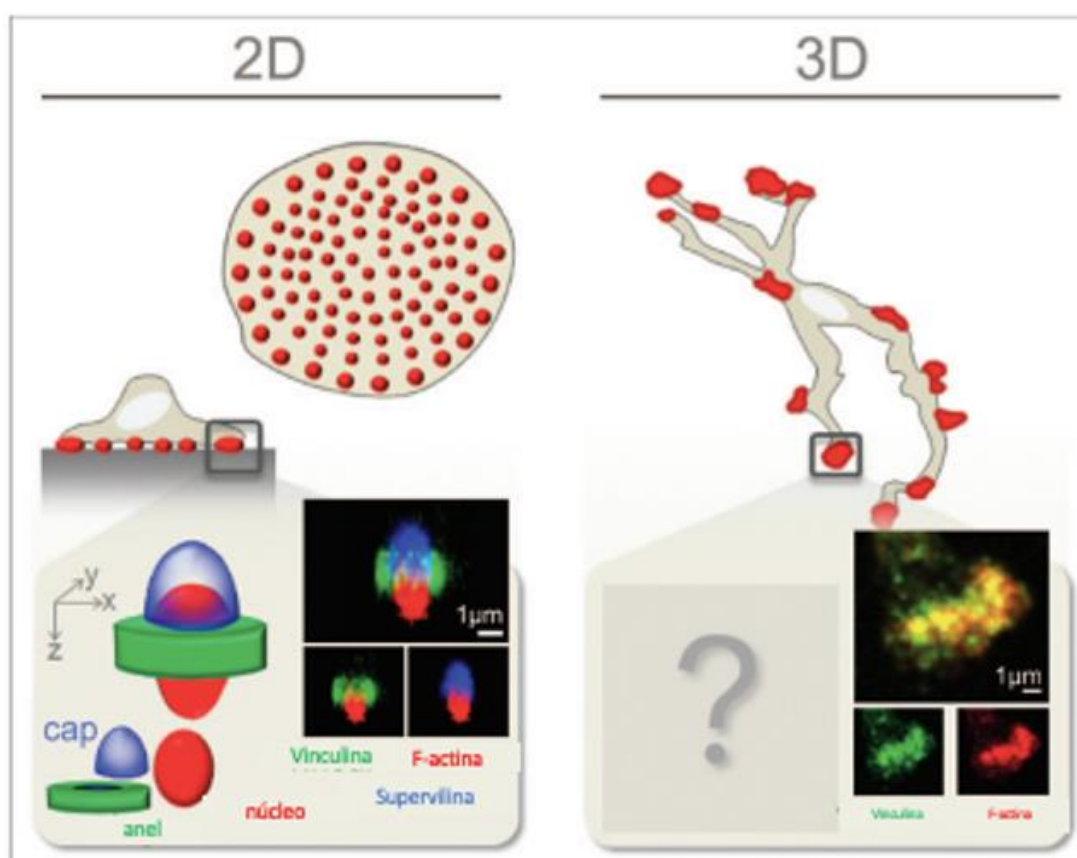


Figura 7 - Podossomos em ambiente 2D e 3D. Em ambiente 2D, os macrófagos formam vários podossomos semelhantes a pontos. Em ambientes 3D densos, macrófagos humanos formam podossomos 3D em saliências.

Fonte: (Wiesner et al, 2014).

A degradação eficiente da matriz extracelular por podossomos requer a regulação da secreção de proteases por essas estruturas (Van Goethem et al., 2010). Estudo anterior demonstrou que a deleção de proteínas envolvidas na estabilidade e organização de podossomos, como filamina A e Hck, afetou a degradação da matriz 3D por proteases, o que implica em uma migração mesenquimal menos eficaz (Cougoule et al., 2010; Guet et al., 2012). Trabalhos publicados anteriormente demonstraram diferentes proteases envolvidas na lise de componentes de matriz extracelular, incluindo metaloproteases, como metaloproteases de matriz (MMPs), bem como serina proteases e seus receptores associados, o sistema de ativação de plasmina e cisteína proteases (Kessenbrock et al., 2010; Mohamed et al., 2006).

Em ambiente bidimensional, foi mostrado que fagócitos formam podossomos constitutivamente, apesar desse processo estar relacionado aos seus subtipos celulares (Wiesner et al., 2014). Em macrófagos, os podossomos tendem a ser bem distribuídos por todo o corpo celular, no entanto, em resposta a fatores quimiotáticos, essa distribuição uniforme pode ser perdida (Alonso et al., 2019). Estudo demonstrou que macrófagos M2 são mais capazes de formar uma maior quantidade de podossomos, do que os subtipos M1 (Cougoule et al., 2012). Ainda, em macrófagos e monócitos, os podossomos podem estar uniformemente distribuídos em podossomos “dot-like”, podendo exibir um padrão e variando de dez a centenas de estruturas em forma de ponto por célula (Corfine et al., 2011; Bhuwania et al., 2012). Além de podossomos individuais, já foi descrita a formação de superestruturas conhecidas como rosetas de podossomos, mais comumente encontradas em macrófagos ativados por M-CSF ou LPS (Yamaguchi et al., 2006; Poincloux et al., 2006). Adicionalmente, foi demonstrado que a maior motilidade de células RAW está associada à presença de podossomos altamente ativos (Hind et al., 2014). Diferente do observado em macrófagos, células dendríticas podem formar podossomos alongados e direcionados, estabelecendo os chamados invadosomos ou podossomos protrusivos com áreas de formação de contatos focais (Linder et al., 2000; Gawden-Bone et al., 2010; Luxenburg et al., 2007; Akisaka et al., 2008; Martin-Granados et al., 2015). Sabe-se ainda que células dendríticas maduras formam menos podossomos e mais conjunturas alongadas na periferia celular, ao passo que subtipos imaturos formam podossomos “dot-like”, demonstrando que características associadas a maturação de células dendríticas fazem parte do processo de adesão e migração dessas células (Burns et al., 2001).

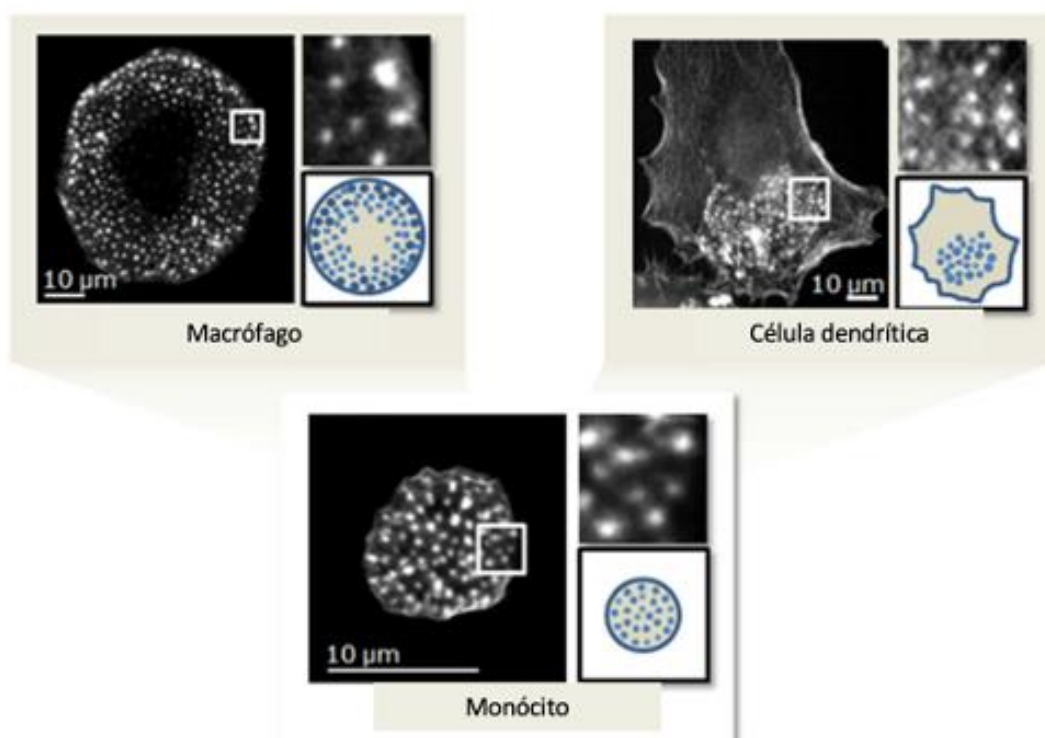


Figura 8 - Podossomos em células mononucleares. Organização de podossomos em diferentes tipos celulares.
Fonte: (Linder et al, 2014).

1.4.5 Vias de sinalização na migração celular

Para que células hospedeiras como macrófagos, monócitos ou células dendríticas migrem frente a homeostase ou a processos inflamatórios, a ativação de vias de sinalização específicas é fundamental (Welf; Haugh, 2011). Além disso, para organizar a migração contínua das células, estas estabelecem padrões dianteiros e traseiros, onde diferentes vias de sinalização como PI3K, ROCK ou JAK/STAT podem ser ativadas, favorecendo a extensão e retração da membrana.

A proteína cinase fosfatidilinositol 3-cinase (PI3K) é um componente essencial na regulação de diversos processos celulares, como a sobrevivência, o crescimento e migração celular. Funcionalmente, estas proteínas estão envolvidas em uma variedade de processos celulares, incluindo regulação do citoesqueleto, tráfego de vesículas, sinalização de insulina e migração celular, através da produção de fosfatidilinositol 3-fosfato (PtdIns3P) (Sharif et al., 2019). Elas são classificadas em três subgrupos distintos com base em sua especificidade de substrato e também na similaridade de sequência: classe I (A e B), classe II e classe III (Cain; Ridley, 2009).

A atividade da classe I das PI3K está associada a uma dinâmica cortical da F-actina, processos quimiotáticos e fagocitose, ao passo que PI3Ks da Classe II abrangem diferentes funções como migração celular, transporte de glicose e sinalização de insulina além de fatores associados a endocitose e exocitose nas células (Falasca; Mafucci, 2012; Jean; Kiger, 2014). A fosfatidilinositol 3-cinase, classe III ou PI3KC3, está associada à regulação da autofagia nas células, formação de vesículas autofágicas e nucleação do autofagossomo (Jean; Kiger, 2014). Adicionalmente, as PI3Ks de Classe II também têm sido implicadas em processos metabólicos, como a regulação do tráfego de vesículas em células secretoras de insulina, desempenhando um papel importante na secreção de insulina. Além disso, essas enzimas podem influenciar a regulação do tráfego de vesículas em outras células, afetando a distribuição de proteínas e lipídios na membrana celular (Yano et al., 2018).

A via de sinalização da fosfoinositídeo-3-cinase (PI3K) classe I emergiu como um regulador-chave da função dos macrófagos em resposta a estímulos metabólicos e, a ativação da via PI3K/Akt tem sido demonstrada como promotora da polarização do tipo M2 em macrófagos (Sharif et al., 2019). Estudo anterior demonstrou que a ativação da PI3K é essencial para a quimiotaxia de macrófagos em locais de inflamação, uma vez que a inibição da quimiotaxia de macrófagos foi completamente revertida pelo inibidor de PI3K (Yano et al., 2018). Adicionalmente, trabalho recente mostrou que eventos intracelulares são gerados durante o processo de ativação da PI3K em macrófagos, incluindo a ativação de AKT, uma serina cinase importante para a dinâmica do citoesqueleto de actina em macrófagos no processo de formação de protrusões citoplasmáticas. Os autores mostraram que a F-actina se acumula durante as fases iniciais da internalização de partículas, mas durante a formação dos pseudópodos no processo migratório, é clivada na presença do inibidor de PI3K (Schlam et al., 2015).

Uma outra importante via associada a processos de motilidade celular é a via JAK-STAT (*Janus kinase-signal transducer and activator of transcription*), que desempenha um papel fundamental na regulação de respostas imunológicas, inflamação, crescimento celular e diferenciação. Essa via é ativada por uma variedade de citocinas, envolvendo várias proteínas-chave, incluindo Janus cinases (JAKs) e fatores de transcrição STAT (*signal transducers and activators of transcription*) (Harrisson, 2012). A migração celular envolve mudanças na morfologia e na dinâmica do citoesqueleto, como a reorganização da actina, que é crucial para a formação de protrusões celulares, como lamelipódios e filopódios. A via JAK-STAT pode influenciar a remodelagem do citoesqueleto, facilitando a formação dessas estruturas e, portanto, a migração das células. Adicionalmente, foi demonstrado que a expressão de genes

envolvidos na motilidade celular e reguladores do citoesqueleto são efetivamente regulados pela via JAK-STAT (Seif et al., 2017; Hu et al., 2021). Além disso, estudo recente demonstrou que IFN- γ inibe diretamente a migração de monócitos, suprimindo a remodelação do citoesqueleto de actina e a polarização celular, sendo esta inibição dependente de STAT1 (Yang et al., 2008; Xin et al., 2020).

Outra via de sinalização associada a processos migratórios é a via ROCK (*Rho-Associated Protein Kinase*), uma cascata de sinalização intracelular que participa da regulação do citoesqueleto e da motilidade celular. Essa via é mediada por duas isoformas de cinases, ROCK1 e ROCK2, que são ativadas pela pequena proteína GTPase Rho (Guan et al., 2023). Essas cinases medeiam uma grande parte dos sinais, além de regular a dinâmica do citoesqueleto de actina e a contratilidade celular (Amano et al., 2010; Alblas et al., 2001). Como resultado, essa regulação afeta processos como a migração celular, adesão célula-célula e o comportamento invasivo de células cancerígenas (Narumiya et al., 2009). Recentemente, foi demonstrado que ROCK2 estimula a migração e adesão de monócitos em modelo murino, modulando a expressão de citocinas inflamatórias (Takeda et al., 2019).

1.5 Migração de células hospedeiras na infecção por diferentes patógenos

A migração celular constitui um mecanismo importante para a manutenção de processos fisiológicos celulares, além de estar amplamente relacionada a processos inflamatórios e de respostas a diversas infecções (Friedl; Weigeling, 2008). Células dendríticas ativadas, por exemplo, migram do sítio inflamatório para os linfonodos, onde orientam a apresentação de antígenos e a proliferação de células T (Guak; Krawczyk, 2020). Diversos estudos tem mostrado que patógenos são capazes de modular a migração de células hospedeiras. Recentemente, foi demonstrado que diferentes patógenos são capazes de modular não só o citoesqueleto, associado a motilidade e estrutura celular, como mecanismos associados a adesão de células hospedeiras, importantes no processo migratório (Murphy; Brinkworth, 2021). Adicionalmente, foi demonstrado que a infecção por *Chlamydia trachomatis* modula o recrutamento de actina dependente de RhoA e que *Rickettsia rickettsii* e *Shigella flexneri* modulam estruturalmente a vinculina, reduzindo a adesão das células (Dowd et al., 2020; Caven et al., 2020).

No modelo de infecções virais, foi demonstrado que células dendríticas infectadas por HIV apresentam a expressão e a distribuição espacial de vinculina alteradas (Quaranta et al., 2003). Trabalho anterior também demonstrou que a infecção de macrófagos por HIV-1 afeta tanto a

motilidade em ambiente 2D, quanto a migração mesenquimal em ambiente 3D dessas células (Verollet et al., 2015). Ainda em modelo viral, Nogalski e colaboradores demonstraram que monócitos deficientes para paxilina, apresentaram uma redução da interação e entrada do citomegalovírus (HCMV), evidenciando a importância dessa proteína no processo de captura viral e motilidade celular (Nogalski et al., 2011).

Com relação às infecções parasitárias, em modelo de infecção de células HeLa por *Trypanosoma cruzi*, foi observado que células deficientes para FAK eram mais susceptíveis à infecção pelo parasito quando comparadas com as células selvagens, evidenciando que o rearranjo da adesão focal é importante para processos invasivos de *T. cruzi* (Onofre et al., 2019). Adicionalmente, foi demonstrado que a infecção por *Toxoplasma gondii* leva ao aumento da migração de células dendríticas (Lambert et al., 2006). Estudo recente mostrou ainda que, durante a infecção por *T. gondii*, macrófagos expressam CCR7 em ensaio de quimiotaxia *in vitro*, e em estudo *in vivo*, macrófagos de medula óssea infectados tiveram sua migração aumentada quando comparados com macrófagos não-infectados (Hoeve et al., 2022). Adicionalmente, foi demonstrado que a infecção por *T. cruzi* reduz os níveis e rompe a distribuição de vinculina em costâmeros celulares, além da infecção por este parasito alterar a distribuição espacial de talina e paxilina e induzir a expressão de FAK, levando a um distúrbio na mecanotransdução durante a cardiomiopatia chagásica (Melo et al., 2014; Melo et al., 2019).

1.6 Migração de células hospedeiras na infecção por *leishmania*

Uma resposta imune eficaz depende da capacidade dos leucócitos em migrar através da vasculatura e tecidos para locais de infecção e inflamação. Assim como outros patógenos, uma vez internalizados, parasitos de *Leishmania* podem modular migração de células hospedeiras.

Na patogênese da leishmaniose, os monócitos se desenvolvem na medula óssea e migram para os tecidos periféricos em resposta a sinais quimiotáticos ou danos causados por infecção e inflamação (Martinez; Helming; Gordon, 2009). Estudo publicado anteriormente demonstrou que a infecção por *L. amazonensis* reduziu a área de espalhamento de células de linhagem monocítica, sugerindo uma modulação da adesão destas células (Figueira et al., 2015). Em contrapartida, foi demonstrado que monócitos intermediários expressavam CCR2 com níveis aumentados de proteína CCL2 em lesões de pacientes infectados por *L. braziliensis*, sugerindo ainda que essas células têm maior probabilidade do que monócitos não clássicos de migrarem para o local da lesão e que a frequência dos monócitos intermediários aumenta logo após a infecção (Passos et al, 2015).

Apesar de ser a principal célula hospedeira da *Leishmania*, pouco se conhece sobre a adesão e migração de macrófagos na infecção por este parasito. Dados da literatura descrevem que a infecção por *L. major* reduz a migração de macrófagos em ensaio *in vivo* (Moll et al., 1993). Adicionalmente, estudo anterior relatou a redução da migração de macrófagos após a infecção por *L. mexicana* (Bray et al., 1983). Foi demonstrado ainda que a infecção por *L. amazonensis* reduz a adesão de macrófagos peritoneais de camundongos ao tecido conjuntivo, reduzindo a adesão à fibronectina, colágeno e laminina (Pinheiro et al., 2006). Em trabalho mais recente, foi demonstrado que a infecção por *L. amazonensis* reduz a migração de macrófagos murinos em ambiente bidimensional. Os autores mostraram ainda que essa redução está associada a uma modulação da dinâmica de actina nas células e a uma redução da formação de complexos de adesão nessas células (Menezes et al., 2017). Entretanto, em estudo realizado por Rocha et al. (2020), foi demonstrado uma redução da migração de macrófagos murinos após a infecção por *L. major*, mas um aumento nas células infectadas por *L. infantum*.

As células dendríticas, células apresentadoras profissionais de antígeno, também são descritas por terem sua migração moduladas por *Leishmania*. Estudos prévios demonstraram que as células de Langerhans (MHC II+, CD11c, CD205+) fagocitam *L. major in vivo* e migram para os linfonodos drenantes (dLNs) (Moll et al., 1993; Ritter et al., 2004). Por outro lado, estudo adicional em modelo murino de LC evidenciou que há uma redução na população migratória de células dendríticas infectadas quando comparadas com células não infectadas, indicando que o parasito pode alterar o padrão migratório dessas células *in vivo* (Hermida et al., 2014). Ainda hoje, pouco se conhece sobre o processo de migração de células hospedeiras na infecção por *Leishmania* e o papel destas células no estabelecimento ou disseminação do parasito.

2 JUSTIFICATIVA

A leishmaniose é uma doença endêmica em 90 países entre os trópicos e subtropicais, e estima-se a incidência de 1.2 milhões de novos casos anualmente (CDC, 2020). A presença da leishmaniose está diretamente ligada a fatores sociais e econômicos, ambientais, climáticos e nutrição precária, onde juntos, influenciam diretamente na epidemiologia da doença (WHO, 2023).

Leishmania spp. infectam diversos hospedeiros vertebrados, incluindo o homem (Banuls et al., 2007). As doenças causadas por este protozoário, as leishmanioses, podem apresentar diferentes tipos de manifestações clínicas, desde lesões localizadas na pele que cicatrizam espontaneamente, lesões de pele que se espalham de forma disseminada, a forma mucocutânea da doença, além da leishmaniose visceral, forma mais grave que podem levar à morte se não tratada (Ashford, 2000; Desjeux, 2004). Nesse cenário de diferentes manifestações clínicas da doença, ainda não se conhece como o parasito dissemina no hospedeiro vertebrado e o papel de diferentes células hospedeiras nesse processo.

Sabe-se que patógenos são capazes de modular a migração de células hospedeiras. Com relação à leishmaniose, foi mostrado que a infecção por *L. mexicana* reduz a migração de macrófagos (Bray et al., 1983). Adicionalmente, trabalhos mais recentes demonstraram que a infecção por *L. amazonensis* reduz adesão e migração de macrófagos (Carvalho et al., 2004; Pinheiro et al., 2006; De Menezes et al., 2017). Entretanto, em trabalho recente, Rocha et al. (2020) mostraram que há uma modulação diferente na migração de macrófagos após a infecção por diferentes espécies de *Leishmania*. No entanto, pouco se sabe sobre o papel de diferentes células hospedeiras na disseminação do parasito no hospedeiro vertebrado e seus mecanismos moleculares envolvidos. Adicionalmente, com relação à migração de células dendríticas, trabalhos publicados demonstraram que a infecção por *Leishmania amazonensis* leva à redução da migração por essas células (Hermida et al., 2014). Outros trabalhos, no entanto, avaliaram o perfil migratório de células dendríticas após infecção por *Leishmania major* em modelo murino e demonstraram um aumento da capacidade motora destas células. Esses dados evidenciam a necessidade de estudos mais aprofundados acerca da migração de células hospedeira, do processo de disseminação do parasito e sua relação com as diferentes espécies de *Leishmania*.

Na leishmaniose, a relação entre disseminação do parasito e a gravidade da doença é particularmente estreita. Neste trabalho, buscamos elucidar o papel de células hospedeiras na disseminação de *Leishmania* sp. no hospedeiro vertebrado. Nossa hipótese é que a infecção por *Leishmania* induz a migração de células dendríticas, mas não de monócitos e macrófagos.

Estudos para melhor entender o processo de migração de células hospedeiras infectadas e sua relação com a disseminação da doença são fundamentais para a concepção de novas estratégias para terapêutica e prevenção do desenvolvimento de formas graves e desfigurantes da leishmaniose.

3 CAPÍTULO 1

3.1 Hipótese

A infecção por *Leishmania* induz a migração de células dendríticas humanas.

3.2 Objetivos

3.2.1 Objetivo geral

Avaliar a migração de células dendríticas humanas infectadas por *Leishmania* e os mecanismos envolvidos neste processo.

3.2.2 Objetivos específicos

- Avaliar a migração de células dendríticas infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum* em ambiente bidimensional;
- Investigar a formação complexos de adesão em células dendríticas, por *L. amazonensis*, *L. braziliensis* ou *L. infantum* em ambiente bidimensional;
- Determinar a atividade de moléculas sinalizadoras e a polimerização dos filamentos de actina durante o processo de migração de células dendríticas, humanos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum* em ambiente bidimensional;
- Avaliar a expressão de CCR7 em células dendríticas humanas infectadas por diferentes espécies de *Leishmania*.

4 MANUSCRITO 1

***Leishmania*-induced dendritic cell migration and its potential contribution to parasite dissemination**

O presente trabalho publicado avaliou a migração de células dendríticas humanas infectadas por diferentes espécies de *Leishmania*.

Abstract: *Leishmania*, an intracellular parasite species, causes lesions on the skin and in the mucosa and internal organs. The dissemination of infected host cells containing *Leishmania* is crucial to parasite survival and the establishment of infection. Migratory phenomena and the mechanisms underlying the dissemination of *Leishmania*-infected human dendritic cells (hDCs) remain poorly understood. The present study aimed to investigate differences among factors involved in hDC migration by comparing infection with visceral leishmaniasis (VL) induced by *Leishmania infantum* with diverse clinical forms of tegumentary leishmaniasis (TL) induced by *Leishmania braziliensis* or *Leishmania amazonensis*. Following the infection of hDCs by isolates obtained from patients with different clinical forms of *Leishmania*, the formation of adhesion complexes, actin polymerization, and CCR7 expression were evaluated. We observed increased hDC migration following infection with isolates of *L. infantum* (VL), as well as disseminated (DL) and diffuse (DCL) forms of cutaneous leishmaniasis (CL) caused by *L. braziliensis* and *L. amazonensis*, respectively. Increased expression of proteins involved in adhesion complex formation and actin polymerization, as well as higher CCR7 expression, were seen in hDCs infected with *L. infantum*, DL and DCL isolates. Together, our results suggest that hDCs play an important role in the dissemination of *Leishmania* parasites in the vertebrate host.

Keywords: Dendritic cell; migration; *Leishmania* sp.; dissemination

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Article

Leishmania-Induced Dendritic Cell Migration and Its Potential Contribution to Parasite Dissemination

Amanda Rebouças ¹, Thailla S. Silva ¹, Lilian S. Medina ², Bruno D. Paredes ^{3,4}, Luciana S. Aragão ^{3,4}, Bruno S. F. Souza ^{3,4,5}, Valéria M. Borges ⁶, Albert Schriefer ^{2,7} , Patricia S. T. Veras ¹, Claudia I. Brodskyn ¹ and Juliana P. B. de Menezes ^{1,*}

- ¹ Laboratory of Host—Parasite Interaction and Epidemiology, Gonçalo Moniz Institute, Salvador 40296-710, BA, Brazil; amandareboucas95@gmail.com (A.R.); thaillasilva16.1@bahiana.edu.br (T.S.S.); patricia.veras@fiocruz.br (P.S.T.V.); claudia.brodskyn@fiocruz.br (C.I.B.)
- ² Immunology Service, Professor Edgard Santos Hospital, Federal University of Bahia, Salvador 40170-110, BA, Brazil; lilimedina25@hotmail.com (L.S.M.); nab.schriefer@gmail.com (A.S.)
- ³ Center for Biotechnology and Cell Therapy, São Rafael Hospital, Salvador 41253-900, BA, Brazil; brunoparedes@gmail.com (B.D.P.); luaragao@gmail.com (L.S.A.); bruno.solano@fiocruz.br (B.S.F.S.)
- ⁴ D'Or Institute for Research and Education, Salvador 41253-900, BA, Brazil
- ⁵ Laboratory of Tissue Engineering and Immunopharmacology, Gonçalo Moniz Institute, Salvador 40296-710, BA, Brazil
- ⁶ Laboratory of Inflammation and Biomarkers, Gonçalo Moniz Institute, Salvador 40296-710, BA, Brazil; valeria.borges@fiocruz.br
- ⁷ Department of Bio-Interaction, Institute of Health Sciences, Federal University of Bahia, Salvador 40110-902, BA, Brazil
- * Correspondence: juliana.fullam@fiocruz.br



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Abstract: *Leishmania*, an intracellular parasite species, causes lesions on the skin and in the mucosa and internal organs. The dissemination of infected host cells containing *Leishmania* is crucial to parasite survival and the establishment of infection. Migratory phenomena and the mechanisms underlying the dissemination of *Leishmania*-infected human dendritic cells (hDCs) remain poorly understood. The present study aimed to investigate differences among factors involved in hDC migration by comparing infection with visceral leishmaniasis (VL) induced by *Leishmania infantum* with diverse clinical forms of tegumentary leishmaniasis (TL) induced by *Leishmania braziliensis* or *Leishmania amazonensis*. Following the infection of hDCs by isolates obtained from patients with different clinical forms of *Leishmania*, the formation of adhesion complexes, actin polymerization, and CCR7 expression were evaluated. We observed increased hDC migration following infection with isolates of *L. infantum* (VL), as well as disseminated (DL) and diffuse (DCL) forms of cutaneous leishmaniasis (CL) caused by *L. braziliensis* and *L. amazonensis*, respectively. Increased expression of proteins involved in adhesion complex formation and actin polymerization, as well as higher CCR7 expression, were seen in hDCs infected with *L. infantum*, DL and DCL isolates. Together, our results suggest that hDCs play an important role in the dissemination of *Leishmania* parasites in the vertebrate host.

Keywords: dendritic cell; migration; leishmania sp.; dissemination

1. Introduction

Leishmaniasis is a broad group of diseases caused by approximately 20 species of *Leishmania* parasites. The WHO has estimated that 30,000 new cases of visceral leishmaniasis (VL) and more than one million new cases of cutaneous leishmaniasis (CL) occur annually, with more than one billion people at risk of infection worldwide [1]. Leishmaniasis results in a wide spectrum of clinical manifestations, comprising two major forms delineated in accordance with clinical symptoms and manifestations. TL is characterized by the presence

of skin and/or mucosal lesions. VL is a chronic infection affecting internal organs, such as the liver, spleen, and bone marrow, which can be fatal if untreated. Specific clinical manifestations of leishmaniasis are dependent on the infecting parasite species and host immune response [2].

In the New World, specifically Brazil, VL caused by *L. infantum* is a serious public health problem. TL is considered a neglected tropical disease, resulting in more than one million infections annually [1]. Diverse manifestations of TL are classically categorized as localized cutaneous leishmaniasis (LCL), mucocutaneous leishmaniasis (ML), diffuse cutaneous leishmaniasis (DCL), and disseminated leishmaniasis (DL). LCL, which is caused by *L. braziliensis* (*Lb*) and *L. amazonensis* (*La*), is characterized by the presence of a single or a few well-delimited ulcers with raised borders. DL, a severe form of disease caused by *Lb* infection, is defined by the presence of at least 10 and up to more than 1000 skin lesions distributed over two or more noncontiguous parts of the body. DCL, a rare anergic form of disease caused by *La*, is characterized by numerous non-ulcerating nodules at one or more sites of the body [3]. ML, a variant form of CL, is characterized by the destruction of mucous membranes in the nose, mouth, and throat cavities, as well as surrounding tissues.

The biological cycle of *Leishmania* is initiated when parasite promastigote forms are inoculated into the skin of a mammalian host during blood feeding by phlebotomine sandflies. Inside the vertebrate host, macrophages and dendritic cells (DC) take up parasites, which can then survive within host cells [4]. Once inside the host, the parasite can persist at the original site of infection on the skin, and may disseminate to different host tissues. The mechanisms involved in parasite dissemination and the accompanying role played by host cells remains poorly understood [5]. The ability of *Leishmania*-infected host cells to migrate may be important to lesion distribution on the host and the dissemination of disease.

Previously published work has shown that infection with *Leishmania* parasites modulates different phagocyte functions associated with cell migration, such as signaling, spreading, and adhesion to substrates [5–9]. Using a murine model, Hermida et al. showed that *L. amazonensis* infection reduces dendritic cell migration [7]. In addition, the blockade of adhesion molecules was found to lead to reduced migration in *L. major*-infected dendritic cells [10]. However, other studies have demonstrated that *L. major* infection leads to increased CCR7 expression and DC migration in response to CCL21 [11]. Thus, the mechanisms underlying the modulation of DC migration induced by *Leishmania*, as well as impacts on parasite dissemination, remain unelucidated.

Cell migration constitutes a complex process and is essential to a variety of cell-mediated processes, such as immune response, tissue repair, and homeostasis [12]. This process consists of five steps: the formation of a leading pseudopod; cellular adhesion to substrate; cell body translocation; release of the rear edge of the cell; and the retraction and recycling of the membrane and receptors from the rear to the front of the cell [13]. The ability of cells to migrate effectively is dependent on adhesion signaling mediated by integrin receptors, as well as interdependent feedback between F-actin polymerization/depolymerization and motility-activated myosin II and focal adhesion assembly/disassembly [14,15].

Integrin-mediated adhesion complexes assemble at the leading edge of migrating cells, establishing a structural link between the extracellular matrix and the actin cytoskeleton [16]. Upon integrin engagement, signaling is initiated through focal adhesion kinase (FAK) and the Src family kinases, as well as scaffolding molecules, such as talin and paxillin. Paxillin, an adaptor phosphoprotein that localizes to focal adhesions, recruits and binds to many signaling and structural proteins [17]. The phosphorylation of paxillin by activated kinases, such as FAK, recruits effector molecules, leading to changes in cell motility [18]. During the migratory process, the assembly of focal adhesions associated with actin stress fibers requires the activation of Rho-GTPase family proteins, which regulate complex interplay between integrins and the actin cytoskeleton [19]. The roles played by Rho GTPases in cell migration are well-established and rely on Cdc42- and Rac-dependent cell protrusion at the leading edge, in addition to Rho-dependent contractility, which is required to move the cell body forward [20]. In this study, we investigated the effects

of infection by different strains of *Leishmania* parasites associated with diverse forms of disease presentation on the migration of hDCs. Our results indicate that these cells play an important role in the visceralization of disease and the dissemination of *Leishmania* parasites in the vertebrate host.

2. Materials and Methods

2.1. Ethics Statements

Whole blood monocytes were isolated from healthy donor buffy coats (Hemoba, Bahia, Brazil). This project was approved by the Institutional Review Board of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (CEP/IGM-FIOCRUZ) (protocol no. 2.751.345).

2.2. *Leishmania* Parasites

Promastigotes of *L. infantum* (MCAN/BR/89/BA262), *L. braziliensis* (MHOM/BR/01/BA788) and *L. amazonensis* (MHOM/Br88/BA125) maintained at our institution (IGM-FIOCRUZ), as well as *L. amazonensis* isolated from a DCL (La DCL) patient (BA336) residing in Maranhão and *L. braziliensis* (BA9432/BA19689) isolates obtained from patients with DL (Lb DL) and CL (Lb LCL) who lived in Corte de Pedra (Bahia-Brazil), an area endemic for CL, were maintained for up to six successive passages in Schneider's Insect Medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 50 µg/mL gentamicin (Gibco, Waltham, MA, USA) and 10–20% (*v/v*) fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). Promastigotes were grown in an incubator at 24 °C and monitored daily by counting in a Neubauer chamber. Upon reaching stationary phase, promastigotes were used in experiments.

2.3. Dendritic Cell Cultures

Immature monocyte-derived hDCs were obtained from freshly isolated healthy peripheral blood monocytes using Ficoll–Histopaque density gradient separation (Sigma-Aldrich, Saint Louis, MO, USA). Peripheral blood mononuclear cells (PBMCs) were washed three times, and the CD14+ cell population was enriched by positive selection using magnetic cell sorting (Miltenyi Biotec, Leiden, The Netherlands) to isolate the CD14+ subset. Cells were then resuspended and plated in RPMI medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL) and interleukin 4 (IL4) (100 UI/mL) (PeproTech, Rocky Hill, NJ, USA) for 7 days.

2.4. hDC Infection

hDCs (2×10^5 /well) were plated on 24-well plates for immunofluorescence and migration assays 24 h prior to experimentation. *L. amazonensis* (10:1), *L. braziliensis* (10:1) or *L. infantum* (20:1) promastigotes were added to the hDCs and incubated for 4 h at 37 °C. Cells were then washed in PBS to remove non-internalized parasites, then re-incubated for 6, 12, 24 or 48 h for cell migration assays, or 48 h at 37 °C for immunofluorescence and flow cytometry analyses.

2.5. Migration Assay

The evaluation of chemotaxis was assayed in Boyden chambers containing polycarbonate membranes (24 well, 5 µm pores, Corning® Transwell® polycarbonate membrane cell culture inserts). Infected hDCs and controls were seeded in RPMI and allowed to migrate toward RPMI containing chemokine ligand 3 (CCL3) (300 ng/mL) in the lower compartment for 4 h. The membranes were then washed three times with PBS, fixed with 4% (*v/v*) paraformaldehyde for 15 min, washed twice with PBS, and finally incubated with 10 mg/mL DAPI for nuclear staining. The top of the membrane was scraped to remove any residual non-migrating cells. hDCs were counted using a fluorescence microscope in 10 random fields across the bottom of the membrane in each well.

2.6. Zymosan Phagocytosis

Control hDCs were plated as described above and incubated with zymosan A derived from *Saccharomyces cerevisiae* (Sigma-Aldrich, Saint Louis, MO, USA) at a ratio of 10 or 20 particles per cell for 4 h, then washed in PBS to remove any non-internalized particles, and re-incubated for 6, 12, 24 or 48 h prior to performing migration analysis.

2.7. Immunofluorescence Assay

hDCs infected or not with *L. amazonensis*, *L. braziliensis* or *L. infantum* were fixed with 4% (v/v) paraformaldehyde for 15 min, washed with PBS, and submitted to cytospin centrifugation. Coverslips were quenched with 15 mM NH₄Cl for 20 min and washed three times with PBS, incubated in a blocking solution (3% (v/v) bovine serum albumin (BSA) in PBS) for 1 h, washed three times with PBS, permeabilized with 0.15% (v/v) saponin–PBS (Sigma-Aldrich, Saint Louis, MO, USA) for 15 min and then incubated with 1:500 rabbit anti-FAK (0.5 µg/mL) (Invitrogen, catalog number RC222574) or 1:100 rabbit anti-paxillin (0.1 µg/mL) (Invitrogen, catalog number QF221230) diluted in 1% (v/v) PBS + 0.15% (v/v) BSA saponin for 1 h. Next, anti-rabbit Alexa fluor 594 (Molecular Probes, catalog number A1011) was added and incubated for 1 h. Cells were then mounted with Prolong Gold antifade reagent and DAPI for nuclear staining (Invitrogen, Carlsbad, CA, USA). Images were acquired on a Leica confocal microscope using a 63×/1.4 objective and analyzed using Fiji Image J software.

2.8. Actin Polymerization Assay

Dendritic cells infected or not with *L. amazonensis*, *L. braziliensis* or *L. infantum* were fixed with 4% (v/v) paraformaldehyde for 15 min, washed with PBS, and submitted to cytospinning. Coverslips were quenched with 15 mM NH₄Cl for 20 min and washed three times with PBS, incubated in blocking solution (3% (v/v) BSA in PBS) for 1 h, washed three times with PBS, permeabilized with 0.15% (v/v) saponin–PBS for 15 min, and then incubated with 1:500 rabbit anti-Rac1 (2.5 ng/mL) (BD Biosciences, catalog number 610650), 1:200 mouse anti-Cdc42 (1 ng/mL) (Invitrogen, catalog number PA1-092X), 1:200 rabbit anti-RhoA (1 µg/mL) (Invitrogen, catalog number 0SR00266W) and 1:1200 phalloidin (1 µg/mL) (Invitrogen, catalog number A12379). All antibodies were diluted in 1% (v/v) PBS + 0.15% (v/v) BSA saponin for 1 h. Subsequently, anti-rabbit Alexa fluor 552 (0.02 µg/µL) (Molecular Probes, catalog number A32740), anti-mouse Alexa fluor 594 (0.02 µg/µL) (Molecular Probes, catalog number A1011) or anti-rabbit Alexa fluor 488 (0.02 µg/µL) (Molecular Probes, catalog number A32732) was added for 1 h. Cells were mounted with Prolong Gold antifade reagent with DAPI for nuclear staining (Invitrogen). Images were acquired on a Leica confocal microscope using a 63×/1.4 objective and analyzed using Fiji Image J software.

2.9. CCR7 Analysis

hDCs infected or not with *L. amazonensis*, *L. braziliensis* or *L. infantum* were stained with FITC anti-human CD197 [CCR7] (2 µg/mL) (Biolegend, catalog number 353215) and anti-CD11c (5 µg/mL) (Biolegend, catalog number 117301) and incubated on ice for 30 min. Cells were then washed twice with 1 mL of cold stain buffer (2% (v/v) FBS in PBS 1X) and centrifuged at 300× g for 10 min at 4 °C. Finally, the supernatant was aspirated, and the pellet was resuspended in 200 µL of cold stain buffer. Data were collected on an LSR Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FACSDiva software version 8.0 (BD Biosciences, Franklin Lakes, NJ, USA).

2.10. Statistical Analysis

All experiments were repeated three times and, after verifying data normality, data were analyzed by two-tailed Student's *t*-test or ANOVA using GraphPad Prism software. Results were considered significant when *p* < 0.05.

3. Results

3.1. Kinetics of hDC Infection with *L. amazonensis*, *L. braziliensis* or *L. infantum*

The kinetic analysis of hDC infection involving promastigotes of *L. amazonensis* (10:1), *L. braziliensis* (10:1) or *L. infantum* (20:1) revealed that the percentage of infected cells and the number of parasites per infected cell were similar among all infected cell groups at 4 h after infection. Cells were then washed and re-incubated for 6, 12, 24 or 48 h. On average, 4–5 parasites were observed per cell in all groups, and around 55% infected cells observed per group (Figure 1).

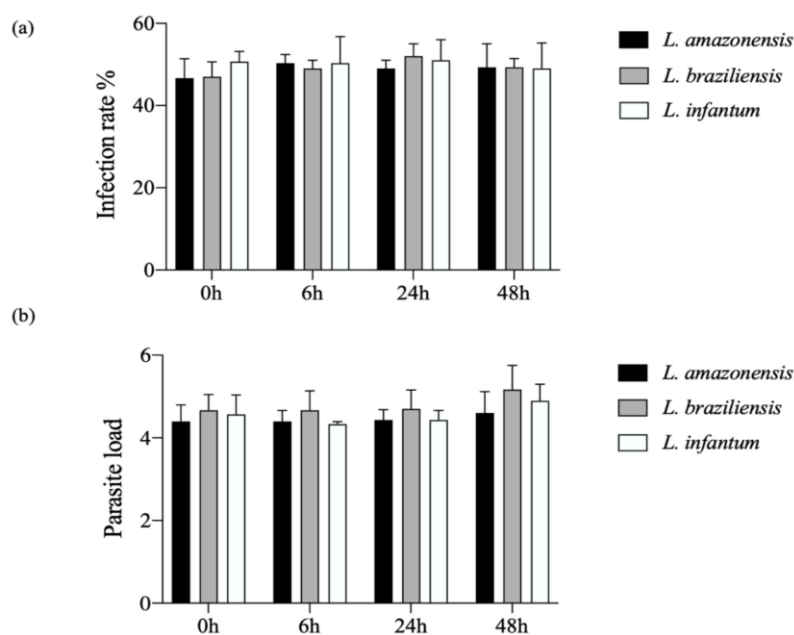


Figure 1. Kinetics of hDC infection with *L. amazonensis*, *L. braziliensis* or *L. infantum*. Human dendritic cells were infected with *L. amazonensis* (10:1), *L. braziliensis* (10:1) or *L. infantum* (20:1) for 4 h. (a) Percentage of infection; (b) parasitic burden in 400 cells randomly evaluated by fluorescence microscopy. (Student's *t*-test). Data are representative of three independent experiments.

3.2. Increased hDC Migration in *L. infantum* Infection

To evaluate the impact of infection by *L. amazonensis*, *L. braziliensis* or *L. infantum* on hDC migration, the ability of these cells to migrate directionally in response to CCL3, a chemokine that regulates DC migration, was analyzed. Our results demonstrated significantly greater migration in *L. infantum*-infected hDCs compared to uninfected DCs. A transient increase was seen in the migration of *L. amazonensis*-infected DCs for up to 12 h after infection. Notably, the migration of *L. braziliensis*-infected hDCs was reduced at all timepoints analyzed. Importantly, no changes were observed in migration rates among uninfected groups compared to those incubated with zymosan (Figure 2).

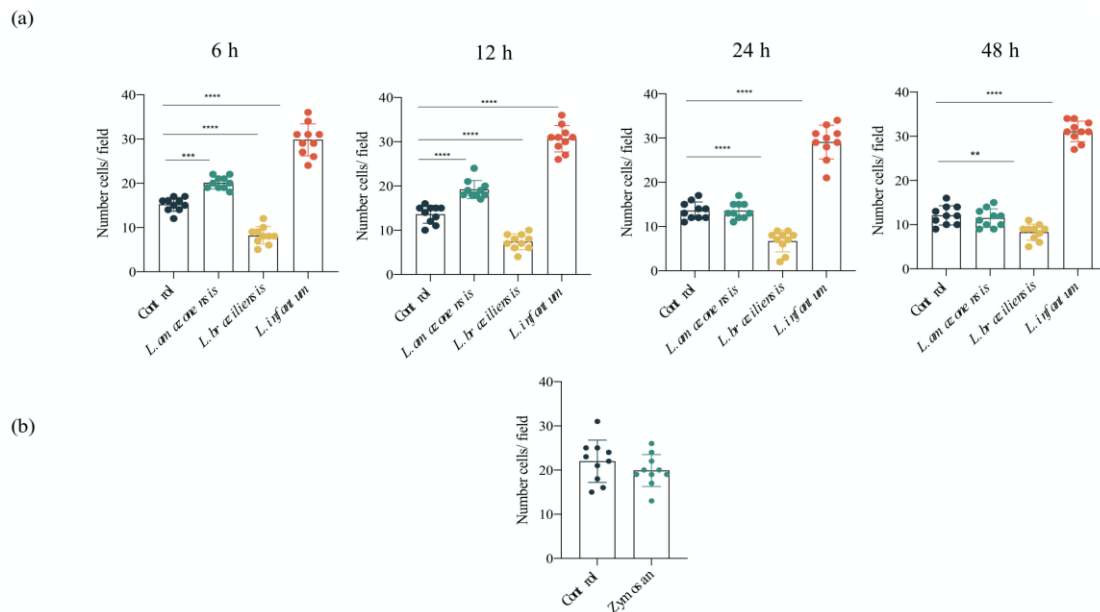


Figure 2. Increased hDC migration in *L. infantum* infection. Effects of *L. amazonensis*, *L. braziliensis* and *L. infantum* infection (4 h) on hDC migration in a Boyden chamber assay employing CCL3 in the lower chamber. At 6, 12, 24 and 48 h post-infection, cells were allowed to migrate for an additional 4 h. Following DAPI nuclear staining, migrating cells were randomly counted (10 fields) by fluorescence microscopy. (a) Numbers of migrating cells at 6, 12, 24 and 48 h post-infection. (b) Numbers of migratory cells after exposure to zymosan. ** $p < 0.0015$, *** $p < 0.0002$, **** $p < 0.0001$ (ANOVA). Results were obtained from three independent experiments.

3.3. *L. infantum* Induces hDC Expression of Adhesion Complex Proteins and Actin Polymerization

To investigate adhesion complex formation in hDCs and the potential role of this complex in migration induced by *L. infantum*, we analyzed pFAK and p-paxillin expression in the context of infection by *L. amazonensis*, *L. braziliensis* and *L. infantum*. Fluorescence analysis indicated increased FAK and paxillin expression following *L. infantum* infection compared to uninfected controls. In contrast, reduced levels of FAK and paxillin were found in response to *L. amazonensis* or *L. braziliensis* infection, compared to the control group (Figure 3).

Considering its essential role in cell migration, we analyzed the association between actin polymerization and the induction of migration by staining hDCs with phalloidin, which binds to filamentous actin (F-actin) bundles. Fluorescence analysis revealed no significant differences in levels of F-actin between each infected group and uninfected controls. However, we observed higher concentrations of polymerized actin bundles near cell borders in both control cells and hDCs infected by *L. infantum*, which was not the case in *L. amazonensis*- or *L. braziliensis*-infected cells (Figure 4a). To further investigate the process underlying cytoplasmic actin polymerization in *Leishmania*-infected hDCs, we evaluated the expression of protein members of the Rho GTPases family, which regulate actin cytoskeleton polymerization and dynamics. Increased expression of Rac1, RhoA and Cdc42 was seen in hDCs infected by *L. infantum*, but not *L. amazonensis* or *L. braziliensis*, in comparison to uninfected controls (Figure 4b,c).

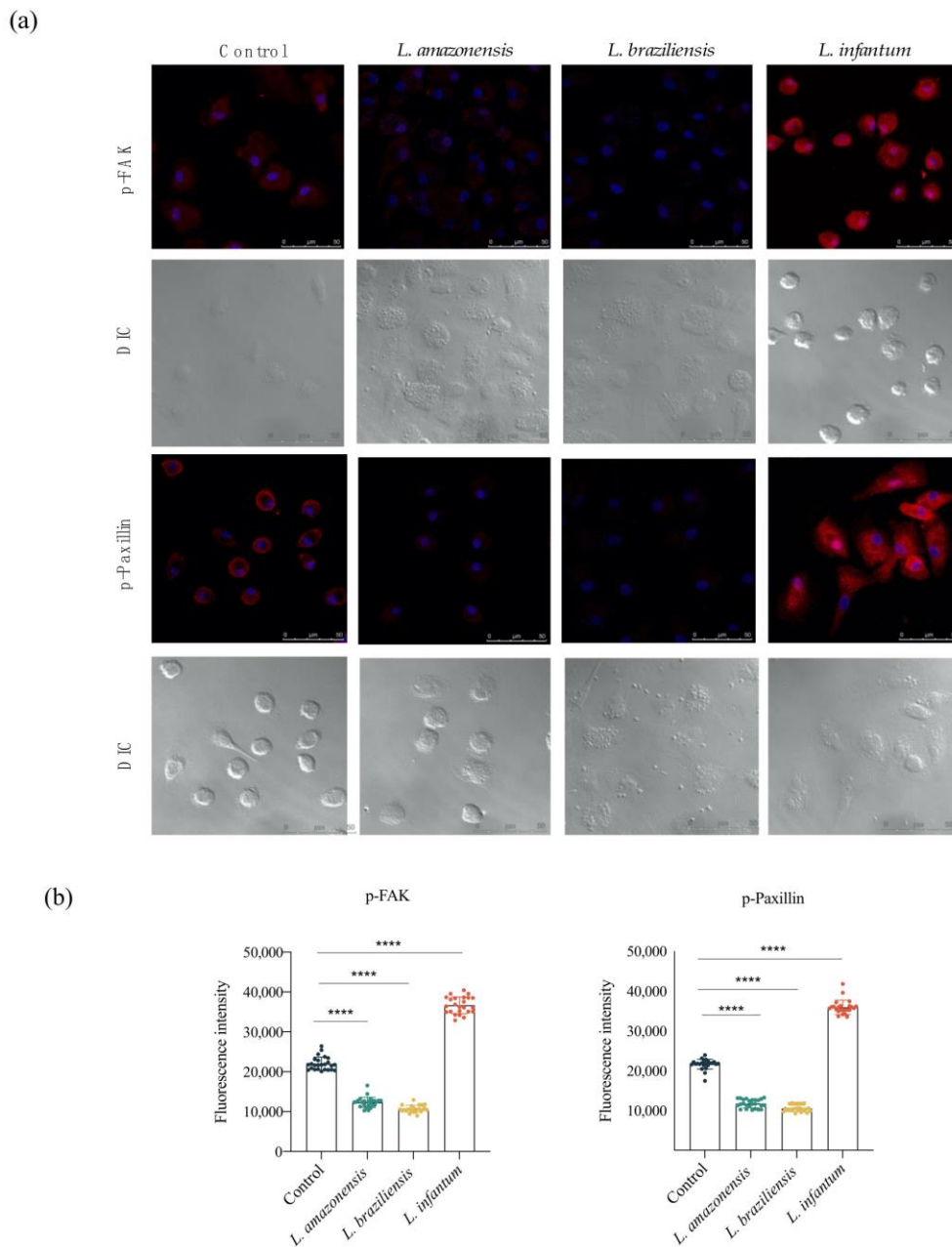


Figure 3. *L. infantum* induces hDC expression of adhesion complex proteins. Effects of *Leishmania* infection on phosphorylated FAK and paxillin protein levels in hDCs infected or not with *L. amazonensis*, *L. braziliensis* or *L. infantum*. (a) Fluorescence intensity of FAK and paxillin labeling in hDCs infected with *L. amazonensis*, *L. braziliensis* or *L. infantum*. (b) Quantification of fluorescence intensity of FAK and paxillin after *L. amazonensis*, *L. braziliensis* or *L. infantum* infection. Fluorescence intensity was quantified in 30 cells from each infection condition. Red: anti-FAK or anti-paxillin; blue: DAPI; grayscale: differential interference contrast (DIC); Control, uninfected cells. **** $p < 0.001$ (ANOVA). Data are representative of three independent experiments.

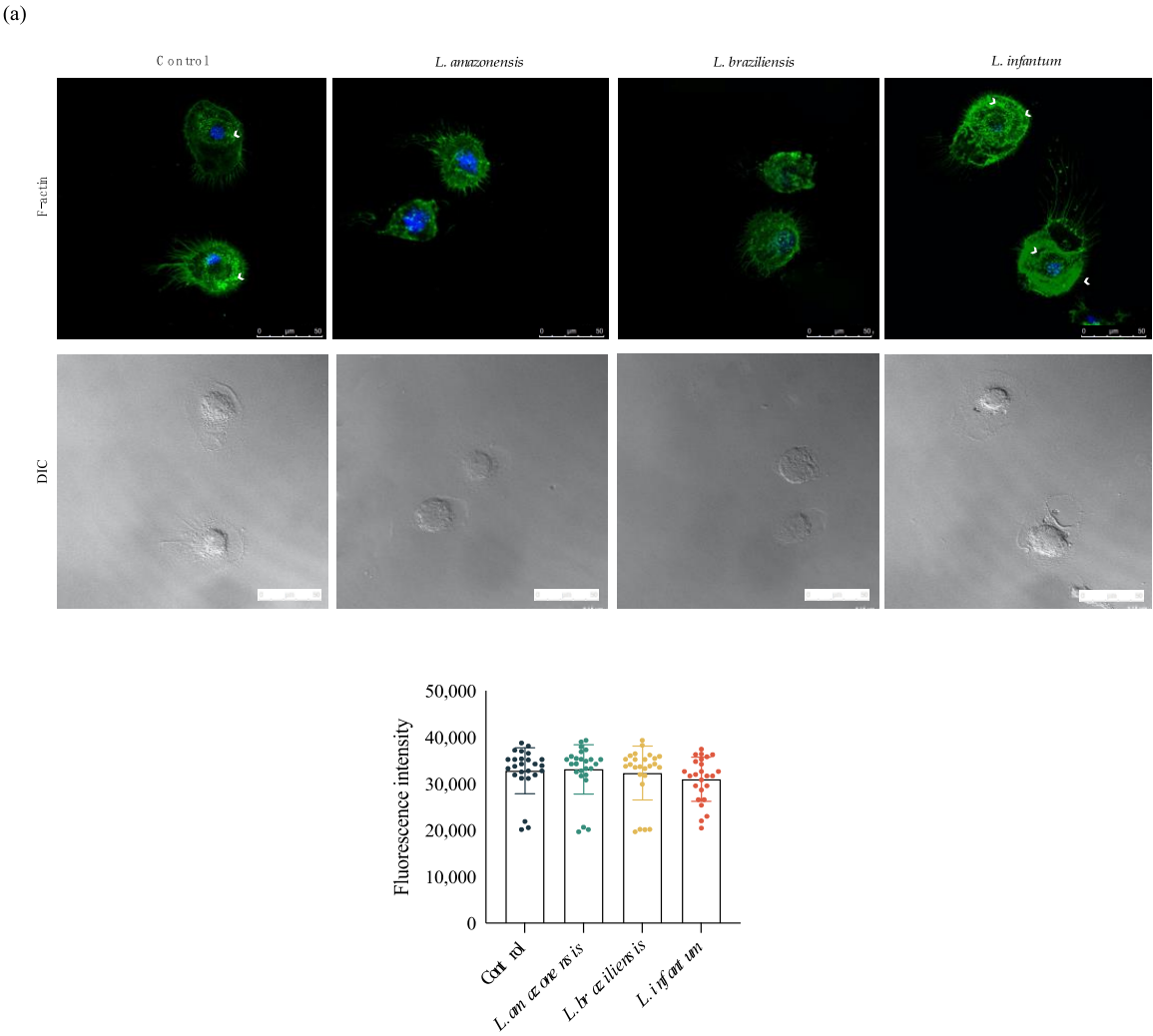
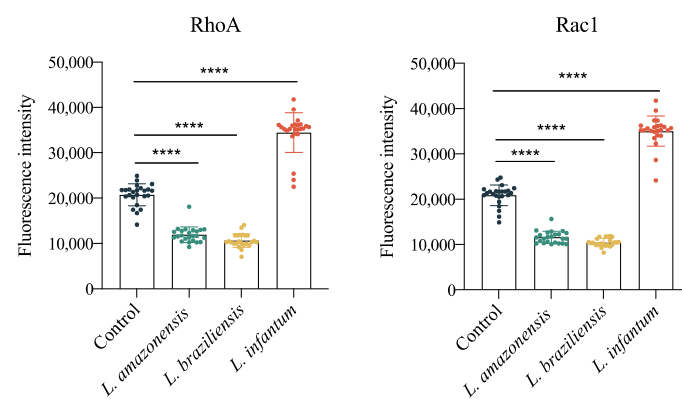


Figure 4. Cont.

Figure 3 displays a 4x4 grid of fluorescence microscopy images showing the localization of RhoA and Rac1 in *Leishmania* species. The columns are labeled 'RhoA', 'Rac1', 'Merge', and 'DIC'. The rows represent different *Leishmania* species: 'Control', '*L. amazonensis*', '*L. braziliensis*', and '*L. infantum*'. Each image includes a 50 µm scale bar. The 'RhoA' column shows red fluorescence, 'Rac1' shows green fluorescence, and 'Merge' shows both with blue DAPI-stained nuclei. The 'DIC' column shows brightfield images of the cells. The images illustrate that RhoA and Rac1 are primarily localized in the cytoplasm and nucleus, with some punctate localization observed in the control and *L. infantum* cells.



