Universidade Estadual de Londrina

TAYLON FELIPE SILVA

EFEITO DO DIABETES E EXPOSIÇÃO HIPERGLICÊMICA NA SUSCEPTIBILIDADE À LEISHMANIOSE EXPERIMENTAL

Londrina
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Orientadora: Profª. Drª. Ivete Conchon-Costa

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Londrina, 04 de março de 2022.

Dedico este trabalho a minha mãe, por ser meu alicerce.

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“Eu sei. Tá tudo errado! Nós nem deveríamos estar aqui… mas estamos. É como nas grandes histórias Senhor Frodo. As realmente importantes. Eram cheias de perigo e de escuridão. E, às vezes, não queria nem saber o final… porque como o fim poderia ser feliz? Como o mundo poderia voltar a ser o que sempre foi quando tanta coisa ruim aconteceu? Mas no final, essa sombra vai passar… com certeza… até mesmo a escuridão acabará… um novo dia virá, e quando o sol nascer ele brilhará ainda mais.”

J. R. R. Tolkien

SILVA, Taylon Felipe. **Efeito do diabetes e exposição hiperglicêmica na susceptibilidade à leishmaniose experimental**. 2022. 105 pág. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2022.

RESUMO

A leishmaniose é um grupo de doenças infecciosas causadas por protozoários do gênero *Leishmania* e sua imunopatogênese resulta de fatores do hospedeiro e do parasita que desencadeiam uma resposta imune desequilibrada durante a infecção. O diabetes é uma doença crônica resultante da disfunção da produção de insulina pelo organismo ou da capacidade de utilizá-la adequadamente, levando à hiperglicemia, cujo descontrole crônico causa diversas lesões nos tecidos e prejudica o sistema imunológico. Assim, devido ao delicado equilíbrio imunológico necessário no combate e resistência à infecção por *Leishmania* e a desregulação crônica da resposta inflamatória observada no diabetes, o objetivo deste trabalho foi avaliar a resposta de PBMC de pacientes diabéticos, de celulas de linhagem THP-1 exposta a um ambiente hiperglicêmico e camundongos C57BL/6 diabéticos contra a infecção por *Leishmania amazonensis* e como essas condições alteram a resposta imune frente ao parasito. Para isso, foi coletado sangue periférico de 25 pacientes diabéticos e 25 controles saudáveis para extração de PBMC e posterior infecção experimental com *Leishmania amazonensis* por 2 ou 24 h, após isso foi analisada a capacidade leishmanicida e a resposta imune dessas células. Também realizamos um modelo de estímulo hiperglicêmico *in vitro* com células THP-1 e as infectamos com a mesma espécie de parasito por diversos tempos. Além disso, induzimos diabetes experimental com streptozotocina (STZ) em camundongos C57BL/6 e avaliamos a infecção cutânea no coxim plantar por 5 semanas. Observamos que o diabetes e a hiperglicemia prejudicam a capacidade leishmanicida de PBMC e macrófagos derivados de células THP-1, principalmente por manter o efeito antioxidante do NRF2 e aumentar a atividade das enzimas sequestradoras de radicais livres SOD e catalase, respectivamente, além de reverter o perfil de resistencia que camundongos C57BL/6 apresentam frente a infecção por *L. amazonensis,* induzindo lesões mais exacerbadas em comparação a animais não diabéticos. Além disso, o estímulo hiperglicêmico favoreceu o aumento de marcadores relacionados ao fenótipo de macrófagos M2 de maneira mediada pelo parasito. No mesmo sentido, a indução de diabetes experimental em camundongos C57BL/6 resultou em falha na produção de oxido nitrico (NO) frente a infecção, além de gerar uma respota semelhante a Th2/Th2 nestes animais, em comparação com animais controle que geraram uma respota semelhantente o perfil Th1. Ainda observamos que macrófagos de animais diabéticos falharam em processar e apresentar antígenos de *Leishmania* durante a infecção, sendo incapazes de ativar e induzir a proliferação de linfócitos antígeno-específicos. Juntos, esses dados demonstram que o diabetes e a hiperglicemia podem prejudicar a resposta imune celular, principalmente dos macrófagos, contra a infecção por parasitas do gênero *Leishmania*.

Palavras-chave: *Leishmania amazonensis*; hiperglicemia; macrófagos; oxido nitrico; NFR2; IL-17A; arginase; estresse oxidativo; apresentação de antigeno.

SILVA, Taylon Felipe. **Effect of diabetes and hyperglycemic exposure on susceptibility to experimental leishmaniasis**. 2022. 105 pag. Thesis (Doctorate in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2022.

ABSTRACT

Leishmaniasis is a group of infectious diseases caused by protozoa of the *Leishmania* genus and it's immunopathogenesis results from host and parasite factors that triggers an unbalanced immune response during infection. Diabetes is a chronic disease resulting from dysfunction of the body's production of insulin or the ability to use it properly, leading to hyperglycemia whose chronic lack of control causes several tissue injuries and impairs immune system. Thus, due to the delicate immune balance required in the combat and resistance to *Leishmania* infection and the chronic deregulation of the inflammatory response observed in diabetes, the objective of this work was to evaluate the PBMC response of diabetic patients, THP-1 cells exposed to a hyperglycemic environment and C57BL/6 diabetic mice against *Leishmania amazonensis* infection and how these conditions alter the immune response to the parasite. For this, peripheral blood was collected from 25 diabetic patients and 25 healthy controls for PBMC extraction and subsequent experimental infection with *L. amazonensis* for 2 or 24 h, after which the leishmanicidal capacity and immune response of these cells were analyzed. We also performed an in vitro hyperglycemic stimulus model with THP-1 cells and infected them with the same species of parasite for several times. In addition, we induced experimental diabetes with streptozotocin (STZ) in C57BL/6 mice and evaluated cutaneous infection in the footpad for 5 weeks. We observed that diabetes and hyperglycemia impair the leishmanicidal capacity of PBMC and macrophages derived from THP-1 cells, mainly by maintaining the antioxidant effect of NRF2 and increasing the activity of the free radical scavenging enzymes SOD and catalase, respectively, in addition to reversing the resistance profile that C57BL/6 mice present against infection by *L. amazonensis*, inducing more exacerbated lesions compared to non-diabetic animals. In addition, the hyperglycemic stimulus favored the increase of markers related to the phenotype of M2 macrophages in a parasite-mediated manner. In the same sense, the induction of experimental diabetes in C57BL/6 mice resulted in a failure in the production of nitric oxide (NO) in the face of infection, in addition to generating a Th2/Th17-like response in these animals compared to control animals that generated a response similarly to the Th1 profile. We also observed that macrophages from diabetic animals failed to process and present *Leishmania* antigens during infection, being unable to activate and induce proliferation of antigen-specific lymphocytes. Together, these data demonstrate that diabetes and hyperglycemia can impair the cellular immune response, mainly of macrophages, against infection by parasites of the genus *Leishmania.*

Key words: *Leishmania amazonensis*; Hyperglycemia; Macrophages; nitric oxide; NRF2; IL-17A; arginase; oxidative stress; antigen presentation.

LISTA DE ABREVIATURAS E SIGLAS

ADA	American Diabetes Association
AGE	Advanced glycation end-products
ATP	Adenosina trifosfato
CCL	CC chemokine ligand)
CD	Cluster of ifferentiation
CXCL	CXC chemokine ligand
DM1	Diabete Mellitus tipo 1
DM2	Diabete Mellitus tipo 2
DTH	Delayed-type hypersensitivity
eGFP	Enhanced green fluorescent protein
GLUT	Glucose transporter type
gp63	Glicoproteina 63
GPI	Glicofosfatidilinositol
HLA-DR	Human leukocyte antigen DR
IFN	Interferon
Ig	Imunoglobulina
IL	Interleucina
iNOS	Oxido nítrico sintase induzível
IR	Insulin receptor
LADA	Latent autoimmune diabetes in adults
LC	Leishmaniose cutânea
LMC	Leishmaniose mucocutânea
LPG	Lipofosfoglicano
LTA	Leishmaniose tegumentar americana
LUC	Luciferase
LV	Leishmaniose visceral
MyD88	Myeloid differentiation primary response 88
NAG	N-acetyl-β-d-glucosaminidase
NF-kB	Nuclear factor kappa B
NFR2	Nuclear factor erythroid 2
NK	Natural Killer

NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing
NO	Oxido nitrico
OMS	Organização Mundial de Saúde
PAHO	Organização Panamericana de Saúde
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PCR	Reação em cadeira da polimerase
PPAR	Peroxisome proliferator-activated receptor
PPG	Proteofosfoglicano
PRR	Pattern Recognition Receptors
RAGE	AGE receptors
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STZ	Streptozotocin
TGF	Transforming growth factor
Th	Células T helper
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TOTG	Teste oral de tolerância a glicose
Treg	Células T regulatórias

SUMÁRIO

1. INTRODUÇÃO

1.1. Leishmaniose

As leishmanioses são um complexo grupo de doenças causadas por mais de 20 espécies de parasitos do gênero *Leishmania*, que afetam tanto humanos como outros animais. As diferentes espécies de *Leishmania* causam uma série de manifestações clínicas, que variam desde lesões cutâneas auto curativas até acometimento visceral com risco de morte (BURZA; CROFT; BOELAERT, 2018; PAHO, 2022). No mundo, a leishmaniose é considerada uma das 10 doenças negligenciadas, com mais de 12 milhões de pessoas infectadas, cerca de 1 milhão de potenciais novos casos e aproximadamente 30 mil mortes anualmente. Além disso, mais de 350 milhões de pessoas vivem em áreas de risco eminente de infecção (PAHO, 2022).

Dentre as principais formas clínicas da leishmaniose, destacam-se a forma visceral, cutânea e mucocutânea, as quais variam em incidência intra e inter-regional, a depender das espécies do parasito circulante, dos vetores regionais, do estado imunológico e *background* genético do indivíduo infectado (WHO, 2020).

A leishmaniose visceral (LV), também conhecida como Kala-azar, geralmente está associada a infecção pelas espécies *Leishmania (Leishmania) donovani* e *L. (L.) infantum*, sendo fatal em 95% dos casos não tratados. Esta forma clínica da doença é caracterizada por febre, perda de peso e hepatoesplenomegalia (WHO, 2020). Por outro lado, a leishmaniose cutânea (LC) é a forma mais comum da doença e se manifesta pelo aparecimento na derme de lesões ulceradas, únicas ou múltiplas, caracterizadas por apresentarem bordas elevadas, contornos regulares e fundo granulomatoso, com ou sem presença de exsudato purulento. A infecção pode ser assintomática ou manifestar-se clinicamente após um período variável de latência. Em geral, as lesões cutâneas, dependendo da resposta do indivíduo frente à infecção, podem durar entre 3 e 18 meses, além de apresentarem altas taxas de recidiva se não tratadas (BURZA; CROFT; BOELAERT, 2018; HANDLER et al., 2015).