2

(c)

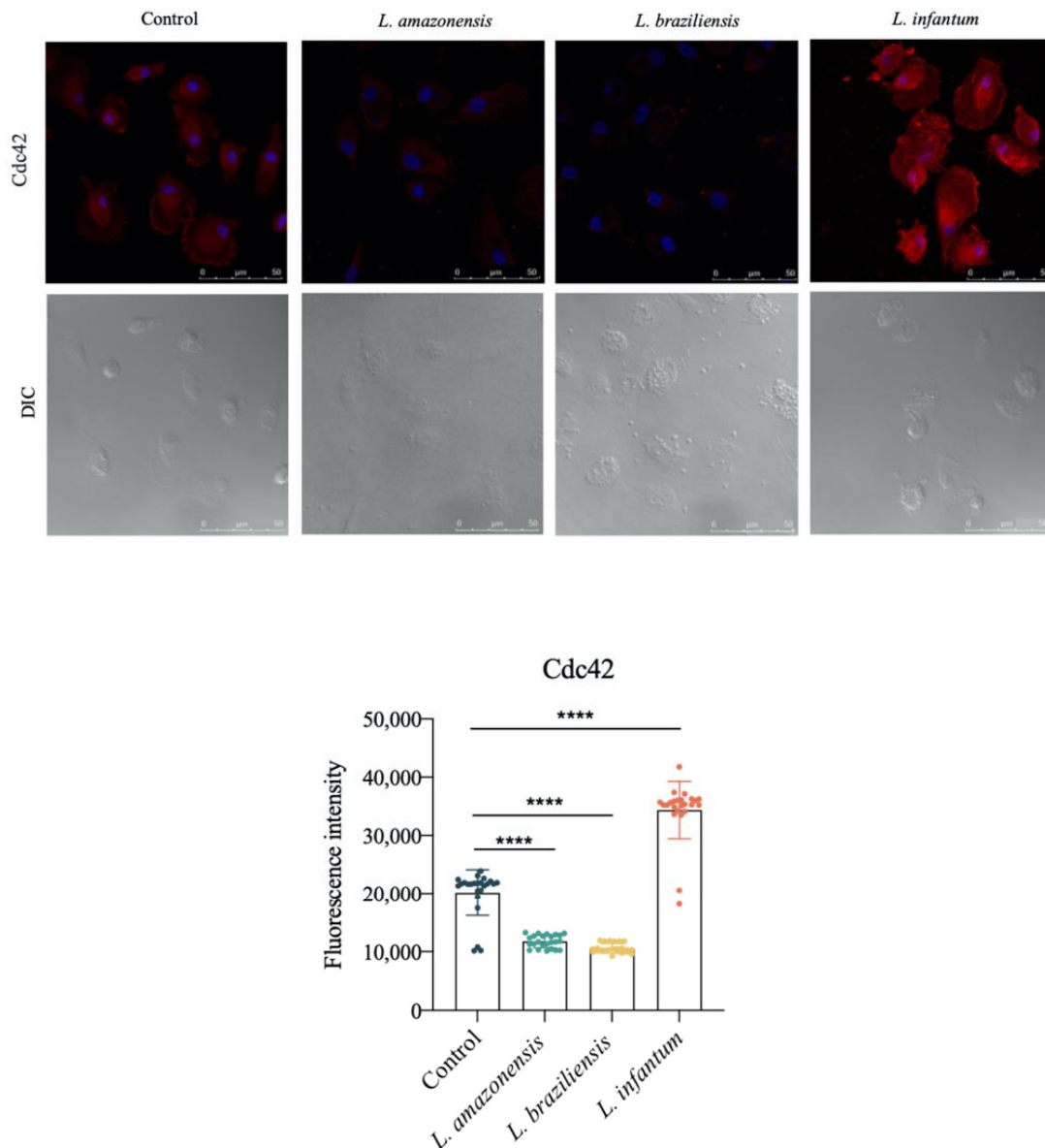


Figure 4. *L. infantum* infection induces actin polymerization in hDC. Effects of *Leishmania* infection on Rac1, RhoA, and Cdc42 protein levels in hDCs infected or not with *L. amazonensis*, *L. braziliensis* or *L. infantum*. (a) Fluorescence intensity of phalloidin labeling in hDCs infected with *L. amazonensis*, *L. braziliensis* or *L. infantum*. (b) Fluorescence intensity of Rac1 and RhoA after *L. amazonensis*, *L. braziliensis* or *L. infantum* infection. (c) Fluorescence intensity of Cdc42 in hDCs infected with *L. amazonensis*, *L. braziliensis* or *L. infantum*. Fluorescence intensity quantified in 30 cells from each group. Red: anti-Rac1, anti-RhoA or anti-Cdc42; blue: DAPI, cell nuclei; grayscale: DIC; Control, uninfected cells; white arrowheads: actin bundles. **** $p < 0.001$ (Student's *t*-test). The data are representative of three independent experiments.

3.4. Infection with *L. braziliensis* (DL) and *L. amazonensis* (DCL) Isolates Increases hDC Migration

Considering that the mechanisms underlying parasite dissemination in *Lb* DL and *La* DCL remain unelucidated, we aimed to further examine the role of hDCs in the dissemination of *Leishmania* parasites. Our assessment of the ability of these cells to migrate directionally in response to dendritic cell chemoattractant CCL3 revealed increased hDC migration following infection with isolates from *Lb* DL and *La* DCL patients, while this was not observed under infection by *Lb* or *La* LCL isolates at all timepoints analyzed (Figure 5).

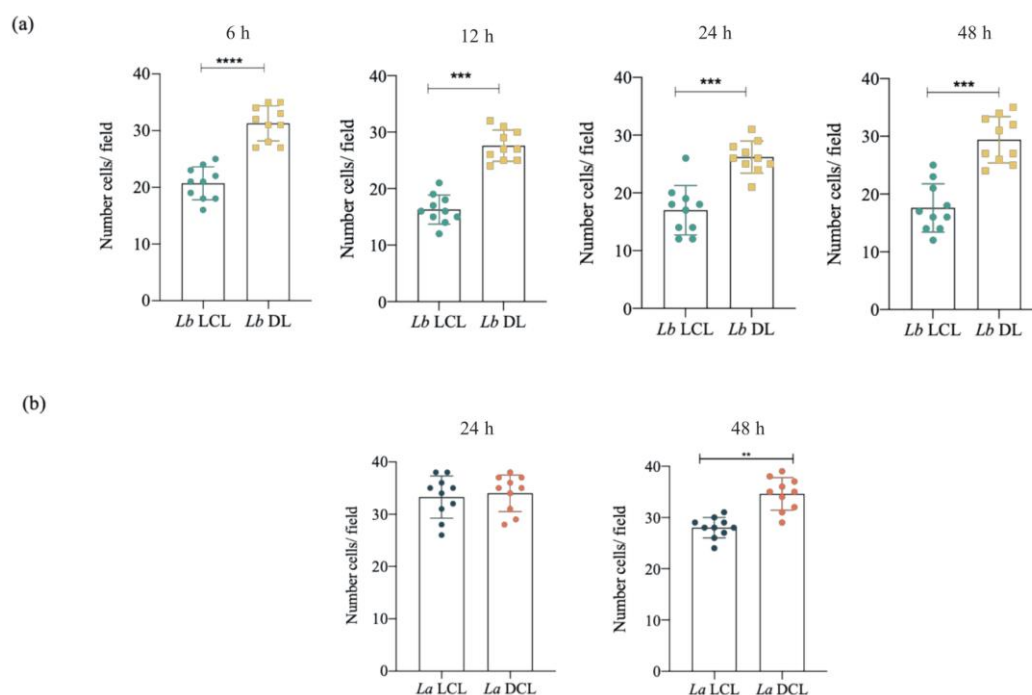


Figure 5. Infection with *L. braziliensis* (DL) and *L. amazonensis* (DCL) isolates increases hDC migration. Effects of *Lb* LCL, *Lb* DL, *La* LCL, or *La* DCL infection on hDC migration using a Boyden chamber assay with CCL3 in the lower chamber. hDCs infected (4 h) with *Lb* LCL, *Lb* DL, *La* LCL or *La* DCL were allowed to migrate for 4 h at 6, 12, 24 or 48 h post-infection. Boyden chamber membranes were washed, fixed, and incubated with DAPI for nuclear staining. Migrating cells were randomly counted (10 fields) by fluorescence microscopy. (a) Number of migrating cells following *Lb* LCL or *Lb* DL infection. (b) Number of migrating cells following *La* LCL or *La* DCL infection. ** $p < 0.05$; *** $p < 0.001$, **** $p < 0.0001$ (Student's *t*-test). Data are representative of three independent experiments.

3.5. *Lb* DL and *La* DCL Isolates Induce hDC Expression of Adhesion Complex Proteins and Actin Polymerization

Cell adhesion is a key factor in infected hDC cell migration; therefore, we examined adhesion complex formation to further investigate the increased migration observed in *Lb* DL and *La* DCL-infected cells. Markedly increased levels (three-fold higher) of paxillin and FAK were observed in *Lb* DL and *La* DCL when compared to hDCs infected with *Lb* or *La* LCL isolates, respectively (Figure 6).

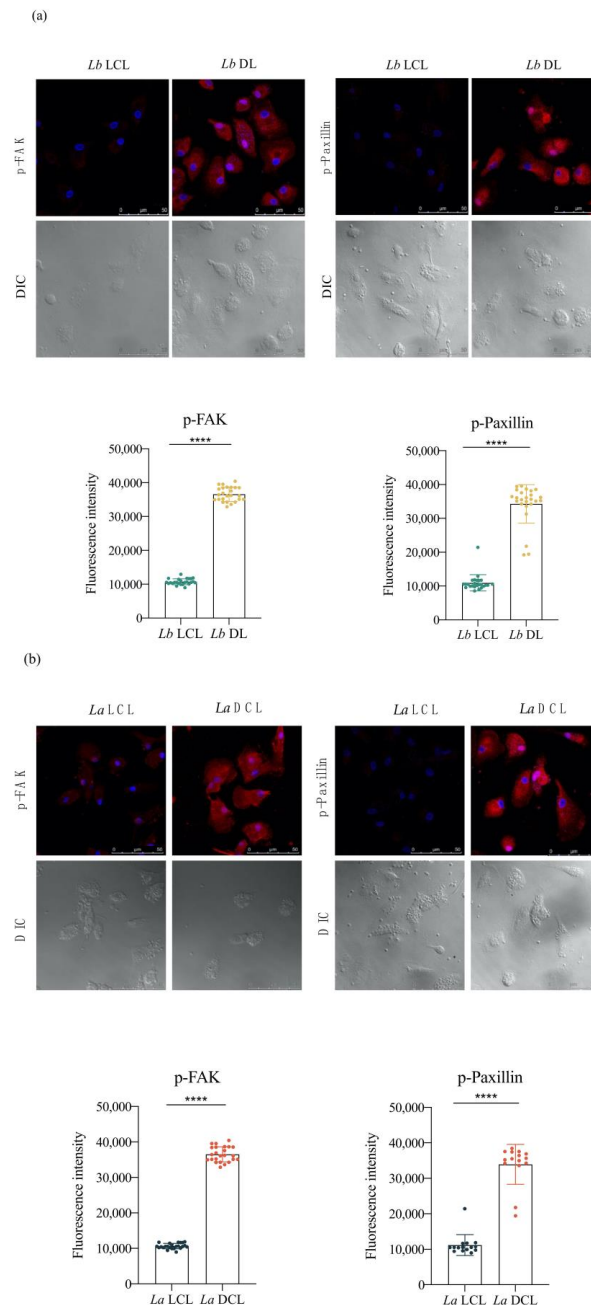


Figure 6. *Lb* DL and *La* DCL isolates induce hDC expression of adhesion complex proteins. Effects of *Leishmania* infection using isolates obtained from patients with localized or disseminated forms on phosphorylated FAK and paxillin protein levels. hDCs were infected with *Lb* LCL, *Lb* DL, *La* LCL, or *La* DCL and stained with anti-pFAK and anti-p-Paxillin. Fluorescence intensity was quantified in 30 cells from each experimental group. (a) Fluorescence intensity of p-FAK and p-Paxillin in cells infected with *Lb* LCL or *Lb* DL. (b) Fluorescence intensity of p-FAK and p-Paxillin in cells infected with *La* LCL or *La* DCL. Red: anti-FAK or phosphorylated paxillin; blue, DAPI (cell nuclei); grayscale, DIC. **** $p < 0.001$ (Student's *t*-test). Data are representative of three independent experiments.

Our investigation of alterations in actin filament dynamics revealed a significantly higher fluorescence intensity of Rac1, RhoA and Cdc42 in *Lb* DL and *La* DCL-infected cells in comparison to hDCs infected with *Lb* or *La* LCL isolates, respectively (Figure 7).

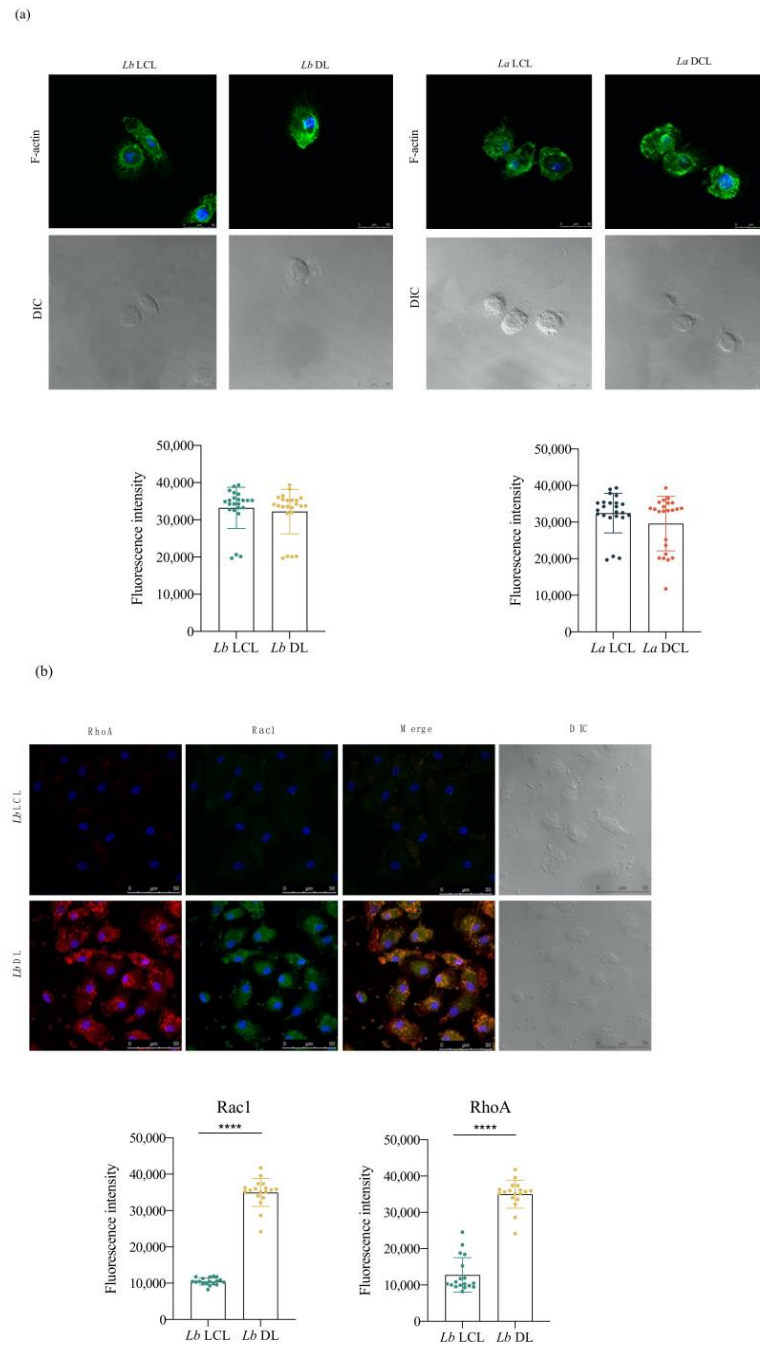


Figure 7. *Cont.*

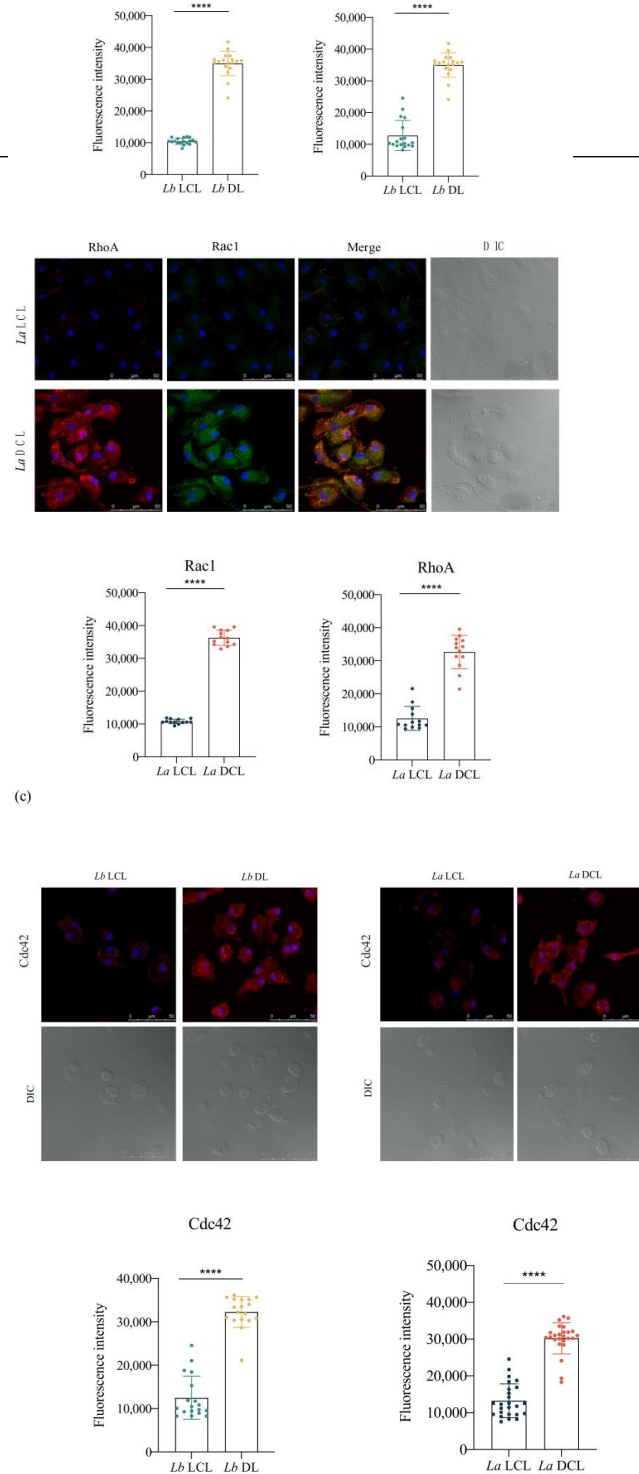


Figure 7. *Lb* DL and *La* DCL isolates induce actin polymerization in hDC. Effects of *Leishmania* infection using isolates obtained from patients with localized or disseminated forms on Rac1, RhoA and Cdc42 protein levels. hDCs were infected with *Lb* (LCL), *Lb* (DL), *La* (LCL) or *La* (DCL) and stained with phalloidin, anti-Rac1, anti-RhoA or anti-Cdc42. (a) Fluorescence intensity of phalloidin labeling in hDCs infected with *Lb* (LCL), *Lb* (DL), *La* (LCL) or *La* (DCL). (b) Fluorescence intensity of Rac1 and RhoA following *Lb* (LCL), *Lb* (DL), *La* (LCL) and *La* (DCL) infection. (c) Fluorescence intensity of Cdc42 following *Lb* (LCL), *Lb* (DL), *La* (LCL) and *La* (DCL) infection. Fluorescence intensity quantified in 30 cells from each group. Red: anti-Rac1, anti-RhoA or anti-Cdc42; blue: DAPI (cell nuclei); grayscale, DIC. **** $p < 0.001$ (Student's *t*-test). Data are representative of three independent experiments.

3.6. Infection with *L. infantum*, *Lb* DL and *La* DCL Isolates Induces CCR7 Expression in hDCs

CCR7 is involved hDC migration to the draining lymph nodes; therefore, we investigated the expression of this molecule in *Leishmania*-infected hDCs. Our results showed increased CCR7 expression 24 h after *L. infantum* infection in comparison to *L. amazonensis* or *L. braziliensis* infection and uninfected controls (Figure 8a). To investigate whether the enhanced migration observed in *Lb* DL and *La* DCL-infected cells was also associated with higher CCR7 expression in infected hDCs, we evaluated the expression of this surface molecule on hDCs infected with *Lb* DL, *Lb* LCL, *La* DCL or *La* LCL isolates. As shown in Figure 8b, hDCs infected with *Lb* DL and *La* DCL expressed higher amounts of CCR7 compared to *Lb* or *La* LCL-infected cells.

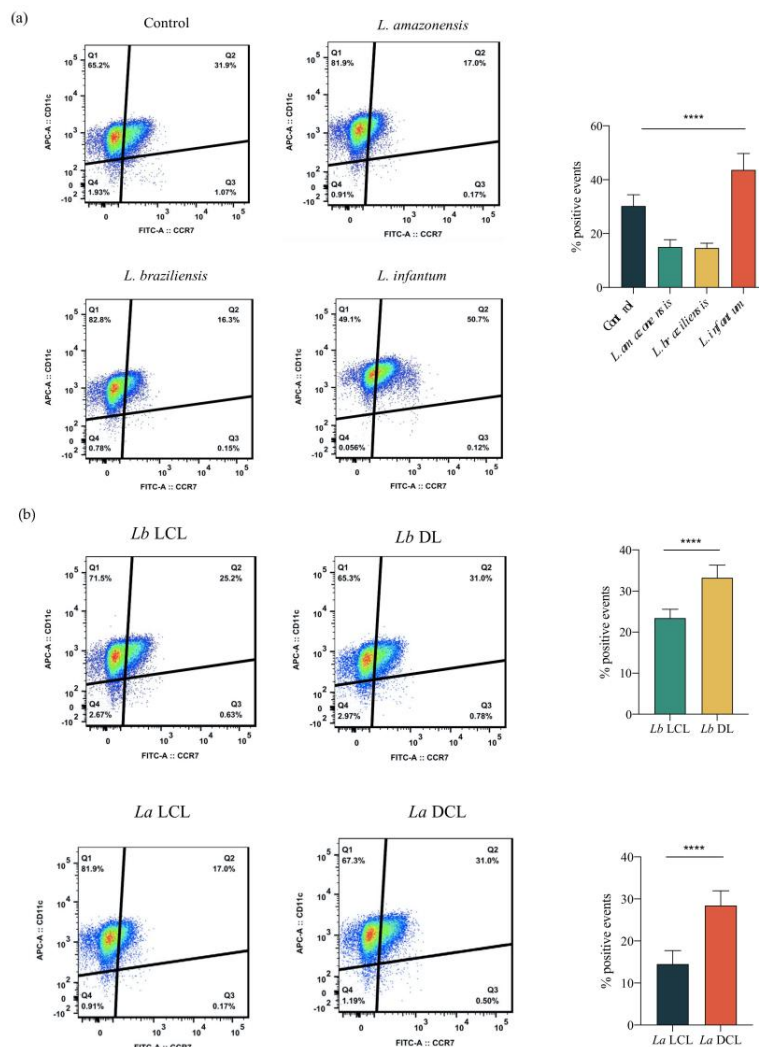


Figure 8. Infection using *L. infantum*, *Lb* DL and *La* DCL isolates induces CCR7 expression in hDCs. Representative gating strategy illustrating CCR7 expression by hDCs infected or not with isolates of different *Leishmania* species at 6 h post-infection, as assessed by flow cytometry. (a) CCR7 expression following DC infection with *L. amazonensis*, *L. braziliensis* or *L. infantum*. (b) CCR7 expression following DC infection with *Lb* (LCL), *Lb* (DL), *La* (LCL) or *La* (DCL). **** $p < 0.001$ (ANOVA). Data are representative of two independent experiments.

4. Discussion

Once inoculated in the skin of the vertebrate host, *Leishmania* parasites may remain at the inoculation site or disseminate to different tissues. Parasite dissemination in the host may result in a wide spectrum of clinical manifestations, ranging from self-healing skin ulcers to disfiguring mucosal lesions, or even fatal VL [7]. Previous studies have shown that *Leishmania*-infected DCs migrate through the skin, transporting antigens to the draining lymph nodes [21,22]. The present study demonstrated that infection by *L. infantum*, but not *L. amazonensis* or *L. braziliensis*, increases hDC directional migration driven by chemotaxis in comparison to uninfected controls. Our findings further suggest that *L. infantum* infection may actually increase the ability of hDCs to migrate from the site of infection, which is consistent with the visceral form associated with this parasite species. Other authors have reported reduced DC migration following *L. amazonensis* infection [7]. Moreover, *L. donovani* infection was previously shown to increase the presence of DCs in the lymph nodes at early stages of infection [23].

Cell–substrate adhesion is crucial to migration and is required for cells to propel their leading edge forward [17]. Over the years, the complexity of adhesion complex domains has been revealed, with around 125 proteins currently known to be involved in focal adhesions [18]. FAK signaling promotes cellular adhesion, whereas its inhibition results in a defective migration process [24,25]. Paxillin is another essential signaling protein involved in adhesion dynamics and migration [25–27]. We found higher levels of FAK and paxillin in *L. infantum*-infected cells, but not *L. amazonensis*- or *L. braziliensis*-infected hDCs. These results indicate that *L. infantum*, but not *L. amazonensis* or *L. braziliensis*, induces adhesion complex formation in hDCs, which suggests a possible mechanism for the increased cell migration observed in these cells. A previous study demonstrated the association between adhesion complex formation and the modulation of cell migration in *Leishmania*-infected murine macrophages, demonstrating reduced migration in *L. amazonensis*-infected macrophages in association with lower levels of FAK and paxillin [28].

For cells to efficiently perform cellular functions, a properly organized internal structure is necessary. The cytoskeleton is highly dynamic and adaptable, providing the essential structure and components for cell-oriented motility, such as the availability of actin filaments that are crucial to cell survival and migration [29,30]. The present study demonstrated higher expression of Rac1, RhoA and Cdc42 in hDCs infected by *L. infantum*, but not *L. amazonensis* or *L. braziliensis*, in comparison to uninfected controls. The importance of the Rho GTPases in actin dynamics and cell migration has already been demonstrated [31]. The reduced motility of *Mycobacterium*-infected fibroblasts was found to be associated with actin dynamics, as well as Rac and Rho modulation [32]. In addition, reduced invasion was demonstrated in macrophages knocked out for Rac1 [33], and lower levels of Cdc42 were also shown to lead to reduced cell migration [34].

The disseminated manifestations of CL include ML, DL and DCL [35]. To investigate the role played by hDCs in the dissemination of *Leishmania* parasites in vertebrate hosts, we evaluated cell migration, as well as adhesion complex formation and actin polymerization in DCs infected with DL, DCL or LCL isolates. We found increased hDC migration following infection with isolates obtained from *Lb* DL and *La* DCL patients in comparison to *Lb* or *La* LCL isolates. We further associated the enhanced migration observed in these cells with increased adhesion complex formation and actin polymerization. While the clinical course of LCL is well characterized, the molecular mechanisms underlying DL and DCL pathogenesis are still unclear. In contrast to a single or a few skin ulcers more commonly found in LCL patients, multiple ulcerated and nonulcerated skin lesions are currently found in more than one area of the DL patient's body, and several nonulcerated nodular skin lesions are found in DCL patients [35]. Our data suggest that the increased migration observed in hDCs infected with isolates obtained from DL and DCL patients, but not with LCL isolates, could play a role in parasite dissemination in the vertebrate host and the pathogenesis of chronic disseminated forms of leishmaniasis.

The migration of DCs to lymph nodes is coordinated by recruitment signaling involving the specific bonding of chemokines to their respective membrane receptors [36]. Chemokine receptors are transmembrane proteins associated with G protein, whose activation triggers signaling pathways responsible for cell recruitment [37]. Immature DCs express CCR2, CCR5, and CCR6, and, following activation and maturation, CCR7, which favors the migration of these cells to the draining lymph nodes [38]. The induction of CCR7 expression has already been shown to induce hDC migration [11]. Our results show that infection by *L. infantum*, *Lb* DL and *La* DCL isolates induces CCR7 expression in hDCs, suggesting the participation of this molecule with parasite dissemination in the vertebrate host.

Studies investigating factors associated with the migration of *Leishmania*-infected cells and the potential role of migrating DCs in lesion development are important to elucidating the pathogenesis of this inflammatory disease. Our results contribute to the understanding of the phenomenon of hDC migration during *Leishmania* infection and shed light on their role in parasite dissemination in the vertebrate host.

5. Conclusions

Infection with *L. infantum* leads to an increase in hDC migration, suggesting an association between these cells and disease visceralization. We found that the infection of hDCs with isolates obtained from DL and DCL patients, but not LCL, leads to higher hDC migration rates, suggesting the important role of these cells in the dissemination of *Leishmania* parasites in vertebrate hosts.

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References

1. WHO. *Control of the Leishmaniasis*; WHO: Geneva, Switzerland, 2010; Volume xii–xiii, pp. 1–186.
2. Podinovskaia, M.; Descoteaux, A. Leishmania and the macrophage: A multifaceted interaction. *Future Microbiol.* **2015**, *10*, 111–129. [\[CrossRef\]](#)
3. Silva, J.; Queiroz, A.; Moura, I.; Sousa, R.S.; Guimarães, L.H.; Machado, P.R.; Lessa, M.; Lago, E.; Wilson, M.E.; Schriefer, A. Dynamics of American tegumentary leishmaniasis in a highly endemic region for *Leishmania* (Viannia) *braziliensis* infection in northeast Brazil. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0006015. [\[CrossRef\]](#)

4. Arango Duque, G.; Descoteaux, A. Leishmania survival in the macrophage: Where the ends justify the means. *Curr. Opin. Microbiol.* **2015**, *26*, 32–40. [[CrossRef](#)]
5. Bray, R.S.; Heikal, B.; Kaye, P.M.; Bray, M.A. The effect of parasitization by *Leishmania mexicana mexicana* on macrophage function in vitro. *Acta Trop.* **1983**, *40*, 29–38. [[PubMed](#)]
6. Pinheiro, N.F., Jr.; Hermida, M.D.; Macedo, M.P.; Mengel, J.; Bafica, A.; dos-Santos, W.L. Leishmania infection impairs beta 1-integrin function and chemokine receptor expression in mononuclear phagocytes. *Infect. Immun.* **2006**, *74*, 3912–3921. [[CrossRef](#)]
7. Hermida, M.D.; Doria, P.G.; Taguchi, A.M.; Mengel, J.O.; dos-Santos, W. Leishmania amazonensis infection impairs dendritic cell migration from the inflammatory site to the draining lymph node. *BMC Infect. Dis.* **2014**, *14*, 450. [[CrossRef](#)] [[PubMed](#)]
8. De Almeida, M.C.; Vilhena, V.; Barral, A.; Barral-Netto, M. Leishmanial infection: Analysis of its first steps. A review. *Mem. Inst. Oswaldo Cruz* **2003**, *98*, 861–870. [[CrossRef](#)] [[PubMed](#)]
9. Carvalhal, D.G.; Barbosa, A., Jr.; D’El-Rei Hermida, M.; Clarencio, J.; Pinheiro, N.F., Jr.; Veras, P.S.; dos-Santos, W.L. The modelling of mononuclear phagocyte-connective tissue adhesion in vitro: Application to disclose a specific inhibitory effect of *Leishmania* infection. *Exp. Parasitol.* **2004**, *107*, 189–199. [[CrossRef](#)]
10. Ballet, R.; Emre, Y.; Jemelin, S.; Charmoy, M.; Tacchini-Cottier, F.; Imhof, B.A. Blocking junctional adhesion molecule C enhances dendritic cell migration and boosts the immune responses against *Leishmania major*. *PLoS Pathog.* **2014**, *10*, e1004550. [[CrossRef](#)]
11. Steigerwald, M.; Moll, H. *Leishmania major* modulates chemokine and chemokine receptor expression by dendritic cells and affects their migratory capacity. *Infect. Immun.* **2005**, *73*, 2564–2567. [[CrossRef](#)]
12. Trepatt, X.; Chen, Z.; Jacobson, K. Cell migration. *Compr. Physiol.* **2012**, *2*, 2369–2392. [[CrossRef](#)] [[PubMed](#)]
13. Sheetz, M.P.; Felsenfeld, D.; Galbraith, C.G.; Choquet, D. Cell migration as a five-step cycle. *Biochem. Soc. Symp.* **1999**, *65*, 233–243. [[PubMed](#)]
14. Gupton, S.L.; Waterman-Storer, C.M. Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell* **2006**, *125*, 1361–1374. [[CrossRef](#)]
15. De Fougères, A.R.; Kotliansky, V.E. Regulation of monocyte gene expression by the extracellular matrix and its functional implications. *Immunol. Rev.* **2002**, *186*, 208–220. [[CrossRef](#)]
16. Cortesio, C.L.; Boateng, L.R.; Piazza, T.M.; Bennin, D.A.; Huttenlocher, A. Calpain-mediated proteolysis of paxillin negatively regulates focal adhesion dynamics and cell migration. *J. Biol. Chem.* **2011**, *286*, 9998–10006. [[CrossRef](#)]
17. Huveneers, S.; Danen, E.H. Adhesion signaling—Crosstalk between integrins, Src and Rho. *J. Cell Sci.* **2009**, *122*, 1059–1069. [[CrossRef](#)] [[PubMed](#)]
18. Turner, C.E. Paxillin and focal adhesion signalling. *Nat. Cell Biol.* **2000**, *2*, E231–E236. [[CrossRef](#)]
19. Ren, X.D.; Kiosses, W.B.; Schwartz, M.A. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **1999**, *18*, 578–585. [[CrossRef](#)]
20. Zegers, M.M.; Friedl, P. Rho GTPases in collective cell migration. *Small GTPases* **2014**, *5*, e28997. [[CrossRef](#)]
21. Ng, L.G.; Hsu, A.; Mandell, M.A.; Roediger, B.; Hoeller, C.; Mrass, P.; Iparraguirre, A.; Cavanagh, L.L.; Triccas, J.A.; Beverley, S.M.; et al. Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS Pathog.* **2008**, *4*, e1000222. [[CrossRef](#)]
22. Leon, B.; Lopez-Bravo, M.; Ardavin, C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against *Leishmania*. *Immunity* **2007**, *26*, 519–531. [[CrossRef](#)] [[PubMed](#)]
23. Ato, M.; Stager, S.; Engwerda, C.R.; Kaye, P.M. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat. Immunol.* **2002**, *3*, 1185–1191. [[CrossRef](#)]
24. Yano, H.; Mazaki, Y.; Kurokawa, K.; Hanks, S.K.; Matsuda, M.; Sabe, H. Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. *J. Cell Biol.* **2004**, *166*, 283–295. [[CrossRef](#)]
25. Mitra, S.K.; Hanson, D.A.; Schlaepfer, D.D. Focal adhesion kinase: In command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 56–68. [[CrossRef](#)]
26. Playford, M.P.; Schaller, M.D. The interplay between Src and integrins in normal and tumor biology. *Oncogene* **2004**, *23*, 7928–7946. [[CrossRef](#)] [[PubMed](#)]
27. Huttenlocher, A.; Horwitz, A.R. Integrins in cell migration. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a005074. [[CrossRef](#)] [[PubMed](#)]
28. De Menezes, J.P.; Koushik, A.; Das, S.; Guven, C.; Siegel, A.; Laranjeira-Silva, M.F.; Losert, W.; Andrews, N.W. *Leishmania* infection inhibits macrophage motility by altering F-actin dynamics and the expression of adhesion complex proteins. *Cell Microbiol.* **2017**, *19*. [[CrossRef](#)] [[PubMed](#)]
29. Tang, D.D.; Gerlach, B.D. The roles and regulation of the actin cytoskeleton, intermediate filaments and microtubules in smooth muscle cell migration. *Respir. Res.* **2017**, *18*, 54. [[CrossRef](#)]
30. Rossier, O.M.; Gauthier, N.; Biais, N.; Vonnegut, W.; Fardin, M.A.; Avigan, P.; Heller, E.R.; Mathur, A.; Ghassemi, S.; Koeckert, M.S.; et al. Force generated by actomyosin contraction builds bridges between adhesive contacts. *EMBO J.* **2010**, *29*, 1055–1068. [[CrossRef](#)] [[PubMed](#)]
31. Sander, E.E.; ten Klooster, J.P.; van Delft, S.; van der Kammen, R.A.; Collard, J.G. Rac downregulates Rho activity: Reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J. Cell Biol.* **1999**, *147*, 1009–1022. [[CrossRef](#)]

-
32. Sanchez-Barinas, C.D.; Ocampo, M.; Vanegas, M.; Castaneda-Ramirez, J.J.; Patarroyo, M.A.; Patarroyo, M.E. Mycobacterium tuberculosis H37Rv LpqG Protein Peptides Can Inhibit Mycobacterial Entry through Specific Interactions. *Molecules* **2018**, *23*, 526. [[CrossRef](#)] [[PubMed](#)]
 33. Wheeler, A.P.; Wells, C.M.; Smith, S.D.; Vega, F.M.; Henderson, R.B.; Tybulewicz, V.L.; Ridley, A.J. Rac1 and Rac2 regulate macrophage morphology but are not essential for migration. *J. Cell Sci.* **2006**, *119*, 2749–2757. [[CrossRef](#)] [[PubMed](#)]
 34. Lavina, B.; Castro, M.; Niaudet, C.; Cruys, B.; Alvarez-Aznar, A.; Carmeliet, P.; Bentley, K.; Brakebusch, C.; Betsholtz, C.; Gaengel, K. Defective endothelial cell migration in the absence of Cdc42 leads to capillary-venous malformations. *Development* **2018**, *145*. [[CrossRef](#)] [[PubMed](#)]
 35. Franca-Costa, J.; Wanderley, J.L.; Deolindo, P.; Zarattini, J.B.; Costa, J.; Soong, L.; Barcinski, M.A.; Barral, A.; Borges, V.M. Exposure of phosphatidylserine on *Leishmania amazonensis* isolates is associated with diffuse cutaneous leishmaniasis and parasite infectivity. *PLoS ONE* **2012**, *7*, e36595. [[CrossRef](#)]
 36. Tiberio, L.; Del Prete, A.; Schioppa, T.; Sozio, F.; Bosisio, D.; Sozzani, S. Chemokine and chemotactic signals in dendritic cell migration. *Cell Mol. Immunol.* **2018**, *15*, 346–352. [[CrossRef](#)] [[PubMed](#)]
 37. Hughes, C.E.; Nibbs, R.J.B. A guide to chemokines and their receptors. *FEBS J.* **2018**, *285*, 2944–2971. [[CrossRef](#)] [[PubMed](#)]
 38. Ohl, L.; Mohaupt, M.; Czeloth, N.; Hintzen, G.; Kiafard, Z.; Zwirner, J.; Blankenstein, T.; Henning, G.; Forster, R. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* **2004**, *21*, 279–288. [[CrossRef](#)] [[PubMed](#)]

5 CAPÍTULO 2

5.1 Hipótese

A infecção por *Leishmania* induz a migração de células dendríticas, mas não de monócitos e macrófagos.

5.2 Objetivos

5.2.1 Objetivo geral

Avaliar a migração de macrófagos, monócitos e células dendríticas humanas infectadas por *Leishmania* e os mecanismos envolvidos neste processo.

5.2.2 Objetivos específicos

- Avaliar a migração de macrófagos, monócitos e células dendríticas infectados por *L. amazonensis*, *L. braziliensis*, ou *L. infantum* em ambiente bidimensional;
- Investigar a formação complexos de adesão em macrófagos, monócitos e células dendríticas infectadas por *L. amazonensis*, *L. braziliensis* ou *L. infantum*, em ambiente bidimensional;
- Determinar a atividade de moléculas sinalizadoras e a polimerização dos filamentos de actina durante o processo de migração de macrófagos, monócitos, células dendríticas humanas infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*, em ambiente bidimensional;
- Avaliar o papel de podossomos durante o processo de migração de macrófagos, monócitos, células dendríticas humanas infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*, em ambiente bidimensional;
- Avaliar o papel de vias de sinalização na migração de macrófagos, monócitos e células dendríticas infectadas por *L. amazonensis*, *L. braziliensis* ou *L. infantum* em ambiente bidimensional.

6 MANUSCRITO 2

***Leishmania* orchestrates 2D host cell migration by different cellular mechanisms.**

Resumo: *Leishmania* spp. infectam diversos hospedeiros vertebrados, incluindo o homem. As doenças causadas por este protozoário, as leishmanioses, podem apresentar diferentes tipos de manifestações clínicas, desde lesões localizadas na pele que cicatrizam espontaneamente, lesões de pele, que se espalham de forma disseminada, a forma mucocutânea da doença, além da leishmaniose visceral, forma mais grave que podem levar à morte se não tratada. O presente estudo tem como objetivo investigar a migração de células hospedeiras como macrófagos, monócitos e células dendríticas em modelo humano. Para isso, células hospedeiras foram infectadas por diferentes espécies do parasito e submetidas a análise de migração direcional e em tempo real, análise da formação de complexos de adesão e podossomos, dinâmica de actina, bem como os mecanismos associados à via de sinalização importante para migração celular. Foi mostrado que a infecção por *Leishmania* modula diferentemente a migração de células hospedeiras, levando à um aumento na migração de células dendríticas infectadas por *L. infantum*, ao passo que foi observado uma redução na motilidade de macrófagos e monócitos humanos infectados por todas as espécies do parasito estudadas. Adicionalmente, foi mostrado que este processo está associado à modulação da formação de complexo de adesão dinâmica de actina e formação de podossomos, bem como a expressão de Akt/PI3K. Nossos resultados mostram que *Leishmania* modula diferentemente a migração de células hospedeiras, sugerindo um diferente papel dessas células na disseminação do parasito e controle da infecção.

Palavras-chave: Migração celular. célula hospedeira. *Leishmania*.

Manuscrito em construção

INTRODUÇÃO

Leishmania sp são parasitos protozoários intracelulares que causam a leishmaniose. A doença afeta cerca de 12 milhões de pessoas em 98 países, com aproximadamente 350 milhões de pessoas em risco (WHO, 2023). A leishmaniose pode apresentar diferentes manifestações clínicas a depender da espécie do parasito e do sistema imune do hospedeiro, podendo ser dividida em leishmaniose cutânea (LC) ou leishmaniose visceral (LV). A LC é caracterizada por lesões de pele em forma de úlceras e é subclassificada em leishmaniose cutânea localizada (LCL), com presença de uma ou mais lesões ulcerada com tendência à cicatrização espontânea; leishmaniose mucocutânea (LMC), que atinge mucosas; leishmaniose cutânea difusa (LCD), ocasionando inúmeras lesões nodulares anérgicas não-ulceradas ou leishmaniose disseminada (LD), caracterizada pela presença de nódulos espalhados pelo corpo, que podem ser em menor ou maior quantidade, abrangendo de 10 a mais de 100 lesões espalhadas. A LV, forma mais

grave da doença, atinge por sua vez órgãos internos como baço, medula óssea e fígado, podendo levar ao óbito se não tratada (Torres-guerrero et al., 2017).

A infecção por *Leishmania* se inicia durante o repasto sanguíneo de flebotomíneos no hospedeiro. Formas promastigotas metacíclicas são internalizadas por células hospedeiras como neutrófilos, macrófagos, monócitos ou células dendríticas, iniciando o processo de replicação no interior de compartimentos fagolisossomais ou vacúolos parasitóforos. Uma vez dentro de células hospedeiras, os parasitos internalizados podem permanecer no local da infecção ou migrar para outros tecidos. Estudo prévio em modelo murino demonstrou que células de Langerhans infectadas por *L. major* apresentaram uma redução da migração para linfonodos (Moll et al., 1993). Adicionalmente, foi demonstrado que a infecção por *L. amazonensis* ou *L. major* reduzem a adesão e migração de macrófagos e monócitos. Adicionalmente, foi demonstrado que a infecção por *L. donovani* aumenta a taxa de células dendríticas presentes em linfonodos (Steigerwald; Moll, 2005; Carvalhal et al., 2004; Pinheiro et al., 2006; Menezes et al., 2017; Ato et al., 2002).

Apesar de se conhecer diferentes mecanismos que levam a sobrevivência do parasito, pouco se conhece sobre o processo de estabelecimento de lesões ou disseminação de *Leishmania* no hospedeiro vertebrado. De forma geral, para que a célula migre de forma eficaz, vias de transdução de sinal são ativadas em cascatas. A migração celular e seus mecanismos são oriundos de diferentes vias de sinalização, como PI3K/AKT, JAK/STAT e ROCK e proteínas oriundas dessas vias têm sido relacionadas ao remodelamento do citoesqueleto (Cain and Ridley, 2009; Deng et al., 2022; Hu et al., 2023; Guan et al., 2023). A capacidade de migração de células hospedeiras está associada ainda à mecanismos de adesão celular, que desempenham funções primordiais na migração, como a conexão do substrato ao citoesqueleto celular e o controle do processo de deslocamento (Veillat et al., 2015; Eddy et al., 2017). Durante a adesão celular, as adesões focais são formadas por um ponto de integrina ancorada à membrana, contendo entre outras proteínas, paxilina e a cinase de adesão focal (FAK), além de uma região intermediária de transdução de força contendo talina e vinculina, associada à um feixe de actina que culmina na formação de podossomos (Case et al., 2015; Liu et al., 2015; Zaidel-Bar et al., 2010). Uma vez aderidas, o complexo de adesão celular é ancorado ao citoesqueleto de actina, que participa da estrutura da célula e sua dinâmica é regulada por pequenas GTPases como RhoA, Rac1 e Cdc42. Juntas, essas proteínas desempenham papéis essenciais na coordenação dos movimentos da célula (Jacquemet et al., 2013; Bass et al., 2007).

Neste estudo investigamos os mecanismos envolvidos na migração de células hospedeiras infectadas por diferentes espécies de *Leishmania*, em ambiente bidimensional. Nossos

resultados indicam que células dendríticas migram mais que macrófagos e monócitos humanos e este processo está associado a fatores de adesão, dinâmica de actina e sinalização celular via AKT/PI3K.

MATERIAIS E MÉTODOS

Comitê de ética

O projeto está de acordo com os princípios de ética na pesquisa adotado pela Resolução CNS 466/12 II.4 e foi aprovado pela Comissão de Ética em Pesquisas (CEP) do IGM, conforme parecer de número 129311/2021.

Cultivo de células hospedeiras

Células dendríticas, macrófagos e monócitos humanos foram obtidos a partir de amostras de sangue de doadores saudáveis, por separação em Ficoll-Hypaque. A partir das células mononucleares do sangue periférico, a separação de células dendríticas foi realizada utilizando coluna magnética para isolamento de células CD14+, que então, foram ressuspensas e plaqueadas em meio RPMI com GM-CSF (Peprotech) [50ng/ml] + IL4 [100UI/ml] por 7 dias. Os monócitos foram obtidos por adesão, e os macrófagos diferenciados a partir dos monócitos em meio RPMI por 7 dias.

Cultivo de parasitos

Parasitos de *L. amazonensis* (MHOM/BR88/Ba-125), *L. braziliensis* (MHOM/BR/01/BA788) e *L. infantum* (MCAN/BR/89/BA262) foram mantidos em meio ágar-sangue NNN em estufa B.O.D. a 24°C. Após a cultura atingir a fase estacionária de crescimento, os parasitos foram utilizados para realização dos experimentos até a 7ª passagem em meio Schneider completo.

Determinação da taxa de infecção

Para realização da determinação da taxa de infecção e carga parasitária, macrófagos e monócitos foram obtidos e plaqueados em quadruplicata em placas de 24 poços, na concentração de 2×10^5 /poço, e infectados por *L. amazonensis* na proporção de 10:1 por 6 horas e *L. braziliensis* ou *L. infantum* na proporção de 20:1 por 24 horas. Células dendríticas foram infectadas por *L. amazonensis* (10:1), *L. braziliensis* ou *L. infantum* na proporção de 20:1 por 4 horas. Em seguida, as células foram lavadas e posteriormente, fixadas com PFA 4% nos

tempos de 0, 6, 24 e 48h, seguidamente coradas com DAPI (Invitrogen) para marcação nuclear das células e dos parasitos. Um total de 400 células foram contadas por grupo de forma randomizada por microscopia de fluorescência convencional (Microscópio Olympus).

Avaliação da migração de células hospedeiras infectadas por *Leishmania*

Inicialmente, avaliamos a migração de macrófagos, monócitos e células dendríticas de isolados de sangue periférico de doadores saudáveis. Para avaliar a migração destas células, utilizamos duas metodologias: (1) Migração direcional induzida por quimioatrator em boyden chamber e videomicroscopia; (2) Migração randômica, através da análise da trajetória de migração destas células, como publicado anteriormente (Menezes *et al.*, 2017). A avaliação da migração direcional de células dendríticas e macrófagos infectados ou não por diferentes espécies de *Leishmania* foi avaliada na presença de um quimioatrator (CCL3 para células dendríticas e MCP-1 para macrófagos e monócitos), utilizando câmaras *Transwell*. Nos ensaios de migração direcional, células dendríticas, macrófagos e monócitos humanos foram plaqueados na concentração de 2×10^5 células por poço e infectados por *L. amazonensis*, *L. braziliensis* e *L. infantum* e fixadas com paraformaldeído a 4%, em diferentes tempos após a infecção (6, 24 e 48h). A parte superior da membrana foi raspada com *swab*, a fim de remover as células que não migraram e, em seguida, as membranas foram removidas do inserto com auxílio de um bisturi. A análise da migração das células foi realizada pela contagem do núcleo de células, corados por DAPI, que atravessaram a membrana do inserto da *transwell*, com auxílio de microscopia de fluorescência convencional. Para a análise da migração randômica, as células foram plaqueadas na concentração de 2×10^5 células por poço, em câmaras *mattek*, infectadas, lavadas e posteriormente submetidas a análise de migração em tempo real, 24h após a infecção. Para isso, utilizamos o sistema *timelapse*, obtendo uma foto a cada minuto, por um período de 1h, na presença de CO₂. A trajetória de migração foi analisada por software *Volocity CellTracker* e *imageJ*.

Avaliação da formação de complexos de adesão e dinâmica de actina em células hospedeiras infectadas por *Leishmania* por imunofluorescência

Células infectadas ou não por diferentes espécies de *Leishmania* foram lavadas com PBS 1x, fixadas com PFA 4% por 15 minutos e, posteriormente, incubadas com NH₄CL₂ [15 mM] por 20 minutos, lavadas 3 vezes com PBS 1x, incubadas com solução de bloqueio (3% bovino

serum albumin [BSA] em PBS 1x por 1 hora, lavadas 3 vezes com PBS 1x, permeabilizada com PBS-Saponina 0.15% (Sigma) por 15 min e incubadas com anti-p-paxilina 1:100 ou anti-p-FAK 1:500 (pY¹¹⁸) (Invitrogen) para análise do complexo de adesão. Para análise da dinâmica de actina, as células foram incubadas com anti-Rac-1, anti-RhoA, anti-Cdc42 e faloidina (Invitrogen) por 1 hora. Posteriormente, as células foram lavadas 3x com PBS 1x, incubadas com anti-rabbit Alexa Fluor 552/488 ou anti-mouse Alexa Fluor 552 (Molecular Probes) por 45 minutos, lavadas com PBS 1x e incubadas com DAPI Prolong Gold Antifade (Invitrogen) [10 mg/mL] para marcação nuclear. As imagens foram adquiridas com auxílio de microscópio confocal (Leica SP8) e a intensidade de fluorescência das imagens foi quantificada por software FIJI.

Avaliação da formação de podossomos em células hospedeiras infectadas por *Leishmania* por imunofluorescência

Macrófagos, monócitos e células dendríticas foram plaqueados em placas de 24 poços 2×10^5 em lamínulas cobertas com fibronectina [100ng/mL], infectadas como descrito anteriormente, e em seguida fixadas por 15 min com PFA 4%. Em seguida, lavadas 3x com TBS 1%, permeabilizadas com Triton-X 0,1% por 15 min, incubadas com BSA 2% diluído em TBS 1% por 1 hora, a 4°C. Posteriormente, as células foram lavadas 3x com TBS 1% e incubadas com anti-vinculina [1:1000] em BSA 0,1% overnight a 4°C (Millipore MAB3574). As lamínulas foram então lavadas 3x com TBS 1%, incubadas com DAPI [1:2500] a 37°C, lavadas 3x com TBS 1% e montadas com DAKO. A análise da formação de podossomos foi realizada com auxílio do *Leica laser 5 scanning confocal microscope* e *Spinning disk confocal waveFx* da Universidade de Toronto, o número e a circularidade de podossomos foram quantificados pelo *Focal adhesion server* (North Carolina University, USA) e image J.

Análise da expressão de proteínas associadas a vias de sinalização em migração celular (AKT/PI3K)

Para análise da expressão de AKT e PI3K por western blot, macrófagos, monócitos ou células dendríticas [1×10^6], infectados ou não por diferentes espécies de *Leishmania* foram lavados 2x com PBS 1x, lisados em 50 μ L de tampão RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0,50% de desoxicolato de sódio, 0,10% de SDS com inibidores de protease, centrifugados 1500 RPM 4°C. Os lisados foram então submetidos a quantificação de

proteína (método BSA Pierce) e 100 mg de proteína por amostra foram adicionados ao tampão de amostra SDS, a temperatura ambiente por 30 minutos, separados por SDS-PAGE e transferidos para uma membrana de PVDF. As membranas foram incubadas *overnight* com anti-PI3K [1:1000] ou anti-pAKT [1:2000] em tampão de bloqueio (BSA 3% leite, 0,1% tween), lavadas 3x com TBS e em seguida incubadas com anticorpo secundário anti-rabbit [1:1000] (Invitrogen) acoplados a HRP. Por fim, as membranas foram reveladas utilizando o ImageQuant LAS 4000 e a quantificação digital da expressão de proteínas foi realizada pelo software ImageJ.

Avaliação do papel de PI3K na migração de células hospedeiras infectadas por *Leishmania*

Células hospedeiras foram plaqueadas [2×10^5] e infectadas como descrito anteriormente, e em seguida, tratadas com inibidor de PI3K AS60925 [100 nM] por 24h. As células foram então lavadas 3x com PBS 1x, e reincubadas por adicionais 24h em RPMI completo contendo 10% SBF. Posteriormente, foram submetidas a ensaios de migração direcional em sistema transwell, como previamente descrito, 24h após o tratamento. As células que migraram foram contadas randomicamente por microscopia de fluorescência convencional (Olympus) em um total de 10 campos por inserto.

Análise estatística

O programa GraphPad Prism 8.0 foi utilizado para a realização das análises estatísticas. Os dados foram submetidos ao teste de normalidade, Kolmogorov-Smirnov, Shapiro-Wilk e D'Agostino e Person, para determinação do uso de testes paramétricos ou não paramétricos. Os resultados foram considerados estatisticamente significantes quando o valor de $p < 0,05$.

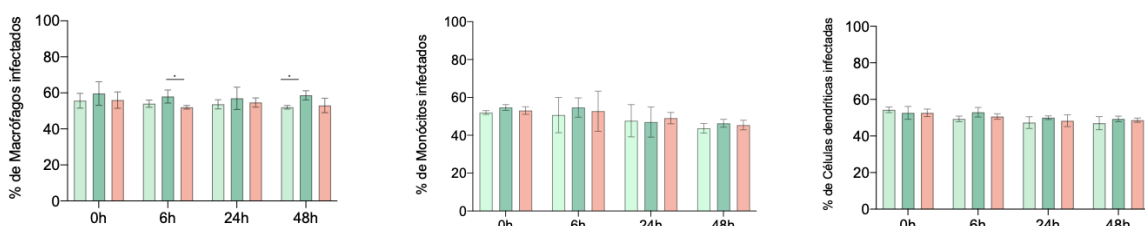
RESULTADOS

Determinação da taxa de infecção em células hospedeiras

A análise da cinética de infecção feita para as três células hospedeiras nas infecções por diferentes parasitos de *Leishmania* mostrou que tanto o percentual de células e o número de parasitos por célula foi semelhante entre os grupos, seguindo a proporção de 10:1 para *L. amazonensis* e *L. braziliensis* ou 20:1 *L. infantum* para células dendríticas por um período de

4h. Para macrófagos ou monócitos, utilizamos a proporção 10:1 para *L.amazonensis* por 6h , 10:1 para *L.braziliensis* e 20:1 para *L. infantum* por 24h. Após a infecção, as células foram então lavadas e reincubadas por adicionais 6, 24 ou 48h e fixadas nos tempos determinados previamente (Figura 1).

A



B

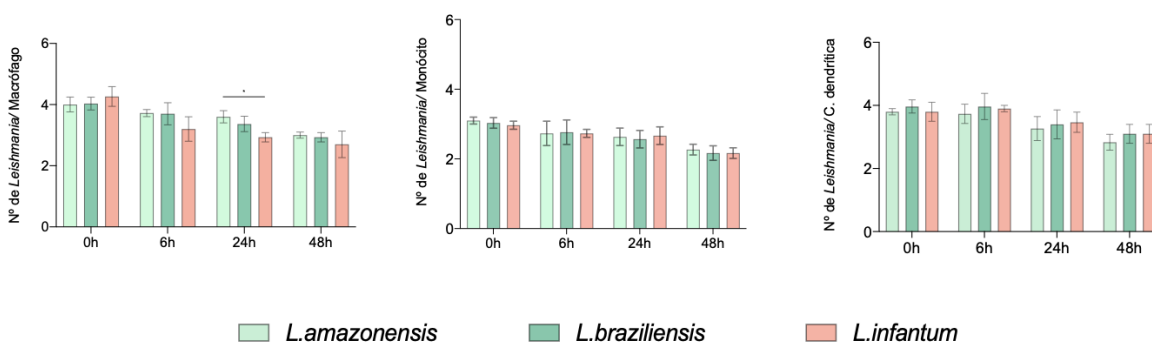


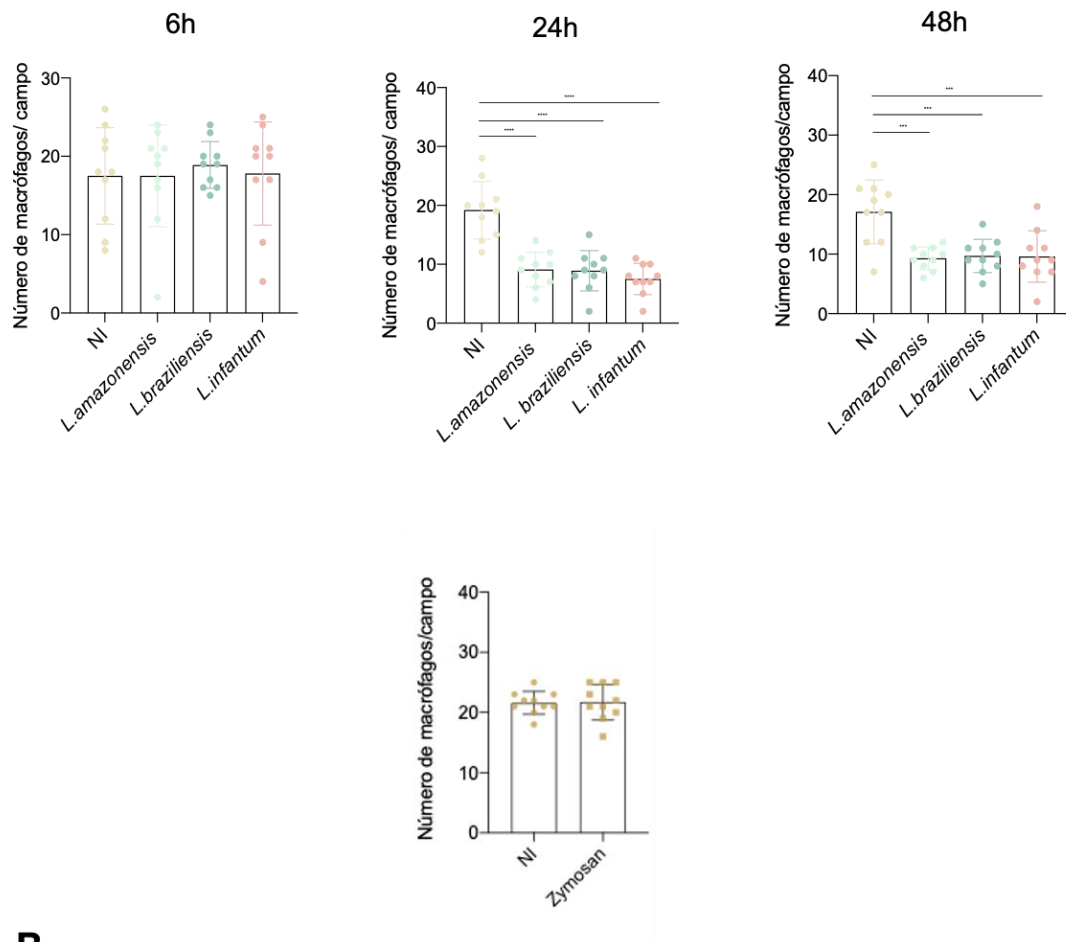
Figura 1. Avaliação da taxa de infecção de células hospedeiras infectadas por *Leishmania*. Macrófagos, monócitos [2×10^5 /poço] foram infectados por *L. amazonensis* 10:1 por 6 horas, por *L. braziliensis* 10:1 por 24h ou *L. infantum* na proporção de 20:1 por 24 horas. Células dendríticas [2×10^5 /poço] foram infectadas por *L. amazonensis* ou *L. braziliensis* na proporção de 10:1 ou *L. infantum* na proporção de 20:1 por 4 horas. Um total de 400 células foram avaliadas randomicamente por microscopia de fluorescência. (A) Percentual de infecção (B) Carga parasitária.* $p < 0,05$ (Anova). Resultado representativo de 3 experimentos.