A leishmaniose mucocutânea (LMC) é uma forma mais agressiva da LC, geralmente associada à habilidade do parasito em evadir da derme e linfonodos

proximais atingindo áreas de mucosas orais e nasais, causando lesões desfigurantes que impactam significativamente a vida do indivíduo acometido por levarem ao crescimento progressivo, com destruição parcial ou total dessas áreas, sem evolução para cura espontânea (5).Seu desenvolvimento está associado à espécie de *Leishmania* e tratamento ineficaz, permitindo sucessivas recidivas da LC e, consequentemente, levar a morte (HANDLER et al., 2015).

Em relação a epidemiologia da LV, estima-se uma incidência anual entre 50 e 90 mil novos casos mundialmente. No ano de 2019, a Organização Mundial da Saúde (OMS) reportou mais de 90% de casos desta doença em 10 países, incluindo o Brasil, o qual foi considerado a área endêmica de maior extensão territorial, contendo cerca de 97% de acometidos com LV das américas (PAHO, 2022; WHO, 2020).

A estimativa para LC está entre 600 mil e 1 milhão de novos casos anualmente (WHO, 2020). As Américas, bacia do Mediterrâneo, Oriente Médio e Ásia Central abrigam cerca de 95% dos casos mundiais, e em 2020, mais de 85% dos novos casos ocorreram em 10 países, incluindo o Brasil. No país, entre 1995 e 2014, verificou-se uma média anual de 25.763 casos novos registrados e coeficiente de detecção médio de 14,7 casos/100 mil habitantes, e entre 2000 e 2020 foram notificados mais de 463 mil casos da doença. Atualmente, todos os estados brasileiros apresentam casos de leishmaniose, sendo a região norte com maior índice (MINISTÉRIO DA SAÚDE, 2022), causados principalmente pelas espécies *L. (Viannia) braziliensis, L. (L.) amazonensis* e *L. (V.) shawi*, está última localizada apenas em regiões de floresta amazônica (MINISTÉRIO DA SAÚDE et al., 2017).

Quanto à LMC, mais de 90% dos casos mundiais ocorrem no Brasil, Bolívia, Etiópia e Peru, com uma taxa de mortalidade de 7%. Entretanto, as leishmanioses de modo geral, são doenças subnotificadas e seu real quadro epidemiológico pode ser mais intenso. A LMC possui impacto significativo na vida social dos indivíduos acometidos, uma que gera lesões em regiões de mucosa, principalmente face, nariz e boca, causando desfiguração irreversível nestes indivíduos (PAHO, 2022; SAÚDE, 2020).

Apesar dos avanços nos estudos acerca do parasito, bem como no conhecimento sobre a doença e sua caracterização epidemiológica, a leishmaniose continua sendo um grave problema de saúde pública, não apenas

pela sua alta incidência, ampla distribuição e complexidade, mas também devido ao aparecimento de lesões destrutivas e incapacitantes. Além disso, vale ressaltar que as medidas de controle, incluindo a atual terapia contra leishmaniose são insatisfatórias, devido à ineficácia, tratamento prologado, alto custo e toxicidade dos atuais fármacos disponíveis (BURZA; CROFT; BOELAERT, 2018).

1.1.2. Agente etiológico da leishmaniose e ciclo biológico

As leishmanioses são causadas por diferentes espécies de protozoários do gênero *Leishmania,* pertencentes à classe *Kinetoplastida* e família *Trypanosomatidae*. Esses parasitos são ainda subdivididos em dois subgêneros: *Leishmania* e *Viannia*, com diferença taxonômica baseada no local de desenvolvimento e fixação de promastigotas no intestino do vetor. As espécies que causam a LC e LMC são comumente divididas entre espécies do Velho Mundo (*L. major, L. tropica* e *L. aethiopica*), prevalentes nas regiões da bacia do Mediterrâneo, Oriente Médio e subcontinente Indiano, e espécies do Novo Mundo (*L. amazonensis, L. mexicana, L. braziliensis* e *L. guyanensis*), endêmicas nas áreas do México e Américas Central e do Sul, responsáveis pela Leishmaniose Tegumentar Americana (LTA) (DE VRIES; REEDIJK; SCHALLIG, 2015; PETERS; 1924-; KILLICK-KENDRICK, 1987).

Os parasitos do gênero *Leishmania* são seres unicelulares com ciclo de vida heteroxeno, ou seja, necessitam da presença de dois hospedeiros, podendo ter disseminação de caráter antroponótico (*L. tropica*) ou zoonótico (*L. major, L. aethiopica* e todas as espécies do Novo Mundo). A transmissão dos parasitos ocorre através do repasto sanguíneo de fêmeas hematófagas do gênero *Phlebotomus*, no Velho Mundo, e *Lutzomyia*, no Novo Mundo. O hospedeiro invertebrado é essencial para a disseminação do parasito devido à adaptação evolutiva simbiótica de ambos, mas tem papel limitante na distribuição da doença por conta da necessidade de adaptação às condições climáticas de regiões tropicais (BURZA; CROFT; BOELAERT, 2018).