Avaliação da migração de células hospedeiras infectadas por *Leishmania*

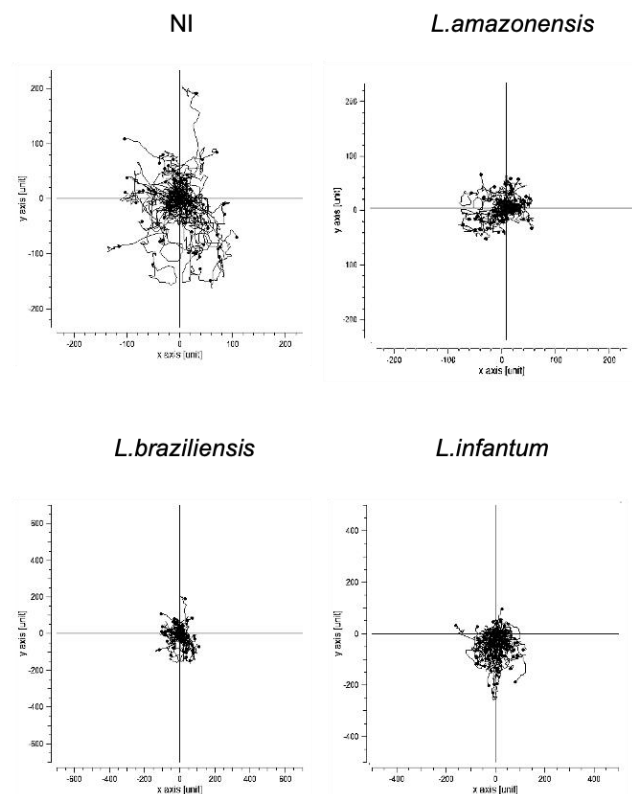
Para avaliar a migração de células hospedeiras infectadas ou não por *Leishmania*, foram realizados ensaios de migração direcional na presença do quimioatratante específico (MCP-1) para macrófagos e monócitos ou (CCL3) para células dendríticas, utilizando câmaras Transwell, 6, 24 ou 48h após a infecção. Adicionalmente, realizamos ensaios de videomicroscopia em tempo real utilizando microscopia acoplada a câmaras de CO₂. Os resultados demonstraram que, para macrófagos e monócitos, 24 ou 48 horas após a infecção,

há uma redução na taxa de migração nos grupos infectados quando comparados com o grupo controle. No entanto, observamos um aumento transitório na migração de células dendríticas infectadas por *L. amazonensis* 6h após a infecção e uma redução significativa no grupo infectado com *L. braziliensis* nos tempos avaliados. Nos tempos de 24 e 48 horas após a infecção, observamos uma redução da migração de células dendríticas infectadas por *L. braziliensis*. Para todos os tempos, no entanto, observamos uma indução na migração destas células na infecção por *L. infantum*. Observamos ainda uma redução na trajetória percorrida por macrófagos e monócitos humanos infectados por *L. braziliensis* e um aumento em células dendríticas infectadas por *L. infantum*. Adicionalmente, não foi observada alteração entre a taxa de migração dos grupos não infectados e expostos à fagocitose por zymosan (Figura 2 A-F).

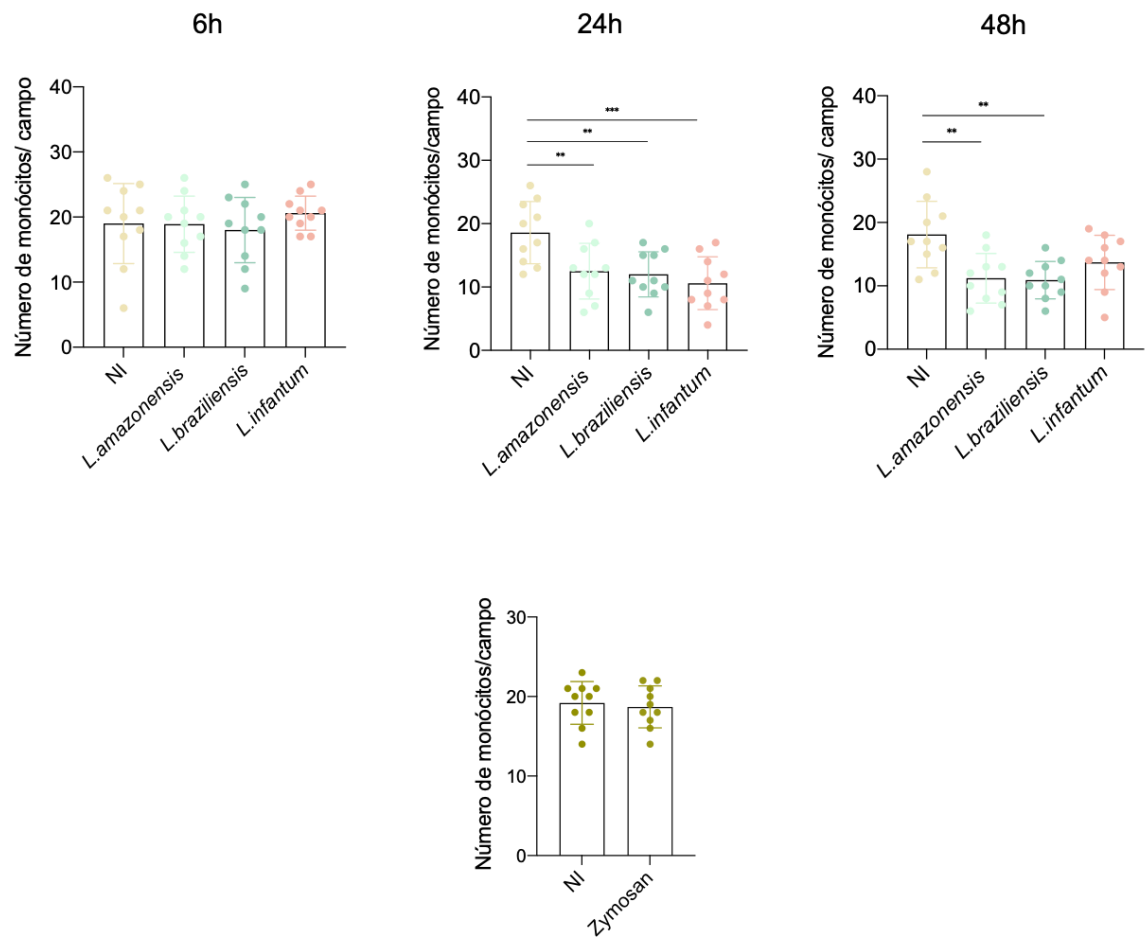
A



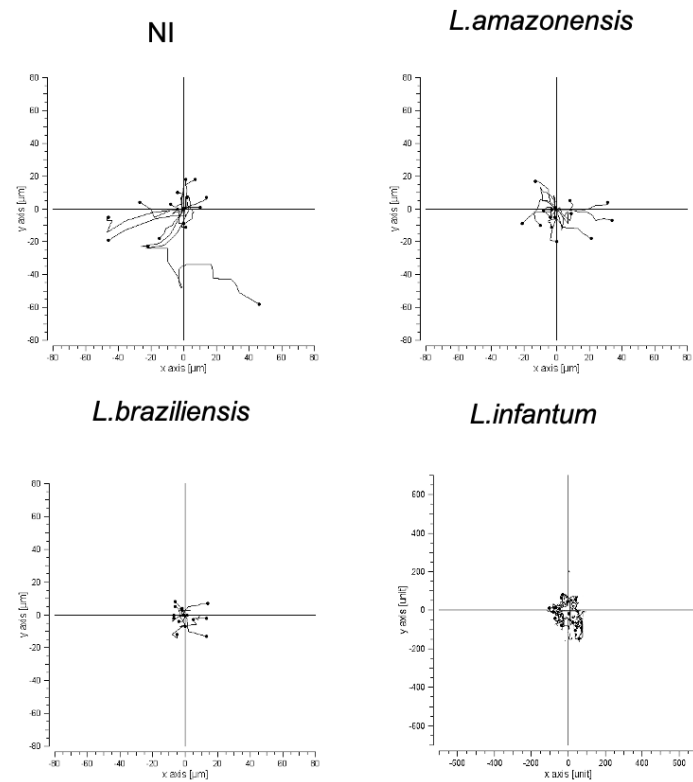
B



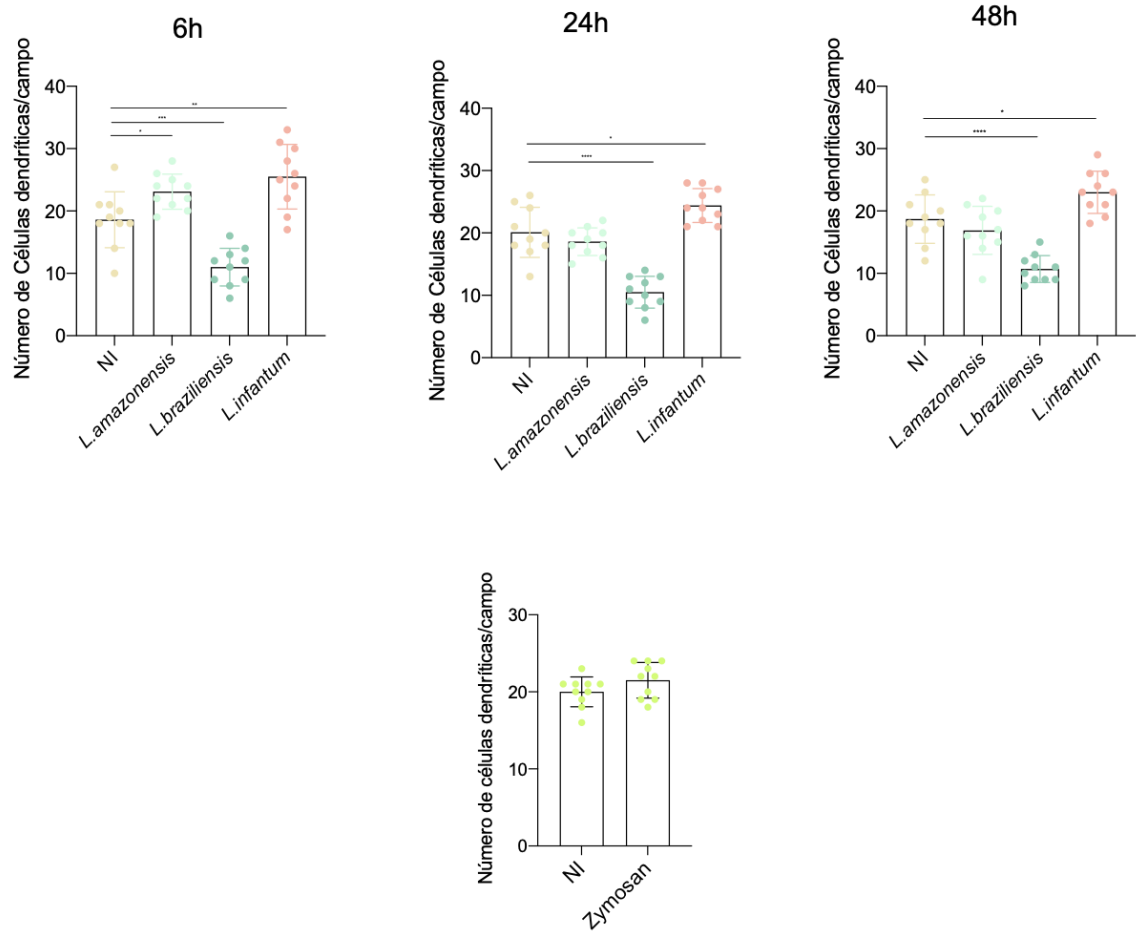
C



D



E



F

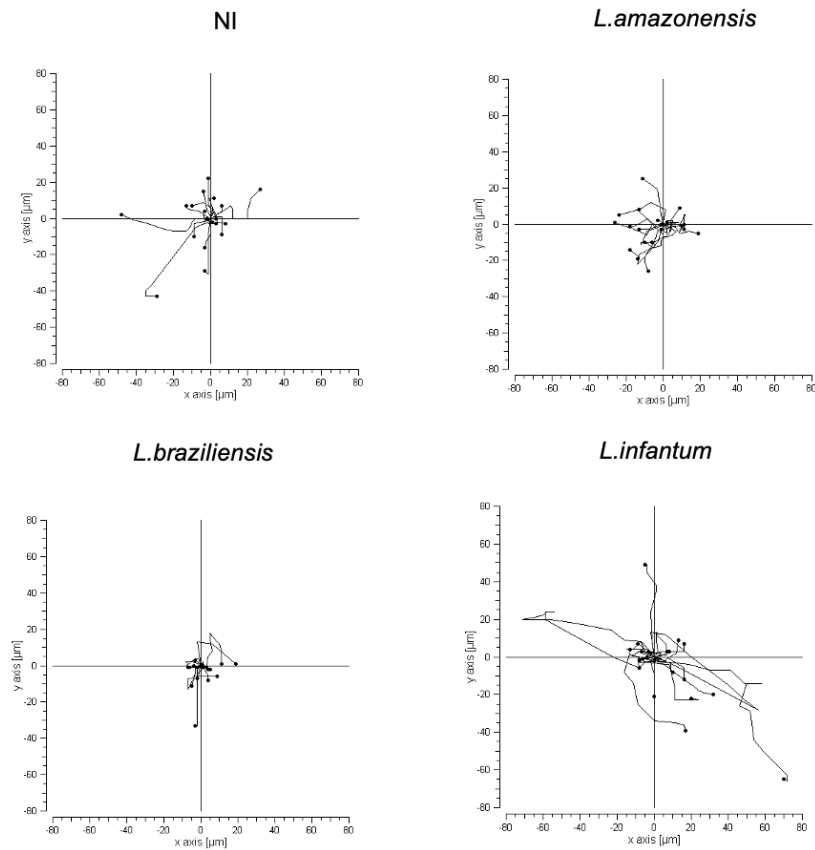
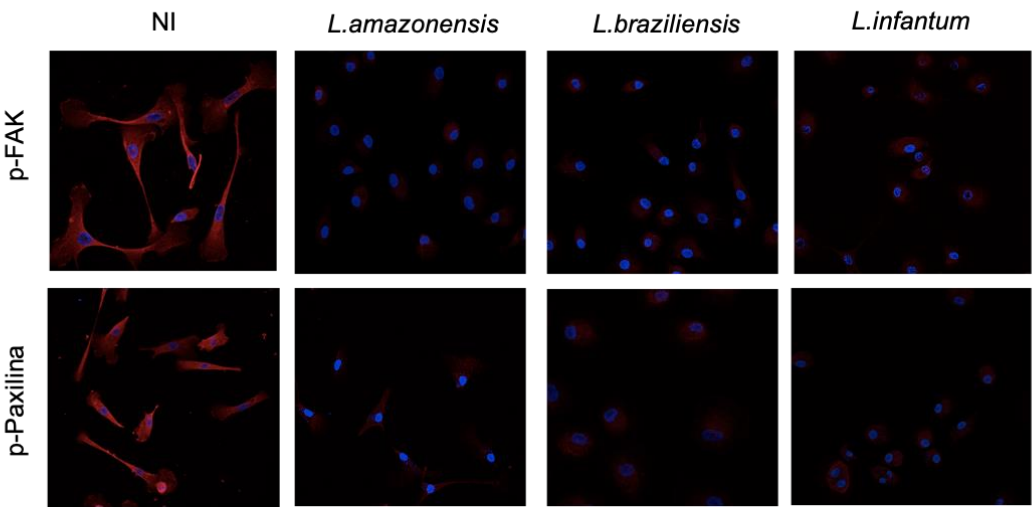


Figura 2. Avaliação da migração de células hospedeiras na infecção por *Leishmania*. Células hospedeiras humanas foram infectadas por *L. amazonensis*, *L. braziliensis* ou *L. infantum* e outro grupo foi exposto a fagocitose por partículas de zymosan FITC (Zy). 6, 24 ou 48h após a infecção, as células foram submetidas a migração na presença de MCP-1 ou CCL3, em sistema *Transwell*. Os insertos foram lavados e fixados, retirados e corados com DAPI para marcação nuclear. As células migrantes foram contadas (10 campos) aleatoriamente por microscopia de fluorescência. (A) Número de macrófagos migrantes 6, 24 ou 48h após infecção ou exposição ao zymosan (B) Trajetória percorrida pelos macrófagos (C) Número de monócitos migrantes 6, 24 ou 48h após infecção ou exposição ao zymosan (D) Trajetória percorrida pelos monócitos (E) Número de células dendríticas migrantes 6, 24 ou 48h após infecção ou exposição ao zymosan (F) Trajetória percorrida pelas células dendríticas. ** $p < 0,05$; ***, **** $p < 0,001$ (Anova). Resultado representativo de 3 experimentos.

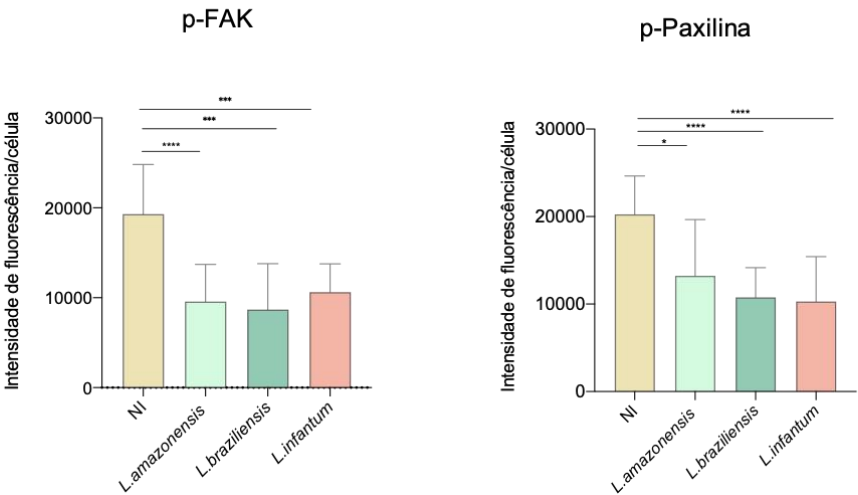
Avaliação da formação de complexos de adesão em células hospedeiras infectadas por *Leishmania*

A migração de células pode ser dividida em diferentes etapas: protrusão da parte anterior da célula, adesão da área de protrusão ao substrato e contração da actina citoplasmática na parte traseira da célula (Allen et al, 1998). Para investigar os mecanismos envolvidos na modulação da migração induzida por *Leishmania*, avaliamos a formação de complexos de adesão a partir da marcação de proteínas como FAK e Paxilina fosforiladas. Nossos resultados mostram que a infecção por *Leishmania* reduz a expressão de FAK e Paxilina fosforiladas em macrófagos e monócitos humanos em ambiente bidimensional (Figura 3A-D). Em contraste, observamos um aumento na expressão destas proteínas em células dendríticas infectadas por *L. infantum*, mas não naquelas infectadas por *L. amazonensis* ou *L. braziliensis* (Figura 3E-F).

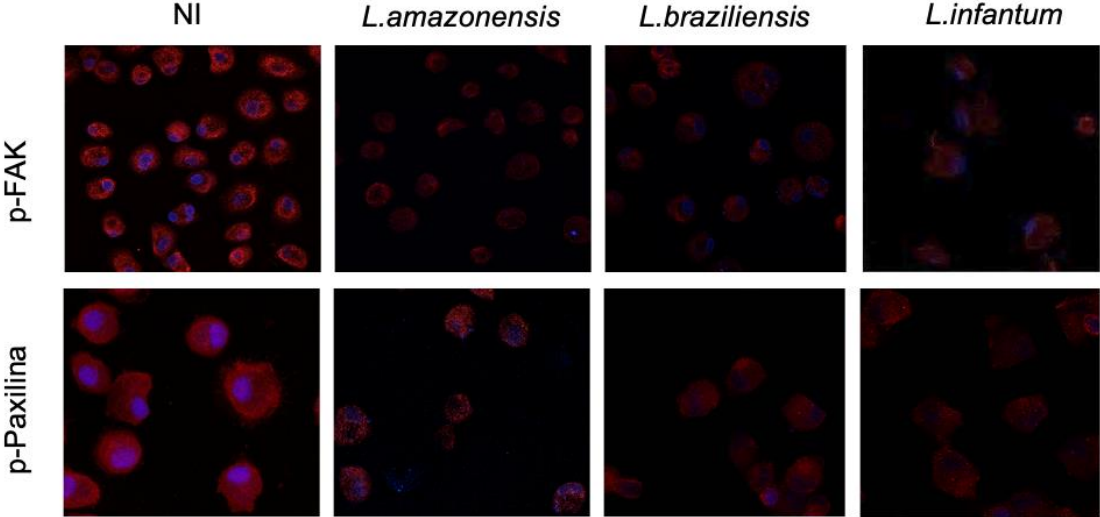
A



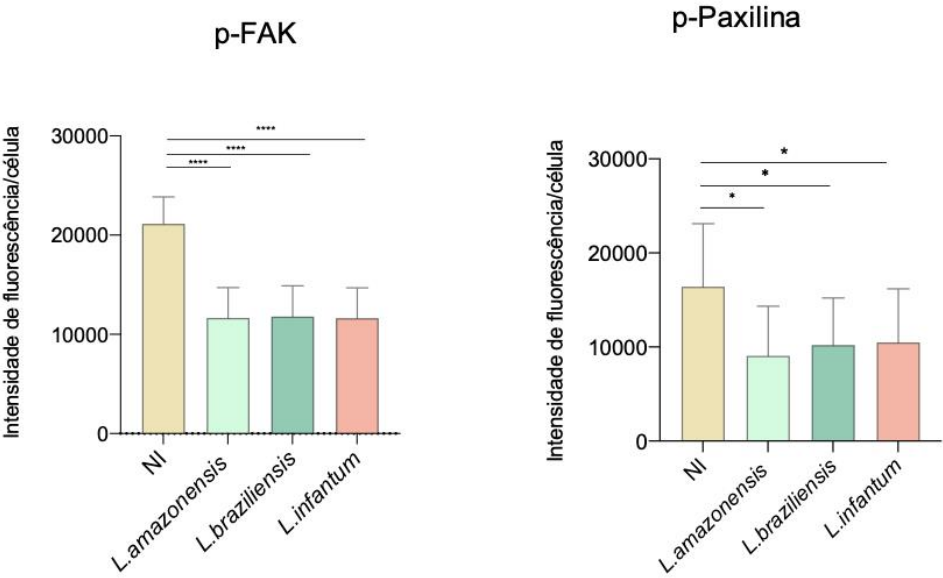
B



C



D



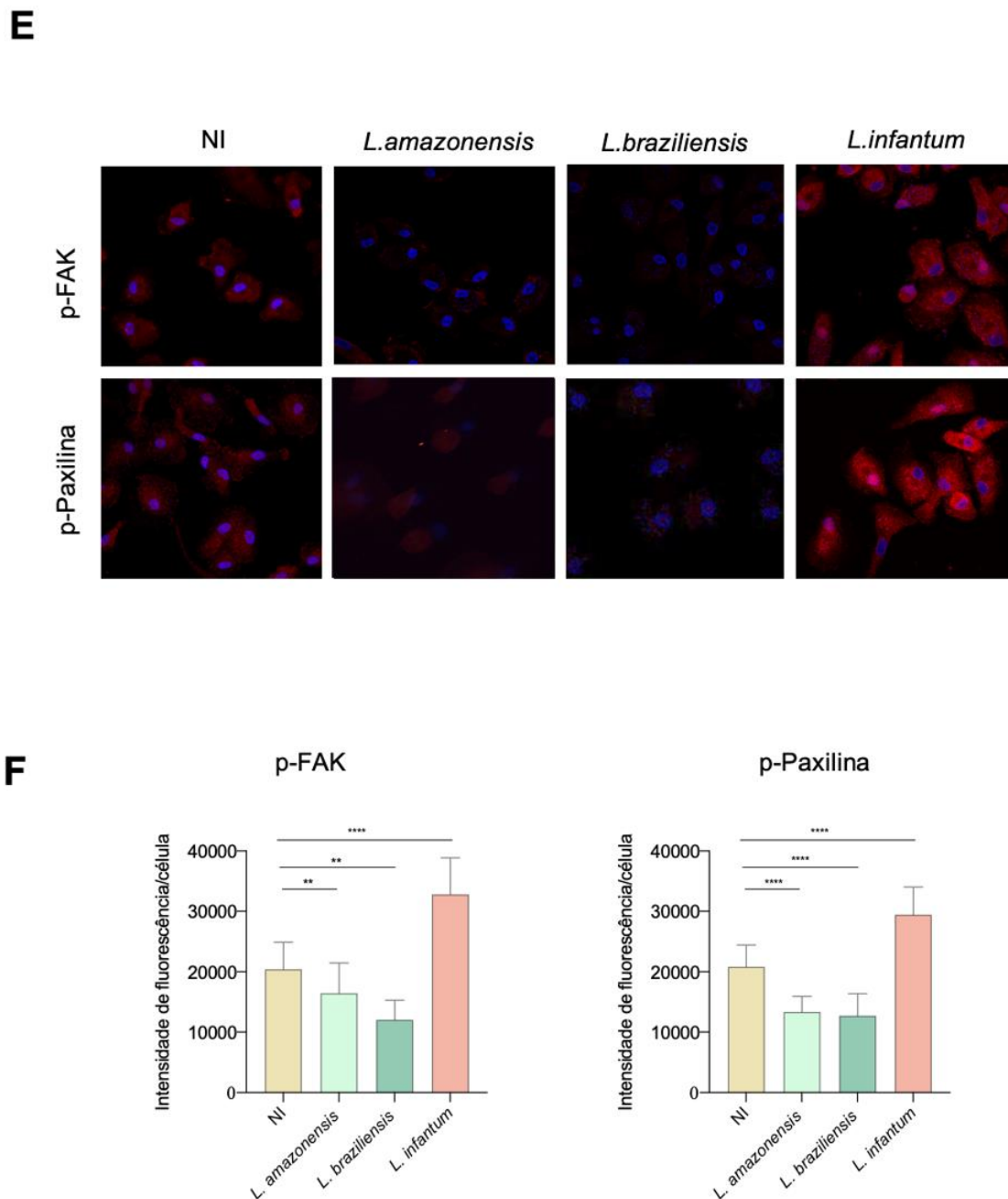
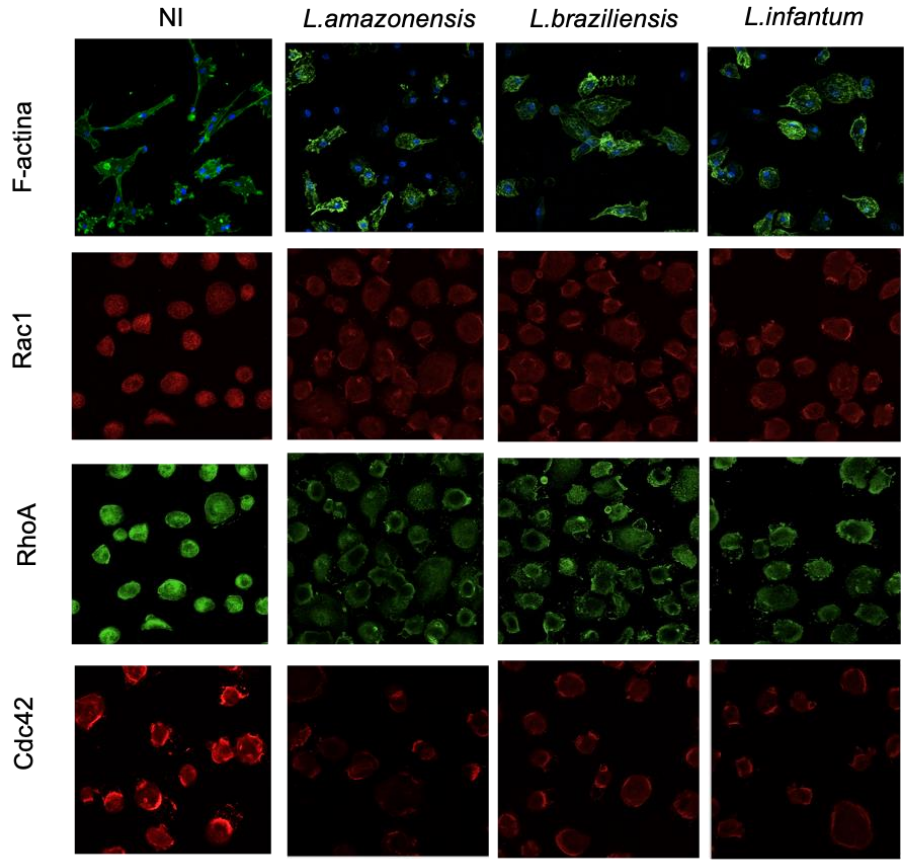


Figura 3. Avaliação da formação de complexos de adesão em células hospedeiras infectadas por *Leishmania*. Células hospedeiras infectadas ou não por *L. amazonensis*, *L. braziliensis* ou *L. infantum* foram marcadas com anticorpo anti-FAK e anti-paxilina fosforiladas. (A) Expressão da intensidade de fluorescência para p-FAK ou p-paxilina em macrófagos humanos. (B) Quantificação da intensidade de fluorescência em macrófagos humanos (C) Expressão da intensidade de fluorescência para p-FAK ou p-paxilina em monócitos humanos (D) Quantificação da intensidade de fluorescência em monócitos humanos (E) Expressão da intensidade de fluorescência para p-FAK ou p-paxilina em células dendríticas humanas (F) Quantificação da intensidade de fluorescência em células dendríticas humanas. A fluorescência foi quantificada utilizando o software FIJI. Vermelho, anti-FAK ou paxilina fosforilada; azul, DAPI. **** $p < 0,001$ (Anova). Resultado representativo de 3 experimentos.

Avaliação da dinâmica de actina em células hospedeiras infectadas por *Leishmania*

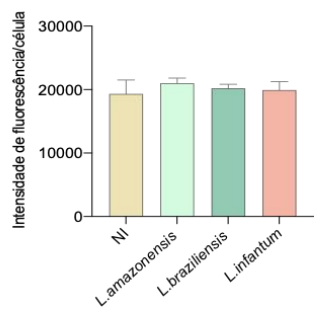
Com o objetivo de investigar a organização dos filamentos de actina, realizamos marcação de F-actina utilizando faloidina. Mostramos que não há uma diferença na análise da actina filamentosa quando quantificamos a intensidade de fluorescência entre os grupos, para todas as células analisadas. A fim de melhor compreender o processo da polimerização da actina citoplasmática em células infectadas por *Leishmania*, e visto que o processo de polimerização de actina é altamente dinâmico, partimos para avaliar a expressão de proteínas da família Rho GTPases, fundamentais na regulação da polimerização do citoesqueleto de actina. Mostramos que há uma redução significativa na expressão de Rac1, RhoA e Cdc42 em macrófagos ou monócitos infectados por todas as espécies de *Leishmania* estudadas, quando comparados com os grupos controle não infectados. Adicionalmente, em células dendríticas, somente a infecção por *L. infantum* induziu a expressão dessas proteínas associadas a modulação de actina, ao passo que células infectadas por *L. amazonensis* ou *L. braziliensis* mostram uma redução na expressão das mesmas (Figura 4A-F).

A

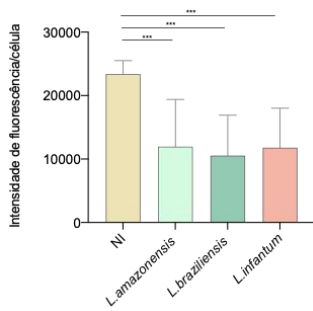


B

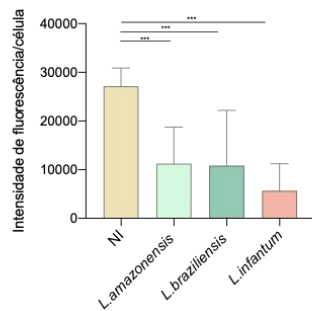
F-actina



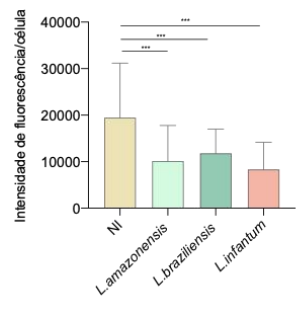
Rac1



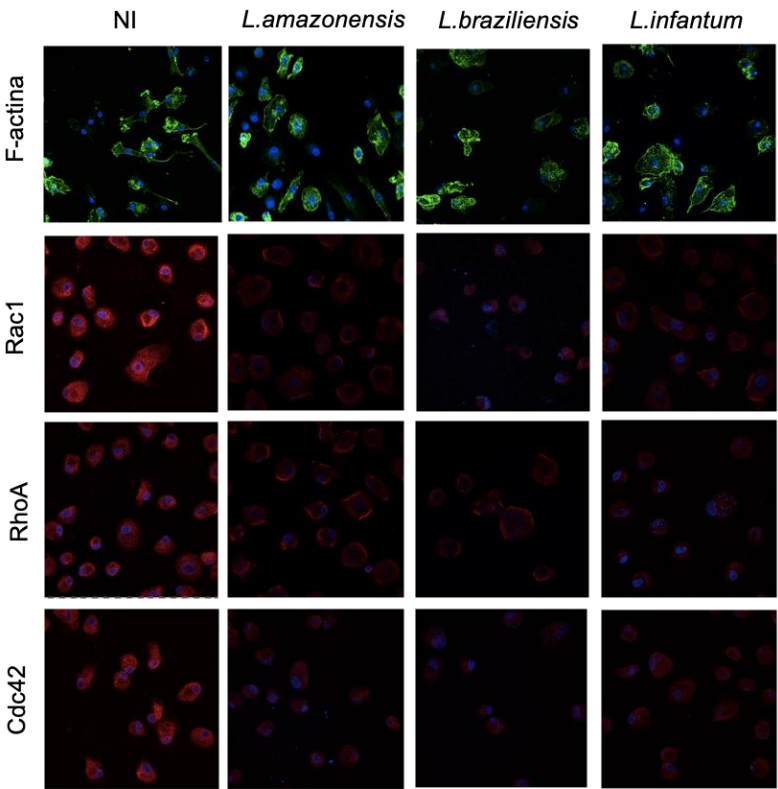
Rho-A



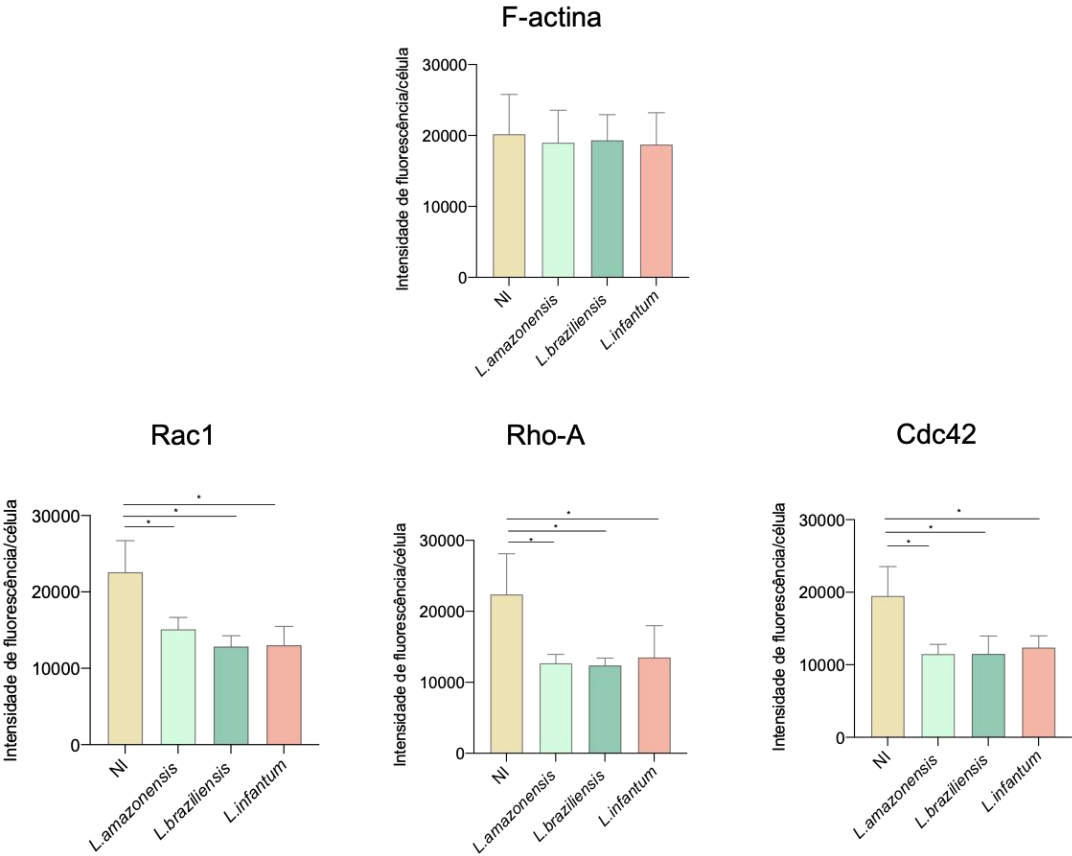
Cdc42



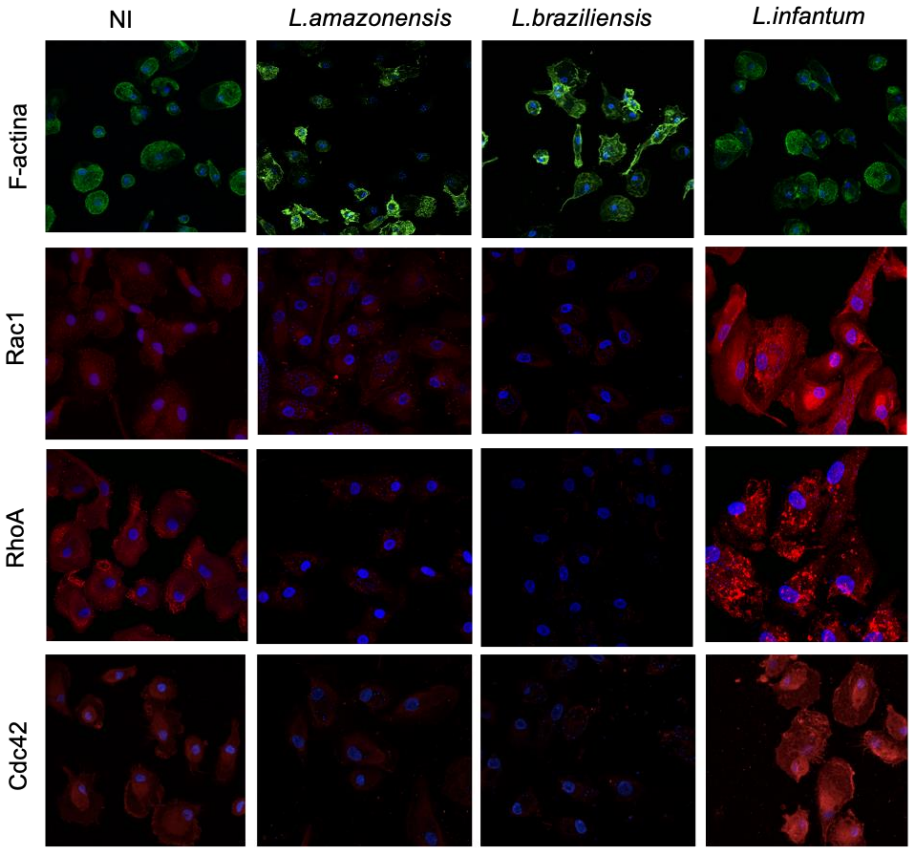
C



D



E



F

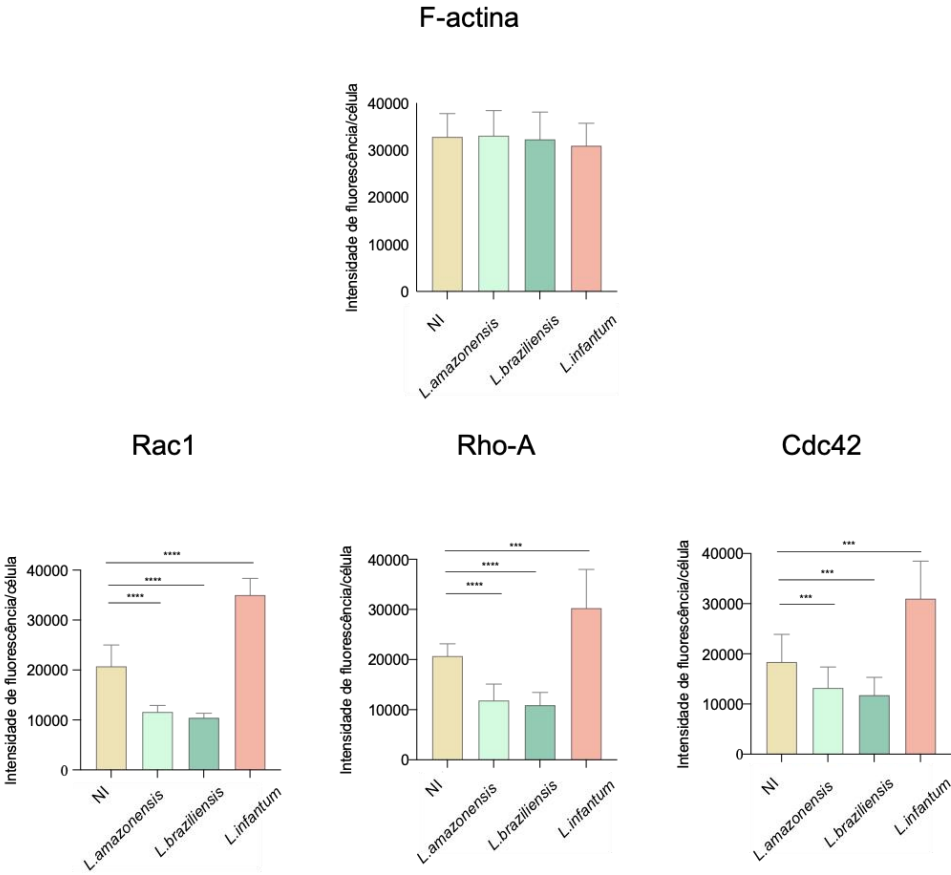
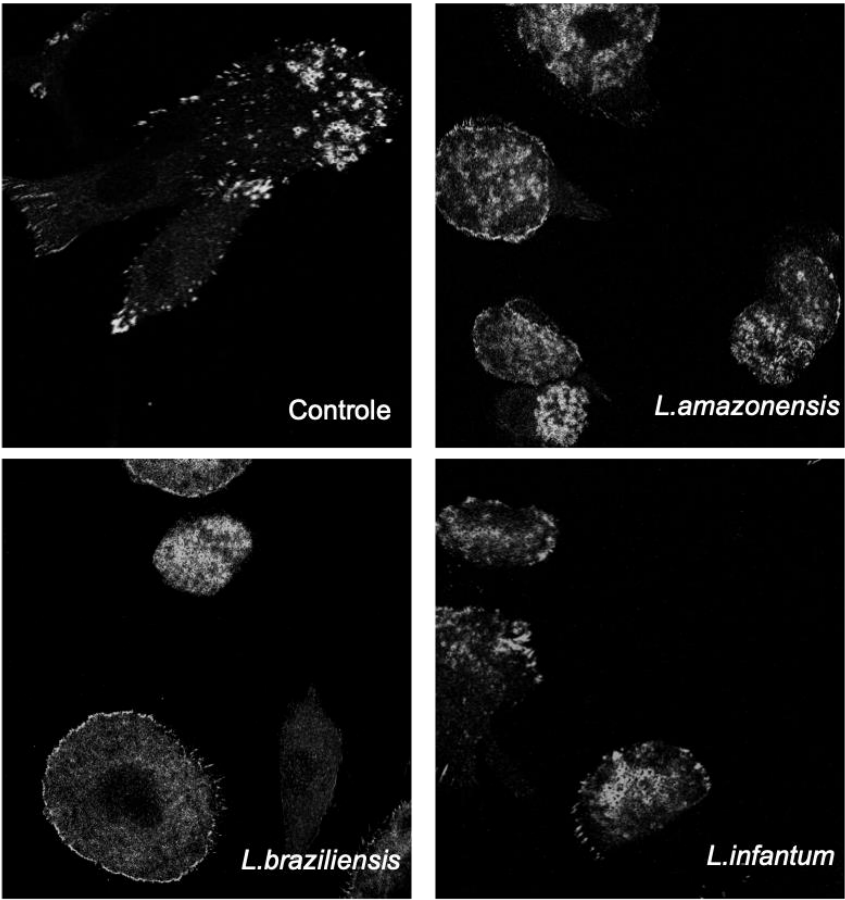


Figura 4. Avaliação da dinâmica de actina em células hospedeiras infectadas por *Leishmania*. Células hospedeiras infectadas ou não por *L. amazonensis*, *L. braziliensis* ou *L. infantum* foram marcadas com anticorpo anti-Rac1, anti-RhoA e anti-Cdc42 ou faloidina. (A) Expressão da intensidade de fluorescência de F-actina, Rac1, RhoA e Cdc42 em macrófagos (B) Quantificação da intensidade de fluorescência em macrófagos (C) Expressão da intensidade de fluorescência de F-actina, Rac1, RhoA e Cdc42 em monócitos (D) Quantificação da intensidade de fluorescência em monócitos (E) Expressão da intensidade de fluorescência de F-actina, Rac1, RhoA e Cdc42 em células dendríticas (F) Quantificação da intensidade de fluorescência em células dendríticas. A intensidade de fluorescência das marcações foi quantificada utilizando o software FIJI. Vermelho, anti-Rac1, anti-RhoA ou anti-Cdc42; verde, faloidina; azul, DAPI, núcleo da célula. **** $p < 0,001$ (Anova; Kruskal-wallis). Resultado representativo de 3 experimentos.

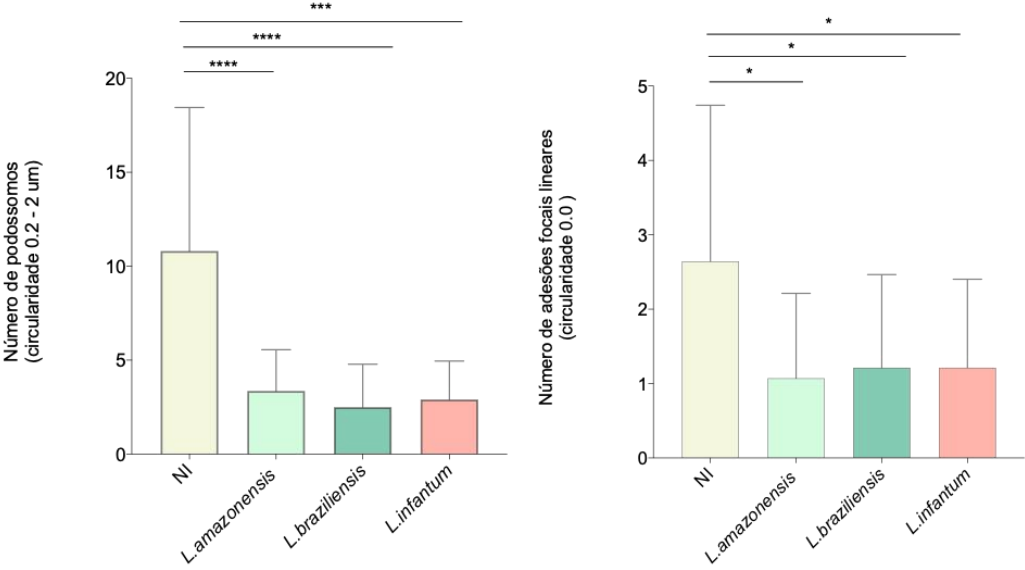
Avaliação da formação de podossomos em células hospedeiras infectadas por *Leishmania*

Com o objetivo de investigar a formação de podossomos em células hospedeiras infectadas por *Leishmania*, analisamos o número de podossomos nestas células por marcação de vinculina. Avaliamos ainda a circularidade ou linearidades das regiões marcadas por vinculina em células aderidas. Nossos dados mostram que, em macrófagos, a infecção por todas as espécies de *Leishmania* estudadas reduz o número de podossomos circulares em conformação inativa e ativa (Figura 5A-B). Entretanto, não foi observado diferença significativa na formação de podossomos circulares ou lineares em monócitos na infecção por *L. amazonensis* e *L. infantum*, ao passo que em *L. braziliensis* observamos uma redução na linearidade (Figura 5C-D). Adicionalmente, foi observada uma indução na formação destas conformações protrusivas nas bordas celulares, com diminuição de circularidade em células dendríticas infectadas por *L. infantum*, mas não por *L. amazonensis* ou *L. braziliensis* (Figura 5E-F).

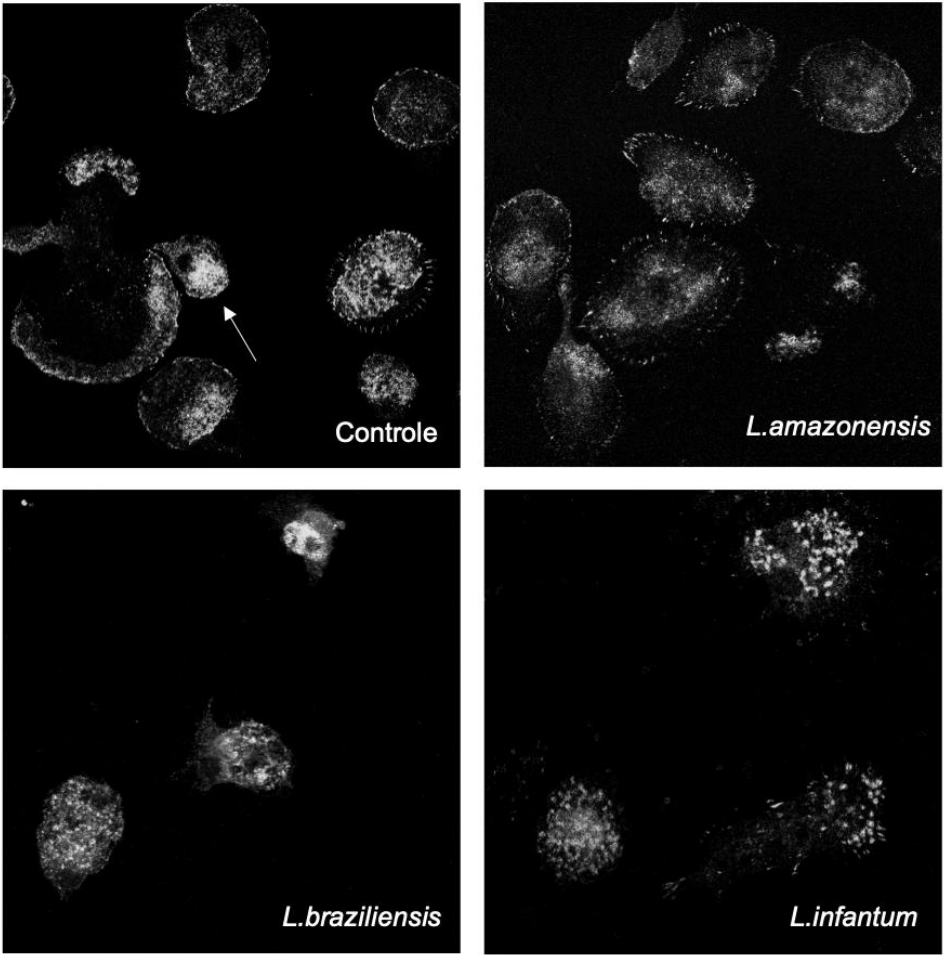
A



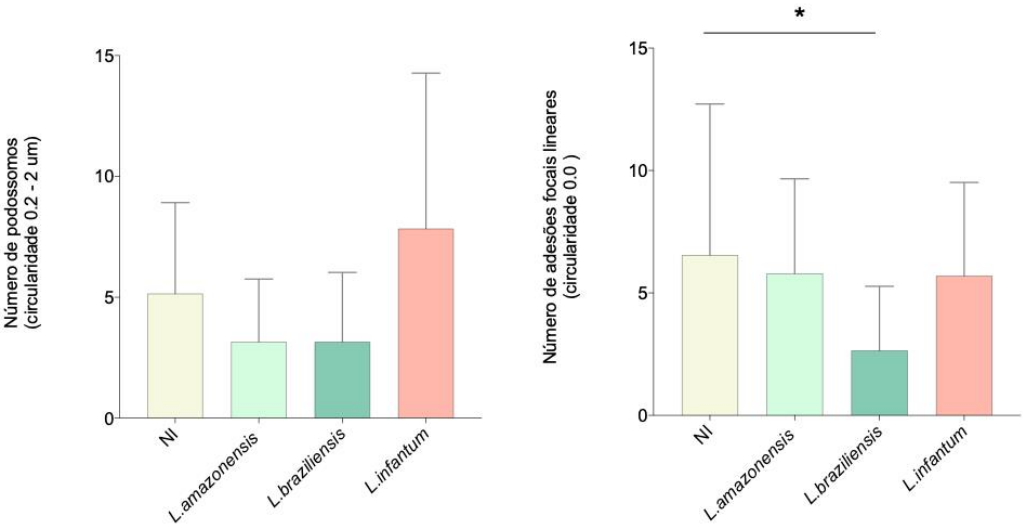
B



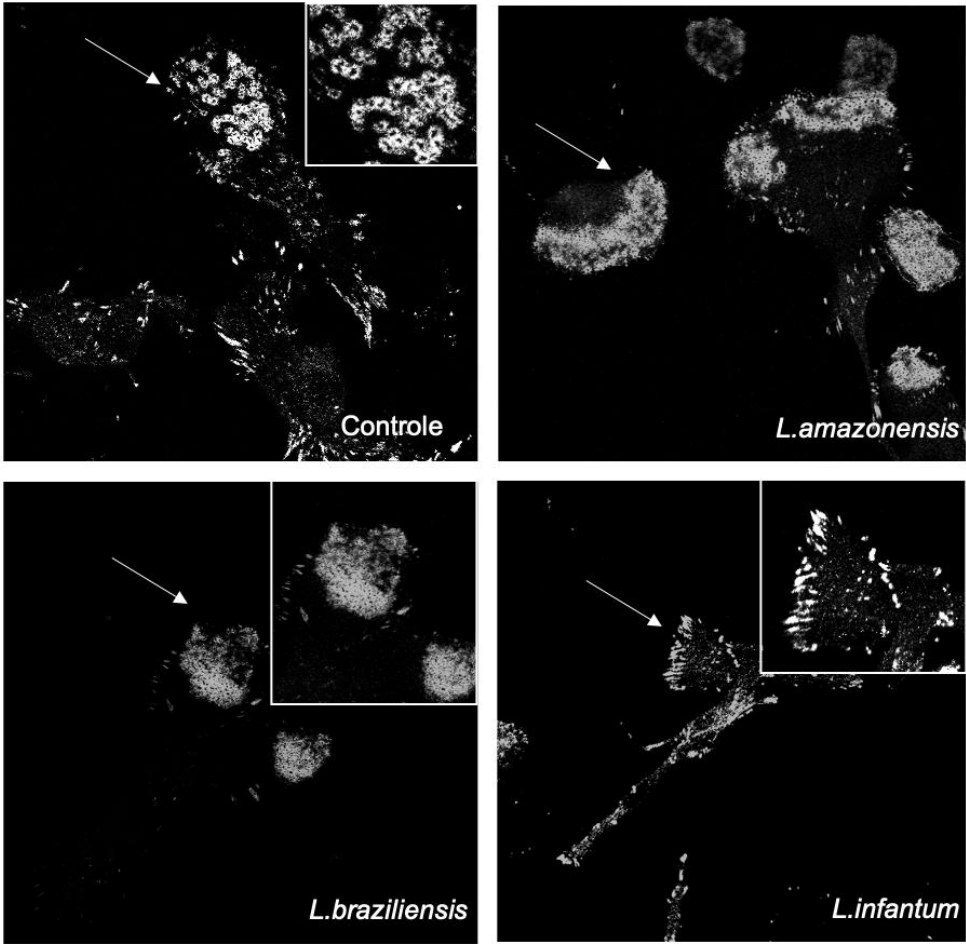
C



D



E



F

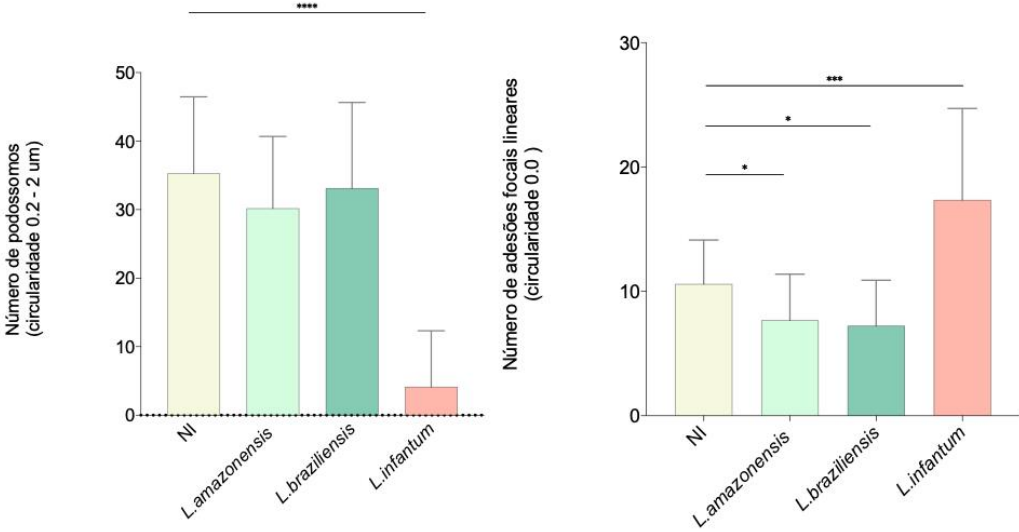


Figura 5. Avaliação da formação de podossomos em células hospedeiras infectadas por *Leishmania*. Células hospedeiras [2×10^5] infectadas ou não, plaqueadas em lamínulas cobertas com fibronectina [100ng/mL] foram fixadas com PFA 4% e imunomarcadas com anti-vinculina. Os efeitos da infecção por diferentes parasitos em diferentes células na formação de podossomos foram avaliados por microscopia de fluorescência. (A) Podossomos em macrófagos humanos infectados ou não por *Leishmania* (B) Número e circularidade dos podossomos em macrófagos humanos infectados ou não por *Leishmania* (C) Podossomos em monócitos humanos infectados ou não por *Leishmania* (D) Número e circularidade dos podossomos em monócitos humanos infectados ou não por *Leishmania* (E) Podossomos em células dendríticas humanas infectadas ou não por *Leishmania* (F) Número e circularidade dos podossomos em células dendríticas humanas infectadas por *Leishmania*. As células foram analisadas utilizando os microscópios *Leica laser 5 scanning confocal microscope* e *Spinning disk confocal waveFx* da Universidade de Toronto e o número, área total e a circularidade de podossomos foram quantificados pelo *Focal adhesion server* (North Carolina University, USA) e image J. $p^{***} < 0.001$ (Kruskal-Wallis). Resultado representativo de 1 experimento.