Durante as etapas de infecção os parasitos exibem diferentes formas morfológicas. No vetor, o parasito apresenta-se como forma promastigota, caracterizada pelo corpo alongado e flagelo livre na região anterior do corpo celular (LANG; LECOEUR; PRINA, 2009). O ciclo inicia-se quando insetos

transmissores se contaminam durante o processo de alimentação hematófaga em indivíduos ou animais contaminados. Uma vez que alcançam o tubo digestório do inseto, macrófagos infectados absorvidos através do sangue são rompidos, liberando os parasitos que se diferenciam em formas promastigotas procíclicas nas primeiras 48 h. Estas formas são capazes de sobreviver ao ambiente hostil do sistema digestivo do inseto, onde iniciam o processo de multiplicação por divisão binária e consequente colonização do vetor.

Cinco dias pós infecção, inicia-se o processo de metaciclogênese, no qual há modificações estruturais de moléculas de lipofosfoglicanos (LPG) presentes na superfície celular dos parasitos, diferenciando-os em promastigotas metacíclicas, forma infectante do parasito para hospedeiros vertebrados (SUNTER; GULL, 2017). Promastigotas metacíclicas, por sua vez, são caracterizadas pela presença de um espesso glicocálice na superfície celular, constituído por diferentes proteínas, lipídeos e carboidratos, dos quais destacam-se o LPG, glicosilfosfatidilinositol (GPI), proteofosfoglicanos (PPG) e a glicoproteína metaloproteinase 63 (gp63), que possuem papel fundamental no estabelecimento de uma infecção bem-sucedida (CARNEIRO; PETERS, 2021).

Uma vez diferenciados, os parasitos migram para as porções anteriores do estômago e proventriculo do inseto e no repasto sanguíneo posterior, ocorre a regurgitação destas formas infectivas no hospedeiro. Vale ressaltar que no processo de colonização do inseto, os parasitos produzem um gel formado por PPG de origem protista que assume importante função na transmissão do parasito para o hospedeiro vertebrado, uma vez bloqueia parcialmente a válvula estamodeal do sistema digestório e dificulta a ingestão de sague pelo inseto, o que induz a necessidade de alimentações mais frequentes e maior tempo de repasto sanguíneo, acumulando chances de uma infecção bem-sucedida (ROGERS et al., 2004).

Após a infecção, células do sistema fagocítico do hospedeiro, como macrófagos, monócitos e células dendríticas, fagocitam promastigotas metacíclicas e, uma vez internalizados por estas células, os parasitos modificam o fagossomo, tornando-o um ambiente favorável para sua sobrevivência e replicação, agora denominado vacúolo parasitóforo (VP). O ambiente ácido do VP e a temperatura mais elevada do hospedeiro vertebrado induzem o processo de diferenciação do parasito para forma intracelular, denominada amastigota,

caracterizada pelo seu formato arredondado e flagelo atrofiado (SUNTER; GULL, 2017).

Este controle e modificação estrutural do fagossomo é fundamental para sobrevivência do parasito, os quais têm desenvolvido importantes mecanismos de controle e escape que levam à fusão do VP com vesículas da via endocítica da célula hospedeira, incluindo endossomos de reciclagem, endossomos tardios e o retículo endoplasmático. A maturação do VP leva à proliferação intracelular das formas amastigotas de *Leishmania* pela aquisição de nutrientes da célula hospedeira, enquanto escapa das respostas de defesa do hospedeiro (LIÉVIN-LE MOAL; LOISEAU, 2016; YOUNG; KIMA, 2019).

Uma vez estabilizada a infecção, vetores que se alimentarem destes indivíduos ou animais infectados, farão a ingestão de macrófagos contendo formas amastigotas juntamente com o sangue e os parasitos serão liberados no sistema digestivo do inseto, reiniciando o ciclo biológico (BURZA; CROFT; BOELAERT, 2018).