Avaliação da expressão de AKT/PI3K em células hospedeiras infectadas por *Leishmania*

A fim de investigar os mecanismos associados à modulação da migração celular induzida por *Leishmania*, partimos para analisar a participação de vias de sinalização importantes para a motilidade celular. Desta forma, extratos proteicos de células hospedeiras infectadas ou não por *Leishmania* foram analisadas por western blot. Nossos resultados mostraram que a infecção de macrófagos, monócitos e células dendríticas humanas induzem a expressão de proteínas da via AKT/PI3K (Figura 6A-B).

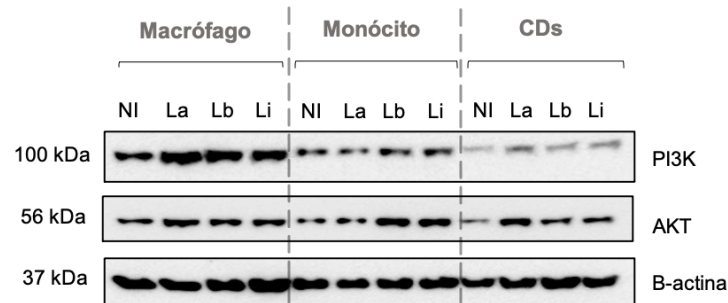
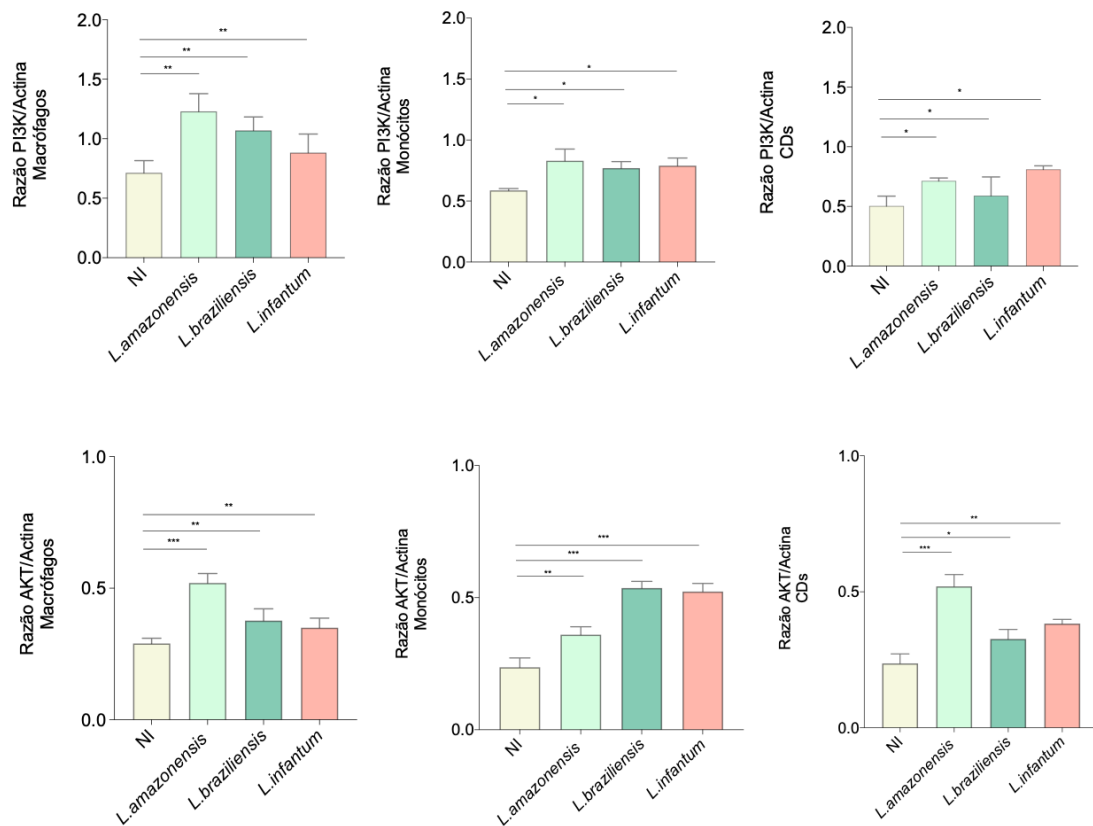
A**B**

Figura 6. Avaliação da expressão de AKT/PI3K em células hospedeiras infectadas por *Leishmania*. Células hospedeiras infectadas ou não por *L. amazonensis*, *L. braziliensis* ou *L. infantum*, 24h após a infecção, foram submetidas a extração e análise de expressão proteica por western blot. (A) Expressão de AKT/PI3K e actina em células hospedeiras infectadas por *L. amazonensis*, *L. braziliensis* ou *L. infantum* (B) As bandas de proteína foram quantificadas por densitometria através da razão PI3K/Actina ou AKT/Actina. * p < 0,05; ** p < 0,01 (Anova). Resultado representativo de 2 experimentos.

Avaliação do papel de AKT/PI3K na migração de células hospedeiras infectadas por *Leishmania*

Para avaliar o papel de AKT/PI3K na migração de células infectadas por *Leishmania*, utilizamos inibidores específicos para as mesmas e realizamos ensaios de migração direcional. Células hospedeiras foram infectadas por diferentes espécies de *Leishmania*, e tratadas com inibidor de PI3K por 24h. Em seguida os grupos foram submetidos a ensaio de migração direcional conforme previamente descrito. Nossos dados mostram que a inibição da via PI3K leva à redução da migração de macrófagos, monócitos 24h após o tratamento, nas células infectadas ou não. Para células dendríticas, mostramos que após o tratamento, a inibição da via PI3K reduz a migração nos grupos controle e infectados, exceto na infecção por *L. braziliensis* (Figura 7A-C).

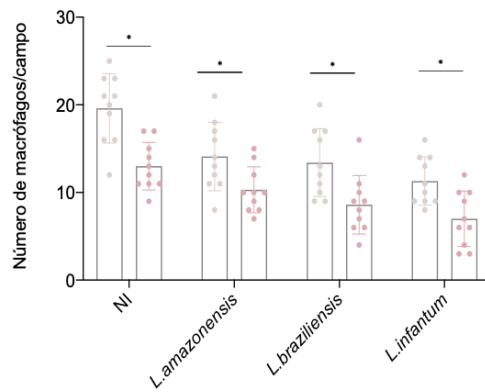
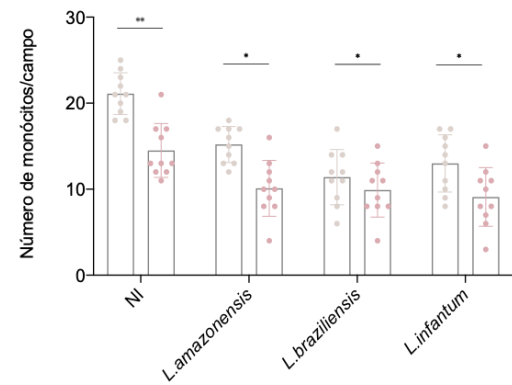
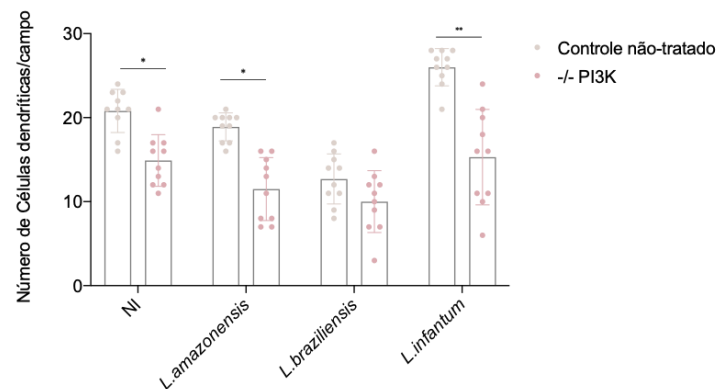
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Figura 7. Avaliação da expressão de AKT/PI3K em células hospedeiras infectadas por *Leishmania*. Macrófagos, monócitos ou células dendríticas humanas, infectados ou não por *L. amazonensis*, *L. braziliensis* ou *L. infantum* foram submetidos a tratamento com inibidor farmacológico específico das vias AKT/PI3K, AS605240 [100 nM] por 24h. Após este período, a migração direcional por sistema transwell foi analisada. (A) Número de macrófagos migrantes tratados e não-tratados. (B) Número de monócitos migrantes tratados e não-tratados. (C) Número de células dendríticas migrantes tratadas e não-tratadas. As células migrantes foram contadas aleatoriamente por microscopia de fluorescência. * $p < 0,05$ (Anova). Resultado representativo de 2 experimentos.

7 DISCUSSÃO

O presente estudo buscou investigar como diferentes espécies de *Leishmania* modulam a migração de células hospedeiras, bem como elucidar o papel destas diferentes células no contexto da disseminação do parasito no hospedeiro. Mostramos que monócitos e macrófagos apresentam migração reduzida após a infecção por *Leishmania*. Entretanto, nossos dados mostram que células dendríticas, por sua vez, apresentam um aumento na migração quando infectadas por espécies associadas a leishmaniose disseminada, difusa ou visceral, sugerindo que estas células podem ter um importante papel na visceralização ou disseminação da doença.

Inicialmente investigamos a migração randômica e direcional de monócitos, macrófagos e células dendríticas humanas na infecção por *L. amazonensis*, *L. braziliensis* ou *L. infantum*. Nossos dados demonstram que, em macrófagos e monócitos humanos, a infecção pelas espécies de *Leishmania* estudadas reduz a migração randômica e direcional dessas células (Figura 2 A-D, capítulo 2, manuscrito 2). Trabalhos prévios corroboram com nossos achados, mostrando uma redução da migração de macrófagos no modelo de LC. Os diferentes autores mostraram que macrófagos infectados por amastigotas de *L. amazonensis* ou *L. mexicana* tiveram sua migração reduzida, não sofrendo alterações em sua capacidade de secretar enzimas lisossômicas (Blewett, M; Kadivar, M; Soulsby, J, 1971; Bray et al, 1983). Ainda nesse contexto, Menezes e colaboradores (2017) mostraram uma redução na trajetória percorrida e na migração de macrófagos murinos infectados por *L. amazonensis*, independentes da carga parasitária ou de fatores solúveis secretados por estas células (Menezes et al, 2017). Adicionalmente, em publicação recente utilizando modelo *wound healing*, foi demonstrado que macrófagos de camundongos C57BL/6 infectados por *L. major* apresentam menor motilidade quando comparados macrófagos não infectados. No entanto, em contraste com nossos achados, os autores mostraram que macrófagos infectados por *L. major* tem a capacidade de realizar *gap closure* com menor velocidade quando comparados com aqueles infectados por *L. infantum* (Rocha et al, 2020). É possível que as diferenças observadas entre os resultados obtidos por Rocha e colaboradores possam estar relacionadas às diferenças metodológicas dos ensaios de migração e tipo de macrófago estudado.

O presente trabalho mostrou também um aumento da migração em células dendríticas infectadas por *L. infantum*, diferente do observado em macrófagos e monócitos infectados (Figura 2, capítulo 1, manuscrito 1; Figura 2 A-F, capítulo 2, manuscrito 2). Esses achados estão de acordo com estudos prévios que relataram uma redução da migração de células dendríticas infectadas por espécies causadoras de LC, em contraste com um aumento da

migração destas células após a infecção por espécies causadoras da forma visceral da doença. Trabalho prévio, utilizando modelo murino de peritonite crônica e rastreamento de células hospedeiras do local da infecção para linfonodos, mostrou uma redução na migração de células dendríticas mielóides infectadas por *L. amazonensis* (Hermida et al., 2014). Adicionalmente, trabalhos anteriores mostraram que a migração de células dendríticas infectadas por *L. major* foi reduzida, enquanto outros autores sugerem que *L. donovani* leva a um aumento do número de células dendríticas nos linfonodos drenantes em tempos iniciais *in vitro* (Ponte Sucre e Moll, 2001; Ato et al., 2002). Com o objetivo de investigar melhor o papel de células dendríticas na disseminação do parasito no hospedeiro vertebrado, avaliamos a migração dessas células após a infecção por isolados de *L. braziliensis* de obtidos de pacientes com a forma disseminada da doença e isolados de *L. amazonensis* obtidos de pacientes com a forma difusa da doença. A análise dos nossos dados mostra que a infecção por isolados de *L. braziliensis* ou *L. amazonensis* oriundas de pacientes com as formas disseminada ou difusa, respectivamente, induzem a migração de células dendríticas, em comparação com células infectadas por isolados obtidos de pacientes com LCL. Esses achados sugerem que, independentemente da espécie, a infecção por isolados de *Leishmania* associados às formas visceral ou que disseminam no hospedeiro vertebrado leva a um aumento da migração de células dendríticas (Figura 5, capítulo 1, manuscrito 1). Assim, é plausível sugerir que células dendríticas apresentam um papel importante na disseminação de *Leishmania* no hospedeiro vertebrado. Podemos hipotetizar ainda que o fato de monócitos e macrófagos apresentarem migração reduzida após a infecção por *Leishmania* poderia ser devido ao seu papel na manutenção da lesão no hospedeiro vertebrado.

Diversos estudos demonstraram com sucesso que os complexos de adesão celulares representam componentes moleculares altamente organizados e dinâmicos, que desempenham um papel crucial na interação célula-célula e célula-matriz extracelular (Turner et al, 2000; Schachtner et al, 2013). Essas estruturas são compostas por um grupo extenso de proteínas, incluindo FAK e paxilina, que formam pontos de ancoragem estratégicos na membrana celular (Hu et al, 2014). A fosforilação de FAK induz a ativação de paxilina, processo chave para que ocorra a adesão da célula a determinados substratos, e sua inibição, por consequência, reduz significativamente a formação de estruturas adesivas e pode impactar diretamente na motilidade da célula (Deakin; Turner, 2008; Deramautdt et al, 2014). Com o objetivo de aprofundar os conhecimentos acerca dos mecanismos associados à modulação da migração celular de células hospedeiras induzida por *Leishmania*, investigamos a formação de complexos de adesão nessas células após a infecção por diferentes isolados de *Leishmania*. Mostramos uma redução da

expressão de pFAK e pPaxilina nessas células, sugerindo que a redução da migração observada em monócitos e macrófagos humanos infectados por todas as espécies de *Leishmania* estudadas está associada a uma redução da formação de complexos de adesão nestas células (Figura 3 A-D, capítulo 2, manuscrito 2). Trabalhos prévios da literatura já mostraram que diferentes patógenos são capazes de modular a adesão de células hospedeiras (Murphy; Brinkworth, 2021; Clé et al, 2020; Colomé et al, 2017). Apesar deste ser o primeiro estudo que avalia a expressão de FAK e paxilina fosforiladas em diferentes células hospedeiras na infecção por *Leishmania*, outros autores mostraram que este parasito é capaz de modular a adesão da célula hospedeira. Consistente com nossos achados, estudos mostraram que a infecção de fagócitos mononucleares oriundos do exsudato peritoneal de camundongos por *L. amazonensis* reduz a adesão celular a diferentes substratos como colágeno, laminina e fibronectina. Adicionalmente, foi demonstrado que a infecção por *L. amazonensis* e *L. chagasi* induziu modificações na expressão de moléculas de adesão em monócitos e macrófagos, impactando sua habilidade de aderir ao tecido conjuntivo, o que pode implicar na migração destas células (Pinheiro et al, 2006; Carvalhal et al, 2004). Resultado semelhante foi descrito por Figueira e colaboradores (2015), que mostraram uma redução da adesão e espalhamento citoplasmático de monócitos infectados por *L. amazonensis*, sugerindo uma menor adesão dessas células (Figueira et al, 2015). Em estudo mais recente, Menezes e colaboradores (2017) mostraram também que *L. amazonensis* inibe a expressão de FAK e paxilina fosforiladas em macrófagos murinos, corroborando com nossos dados (Menezes et al, 2017). Interessantemente, nossos achados mostram ainda, ao contrário do observado em macrófagos e monócitos infectados por *Leishmania*, que em células dendríticas houve uma indução da expressão de proteínas formadoras do complexo de adesão, FAK e paxilina fosforiladas, após a infecção por *L. infantum* (Figura 3, capítulo 1, manuscrito 1; Figura 3 E-F, capítulo 2, manuscrito 2) e isolados de pacientes com a forma disseminada ou difusa da LC, mas não por isolados associados à forma localizada da doença (Figura 6, capítulo 1, manuscrito 1). Em conjunto, esses achados sugerem que a modulação da formação de moléculas de adesão em células hospedeiras infectadas por *Leishmania* é um mecanismo essencial para a modulação da migração nestas células. Esses dados estão de acordo com dados da literatura que mostram que a adesão celular é fundamental para que as células migrem de forma eficaz (Parsons et al, 2010; Owen et al., 2007).

Outro fator fundamental para que o processo de migração ocorra de forma eficaz é a dinâmica do citoesqueleto de actina (Driscoll et al., 2014; Schacks et al., 2019). Dada a importância da modulação desses filamentos no processo de migração celular, avaliamos a

marcação para F-actina em monócitos, macrófagos e células dendríticas infectadas por *Leishmania*. Entretanto, curiosamente, nossa análise não demonstrou diferença significativa na marcação por faloidina após a infecção por *Leishmania* em nenhuma das células estudadas (Figura 4 A, capítulo 1, manuscrito 1; Figura 4 A-F, parte superior “F-actina”, capítulo 2, manuscrito 2). A fim de melhor compreender o processo da polimerização da actina citoplasmática em células infectadas por *Leishmania*, e visto que o processo de polimerização de actina é altamente dinâmico, avaliamos a expressão de proteínas da família Rho GTPases, fundamentais na regulação da polimerização do citoesqueleto de actina. Demonstramos que a infecção por *Leishmania*, em macrófagos e monócitos, leva à redução da expressão de Rac1, RhoA e Cdc42, em comparação com células controle não-infectadas (Figura 4 A-D, capítulo 2, manuscrito 2). Resultado semelhante foi descrito por Menezes e colaboradores (2017), que mostraram que a infecção por *L. amazonensis* altera a dinâmica de actina em macrófagos através da reorganização da distribuição desses filamentos nas células infectadas. Este mecanismo pode estar associado ao processo de redução da capacidade migratória destas células, uma vez que o direcionamento da actina é um fator essencial para a adesão e protusão de membrana (Menezes et al, 2017). No entanto, diferente do observado em monócitos e macrófagos, observamos um aumento da expressão de Rho GTPases em células dendríticas infectadas por *L. infantum* (Figura 4 B-C, capítulo 1, manuscrito 1; Figura 4 E-F, capítulo 2, manuscrito 2), ou isolados de pacientes com a forma disseminada ou difusa da LC, mas não por isolados da forma localizada da doença (Figura 7 A, capítulo 1, manuscrito 1). Observamos ainda, em análise qualitativa, uma maior concentração de F-actina nas bordas dessas células, sugerindo uma distribuição diferente de actina polimerizada entre essas células e aquelas infectadas por isolados de LCL de *L. amazonensis* ou *L. braziliensis* (Figura 4 A, capítulo 1, manuscrito 1; Figura 4 E, F-actina, capítulo 2, manuscrito 2). Embora a avaliação da expressão de GTPases apenas não seja suficiente para demonstrar uma alteração da dinâmica de actina, esses achados em conjunto com uma distribuição diferente de filamentos de actina nos grupos estudados sugere uma modulação da polimerização desses filamentos. Estudos adicionais para avaliar a atividade dessas GTPases estão sendo realizados para reforçar a ideia de que *Leishmania* é capaz de modular a dinâmica de filamentos actina em diferentes células hospedeiras infectadas, alterando, consequentemente a migração destas células.

Ainda com o objetivo de investigar os mecanismos associados a modulação da migração observada em células hospedeiras após a infecção por *Leishmania*, avaliamos também a formação de podossomos nessas células. Os podossomos são estruturas celulares conhecida como complexos adesivos dinâmicos que desempenham papel crucial na

adesão celular, migração e remodelamento da matriz extracelular (Wiesner et al, 2014). Nossos achados indicam que, em macrófagos humanos, a infecção por *Leishmania* reduz a formação de podossomos inativos com maior circularidade, além de reduzir o aparecimento da forma ativa linear (Figura 5 A-B, capítulo 2, manuscrito 2). Adicionalmente, apesar de não ter sido observada diferença na formação de podossomos circulares em monócitos infectados pelas espécies de *Leishmania* estudadas, observamos uma redução dos podossomos com baixa circularidade nessas células após a infecção por *L. braziliensis* (Figura 5 B-C, capítulo 2, manuscrito 2). A modulação da formação de podossomos em células hospedeiras por patógenos foi previamente demonstrada. De maneira semelhante ao observado em nossos achados após a infecção de macrófagos por *Leishmania*, diferentes trabalhos mostram que macrófagos infectados por HIV possuem uma menor taxa de migração associada a redução da formação de podossomos (Vérollet; Le Cabec; Maridonneau-Parini, 2015). Esses dados reforçam a hipótese de que a redução da formação de podossomos nessas células constitui um mecanismo importante associado à redução da migração. No presente estudo, mostramos ainda que, em contraste ao observado em macrófagos e monócitos infectados, há uma maior formação de podossomos protrusivos na periferia em células dendríticas infectadas por *L. infantum* (Figura 5 E-F, capítulo 2, manuscrito 2). O aumento da formação de podossomos protrusivos em células dendríticas, associado ao favorecimento da migração destas células já foi demonstrado na infecção por HIV (Geijtenbeek et al, 2000; Baranov et al, 2014). Entretanto, este é o primeiro estudo que avalia a formação de podossomos e sua associação com a migração de células hospedeiras em células infectadas por *Leishmania*.

Após sua ativação e maturação, células dendríticas passam a expressar CCR7, favorecendo sua migração para linfonodos drenantes (Ohl et al., 2004; Mccoll et al., 2002). Uma vez que nossos achados mostram um aumento da migração de dendríticas infectadas por *L. infantum* ou isolados de *Leishmania* associados às formas difusa ou disseminada da doença, avaliamos a expressão de CCR7 nessas células. A modulação da expressão de CCR7 na infecção por *Leishmania* já foi demonstrada previamente. Trabalho publicado por Moll e colaboradores (2005), demonstrou que o aumento na expressão de CCR7 está associado a um aumento da migração de células dendríticas infectadas por *L. major* (Steigard; Moll, 2005). Esse trabalho vai de encontro aos nossos dados, no qual não observamos um aumento da expressão de CCR7 em células infectadas com as espécies associadas a forma localizada da doença. Entretanto, estudos na literatura demonstraram a participação do CCR7 na migração de células dendríticas em outros modelos experimentais. Anteriormente, foi descrito na literatura que infecções virais como as provocadas pelo citomegalovírus humano (HCMV) é capaz de não só inibir a

regulação do CCR7 ao infectar células dendríticas imaturas, mas também dificultar a migração destas células em resposta ao CCL19 (Grosche, 2017). Adicionalmente, foi demonstrado que o tráfego precoce de células dendríticas dos pulmões para o linfonodo drenante dependia da sinalização mediada pelo CCR7. Além disso, os autores mostraram que a migração prejudicada de células dendríticas em camundongos CCR7 nocaute resultou em disseminação tardia de bactérias para linfonodo e baço (Olmos, 2009). Nesse sentido, propomos que a ativação induzida por *L. infantum* ou isolados de *Leishmania* associados às formas difusa ou disseminada da doença envolve a indução da expressão de CCR7 e migração dessas células para linfonodos drenantes, levando a uma resposta que favorece a disseminação do parasito no hospedeiro vertebrado.

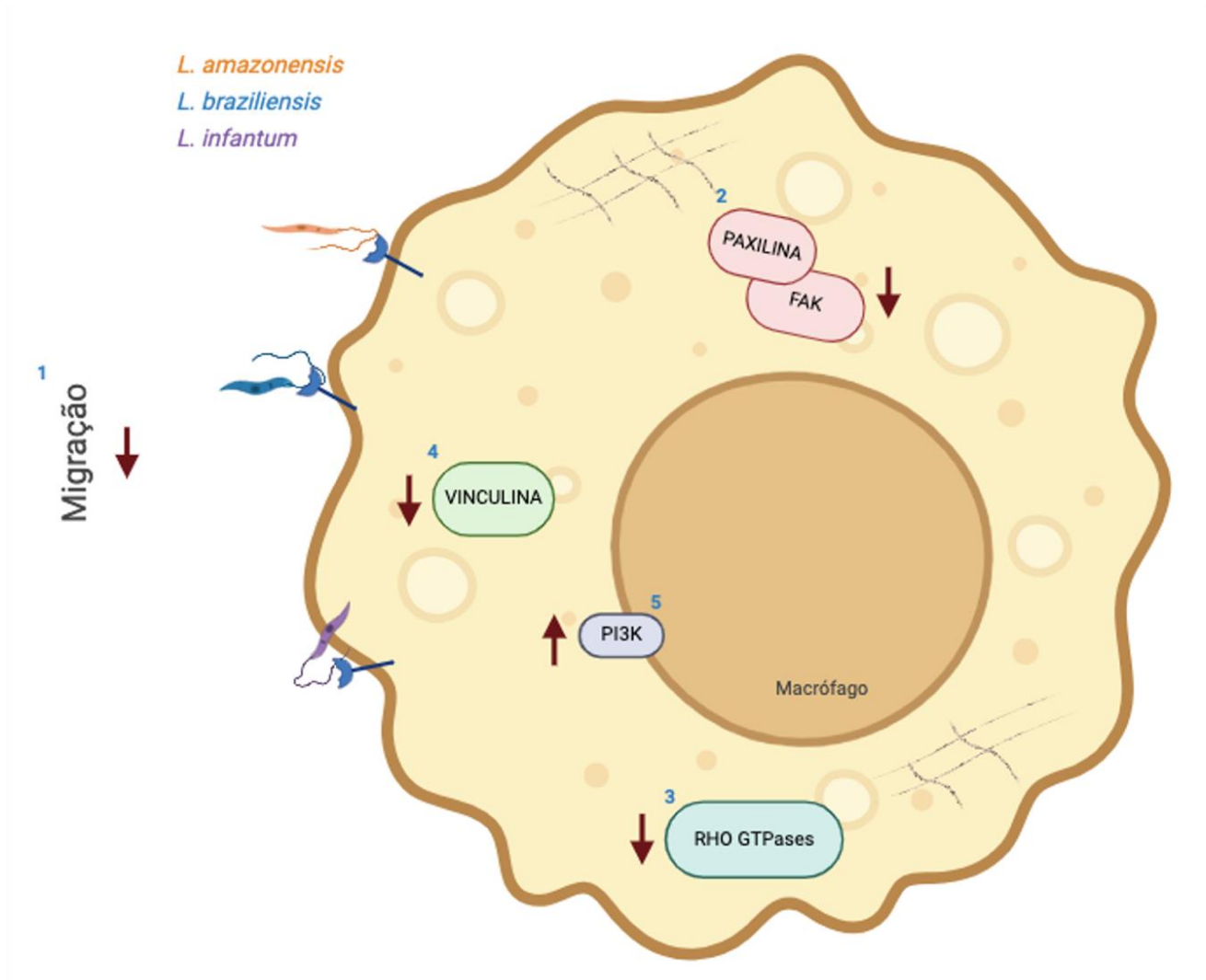
Estudos evidenciam que *Leishmania* modula diversas vias de sinalização em células hospedeiras (Ruhland et al, 2007; Blanchette et al., 1999; Gutiérrez-Kobeh et al., 2013). Assim, investigamos a expressão de proteínas envolvidas na via de PI3K/AKT, via de sinalização envolvida em diversos eventos celulares, incluindo a migração celular (Deng et al, 2022; Jean and Kiger, 2014). A PI3Kg é frequentemente associada à migração celular e sua ativação resulta na produção do lipídio fosfatidilinositol-3,4,5-trifosfato (PIP3), que ativa a proteína cinase Akt, levando à ativação de várias vias adjuntas de sinalização intracelular, incluindo aquelas que afetam o citoesqueleto e a adesão celular, como as Rho GTPases (Kakar; Chinmoy; Sun; 2023; Basta; Menko; Walker, 2022). Mostramos que para todas as células hospedeiras, a infecção por diferentes espécies de *Leishmania* resulta na indução da expressão de PI3K/AKT (Figura 6 A-B, capítulo 2, manuscrito 2). Além disso, nossos achados mostram que a inibição desta via leva à redução da migração de monócitos, macrófagos e células dendríticas infectadas por todas as espécies estudadas (Figura 7, capítulo 2, manuscrito 2), sugerindo que a ativação desta via apenas não é suficiente para induzir ou inibir a migração de células hospedeiras. Em contraste com nossos achados, Rocha et al. (2020) observaram uma indução da ativação desta via em macrófagos murinos infectados por *L. infantum*, associado ao aumento da migração dessas células. Os autores sugerem ainda que este parasito ativa, além da via dependente de PI3Kg em macrófagos, uma via independente da tirosina cinase para aumentar a atividade da PI3Kg, favorecendo assim sua migração (Rocha et al, 2020). Estudos relatam a participação de PI3K/AKT em LC, evidenciando seu papel na invasão parasitária de fagócitos e resistência a apoptose (Ruhland; Leal; Kima, 2006). Entretanto, o papel desta via na modulação da migração de células hospedeiras precisam ser mais bem estudado. É possível que as diferenças observadas entre nossos achados e os observados por Rocha e colaboradores estejam relacionadas às diferentes metodologias empregadas e tipo de macrófago estudado.

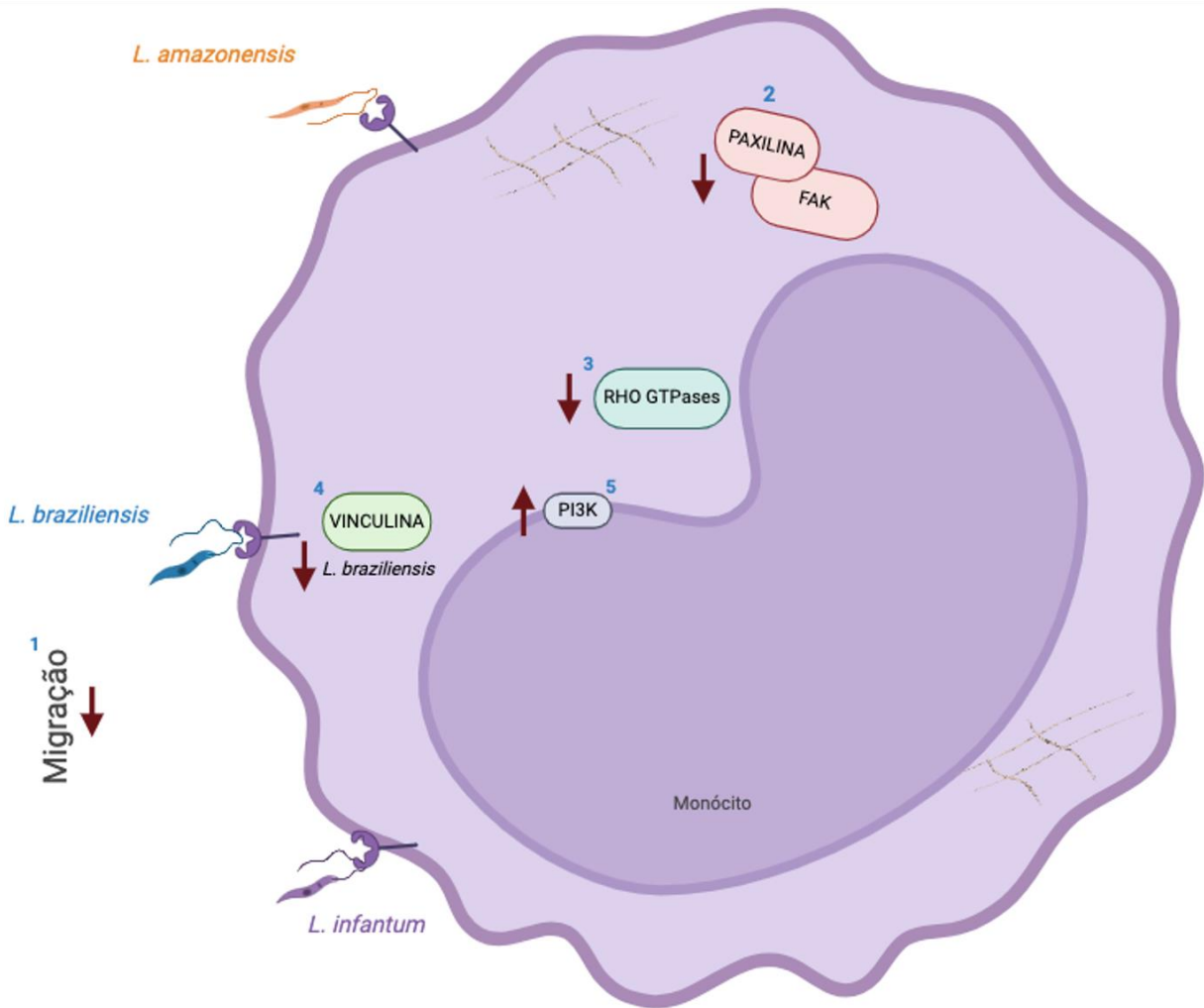
Em conjunto, os nossos achados sugerem que a infecção por *Leishmania* modula diferentemente a migração de células hospedeiras. Em macrófagos ocorre a redução da migração após a infecção por diferentes espécies (1), associada a uma redução na expressão de FAK e paxilina fosforiladas (2), redução na expressão de Rho GTPases (3) e formação de podossomos (4), além de *Leishmania* induzir a expressão de PI3K nessas células (5) (Figura 8A, considerações finais). Em monócitos, observamos um perfil semelhante: redução da migração durante a infecção por diferentes espécies de *Leishmania* (1), redução na expressão de FAK e paxilina fosforiladas (2), redução na expressão de Rho GTPases (3) e da formação de podossomos na infecção por *L. braziliensis* (4) e indução da expressão de PI3K (Figura 8B, considerações finais). Em contraste, em células dendríticas, mostramos que a infecção por *L. amazonensis* apresenta um perfil transitório de migração e redução da expressão de FAK e paxilina (2), redução da formação de podossomos (3) e da expressão de Rho GTPases (4) e indução da expressão de PI3K (5). Durante a infecção por *L. braziliensis*, mostramos que estes parasitos reduzem a migração destas células (1), associado a uma redução na expressão de FAK e paxilina (2), redução da formação de podossomos (3) e da expressão de Rho GTPases (4) e indução de PI3K (5). Em contraste, em células dendríticas infectadas por *L. infantum* ou isolados associados à forma difusa ou disseminada da doença, há uma indução da migração (6), aumento da expressão de FAK e paxilina (7), aumento da expressão de Rho GTPases (8) indução da formação de podossomos e indução da expressão de CCR7 e da expressão de PI3K para *L. infantum* (9,10) (Figura 8C, considerações finais).

8 CONSIDERAÇÕES FINAIS

O resumo dos resultados obtidos neste trabalho está esquematizado na figura abaixo:

A



B

C

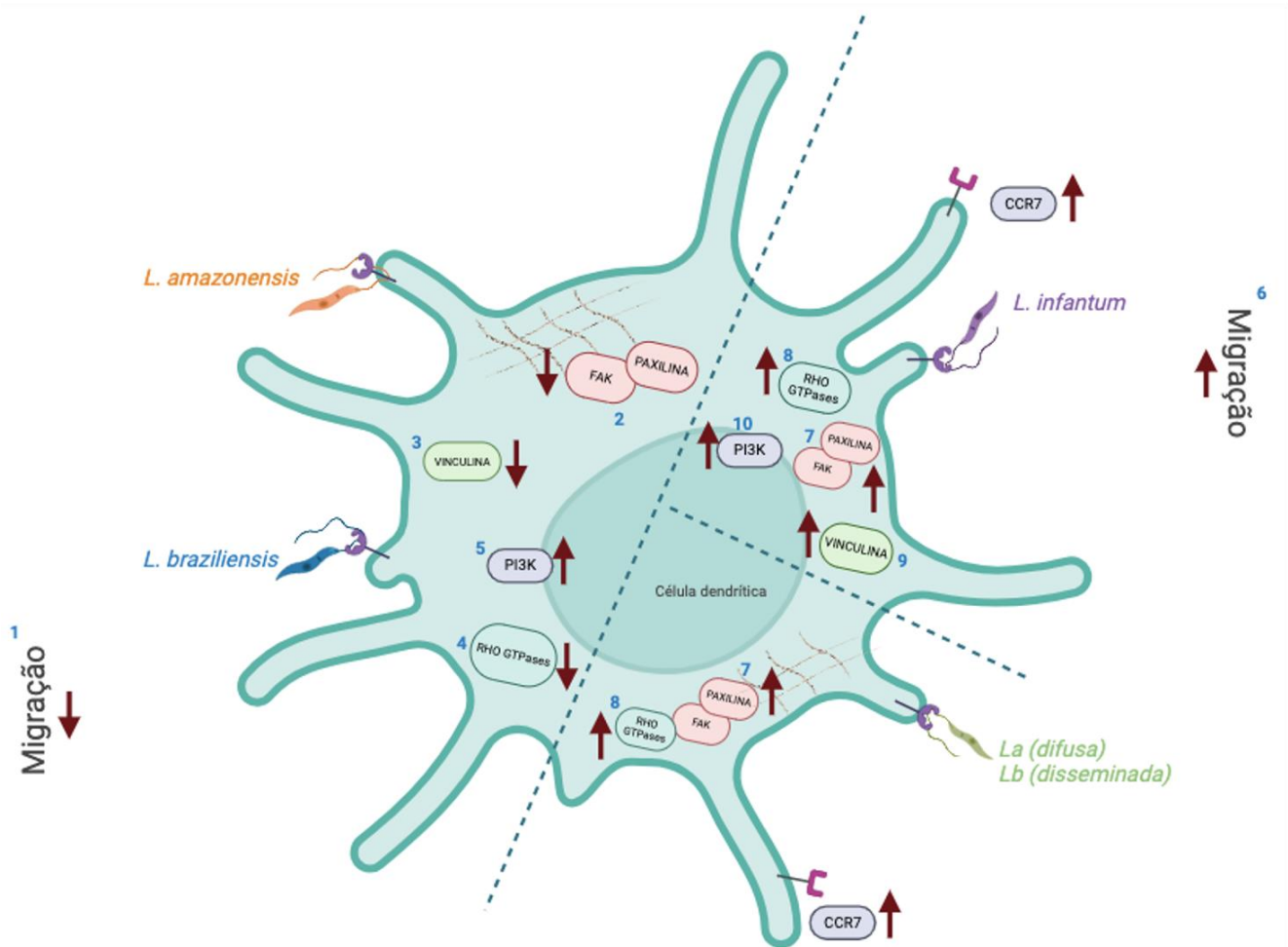


Figura 8. Diagrama esquemático dos resultados. (A) Representação dos resultados para macrófagos humanos, onde: 1. Redução da migração de macrófagos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; 2. Redução da expressão de proteínas formadoras do complexo de adesão em macrófagos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; 3. Redução da expressão de Rho GTPases adesão em macrófagos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; 4. Redução da formação de podossomos através da análise de vinculina em macrófagos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; Expressão induzida de PI3K em macrófagos infectados *L. amazonensis*, *L. braziliensis* ou *L. infantum*. (B) Representação dos resultados para monócitos humanos, onde: Redução da migração de monócitos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; 2. Redução da expressão de proteínas formadoras do complexo de adesão em monócitos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; 3. Redução da expressão de Rho GTPases adesão em monócitos infectados por *L. amazonensis*, *L. braziliensis* ou *L. infantum*; 4. Redução da formação de podossomos através da análise de vinculina em monócitos infectados por *L. braziliensis*; Expressão induzida de PI3K em monócitos infectados *L. amazonensis*, *L. braziliensis* ou *L. infantum*. (C) Representação dos resultados para células dendríticas humanas, onde: 1. Redução da migração de células dendríticas infectadas por *L. braziliensis*, e uma indução transitória por *L. amazonensis*; 2. Redução da expressão de proteínas formadoras do complexo de adesão em células dendríticas infectadas por *L. braziliensis* e *L. amazonensis*; 3. Redução da formação de podossomos através da análise de vinculina em células dendríticas infectadas por *L. amazonensis* ou *L. braziliensis*; 4. Redução da expressão de Rho GTPases em células dendríticas infectadas por *L. amazonensis*

ou *L. braziliensis*; 5. Expressão induzida de PI3K em células dendríticas infectadas por *L. amazonensis* ou *L. braziliensis*; 6. *L. infantum* ou isolados de leishmaniose difusa (La) ou disseminada (Lb) induzem a migração de células dendríticas humanas; 7. *L. infantum* ou isolados de leishmaniose difusa (La) ou disseminada (Lb) induzem a formação de complexos de adesão em células dendríticas humanas; 8. *L. infantum* ou isolados de leishmaniose difusa (La) ou disseminada (Lb) induzem a expressão de Rho GTPases em células dendríticas humanas; 9. *L. infantum* induz a formação de podossomos em células dendríticas humanas; 10. Expressão induzida de PI3K em células dendríticas infectadas por *L. infantum*.

9 CONCLUSÕES

A infecção por *Leishmania* modula diferentemente a migração de células hospedeiras, associado à alteração da formação do complexo de adesão, podossomos e dinâmica de actina nessas células.

A infecção por *L. infantum* e isolados associados às formas disseminada e difusa de LC levam à um aumento da migração de células dendríticas, mas não de macrófagos e monócitos, sugerindo um possível papel para estas células na disseminação do parasito no hospedeiro vertebrado.

REFERÊNCIAS

- ALBLAS, J. et al. Activation of Rhoa and ROCK are essential for detachment of migrating leukocytes. **Molecular Biology of the Cell**, v. 12, n. 7, p. 2137–45, jul. 2001.
- ALONSO, F. et al. Variations on the theme of podosomes: A matter of context. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, v. 1866, n. 4, p. 545–553, abr. 2019.
- AKISAKA, T. et al. Adhesion structures and their cytoskeleton-membrane interactions at podosomes of osteoclasts in culture. **Cell and Tissue Research**, v. 331, n. 3, p. 625–41, mar. 2008.
- AMATANGELO, M. D. et al. Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. **The American Journal of Pathology**, v. 167, n. 2, p. 475–88, ago. 2005.
- AMANO, M.; NAKAYAMA, M.; KAIBUCHI, K. Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. **Cytoskeleton**, v. 67, n. 9, p. 545–54, set. 2010.
- ANTOINE, J. C. et al. The biogenesis and properties of the parasitophorous vacuoles that harbour Leishmania in murine macrophages. **Trends in Microbiology**, v. 6, n. 10, p. 392–401, out. 1998.
- ARGUETA-DONOHUÉ, J. et al. Differential phagocytosis of Leishmania mexicana promastigotes and amastigotes by monocyte-derived dendritic cells. *Microbiology and immunology*, v. 60, n. 6, p. 369–81, jun. 2016.
- ASSREUY, J. et al. Production of nitric oxide and superoxide by activated macrophages and killing of Leishmania major. **European Journal of Immunology**, v. 24, n. 3, p. 672–676, 23 mar. 1994.
- ASHFORD, R. W. The leishmaniasis as emerging and reemerging zoonoses. **International Journal for Parasitology**, v. 30, n. 12–13, p. 1269–81, nov. 2000.
- ATO, M. et al. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. **Nature Immunology**, v. 3, n. 12, p. 1185–1191, 18 dez. 2002.
- AVRAHAM, H. K. et al. HIV-1 Tat-mediated effects on focal adhesion assembly and permeability in brain microvascular endothelial cells. **Journal of Immunology**, Baltimore, Md., v. 173, n. 10, p. 6228–33, 15 nov. 2004.
- BALDWIN, T. et al. Dendritic cell populations in Leishmania major-infected skin and draining lymph nodes. **Infection and Immunity**, v. 72, n. 4, p. 1991–2001, abr. 2004.
- BIANCO, A. et al. Two distinct modes of guidance signalling during collective migration of border cells. **Nature**, v. 448, n. 7151, p. 362–5, 19 jul. 2007.
- BHUWANIA, R. et al. Supravillin couples myosin-dependent contractility to podosomes and enables their turnover. **Journal of Cell Science**, v. 125, n. Pt 9, p. 2300–14, 1 maio 2012.

BADOLATO, R. et al. Leishmania major: Infection of Human Monocytes Induces Expression of IL-8 and MCAF. **Experimental Parasitology**, v. 82, n. 1, p. 21–26, jan. 1996.

BAÑULS, A.-L.; HIDE, M.; PRUGNOLLE, F. Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. **Advances in Parasitology**, v. 64, p. 1–109, 2007.

BARANOV, M. et al. Podosomes of dendritic cells facilitate antigen sampling. **Journal of Cell Science**, v. 127, n. Pt 5, p. 1052–1064, 1 mar. 2014.

BARBIER, L. et al. Myosin II Activity Is Selectively Needed for Migration in Highly Confined Microenvironments in Mature Dendritic Cells. **Frontiers in Immunology**, v. 10, 12 abr. 2019.

BASS, M. D. et al. Syndecan-4-dependent Rac1 regulation determines directional migration in response to the extracellular matrix. **The Journal of Cell Biology**, v. 177, n. 3, p. 527–38, 7 maio 2007.

BEATTIE, L.; KAYE, P. M. Leishmania-host interactions: what has imaging taught us? **Cellular Microbiology**, v. 13, n. 11, p. 1659–67, nov. 2011.

BEHIN, R.; MAUEL, J.; SORDAT, B. Leishmania tropica: pathogenicity and in vitro macrophage function in strains of inbred mice. **Experimental Parasitology**, v. 48, n. 1, p. 81–91, ago. 1979.

BECKER, I. et al. Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. **Molecular and Biochemical Parasitology**, v. 130, n. 2, p. 65–74, 31 ago. 2003.

BURNS, S. et al. Configuration of human dendritic cell cytoskeleton by Rho GTPases, the WAS protein, and differentiation. *Blood*, v. 98, n. 4, p. 1142–9, 15 ago. 2001.

BURRIDGE, K. et al. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. **Annual Review of Cell Biology**, v. 4, p. 487–525, 1988.

BLANCHETTE, J. et al. Leishmania-induced increases in activation of macrophage SHP-1 tyrosine phosphatase are associated with impaired IFN-gamma-triggered JAK2 activation. **European Journal Of Immunology**, v. 29, n. 11, p. 3737–44, nov. 1999.

BLEWETT, T. M.; KADIVAR, D. M.; SOULSBY, E. J. Cutaneous leishmaniasis in the guinea pig. Delayed-type hypersensitivity, lymphocyte stimulation, and inhibition of macrophage migration. **The American Journal of Tropical Medicine and Hygiene**, v. 20, n. 4, p. 546–51, jul. 1971.

BRAY, R. S. et al. The effect of parasitization by *Leishmania mexicana mexicana* on macrophage function in vitro. *Acta tropica*, v. 40, n. 1, p. 29–38, mar. 1983.

CAIN, R. J.; RIDLEY, A. J. Phosphoinositide 3-kinases in cell migration. **Biology of the cell**, v. 101, n. 1, p. 13–29, jan. 2009.

CARVALHAL, D. G. F. et al. The modelling of mononuclear phagocyte-connective tissue adhesion in vitro: Application to disclose a specific inhibitory effect of Leishmania infection. **Experimental Parasitology**, v. 107, n. 3–4, p. 189–199, jul. 2004.

CASE, L. B.; WATERMAN, C. M. Integration of actin dynamics and cell adhesion by a three-dimensional, mechanosensitive molecular clutch. **Nature Cell Biology**, v. 17, n. 8, p. 955–963, 29 ago. 2015.

CAUX, C. et al. Activation of human dendritic cells through CD40 cross-linking. **The Journal of Experimental Medicine**, v. 180, n. 4, p. 1263–72, 1 out. 1994.

CAVEN, L.; CARABEO, R. A. Pathogenic Puppetry: Manipulation of the Host Actin Cytoskeleton by Chlamydia trachomatis. **International Journal of Molecular Sciences**, v. 21, n. 1, 21 dez. 2019.

CHEN, D. Q. et al. Episomal expression of specific sense and antisense mRNAs in Leishmania amazonensis: modulation of gp63 level in promastigotes and their infection of macrophages in vitro. **Infection and Immunity**, v. 68, n. 1, p. 80–6, jan. 2000.

CENTER FOR DISEASE CONTROL AND PREVENTION. **Leishmaniasis epidemiology and risk factors**. 2020. <https://www.cdc.gov/parasites/leishmaniasis/epi.html>.

CHOI, C. K. et al. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. **Nature Cell Biology**, v. 10, n. 9, p. 1039–50, set. 2008.

CLÉ, M. et al. Zika Virus Infection Promotes Local Inflammation, Cell Adhesion Molecule Upregulation, and Leukocyte Recruitment at the Blood-Brain Barrier. **mBio**, v. 11, n. 4, 4 ago. 2020.

CORNFINE, S. et al. The kinesin KIF9 and reggie/flotillin proteins regulate matrix degradation by macrophage podosomes. **Molecular Biology of the Cell**, v. 22, n. 2, p. 202–215, 15 jan. 2011.

COUGOULE, C. et al. Blood leukocytes and macrophages of various phenotypes have distinct abilities to form podosomes and to migrate in 3D environments. **European Journal of Cell Biology**, v. 91, n. 11–12, p. 938–49, 2012.

COURRET, N. et al. Biogenesis of Leishmania-harboring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. **Journal of Cell Science**, v. 115, n. Pt 11, p. 2303–16, 1 jun. 2002.

DE SOUZA CARMO, E. V.; KATZ, S.; BARBIÉRI, C. L. Neutrophils reduce the parasite burden in Leishmania (Leishmania) amazonensis-infected macrophages. *PloS one*, v. 5, n. 11, p. e13815, 3 nov. 2010.

DEAKIN, N. O.; TURNER, C. E. Paxillin comes of age. **Journal of Cell Science**, v. 121, n. Pt 15, p. 2435–44, 1 ago. 2008.

DELORME-WALKER, V. D. et al. Pak1 regulates focal adhesion strength, myosin IIA distribution, and actin dynamics to optimize cell migration. **The Journal of Cell Biology**, v. 193, n. 7, p. 1289–303, 27 jun. 2011.

DOWD, G. C.; MORTUZA, R.; IRETTON, K. Molecular Mechanisms of Intercellular Dissemination of Bacterial Pathogens. **Trends in Microbiology**, v. 29, n. 2, p. 127–141, fev. 2021.

DENG, S. et al. PI3K/AKT Signaling Tips the Balance of Cytoskeletal Forces for Cancer Progression. **Cancers**, v. 14, n. 7, 24 mar. 2022.

DENG, H. et al. PI3K/AKT/mTOR pathway, hypoxia, and glucose metabolism: Potential targets to overcome radioresistance in small cell lung cancer. **Cancer Pathogenesis and Therapy**, v. 1, n. 1, p. 56–66, jan. 2023.

DENG, S. et al. PI3K/AKT Signaling Tips the Balance of Cytoskeletal Forces for Cancer Progression. **Cancers**, v. 14, n. 7, p. 1652, 24 mar. 2022.

DERAMAUDT, T. B. et al. Altering FAK-paxillin interactions reduces adhesion, migration and invasion processes. **PloS One**, v. 9, n. 3, p. e92059, 2014.

DE MENEZES, J. P. B. et al. Leishmania infection inhibits macrophage motility by altering F-actin dynamics and the expression of adhesion complex proteins. **Cellular Microbiology**, v. 19, n. 3, 2017.

DESJARDINS, M. ER-mediated phagocytosis: a new membrane for new functions. **Nature Reviews Immunology**, v. 3, n. 4, p. 280–291, abr. 2003.

DESJEUX, P. Leishmaniasis: current situation and new perspectives. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 27, n. 5, p. 305–318, set. 2004.

DESCOTEAUX, A.; TURCO, S. J. Glycoconjugates in Leishmania infectivity. **Biochimica et Biophysica Acta**, v. 1455, n. 2–3, p. 341–52, 8 out. 1999.

EDDY, R. J. et al. Tumor Cell Invadopodia: Invasive Protrusions that Orchestrate Metastasis. **Trends in Cell Biology**, v. 27, n. 8, p. 595–607, ago. 2017.

EVEN-RAM, S.; YAMADA, K. M. Cell migration in 3D matrix. **Current Opinion in Cell Biology**, v. 17, n. 5, p. 524–32, out. 2005.

FALASCA, M.; MAFFUCCI, T. Regulation and cellular functions of class II phosphoinositide 3-kinases. **The Biochemical Journal**, v. 443, n. 3, p. 587–601, 1 maio 2012.

FAIX, J.; ROTTNER, K. The making of filopodia. **Current Opinion in Cell Biology**, v. 18, n. 1, p. 18–25, fev. 2006.

FAVALI, C. et al. Leishmania amazonensis infection impairs differentiation and function of human dendritic cells. **Journal of Leukocyte Biology**, v. 82, n. 6, p. 1401–6, dez. 2007.

FIGUEIRA, C. P. et al. Leishmania infection modulates beta-1 integrin activation and alters the kinetics of monocyte spreading over fibronectin. **Scientific Reports**, v. 5, n. 1, p. 12862, 7 ago. 2015.

FIORINI, M.; MESSINA, M. F.; BARRACCHIA, A. Peripheral intramonocytic and intraneutrophil leishmanias observed in a chronic myelomonocytic leukemia (CMMoL) patient. **Haematologica**, v. 87, n. 8, p. EIM21, ago. 2002.

FRIEDL, P.; WEIGELIN, B. Interstitial leukocyte migration and immune function. **Nature Immunology**, v. 9, n. 9, p. 960–9, set. 2008.

FRIEDL, P.; GILMOUR, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nature Reviews Molecular Cell Biology*, v. 10, n. 7, p. 445–457, jul. 2009.

COUGOULE, C. et al. Three-dimensional migration of macrophages requires Hck for podosome organization and extracellular matrix proteolysis. **Blood**, v. 115, n. 7, p. 1444–52, 18 fev. 2010.

FRIEDL, P.; WEIGELIN, B. Interstitial leukocyte migration and immune function. **Nature Immunology**, v. 9, n. 9, p. 960–9, set. 2008.

FÖRSTER, R. et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*, v. 99, n. 1, p. 23–33, 1 out. 1999.

FORSCHER, P.; SMITH, S. J. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. **The Journal of Cell Biology**, v. 107, n. 4, p. 1505–16, out. 1988.

FORESTIER, C. L. et al. Imaging host cell-Leishmania interaction dynamics implicates parasite motility, lysosome recruitment, and host cell wounding in the infection process. **Cell Host & Microbe**, v. 9, n. 4, p. 319–30, 21 abr. 2011.

GAWDEN-BONE, C. et al. Dendritic cell podosomes are protrusive and invade the extracellular matrix using metalloproteinase MMP-14. **Journal of Cell Science**, v. 123, n. Pt 9, p. 1427–37, 1 maio 2010.

GEIJTENBEEK, T. B. et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. **Cell**, v. 100, n. 5, p. 587–97, 3 mar. 2000.

GREEN, S. J. et al. Activated macrophages destroy intracellular Leishmania major amastigotes by an L-arginine-dependent killing mechanism. **Journal of immunology**, Baltimore, Md., v. 144, n. 1, p. 278–83, 1 jan. 1990.

GROSCHKE, L. et al. Human Cytomegalovirus-Induced Degradation of CYTIP Modulates Dendritic Cell Adhesion and Migration. **Frontiers in Immunology**, v. 8, p. 461, 2017.

GLENNIE, N. D.; VOLK, S. W.; SCOTT, P. Skin-resident CD4⁺ T cells protect against Leishmania major by recruiting and activating inflammatory monocytes. **PLoS Pathogens**, v. 13, n. 4, p. e1006349, abr. 2017.

GORAK, P. M.; ENGWERDA, C. R.; KAYE, P. M. Dendritic cells, but not macrophages, produce IL-12 immediately following *Leishmania donovani* infection. **European Journal of Immunology**, v. 28, n. 2, p. 687–95, fev. 1998.

GORDON, S.; TAYLOR, P. R. Monocyte and macrophage heterogeneity. **Nature Reviews. Immunology**, v. 5, n. 12, p. 953–64, dez. 2005.

GOUGH, P. J.; GORDON, S. The role of scavenger receptors in the innate immune system. **Microbes and Infection**, v. 2, n. 3, p. 305–11, mar. 2000.

GUAN, G. et al. Effect of the Rho-Kinase/ROCK Signaling Pathway on Cytoskeleton Components. **Genes**, v. 14, n. 2, p. 272, 20 jan. 2023.

GUAK, H.; KRAWCZYK, C. M. Implications of cellular metabolism for immune cell migration. **Immunology**, v. 161, n. 3, p. 200–208, nov. 2020.

GUEIRARD, P. et al. Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. **Cellular Microbiology**, v. 10, n. 1, p. 100–11, jan. 2008.

GUIET, R. et al. The Process of Macrophage Migration Promotes Matrix Metalloproteinase-Independent Invasion by Tumor Cells. **The Journal of Immunology**, v. 187, n. 7, p. 3806–3814, 1 out. 2011.

GUILLIAMS, M.; MILDNER, A.; YONA, S. Developmental and Functional Heterogeneity of Monocytes. **Immunity**, v. 49, n. 4, p. 595–613, 16 out. 2018.

GUPTON, S. L.; WATERMAN-STORER, C. M. Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. **Cell**, v. 125, n. 7, p. 1361–74, 30 jun. 2006.

GUPTON, S. L.; GERTLER, F. B. Filopodia: the fingers that do the walking. **Science's STKE : Signal Transduction Knowledge Environment**, v. 2007, n. 400, p. re5, 21 ago. 2007.

GUPTON, S. L.; GERTLER, F. B. Filopodia: the fingers that do the walking. **Science's STKE: Signal Transduction Knowledge Environment**, v. 2007, n. 400, p. re5, 21 ago. 2007.

GUTIÉRREZ-KOBEH, L. et al. Inhibition of dendritic cell apoptosis by *Leishmania mexicana* amastigotes. **Parasitology Research**, v. 112, n. 4, p. 1755–62, abr. 2013.

GUTIÉRREZ-KOBEH, L. et al. Inhibition of dendritic cell apoptosis by *Leishmania mexicana* amastigotes. **Parasitology Research**, v. 112, n. 4, p. 1755–62, abr. 2013.

HARRIS, A. Behavior of cultured cells on substrata of variable adhesiveness. *Experimental cell research*, v. 77, n. 1, p. 285–97, 15 mar. 1973.

HARRISON, D. A. The Jak/STAT pathway. **Cold Spring Harbor Perspectives in Biology**, v. 4, n. 3, 1 mar. 2012.

HEASMAN, S. J. et al. Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. **The Journal of Cell Biology**, v. 190, n. 4, p. 553–63, 23 ago. 2010.

HERMIDA, M. D. R. et al. Leishmania amazonensis infection impairs dendritic cell migration from the inflammatory site to the draining lymph node. **BMC Infectious Diseases**, v. 14, p. 450, 20 ago. 2014.

HEYDE, S. et al. CD11c-expressing Ly6C+CCR2+ monocytes constitute a reservoir for efficient Leishmania proliferation and cell-to-cell transmission. **PLoS Pathogens**, v. 14, n. 10, p. e1007374, out. 2018.

HIND, L. E. et al. Two-dimensional motility of a macrophage cell line on microcontact-printed fibronectin. Hoboken. **Cytoskeleton**, v. 71, n. 9, p. 542–54, set. 2014.

HU, X. et al. The JAK/STAT signaling pathway: from bench to clinic. Signal transduction and targeted therapy, v. 6, n. 1, p. 402, 26 nov. 2021.

JEAN, S.; KIGER, A. A. Classes of phosphoinositide 3-kinases at a glance. **Journal of Cell Science**, v. 127, n. Pt 5, p. 923–8, 1 mar. 2014.

JEAN, S.; KIGER, A. A. Classes of phosphoinositide 3-kinases at a glance. **Journal of Cell Science**, v. 127, n. Pt 5, p. 923–8, 1 mar. 2014.

JACQUEMET, G. et al. RCP-driven $\alpha 5 \beta 1$ recycling suppresses Rac and promotes RhoA activity via the RacGAP1–IQGAP1 complex. **Journal of Cell Biology**, v. 202, n. 6, p. 917–935, 16 set. 2013.

JAFFE, A. B.; HALL, A. Rho GTPases: biochemistry and biology. **Annual Review of Cell and Developmental Biology**, v. 21, p. 247–69, 2005.

JURADO, C.; HASERICK, J. R.; LEE, J. Slipping or gripping? Fluorescent speckle microscopy in fish keratocytes reveals two different mechanisms for generating a retrograde flow of actin. **Molecular Biology of the Cell**, v. 16, n. 2, p. 507–18, fev. 2005.

LÄMMERMANN, T. et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. **Nature**, v. 453, n. 7191, p. 51–5, 1 maio 2008.

LAUFS, H. et al. Intracellular survival of Leishmania major in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. **Infection and Immunity**, v. 70, n. 2, p. 826–35, fev. 2002.

LEÓN, B.; LÓPEZ-BRAVO, M.; ARDAVÍN, C. Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania. **Immunity**, v. 26, n. 4, p. 519–531, 2007.

LIMA, J. B. et al. Leishmania infantum lipophosphoglycan induced-Prostaglandin E2 production in association with PPAR- γ expression via activation of Toll like receptors-1 and 2. **Scientific Reports**, v. 7, n. 1, p. 14321, 30 out. 2017.

LIN, C. H.; FORSCHER, P. Growth cone advance is inversely proportional to retrograde F-actin flow. **Neuron**, v. 14, n. 4, p. 763–71, abr. 1995.

LINDER, S. The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. **Trends in Cell Biology**, v. 17, n. 3, p. 107–17, mar. 2007.

LINDER, S.; AEPFELBACHER, M. Podosomes: adhesion hot-spots of invasive cells. **Trends in Cell Biology**, v. 13, n. 7, p. 376–85, jul. 2003.

LINDER, S.; KOPP, P. Podosomes at a glance **Journal of Cell Science**, v. 118, n. Pt 10, p. 2079–82, 15 maio 2005.

LINDER, S.; WIESNER, C.; HIMMEL, M. Degrading Devices: Invadosomes in Proteolytic Cell Invasion. **Annual Review of Cell and Developmental Biology**, v. 27, n. 1, p. 185–211, 10 nov. 2011.

LAMBERT, H. et al. Induction of dendritic cell migration upon *Toxoplasma gondii* infection potentiates parasite dissemination. **Cellular Microbiology**, v. 8, n. 10, p. 1611–23, out. 2006.

LIEW, F. Y. et al. Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. **Journal of Immunology**, Baltimore, Md., v. 144, n. 12, p. 4794–7, 15 jun. 1990.

LIMA, J. B. et al. *Leishmania infantum* lipophosphoglycan induced-Prostaglandin E2 production in association with PPAR- γ expression via activation of Toll like receptors-1 and 2. **Scientific Reports**, v. 7, n. 1, p. 14321, 30 out. 2017.

LÓPEZ-COLOMÉ, A. M. et al. Paxillin: a crossroad in pathological cell migration. **Journal of Hematology & Oncology**, v. 10, n. 1, p. 50, 18 fev. 2017.

LINDER, S. et al. Microtubule-dependent formation of podosomal adhesion structures in primary human macrophages. v. 113 Pt 23, p. 4165–76, dez. 2000.

LIU, D.; UZONNA, J. E. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. **Frontiers in Cellular and Infection Microbiology**, v. 2, p. 83, 2012.

LUXENBURG, C. et al. The Architecture of the Adhesive Apparatus of Cultured Osteoclasts: From Podosome Formation to Sealing Zone Assembly. **Plos One**, v. 2, n. 1, p. e179, 31 jan. 2007.

LIU, Y.-J. et al. Confinement and low adhesion induce fast amoeboid migration of slow mesenchymal cells. **Cell**, v. 160, n. 4, p. 659–672, 12 fev. 2015.

KAKAR, R.; GHOSH, C.; SUN, Y. Phosphoinositide Signaling in Immune Cell Migration. **Biomolecules**, v. 13, n. 12, p. 1705, 24 nov. 2023.

KUPANI, M. et al. IL-10 and TGF- β Induced Arginase Expression Contributes to Deficient Nitric Oxide Response in Human Visceral Leishmaniasis. **Frontiers in cellular and infection microbiology**, v. 10, p. 614165, 2020.

KAYE, P.; SCOTT, P. Leishmaniasis: complexity at the host-pathogen interface. *Nature reviews. Microbiology*, v. 9, n. 8, p. 604–15, 11 jul. 2011.

KOLE, T. P. et al. Intracellular mechanics of migrating fibroblasts. ***Molecular Biology of the Cell***, v. 16, n. 1, p. 328–38, jan. 2005.

KELLEHER, M. et al. Lipophosphoglycan blocks attachment of *Leishmania major* amastigotes to macrophages. ***Infection and Immunity***, v. 63, n. 1, p. 43–50, jan. 1995.

KESSENBROCK, K.; PLAKS, V.; WERB, Z. Matrix metalloproteinases: regulators of the tumor microenvironment. ***Cell***, v. 141, n. 1, p. 52–67, 2 abr. 2010.

MOLL, H. et al. Langerhans cells transport *Leishmania major* from the infected skin to the draining lymph node for presentation to antigen-specific T cells. ***European Journal of Immunology***, v. 23, n. 7, p. 1595–601, jul. 1993.

MACHADO, G. U.; PRATES, F. V.; MACHADO, P. R. L. Disseminated leishmaniasis: clinical, pathogenic, and therapeutic aspects. ***Anais Brasileiros de Dermatologia***, v. 94, n. 1, p. 9–16, fev. 2019.

MARTINEZ, F. O.; HELMING, L.; GORDON, S. Alternative Activation of Macrophages: An Immunologic Functional Perspective. ***Annual Review of Immunology***, v. 27, n. 1, p. 451–483, 1 abr. 2009.

MELO, T. G. et al. The involvement of FAK and Src in the invasion of cardiomyocytes by *Trypanosoma cruzi*. ***Experimental Parasitology***, v. 139, p. 49–57, abr. 2014.

MELO, T. G. et al. *Trypanosoma cruzi* down-regulates mechanosensitive proteins in cardiomyocytes. ***Memórias do Instituto Oswaldo Cruz***, v. 114, 2019.

MOHAMED, M. M.; SLOANE, B. F. Cysteine cathepsins: multifunctional enzymes in cancer. *Nature reviews. Cancer*, v. 6, n. 10, p. 764–75, out. 2006.

MARTIN-GRANADOS, C. et al. A key role for PTP1B in dendritic cell maturation, migration, and T cell activation. ***Journal of Molecular Cell Biology***, v. 7, n. 6, p. 517–528, dez. 2015.

MURPHY, K. N.; BRINKWORTH, A. J. Manipulation of Focal Adhesion Signaling by Pathogenic Microbes. ***International Journal of Molecular Sciences***, v. 22, n. 3, 29 jan. 2021.

MCCOLL, S. R. Chemokines and dendritic cells: A crucial alliance. ***Immunology & Cell Biology***, v. 80, n. 5, p. 489–496, out. 2002.

MELO, T. G.; COUTINHO, E. A.; PEREIRA, M. C. S. Heparan sulfate proteoglycan triggers focal adhesion kinase signaling during *Trypanosoma cruzi* invasion. ***Memórias do Instituto Oswaldo Cruz***, v. 115, 2020.

MUKBEL, R. M. et al. Macrophage killing of *Leishmania amazonensis* amastigotes requires both nitric oxide and superoxide. **The American Journal of Tropical Medicine and Hygiene**, v. 76, n. 4, p. 669–75, abr. 2007.

MURRAY, H. W.; NATHAN, C. F. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. **The Journal of Experimental Medicine**, v. 189, n. 4, p. 741–6, 15 fev. 1999.

MACHACEK, M. et al. Coordination of Rho GTPase activities during cell protrusion. **Nature**, v. 461, n. 7260, p. 99–103, 3 set. 2009.

MCLEAN, G. W. et al. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nature reviews*. **Cancer**, v. 5, n. 7, p. 505–15, jul. 2005.

MCCONVILLE, M. J. et al. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. **The EMBO Journal**, v. 11, n. 10, p. 3593–600, out. 1992.

MILDNER, A.; MARINKOVIC, G.; JUNG, S. Murine Monocytes: Origins, Subsets, Fates, and Functions. **Microbiology Spectrum**, v. 4, n. 5, out. 2016.

MÓCSAI, A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. **The Journal of Experimental Medicine**, v. 210, n. 7, p. 1283–99, 1 jul. 2013.

MOLL, H. et al. Langerhans cells transport *Leishmania major* from the infected skin to the draining lymph node for presentation to antigen- specific T cells. **European Journal of Immunology**, v. 23, p. 1595–1601, 1993.

MOLLINEDO, F. et al. Selective Fusion of Azurophilic Granules with *Leishmania*-containing Phagosomes in Human Neutrophils. **Journal of Biological Chemistry**, v. 285, n. 45, p. 34528–34536, 5 nov. 2010.

MONYPENNY, J. et al. Cdc42 and Rac family GTPases regulate mode and speed but not direction of primary fibroblast migration during platelet-derived growth factor-dependent chemotaxis. **Molecular and Cellular Biology**, v. 29, n. 10, p. 2730–47, maio 2009.

MOREAU, V. et al. Actin can reorganize into podosomes in aortic endothelial cells, a process controlled by Cdc42 and RhoA. *Molecular and cellular biology*, v. 23, n. 19, p. 6809–22, out. 2003.

MOSSER, D. M.; ROSENTHAL, L. A. *Leishmania*-macrophage interactions: multiple receptors, multiple ligands and diverse cellular responses. **Seminars in Cell Biology**, v. 4, n. 5, p. 315–22, out. 1993.

NARUMIYA, S.; TANJI, M.; ISHIZAKI, T. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. **Cancer and Metastasis Reviews**, v. 28, n. 1–2, p. 65–76, 22 jun. 2009.

NARUMIYA, S.; TANJI, M.; ISHIZAKI, T. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. **Cancer and Metastasis Reviews**, v. 28, n. 1–2, p. 65–76, 22 jun. 2009.

NADERER, T. et al. Intracellular Survival of *Leishmania major* Depends on Uptake and Degradation of Extracellular Matrix Glycosaminoglycans by Macrophages. *PLoS pathogens*, v. 11, n. 9, p. e1005136, set. 2015.

NANDAN, D.; REINER, N. E. Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania donovani*: selective inhibition of signaling through Janus kinases and Stat1. **Infection and Immunity**, v. 63, n. 11, p. 4495–500, nov. 1995.

NAVARRO-GARCIA, F. et al. EspC promotes epithelial cell detachment by enteropathogenic *Escherichia coli* via sequential cleavages of a cytoskeletal protein and then focal adhesion proteins. **Infection and Immunity**, v. 82, n. 6, p. 2255–65, jun. 2014.

NAYAL, A. et al. Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics. **The Journal of Cell Biology**, v. 173, n. 4, p. 587–9, 22 maio 2006.

NEWMAN, S. L.; MIKUS, L. K.; TUCCI, M. A. Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. Baltimore. **Journal of Immunology**, v. 146, n. 3, p. 967–74, 1 fev. 1991.

NOGALSKI, M. T. et al. Human cytomegalovirus-regulated paxillin in monocytes links cellular pathogenic motility to the process of viral entry. **Journal of Virology**, v. 85, n. 3, p. 1360–9, fev. 2011.

NOVAIS, F. O. et al. Human classical monocytes control the intracellular stage of *Leishmania braziliensis* by reactive oxygen species. **The Journal of Infectious Diseases**, v. 209, n. 8, p. 1288–96, 15 abr. 2014.

NOGALSKI, M. T. et al. Human cytomegalovirus-regulated paxillin in monocytes links cellular pathogenic motility to the process of viral entry. **Journal of Virology**, v. 85, n. 3, p. 1360–9, fev. 2011.

OHL, L. et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. **Immunity**, v. 21, n. 2, p. 279–88, ago. 2004.

OLMOS, S.; STUKES, S.; ERNST, J. D. Ectopic activation of *Mycobacterium tuberculosis*-specific CD4⁺ T cells in lungs of CCR7^{-/-} mice. **Journal of Immunology**, Baltimore, Md., v. 184, n. 2, p. 895–901, 15 jan. 2010.

ORGANIZAÇÃO PAN-AMERICANA DA SAÚDE. **Leishmanioses**: Informe epidemiológico das Américas. nº 11. 2022. <https://iris.paho.org/handle/10665.2/56832>.

ONOFRE, T. S.; RODRIGUES, J. P. F.; YOSHIDA, N. Depletion of Host Cell Focal Adhesion Kinase Increases the Susceptibility to Invasion by *Trypanosoma cruzi* Metacyclic Forms. **Frontiers in Cellular and Infection Microbiology**, v. 9, p. 231, 2019.

OWEN, R. M. et al. Bifunctional ligands that target cells displaying the alpha v beta3 integrin. **Chembiochem European Journal of Chemical Biology**, v. 8, n. 1, p. 68–82, 2 jan. 2007.

PANARO, M. A. et al. Nitric oxide production by Leishmania-infected macrophages and modulation by prostaglandin E2. **Clinical and Experimental Medicine**, v. 1, n. 3, p. 137–43, set. 2001.

PETERS, N. C. et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. New York. **Science**, v. 321, n. 5891, p. 970–4, 15 ago. 2008.

POINCLOUX, R. et al. Re-arrangements of podosome structures are observed when Hck is activated in myeloid cells. **European Journal of Cell Biology**, v. 85, n. 3–4, p. 327–32, abr. 2006.

PASSOS, S. et al. Intermediate monocytes contribute to pathologic immune response in Leishmania braziliensis infections. **The Journal of Infectious Diseases**, v. 211, n. 2, p. 274–82, 15 jan. 2015.

PINHEIRO, N. F. et al. Leishmania infection impairs beta 1-integrin function and chemokine receptor expression in mononuclear phagocytes. **Infection and immunity**, v. 74, n. 7, p. 3912–21, jul. 2006.

PASSOS, S. et al. Intermediate monocytes contribute to pathologic immune response in Leishmania braziliensis infections. **The Journal of Infectious Diseases**, v. 211, n. 2, p. 274–82, 15 jan. 2015.

PONTE-SUCRE, A.; HEISE, D.; MOLL, H. Leishmania major lipophosphoglycan modulates the phenotype and inhibits migration of murine Langerhans cells. **Immunology**, v. 104, n. 4, p. 462–7, dez. 2001.

PARSONS, J. T. Focal adhesion kinase: the first ten years. **Journal of Cell Science**, v. 116, n. Pt 8, p. 1409–16, 15 abr. 2003.

PARSONS, J. T.; HORWITZ, A. R.; SCHWARTZ, M. A. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nature reviews. **Molecular Cell Biology**, v. 11, n. 9, p. 633–43, set. 2010.

PATERSON, N.; LÄMMERMANN, T. Macrophage network dynamics depend on haptokinesis for optimal local surveillance. **eLife**, v. 11, 28 mar. 2022.

PERGOLA, C. et al. Modulation of actin dynamics as potential macrophage subtype-targeting anti-tumour strategy. **Scientific Reports**, v. 7, p. 41434, 30 jan. 2017.

POLLARD, T. D.; BORISY, G. G. Cellular motility driven by assembly and disassembly of actin filaments. **Cell**, v. 112, n. 4, p. 453–65, 21 fev. 2003a.

POLLARD, T. D.; BORISY, G. G. Cellular motility driven by assembly and disassembly of actin filaments. **Cell**, v. 112, n. 4, p. 453–65, 21 fev. 2003.

PONTI, A. et al. Two distinct actin networks drive the protrusion of migrating cells. New York, **Science**, v. 305, n. 5691, p. 1782–6, 17 set. 2004.

PUENTES, S. M. et al. Complement binding by two developmental stages of *Leishmania* major promastigotes varying in expression of a surface lipophosphoglycan. **The Journal of Experimental Medicine**, v. 167, n. 3, p. 887–902, 1 mar. 1988.

QUARANTA, M. G. et al. HIV-1 Nef triggers Vav-mediated signaling pathway leading to functional and morphological differentiation of dendritic cells. **The FASEB Journal**, v. 17, n. 14, p. 2025–2036, nov. 2003.

REMALEY, A. T. et al. Leishmanial phosphatase blocks neutrophil O-2 production. **The Journal of Biological Chemistry**, v. 259, n. 18, p. 11173–5, 25 set. 1984.

RIDLEY, A. J. et al. Cell migration: integrating signals from front to back. New York. **Science**, v. 302, n. 5651, p. 1704–9, 5 dez. 2003.

RITTER, U. et al. CD8 α - and Langerin-negative dendritic cells, but not Langerhans cells, act as principal antigen-presenting cells in leishmaniasis. **European Journal of Immunology**, v. 34, n. 6, p. 1542–1550, 18 jun. 2004.

RICART, B. G. et al. Dendritic cells distinguish individual chemokine signals through CCR7 and CXCR4. Baltimore. **Journal of Immunology**, v. 186, n. 1, p. 53–61, 1 jan. 2011.

RIOL-BLANCO, L. et al. The chemokine receptor CCR7 activates in dendritic cells two signaling modules that independently regulate chemotaxis and migratory speed. Baltimore. **Journal of Immunology**, v. 174, n. 7, p. 4070–80, 1 abr. 2005

RITTER, U.; MOLL, H. Monocyte chemotactic protein-1 stimulates the killing of *leishmania* major by human monocytes, acts synergistically with IFN- γ and is antagonized by IL-4. **European Journal of Immunology**, v. 30, n. 11, p. 3111–20, nov. 2000.

ROCHA, M. I. et al. *Leishmania infantum* Enhances Migration of Macrophages via a Phosphoinositide 3-Kinase γ -Dependent Pathway. **ACS Infectious Diseases**, v. 6, n. 7, p. 1643–1649, 10 jul. 2020.

RUHLAND, A.; LEAL, N.; KIMA, P. E. *Leishmania* promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. **Cellular Microbiology**, v. 9, n. 1, p. 84–96, jan. 2007.

ROMANO, A. et al. Divergent roles for Ly6C+CCR2+CX3CR1+ inflammatory monocytes during primary or secondary infection of the skin with the intra-phagosomal pathogen *Leishmania* major. **PLoS Pathogens**, v. 13, n. 6, p. e1006479, jun. 2017.

ROSENTHAL, L. A. et al. *Leishmania* major-human macrophage interactions: cooperation between Mac-1 (CD11b/CD18) and complement receptor type 1 (CD35) in promastigote adhesion. **Infection and Immunity**, v. 64, n. 6, p. 2206–15, jun. 1996.

ROTUREAU, B. et al. The flagellum-mitogen-activated protein kinase connection in Trypanosomatids: a key sensory role in parasite signalling and development? **Cellular Microbiology**, v. 11, n. 5, p. 710–8, maio 2009.

ROY, K. et al. Class II MHC/peptide interaction in *Leishmania donovani* infection: implications in vaccine design. Baltimore. **Journal of Immunology**, v. 192, n. 12, p. 5873–80, 15 jun. 2014.

SHARILI, A. S. et al. Nuclear actin modulates cell motility via transcriptional regulation of adhesive and cytoskeletal genes. **Scientific Reports**, v. 6, p. 33893, 21 set. 2016.

SACKS, D. L. Metacyclogenesis in *Leishmania* promastigotes. **Experimental parasitology**, v. 69, n. 1, p. 100–3, jul. 1989.

SACKS, D.; KAMHAWI, S. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. **Annual Review of Microbiology**, v. 55, p. 453–83, 2001.

SALGADO, V. R. et al. The microbiological signature of human cutaneous leishmaniasis lesions exhibits restricted bacterial diversity compared to healthy skin. **Memórias do Instituto Oswaldo Cruz**, v. 111, n. 4, p. 241–251, abr. 2016.

SANTOS, D. et al. IL-1 β Production by intermediate monocytes is associated with immunopathology in cutaneous leishmaniasis. **The Journal of Investigative Dermatology**, v. 138, n. 5, p. 1107–1115, maio 2018.

SAUNDERS, E. C. et al. *Leishmania mexicana* can utilize amino acids as major carbon sources in macrophages but not in animal models. **Molecular Microbiology**, v. 108, n. 2, p. 143–158, abr. 2018.

SCHAKS, M.; GIANNONE, G.; ROTTNER, K. Actin dynamics in cell migration. **Essays in Biochemistry**, v. 63, n. 5, p. 483–495, 31 out. 2019.

SCOTT, P.; NOVAIS, F. O. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. **Nature Reviews Immunology**, v. 16, n. 9, p. 581–92, set. 2016.

SERBINA, N. V et al. Monocyte-mediated defense against microbial pathogens. **Annual Review of Immunology**, v. 26, p. 421–52, 2008.

SABEH, F.; SHIMIZU-HIROTA, R.; WEISS, S. J. Protease-dependent versus -independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. **The Journal of Cell Biology**, v. 185, n. 1, p. 11–9, 6 abr. 2009.

SHARIF, O. et al. Macrophage Rewiring by Nutrient Associated PI3K Dependent Pathways. **Frontiers in Immunology**, v. 10, p. 2002, 2019.

SCHLAM, D. et al. Phosphoinositide 3-kinase enables phagocytosis of large particles by terminating actin assembly through Rac/Cdc42 GTPase-activating proteins. **Nature Communications**, v. 6, p. 8623, 14 out. 2015.

SEIF, F. et al. The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells. *Cell communication and signaling*: **CCS**, v. 15, n. 1, p. 23, 21 jun. 2017.

SKRUBER, K.; READ, T.-A.; VITRIOL, E. A. Reconsidering an active role for G-actin in cytoskeletal regulation. **Journal of Cell Science**, v. 131, n. 1, 10 jan. 2018.

STAFFORD, J. L.; NEUMANN, N. F.; BELOSEVIC, M. Macrophage-mediated innate host defense against protozoan parasites. **Critical Reviews in Microbiology**, v. 28, n. 3, p. 187–248, 2002.

STEVERDING, D. The history of leishmaniasis. **Parasites & Vectors**, v. 10, n. 1, p. 82, 15 dez. 2017.

STEIGERWALD, M.; MOLL, H. Leishmania major modulates chemokine and chemokine receptor expression by dendritic cells and affects their migratory capacity. **Infection and Immunity**, v. 73, n. 4, p. 2564–7, abr. 2005.

SUTER, D. M.; FORSCHER, P. Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance. **Journal of Neurobiology**, v. 44, n. 2, p. 97–113, ago. 2000.

SUZUE, K. et al. Critical role of dendritic cells in determining the Th1/Th2 balance upon Leishmania major infection. **International immunology**, v. 20, n. 3, p. 337–43, mar. 2008.

TAVARES, N. M. et al. **Understanding the mechanisms controlling leishmania amazonensis infection in vitro**: the role of LTB 4 derived from human neutrophils. 2014.

TAKEDA, Y. et al. ROCK2 Regulates Monocyte Migration and Cell to Cell Adhesion in Vascular Endothelial Cells. **International Journal Of Molecular Sciences**, v. 20, n. 6, 16 mar. 2019.

TEN HOEVE, A. L. et al. The Toxoplasma effector GRA28 promotes parasite dissemination by inducing dendritic cell-like migratory properties in infected macrophages. **Cell Host & Microbe**, v. 30, n. 11, p. 1570- 1588.e7, 9 nov. 2022.

TORRES-GUERRERO, E. et al. Leishmaniasis: a review. **F1000Research**, v. 6, p. 750, 2017.

TIBÚRCIO, R. et al. Molecular Aspects of Dendritic Cell Activation in Leishmaniasis: An Immunobiological View. **Frontiers in immunology**, v. 10, p. 227, 2019.

TREPAT, X.; CHEN, Z.; JACOBSON, K. Cell migration. *Comprehensive Physiology*, v. 2, n. 4, p. 2369–92, out. 2012.

TURCO, S. J.; DESCOTEAUX, A. The lipophosphoglycan of Leishmania parasites. **Annual Review of Microbiology**, v. 46, p. 65–94, 1992.

TURNER, C. E. Paxillin and focal adhesion signalling. **Nature Cell Biology**, v. 2, n. 12, p. E231-6, dez. 2000.

VAN GOETHEM, E. et al. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. **Journal of Immunology**, v. 184, n. 2, p. 1049–61, 15 jan. 2010.

VAN ZANDBERGEN, G. et al. Leishmania promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10

production by neutrophil granulocytes. **Infection and Immunity**, v. 70, n. 8, p. 4177–84, ago. 2002.

VAN ZANDBERGEN, G. et al. Leishmania promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. **Infection and Immunity**, v. 70, n. 8, p. 4177–84, ago. 2002.

VAN ZANDBERGEN, G. et al. Leishmania promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. **Infection and immunity**, v. 70, n. 8, p. 4177–84, ago. 2002.

VÉROLLET, C. et al. HIV-1 reprograms the migration of macrophages. **Blood**, v. 125, n. 10, p. 1611–22, 5 mar. 2015.

VARGAS-INCHAUSTEGUI, D. A. et al. CXCL10 production by human monocytes in response to Leishmania braziliensis infection. **Infection and Immunity**, v. 78, n. 1, p. 301–8, jan. 2010.

VILLALONGA, P.; RIDLEY, A. J. Rho GTPases and cell cycle control. Chur, Switzerland. **Growth factors**, v. 24, n. 3, p. 159–64, set. 2006.

VERAS, P. S.; DE CHASTELLIER, C.; RABINOVITCH, M. Transfer of zymosan (yeast cell walls) to the parasitophorous vacuoles of macrophages infected with Leishmania amazonensis. **The Journal of Experimental Medicine**, v. 176, n. 3, p. 639–46, 1 set. 1992.

VEILLAT, V. et al. Podosomes: Multipurpose organelles? **The International Journal of Biochemistry & Cell Biology**, v. 65, p. 52–60, ago. 2015.

VÉROLLET, C. et al. Extracellular proteolysis in macrophage migration: losing grip for a breakthrough. **European journal of immunology**, v. 41, n. 10, p. 2805–13, out. 2011.

VON STEBUT, E. et al. Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity. **The Journal of Experimental Medicine**, v. 188, n. 8, p. 1547–52, 19 out. 1998.

WEBB, D. J. et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nature cell biology*, v. 6, n. 2, p. 154–61, fev. 2004.

WELF, E. S.; HAUGH, J. M. Signaling pathways that control cell migration: models and analysis. Wiley interdisciplinary reviews. **Systems Biology and Medicine**, v. 3, n. 2, p. 231–40, 2011.

WIESNER, C. et al. Podosomes in space: Macrophage migration and matrix degradation in 2D and 3D settings. **Cell Adhesion and Migration**, v. 8, n. 3, p. 179–191, 2014.

WIESNER, C. et al. Podosomes in space: macrophage migration and matrix degradation in 2D and 3D settings. **Cell Adhesion & Migration**, v. 8, n. 3, p. 179–91, 2014.

WEI, X. Q. et al. Altered immune responses in mice lacking inducible nitric oxide synthase. **Nature**, v. 375, n. 6530, p. 408–11, 1 jun. 1995.

WORLD HEALTH ORGANIZATION. Leishmaniasis. **World Health Organization technical report series**. 2023. <https://www.who.int/news-room/fact-sheets/detail/leishmaniasis>

WYNN, T. A.; CHAWLA, A.; POLLARD, J. W. Macrophage biology in development, homeostasis and disease. **Nature**, v. 496, n. 7446, p. 445–55, 25 abr. 2013.

YAMAGUCHI, H.; PIXLEY, F.; CONDEELIS, J. Invadopodia and podosomes in tumor invasion. **European Journal of Cell Biology**, v. 85, n. 3–4, p. 213–8, abr. 2006.

YANG, N. et al. Blockage of JAK/STAT signalling attenuates renal ischaemia-reperfusion injury in rat. Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association. **European Renal Association**, v. 23, n. 1, p. 91–100, jan. 2008.

YAMADA, K. M. et al. Extracellular matrix dynamics in cell migration, invasion and tissue morphogenesis. **International Journal of Experimental Pathology**, v. 100, n. 3, p. 144–152, 10 jun. 2019.

YANO, H. et al. Reduction of Real-Time Imaging of M1 Macrophage Chemotaxis toward Damaged Muscle Cells is PI3K-Dependent. **Antioxidants**, Basel, Switzerland, v. 7, n. 10, 8 out. 2018.

YOUNG, J.; KIMA, P. E. The Leishmania Parasitophorous Vacuole Membrane at the Parasite-Host Interface. **The Yale Journal of Biology and Medicine**, v. 92, n. 3, p. 511–521, set. 2019.

XIN, L.; LI, K.; SOONG, L. Down-regulation of dendritic cell signaling pathways by Leishmania amazonensis amastigotes. **Molecular Immunology**, v. 45, n. 12, p. 3371–82, jul. 2008.

XIN, P. et al. The role of JAK/STAT signaling pathway and its inhibitors in diseases. **International Immunopharmacology**, v. 80, p. 106210, mar. 2020.

ZAIDEL-BAR, R. et al. A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. **Journal of Cell Science**, v. 120, n. Pt 1, p. 137–48, 1 jan. 2007.

ZAIDEL-BAR, R.; GEIGER, B. The switchable integrin adhesome. **Journal of Cell Science**, v. 123, n. Pt 9, p. 1385–8, 1 maio 2010.

ANEXOS

Manuscrito I - *Leishmania* infection alters macrophage and dendritic cell migration in a three-dimensional environment (Publicado).

Manuscrito II - Cell migration and cell adhesion assays to investigate *Leishmania*-host cell interactions (Publicado).

Manuscrito III – In vitro differentiation of human dendritic cells and their markers in *Leishmania* infection (Publicado)

Manuscrito IV – Investigating the phagocytosis of *Leishmania* using confocal microscopy (Publicado)

Anexo A - Manuscrito 1: *Leishmania* infection alters macrophage and dendritic cell migration in a three-dimensional environment

Abstract: Leishmaniasis results in a wide spectrum of clinical manifestations, ranging from skin lesions at the site of infection to disseminated lesions in internal organs, such as the spleen and liver. While the ability of *Leishmania*-infected host cells to migrate may be important to lesion distribution and parasite dissemination, the underlying mechanisms and the accompanying role of host cells remain poorly understood. Previously published work has shown that *Leishmania* infection inhibits macrophage migration in a 2-dimensional (2D) environment by altering actin dynamics and impairing the expression of proteins involved in plasma membrane-extracellular matrix interactions. Although it was shown that *L. infantum* induces the 2D migration of dendritic cells, *in vivo* cell migration primarily occurs in 3-dimensional (3D) environments. The present study aimed to investigate the migration of macrophages and dendritic cells infected by *Leishmania* using a 3-dimensional environment, as well as shed light on the mechanisms involved in this process. Following the infection of murine bone marrow-derived macrophages (BMDM), human macrophages and human dendritic cells by *L. amazonensis*, *L. braziliensis*, or *L. infantum*, cellular migration, the formation of adhesion complexes and actin polymerization were evaluated. Our results indicate that *Leishmania* infection inhibited 3D migration in both BMDM and human macrophages. Reduced expression of proteins involved in adhesion complex formation and alterations in actin dynamics were also observed in *Leishmania*-infected macrophages. By contrast, increased human dendritic cell migration in a 3D environment was found to be associated with enhanced adhesion complex formation and increased actin dynamics. Taken together, our results show that *Leishmania* infection inhibits macrophage 3D migration, while enhancing dendritic 3D migration by altering actin dynamics and the expression of proteins involved in plasma membrane extracellular matrix interactions, suggesting a potential association between dendritic cells and disease visceralization.

Keywords: Macrophage; Dendritic cell; migration; *Leishmania* sp.; dissemination, 3D

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EDITED BY
Claudia Tanja Mierke,
Leipzig University, Germany

REVIEWED BY
Despina Smirlis,
Pasteur Hellenic Institute, Greece
Luca Galluzzi,
University of Urbino Carlo Bo, Italy

*CORRESPONDENCE
Juliana P. B. de Menezes,
✉ juliana.fullam@fiocruz.br

[†]These authors have contributed equally
to this work and share first authorship

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Leishmania infection alters macrophage and dendritic cell migration in a three-dimensional environment

Yasmin Luz^{1†}, Amanda Rebouças^{1†}, Carla Polyana O. S. Bernardes¹, Erik A. Rossi², Taíse S. Machado¹, Bruno S. F. Souza^{2,3,4}, Claudia Ida Brodszyn¹, Patricia S. T. Veras¹, Washington L. C. dos Santos⁵ and Juliana P. B. de Menezes^{1*}

¹Laboratory of Host–Parasite Interaction and Epidemiology, Gonçalo Moniz Institute, Salvador, Brazil, ²Center for Biotechnology and Cell Therapy, São Rafael Hospital, Salvador, Brazil, ³D'Or Institute for Research and Education, Salvador, Brazil, ⁴Laboratory of Tissue Engineering and Immunopharmacology, Gonçalo Moniz Institute, Salvador, Brazil, ⁵Laboratory of Structural and Molecular Pathology, Gonçalo Moniz Institute, Salvador, Brazil

Background: Leishmaniasis results in a wide spectrum of clinical manifestations, ranging from skin lesions at the site of infection to disseminated lesions in internal organs, such as the spleen and liver. While the ability of *Leishmania*-infected host cells to migrate may be important to lesion distribution and parasite dissemination, the underlying mechanisms and the accompanying role of host cells remain poorly understood. Previously published work has shown that *Leishmania* infection inhibits macrophage migration in a 2-dimensional (2D) environment by altering actin dynamics and impairing the expression of proteins involved in plasma membrane-extracellular matrix interactions. Although it was shown that *L. infantum* induces the 2D migration of dendritic cells, *in vivo* cell migration primarily occurs in 3-dimensional (3D) environments. The present study aimed to investigate the migration of macrophages and dendritic cells infected by *Leishmania* using a 3-dimensional environment, as well as shed light on the mechanisms involved in this process.

Methods: Following the infection of murine bone marrow-derived macrophages (BMDM), human macrophages and human dendritic cells by *L. amazonensis*, *L. braziliensis*, or *L. infantum*, cellular migration, the formation of adhesion complexes and actin polymerization were evaluated.

Results: Our results indicate that *Leishmania* infection inhibited 3D migration in both BMDM and human macrophages. Reduced expression of proteins involved in adhesion complex formation and alterations in actin dynamics were also observed in *Leishmania*-infected macrophages. By contrast, increased human dendritic cell migration in a 3D environment was found to be associated with enhanced adhesion complex formation and increased actin dynamics.

Conclusion: Taken together, our results show that *Leishmania* infection inhibits macrophage 3D migration, while enhancing dendritic 3D migration by altering actin dynamics and the expression of proteins involved in plasma membrane extracellular matrix interactions, suggesting a potential association between dendritic cells and disease visceralization.

KEYWORDS

macrophages, dendritic cells, 3D migration, *Leishmania*, dissemination

1 Introduction

Leishmaniasis is a neglected tropical disease endemic in 98 countries. Every year, it is estimated that between 700,000 and 1 million new cases occur, leading to approximately 30,000 deaths (Alvar et al., 2012). Cutaneous leishmaniasis (CL) in the New World, typically caused by *L. amazonensis* or *L. braziliensis*, results in ulcerative skin lesions. By contrast, visceral leishmaniasis (VL), which is caused by *L. infantum*, can affect the internal organs, such as the liver, spleen, and bone marrow, and may lead to more severe clinical manifestations, even death, if left untreated (Ashford, 2000; Desjeux, 2004; Kaye and Scott, 2011; Podinovskaia and Descoteaux, 2015).

Infection in the vertebrate host occurs through the inoculation of metacyclic promastigotes in the host's skin by female sandflies during bloodfeeding (Veras and Bezerra de Menezes, 2016). Once inside the mammalian host, *Leishmania* parasites can survive and multiply inside acidic parasitophorous vacuoles within macrophages (Afrin et al., 2019). Subsequently, parasites may either persist at the local inoculation site or become disseminated to other tissues. The detection of *Leishmania*-infected mononuclear phagocytes in the bloodstream suggests that migration by infected macrophages may potentially contribute to the spread of disease (Liarte et al., 2001). Additionally, previous studies have shown that dendritic cells carrying *Leishmania* parasites can migrate, consequently transporting parasite antigens to lymph nodes (Ato et al., 2002). However, the mechanisms involved in the dissemination of *Leishmania* to different host tissues, as well as the homing and persistence of infected cells *in vivo*, remain poorly understood.

The cell migration carried out by a wide variety of cells plays an essential role in many physiological processes, such as leukocyte trafficking and immune response (Ridley and Hall, 1992; Ridley et al., 2003; Friedl and Wolf, 2010; Gardel et al., 2010). Integrated molecular events mediated primarily by actin filaments in the cytoplasm, in addition to the formation of adhesion complexes, are the main aspects involved in cell migration (Sheetz et al., 1999; Wiesner et al., 2014). Initially, a process mediated by integrins (Ridley et al., 2003), actin-binding proteins (talin, vinculin, and α -actinin) and adapter proteins (Paxillin, FAK, and Src) results in the formation of adhesion complexes (Turner, 2000; Zamir and Geiger, 2001), allowing the plasma membrane to anchor itself to the substrate. FAK protein functions as a focal adhesion regulatory factor during cell movement (Zaidel-Bar et al., 2004), leading to the phosphorylation of members of the Src family of tyrosine kinases, Paxillin, and p130Cas, thus creating binding sites for the formation of adhesion complexes (Toutant et al., 2002; Playford and Schaller, 2004; Mitra and Schlaepfer, 2006; López-Colomé et al., 2017). Additionally, proteins from the Rho family of small GTPases (Rac1, Cdc42, and RhoA) (Ridley and Hall, 1992; DeMali and Burridge, 2003; Huveneers and Danen, 2009; Huttenlocher and Horwitz, 2011; López-Colomé et al., 2017) are responsible for the regulation of actin dynamics within cells. Rac-1 controls the extension of lamellipodia in the anterior region of the cell, while

the extension of filopodia is regulated by Cdc-42 (DeMali and Burridge, 2003; Huveneers and Danen, 2009; López-Colomé et al., 2017). RhoA controls retraction of the rear end of the cell, in addition to the formation of stress fibers throughout the cell structure (Ridley and Hall, 1992).

Previous studies have evidenced that *Leishmania* infection reduces macrophage adhesion by altering the function of molecules involved in adhesion (Carvalho et al., 2004; Pinheiro et al., 2006). In addition, decreased macrophage migration has been observed following *Leishmania* infection in a two-dimensional environment (Blewett et al., 1971; Bray et al., 1983). A report published by de Menezes et al. (2017) also demonstrated a reduction in macrophage migration due to *L. amazonensis* infection in a two-dimensional environment, which was further associated with diminished adhesion complex formation and a greater frequency of polymerization and actin filament turnover (de Menezes et al., 2017). In contrast, a recent study published by our group identified increased human dendritic cell migration following infection with *L. infantum*, but not *L. braziliensis* and *L. amazonensis*, the latter two being localized forms of CL. Additionally, we found increased expression of proteins involved in adhesion complex formation and actin polymerization, as well as higher CCR7 expression in the human dendritic cells infected with all three isolates (Rebouças et al., 2021).

While most studies investigating cell migration are performed in a two-dimensional environment, the study of cellular infiltration into tissue requires the use of a three-dimensional environment. Within a 3D environment, leukocytes can migrate via amoeboid or mesenchymal modes (Friedl and Wolf, 2010; Huttenlocher and Horwitz, 2011; Wiesner et al., 2014). Each mode of migration is determined by the mechanical, structural, and biochemical properties of a given extracellular matrix. Amoeboid migration is dependent on the Rho/ROCK pathway, which is responsible for the regulation of actomyosin. In denser environments, the cell assumes a mode of mesenchymal migration dependent on the secretion of proteases and the formation of podosomes (Wiesner et al., 2014). In an attempt to better understand the mechanisms involved in the spread of *Leishmania* within the host, the present study aimed to evaluate the migration of *Leishmania* spp.-infected macrophages and dendritic cells through the use of a 3D environment.

2 Materials and methods

2.1 Ethics statement

The isogenic mice (*Mus musculus*) of the Balb/c strain used in the present study were provided by the animal care facility of the Gonçalo Moniz Institute (IGM-FIOCRUZ/BA), following approval by the Institutional Animal Experimentation Review Board (CEUA) under protocol number 014/2019.

Whole blood monocytes were isolated from healthy blood donors following approval by the Institutional Review Board of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (CEP/IGM-FIOCRUZ) (protocol No. 5.138.962).

2.2 *Leishmania* culturing

Promastigotes of *L. amazonensis* (MHOM/Br88/BA125), *L. braziliensis* (MHOM/BR/01/BA788), and *L. infantum* (MCAN/BR/89/BA262) were maintained for up to six successive passages in Schneider's Insect Medium (Sigma-Aldrich, Saint Louis, MO, United States), or modified essential minimum medium hemoflagellate (MEM Medium), both supplemented with 50 µg/mL gentamicin (Gibco, Waltham, MA, United States) and 10%–20% (v/v) FBS (Gibco, Waltham, MA, United States). Promastigotes were grown in an incubator at 24°C and monitored daily by counting in a Neubauer chamber. Upon reaching stationary phase, promastigotes were then used in the experiments.

2.3 Mouse bone marrow-derived macrophage cultures

Bone marrow-derived macrophages (BMDM) obtained from Balb/c mice were cultured for 7 days in RPMI medium containing 10% FBS (Gibco, Waltham, MA, United States), 30% L929 cell supernatant containing macrophage colony-stimulating factor (M-CSF), 200 mM glutamine (Sigma-Aldrich, Saint Louis, MO, United States) and 10 µg/mL ciprofloxacin (Isopharma, Eusébio, CE, BR). Mature, adherent BMDM were detached using 0.05% EDTA/PBS (Gibco, Waltham, MA, United States). Cell viability was determined via trypan blue exclusion.

2.4 Human monocyte-derived macrophage and dendritic cell cultures

Human monocyte-derived macrophages were obtained from freshly isolated healthy peripheral blood monocytes using Ficoll-Histopaque density gradient separation (Sigma-Aldrich, Saint Louis, MO, United States). Peripheral blood mononuclear cells (PBMCs) were washed three times and then plated at 2×10^6 in 500 mL of Roswell Park Memorial Institute (RPMI) supplemented with 25 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid] (HEPES), 2 g/L sodium bicarbonate, 2 mM glutamine, 20 g/mL ciprofloxacin and 10% inactivated FBS (complete RPMI medium) for 7 days at 37°C under 5% CO₂, on 24-well plates to allow monocytes to differentiate into macrophages. For human dendritic cells, peripheral blood monocytes were collected by positive selection using a magnetic cell sorter to isolate CD14⁺ subtypes. Positive cells were then plated in RPMI with granulocyte-macrophage colony-stimulating (GM-CSF) [50 ng/mL] and interleukin-4 (IL-4) [100 UI/mL] for 7 days to allow dendritic cell differentiation.

2.5 Preparation of 3D collagen matrix

Cells were embedded in a collagen type I matrix derived from rat tail collagen (Gibco), as described (Van Goethem et al., 2010). Cells (5×10^4) were added to a solution containing collagen I [2 mg/mL], PBS 1X, and NaOH [2.6 mM] before polymerization at 37°C for 24 h.

To investigate the surface and shape of the 3D collagen matrix with embedded macrophages, scanning electron microscopy (SEM) was employed, following a previously published protocol (Cruz et al., 2021).

2.6 Bone marrow-derived macrophages, human macrophages and dendritic cell infection

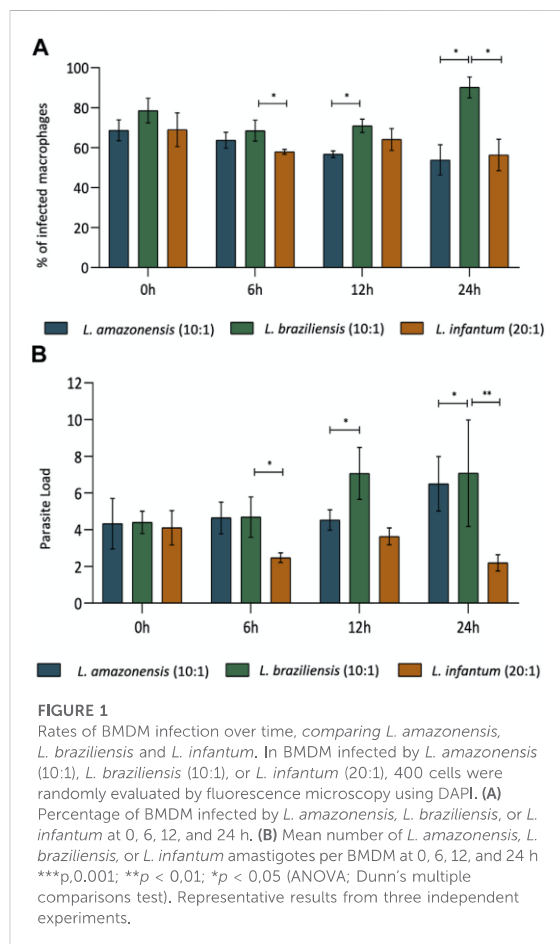
BMDM and human macrophages were plated on 24-well plates for immunofluorescence (2×10^5) and migration assays (5×10^4) 24 h prior to experimentation. *L. braziliensis* (10:1) or *L. infantum* (20:1) promastigotes were added to both macrophage cultures and incubated for 24 h at 37°C, while cultures with added *L. amazonensis* (10:1) promastigotes were incubated for 6 h at 37°C. BMDM and human macrophages were then washed in PBS to remove any non-internalized parasites, and then re-incubated for 24 or 48 h for cell migration assays, or 24 h for immunofluorescence. For dendritic cells, *L. amazonensis* (10:1), *L. braziliensis* (10:1) and *L. infantum* (20:1) promastigotes were added to dendritic cell cultures and incubated for 4 h at 37°C. Cells were then washed in PBS to remove non-internalized parasites, then re-incubated in RPMI for 6, 12, 24, and 48 h (for BMDM and human macrophages) and for 6, 24, and 48 h (for dendritic cells) at 37°C.

2.7 Parasite burden

BMDM, human macrophages and dendritic cells were infected as described earlier. Subsequently, the cells were washed with PBS to remove non-internalized parasites and then either fixed (0 h) or re-incubated in RPMI at 37°C for 6, 12, 24, and 48 h (for BMDM and human macrophages) and for 6, 24, and 48 h (for dendritic cells). After the specified incubation period, coverslips were fixed using 4% paraformaldehyde, washed, and mounted using the ProLong Gold Antifade kit with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). The number of intracellular parasites was determined by counting the total macrophages and the total intracellular parasites per microscopic field using a fluorescence microscope. At least 400 host cells, in triplicate, were analyzed for each time point.

2.8 Migration assay

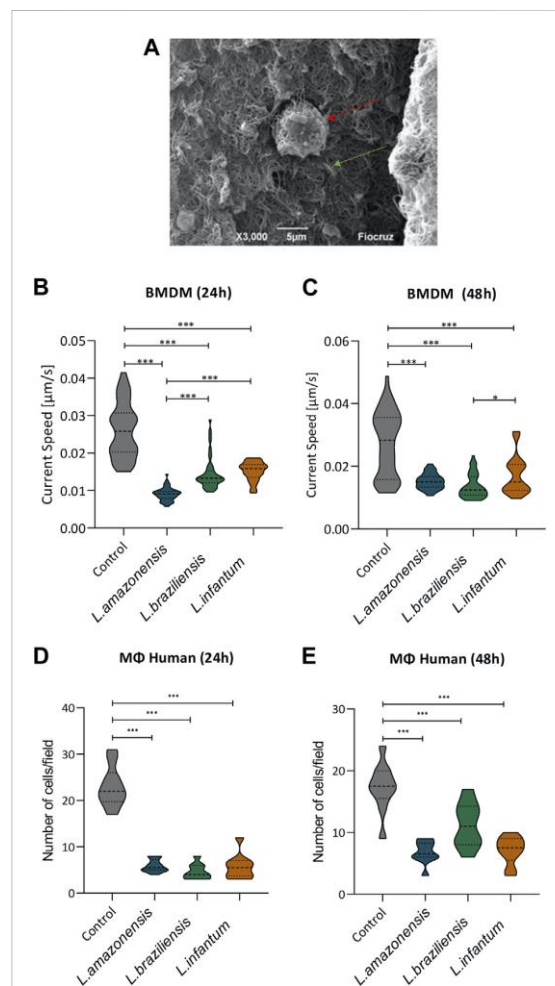
BMDM, human macrophages, and dendritic cells were infected as previously described and then washed to remove non-internalized parasites. Following the infection period, BMDM (5×10^4 /well) were incorporated into a collagen I matrix (Gibco) and plated on 96-well plates for matrix polymerization. The matrices were then incubated for 24 or 48 h at 37°C. Time-lapse evaluations were then performed using an Operetta High-Content Analysis System (PerkinElmer, Waltham, MA, United States) at this timepoints, with image acquisition performed every 2 min for a period of 1 h to analyze cell migration rates. For human macrophages and human dendritic cells (5×10^4), a chemotaxis assay was performed in Boyden chambers containing polycarbonate membranes (24-well, 5 µm pores, Corning® Transwell® polycarbonate membrane cell culture inserts). Each 3D collagen I matrix containing human macrophages or dendritic cells



was incubated for 24 or 48 h. After this incubation period, the matrices were then placed within a Transwell insert, and allowed to migrate for an additional 4 h toward RPMI containing 100 ng/mL MCP1 (Monocyte chemoattractant protein-1) for macrophages and 300 ng/mL CCL3 (C-C motif ligand 3) for dendritic cells. Next, the matrix was removed, and membranes were washed with PBS, fixed with 4% paraformaldehyde for 15 min, washed again with PBS and incubated with 10 mg/mL DAPI for nuclear staining. The upper side of the filter was scraped to remove any residual non-migrating cells. Cells in 10 random fields from each membrane were counted using a fluorescence microscope.

2.9 Immunofluorescence assay

Following migration, 3D collagen I matrices containing BMDM, human macrophages or human dendritic cells infected or not with *L. amazonensis*, *L. braziliensis*, or *L. infantum*, were fixed with 4% (v/v) paraformaldehyde for 15 min and washed five times in PBS. The matrices were then quenched for 20 min with 15 mM NH_4Cl and washed five times with PBS, incubated in a pre-blocking solution (2% (v/v) bovine



serum albumin (BSA) in 1% glycine) for 1 h, washed five times with PBS, permeabilized with 0.15% (v/v) Triton X-100/PBS (Sigma-Aldrich, Saint Louis, MO, United States) for 15 min, incubated in a blocking solution (1% BSA in PBS) for 45 min, and then incubated for 1 h with 1:500 rabbit anti-pFAK (0.5 $\mu\text{g/mL}$) (Invitrogen, catalog number RC222574), 1:100 rabbit anti-pPaxillin (0.1 $\mu\text{g/mL}$) (Invitrogen, catalog number QF221230), 1:200 rabbit anti-Rac1 (2.5 ng/mL) (BD Biosciences,

catalog number 610650), 1:100 mouse anti-Cdc42 (1 ng/mL) (Invitrogen, catalog number PA1-092X), 1:100 rabbit anti-RhoA (1 µg/mL) (Invitrogen, catalog number 0SR00266W), mouse anti-Geosolin (1 µg/mL) (Sigma, catalog number G4896) or 1:1200 phalloidin (1 µg/mL) (Invitrogen, catalog number A12379). All antibodies were diluted in 1% (v/v) PBS + 1% (v/v) BSA. Subsequently, anti-mouse Alexa fluor 594 (0.02 µg/µL) (Molecular Probes, catalog number A1011) or anti-rabbit Alexa fluor 488 (0.02 µg/µL) (Molecular Probes, catalog number A32732) was added and reincubated for 1 h. Cells were mounted using Prolong Gold antifade reagent with DAPI for nuclear staining (Invitrogen). Images were acquired using a Leica TCS SP8 confocal microscope with a 63x/1.4 objective. The Z-stack tool was employed with 0.3 µm intervals. Fiji software was used for digital fluorescence quantification. Maximal projection images were utilized, and the quantification considered the cell area. Several parameters, including Area, Min and Max gray value, integrated density, and mean gray value, were quantified for individual cells. To calculate the corrected total cellular fluorescence (CTCF), the following formula was applied: CTCF = integrated density - (selected cell area x mean fluorescence of background readings).

2.10 Statistical analysis

All experiments were repeated three times. After verifying data normality using D'Agostino, Kolmogorov-Smirnov and Shapiro-Wilk tests, the use of parametric or non-parametric tests for data analysis was determined. Parametric data were submitted to ANOVA testing (unpaired), while the Kruskal-Wallis test (unpaired) was used for non-parametric data. All analysis was performed using GraphPad Prism software. Results were considered significant when $p < 0.05$.

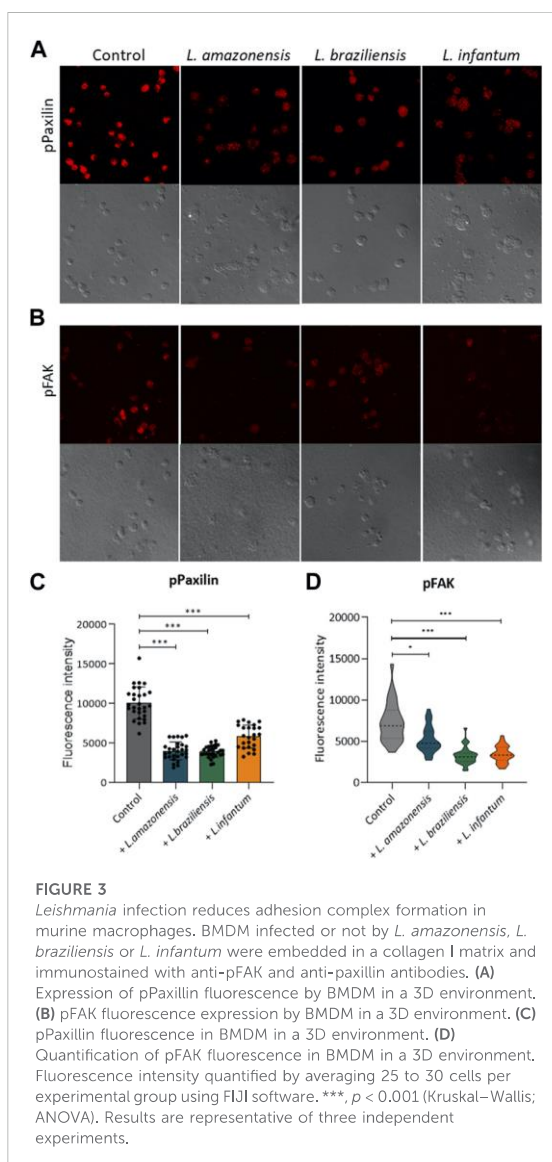
3 Results

3.1 Rates of macrophage infection over time, comparing *L. amazonensis*, *L. braziliensis* and *L. infantum*

The rates of BMDM infection were compared, considering *L. amazonensis* (10:1), *L. braziliensis* (10:1), and *L. infantum* (20:1), in an attempt to standardize the percentage of macrophages infection between species. Our results indicate that similar rates of infection (70%–80%) were achieved between macrophages infected by *L. amazonensis* (10:1), *L. braziliensis* (10:1), or *L. infantum* (20:1) at 0 h (Figure 1A). Accordingly, the number of *Leishmania* per macrophage was similar, with an average of 4 *Leishmania* per cell (Figure 1B). Similar results were found for human macrophages (Supplementary Figure S1).