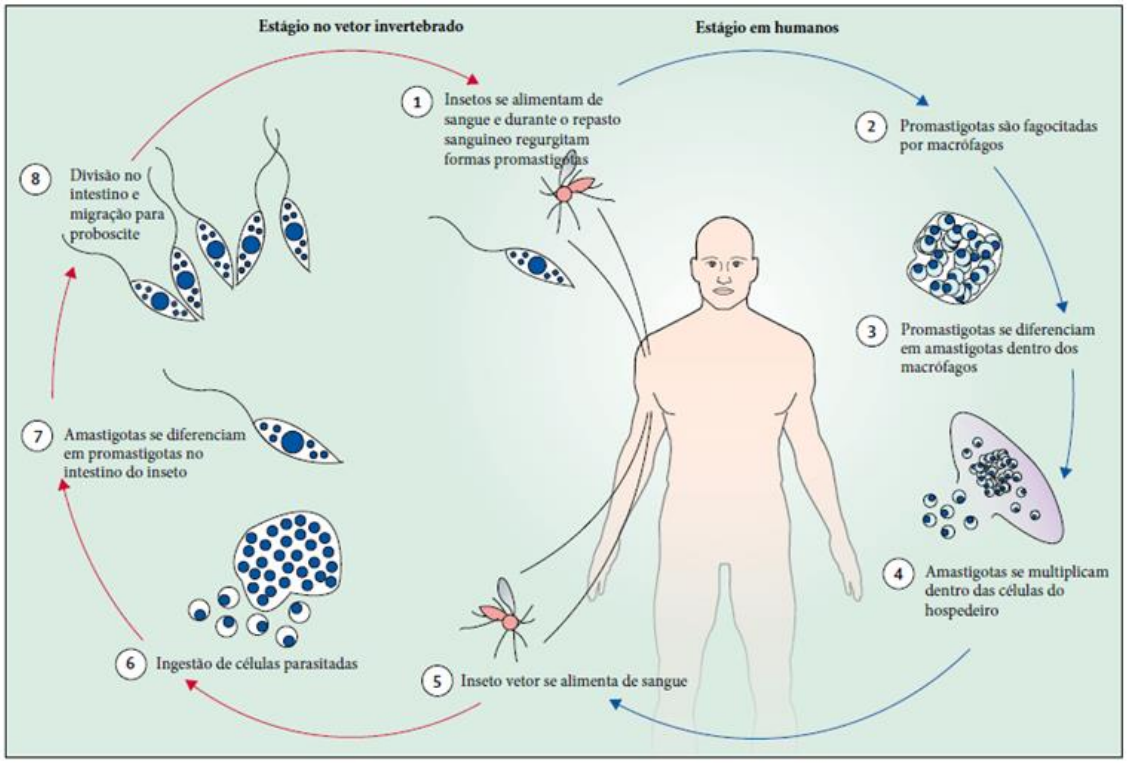

Figura 1. Ciclo biológico de parasitos do gênero *Leishmania.* (1) Durante o repasto sanguíneo, fêmeas hematófagas do gênero *Lutzomyia* ou *Phlebotomus* contaminados com *Leishmania*, regurgitam formas promastigotas metacíclicas no hospedeiro vertebrado; (2) As promastigotas são fagocitados por macrófagos e (3) se diferenciam em formas amastigotas intracelulares, (4) que se multiplicam e dentro das células

podendo rompê-las, liberando os parasitos para serem fagocitados por mais macrófagos. (5) Quando insetos vetores se alimentam de indivíduos ou animais contaminados, (6) ingerem células infectadas que se rompem no sistema digestivo do inseto, (7) liberando os parasitos que se diferenciam em formas promastigotas. (8) Após colonizarem o vetor, as promastigotas de migram para a proboscite, onde serão transmitidas para o hospedeiro vertebrado durante a próxima alimentação (Adaptado de: BURZA; CROFT; BOELAERT, 2018)

1.1.3. Interação parasito-hospedeiro e imunopatogênese da leishmaniose

A imunopatologia da leishmaniose é determinada pela combinação de fatores relacionados à virulência do parasito e da resposta imunológica do hospedeiro. Assim que invadem o hospedeiro, a primeira linha de defesa contra os parasitos é o sistema complemento, que consiste em uma cascata enzimática de proteínas solúveis, que participam das respostas imune inata e adquirida ao opsonizar patógenos, formar poros em suas membranas e induzir respostas inflamatórias que auxiliam no combate à infecção (ABBAS, 2019).

Entretanto, parasitos do gênero *Leishmania* desenvolveram resistência contra o sistema complemento por dois mecanismos conhecidos, o primeiro sendo a quebra do componente C3b em iC3b pela gp63. Quando o fator de complemento I cliva C3b, é produzido o fragmento de proteína iC3b, que atua como uma opsonina, facilitando o reconhecimento e entrada dos parasitos no interior das células através da ligação com os receptores CR1/CD11b presentes nas células fagocíticas. Ao contrário do C3b intacto, o iC3b não é capaz de se associar ao fator B, evitando assim a amplificação da cascata do complemento pela via alternativa. Do mesmo modo, o parasito também inibe a ligação da C9 no final da cascata de ativação do complemento. Em ambos os casos, a sinalização é bloqueada e falha em gerar o complexo de ataque a membrana que resultaria em lise do parasito (CARNEIRO; PETERS, 2021).

Uma vez que infectam o hospedeiro e evadem o sistema complemento, formas promastigotas do parasito são fagocitadas por células imunes, como macrófagos residentes e neutrófilos. Nesta etapa, o principal desafio dos parasitos é estabelecer uma infecção bem-sucedida e inibir a liberação de alarminas pelas células infectadas que podem resultar em um ambiente pró-inflamatório desfavorável ao parasito (SCOTT; NOVAIS, 2016).

As primeiras células a chegarem rapidamente ao local da infecção são os neutrófilos, atraídos tanto por quimiocinas do hospedeiro frente à lesão tecidual causada pelo repasto sanguíneo, tais quais CXCL1, CXCL2 e CCL4, quanto por quimioatrativos presentes na saliva do vetor, que promovem vasodilatação local, favorecendo a migração celular. O papel dos neutrófilos na leishmaniose é dualístico, se por um lado são essenciais na eliminação dos parasitos através da geração do *burst* oxidativo, com intensa produção de NO (do inglês, *nitric oxide*) e radicais livres derivados de oxigênio, por outr, também atuam como importantes reservatórios e fontes de disseminação do parasito no organismo, uma vez que leishmanias têm evolutivamente desenvolvido mecanismos de combate e resistência à moléculas microbicidas produzidas por estas células (GUIMARAES-COSTA et al., 2021; TOMIOTTO-PELLISSIER et al., 2018).

Os neutrófilos são conhecidos pelo seu curto período de vida após migrarem para os locais de infecção nos tecidos (entre 4 e 8 h). Entretanto, tem sido descrito que neutrófilos infectados com *Leishmania* apresentam uma sobrevida de até 48 h e produzem grandes quantidades de quimiocinas atrativas para macrófagos, como CCL4. A chegada de macrófagos para o local da infecção ocorre concomitantemente com o estímulo de morte dos neutrófilos, que sofrem apoptose e são fagocitados de maneira silenciosa pelos macrófagos. Assim, os corpos apoptóticos dos neutrófilos atuam como “cavalos de Tróia”, pois contém amastigotas que, ao serem internalizadas pelos macrófagos, os infectam sem disparo de resposta imune adequada (VAN ZANDBERGEN et al., 2004).