3.2 *Leishmania* infection reduces macrophage migration in a 3D environment

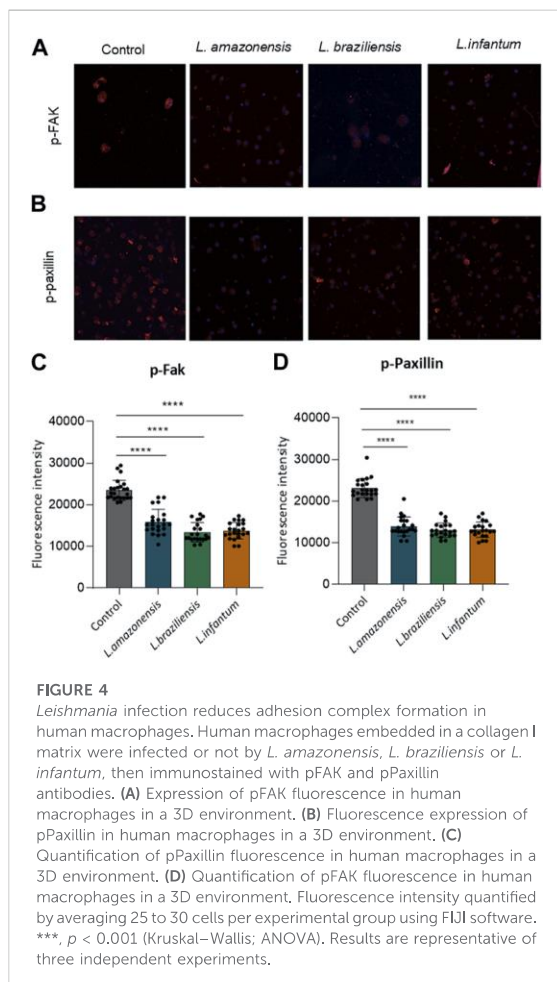
To assess macrophage migration in a 3D environment, we incorporated BMDM into a collagen I matrix (Figure 2A) and allowed cells to migrate for 24 or 48 h. Our results revealed significantly reduced BMDM migration following infection with all *Leishmania* spp. Evaluated compared to uninfected controls at



both 24 (Figure 2B) and 48 h (Figure 2C). To further confirm these findings, we then evaluated human macrophages in migration assays using a transwell system with MCP-1 chemoattractant. These results again indicated a significant reduction in macrophage migration following infection with all three *Leishmania* spp. at both 24 (Figure 2D) and 48 h post-infection (Figure 2E).

3.3 *Leishmania* infection reduces adhesion complex formation within macrophages in a 3D environment

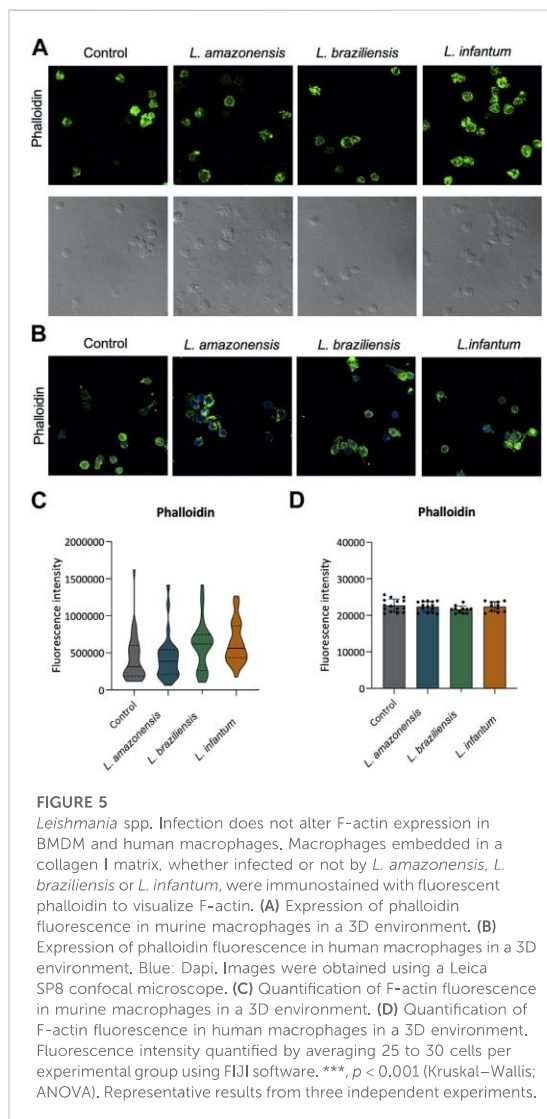
To investigate the mechanisms underlying migration, we analyzed adhesion complex formation using phosphorylated FAK



and paxillin fluorescence expression in murine and human macrophages infected with *Leishmania* in a 3D environment. Our results revealed significant reductions in both phosphorylated FAK and paxillin expression in murine (Figure 3) and human macrophages (Figure 4) infected with all *Leishmania* spp. Evaluated.

3.4 *Leishmania* infection alters actin dynamics in macrophages in a 3D environment

To investigate the role of actin cytoskeleton dynamics with respect to macrophage migration, we evaluated F-actin expression, as well as the expression of Rac1, Cdc42, RhoA, and gelsolin, in a 3D environment. Our findings indicated no differences in F-actin expression in BMDM (Figures 5A,C) or human macrophages (Figures 5B,D) infected with any of the three *Leishmania* spp. Evaluated. By contrast, murine macrophages exhibited increased Rac-1 expression after infection with *L. braziliensis* and



L. infantum (Figures 6A,E), while *L. amazonensis* infection was found to increase Cdc42 expression (Figures 6B,F). Additionally, infection with *L. amazonensis* or *L. braziliensis* led to reduced RhoA and gelsolin expression in BMDM (Figures 6C,D,G,H). Similarly, in human macrophages, infection with *L. amazonensis*, *L. braziliensis* or *L. infantum* resulted in reduced Rac1, Cdc42, and RhoA expression in a 3D environment compared to uninfected controls (Figure 7).

3.5 Rates of dendritic cell infection over time, comparing *L. amazonensis*, *L. braziliensis* and *L. infantum*

Human dendritic cell infection rates were compared following infection by *L. amazonensis* (10:1), *L. braziliensis* (10:1) or *L.*

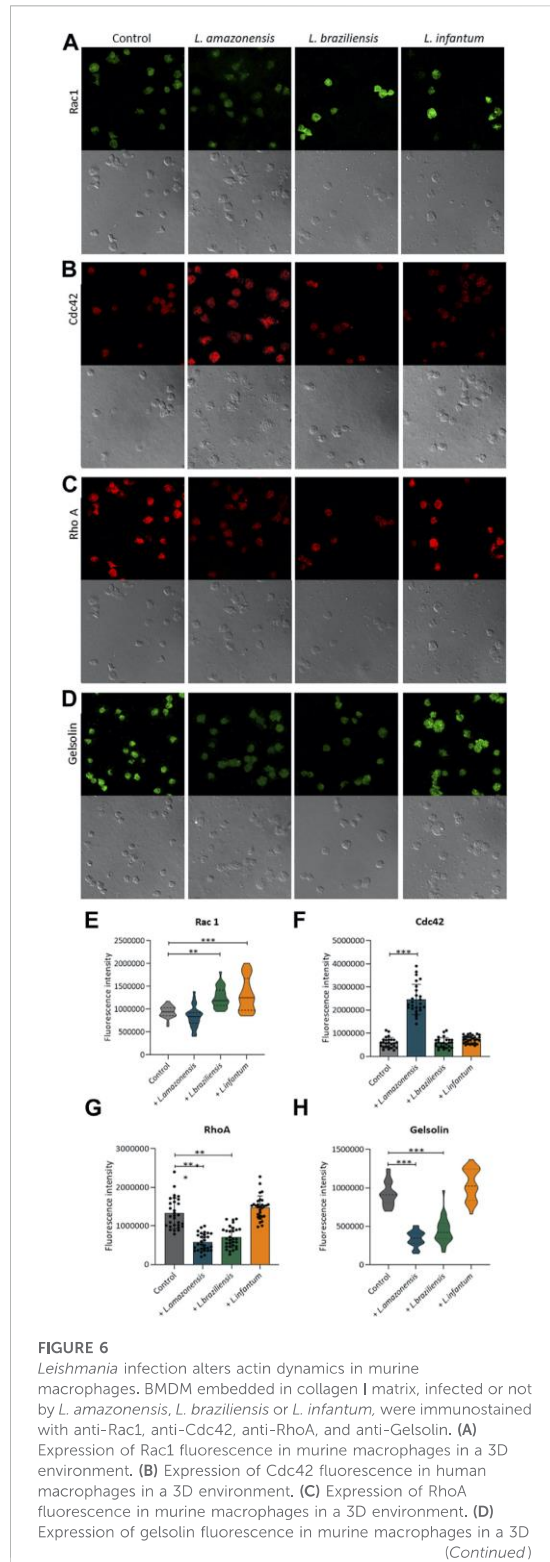


FIGURE 6 (Continued)

environment. Images obtained using a Leica SP8 confocal microscope. (E) Quantification of Rac1 (E), Cdc42 (F), RhoA (G) and Gelsolin (H) fluorescence in murine macrophages in a 3D environment. Fluorescence intensity quantified by averaging 25 to 30 cells per experimental group using FIJI software. ***, $p < 0.001$ (Kruskal–Wallis; ANOVA). Representative results from three independent experiments.

infantum (20:1). Our results indicate that similar rates of infection (60%) were achieved between human dendritic cells infected by *L. amazonensis* (10:1), *L. braziliensis* (10:1) or *L. infantum* (20:1) at 0 h (Figure 8A). Furthermore, the number of *Leishmania* per cell was similar, on average 2 parasites per cell (Figure 8B).

3.6 *Leishmania infantum* induces dendritic cell migration in a 3D environment

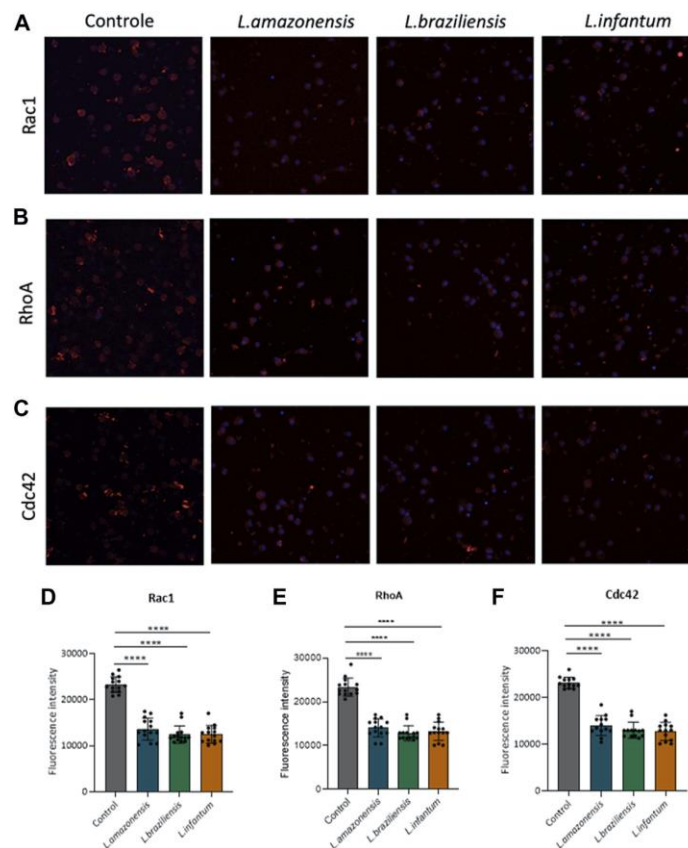
To assess dendritic cell migration in a 3D environment, these cells were incorporated into a collagen I matrix and allowed to migrate for 24 or 48 h in migration assays using a transwell system. Our results revealed significantly enhanced dendritic cell migration following infection with *L. infantum* compared to *L. amazonensis* and *L. braziliensis*-infected cells, as well as uninfected controls, at both 24 (Figure 9A) and 48 h (Figure 9B). Additionally, infection with *L. braziliensis* led to reduced dendritic cell migration at both 24 (Figure 9A) and 48 h (Figure 9B).

3.7 *Leishmania infantum* induces adhesion complex formation within dendritic cells in a 3D environment

To investigate the mechanisms underlying dendritic cell migration, we evaluated adhesion complex formation in human dendritic cells infected with *Leishmania infantum* in a 3D environment by analyzing the expression of FAK and paxillin. Our fluorescence data analysis revealed significant increases in both FAK and paxillin in *L. infantum*-infected dendritic cells compared to uninfected controls. Additionally, a significant decrease in the expression of these proteins was noted in dendritic cells infected by either *L. braziliensis* or *L. amazonensis* compared to controls (Figures 10A–D).

3.8 *Leishmania infantum* alters actin dynamics in dendritic cells in a 3D environment

We assessed the expression of F-actin, Rac1, Cdc42, RhoA, and gelsolin in a 3D environment to study the participation of actin cytoskeleton dynamics in dendritic cell migration. Our results showed no differences in F-actin expression between human dendritic cells infected with either of the three *Leishmania* spp. Evaluated (Figures 11A,B). However, infection with *L.*

**FIGURE 7**

Leishmania infection in human macrophages reduces the expression of proteins involved in actin dynamics. Human macrophages embedded in a collagen I matrix, infected or not by *L. amazonensis*, *L. braziliensis* or *L. infantum*, were immunostained with anti-Rac1, anti-Cdc42, and anti-RhoA antibodies. (A) Expression of Rac1 fluorescence in human macrophages in a 3D environment. (B) Fluorescence expression of Cdc42 in human macrophages in a 3D environment. (C) Expression of RhoA fluorescence in human macrophages in a 3D environment. Images obtained using a Leica SP8 confocal microscope. (D) Significantly reduced Rac1 fluorescence intensity in infected human macrophages compared to controls in a 3D environment. (E) Significantly reduced Cdc42 fluorescence intensity in infected human macrophages compared to controls in a 3D environment. (F) Significantly reduced RhoA fluorescence intensity in infected human macrophages compared to controls in a 3D environment. Fluorescence intensity quantified by averaging 25 to 30 cells per experimental group using FIJI software. Blue: Dapi. ***, $p < 0.001$ (Kruskal–Wallis; ANOVA). Representative results from three independent experiments.

infantum, but not with *L. amazonensis* or *L. braziliensis*, led to increased Rac1, Cdc42, and RhoA expression compared to uninfected controls in a 3D environment (Figures 11C–F).

4 Discussion

The dissemination of *Leishmania* within the host subsequent to infection can result in a diverse range of clinical manifestations, varying from spontaneously healing skin ulcers to widespread lesions or disfiguring mucosal lesions; in extreme cases, visceral leishmaniasis can be lethal. The present study demonstrated that infection by three different species of *Leishmania* resulted in reduced macrophage migration in a 3D environment. In contrast, our results

demonstrated that *L. infantum* infection resulted in increased dendritic cell migration, while *L. braziliensis* infection led to reduced dendritic cell migration. However, infection with *L. amazonensis* did not significantly affect cell migration compared to uninfected controls. Our findings, which show that *L. infantum* infection can potentially enhance the migratory capacity of dendritic cells from the site of infection, are consistent with the visceral form of the disease associated with this parasite species. Moreover, the observed changes were associated with marked differences in adhesion complex formation and actin dynamics in infected macrophages and dendritic cells.

Previous studies have shown that *Leishmania* infection inhibits the macrophage's ability to adhere to different substrates, such as collagen, laminin, and fibronectin (Carvalho et al., 2004; Pinheiro et al., 2006). It has also been demonstrated that infection with *L.*

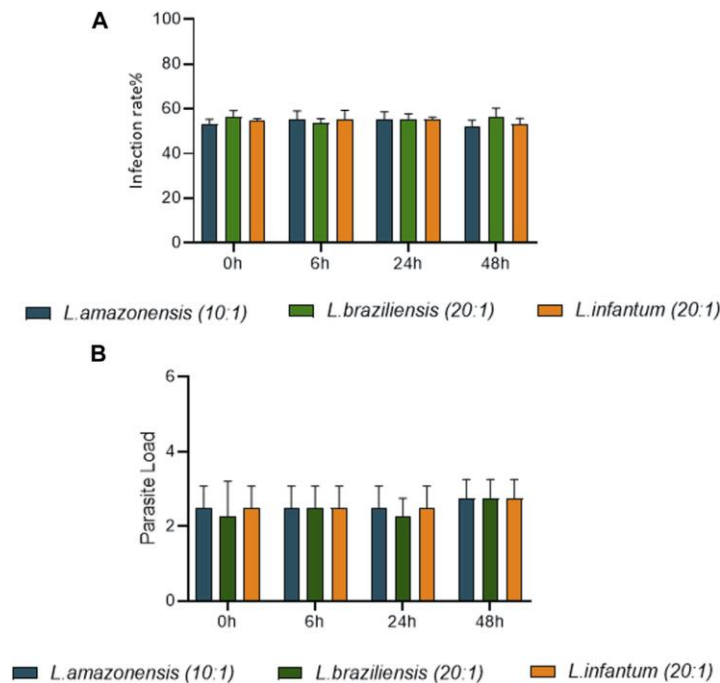


FIGURE 8

Rates of human dendritic cell infection over time, comparing *L. amazonensis*, *L. braziliensis* and *L. infantum*. Human dendritic cells infected by *L. amazonensis* (10:1), *L. braziliensis* (10:1), or *L. infantum* (20:1), 400 cells were randomly evaluated by fluorescence microscopy using DAPI. (A) Percentage of human dendritic cells infected by *L. amazonensis*, *L. braziliensis*, or *L. infantum* at 0, 6, 24 and 48 h. (B) Mean number of *L. amazonensis*, *L. braziliensis*, or *L. infantum* amastigotes per dendritic cell at 0, 6, 24 and 48 h (ANOVA). Representative results from three independent experiments.

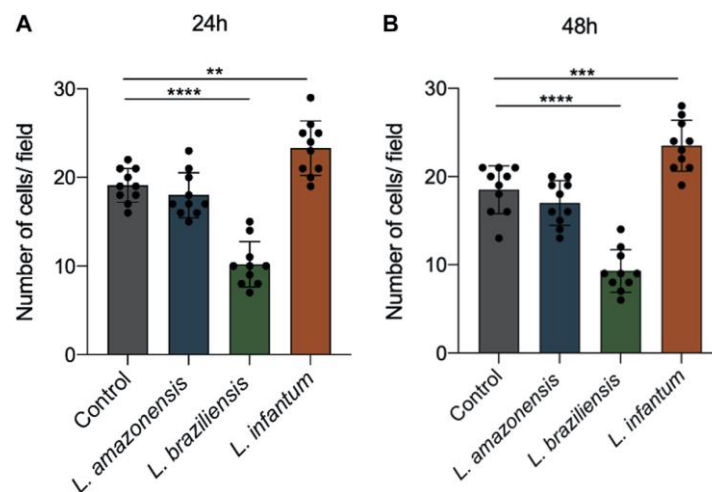
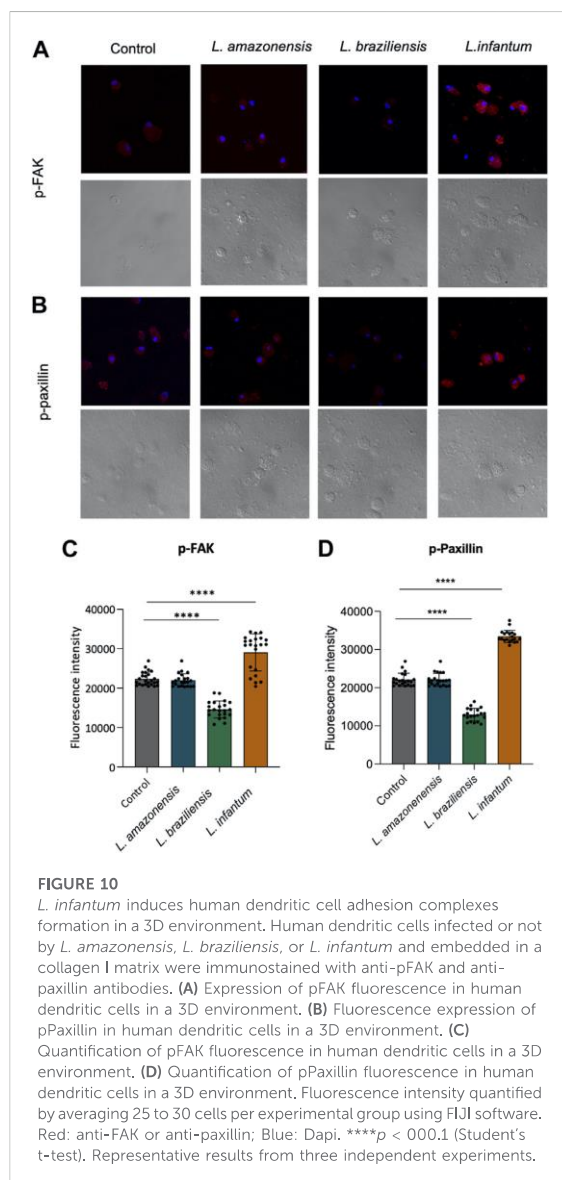


FIGURE 9

L. infantum increases human dendritic cell 3D migration in a 3D environment. Cells (infected or not) were incorporated to a collagen matrix. At 24 and 48 h after infection, the cells were placed in a Boyden chamber and allowed to migrate towards CCL3. Migrated cells were randomly counted with nuclear staining with DAPI through fluorescence microscopy. (A) Number of human dendritic cells that migrated after 24 h, (B) Number of human dendritic cells that migrated after 48 h **** $p < 0.0001$ (Student's t-test). Representative results from three independent experiments.

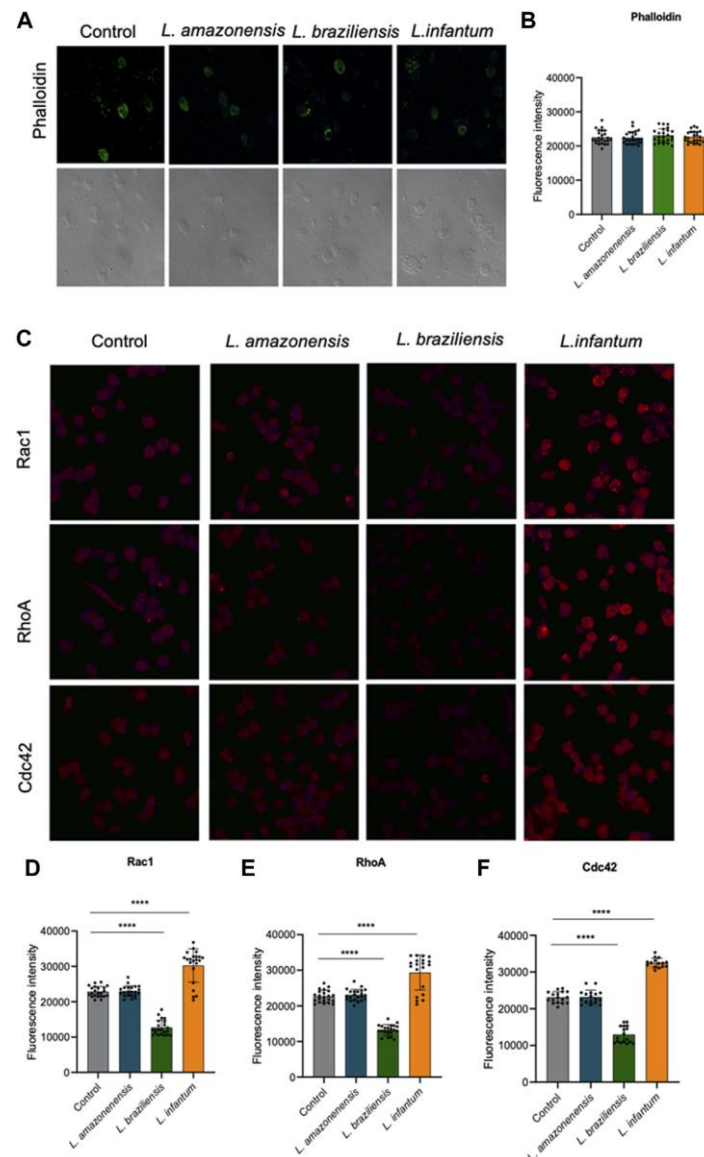


amazonensis leads to a reduction in macrophage migration in a two-dimensional environment (Blewett et al., 1971; Bray et al., 1983; de Menezes et al., 2017). In contrast, studies using *L. infantum* have shown an increase in BMDM migration following infection, via a PI3K-dependent pathway (Rocha et al., 2020). Additionally, studies have demonstrated that, following *Leishmania* infection, dendritic cells are able to migrate to draining lymph nodes, enabling antigen transportation (León et al., 2007; Lai et al., 2008). Furthermore, a recent work published by our group showed that *L. infantum*, but not *L. amazonensis* or *L. braziliensis*, the latter two being localized forms of CL, induces dendritic cell migration in a two-dimensional environment (Rebouças et al., 2021). Alterations in the migration of infected dendritic cells may significantly impact host immune

response to *Leishmania*. This was highlighted by a recent study reporting that *L. amazonensis* infection inhibited the migration of dendritic cells from the site of infection to draining lymph nodes (Hermida et al., 2014). Although numerous reports have investigated leukocyte migration in two-dimensional environments, the study of migration using a 3D matrix has been relatively limited. Given that 3D migration occurs within tissues, *in vitro* models that mimic three-dimensional extracellular environments can provide valuable insight into phagocyte migration. To our knowledge, this is the first study attempting to examine the migration of both *Leishmania*-infected macrophages and dendritic cells in an three-dimensional environment *in vitro* (Ley et al., 2007; Vérollet et al., 2011).

Our results evidenced reduced 3D migration in both murine and human *Leishmania*-infected macrophages, in association with lower FAK and paxillin expression, which stands in agreement with the data obtained from experimentation in a 2D environment by de Menezes et al. (2017). We also identified increased 3D migration of human dendritic cells associated with the expression of the same adhesion complex proteins, which is consistent with data previously published by our group in a 2D environment (Rebouças et al., 2021). The phosphorylation of FAK plays a fundamental role in cell adhesion, since its activation leads to the phosphorylation and the consequent activation of paxillin, a key protein in cellular adhesion and migration (Schaller and Parsons, 1995). Studies using embryonic mouse fibroblasts mutated for FAK, to which paxillin cannot bind, have demonstrated the loss of localization and phosphorylation of FAK in focal adhesion, resulting in altered migration dynamics and inhibited cell adhesion (Deramaudt et al., 2014). In a study involving *T. cruzi*, it was observed that this pathogen is capable of increasing FAK activation, thus increasing the incidence of cardiomyopathy as well as suggesting an increase in disease dissemination (Melo et al., 2019).

The actin cytoskeleton is crucial for cell migration, with the Rho GTPase family of proteins playing a key role in the regulation of this dynamic process (Ridley and Hall, 1992). The inhibition of Rho GTPases was shown to provoke deficiencies in cell migration (Sander et al., 1999). Herein we observed increased Rac1 expression in BMDMs infected with *L. braziliensis* and *L. infantum*; by contrast, infection by *L. amazonensis* resulted in increased Cdc42 expression. It is possible that the enhanced expression of these GTPases in macrophages may be associated with increased actin dynamics at the leading edge of murine macrophages. However, we also identified reduced expression of Rac1 and Cdc42 following infection by all three *Leishmania* spp. In human macrophages, which suggests a reduction in actin dynamics at the leading edge of these cells. The alterations in actin dynamics observed in murine macrophages infected by *Leishmania* in a three-dimensional environment corroborate previously published data from experiments conducted in a two-dimensional environment by de Menezes et al. (2017). Additionally, it has been demonstrated that Cdc42 and Rac1 promote actin polymerization through the Arp2/3 complex by activating their effector proteins, N-WASP and WAVE-2, respectively (Spiering and Hodgson, 2011). Inhibition of Cdc42 has also been associated with reduced recruitment and chemotaxis of macrophages during invasion and metastasis (Zhang et al., 2021). Furthermore, a previous study has reported that macrophages deficient in Rac1 lose their ability to invade dense matrix environments such as Matrigel (Wheeler et al., 2006). Moreover, the differences observed in GTPases between murine and human macrophages could be related to the fact that murine

**FIGURE 11**

L. infantum increases the expression of proteins involved in human dendritic cells 3D actin polymerization, Human dendritic embedded in a collagen I matrix, whether infected or not by *L. amazonensis*, *L. braziliensis* or *L. infantum*, were immunostained with fluorescent phalloidin to visualize F-actin and anti-Rac1, anti-Cdc42 and anti-RhoA. (A) Expression of phalloidin fluorescence in human dendritic cells in a 3D environment. (B) Quantification of F-actin fluorescence in human dendritic cells in a 3D environment. (C) Expression of Rac1, RhoA and Cdc42 fluorescence in human dendritic cells in a 3D environment. Quantification of Rac1 (D), RhoA (E) and Cdc42 (F) fluorescence in human dendritic cells in a 3D environment. Fluorescence intensity quantified by averaging 25 to 30 cells per experimental group using Fiji software. Green: phalloidin, Red: anti-Rac1, anti-RhoA, anti-Cdc42; Blue: Dapi. *** $p < 0.001$ (Student's t-test). Representative results from three independent experiments.

macrophages were stimulated with MCSF during their differentiation (Pixley, 2012). Further studies are required to investigate the state of activation of these GTPases in both cell types. In addition, the present results further identified increased Rac1 and Cdc42 expression in human dendritic cells infected with *L. infantum*, but not *L. amazonensis* or *L.*

braziliensis, which suggests increased F-actin turnover in these cells. This finding is consistent with our previous results conducted in a two-dimensional environment (Rebouças et al., 2021), leading us to speculate that *L. infantum* may facilitate the migration of infected dendritic cells to the draining lymph nodes. Of note, previously

published work was shown to induce dendritic cell migration upon *Toxoplasma gondii* infection, thereby potentiating parasite dissemination (Lambert et al., 2006). In addition, RhoA, a member of the Rho GTPase family, plays a crucial role in regulating cell migration by mediating the formation of stress fibers in the cell body and posterior region (Ridley and Hall, 1992). Previous studies have also shown that RhoA activity is associated with reduced cell protrusion (Nobes and Hall, 1999; Rottner et al., 1999). Additionally, ROCK activation by RhoA can inhibit actin polymerization (Nobes and Hall, 1999). Furthermore, decreased levels of active GTP-bound RhoA in macrophages, caused by increased cholesterol in the cell membrane, hinder cell migration (Nagao et al., 2007). Our findings indicate reduced RhoA expression in murine macrophages infected with *L. amazonensis* and *L. braziliensis*, as well as in human macrophages infected with all three *Leishmania* spp. Evaluated. This finding provides evidence of the potential suppression of RhoA activity by these *Leishmania* species, which may result in decreased formation of stress fibers and cell protrusion, potentially impairing cell migration. In contrast, we also demonstrated that infection with *L. infantum* led to increased RhoA expression in human dendritic cells, which could favor cell migration. Our study further investigated the expression of gelsolin, an actin-binding protein known to facilitate F-actin breakage (McGough et al., 2003; Silacci et al., 2004; Khaitlina et al., 2013). Previous studies have reported that *Yersinia*'s YopO protein can phosphorylate gelsolin, leading to actin binding and separation, which ultimately results in the alteration of actin dynamics in phagocytic cells (Singaravelu et al., 2017). As our study demonstrated a reduction in gelsolin expression in murine macrophages infected with *L. amazonensis* and *L. braziliensis*, this could lead to decreased F-actin breakage in these cells, which may also be responsible for the reduced migration capacity observed in BMDM.

Taken together, the present study showed that *Leishmania* spp. infection leads to decreased cell migration in both murine and human macrophages, indicating the critical role of these cells in controlling parasites at the lesion site. The present findings also provide evidence of increased human dendritic cell migration following *L. infantum* infection, suggesting a potential association between this cell type and disease visceralization. Elucidating the mechanisms underlying the migration of *Leishmania*-infected cells and their potential role in lesion development and parasite dissemination is crucial to understanding the pathogenesis of leishmaniasis. The findings presented herein serve to expand the base of knowledge on host cell migration during the course of *Leishmania* infection, as well as the consequent dissemination of the parasites within the vertebrate host.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Gonçalo Moniz Institute, Oswaldo Cruz Foundation (CEP/FIOCRUZ/BA). Written

informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Institutional Animal Experimentation Review Board (CEUA—FIOCRUZ/BA).

Author contributions

Conceptualization: JdM; methodology: JdM, YL, AR, CB, ER, and TM; formal analysis: YL, AR, CB., ER, BS, and JdM; investigation: YL, AR, CB, and JdM; resources: JdM, WdS, PV, CB, and BS; writing original draft preparation: YL, AR, CB, and JdM; writing review and editing: BS, CB, PV, WdS, and JdM; supervision: JdM; project administration: JdM, funding acquisition: JdM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2023.1206049/full#supplementary-material>

References

- Afrin, F., Khan, I., and Hemeg, H. A. (2019). Leishmania-host interactions-an epigenetic paradigm. *Front. Immunol.* 10, 492. doi:10.3389/fimmu.2019.00492
- Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7 (5), e35671. doi:10.1371/journal.pone.0035671
- Ashford, R. (2000). The leishmaniases as emerging and reemerging zoonoses. *Int. J. Parasitol.* 30 (12–13), 1269–1281. doi:10.1016/s0020-7519(00)00136-3
- Ato, M., Stäger, S., Engwerda, C. R., and Kaye, P. M. (2002). Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat. Immunol.* 3 (12), 1185–1191. doi:10.1038/ni861
- Blewett, T. M., Kadivar, D. M., and Soulsby, E. J. (1971). Cutaneous leishmaniasis in the Guinea pig. Delayed-type hypersensitivity, lymphocyte stimulation, and inhibition of macrophage migration. *Am. J. Trop. Med. Hyg.* 20 (4), 546–551. doi:10.4269/ajtmh.1971.20.546
- Bray, R. S., Heikal, B., Kaye, P. M., and Bray, M. A. (1983). The effect of parasitization by *Leishmania mexicana mexicana* on macrophage function *in vitro*. *Acta Trop.* 40 (1), 29–38.
- Carvalho, D. G. F., Barbosa, A., D'El-Rei Hermida, M., Clarenco, J., Pinheiro, N. F., Veras, P. S. T., et al. (2004). The modelling of mononuclear phagocyte-connective tissue adhesion *in vitro*: Application to disclose a specific inhibitory effect of *Leishmania* infection. *Exp. Parasitol.* 107 (3–4), 189–199. doi:10.1016/j.exppara.2004.06.003
- Cruz, K. P., Patricio, B. F. C., Pires, V. C., Amorim, M. F., Pinho, A. G. S. F., Quadros, H. C., et al. (2021). Development and characterization of PLGA nanoparticles containing 17-DMAG, an Hsp90 inhibitor. *Front. Chem.* 9, 644827. doi:10.3389/fchem.2021.644827
- de Menezes, J. P. B., Koushik, A., Das, S., Guven, C., Siegel, A., Laranjeira-Silva, M. F., et al. (2017). *Leishmania* infection inhibits macrophage motility by altering F-actin dynamics and the expression of adhesion complex proteins. *Cell Microbiol.* 19 (3), e12668. doi:10.1111/cmi.12668
- DeMali, K. A., and Burridge, K. (2003). Coupling membrane protrusion and cell adhesion. *J. Cell Sci.* 116, 2389–2397. doi:10.1242/jcs.00605
- Deramaut, T. B., Dujardin, D., Noulet, F., Martin, S., Vauchelles, R., Takeda, K., et al. (2014). Altering FAK-paxillin interactions reduces adhesion, migration and invasion processes. *PLoS One* 9 (3), e92059. doi:10.1371/journal.pone.0092059
- Desjeux, P. (2004). Leishmaniasis: Current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 27 (5), 305–318. doi:10.1016/j.cimid.2004.03.004
- Friedl, P., and Wolf, K. (2010). Plasticity of cell migration: A multiscale tuning model. *J. Cell Biol.* 188, 11–19. doi:10.1083/jcb.200909003
- Gardel, M. L., Schneider, I. C., Aratyn-Schaus, Y., and Waterman, C. M. (2010). Mechanical integration of actin and adhesion dynamics in cell migration. *Annu. Rev. Cell Dev. Biol.* 26, 315–333. doi:10.1146/annurev.cellbio.011209.122036
- Hermida, M. D. R., Doria, P. G., Taguchi, A. M. P., Mengel, J. O., and dos-Santos, W. L. C. (2014). *Leishmania amazonensis* infection impairs dendritic cell migration from the inflammatory site to the draining lymph node. *BMC Infect. Dis.* 14 (1), 450. doi:10.1186/1471-2334-14-450
- Huttenlocher, A., and Horwitz, A. R. (2011). Integrins in cell migration. *Cold Spring Harb. Perspect. Biol.* 3 (9), 0050744–a5116. doi:10.1101/cshperspect.a005074
- Huveneers, S., and Danen, E. H. J. (2009). Adhesion signaling - crosstalk between integrins, Src and rho. *J. Cell Sci.* 122, 1059–1069. doi:10.1242/jcs.039446
- Kaye, P., and Scott, P. (2011). Leishmaniasis: Complexity at the host-pathogen interface. *Nat. Rev. Microbiol.* 9 (8), 604–615. doi:10.1038/nrmicro2608
- Khaitlina, S., Fitz, H., and Hinssen, H. (2013). The interaction of gelsolin with tropomyosin modulates actin dynamics. *FEBS J.* 280 (18), 4600–4611. doi:10.1111/febs.12431
- Lai, G. N., Hsu, A., Mandell, M. A., Roediger, B., Hoeller, C., Mrass, P., et al. (2008). Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS Pathog.* 4 (11), e1000222. doi:10.1371/journal.ppat.1000222
- Lambert, H., Hitziger, N., Dellacasa, I., Svensson, M., and Barragan, A. (2006). Induction of dendritic cell migration upon *Toxoplasma gondii* infection potentiates parasite dissemination. *Cell Microbiol.* 8 (10), 1611–1623. doi:10.1111/j.1462-5822.2006.00735.x
- León, B., López-Bravo, M., and Ardavin, C. (2007). Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against *Leishmania*. *Immunity* 26 (4), 519–531. doi:10.1016/j.immuni.2007.01.017
- Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007). Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–689. doi:10.1038/nri2156
- Liarte, D. B., Mendonça, I. L., Luz, F. C., Abreu, E. A., Mello, G. W., Farias, T. J., et al. (2001). QBC for the diagnosis of human and canine American visceral leishmaniasis: Preliminary data. *Rev. Soc. Bras. Med. Trop.* 34 (6), 577–581. doi:10.1590/s0037-86822001000600013
- López-Colomé, A. M., Lee-Rivera, I., Benavides-Hidalgo, R., and Paxillin, L. E. (2017). Paxillin: A crossroad in pathological cell migration. *J. Hematol. Oncol.* 10, 50. doi:10.1186/s13045-017-0418-y
- McGough, A. M., Staiger, C. J., Min, J. K., and Simonetti, K. D. (2003). The gelsolin family of actin regulatory proteins: Modular structures, versatile functions. *FEBS Lett.* 552, 75–81. doi:10.1016/s0014-5793(03)00932-3
- Melo, T. G., Adesse, D., Meirelles, M. de N., and Pereira, M. C. S. (2019). *Trypanosoma cruzi* down-regulates mechanosensitive proteins in cardiomyocytes. *Mem. Inst. Oswaldo Cruz* 114, e180593. doi:10.1590/0074-02760180593
- Mitra, S. K., and Schlaepfer, D. D. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr. Opin. Cell Biol.* 18, 516–523. doi:10.1016/j.ceb.2006.08.011
- Nagao, T., Qin, C., Grosheva, I., Maxfield, F. R., and Pierini, L. M. (2007). Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. *Arterioscler. Thromb. Vasc. Biol.* 27 (7), 1596–1602. doi:10.1161/ATVBAHA.107.145086
- Nobes, C. D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol.* 144 (6), 1235–1244. doi:10.1083/jcb.144.6.1235
- Pinheiro, N. F., Hermida, M. D. R., Macedo, M. P., Mengel, J., Bafica, A., and Dos-Santos, W. L. C. (2006). *Leishmania* infection impairs β 1-integrin function and chemokine receptor expression in mononuclear phagocytes. *Infect. Immun.* 74 (7), 3912–3921. doi:10.1128/IAI.02103-05
- Pixley, F. J. (2012). Macrophage migration and its regulation by CSF-1. *Int. J. Cell Biol.* 2012, 501962. doi:10.1155/2012/501962
- Playford, M. P., and Schaller, M. D. (2004). The interplay between Src and integrins in normal and tumor biology. *Oncogene* 23, 7928–7946. doi:10.1038/sj.onc.1208080
- Podinovskaia, M., and Descoteaux, A. (2015). *Leishmania* and the macrophage: A multifaceted interaction. *Future Microbiol.* 10 (1), 111–129. doi:10.2217/fmb.14.103
- Rebouças, A., Silva, T. S., Medina, L. S., Paredes, B. D., Aragão, L. S., Souza, B. S. F., et al. (2021). *Leishmania*-induced dendritic cell migration and its potential contribution to parasite dissemination. *Microorganisms* 9 (6), 1268. doi:10.3390/microorganisms9061268
- Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70 (3), 389–399. doi:10.1016/0092-8674(92)90163-7
- Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., et al. (2003). Cell migration: Integrating signals from front to back. *Science* 302, 1704–1709. doi:10.1126/science.1092053
- Rocha, M. I., Dias, F., Resende, M., Sousa, M., Duarte, M., Tomás, A. M., et al. (2020). *Leishmania infantum* enhances migration of macrophages via a phosphoinositide 3-kinase γ -dependent pathway. *ACS Infect. Dis.* 6 (7), 1643–1649. doi:10.1021/acsinfectdis.0c00080
- Rottner, K., Hall, A., and Small, J. V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* 9 (12), 640–648. doi:10.1016/s0960-9822(99)80286-3
- Sander, E. E., Ten Klooster, J. P., Van Delft, S., Van Der Kammen, R. A., and Collard, J. G. (1999). Rac downregulates Rho activity: Reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J. Cell Biol.* 147 (5), 1009–1022. doi:10.1083/jcb.147.5.1009
- Schaller, M. D., and Parsons, J. T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol. Cell Biol.* 15 (5), 2635–2645. doi:10.1128/MCB.15.5.2635
- Sheetz, M. P., Felsenfeld, D., Galbraith, C. G., and Choquet, D. (1999). Cell migration as a five-step cycle. *Biochem. Soc. Symp.* 65, 233–243.
- Silacci, P., Mazzolai, L., Gauci, C., Stergiopoulos, N., Yin, H. L., and Hayoz, D. (2004). Gelsolin superfamily proteins: Key regulators of cellular functions. *Cell. Mol. Life Sci.* 61, 2614–2623. doi:10.1007/s00018-004-4225-6
- Singaravelu, P., Lee, W. L., Wee, S., Ghoshdastider, U., Ding, K., Gunaratne, J., et al. (2017). Yersinia effector protein (YopO)-mediated phosphorylation of host gelsolin causes calcium-independent activation leading to disruption of actin dynamics. *J. Biol. Chem.* 292 (19), 8092–8100. doi:10.1074/jbc.M116.757971
- Spiering, D., and Hodgson, L. (2011). Dynamics of the rho-family small GTPases in actin regulation and motility. *Cell Adhesion Migr.* 5, 170–180. doi:10.4161/cam.5.2.14403
- Toutant, M., Costa, A., Studler, J.-M., Kadaré, G., Carnaud, M., and Girault, J.-A. (2002). Alternative splicing controls the mechanisms of FAK autophosphorylation. *Mol. Cell Biol.* 22 (22), 7731–7743. doi:10.1128/MCB.22.22.7731-7743.2002
- Turner, C. E. (2000). Paxillin and focal adhesion signalling. *Nat. Cell Biol.* 2, E231–E236. doi:10.1038/35046659
- Van Goethem, E., Poincloux, R., Gauffre, F., Maridonneau-Parini, I., and Le Cabec, V. (2010). Matrix architecture dictates three-dimensional migration modes of human

- macrophages: Differential involvement of proteases and podosome-like structures. *J. Immunol.* 184 (2), 1049–1061. doi:10.4049/jimmunol.0902223
- Veras, P., and Bezerra de Menezes, J. (2016). Using proteomics to understand how *Leishmania* parasites survive inside the host and establish infection. *Int. J. Mol. Sci.* 17 (8), 1270. doi:10.3390/ijms17081270
- Vérollet, C., Charrière, G. M., Labrousse, A., Cougoule, C., Le Cabec, V., and Maridonneau-Parini, I. (2011). Extracellular proteolysis in macrophage migration: Losing grip for a breakthrough. *Eur. J. Immunol.* 41 (10), 2805–2813. doi:10.1002/eji.201141538
- Wheeler, A. P., Wells, C. M., Smith, S. D., Vega, F. M., Henderson, R. B., Tybulewicz, V. L., et al. (2006). Rac1 and Rac2 regulate macrophage morphology but are not essential for migration. *J. Cell Sci.* 119 (13), 2749–2757. doi:10.1242/jcs.03024
- Wiesner, C., Le-Cabec, V., El Azzouzi, K., Maridonneau-Parini, I., and Linder, S. (2014). Podosomes in space: Macrophage migration and matrix degradation in 2D and 3D settings. *Cell Adhesion Migr.* 8, 179–191. doi:10.4161/cam.28116
- Zaidel-Bar, R., Cohen, M., Addadi, L., and Geiger, B. (2004). Hierarchical assembly of cell-matrix adhesion complexes. *Biochem. Soc. Trans.* 32, 416–420. doi:10.1042/BST0320416
- Zamir, E., and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *J. Cell Sci.* 114 (20), 3583–3590. doi:10.1242/jcs.114.20.3583
- Zhang, B., Zhang, J., Xia, L., Luo, J., Zhang, L., Xu, Y., et al. (2021). Inhibition of CDC42 reduces macrophage recruitment and suppresses lung tumorigenesis *in vivo*. *J. Recept Signal Transduct. Res.* 41 (5), 504–510. doi:10.1080/10799893.2020.1828916

Anexo B - Manuscrito 2 : Cell migration and cell adhesion assays to investigate *Leishmania*-host cell interaction

Abstract: *Leishmania* is an intracellular protozoan parasite that causes a broad spectrum of clinical manifestations, ranging from self-resolving localized cutaneous lesions to a highly fatal visceral form of the disease. An estimated 12 million people worldwide are currently infected, and another 350 million face risk of infection. It is known that host cells infected by *Leishmania* parasites, such as macrophages or dendritic cells, can migrate to different host tissues, yet how migration contributes to parasite dissemination and homing remains poorly understood. Therefore, assessing these parasites' ability to modulate host cell response, adhesion, and migration will shed light on mechanisms involved in disease dissemination and visceralization. Cellular migration is a complex process in which cells undergo polarization and protrusion, allowing them to migrate. This process, regulated by actin and tubulin-based microtubule dynamics, involves different factors, including the modulation of cellular adhesion to the substrate. Cellular adhesion and migration processes have been investigated using several models. Here, we describe a method to characterize the migratory aspects of host cells during *Leishmania* infection. This detailed protocol presents the differentiation and infection of dendritic cells, the analysis of host cell motility and migration, and the formation of adhesion complexes and actin dynamics. This in vitro protocol aims to further elucidate mechanisms involved in *Leishmania* dissemination within vertebrate host tissues and can also be modified and applied to other cell migration studies.

Keywords: *Leishmania*, migration, host cell

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Cell Migration and Cell Adhesion Assays to Investigate *Leishmania*-Host Cell Interaction

Yasmin da Silva Luz^{*1}, Amanda R. Paixão^{*1}, Carla Polyana O. S. Bernardes¹, Thaílla Souza da Silva¹, Natalia Machado Tavares¹, Claudia I. Brodskyn¹, Patricia S. T. Veras¹, Juliana P. B. de Menezes¹

¹ Laboratory of Host - Parasite Interaction and Epidemiology, Gonçalo Moniz Institute

* These authors contributed equally

Corresponding Author

Juliana P. B. de Menezes
juliana.fullam@fiocruz.br

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Abstract

Leishmania is an intracellular protozoan parasite that causes a broad spectrum of clinical manifestations, ranging from self-resolving localized cutaneous lesions to a highly fatal visceral form of the disease. An estimated 12 million people worldwide are currently infected, and another 350 million face risk of infection. It is known that host cells infected by *Leishmania* parasites, such as macrophages or dendritic cells, can migrate to different host tissues, yet how migration contributes to parasite dissemination and homing remains poorly understood. Therefore, assessing these parasites' ability to modulate host cell response, adhesion, and migration will shed light on mechanisms involved in disease dissemination and visceralization. Cellular migration is a complex process in which cells undergo polarization and protrusion, allowing them to migrate. This process, regulated by actin and tubulin-based microtubule dynamics, involves different factors, including the modulation of cellular adhesion to the substrate. Cellular adhesion and migration processes have been investigated using several models. Here, we describe a method to characterize the migratory aspects of host cells during *Leishmania* infection. This detailed protocol presents the differentiation and infection of dendritic cells, the analysis of host cell motility and migration, and the formation of adhesion complexes and actin dynamics. This *in vitro* protocol aims to further elucidate mechanisms involved in *Leishmania* dissemination within vertebrate host tissues and can also be modified and applied to other cell migration studies.

Introduction

Leishmaniasis, a neglected tropical disease caused by protozoan parasites belonging to the genus *Leishmania*, results in a wide-ranging spectrum of clinical manifestations, from self-healing localized cutaneous lesions to fatal visceral forms of the disease. It has been estimated that up to one million new leishmaniasis cases arise annually, with

a reported 12 million people currently infected worldwide¹. Visceral leishmaniasis (VL), which can be fatal in over 95% of cases when left untreated, causes more than 50,000 deaths annually, affecting millions in South America, East Africa, South Asia, and the Mediterranean region². The main etiological agent of VL in the new world, *Leishmania infantum*, is transmitted to humans by infected female sandflies during blood-feeding³. These parasites are recognized and internalized by phagocytes, e.g., macrophages and dendritic cells^{3,4,5}. Inside these cells, parasites differentiate into their intracellular forms, known as amastigotes, which will then multiply and be transported via the lymphatic system and bloodstream to different host tissues^{6,7}. However, the mechanisms by which *Leishmania* parasites are disseminated in the vertebrate host, as well as the role played by host cell migration in this process, remain unclear.

Cell migration is a complex process executed by all nucleated cells, including leukocytes⁸. According to the classic cycling model of cell migration, this process involves several integrated molecular events that can be divided into five steps: leading-edge protrusion; adhesion of the leading edge to matrix contacts; contraction of cellular cytoplasm; release of the rear edge of the cell from contact sites; and the recycling of membrane receptors from the rear to the front of the cell⁹.

For cell migration to occur, protrusions must be formed and then stabilized through attachment to the extracellular matrix. Among the different receptor types involved in the promotion of cell migration, integrins are notable. Integrin activation results in migration-related signaling; intracellular signaling then occurs via focal adhesion kinase (FAK) and Src family kinases, in addition to talin, vinculin, and paxillin molecules^{10,11,12}. The phosphorylation of paxillin by activated kinases, including FAK, leads to the recruitment

of effector molecules, which transduces external signals that prompt cell migration. It has been shown that paxillin is an intracellular molecule that is crucial to cell adhesion, actin polymerization, and cell migration processes^{13,14,15}.

The actin cytoskeleton plays a central role in the polarization and migration of phagocytes¹⁶. During cell migration process, protrusions formed due to actin polymerization become stabilized through cell adhesion to the extracellular matrix. This process may be modulated by integrin receptors associated with the actin cytoskeleton^{17,18,19}. Several actin-binding proteins regulate the rate and organization of actin polymerization in cellular protrusions²⁰. Studies have shown that RhoA, Rac, and Cdc42 regulate actin reorganization after the stimulation of adherent cells by extracellular factors^{21,22}. During migration, Rac1 and Cdc42 are located at the leading edge of the cell, controlling the extension of lamellipodia and filopodia, respectively, while RhoA, located at the rear of the cell, regulates the contraction of the actomyosin cytoskeleton^{15,23,24,25}.

Studies have shown that *Leishmania* infection modulates host cell functions, such as adhesion to the cellular substrate and migration^{26,27,28,29,30,31}. Immature DCs reside in peripheral tissues; upon interaction with PAMPS, these cells become activated and migrate to the draining lymph nodes, prompting antigen presentation to T cells. A previous study using a mouse model showed that *L. amazonensis* infection provokes a reduction in the migration of DCs to draining lymph nodes²⁹. It was also demonstrated that the inhibition of the adhesion process reduced DC migration after infection with *L. major*³⁰. Nonetheless, the impact of DC migration on parasite dissemination in the host, as well as the mechanisms involved in this process, remain poorly understood.

Here we present a compiled step-by-step protocol to perform an *in vitro* adhesion and migration assay involving human DCs infected by *Leishmania*. This method comprises not only the differentiation and infection of DCs, but also permits the analysis of host cell motility and migration, the formation of adhesion complexes, as well as actin dynamics. The presently described *in vitro* protocol allows researchers to further investigate the mechanisms involved in *Leishmania* dissemination within vertebrate host tissues and can also be manipulated and applied to other cell migration studies.

Protocol

The procedures described herein were approved by the Institutional Review Board of the Gonçalo Moniz Institute (IGM-FIOCRUZ, protocol no. 2.751.345). Blood samples were obtained from healthy volunteer donors. Animal experimental procedures were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian law 11.784/2008 and were approved and licensed by the Ethical Committee for Animal Research of the Gonçalo Moniz Institute (IGM-FIOCRUZ, protocol no. 014/2019).

1. Isolation and differentiation of human dendritic cells

1. Pipette 10 mL of the density gradient medium in 50 mL centrifuge tubes.
2. Label the 50 mL tubes according to each donor's sample.
3. Collect ~ 50 mL of venous blood from healthy donors and after collection, follow the procedures described below in the flow cabinet.
4. Carefully transfer the collected blood into centrifuge tubes and dilute in saline solution (0.9% sodium chloride) in a ratio of 1:1 at room temperature.
5. Slowly overlay the diluted blood on the top of the density gradient medium.
6. Centrifuge tubes containing the blood and density gradient medium at 400 x *g* for 30 min at room temperature.
NOTE: Switch off the brake before centrifuging to prevent the mixing of gradient layers. After the 1st centrifugation, lower the centrifuge temperature to 4 °C.
7. Carefully remove the tubes from the centrifuge.
8. Identify the ring of peripheral blood mononuclear cells (PBMC) in the sample (buffy coat); gently remove the residual plasma with a pipette.
NOTE: After centrifugation, the following gradient layers will have formed: erythrocytes, density gradient medium, PBMC ring, and plasma. The PBMC ring is located between the density gradient medium and plasma layers.
9. Transfer the cloud-like PBMC layer to a new tube and bring up the volume to 30 mL with saline solution.
10. Centrifuge the tubes containing cell suspension at 250 x *g* for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 1 mL of saline solution.
11. Collect an aliquot for cell counting with the trypan blue exclusion method. First, dilute at a ratio of 1:1,000. Then, use 10 µL of the diluted cells for trypan blue staining and count cells using a Neubauer chamber to determine cell viability.
12. Centrifuge again at 200 x *g* for 10 min under 4 °C.
13. Resuspend the pellet in MACS buffer. Use 80 µL of the buffer for every 1×10^7 cells.
NOTE: MACS buffer composition: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA.

Dilute the buffer solution at a 1:20 ratio with the rinsing solution. Keep the buffer cold and store at 2-8 °C.

14. Add CD14 microbeads to the cell suspension prepared in step 1.13. Use 20 μL of CD14 microbeads for every 1×10^7 cells.

NOTE: CD14 microbeads are used for the positive selection of human monocytes from PBMCs, as beads bind to CD14-positive cells expressed on most monocytes.

15. Homogenize the solution containing the pellet and microbeads using a pipette. Keep on ice for 15 min.
16. Centrifuge the suspension at $300 \times g$ for 10 min under 4 °C.
17. Resuspend cells in MACS buffer. Use 1-2 mL for 1×10^7 cells in the cell microbead mixture.
18. Centrifuge the suspension at $300 \times g$ for 10 min under 4 °C.
19. Aspirate the supernatant and resuspend the pellet in 500 μL of MACS buffer.

NOTE: This is the maximum volume of the cell suspension that can be processed through one column.

20. Assemble the magnetic column.
21. Wash the column once by adding 500 μL of MACS buffer. Allow buffer to flow under gravity through the column.
NOTE: Do not allow the column to dry, as any air that enters can obstruct the column.
22. Add 500 μL of the cell sample solution from step 1.19 per column. Allow the cell sample solution to flow under gravity through the column.
23. Wash the column by adding 500 μL of MACS buffer (2x). Only add the fresh buffer when the column reservoir is empty. Avoid drying.

24. Pipette 1 mL of the MACS buffer onto the column and place it in a new tube underneath. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

25. Centrifuge the CD14 enriched cells at $300 \times g$ for 10 min under 4 °C.

26. Count cells using a Neubauer chamber.

27. Resuspend cells in 1 mL complete RPMI with interleukin-4 (IL-4) (100 $\mu\text{L}/\text{mL}$) + granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL).

NOTE: GM-CSF is a cytokine secreted by macrophages, T cells, mast cells, natural killer cells, endothelial cells, and fibroblasts that induces the differentiation and proliferation of myeloid progenitors in the bone marrow. GM-CSF and IL-4 are used to induce dendritic cell differentiation³².

28. Seed cells in a 24 well plate at a concentration of 2×10^5 cells per well in 500 μL of complete RPMI medium containing and incubate the cells for 7 days in the cell culture incubator at 37 °C.

2. *Leishmania infantum* cultivation

NOTE: *Leishmania infantum* (MCAN/BR/89/BA262) parasites are used in this assay. Hamsters were intravenously infected with 20 μL of the solution containing 1×10^6 *L. infantum* promastigotes in sterile saline. After 1 to 2 months, animals were euthanized and the amastigote forms of *Leishmania* were recovered from their spleens and differentiated into promastigotes³³.

1. Grow *L. infantum* promastigotes isolated from previously infected hamster spleens in an inclined 25 cm^2 cell

culture flask containing 3 mL of Novy-Nicolle-MacNeal medium (NNN) and 5 mL of supplemented Minimum Essential Medium (MEM).

NOTE: In this assay, MEM medium was supplemented with 10% fetal bovine serum (FBS) and 24.5 nM hemin bovine.

2. Incubate at 24 °C for 7 days in BOD (Bio-Oxygen Demand) incubator.
3. Pipette 100 µL of promastigote culture into a new 25 cm² cell culture flask containing 5 mL of supplemented MEM medium.
4. Incubate the promastigote culture at 24 °C in BOD incubator until promastigotes reach the stationary phase. Periodically count cultured promastigotes by transferring an aliquot of parasite suspension diluted in saline to a Neubauer chamber (i.e., hemocytometer) to determine the stationary phase of growth.
5. Transfer 1 x 10⁵ of stationary growth phase culture of *L. infantum* promastigotes to a new 25 cm² cell culture flask and add 5 mL of MEM supplemented medium.
6. Monitor the growth of the culture periodically for approximately 7 days using a Neubauer chamber until the stationary phase is achieved. Parasites are now ready for use in experimental infection procedures.

NOTE: Promastigote cultures are considered viable for infection for up to 7 passages *in vitro*; additional passages may induce loss of virulence.

3. Human dendritic cell infection

1. Inside a biological laminar flow cabinet, transfer all the contents from the parasite culture flask to 50 mL centrifuge tubes.
2. Add cold saline solution to a final volume of 40 mL.
3. Centrifuge at 1,600 xg for 10 min under 4 °C.
4. Discard the supernatant following centrifugation and resuspend the pellet in 1 mL of cold saline solution (Repeat steps 3.3-3.4 three times).
5. After washing the cells to remove any non-viable parasites, resuspend the pellet in 1 mL of cold saline solution.
6. Pass the contents slowly through a 1 mL syringe with a 16 G needle 5 times to separate the parasite clusters.
7. Remove an aliquot to determine the parasite concentration in a hemocytometer (calculate the mean number of parasites in 4 quadrants x dilution factor x 10⁴).
8. Wash dendritic cells by adding 1 mL of saline solution and centrifuging cells at 300 x g for 10 min at room temperature (3 times).
9. Calculate the quantity of *L. infantum* required for the experimental infection; ratio: 20 parasites per cell.
10. Place the required volume of *L. infantum* in each well of cell culture plates.
11. Incubate dendritic cells with parasites for 4 h in an incubator at 37 °C under 5% CO₂.
12. After step 3.11, centrifuge plates at 300 x g for 10 min under 4 °C.

4. Migration assay using cell culture inserts

1. After infection, wash dendritic cells (infected or not) 3 times with 1 mL of saline solution at room temperature to remove non-internalized parasites. After each wash, centrifuge plates at 300 x g for 10 min under 4 °C.
2. Once non-internalized parasites are removed, detach cells by adding 200 µL of the cell dissociation reagent in each well and incubate for 15 min. Keep the cells in an incubator at 37 °C under 5% CO₂.
3. Homogenize cells using a 1,000 µL pipette tip to allow cells to loosen.
4. Transfer the dissociated cells to a 50 mL centrifuge tube.
5. Centrifuge at 300 x g for 10 min under 4 °C.
6. Resuspend the pellet in 1 mL of RPMI medium supplemented with 10% FBS.
7. Pass the contents of each tube slowly through a 1 mL syringe with a 25 G needle 5 times to separate cells.
8. Remove an aliquot, dilute using trypan blue to determine the cell concentration using a hemocytometer (mean of viable cells in 4 quadrants × dilution factor × 10⁴).
9. Preparation of cell culture microplate inserts:
NOTE: Cell culture inserts (5.0 µm pore polycarbonate membrane) are recommended for migration assays using dendritic cells, macrophages, and monocytes, as these inserts allow phagocytes to pass through the membranes.
 1. Remove inserts with sterile tweezers and place in empty wells.
 2. Add 600 µL of RPMI medium supplemented with 10% FBS to each well; add chemoattractant chemokine (C-C motif) ligand 3 (CCL3) (1 µL for every 1 mL of medium).
- NOTE:** CCL3 is a chemokine that regulates DC migration³⁴.
3. Using sterile tweezers, place inserts in wells containing the medium.
10. Add 2 x 10⁵ dendritic cells (infected or not) in 100 µL of medium to each insert.
11. Incubate for 4 h to allow cells to migrate.
12. After 4 h, take the plate out and aspirate the medium. Add 100 µL of 4% paraformaldehyde and incubate for 15 min.
13. Remove paraformaldehyde and add 100 µL of saline solution.
NOTE: Plates can be kept at 4 °C for later assembly.
14. Collect the supernatant from inserts or from wells to count non-migrating cells or those that crossed the membrane, respectively. Incubate with paraformaldehyde 4% for 15 min.
15. Concentrate cells using a cytocentrifugation technique³⁵.
16. Add 10 µL of the mounting medium with DAPI to the membranes and place coverslips over the wells.
17. Assembly of the insert membrane.
NOTE: This step can be performed outside the biosafety cabinets.
 1. Remove insert membranes from wells.
 2. Scrape the surface of the insert with a swab to remove any cells that have not migrated.
 3. Using a scalpel, remove the membrane from the insert.
 4. Place the membrane on a slide, with cells facing up.

5. Add 10 μ L of mounting medium with DAPI to each membrane and then place overlay coverslips.
6. Cover plates with aluminum foil.
7. Incubate plates at room temperature for 30 min, then store at -20 °C
18. To analyze cell migration, count the number of cells per field (no less than 10 fields) using a fluorescence microscope at a laser excitation wavelength of 405 nm.
1. After infecting cells for 4 h, wash each well 3 times with sterile saline solution at room temperature to remove any non-internalized parasites.
2. Centrifuge the plate at 300 x g for 10 min under 4°C. Add 500 μ L of 4% paraformaldehyde to each well for 15 min.
3. Remove paraformaldehyde and add 1 mL of saline solution.
4. Prepare solutions as described in **Table 1**.

5. Adhesion assay and evaluation of actin polymerization by immunofluorescence

NOTE: For this assay, use 24-well plates with coverslips.

Primary solutions	Chemical compound	Diluent	
Ammonium chloride solution	0,134 g of NH ₄ Cl	50 ml of PBS 1X	
Saponin 15%	150 mg of saponin	1 mL de PBS 1X	
Albumin from bovine serum (ABS) 10%	1 g of ABS	10 mL of PBS 1X	
Secondary solutions	Component 1	Component 2	Component 3
Saponin 0,15%	1mL of saponin 15%	100 mL of PBS 1X	-
PBS 1X / ABS 3% / Saponin 0,15%	13,8 mL of PBS 1X	6 mL of ABS 10%	200 μ L of Saponin 15%
PBS1X / ABS 0,3% / Saponin 0,15%:	19,2 mL of PBS 1X	0,6 mL of ABS 10%	200 μ L of Saponin 15%
PBS 1X / ABS 1% / Saponin 0,15%	17,8 mL of PBS 1X	2 mL of ABS 10%	200 μ L of Saponin 15%

Table 1: Buffer recipes.

5. Immunostaining

NOTE: The following steps must be performed under agitation.

 1. Wash coverslips 3 times with 1x PBS for 5 min.
 2. Add 500 μ L of ammonium chloride solution per well for 10 min.

3. Wash coverslips 3 times with 1x PBS for 5 min.
4. Permeabilize the membrane with Saponin 0.15% for 15 min.
5. Incubate cells with the blocking solution (PBS 1x / ABS 3% / Saponin 0.15%) for 1 h.
6. Wash coverslips 3 times with PBS 1x/Saponin 0.15% for 5 min.
7. Dilute primary antibodies in PBS 1x /ABS 1% / Saponin 0.15% as: Rabbit anti-FAK (pTyr397): 1:500 dilution, Rabbit anti-paxilin (pTyr118): 1:100 dilution. Mouse Anti-Rac1: 1:100 dilution. Rabbit Anti-Cdc42: 1:200 dilution. Rabbit Anti-RhoA: 1:200 dilution.
8. Incubate cells with primary antibody diluted in PBS 1x/ABS 1% /Saponin 0.15% for 1 h.
NOTE: FAK and Paxillin are key molecules involved in the formation of adhesion complexes^{13,14,15}. The Rho GTPase family is responsible for the organization of the actin cytoskeleton. RAC1 and Cdc42, located at the front edge of the cell, control the formation of lamellipodia and filopodia, respectively; RhoA is located at the rear of the cell and is involved in cytoskeleton contraction^{15,23,24,25}.
9. Wash coverslips 3 times with 1x PBS/Saponin 0.15% for 5 min.
10. Dilute secondary antibody or phalloidin as follows: Anti-rabbit IgG, Alexa Fluor 568: 1:500 dilution; Anti-mouse IgG, Alexa Fluor 488: 1:500 dilution; Anti-mouse IgG, Alexa Fluor 594: 1:500 dilution; Phalloidin Alexa Fluor 488: 1:1200 dilution.

11. Incubate cells with secondary antibody or phalloidin diluted in 1x PBS/ABS 0.3% Saponin 0.15% for 45 min.

NOTE: Incubation of the secondary antibody or phalloidin should be performed in the absence of light. Cover plates with aluminum foil to protect from light during the following steps.

12. Wash coverslips 3 times with 1x PBS for 5 min.
13. Remove coverslips from wells.
14. In the absence of light, add 10 μ L of mounting medium with DAPI onto clean microscopic glass slides.
15. Place coverslips with cells facing down to allow contact between cells and DAPI solution.
NOTE: Cover slides with aluminum foil to protect from light.
16. Incubate for 30 min at room temperature.
17. Store at -20 °C.

6. Confocal microscopy, image acquisition, and quantification using FIJI

NOTE: To acquire/capture immunofluorescence images, use a confocal laser scanning microscope. Oil-immersion 63x objective lens is recommended for optimal resolution.

1. Allow coverslips to reach room temperature, protected from light, for at least 30 min prior to image acquisition.
2. Clean the coverslips with absorbent tissue.
3. Add a drop of immersion oil to the objective and place each slide under the microscope.
4. Move the objective until the oil touches the slide.

5. Observe and adjust the microscope focus; select the **63x objective with oil**.
6. Turn on lasers at **488 nm, 552 nm, and 405 nm** wavelengths.
7. Select image resolution: **1024 x 1024**.
8. Click on the **Live** button, set the Z stack, and press **Begin**. Repeat the process and then press **End**.
9. After selecting the **Maximum Projection** option in the **Tool** menu, wait for the image to be acquired.
10. **Save** the experiment.
11. **Export** images in .lif format on a computer. Use FIJI open-source image analysis software to analyze images.
12. Select the images to be analyzed and select **Duplicate Image**.
13. To have the image in grayscale, select **Image | Adjust | Color Threshold**. Select **0** and **255** (saturation).
14. Select the threshold method: **Default**.
15. Select threshold color: **B and W** (black and white).
16. Do not select **Dark background**.
17. Select: **Process | Binary | Options** and then select the relevant data to be measured: **Area, Min, Max, gray value, integrate density**.
18. Select the free hands' tool in the Fiji toolbar and trace each cell manually carefully.
19. From the **Analyze** menu, select **Set measurements**. Make sure to have area integrated intensity and mean grey value selected. Repeat this process for each cell.
20. Select all data in the Results window and copy and paste into a spreadsheet file.

21. Calculate the corrected total cell fluorescence (CTCF):
CTCF (Integrate density = Area of selected cell x Mean fluorescence of background readings).
22. Open the file containing data using statistical analysis software to perform statistical analysis.

7. Statistical analysis

1. Open a **New Project** when a welcome dialog appears.
2. Choose the type of graph and medium with SD.
3. Apply the values obtained from the experimental results to the table.
4. Select **Descriptive Statistics** and choose the option column **Statistics [all tests]** to analyze data distribution.
5. If data follow a Gaussian distribution, choose the t-test to analyze samples by comparing two pairs. If the distribution is non-Gaussian, analyze data using the Mann-Whitney test.
6. Choose the best graph option for optimal data representation.

Representative Results

This protocol described herein enables the evaluation of cell migration and its associated mechanisms, such as actin dynamics and adhesion, thereby providing a tool to determine the migration of *Leishmania*-infected host cells within the vertebrate host. The results presented here demonstrate that this *in vitro* assay provides rapid and consistent indications of changes in cellular adhesion, migration, and actin dynamics prior to *in vivo* experimentation.

First, cells were successfully cultured following aseptic techniques and lab protocols. Data generated via migration

analysis using cell culture membrane inserts allowed us to evaluate the migration of *L. infantum*-infected or uninfected human dendritic cells. DAPI staining permitted the facile visualization of migratory cells, enabling us to discriminate between infected and non-infected cells as staining procedures incorporate both dendritic cell and parasite nuclei. Infected cells can be identified by visualizing the large macrophage nuclei and the number of smaller amastigote nuclei clustered around each macrophage nucleus. Next, infected cells (*L. infantum* infected group) and uninfected cells (control group) were then counted for each field of vision using a manual counter. Finally, the number of migratory infected cells was compared to the number of uninfected cells (control group) that migrated. Our results indicate higher rates of cell migration following *L. infantum* infection when compared to non-infected controls (**Figure 1**).

The evaluation of actin dynamics and the formation of adhesion complexes, factors critical to cellular migration, allows for an enhanced understanding of how infection may modulate host cell migration. To assess these mechanisms, we performed immunostaining for molecules involved in actin dynamics (phalloidin, Rac1, Cdc42, and RhoA) and adhesion complex formation (FAK and paxillin). The expression of each protein was evaluated using confocal microscopy. The differences in protein expression were

assessed by comparing the fluorescence intensity between infected and uninfected cells for each protein analyzed. Our results demonstrate actin polymerization in infected and non-infected cells and the formation and localization of adhesion complexes. DAPI staining enabled the identification of infected cells through the staining of parasite nuclei. Fluorescence analysis indicated increased FAK and paxillin expression in DCs following *L. infantum* infection (**Figure 2**). To evaluate the organization of actin filaments in DCs, actin was labeled with fluorescent phalloidin. The resulting images revealed more areas with actin polymerization, yet no differences in phalloidin staining comparing infected and non-infected cells (**Figure 3A**). However, considering that the structure of actin is highly dynamic, the evaluation of actin-associated molecules may provide additional insight into the organization of this structural protein. Thus, we evaluated the expression of Rho GTPase proteins. Although phalloidin staining yielded similar results in infected and non-infected cells, increases were noted in Rac1, Cdc42 and RhoA expression after *L. infantum* infection when compared to uninfected controls (**Figure 3B,C,D**). These results reinforce the need for further investigation of molecules involved in actin polymerization to gain a more comprehensive understanding of its associated dynamics.

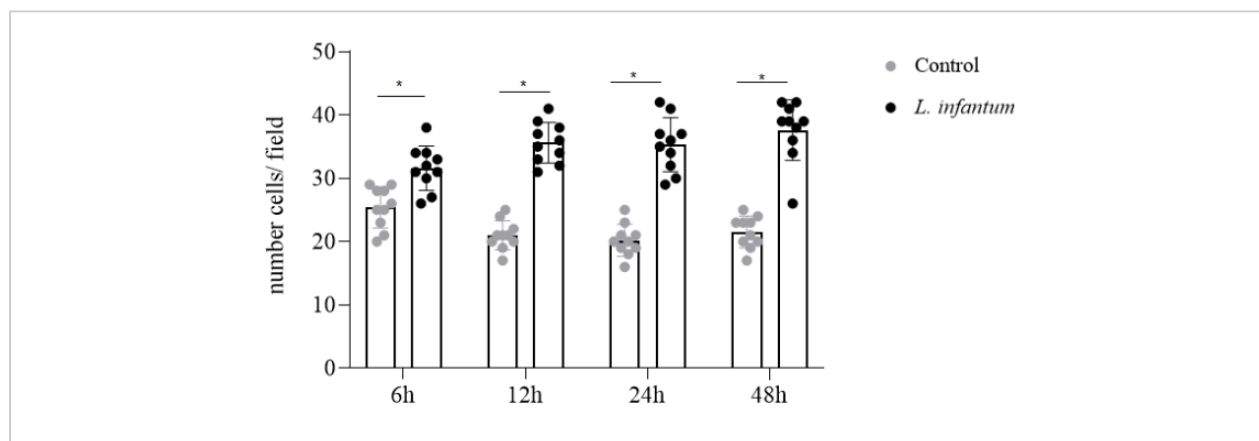


Figure 1: Evaluation of dendritic cell migration in *L. infantum* infection. Dendritic cells were infected by *L. infantum* at a ratio of 20:1 for 4 h. At 6, 12, 24 or 48 h after infection, dendritic cells could migrate in the presence of CCL3 chemoattractant through the cell culture insert system for an additional 4 h. Migrating cells were washed, fixed, and stained with DAPI. Bars represent numbers of migratory cells after *L. infantum* infection from random counts in 10 fields using confocal microscopy. Each dot represents one cell. * $p < 0.05$ (Student's *t*-test). [Please click here to view a larger version of this figure.](#)

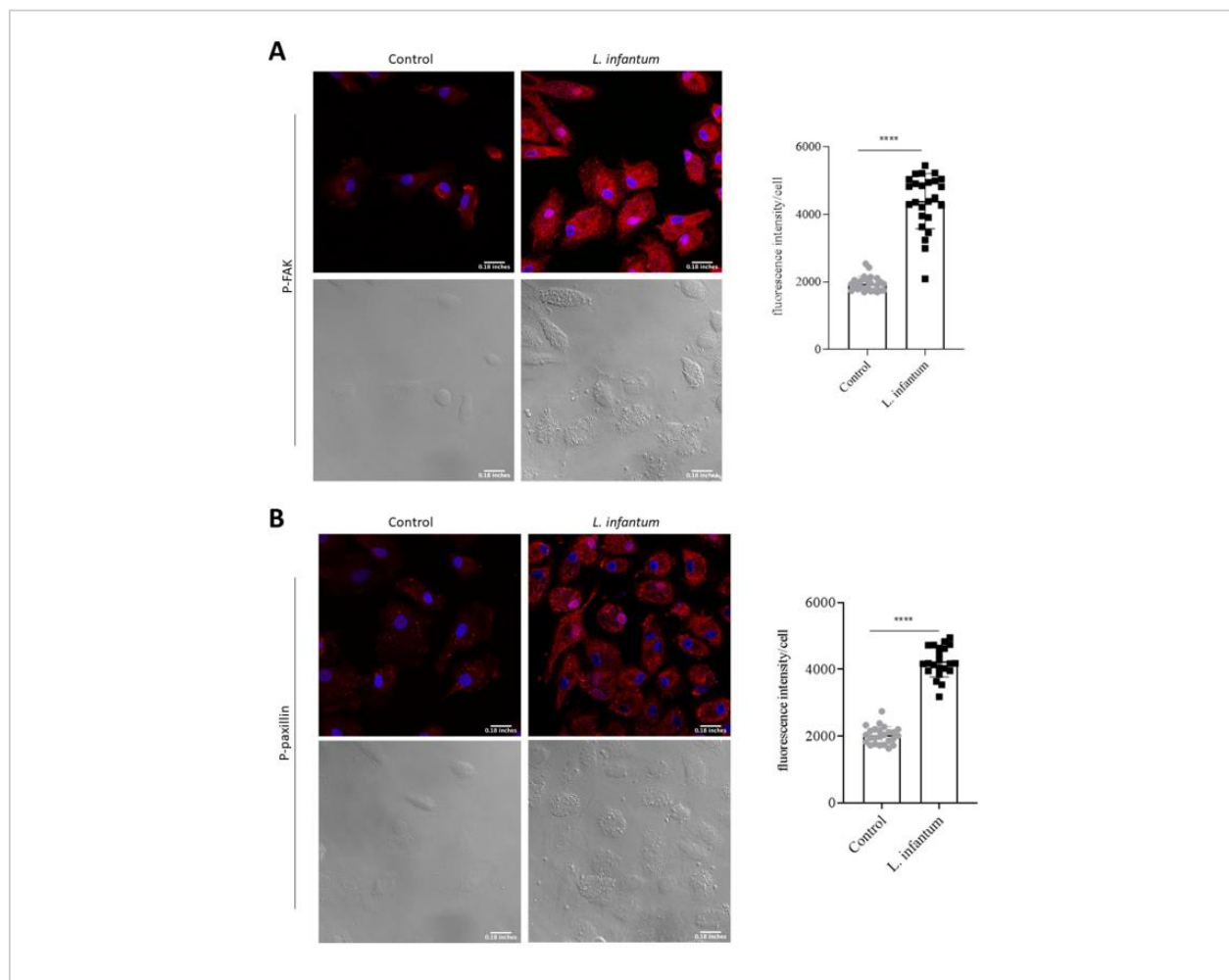


Figure 2: Evaluation of adhesion complex formation in *L. infantum*-infected dendritic cells. Dendritic cells infected or not with *L. infantum* were stained with anti-pFAK or anti-paxillin antibodies. **(A)** Fluorescence intensity of FAK expression. **(B)** Fluorescence intensity of paxillin expression. For each group, 30 cells were analyzed using FIJI software. Red: anti-pFAK, or anti-paxillin; Blue: DAPI; Grayscale: differential interference contrast (DIC). Scale bar = 0.18 inches. * $p < 0.05$ (Student's *t*-test). [Please click here to view a larger version of this figure.](#)

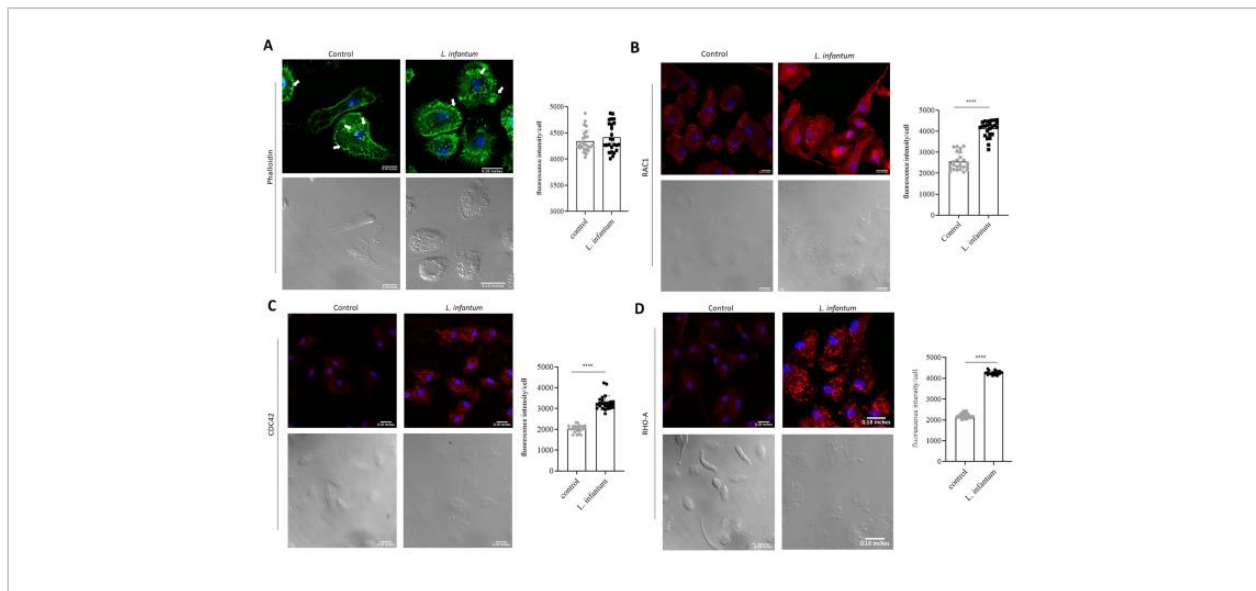


Figure 3: Evaluation of actin dynamics in *L. infantum*-infected dendritic cells. Dendritic cells infected or not with *L. infantum* were stained with anti-Rac1, anti-RhoA and anti-Cdc42 antibodies or fluorescent phalloidin. **(A)** Fluorescence intensity of phalloidin expression (green). **(B)** Fluorescence intensity of Rac1 expression (red). **(C)** Fluorescence intensity of Cdc42 expression (red) **(D)** Fluorescence intensity of RhoA expression (red). For each group, 30 cells were analyzed using FIJI software. Red: anti-Rac1 or anti-Cdc42; Green: anti-RhoA or phalloidin; Blue: DAPI; Grayscale: differential interference contrast (DIC). Scale bar = 0,18 inches. *, $p < 0,05$ (Student's t-test). [Please click here to view a larger version of this figure.](#)

Discussion

The method described here for evaluating cell migration using the cell culture membrane inserts system allows researchers to study the migratory response of cells in a two-dimensional environment. In this technique, some steps are considered critical. Firstly, the differentiation of human DCs and infection with *Leishmania* are determinative since the infection rate is donor-dependent. Using more than one donor per experiment and healthy *Leishmania* cultures will allow for more consistent results. It is also crucial that parasites be maintained in host animals, which favors the selection and maintenance of virulent strains and the ability to readily colonize host cells.

Following DC differentiation, we recommend checking the expression of surface markers CD80 and CD11c to verify that the cells being used in experimentation are, in fact, dendritic cells. Despite variability in infection rates, increased DC migration following *L. infantum* infection was observed in all experiments.

The cell culture membrane inserts system assay, employed herein, entails cell migration through a porous polycarbonate membrane from the upper to the lower compartment of the cell culture membrane inserts system. A chemoattractant is placed in the bottom of each well to direct cell migration through the porous membrane during incubation³⁶. It is

important to use an appropriate pore size for the cell type of interest. For DCs, we used a cell culture membrane inserts system with a 5µm pore size. Of note, after the fixation step, the surfaces of the insert membranes were scraped with a swab to remove cells that had not migrated. This step was implemented to ensure that only those cells that successfully migrated through the membrane were evaluated. Another critical point that warrants consideration is the use of DAPI staining, a rapid procedure that allows investigators to not only identify host cells but also parasite nuclei, thus enabling the convenient identification of infected cells.

Although the cell culture membrane inserts system has been extensively used as an effective tool for assessing migration^{28,36,37,38,39}, there are some limitations associated with this technique. During cell washing and fixation steps, less-adherent cells may be lost when analyzing the membrane, resulting in an underestimation of the number of cells that migrated. Another limitation is time related. After long incubation periods, gradient loss may occur due to diffusion through the porous membrane. Thus, this system should be considered more efficient for shorter incubation periods³⁶. On the other hand, the use of the cell culture membrane inserts system is advantageous compared to other methods such as the scratch assay or random migration since it allows the study of directional migration in the presence of a chemoattractant.

Another essential component of this protocol is the use of immunofluorescence to investigate mechanisms involved in cellular migration, such as adhesion complex formation and actin dynamics. This technique allows investigators to visualize specific targets in tissues or cells using specific antibodies for proteins of interest. In this protocol, to assess cellular adhesion, we evaluated the expression

of phosphorylated FAK and paxillin, both crucial proteins involved in the formation of adhesion complexes in different cell types, including leukocytes¹³, and consequently excellent tools for studying cell adhesion. Previous studies have shown that increased FAK signaling promotes cellular motility¹¹. Also, the use of this technique to evaluate cell adhesion does not require the use of more labor-intensive techniques, such as the use of inflamed connective tissue²⁶.

The phalloidin staining technique^{40,41} provides information about the expression of polymerized actin, F-actin, and the regions of cells with higher polymerization rates. To gain further insight into actin dynamics in *L. infantum*-infected cells, we also evaluated the expression of Rac1, Cdc42, RhoA, and Rho GTPase, which participate in the polymerization of actin filaments⁴². The expression of these molecules was also performed using immunofluorescence. This technique is not only an alternative to the use of transgenic mice expressing fluorescent actin²⁸ but also provides further information about molecules modulated during the actin polymerization process.

Several factors can affect immunofluorescence quality and efficacy. Antibody dilution, for example, when not carefully determined, can impair the acquisition of images and lead to non-specific staining due to elevated levels of background signals. Samples must also always be protected from light to avoid any loss of cell fluorescence or staining⁴³.

In summary, here we describe an *in vitro* protocol that allows for the evaluation of cell adhesion and migration processes in the context of *Leishmania* infection. Our primary focus was on DCs, which are known to play a significant role in the immunopathogenesis of leishmaniasis; however, how these cells participate in parasite dissemination in the vertebrate host remains poorly understood. This protocol can also be

modified to investigate cellular migration in other types of host cells for the investigation of other species of intracellular parasites. Also, extracellular matrix, such as collagen I or Matrigel, can be added to the cell culture membrane inserts system to evaluate 3D migration and cellular invasion in different fields of study.

Disclosures

The authors declare they have no competing financial interests.

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References

- Burza, S., Croft, S. L., Boelaert, M. Leishmaniasis. *The Lancet*. **392** (10151), 951-970 (2018).
- Bi, K., Chen, Y., Zhao, S., Kuang, Y., John Wu, C. H. Current Visceral Leishmaniasis Research: A Research Review to Inspire Future Study. *BioMed Research International*. **2018** (2018).
- Serafim, T. D., Iniguez, E., Oliveira, F. Leishmania infantum. *Trends in Parasitology*. **36** (1), 80-81 (2020).
- Van Assche, T., Deschacht, M., da Luz, R. A. I., Maes, L., Cos, P. Leishmania-macrophage interactions: Insights into the redox biology. *Free Radical Biology and Medicine*. **51** (2), 337-351 (2011).
- Podinovskaia, M., Descoteaux, A. Leishmania and the macrophage: A multifaceted interaction. *Future Microbiology*. **10** (1), 111-129 (2015).
- Antoine, J. C., Prina, E., Jouanne, C., Bongrand, P. Parasitophorous vacuoles of Leishmania amazonensis-infected macrophages maintain an acidic pH. *Infection and Immunity*. **58** (3) (1990).
- Antoine, J. C., Prina, E., Lang, T., Courret, N. The biogenesis and properties of the parasitophorous vacuoles that harbour Leishmania in murine macrophages. *Trends in Microbiology*. **6** (10), 392-401 (1998).
- Friedl, P., Wolf, K. Plasticity of cell migration: A multiscale tuning model. *Journal of Cell Biology*. **188** (1), 11-19 (2010).
- Sheetz, M. P., Felsenfeld, D., Galbraith, C. G., Choquet, D. Cell migration as a five-step cycle. *Biochemical Society symposium*. **65**, 233-243 (1999).
- Yano, H. et al. Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. *Journal of Cell Biology*. **166** (2), 283-295 (2004).
- Mitra, S. K., Hanson, D. A., Schlaepfer, D. D. Focal adhesion kinase: In command and control of cell motility. *Nature Reviews Molecular Cell Biology*. **6** (1), 56-68 (2005).
- Deramandt, T. B. et al. Altering FAK-Paxillin Interactions Reduces Adhesion, Migration and Invasion Processes. *PLoS One*. **9** (3) (2014).
- Turner, C. E. Paxillin and focal adhesion signalling. *Nature Cell Biology*. **2** (12) (2000).

14. Linder, S., Aepfelbacher, M. Podosomes: Adhesion hot spots of invasive cells. *Trends in Cell Biology*. **13** (7), 376-385 (2003).
15. Huveneers, S., Danen, E. H. J. Adhesion signaling - Crosstalk between integrins, Src and Rho. *Journal of Cell Science*. **122** (8), 1059-1069 (2009).
16. Jones, G. E. Cellular signaling in macrophage migration and chemotaxis. *Journal of Leukocyte Biology*. **68** (5), 593-602 (2000).
17. De Fougères, A. R., Kotliansky, V. E. Regulation of monocyte gene expression by the extracellular matrix and its functional implications. *Immunological Reviews*. **186** (1), 208-220 (2002).
18. Cortesio, C. L., Boateng, L. R., Piazza, T. M., Bennin, D. a, Huttenlocher, A. Calpain-mediated proteolysis of paxillin negatively regulates focal adhesion dynamics and cell migration. *The Journal of Biological Chemistry*. **286** (12), 9998-10006 (2011).
19. Verkhovsky, A. B. et al. Orientational order of the lamellipodial actin network as demonstrated in living motile cells. *Molecular Biology of the Cell*. **14** (11), 4667-4675 (2003).
20. Ridley, A. J. et al. Cell Migration: Integrating signals from front to back. *Science*. **302** (5651), 1704-1709 (2003).
21. Hall, a Small GTP-binding proteins, and the regulation of the actin cytoskeleton. *Annual Review of Cell Biology*. **10**, 31-54, (1994).
22. Machesky, L.M., Hall, A. Rho: A connection between membrane receptor signalling and the cytoskeleton. *Trends in Cell Biology*. **6** (8), 304-310 (1996).
23. Ridley, A. J., Hall, A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. **70** (3), 389-399 (1992).
24. DeMali, K. A., Burridge, K. Coupling membrane protrusion and cell adhesion. *Journal of Cell Science*. **116** (12), 2389-2397 (2003).
25. López-Colomé, A. M., Lee-Rivera, I., Benavides-Hidalgo, R., López, E. Paxillin: A crossroad in pathological cell migration. *Journal of Hematology and Oncology*. **10** (1) (2017).
26. Carvalho, D. G. F. et al. The modelling of mononuclear phagocyte-connective tissue adhesion in vitro: Application to disclose a specific inhibitory effect of Leishmania infection. *Experimental Parasitology*. **107** (3-4), 189-199 (2004).
27. Pinheiro, N. F. et al. Leishmania infection impairs β 1-integrin function and chemokine receptor expression in mononuclear phagocytes. *Infection and Immunity*. **74** (7), 3912-3921 (2006).
28. de Menezes, J. P. B. et al. Leishmania infection inhibits macrophage motility by altering F-actin dynamics and the expression of adhesion complex proteins. *Cellular Microbiology*. **19** (3) (2017).
29. Hermida, M. D. R., Doria, P. G., Taguchi, A. M. P., Mengel, J. O., dos-Santos, W. L. C. Leishmania amazonensis infection impairs dendritic cell migration from the inflammatory site to the draining lymph node. *BMC Infectious Diseases*. **14** (1), 450 (2014).
30. Ballet, R. et al. Blocking junctional adhesion molecule c enhances dendritic cell migration and boosts the immune responses against Leishmania major. *PLoS Pathogens*. **10** (12) (2014).

31. Rocha, M. I. et al. Leishmania infantum enhances migration of macrophages via a phosphoinositide 3-Kinase $\bar{\iota}$ -dependent pathway. *ACS Infectious Diseases*. **6** (7), 1643-1649 (2020).
32. Hiasa, M. et al. GM-CSF and IL-4 induce dendritic cell differentiation and disrupt osteoclastogenesis through M-CSF receptor shedding by up-regulation of TNF- α converting enzyme (TACE). *Blood*. **114** (20), 4517-4526 (2009).
33. Melo, C. V. B. de et al. Phenotypical characterization of spleen remodeling in murine experimental visceral leishmaniasis. *Frontiers in Immunology*. **11**, 653 (2020).
34. Gibaldi, D. et al. CCL3/macrophage inflammatory protein-1 α is dually involved in parasite persistence and induction of a TNF- and IFN γ -enriched inflammatory milieu in Trypanosoma cruzi-induced chronic cardiomyopathy. *Frontiers in Immunology*. **11**, 306 (2020).
35. Finger, P. T., Papp, C., Latkany, P., Kurli, M., Iacob, C. E. Anterior chamber paracentesis cytology (cytospin technique) for the diagnosis of intraocular lymphoma. *British Journal of Ophthalmology*. **90** (6), 690-692 (2006).
36. Zhang, C., Barrios, M. P., Alani, R. M., Cabodi, M., Wong, J. Y. A microfluidic Transwell to study chemotaxis. *Experimental Cell Research*. **342** (2), 159-165 (2016).
37. Glynn, S. A., O'Sullivan, D., Eustace, A. J., Clynes, M., O'Donovan, N. The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin, lovastatin and mevastatin inhibit proliferation and invasion of melanoma cells. *BMC Cancer*. **8** (2008).
38. van de Merbel, A. F., van der Horst, G., Buijs, J. T., van der Pluijm, G. Protocols for migration and invasion studies in prostate cancer. *Methods in Molecular Biology*. **1786**, 67-79 (2018).
39. Omar Zaki, S. S., Kanesan, L., Leong, M. Y. D., Vidyadaran, S. The influence of serum-supplemented culture media in a transwell migration assay. *Cell Biology International*. **43** (10), 1201-1204 (2019).
40. Borovikov, I. S. Izuchenie strukturnykh izmenenii sokratitel'nykh belkov myshechnogo volokna s pomoshch'iu poliarizatsionnoi ul'trafiol'etovoi fluorestsentnoi mikroskopii. VIII. Vlianie glutaral'degida i falloidina na konformatsiiu F-aktina. *Tsitologiya*. **26** (11), 1262-1266 (1984).
41. Cooper, J. A. Effects of cytochalasin and phalloidin on actin. *The Journal of Cell Biology*. **105** (4), 1473-1478 (1987).
42. Allen, W. E., Jones, G. E., Pollard, J. W., Ridley, A. J. Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *Journal of Cell Science*. **110**, 707-720 (1997).
43. Lichtman, J. W., Conchello, J. A. Fluorescence microscopy. *Nature Methods*. **2** (12), 910-919 (2005).

Anexo C - Manuscrito 3: In vitro differentiation of human dendritic cells and their markers in *Leishmania* infection

Abstract: Leishmaniasis comprises a collection of clinical manifestations associated with the infection of obligate intracellular protozoans, *Leishmania*. The life cycle of *Leishmania* parasites consists of two alternating life stages (amastigotes and promastigotes), during which parasites reside within either arthropod vectors or vertebrate hosts, respectively. Notably, the complex interactions between *Leishmania* parasites and several cells of the immune system largely influence the outcome of infection. Importantly, although macrophages are known to be the main host niche for *Leishmania* replication, parasites are also phagocytosed by other innate immune cells, such as neutrophils and dendritic cells (DCs). DCs play a major role in bridging the innate and adaptive branches of immunity and thus orchestrate immune responses against a wide range of pathogens. The mechanisms by which *Leishmania* and DCs interact remain unclear and involve aspects of pathogen capture, the dynamics of DC maturation and activation, DC migration to draining lymph node (dLNs), and antigen presentation to T cells. Although a large body of studies support the notion that DCs play a dual role in modulating immune responses against *Leishmania*, the participation of these cells in susceptibility or resistance to *Leishmania* remains poorly understood. After infection, DCs undergo a maturation process associated with the upregulation of surface major histocompatibility complex (MHC) II, in addition to costimulatory molecules (namely, CD40, CD80, and CD86). Understanding the role of DCs in infection outcome is crucial to developing therapeutic and prophylactic strategies to modulate the immune response against *Leishmania*. This paper describes a method for the characterization of *Leishmania*-DC interaction. This detailed protocol provides guidance throughout the steps of DC differentiation, the characterization of cell surface molecules, and infection protocols, allowing scientists to investigate DC response to *Leishmania* infection and gain insight into the roles played by these cells in the course of infection.

Keywords: *Leishmania*, dendritic cell, markers

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In Vitro Differentiation of Human Dendritic Cells and their Markers in *Leishmania* Infection

Amanda R. Paixão^{*1}, Rafael Tibúrcio^{*1}, Elaine Carvalho¹, Thailla Silva¹, Patrícia S. T. Veras¹, Natalia M. Tavares¹, Juliana P. B. de Menezes¹, Cláudia I. Brodskyn¹

¹ Laboratory of Host-Parasite Interaction and Epidemiology, Gonçalo Moniz Institute

* These authors contributed equally

Corresponding Author

Cláudia I. Brodskyn
claudia.brodskyn@fiocruz.br

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Abstract

Leishmaniasis comprises a collection of clinical manifestations associated with the infection of obligate intracellular protozoans, *Leishmania*. The life cycle of *Leishmania* parasites consists of two alternating life stages (amastigotes and promastigotes), during which parasites reside within either arthropod vectors or vertebrate hosts, respectively. Notably, the complex interactions between *Leishmania* parasites and several cells of the immune system largely influence the outcome of infection. Importantly, although macrophages are known to be the main host niche for *Leishmania* replication, parasites are also phagocytosed by other innate immune cells, such as neutrophils and dendritic cells (DCs).

DCs play a major role in bridging the innate and adaptive branches of immunity and thus orchestrate immune responses against a wide range of pathogens. The mechanisms by which *Leishmania* and DCs interact remain unclear and involve aspects of pathogen capture, the dynamics of DC maturation and activation, DC migration to draining lymph node (dLNs), and antigen presentation to T cells. Although a large body of studies support the notion that DCs play a dual role in modulating immune responses against *Leishmania*, the participation of these cells in susceptibility or resistance to *Leishmania* remains poorly understood. After infection, DCs undergo a maturation process associated with the upregulation of surface major histocompatibility complex (MHC) II, in addition to costimulatory molecules (namely, CD40, CD80, and CD86).

Understanding the role of DCs in infection outcome is crucial to developing therapeutic and prophylactic strategies to modulate the immune response against *Leishmania*. This paper describes a method for the characterization of *Leishmania*-DC interaction. This detailed protocol provides guidance throughout the steps of DC differentiation, the characterization of cell surface molecules, and infection protocols, allowing scientists

to investigate DC response to *Leishmania* infection and gain insight into the roles played by these cells in the course of infection.

Introduction

Leishmaniasis constitutes a complex of neglected diseases caused by different species of the *Leishmania* genus¹. *Leishmania* is an intracellular protozoan of the Trypanosomatidae family that infects humans and other mammals, causing a spectrum of diseases ranging from skin lesions to visceral forms². The main clinical manifestations of this disease are tegumentary leishmaniasis (TL) and visceral leishmaniasis (VL). The World Health Organization (WHO) estimates that 700,000 to 1 million new cases occur annually, causing 70,000 deaths each year². Worldwide, leishmaniasis affects approximately 12 to 15 million people, and 350 million are at risk of contracting the disease³.

The genus *Leishmania* presents two evolutionary forms: the promastigote and the amastigote⁴. *Leishmania* promastigotes are characterized by the presence of flagella and high motility. These forms are found in the digestive tract of the sand fly, where they undergo differentiation into the infective form (metacyclic promastigotes)⁵. By contrast, amastigotes are found in the intracellular environment of infected mammalian cells. This evolutionary form, in turn, replicates in the phagolysosomes of phagocytic cells⁶.

The transmission cycle of *Leishmania* spp. starts during blood-feeding, when sandflies inoculate metacyclic promastigotes into the host's skin¹. Shortly after *Leishmania* inoculation, innate immune cells, including neutrophils and tissue-resident macrophages, phagocytize the parasites. Inside parasitophorous vacuoles, *Leishmania* differentiate into amastigotes and replicate, culminating in the rupture of the host cell membrane, which allows the infection

of neighboring cells and parasite spread⁴. The cycle is completed when phlebotomines ingest amastigote-containing phagocytes, which differentiate into procyclic promastigotes and later into metacyclic promastigotes in the insect's intestinal tract⁷.

Dendritic cells, professional antigen-presenting cells found in tissues and lymph nodes, act as a sentinel for the immune system⁸. These cells are found in peripheral tissues at immature stages, mainly involved in antigen capture and processing. After contact with pathogens, DCs undergo a maturation process that culminates in their migration to the lymph nodes, subsequently presenting antigens to naïve CD4⁺ T cells. These cells are also essential in orchestrating the innate and adaptive immune responses that generate tolerance or inflammation⁹. The DC maturation process involves several aspects, including increased expression of MHC and costimulatory molecules, such as CD40 and CD86, as well as enhanced cytokine secretion. DCs express different markers, including CD11b and CD11c, and, in humans, the DCs that originate from CD14⁺ monocytes (moDCs) express CD1a¹⁰. CCR7 is highly expressed on DCs and indicates the complex migratory process of these cells¹². CD209 and CD80 also play an important role in the initial contact with DCs and lymphocytes¹³.

In leishmaniasis, studies suggest that moDCs phagocytose parasites and deliver them to the draining lymph nodes (dLNs), where they present antigens to T cells¹³. The parasite capture mechanism is associated with cytoskeletal reorganization by actin filaments during phagocytosis, which

promotes the internalization of the parasite¹⁴. Most studies concerning the roles exercised by DCs in leishmaniasis have focused on *L. major*, *L. amazonensis*, and *L. braziliensis*¹⁵. Interestingly, *in vivo* studies of *Leishmania* infection have demonstrated that the impairment of DC function occurs in a parasite strain-specific manner.

It has been demonstrated that during the early stages of *L. amazonensis* infection, DCs exhibit a decreased ability to constrain parasite infection. Conversely, in an experimental model of *L. braziliensis* infection, DCs were shown to mount appropriate immune responses that restricted *Leishmania* survival¹⁶. The chief aspects known to be associated with differential responses to *Leishmania spp.* infection are the degree of DC maturation and activation. This paper describes a method to investigate the role human DCs play in *Leishmania* infection to further understand how these cells influence disease outcomes.

Protocol

NOTE: Cells were obtained from healthy donor volunteers. The procedure described herein was approved by the National Ethics Committee (number 2.751.345)-Fiocruz, Bahia, Brazil).

1. Differentiation of human dendritic cells

1. Pipette 10 mL of polysucrose-sodium triazoate mixture in 50 mL conical tubes.
2. Label the 50 mL conical tubes respectively for each donor.
3. Collect 30 mL of blood from healthy donors and perform all subsequent steps in the laminar flow hood.
4. Carefully transfer the blood into the conical tubes and dilute the blood in saline solution (0.9% sodium chloride) at a ratio of 1:1 at room temperature.
5. Overlay the slowly diluted blood onto the polysucrose-sodium triazoate mixture in the tubes (step 1.1). Centrifuge the tubes once at $400 \times g$ for 30 min at 25 °C. **NOTE:** Turn off the brake before centrifugation to prevent the mixing of gradient layers. After the first centrifugation, decrease the temperature to 4 °C in the centrifuge.
6. Carefully take the tubes out from the centrifuge.
7. Look for the ring formed by peripheral blood mononuclear cells (PBMCs) in the sample (buffy coat); aspirate the residual plasma carefully with a pipette. **NOTE:** Centrifugation leads to the formation of the following gradient layers: erythrocytes, density gradient medium, PBMC ring, and plasma. The PBMC ring is between the density gradient medium and the plasma layers.
8. Transfer the cloudy PBMC layer to another tube and add saline to a final volume of 30 mL.
9. Centrifuge the tubes containing the cell suspensions at $250 \times g$ for 10 min at 4 °C. Discard the supernatant and add 1 mL of saline to resuspend the pellet.
10. Collect an aliquot for cell counting and dilute it 1:1,000. Use 10 μ L of the diluted cells for counting the cells by trypan blue exclusion method using a Neubauer chamber to determine cell viability.
11. Spin the suspension again at $200 \times g$ for 10 min under 4 °C.
12. Resuspend the pellet in magnetic-activated cell sorting (MACS) buffer. Use 80 μ L of the buffer per 1×10^7 cells.

NOTE: See **Table 1** for the MACS buffer composition. Keep the buffer cold and store it at 2-8 °C.

13. Add CD14 microbeads to the cell suspension prepared in step 1.9. Use 20 µL of CD14 microbeads per 1×10^7 cells.

NOTE: CD14 microbeads are used to positively select human monocytes from PBMCs, as beads containing human anti-CD14 bind to CD14⁺ cells expressed on the surfaces of most monocytes.

14. Pipette up and down to resuspend the pellets and microbeads uniformly. Keep on ice for 15 min.
15. Centrifuge the suspension at $300 \times g$ for 10 min at 4 °C.
16. Resuspend the cells in MACS buffer. Use 1-2 mL per 1×10^7 cells in the cell-microbead mixture.
17. Centrifuge the suspension at $300 \times g$ for 10 min at 4 °C.
18. Remove the supernatant and resuspend the pellet in 500 µL of MACS buffer.

NOTE: This is the maximum volume of the cell suspension to be processed in one column.

19. Assemble the magnetic column.
20. Wash the column once with 500 µL of MACS buffer and allow the buffer to flow under gravity through the column.
NOTE: Take care not to allow the column to become dry, as air can obstruct the column.
21. Add 500 µL of the cell-bead suspension (step 1.19) per column. Allow the cell suspension to flow under gravity through the column.
22. Wash the column with 500 µL of MACS buffer (2x). Add fresh buffer only when the column reservoir is empty. Do not let the column dry.

23. Place a new tube underneath the column, pipette 1 mL of the MACS buffer onto the column, and immediately flush the magnetically labeled cells out of the column by firmly pushing the plunger.
24. Centrifuge the CD14 enriched cells at $300 \times g$ for 10 min at 4 °C.
25. Count the cells using a Neubauer chamber.
26. Resuspend the cells in 1 mL of complete RPMI with 100 µL/mL of interleukin-4 (IL-4) (50 ng/mL) + granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL).
27. Seed cells on a 24-well plate at a concentration of 2×10^5 cells per well in 500 µL of complete RPMI medium containing the above cytokines and incubate for 7 days at 37 °C.

2. *Leishmania* culture

NOTE: *L. amazonensis* (MHOM/BR88/Ba-125) parasites were used in this assay.

1. Maintain promastigotes on Novy-Nicolle-MacNeal (NNN) blood agar medium with 5 mL of Schneider's complete medium or Minimum Essential Medium (MEM) in a 25 cm² culture bottle in a Bio-Oxygen Demand (BOD) incubator at 24 °C.
NOTE: To cultivate the promastigote form of the *L. amazonensis* species, 5 mL of Schneider's complete medium (Schneider's Insect Medium containing 10% inactivated fetal bovine serum (FBS) and gentamicin at a concentration of 50 µg/mL) was used.
2. Incubate for 7 days at 24 °C in the BOD incubator.
3. Pipette 100 µL of promastigote culture into a new 25 cm² cell culture flask.

4. Add 5 mL of the supplemented MEM.
5. Incubate at 24 °C in the BOD incubator and periodically count the promastigotes by transferring an aliquot of saline-diluted parasite cell suspension into a Neubauer chamber (i.e., hemocytometer) until stationary phase has been reached.

NOTE: Do not use the first passage post-NNN Medium for experiments.

6. Transfer 1×10^5 of *Leishmania* stationary growth phase promastigotes into a new 25 cm² cell culture flask and add 5 mL of the supplemented MEM.
7. Periodically monitor the growth of cultures using a Neubauer chamber until the stationary phase is achieved.

NOTE: Use promastigote cultures for up to 7 passages *in vitro* to avoid loss of virulence.

3. *Leishmania* infection

1. After verifying stationary-phase growth of cultured parasites, remove all contents from culture bottles and place them in 50 mL conical tubes. Add cold saline solution to achieve a final volume of 30 mL.
2. Centrifuge for 10 min at 4 °C at $1,600 \times g$ three times. Discard the supernatant following centrifugation and resuspend the pellet in a cold saline solution.
3. Wash the cells to remove any non-viable parasites, and resuspend the pellet in 1 mL of cold saline. Pass the suspension 5 times slowly through a 1 mL syringe fitted with a 16 G needle to separate parasite clusters.
4. Remove an aliquot from the sample to count parasite concentration in a hemocytometer using Eq (1).

Parasite concentration = mean of parasites in 4 quadrants $\times$ dilution factor $\times 10^4$ (1)

5. Calculate the amount of *Leishmania* sp. required for the number of host cells to be plated to maintain a 10:1 parasite: host cell ratio (step 1.27).
6. Wash the DCs by adding 1 mL of saline solution and centrifuging the cells three times at $300 \times g$ for 10 min at room temperature.
7. Place the required volume of *Leishmania* in each well of the 24-well cell culture plate (see step 3.5).
8. Incubate the DCs with parasites for 4 h in an incubator at 37 °C in a 5% CO₂ atmosphere.

4. Immunostaining for flow cytometry analysis

1. After infection, wash cells twice with 1 mL of saline solution to remove any non-internalized parasites.
NOTE: The panel with antibody clones and fluorochromes is listed in the **Table of Materials**.
2. Dilute antibodies (1:50) in fluorescence-activated cell sorting (FACS) buffer (1x PBS with 1% BSA) at a final volume of 50 μ L per experimental condition.
NOTE: It is important to titrate antibodies before experimentation to ensure optimal staining concentrations. In this protocol, 1:50 was used after titration experiments.
3. Prepare a master mix (1x PBS with 1% BSA + antibody mix). Vortex and pipette 50 μ L of the mix in wells containing cells.
4. Briefly incubate the DCs with the antibody mix (anti-human CD1a, anti-human CCR7, anti-human CD83, anti-CD11c, anti-human CD209, anti-human HLA-DR) on ice for 30 min, protected from light.

5. Wash cells twice with 1 mL of cold staining buffer (1% FBS in 1x PBS) and centrifuge at $300 \times g$ for 5 min at 4 °C.

6. Aspirate the supernatant and resuspend the pellet in 200 μ L of cold staining buffer.

7. Proceed with data acquisition on a flow cytometer.

NOTE: To ensure optimal conditions for FACS analysis, data acquisition should be performed within 48 h after sample staining. Data were analyzed using FlowJo software.

1. Open the FlowJo software program and create a new workspace. Add the flow cytometry files to be analyzed by dragging them into the workspace window.

2. Click on the **tube name** to select side scatter (SSC) and forward scatter (FSC) parameters.

NOTE: Flow cytometric analysis of DC maturation was based on the expression of CD1a, CD11c, CD80, CCR7, CD209, and HLA-DR.

3. Using the **polygon gating** tool, draw a gate around the DCs. Double-click on the selected gated cells to display a new window. Perform doublet cleanup by selecting the FSC-A and FSC-H parameters. Using the **polygon** tool, draw a new plot around individual cells.

4. Double-click on the gated cells and select SSC and CD11c parameters to generate a gate containing CD11c⁺ cells.

5. Export the cleaned CD11c⁺ cell gate by selecting it. Right-click on the gate and select the **Select equivalent nodes** option. Right-click and export

the gate along with all compensated parameters by saving it to a recently created folder.

6. Create a new workspace by opening the recently saved files. Click on **Workspace** and select **plugins | Downsample | 30,000 to 50,000 events**; press **OK**.

NOTE: As TSNE is both a time-consuming and computationally demanding process, this step is recommended to reduce the total number of analyzed events.

7. Right-click on a downsampled file and select **equivalent nodes**.

8. Right-click on a downsampled file and select **Export/concatenate**.

9. Click on the **concatenate** window and select **all compensated parameters**. Select the concatenated files and click on **workspace | plugin | tSNE**.

10. Select the desired parameters (CD1a, HLA-DR, CCR7, CD80, CD209, and CD11c) to create clusters. Press **OK**.

11. Click on the concatenated file to visualize the tSNE1 and tSNE2 parameters.

5. Actin immunostaining

1. Plate infected DCs on coverslips placed in 24-well plates. Centrifuge the plates at $300 \times g$ at 4 °C for 10 min. Wash cells 3x with sterile saline after infection at room temperature to remove any extracellular parasites.
2. Incubate the DCs with 500 μ L of 4% paraformaldehyde for 15 min at room temperature. Remove the paraformaldehyde and add 1 mL of saline (see **Table 1**).

6. Immunolabeling

NOTE: Perform the following steps under agitation.

1. Wash the coverslips with 1x PBS for 5 min; repeat 3x. Add 500 μ L of ammonium chloride solution per well; incubate for 10 min at room temperature.
2. Wash the coverslips with 1x PBS for 5 min; repeat 3x.
3. Permeabilize the membrane with 0.15% Saponin for 15 min.
4. Block for 1 h with 1x PBS 1x/3% BSA/0.15% saponin. Wash the coverslips with 1x PBS/0.15% saponin for 5 min; repeat 3x.
5. Incubate the cells for 1 h with phalloidin (1:1,200) diluted in 1x PBS/1% BSA/0.15% saponin. Wash the coverslips with 1x PBS/0.15% saponin for 5 min; repeat 3x.
6. Place the coverslips with cells facing down on mounting medium with 4',6-diamidino-2-phenylindole (DAPI). Cover the slides with aluminum foil to protect them from light.
7. Acquire images using a confocal fluorescence microscope with a 63x/1.4 objective.

7. Confocal microscopy acquisition and Fiji quantification

1. Capture immunofluorescence images using a confocal laser scanning microscope (see the **Table of Materials**).
NOTE: For best resolution, use a 63x objective oil immersion lens.
2. Protect the coverslips from light and leave them at room temperature for at least 30 min prior to acquisition.
3. Use absorbent tissue to clean the coverslips. Add a drop of immersion oil to the objective and place the slide on the

microscope stage. Select the 63x objective with oil, raise the platform until the oil touches the slide, and adjust the focus on the microscope.

4. Open the program and activate the laser wavelengths of 488, 552, and 405 nm.
5. Set the image resolution to 1024 x 1024 pixels.
6. Click on **live bottom**, set the **Z stack**, and press **Begin**. Repeat this process and then press the **End** button.
7. Wait for the image acquisition to complete, and then select the option **Maximum Projection** in **tools**.
8. Save the experiment, and export the .lif format images to a computer.
9. Open the FIJI program, open the experiment in FIJI, and set the **view stack** to **Hyperstack**.
10. Select **open image files individually | stitch tiles**.
11. Select the **free hand** tool in the **Fiji** toolbar and trace each cell carefully by hand. Press **analyze | measure** to visualize fluorescence intensity. Repeat this process for each cell type per group.
12. Save the measurements and export them to a spreadsheet. Open the file containing data using statistical analysis software (see the **Table of Materials**) to perform statistical analysis.

8. Statistical analysis

1. Open a new project in the software.
2. For data with normal distribution, use Student's *t*-test; for nonparametric data, use the Mann-Whitney *U* test.
3. Paste the values obtained from the experimental results in the table.

4. Select **descriptive statistics** and choose the option **column statistics [all tests]** to analyze data distribution.
NOTE: If the data follow Gaussian distribution, choose *t*-test to examine samples by comparing two pairs. If the distribution is non-Gaussian, analyze data using the Mann-Whitney *U* test with central tendency measures (means or medians) and measures of variation.
5. Choose the **best graph** option for optimal data representation.

Representative Results

This report investigates the role of DCs in *Leishmania* infection using flow cytometry and confocal microscopy. Initially, the phenotypic profile of the human monocyte-derived DC was established. Notably, the obtained CD11c⁺ dendritic cell populations were positive for CCR7, CD209, CD80, CD1a, and HLA-DR. The results indicate that the expression of these markers in DC populations is profoundly impacted by *Leishmania* infection. Infected DCs exhibited augmented CD80, CD209, CCR7, and HLA-DR expression. However, downregulation of CD1a was also

observed in *L. amazonensis*-infected DCs. Additionally, principal component analysis revealed substantial differences in the expression of these molecules (**Figure 1B-D**). The T-Distributed Stochastic Neighbor Embedding (t (tSNE) algorithm, a dimensionality reduction technique, was employed to better visualize how *Leishmania* infection affects the expression of maturation-related molecules.

Of note, the tSNE density plots show that infected DCs present differential HLA-DR and CD80 expression, which further suggests the upregulation of molecules involved in antigen presentation (**Figure 2**). To visualize DCs, F-actin immunostaining was performed by labeling the cells with fluorescent phalloidin. To observe *L. amazonensis* infection inside DCs, nuclear staining (DAPI) was used to compare *Leishmania*-infected cells with non-infected cells (**Figure 3**). The kinetic analysis of hDC infection involving *L. amazonensis* promastigotes (10:1) indicates the percentage of infected cells and the number of parasites per infected cell at 4 h after infection. Cells were then washed and re-incubated for 6, 12, or 24h. On average, 4-5 parasites were observed per DC in ~55% of the infected cells.