Por outro lado, o sistema imune é preparado para reconhecer parasitos circulantes através do reconhecimento de sinais não-self, ou PAMPs (do inglês, *pathogen-associated molecular patterns*), como o glicocálice das promastigotas, através de PRR (do inglês, *pattern recognition receptor*), que detectam a presença dos parasitos e iniciam ativação da resposta contra a infecção. A ativação de PRR, como TLR (do inglês, *toll-like receptors*), pode resultar no desencadeamento de respostas tanto anti- como pró-inflamatórias, a depender do tipo do receptor, espécie do parasito, *background* genético do hospedeiro e o tipo celular (CARNEIRO; PETERS, 2021).

O LPG presente no glicocálice, por exemplo, pode ativar diferentes respostas de acordo com o subtipo de TLR ativado. Quando LPG é reconhecido

por TLR4, ocorre a ativação da sinalização mediada pela proteína adaptadora MyD88 (do inglês: *Myeloid differentiation primary response 88*) que faz a transdução do sinal do receptor para dentro da célula, culminando em ativação do fator de transcrição nuclear NF-kB, que induz a transcrição de genes relacionados a produção de interferon gama (IFN-γ), TNF-α (do inglês, *tumor necrosis factor - alpha*) e NO, levando ao consequente controle da infecção. Por outro lado, o reconhecimento de LPG por TLR2 está associado a indução da produção IL-27/IL-10 que desencadeiam a produção de superóxido dismutase (SOD), importante enzima sequestradora de ânion superóxido. O sequestro de ânion superóxido pela SOD, inibe seu efeito microbicida e, consequentemente, favorece a sobrevivência e replicação do parasito (BARRETO-DE-SOUZA et al., 2015; DE CARVALHO VIVARINI et al., 2011; GUERRA et al., 2010; WIETZERBIN et al., 2009; XIN et al., 2010).

Os macrófagos desempenham papel chave na patogênese da doença por serem o principal reservatório dos parasitos no organismo. Existem dois principais tipos de macrófagos, o fenótipo M1 (caracterizados por CD80, CD86, HLA-DR, iNOS, CD68, IL-1R, TLR-2, TLR-4, IL-10^{Lo}, IL-12^{hi}), conhecido por suas características pró-inflamatórias e importante capacidade microbicida, e o M2 (Arg1, CD206, CD163, IL-10^{hi}, IL-12^{Lo}), um fenótipo anti-inflamatório/regulatório que desempenha funções de reparação tecidual e controle da resposta imune. A polarização de macrófagos nos fenótipos M1 e M2 é dependente de sinais provenientes do microambiente infeccioso e podem ser ativados por diferentes mecanismos que resultam em respostas distintas e impactam significativamente nas diferentes manifestações clínicas da doença. Macrófagos ativados por IFN-γ, por exemplo, se diferenciam preferencialmente para o fenótipo M1, enquanto a presença de TGF-β (do inglês, *transforming growth fator - beta*) e interleucina (IL)-4 privilegiam o desenvolvimento de M2 (SHAPOURI-MOGHADDAM et al., 2018; TOMIOTTO-PELLISSIER et al., 2018).

Similarmente, a resposta de células T na leishmaniose é um fator crítico na patogênese e progressão da doença. Espécies do Velho Mundo, como *L. major*, apresentam um perfil claro de padrões de resposta. Na infecção por esta espécie, sabe-se que enquanto indivíduos que desenvolvem padrão do tipo Th1, com produção de IFN-γ, TNF-α e IL-12 são resistentes à infecção, aqueles que desenvolvem resposta do tipo Th2, com produção de IL-4, manifestam os sinais

clínicos clássicos da LC. Entretanto, em espécies do Novo Mundo, como *L. amazonensis*, não se observa uma dicotomia tão clara entre indivíduos resistentes e suscetíveis (SCOTT; NOVAIS, 2016).

A resistência em infecções por esta espécie decorre da geração de uma resposta inicial precoce do tipo Th1 com produção de IFN-γ que estimula macrófagos a apresentarem um fenótipo pró-inflamatório M1 que decai ao longo do tempo e é substituída por uma resposta do tipo Th2 e T regulatória com presença de macrófagos M2 responsáveis pelo reparo tecidual e resolução da inflamação (CARNEIRO; PETERS, 2021; SCOTT; NOVAIS, 2016; TOMIOTTO-PELLISSIER et al., 2018).

Por outro lado, uma resposta baseada apenas no padrão Th1 desencadeia piores quadros clínicos devido a intensa e descontrolada inflamação, enquanto uma resposta baseada apenas no padrão Th2 falha em controlar a infecção e proporciona desenvolvimento progressivo das lesões. Os diferentes padrões de resposta podem ser observados em modelos murinos, nos quais camundongos C57BL/6 são parcialmente resistentes a infecção por *L. amazonensis,* por desenvolverem uma resposta inicial Th1 e tardia Th2, ao passo que camundongos BALB/c, que são suscetíveis, desenvolvem um claro padrão Th2 (SCOTT; NOVAIS, 2016; TOMIOTTO-PELLISSIER et al., 2021).

No mesmo sentido, macrófagos que fagocitam neutrófilos apoptóticos infectados, geralmente assumem um perfil M2, os quais são mais permissivos à infecção por serem maus indutores de estresse oxidativo e apresentarem aumento da atividade da enzima arginase I, a qual é responsável pela quebra do aminoácido L-arginina e geração de poliaminas, essenciais para o aumento da capacidade metabólica das amastigotas internalizadas. Por outro lado, a ativação de M1 via IFN-γ tem sido associada à resistência do hospedeiro e eliminação dos parasitos fagocitados devido a intensa produção de ROS (do inglês, *reactive oxygen species*) e NO (TOMIOTTO-PELLISSIER et al., 2018).

Entretanto, a polarização de macrófagos e linfócitos T em condições patológicas, especialmente na leishmaniose, não apresenta padrões exclusivos de resposta, mas sim um espectro de polarização entre os fenótipos que pode tender a padrões mais pró- ou anti-inflamatórios (SCOTT; NOVAIS, 2016; TOMIOTTO-PELLISSIER et al., 2018). O eixo iNOS/Arg1, por exemplo, tem sido amplamente utilizado para diferenciar fenótipos macrofágicos M1/M2,

respectivamente. Entretanto, macrófagos apresentam alta plasticidade celular e metabólica e podem expressar ao mesmo tempo ambos os marcadores, tornando essa classificação fenotípica mais complexa (KIELER; HOFMANN; SCHABBAUER, 2021; SHAPOURI-MOGHADDAM et al., 2018).

Apesar da arginase I estar envolvida no sucesso e cronicidade da infecção por parasitos do gênero Leishmania (PESSENDA; SILVA, 2020), seu papel também é essencial para capacidade de restauração tecidual exercida por macrófagos, uma vez que atua na produção de poliaminas, essenciais para a síntese de colágeno, proliferação celular, fibrose e outras funções de remodelação tecidual (SHAPOURI-MOGHADDAM et al., 2018). Interessantemente, tem sido descrito que poliaminas geradas pela catalise da L-arginina pela arginase I são potentes indutoras do fenótipo M2 e, portanto, agem de maneira autocrina nas células, o que poderia explicar a presença de um fenótipo dualístico em macrófagos no início do switch fenotípico de M1 para M2, que tende a se tornar cada vez mais M2 conforme a produção de poliaminas se intensifica/acumula nas células (KIELER; HOFMANN; SCHABBAUER, 2021).

Por outro lado, sabe-se que NO, produzido principalmente por macrófagos M1, é fundamental no combate e eliminação da infecção, entretanto parasitos do gênero *Leishmania* têm desenvolvido métodos de regular a produção deste mediador pelas células infectadas. Dentre as principais formas, podemos destacar a competitividade pelo substrato L-arginina, que é utilizado tanto pela célula hospedeira para produção de NO através da iNOS (do inglês, *inducible nitric oxide synthase*), quanto pelo parasito para produção de poliaminas para o seu crescimento e proliferação. No mesmo sentido, Cortez e colaboradores (CORTEZ et al., 2011) demonstraram que *L. amazonensis,* mas não *L. major,* também é capaz de aumentar a produção de CD200 em macrófagos infectados logo na primeira hora pós-infecção, levando à inibição de iNOS e permitindo o estabelecimento da infecção.

Como NO e ROS são os principais mecanismos relacionados à morte dos parasitos, sua indução é essencial para controle da infecção. Embora a *Leishmania* seja sensível a ROS, a quantidade destes mediadores produzidos por células infectadas é insuficiente para induzir morte dos parasitos, principalmente devido a sua capacidade de evadir e neutralizar estes radicais livres, especialmente quando falamos de *L. amazonensis* (PASSELLI; BILLION;

TACCHINI-COTTIER, 2021). Entretanto, a presença de co-estímulos, como IFN-γ e TNF-α, conseguem aumentar o *burst* respiratório em células infectadas, como neutrófilos e macrófagos, gerando radicais livres e NOS suficientes para eliminar o parasito (TOMIOTTO-PELLISSIER et al., 2018).

1.2. Diabetes

O diabetes é um conjunto de distúrbios metabólicos caracterizados por hiperglicemia crônica, resultante de defeitos na secreção e/ou ação da insulina. Embora a hiperglicemia seja uma característica comum a todos os tipos de diabetes, sua etiologia varia, sendo necessária identificação de demais fatores para sua classificação (KAUL et al., 2012).

De maneira geral, a maioria dos casos de diabetes registrados são divididos em diabetes mellitus tipo 1 (DM1), deficiência total de insulina decorrente da destruição de células beta pancreáticas por processos autoimunes, e diabetes mellitus tipo 2 (DM2), resultante da associação de resistência periférica a insulina e resposta compensatória inadequada de sua produção pelas células beta pancreáticas. Existem outras etiologias que desencadeiam diabetes em indivíduos acometidos, como por exemplo gravidez, disfunções genéticas das células beta e mutações nos receptores de insulina, mas estas representam a minoria dos casos de diabetes reportados (AMA, 2008).

DM1 pode ocorrer de forma rápida e progressiva, afetando principalmente crianças e adolescentes entre 10 e 14 anos de idade. Entretanto, esta forma da doença também pode ocorrer de forma lenta e progressiva, conhecida como LADA (do inglês, *Latent autoimmune diabetes in adults*), que apesar de se assemelhar muito com o DM1 que ocorre em indivíduos mais jovens, é erroneamente classificado como DM2 por ocorrer tardiamente em adultos. Assim, estima-se que entre 5 e 10% dos indivíduos diagnosticados com DM2 possivelmente apresentam LADA (29,30).