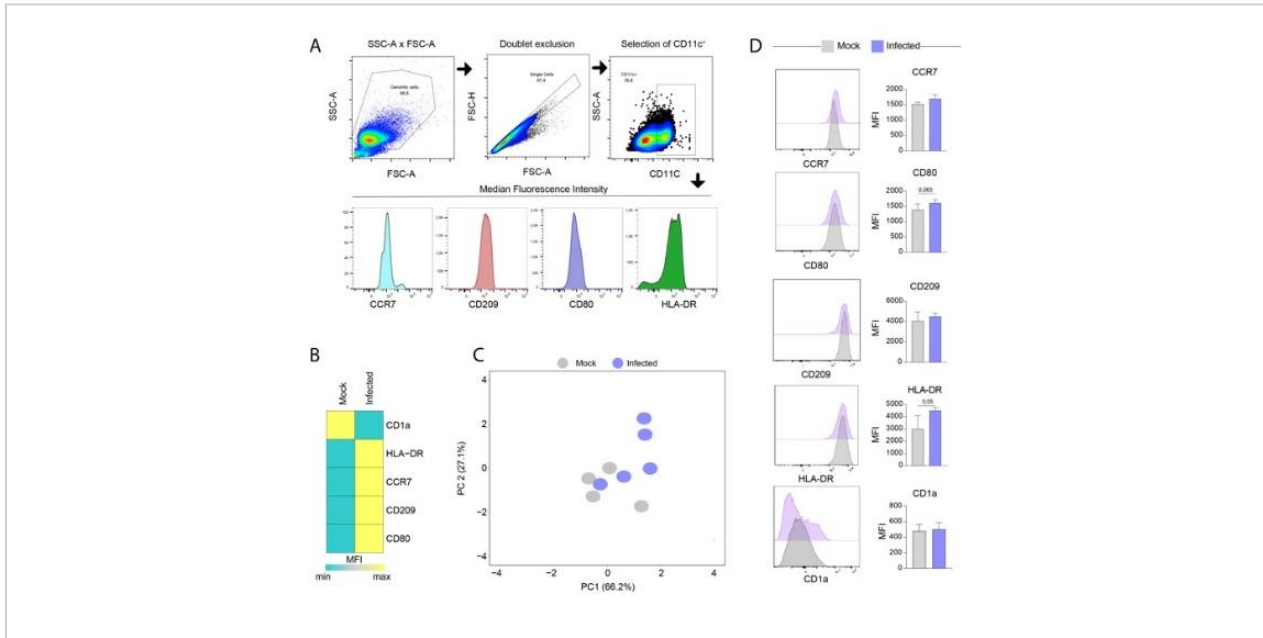


Figure 1: Marker-based human DC characterization after *Leishmania amazonensis* infection; gating strategy for selection of CD11c⁺. (A) Assessment of costimulatory molecule MFI. (B) Heatmap and (C) principal component analysis of costimulatory molecule MFI values. (D) Representative histograms and scatter plots depicting MFI for each molecule. Abbreviations: DC = dendritic cell; MFI = median fluorescence intensity; SSC-A = side scatter area; FSC-A = forward scatter area; FSC-H = forward scatter height. [Please click here to view a larger version of this figure.](#)

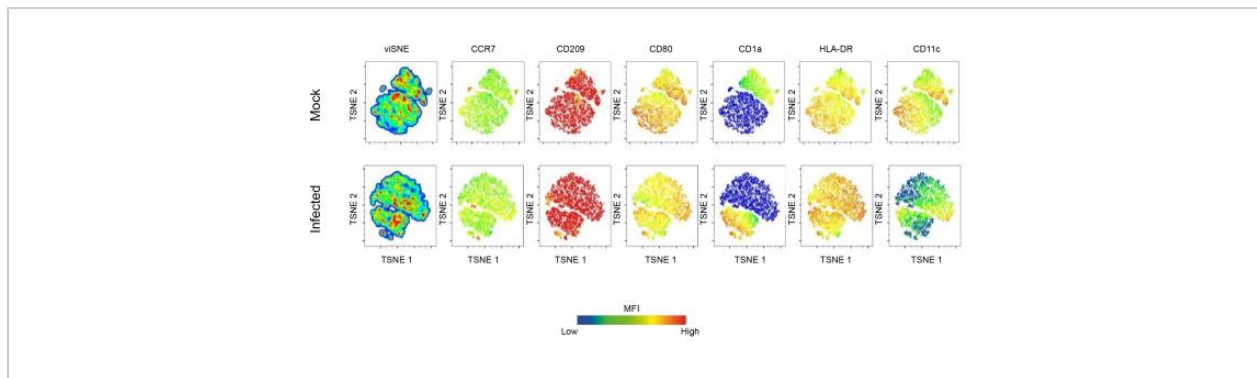


Figure 2: *Leishmania*-induced DC surface molecular expression. In-depth characterization of DC maturation landscape induced by *Leishmania* infection. Briefly, monocyte-derived DCs were obtained after 7 days of differentiation with GM-CSF and IL-4. Fully differentiated DCs were infected with *L. amazonensis* and then stained with a panel of maturation FACS antibodies. TSNE, a dimensionality reduction technique, was employed to perform a high dimensional analysis of flow cytometry data. Abbreviations: DC =dendritic cell; GM-CSF = granulocyte-macrophage colony-stimulating factor; FACS = fluorescence-activated cell sorting; IL-4 = interleukin-4; TSNE = T-Distributed Stochastic Neighbor Embedding. [Please click here to view a larger version of this figure.](#)

human dendritic cell; DAPI = 4',6-diamidino-2-phenylindole; DIC = differential interference contrast. [Please click here to view a larger version of this figure.](#)

MACS buffer
EDTA 2 mM
PBS 1x
BSA 0.5%
Phosphate-buffered saline (PBS) 1x
Ammonium chloride solution: 0.134 g of NH ₄ Cl
Saponin 15%
150 mg of saponin in 1 mL of 1x PBS
1 mL of 15% saponin in 100 mL of 1x PBS
10% Bovine serum albumin
100 mg – 1 mL → 10%
1 g – 100 mL → 1%
For 10% BSA - 1 g of BSA in 10 mL of 1x PBS
1x PBS/ 1% BSA / 0.15% Saponin: 20 mL of 1x PBS
2 mL of 10% BSA
200 µL of 15% Saponin
1x PBS/ 3% BSA / 0.15% Saponin: 20 mL of 1x PBS ; 6 mL of 10% BSA ; 200 µL 15% Saponin
1x PBS / 0.3% BSA / 0.15% Saponin: 20 mL PBS; 0.6 mL of 10% BSA; 200 µL of 15% Saponin

Table 1: Compositions of solutions used in this protocol.

Discussion

Leishmaniasis is a severe public health problem worldwide. The pathogenesis of this disease is quite complex, and the mechanisms favoring parasite survival in vertebrate hosts remain elusive¹⁷. DCs are professional antigen-presenting cells found throughout the body, including filtering and lymphoid organs. Following antigen capture and processing,

immature DCs undergo a complex maturation process that culminates in their migration to lymph nodes, where these cells are responsible for presenting antigens to T lymphocytes¹⁸. This maturation process is characterized by increased MHC-class II molecular expression, notably CD11c, CD86, CD80, and CD1a¹⁹. In leishmaniasis,

monocyte-derived DCs phagocytose parasites and deliver the cargo to dLNs, inducing antigen presentation to T cells¹³.

This paper describes a concise protocol that enables the investigation of the impact of *Leishmania* on the immunobiological functioning of DCs in an *in vitro* model of infection. This protocol encompasses the use of state-of-the-art immunological techniques, including the obtaining and differentiation of human monocytes into DCs, as well as DC characterization via flow cytometry and confocal microscopy¹⁴. Initially, monocytes were purified from the PBMCs of healthy donors. The use of a positive selection method, such as anti-human CD14 microbeads, ensures a high degree of purity (>95%) of differentiated moDCs. These cells are incubated with anti-human CD14 microbeads that bind the antibody to CD14-positive cells in the sample, which are then separated by a high-gradient magnetic field inside the column of a magnetic cell separator²⁰.

Notably, the *in vitro* differentiation of human monocytes into DCs requires the addition of GM-CSF and IL-4. GM-CSF is a pleiotropic growth factor that not only downregulates the expression of macrophage colony-stimulating factor (M-CSF) but also inhibits M-CSF-induced differentiation into macrophages. In addition, IL-4 acts by inhibiting macrophage colony formation, considered a suitable method for culturing moDCs *in vitro*²¹. The present protocol proposes the use of flow cytometry to assess DC differentiation by analyzing the expression of maturation-related markers, such as CCR7, CD209, CD80, CD1a, HLA-DR, and CD11c. Additionally, high-dimensional flow cytometry techniques (e.g., t-SNE) are used to precisely identify expression patterns influenced by *Leishmania* infection²². It is crucial to optimize the antibody dilution during immunostaining.

Confocal microscopy constitutes an excellent tool to analyze and quantify fluorescence intensity in human cells²³. It is important to note that this assay requires careful manipulation before the image acquisition such as washing the coverslips gently and avoiding exposure to light. The main limitation of this method is the acquisition of suitable image resolution in confocal microscopy, which requires experience with microscopy manipulation. However, this method can be used to study the response of DCs to infection by different pathogens. Alternatively, the steps described in this protocol can also be extrapolated to other models of pathogen infection.

Disclosures

The authors have no conflicts of interest to disclose.

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References

1. Torres-Guerrero, E., Qunitanilla-Cedillo, M. R., Ruiz-Esmenhjoud, J., Arenas, R. Leishmaniasis: a review. *F1000 Research*. **6**, 750 (2017).
2. Pace, D. Leishmaniasis. *Journal of Infection*. **69** (Suppl 1), S10-8 (2014).
3. Reithinger, R. et al. Cutaneous leishmaniasis. *Lancet. Infectious Diseases*. **7** (9), 581-596 (2007).

4. Kaye, P., Scott, P. Leishmaniasis: complexity at the host-pathogen interface. *Nature Reviews. Microbiology*. **9** (8), 604-615 (2011).
5. Handman, E., Bullen, D. V. Interaction of Leishmania with the host macrophage. *Trends in Parasitology*. **18** (8), 332-334 (2002).
6. Burza, S., Croft, S. L., Boelaert, M. Leishmaniasis. *Lancet*. **392** (10151), 951-970 (2018).
7. Kamhawi, S. Phlebotomine sand flies and Leishmania parasites: friends or foes? *Trends in Parasitology*. **22** (9), 439-445 (2006).
8. Guilliams, M. et al. Unsupervised high-dimensional analysis aligns dendritic cells across tissues and species. *Immunity*. **45** (3), 669-684 (2016).
9. Moll, H. Dendritic cells and host resistance to infection. *Cellular Microbiology*. **5** (8), 493-500 (2003).
10. Sundquist, M., Rydstrom, A., Wick, M. J. Immunity to Salmonella from a dendritic point of view. *Cellular Microbiology*. **6** (1), 1-11 (2004).
11. Saban, D. The chemokine receptor CCR7 expressed by dendritic cells: A key player in corneal and ocular surface inflammation. *Ocular Surface Journal*. **12**, 87-99 (2014).
12. Relloso, M. et al. DC-SIGN (CD209) Expression is IL-4 dependent and it is negatively regulated by IFN, TGF and inflammatory agents. *Journal of Immunology*. **168** (6), 2634-2643 (2002).
13. Leon, B., Lopez-Bravo, M., Ardavin, C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity*. **26** (4), 519-531 (2007).
14. Gordon, S. Phagocytosis: An immunobiologic process. *Immunity*. **44** (3), 463-475 (2016).
15. Ashok, D., Acha-Orbea, H. Timing is everything: dendritic cell subsets in murine Leishmania infection. *Trends in Parasitology*. **30** (10), 499-507 (2014).
16. Carvalho, A. K. et al. *Leishmania* (V.) *braziliensis* and *L. (L.) amazonensis* promote differential expression of dendritic cells and cellular immune response in murine model. *Parasite Immunology*. **34** (8-9), 395-403 (2012).
17. Bates, P. A. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. *International Journal of Parasitology*. **37** (10), 1097-1106 (2007).
18. Cervantes-Barragan, L. et al. Plasmacytoid dendritic cells control T-cell response to chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America*. **109** (8), 3012-3017 (2012).
19. Caux, C. et al. Activation of human dendritic cells through CD40 cross-linking. *Journal of Experimental Medicine*. **180** (4), 1263-1272 (1994).
20. Suzuki, H. et al. Activities of granulocyte-macrophage colony-stimulating factor and interleukin-3 on monocytes. *American Journal of Hematology*. **75** (4), 179-189 (2004).
21. Akira, S., Takeda, K. Toll-like receptor signalling. *Nature Reviews. Immunology*. **4** (7), 499-511 (2004).
22. McKinnon, K. M. Flow cytometry: An overview. *Current Protocols in Immunology*. **120**, 5.1.1-5.1.11 (2018).
23. Sanderson, M. J. et al. Fluorescence microscopy. *Cold Spring Harbor Protocols*. **10**, 1795 (2014).

Manuscrito 4

Investigating the phagocytosis of *Leishmania* using confocal microscopy

Abstract: Phagocytosis is an orchestrated process that involves distinct steps: recognition, binding, and internalization. Professional phagocytes take up *Leishmania* parasites by phagocytosis, consisting of recognizing ligands on parasite surfaces by multiple host cell receptors. Binding of *Leishmania* to macrophage membranes occurs through complement receptor type 1 (CR1) and complement receptor type 3 (CR3) and Pattern Recognition Receptors. Lipophosphoglycan (LPG) and 63 kDa glycoprotein (gp63) are the main ligands involved in macrophage-*Leishmania* interactions. Following the initial recognition of parasite ligands by host cell receptors, parasites become internalized, survive, and multiply within parasitophorous vacuoles. The maturation process of *Leishmania*-induced vacuoles involves the acquisition of molecules from intracellular vesicles, including monomeric G protein Rab 5 and Rab 7, lysosomal associated membrane protein 1 (LAMP-1), lysosomal associated membrane protein 2 (LAMP-2), and microtubule-associated protein 1A/1B-light chain 3 (LC3). Here, we describe methods to evaluate the early events occurring during *Leishmania* interaction with the host cells using confocal microscopy, including (i) binding (ii) internalization, and (iii) phagosome maturation. By adding to the body of knowledge surrounding these determinants of infection outcome, we hope to improve the understanding of the pathogenesis of *Leishmania* infection and support the eventual search for novel chemotherapeutic targets.

Keywords: Phagocytosis; *Leishmania*; Confocal microscopy

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Investigating the Phagocytosis of *Leishmania* using Confocal Microscopy

Amanda R. Paixão^{*1}, Beatriz R. S. Dias^{*1}, Luana C. Palma¹, Natália M. Tavares¹, Cláudia I. Brodskyn¹, Juliana P. B. de Menezes¹, Patricia S. T. Veras^{1,2}

¹Laboratory of Host-Parasite Interaction and Epidemiology, Gonçalo Moniz Institute ²National Institute of Science and Technology of Tropical Diseases - National Council for Scientific Research and Development (CNPq)

*These authors contributed equally

Corresponding Author

Patricia S. T. Veras
patricia.veras@fiocruz.br

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Abstract

Phagocytosis is an orchestrated process that involves distinct steps: recognition, binding, and internalization. Professional phagocytes take up *Leishmania* parasites by phagocytosis, consisting of recognizing ligands on parasite surfaces by multiple host cell receptors. Binding of *Leishmania* to macrophage membranes occurs through complement receptor type 1 (CR1) and complement receptor type 3 (CR3) and Pattern Recognition Receptors. Lipophosphoglycan (LPG) and 63 kDa glycoprotein (gp63) are the main ligands involved in macrophage-*Leishmania* interactions. Following the initial recognition of parasite ligands by host cell receptors, parasites become internalized, survive, and multiply within parasitophorous vacuoles. The maturation process of *Leishmania*-induced vacuoles involves the acquisition of molecules from intracellular vesicles, including monomeric G protein Rab 5 and Rab 7, lysosomal associated membrane protein 1 (LAMP-1), lysosomal associated membrane protein 2 (LAMP-2), and microtubule-associated protein 1A/1B-light chain 3 (LC3).

Here, we describe methods to evaluate the early events occurring during *Leishmania* interaction with the host cells using confocal microscopy, including (i) binding (ii) internalization, and (iii) phagosome maturation. By adding to the body of knowledge surrounding these determinants of infection outcome, we hope to improve the understanding of the pathogenesis of *Leishmania* infection and support the eventual search for novel chemotherapeutic targets.

Introduction

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*, resulting in a broad spectrum of clinical manifestations in the vertebrate

host, including cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis¹. The World Health Organization (WHO) estimates that over one billion people

are at risk, with more than one million new cases reported per year².

Leishmania spp. are obligate intracellular protozoans that survive inside host cells, including monocytes, macrophages and dendritic cells³. *Leishmania*-macrophage interaction is a complex process that involves multiple host cell receptors and parasite ligands either through direct interaction or by opsonization involving complement receptors^{4,5}. Classical surface receptors, such as CR1, CR3, mannose-fucose, fibronectin, toll-like and scavenger receptors, mediate parasite attachment to macrophages^{6,7,8}. These receptors recognize molecules on the surface of *Leishmania*, including the 63 kDa glycoprotein (gp63) and glycolipid lipophosphoglycan (LPG)⁹. These are the most abundant molecules on the surface of promastigotes and play an essential role in the subversion of host immune response, favoring the establishment of parasite infection in mammalian cells¹⁰. After parasite surface ligands bind to macrophage receptors, F-actin accumulates on mammalian cell surfaces, surrounding parasites as they are phagocytosed. Subsequently, this leads to the formation of a parasite-induced compartment termed parasitophorous vacuole (PV), which presents phagolysosomal features¹¹. Once inside these phagolysosomes, parasites undergo several alterations essential to survival and multiplication³.

The biogenesis of PVs is a highly regulated membrane trafficking process critical to the intracellular survival of this pathogen¹². The formation of this compartment results from sequential fusion events between phagosomes and compartments of the host endocytic pathway. Classical cell biology studies have revealed that the maturation of PVs involves the acquisition of monomeric G protein Rab 5 and Rab 7 proteins, which are mainly associated with

early and late endosome maturation, respectively¹³. In addition, these compartments acquire lysosome-associated membrane proteins 1 and 2 (LAMP 1, LAMP 2), the principal protein constituents of the lysosomal membrane and microtubule-associated protein 1A/1B-light chain 3 (LC3), an autophagosome marker¹⁴. Despite apparent similarities, the kinetics of PV formation^{15,16} and the morphology of these compartments vary depending on *Leishmania* species. For example, infection caused by *L. mexicana* or *L. amazonensis* induces the formation of large compartments containing a great number of parasites¹⁷. By contrast, other species, such as *L. braziliensis* and *L. infantum*, form smaller vacuoles that normally contain only one or two parasites in each vacuole¹⁸.

Despite this knowledge surrounding host cell-*Leishmania* interaction, the initial events triggered by contact between host receptors and parasite ligands have not been fully elucidated. These events are known to be determinants of the outcome of parasite infection and are dependent on parasite species, the type of host cell receptors recruited to recognize parasites and the activation of macrophage signaling pathways^{19,20}. Therefore, it is essential to identify the molecules involved in the biogenesis of *Leishmania*-induced PVs and determine the role(s) played by these molecules in infection establishment and outcome. Here, we describe a method of monitoring early events occurring during the phagocytosis of *Leishmania*, including binding, internalization, phagosome formation and maturation. This work could aid in clarifying the participation of PLC, Akt, Rab5, Rab7 and LC3 in the formation of PVs induced by different *Leishmania* species. Importantly, this protocol can be used to investigate the participation of other proteins involved in PV maturation. Future studies will expand the knowledge surrounding mechanisms involved in *Leishmania*-

host cell interaction and contribute to the design of novel chemotherapeutic strategies.

Protocol

Cells were obtained from healthy donors following the approval of procedures by the National Research Ethics Committees (ID: 94648218.8.0000.0040).

1. Cell cultures

1. Human monocyte-derived macrophages

NOTE: To obtain human monocyte-derived macrophages for *in vitro* differentiation into macrophages, collect blood from healthy donors and purify peripheral blood mononuclear cells (PBMC) as described by D. English and B. R. Andersen²¹.

1. After collecting peripheral blood (50 mL), pour it into a heparinized tube and then dilute the blood 1:1 in a phosphate buffer solution (PBS) at room temperature. Gently place diluted heparinized blood on top of previously distributed density gradient medium.

2. Centrifuge the tubes at $252 \times g$ for 30 min at 24°C to avoid hemolysis.

NOTE: Set centrifuge break-off to avoid mixing of gradient layers. After centrifugation, discontinuous gradient layers are formed from the bottom to the top: erythrocytes, density gradient medium, PBMC ring and plasma.

3. Transfer the PBMC ring, located between the density gradient medium and plasma layers, to a new tube and fill with PBS to wash out excess density gradient medium.

4. Wash cells once and centrifuge at $190 \times g$ for 10 min at 4°C .

5. Discard the supernatant and resuspend pellet in 1 mL of complete RPMI medium.

6. Count the cells and plate 2×10^6 cells in 500 mL of Roswell Park Memorial Institute (RPMI) supplemented with 25 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid] (HEPES), 2 g/L sodium bicarbonate, 2 mM glutamine, 20 g/L ciprofloxacin and 10% inactivated Fetal Bovine Serum (FBS) (complete RPMI medium) for 7 days at 37°C under 5% CO_2 in a 24-well plate to allow monocytes to differentiate into macrophages by adhesion.

2. THP-1 cultures

1. Grow THP-1 cell line at a concentration of 2×10^5 cells in 10 mL of complete RPMI medium in 75 cm² culture flask.

2. Maintain cell cultures in an incubator at 37°C under 5% CO_2 for 7 days.

3. Centrifuge cells at $720 \times g$ for 10 min at 4°C and resuspend the pellet in complete RPMI medium.

4. Count cells in a Neubauer chamber.

5. Plate cells on 13 mm glass coverslips at a concentration of 2×10^5 cells per well in 500 μL of complete RPMI medium containing 100 nM phorbol myristate acetate (PMA) at 37°C under 5% CO_2 to allow differentiation of THP1 cells into macrophages.

6. After three days, wash twice cells with 0.9% NaCl solution to remove medium containing PMA.

7. Incubate differentiated THP-1 cells in PMA-free complete RPMI medium at 37 °C under 5% CO₂ for an additional 2 days before starting experimentation.

2. Parasite cultures and CellTracker Red staining

NOTE: To visualize parasites through fluorescence microscopy, perform staining using CellTracker Red fluorescent dye (CMTPX). Alternatively, other markers, including carboxyfluorescein can be used in accordance with manufacturer instructions or promastigotes constitutively expressing GFP, RFP, or other fluorescent reporter genes. Parasites used to infect cells are those at stationary phase of growth obtained from a promastigote axenic culture of no more than 7 passages.

1. Grow *Leishmania* spp. promastigotes at 1×10^5 parasites per 1,000 μ L of medium in a cell culture flask containing 5 mL of Schneider's medium supplemented with 50 μ g/mL gentamicin and 10% FBS.
2. After incubating parasite axenic cultures in a biochemical oxygen demand (B.O.D.) at 24 °C, perform daily counting in a Neubauer chamber. Check for parasite form (thin, elongated) and mobility for 5 days. Parasites are considered in stationary phase of growth when two consecutive counts with 8 hours of interval display similar amounts.
3. Upon reaching the stationary phase of growth, incubate the parasites in 4 mL of 0.9% NaCl solution with 1 μ M CMTPX for 15 min at 37 °C under 5% CO₂ avoiding contact with light.
4. Add FBS at a 1:1 proportion and incubate parasite suspension for an additional 1 min.
5. Wash parasites thrice with PBS, followed by centrifugation at $1,781 \times g$ for 10 min.
6. Resuspend parasite pellet in 1,000 μ L of RPMI complete medium.
7. Count parasites in a Neubauer chamber.

3. Assessment of *Leishmania* binding to macrophages

1. Seed 2×10^5 THP-1 cells or human monocyte-derived macrophages in 500 μ L of complete RPMI medium per well on a 24-well plate with 13 mm glass coverslips.
2. Cultivate cells at 37 °C under 5% CO₂ for 24 h.
3. Wash the cells twice with 0.9% NaCl solution and incubate in complete RPMI medium at 4 °C for 10 min.
4. Add stationary phase promastigotes as described by A. L. Petersen²² at a 10:1 ratio to well plates, and then centrifuge at $720 \times g$ for 5 min under 4 °C.
5. Incubate at 4 °C for 5 min.
6. Wash the cells twice with 0.9% NaCl solution to remove any non-internalized promastigotes.
7. Fix the cells in 4% paraformaldehyde for 15 min at room temperature.
8. Incubate the coverslips with 15 mM NH₄Cl for 15 min at room temperature.
9. Wash thrice with PBS 0.15% bovine serum albumin (BSA). Incubate with blocking solution (3% BSA in PBS) for 1 h at room temperature.
10. Wash thrice with PBS and then permeabilize with 0.15% PBS-Saponin for 15 min at room temperature.
11. Add phalloidin (diluted 1:1,200) for 1 h at room temperature and protect from light.

12. Mount coverslips using mounting media.
13. Acquire images via a confocal fluorescence microscope using a 63×/1.4 objective.

4. Assessment of *Leishmania* phagocytosis by macrophages

1. Seed 2×10^5 THP-1 cells or human monocyte-derived macrophages in 500 μ L of complete RPMI medium per well on a 24-well plate with 13 mm glass coverslips.
2. Cultivate cells for 24 h at 37 °C under 5% CO₂.
3. Wash cells twice in 0.9% NaCl solution and incubate in complete RPMI medium in 24-well plate at 4 °C for 10 min.
4. Add stationary phase *Leishmania* spp. as described by A. L. Petersen²² at a 10:1 (parasite:host cell) ratio, and then centrifuge at 720 $\times$ g for 10 min under 4 °C.
5. Incubate cells at 4 °C for 5 min.
6. Wash the cells twice with 0.9% NaCl solution to remove any non-internalized promastigotes.
7. Incubate the cells in supplemented RPMI medium at 37 °C for 1 h.
8. Fix the cells with 4% paraformaldehyde for 15 min.
9. Mount coverslips using preferred mounting media.
10. Count no less than 400 cells in random fields under a fluorescence microscope using a 100×/1.4 objective.

5. Evaluation of *Leishmania*-induced vacuole maturation

NOTE: THP-1 cell transfection should be performed as described by M. B. Maess, B. Wittig and S. Lorkowski²³. Here we summarize this protocol, with minimal modifications. Nucleofection is a specific transfection method

that requires a nucleofector. As an alternative method, cells can be transfected using lipofectamine²⁴ and lentivirus transduction²⁵.

1. To investigate the biogenesis of *Leishmania*-induced PV, transfect THP1 cells with PLC^{26,27}, Akt^{26,27}, Rab 5^{28,29,30} or Rab 7^{28,29,31} plasmids.

NOTE: This methodology can be used to transfect THP-1 cells with other genes than those listed above.

2. Seed THP-1 cells at 1.5×10^7 in 75 cm² tissue culture flasks containing 10 mL complete RPMI medium supplemented with 100 ng/mL PMA and 50 μ M 2-mercaptoethanol for 48 h.
3. Wash cells once in 0.9% NaCl solution.
4. Detach cells using a non-enzymatic cell dissociation solution and centrifuge (250 $\times$ g) for 5 min at room temperature.
5. Resuspend THP-1 cells in 1 mL of RPMI medium and perform counts in a Neubauer chamber.
6. Centrifuge THP-1 cells again at 250 $\times$ g for 10 min at room temperature. Discard the supernatant.
7. Resuspend 2×10^6 cells in 100 μ L of Nucleofector solution and incubate with 0.5 μ g of the plasmid coding for the protein of interest, tagged with a fluorescent protein.
8. Transfer the suspension containing THP-1 cells and nucleic acid to the Nucleofector cuvette.
9. Transfect THP1 cells using Nucleofector Program Y-001.
10. Recover the transfected cells (2×10^6) and seed in 500 μ L RPMI medium on 24-well plates with 13 mm glass coverslips (4 wells/transfection).

11. Incubate THP-1 cells in complete RPMI medium at 37 °C for 0.5, 2, 4, 6, 12 and 24 h.
12. Repeat steps 3.13 and 3.13.

6. Evaluation of the recruitment of LC3 to *Leishmania* spp. PVs

NOTE: The autophagic membrane marker LC3 can be used to investigate whether phagosomes present autophagic features. LC3 recruitment to *Leishmania*-induced PVs can be assessed during infection by immunolabelling cells with the anti-LC3 antibody, as previously described by C. Matte³² and B. R. S. Dias³³.

1. Seed 2×10^5 THP-1 cells or human monocyte-derived macrophages in 500 μ L complete RPMI medium on a 24-well plate with 13 mm glass coverslips.
2. Cultivate cells for 24 h at 37 °C under 5% CO₂.
3. Wash cells twice in 0.9% NaCl solution and incubate in complete RPMI medium.
4. Add stationary phase *Leishmania* spp. promastigotes as described by A. L. Petersen²² at a 10:1 (parasite: host cells) ratio and centrifuge cells at 720 $\times$ g for 5 min under 4 °C.
5. Incubate at 37 °C for 30 min or 4 h. Then wash twice and fix the cells to evaluate the LC3 recruitment to *Leishmania*-induced PV membranes at the early stages of infection.
 1. Alternatively, to assess LC3 recruitment to PV membranes at later stages of infection, wash twice another macrophage group at 4 h of infection to remove any non-internalized promastigotes. Incubate infected cells in complete RPMI medium for

an additional 12 h and 24 h, to finally wash twice and fix.

NOTE: Fixed cells can be kept in PBS or 0.9% NaCl solution at 4 °C until labeling.

6. Simultaneously block and permeabilize the fixed cells in 0.1% Triton X-100, 1% BSA, 20% normal goat serum, 6% non-fat dry milk, and 50% FBS for 20 min at room temperature.
7. Incubate the cells with anti-LC3 antibody (1: 200) diluted in PBS for 2 h at room temperature.

NOTE: As a negative control of the immunostaining, a group of cells should be incubated with immunoglobulin G (IgG) from the animal of primary antibody origin in a concentration equivalent to that used for the primary antibody.
8. Wash the cells thrice with 0.9% NaCl solution at room temperature.
9. Incubate the cells with AlexaFluor 488-conjugated goat anti-rabbit IgG (1:500) or the preferred fluorescent-dye conjugated secondary antibodies for 1 h at room temperature.
10. Wash the cells thrice with 0.9% NaCl solution at room temperature.
11. Mount coverslips using preferred mounting media.
12. Acquire images via confocal fluorescence microscope using a 63x/1.4 objective.

7. Confocal microscopy acquisition and Fiji quantification

NOTE: Acquiring immunofluorescence images should be performed using a confocal laser scanning microscope. To reach a better resolution, use an oil-immersion 63x objective lens.

1. Leave the 13 mm glass coverslips at room temperature and protect them from the light at least 30 min before the acquisition.
2. Clean the coverslips with an absorbent tissue.
3. Add a drop of immersion oil to the objective and add the slide.
4. Move the objective up until the oil touches the slide.
5. Observe and adjust the focus on the microscope and choose the option 63x objective with oil.
6. Open the Leica program and adjust the lasers in the 488, 552, and 405 wavelengths.
7. Select the image resolution 1,024 x 1,024.
8. Click on the **Live** button, set the Z stack, and press the **Begin** option. Then, do it again and press the **End** button. We recommend 20 μm for slice thickness to get confocal images with good resolutions.
9. Wait for the image acquisition, and then select the option "Maximum Projection" in the Leica tools.
10. Save the experiment.
11. Export the lif or tiff format images to a computer and open the FIJI program.
12. Open the experiment and set the view stack with the hyper stack. Then select open files individually and stitch tiles.
13. Select the free hands tool in the Fiji toolbar and trace the cell carefully by hand.
14. Press the **Analyze** button and measure to visualize the fluorescence intensity.
15. Repeat this process to each cell per group.

16. Save the measurements and export them to a spreadsheet editor.
17. Add this data to a statistical analysis program and do the statistical analysis.

8. Statistical analysis

NOTE: For data analysis and graphics, use a statistical analysis program.

1. Open the program.
2. Insert the obtained data and test the normality parameters.
3. For data with normal distribution, use the Student *t*-test and for nonparametric tests, Mann-Whitney test.
4. Consider data with a statistically significant difference when the *p-value* is less than 0.05.
5. Prepare graphics representing the data, with central tendency measures (mean or median) and variation measures.

Representative Results

This report aims to evaluate the early events occurring during the phagocytosis of *L. braziliensis* isolated from patients presenting *L. braziliensis*-LCL or *L. braziliensis*-DL form of CL. Using confocal microscopy, we investigated the main events associated with parasites' phagocytosis: binding, internalization, and phagosome maturation. We first evaluated the *L. braziliensis*-LCL or *L. braziliensis*-DL binding and phagocytosis by human monocyte-derived macrophages. The data show that both *L. braziliensis*-LCL and *L. braziliensis*-DL similarly bind to macrophages (**Figure 1**). Also, no differences were observed regarding *L. braziliensis*-LCL and *L. braziliensis*-DL phagocytosis by

host cells (**Figure 2**). Finally, we compared the recruitment of LC3 to the PVs induced by *L. braziliensis*-LCL or *L. braziliensis*-DL in infected cells. After 30 min, 4 and 12 h of infection, we observed similar percentages of LC3 decorated PVs in *L. braziliensis*-LCL and *L. braziliensis*-DL-infected macrophages (**Figure 3**). These representative results showed that *L. braziliensis*-LCL and *L. braziliensis*-

DL similarly interact with macrophages during binding, phagocytosis, and biogenesis of PVs, concerning the LC3 recruitment.

Microscopic images representing THP-1 cells efficiently transfected with PLC-GFP, Rab5-GFP, Rab7-GFP plasmids are shown in **Figure 4**.

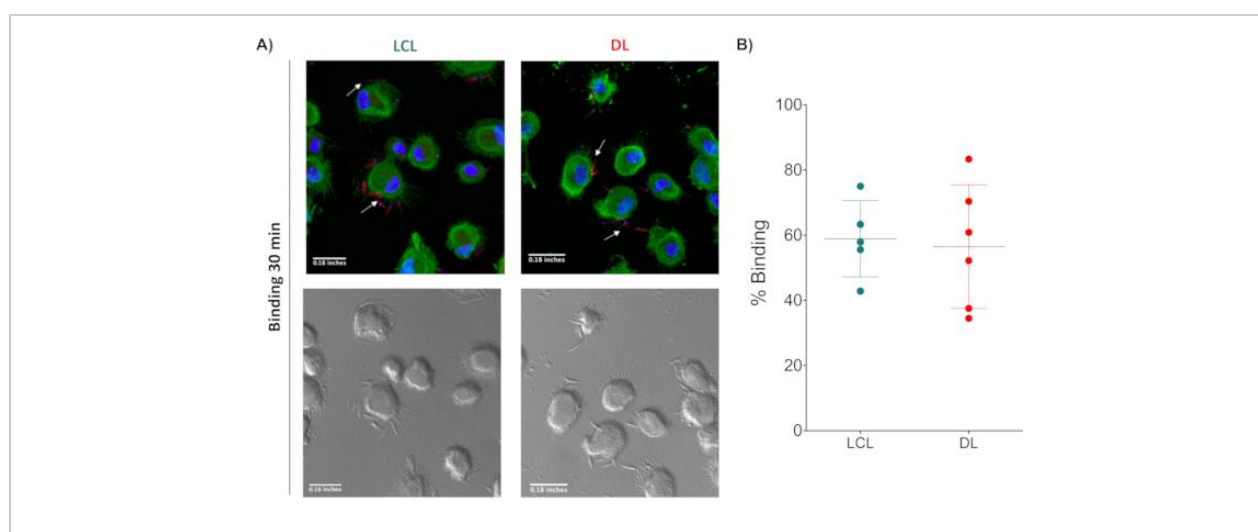


Figure 1. Evaluation of *L. braziliensis*-LCL and *L. braziliensis*-DL binding to human macrophages. Human monocyte-derived macrophages were infected with *L. braziliensis*-LCL- or *L. braziliensis*-DL. After 10 min at 4 °C, the binding was assessed by confocal microscopy. **(A)** Confocal microscopy images of *L. braziliensis*-LCL or *L. braziliensis*-DL (labeled with CMTPIX, red) binding to macrophages (labeled with phalloidin, green). For confocal microscopy, cell nuclei were labeled with DAPI (blue). Arrows depict *Leishmania*-macrophage binding. **(B)** Percentage of *Leishmania* binding to the macrophages. A total of 30 cells per group were analyzed. Data represent each replicate of one experiment performed in quintuplicate (unpaired *t* test, *p* > 0.05). [Please click here to view a larger version of this figure.](#)

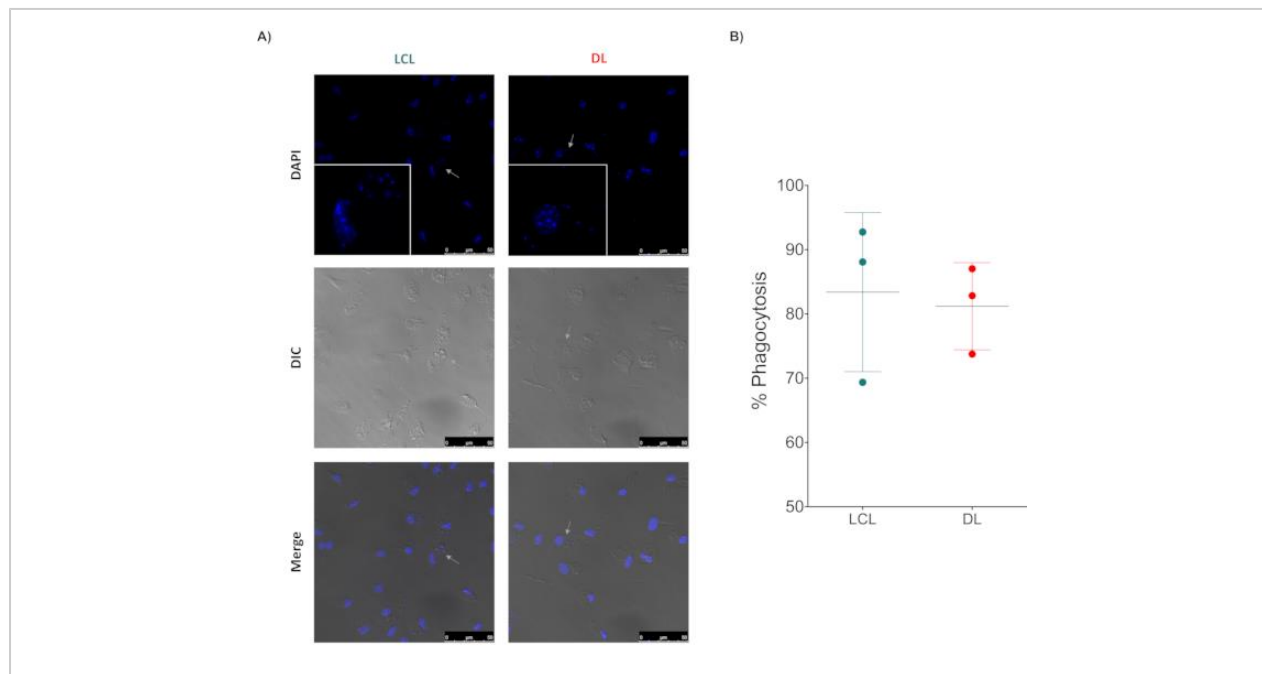


Figure 2. Evaluation of *L. braziliensis*-LCL and *L. braziliensis*-DL phagocytosis by human macrophages. Human monocyte-derived macrophages were incubated with *L. braziliensis*-LCL or *L. braziliensis*-DL for 10 min at 4 °C followed by additional 1 h at 37 °C. Cells were then analyzed by fluorescence microscopy by counting a total of 400 cells. **(A)** Confocal microscopy images of human macrophages infected by *L. braziliensis*-LCL or *L. braziliensis*-DL. For confocal microscopy, cell nuclei were labeled with DAPI (blue). Arrows depict *Leishmania* parasites nuclei. **(B)** Percentage of *Leishmania* phagocytosis. Circles represent data from each replicate of one experiment performed in triplicate (unpaired *t* test, $p > 0.05$). [Please click here to view a larger version of this figure.](#)

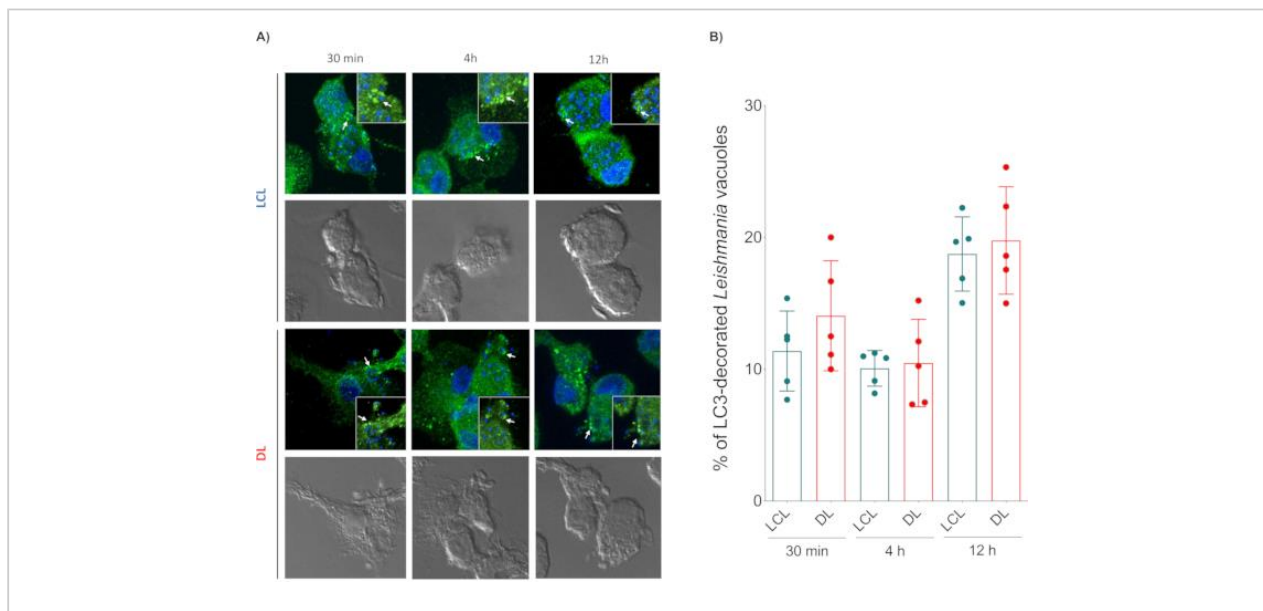


Figure 3. Assessment of LC3 recruitment to PVs induced by *L. braziliensis*-LCL or *L. braziliensis*-DL in macrophages. Human monocyte-derived macrophages were infected and then stained with anti-LC3 antibody for 30 min, 4 and 12 h. **(A)** Confocal microscopy images of *L. braziliensis*-LCL or *L. braziliensis*-DL-infected macrophages labeled with anti-LC3 followed by the secondary anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (green). For confocal microscopy, cell nuclei were labeled with DAPI (blue); **(B)** Percentage of *L. braziliensis*-LCL or *L. braziliensis*-DL-induced PVs decorated with LC3-II. A total of 30 cells per group were analyzed. The circles correspond to each randomly selected field analyzed (unpaired *t* test, $p > 0.05$). [Please click here to view a larger version of this figure.](#)

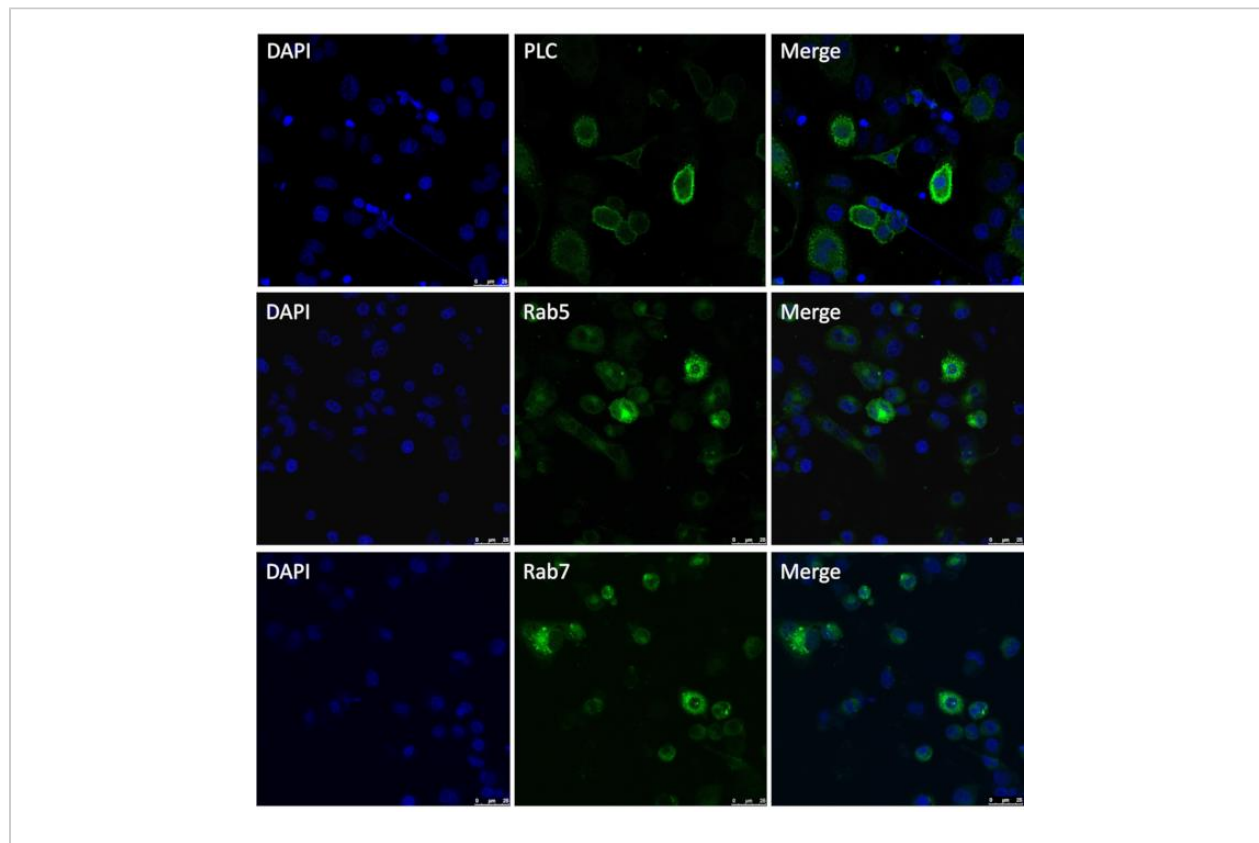


Figure 4. THP-1 cells expressing PLC, Rab5 or Rab7. After differentiating into macrophages, THP-1 cells were subjected to nucleofection with each gene of interest coupled to GFP fluorescent probes: PLC, Rab5 and Rab7. Subsequently, these cells were fixed, had the nucleus stained with DAPI (blue) and were observed under a confocal microscope using a 63x/1.4 objective. [Please click here to view a larger version of this figure.](#)

Discussion

Leishmania-macrophage interaction is a complex process and involves several steps that can influence disease development⁵. To better understand the mechanisms involved in the interaction of unopsonized *Leishmania* and host cells, we have described a protocol that employs confocal fluorescence microscopy to assess phagocytosis from early to late stages of *Leishmania* infection. The use of fluorescence techniques involving two

or more fluorophores to investigate cell biology mechanisms, including immunolabeling and the expression of fluorescent-labeled proteins, allows us to analyze the location of several proteins, as well as to simultaneously evaluate cell morphology. The advantages offered by these methods make them the best tools to monitor pathogen-host cell interaction³⁴.

To better understand the phagocytic process involving different particles, it is crucial to analyze this highly dynamic

process at the molecular level³⁵. Confocal-fluorescence microscopy has been used for decades to this end and has been shown to be an excellent tool for quantifying phagocytosis through the determination of numbers of internalized particles, or the types of proteins known to be involved in early stages of host-pathogen interaction³⁴. The present study proposed the use of confocal microscopy to analyze events occurring during the phagocytosis of *L. braziliensis* isolated from patients with different clinical forms (LCL and DL). This technique enables us to study cells expressing specific fluorescent proteins, including PLC, Akt, Rab 5, and Rab 7, and subsequently evaluate the participation of these proteins in the phagocytosis of *Leishmania* isolates to identify elements relevant to different infection outcomes.

The present study employed primary macrophages and THP1 cells to assess *L. braziliensis* phagocytosis at early stages of infection. The presently described protocol can also be used to study phagocytosis in *Leishmania* spp. by other phagocytes, including dendritic cells, monocytes, macrophage cell-lines, and neutrophils derived from human peripheral blood. During the parasite internalization process, a dynamic change in F-actin occurs at the cell membrane surface¹¹. We then labeled proteins located in the cell membrane using a specific marker of phagocytosis³⁶, such as fluorescent PLC, which allowed us to observe the binding stage of *Leishmania* to host cells, as shown in **Figure 4**. Staining parasites with fluorescent markers, such as CMTPIX or CSFE, is also crucial to assess parasite binding to host cells by immunofluorescence. It is worth noting that this assay requires careful execution: i) wash coverslips gently using washing solutions at room temperature (25 °C), otherwise,

samples can be damaged; ii) prepare reagent dilutions precisely; and iii) protect the samples from light³⁴.

A confocal microscope configured to the optimal laser excitement wavelength is capable of obtaining a high-quality sample image. Labeled cells can be stored for weeks in the dark at 4 °C or frozen until the time of analysis. The use of confocal microscopy to evaluate phagocytosis is limited by prolonged times of exposure and high intensity laser beams, which can damage samples, and, in some cases, lead to high levels of background detection in images^{35,37}.

In the present study, instead of using live imaging to follow the phagocytosis of *Leishmania* spp., we performed a kinetic study by fixing cells at several early times of infection (30 min, 4 h, and 12 h). It must be considered that live imaging offers some advantages, such as the potential to analyze the spatial and temporal dynamics of myriad cellular processes, including phagocytosis, and capturing details that are not observable in static images³⁴. However, live imaging requires that cells be healthy throughout the entire experimentation process, including controlling temperature, pH and oxygen conditions in a microscopic chamber. It is important to note that this cannot be reliably performed at several laboratories around the world.

The nucleofection protocol described has demonstrated efficacy in the transfection of THP-1 cells, as previously reported by M. B. Maess, B. Wittig and S. Lorkowski²³. In this process, it is crucial to gently detach cells to avoid cell damage or loss in cell viability. Based on our experience, we recommend using a non-enzymatic cell dissociation solution to detach cells from plates prior to performing transfection. The authors of the original protocol²³ state that the main limitations of this procedure are the need for cells to be in suspension during the nucleofection process, and the fact

that inadequate detachment can cause stress. Despite these limitations, the protocol does allow for reliable transfection, reaching a 90% successful transfection rate without losing cell viability.

The characterization of PVs using a set of endocytic markers, including PLC, Akt, Rab5, and Rab7, is essential to improving our understanding of *Leishmania* phagocytosis. Identifying new proteins that participate in PV biogenesis and comprehensively characterizing these compartments can clarify differences in macrophage response during *Leishmania* spp. infection. The contribution of our results to the body of knowledge surrounding *Leishmania* infection outcome will undoubtedly advance our understanding of the pathogenesis of *Leishmania* infection and support the eventual search for novel chemotherapeutic targets. It is worth noting that this technique can also be extended to other types of studies, including infection by bacteria, yeasts or bead engulfment by many types of cells^{38,39}.

Disclosures

The funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Goto, H., Lauletta Lindoso, J. A. Cutaneous and mucocutaneous leishmaniasis. *Infectious Disease Clinics of North America*. **26** (2), 293-307 (2012).
2. World Health, O. Control of the leishmaniasis. *World Health Organization Technical Report Series*. (949), xii-xiii, 1-186, back cover (2010).
3. Alexander, J., Russell, D. G. The interaction of *Leishmania* species with macrophages. *Advances in Parasitology*. **31**, 175-254 (1992).
4. Mosser, D. M., Rosenthal, L. A. *Leishmania*-macrophage interactions: multiple receptors, multiple ligands and diverse cellular responses. *Seminars in Cell Biology*. **4** (5), 315-322 (1993).
5. Awasthi, A., Mathur, R. K., Saha, B. Immune response to *Leishmania* infection. *Indian Journal of Medical Research*. **119** (6), 238-258 (2004).
6. Blackwell, J. M. Role of macrophage complement and lectin-like receptors in binding *Leishmania* parasites to host macrophages. *Immunology Letters*. **11** (3-4), 227-232 (1985).
7. Mosser, D. M., Edelson, P. J. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. *Journal of Immunology*. **135** (4), 2785-2789 (1985).
8. Gough, P. J., Gordon, S. The role of scavenger receptors in the innate immune system. *Microbes and Infection*. **2** (3), 305-311 (2000).

9. Russell, D. G., Wilhelm, H. The involvement of the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophages. *Journal of Immunology*. **136** (7), 2613-2620 (1986).
10. Handman, E., Goding, J. W. The *Leishmania* receptor for macrophages is a lipid-containing glycoconjugate. *EMBO J.* **4** (2), 329-336 (1985).
11. Holm, A., Tejle, K., Magnusson, K. E., Descoteaux, A., Rasmussen, B. *Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKC α and defective phagosome maturation. *Cellular Microbiology*. **3** (7), 439-447 (2001).
12. Vergne, I. *et al.* Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*. **102** (11), 4033-4038 (2005).
13. Courret, N., Lang, T., Milon, G., Antoine, J. C. Intradermal inoculations of low doses of *Leishmania major* and *Leishmania amazonensis* metacyclic promastigotes induce different immunoparasitic processes and status of protection in BALB/c mice. *International Journal for Parasitology*. **33** (12), 1373-1383 (2003).
14. Gutierrez, M. G. *et al.* Autophagy induction favours the generation and maturation of the *Coxiella*-replicative vacuoles. *Cellular Microbiology*. **7** (7), 981-993 (2005).
15. Dermine, J. F., Scianimanico, S., Prive, C., Descoteaux, A., Desjardins, M. *Leishmania* promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. *Cellular Microbiology*. **2** (2), 115-126 (2000).
16. Desjardins, M., Descoteaux, A. Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. *Journal of Experimental Medicine*. **185** (12), 2061-2068 (1997).
17. Antoine, J. C., Prina, E., Lang, T., Courret, N. The biogenesis and properties of the parasitophorous vacuoles that harbour *Leishmania* in murine macrophages. *Trends in Microbiology*. **6** (10), 392-401 (1998).
18. Alexander, J. *et al.* An essential role for IL-13 in maintaining a non-healing response following *Leishmania mexicana* infection. *European Journal of Immunology*. **32** (10), 2923-2933 (2002).
19. Aderem, A., Underhill, D. M. Mechanisms of phagocytosis in macrophages. *Annual Review of Immunology*. **17**, 593-623 (1999).
20. Olivier, M., Gregory, D. J., Forget, G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clinical Microbiology Reviews*. **18** (2), 293-305 (2005).
21. English, D., Andersen, B. R. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *Journal of Immunology Methods*. **5** (3), 249-252 (1974).
22. Petersen, A. L. *et al.* 17-AAG kills intracellular *Leishmania amazonensis* while reducing inflammatory responses in infected macrophages. *PLoS One*. **7** (11), e49496 (2012).
23. Maess, M. B., Wittig, B., Lorkowski, S. Highly efficient transfection of human THP-1 macrophages by nucleofection. *Journal of Visualized Experiments*. 10.3791/51960 (91), e51960 (2014).

24. Berges, R. *et al.* End-binding 1 protein overexpression correlates with glioblastoma progression and sensitizes to Vinca-alkaloids in vitro and in vivo. *Oncotarget*. **5** (24), 12769-12787 (2014).
25. Franco, L. H. *et al.* The Ubiquitin Ligase Smurf1 Functions in Selective Autophagy of Mycobacterium tuberculosis and Anti-tuberculous Host Defense. *Cell Host & Microbe*. **22** (3), 421-423 (2017).
26. Corbett-Nelson, E. F., Mason, D., Marshall, J. G., Collette, Y., Grinstein, S. Signaling-dependent immobilization of acylated proteins in the inner monolayer of the plasma membrane. *Journal of Cell Biology*. **174** (2), 255-265 (2006).
27. Yeung, T. *et al.* Receptor activation alters inner surface potential during phagocytosis. *Science*. **313** (5785), 347-351 (2006).
28. Romano, P. S., Gutierrez, M. G., Beron, W., Rabinovitch, M., Colombo, M. I. The autophagic pathway is actively modulated by phase II Coxiella burnetii to efficiently replicate in the host cell. *Cellular Microbiology*. **9** (4), 891-909 (2007).
29. Vieira, O. V. *et al.* Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. *Molecular and Cellular Biology*. **23** (7), 2501-2514 (2003).
30. Roberts, R. L., Barbieri, M. A., Ullrich, J., Stahl, P. D. Dynamics of rab5 activation in endocytosis and phagocytosis. *Journal of Leukocyte Biology*. **68** (5), 627-632 (2000).
31. Vitelli, R. *et al.* Role of the small GTPase Rab7 in the late endocytic pathway. *Journal of Biological Chemistry*. **272** (7), 4391-4397 (1997).
32. Matte, C. *et al.* Leishmania major Promastigotes Evade LC3-Associated Phagocytosis through the Action of GP63. *PLoS Pathogens*. **12** (6), e1005690 (2016).
33. Dias, B. R. S. *et al.* Autophagic Induction Greatly Enhances Leishmania major Intracellular Survival Compared to Leishmania amazonensis in CBA/j-Infected Macrophages. *Frontiers in Microbiology*. **9**, 1890 (2018).
34. Babcock, G. F. Quantitation of phagocytosis by confocal microscopy. *Methods in Enzymology*. **307**, 319-328 (1999).
35. Sanderson, M. J., Smith, I., Parker, I., Bootman, M. D. Fluorescence microscopy. *Cold Spring Harbor Protocols*. **2014** (10), pdb top071795 (2014).
36. Lennartz, M. R. Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis. *International Journal of Biochemistry & Cell Biology*. **31** (3-4), 415-430 (1999).
37. Rashidfarrokhi, A., Richina, V., Tafesse, F. G. Visualizing the Early Stages of Phagocytosis. *Journal of Visualized Experiments*. 10.3791/54646 (120) (2017).
38. Ramarao, N., Meyer, T. F. Helicobacter pylori resists phagocytosis by macrophages: quantitative assessment by confocal microscopy and fluorescence-activated cell sorting. *Infection and Immunity*. **69** (4), 2604-2611 (2001).
39. Bain, J., Gow, N. A., Erwig, L. P. Novel insights into host-fungal pathogen interactions derived from live-cell imaging. *Seminars in Immunopathology*. **37** (2), 131-139 (2015).