Segundo a OMS, mais de 420 milhões de pessoas no mundo vivem com diabetes, dos quais 95% apresentam DM2, com cerca de 1,5 milhão de mortes anuais registradas tendo a doença como causa direta da mortalidade. Estes números têm quadruplicado desde o início do acompanhamento epidemiológico realizado pela OMS em 1980 e estimativas apontam que o número de indivíduos

acometidos pelo diabetes ultrapasse meio bilhão até o fim desta década (WHO, 2022).

De acordo com a *American Diabetes Association* (ADA), em 2018 cerca de 34,2 milhões de pessoas viviam com diabetes nos Estados Unidos, com 1,5 milhão de novos casos sendo registrados todos os anos. Estes índices elevados decorrem, provavelmente, do sedentarismo, consumo de alimentos ultraprocessados e obesidade. Além disso, estima-se que 88 milhões de indivíduos apresentem quadro de pré-diabetes, caracterizado pelo aumento dos níveis glicêmicos no sangue, dos quais não ultrapassam os valores mínimos exigidos para o diagnóstico de diabetes. Entretanto, indivíduos nestas condições apresentam risco elevado de desenvolver posteriormente o diabetes (ADA, 2022). No Brasil, mais de 16 milhões de pessoas vivem com diabetes, correspondendo a cerca de 8,1% da população nacional, sendo a doença responsável diretamente por 6% do total de óbitos registrados no país no ano de 2016 (WHO, 2016).

O diabetes apresenta alta morbimortalidade e acarreta complicações a longo prazo que afetam os rins, olhos, sistema nervoso e vasos sanguíneos. A OMS estima que após 15 anos de doença 2% dos indivíduos acometidos evoluem para cegueira total e 10% apresentarão deficiência visual grave. Além disso, entre 30 e 40% destes indivíduos apresentarão algum grau de retinopatia, 10 a 20% nefropatia, 20 a 30% neuropatia e 10 a 25% terão desenvolvido doenças cardiovasculares, resultando em importante perda de qualidade de vida e alto custo para os serviços de saúde (MINISTÉRIO DA SAÚDE, 2006).

O diagnóstico do diabetes é realizado principalmente pela determinação dos níveis de glicose sanguínea. Indivíduos que apresentem glicemia de jejum superior a 125 mg/dL ou glicemia aleatória de repetição maior que 200 mg/dL são considerados diabéticos, principalmente quando os níveis glicêmicos são acompanhados de sinais e sintomas clássicos como poliúria, polidipsia e polifagia. Além disso, ainda pode ser realizado o teste oral de tolerância a glicose (TOTG), no qual indivíduos diabéticos devem apresentar concentração de glicose igual ou maior que 200 mg/dL 2 h após ingestão de 75 g de glicose (AGUILAR et al., 1999; AMA, 2008).

Geralmente, indivíduos normoglicêmicos apresentam níveis de glicose sérica de 70 a 125 mg/dL e TOTG de 110 a 140 mg/dL. Entretanto, aqueles com

glicose entre 110 e 125 mg/dL, mas com TOTG entre 140 e 199 mg/dL são considerados pré-diabéticos. Indivíduos com comprometimento da tolerância a glicose apresentam risco significativo de evoluir para o diabetes manifesto, além de alto risco cardiovascular decorrente do metabolismo anormal de carboidratos (AGUILAR et al., 1999; AMA, 2008).

1.2.1. Fisiologia do eixo insulina-glicose e patogênese do diabetes

O pâncreas é um órgão com funções endócrinas, constituído por aglomerados de células, denominados ilhotas de Langerhans, que essencialmente contém quatro tipos de células funcionais: células beta, responsáveis pela produção de insulina; células alfa, secretoras de glucagon; células delta, produtoras de somatostatina; e células PP, responsáveis pela produção do peptídeos pancreático. A fisiologia glicêmica é regulada em três etapas correlacionadas que incluem a produção de glicose no fígado, sua captação por tecidos periféricos, principalmente pelo músculo estriado esquelético, e por fim a regulação hormonal desencadeada pela insulina e glucagon (KUMAR et al., 2018).

A insulina tem como papel principal promover o transporte da glicose para dentro das células musculares, a qual, uma vez internalizada, é armazenada como glicogênio ou oxidada para forma de adenosina trifosfato (ATP). Nos adipócitos, a glicose é mantida principalmente na forma de lipídeos, assim, além de promover a lipogênese, a insulina também inibe a lipólise, favorecendo o acúmulo de lipídeos no interior dos adipócitos (MAYER; ZHANG; DIMARCHI, 2007).

Os hormônios polipeptídicos, insulina e glucagon, possuem efeitos antagônicos na regulação da glicose. Em jejum, o glucagon é secretado induzindo a gliconeogênese no fígado e a quebra do glicogênio nos tecidos periféricos, liberando glicose e impedindo a hipoglicemia. Após ingestão de fontes energéticas, os níveis de glucagon reduzem e a insulina se eleva em resposta à carga glicêmica advinda da alimentação, assim o aumento na produção de insulina pelas células beta é regulado pela própria glicose circulante de maneira compensatória (KUMAR et al., 2018; MAYER; ZHANG; DIMARCHI, 2007; POSNER, 2017).

Nos tecidos periféricos, a insulina se liga ao IR (do inglês, *insulin receptor*) desencadeando a sinalização do eixo IRS/PI3K/AKT que culmina na translocação de transportadores de glicose para a membrana, como GLUT4 (do inglês, *glucose transporter type 4*), que internalizará moléculas de glicose para utilização pós prandial. Assim, a desregulação de qualquer fator durante a complexa sinalização envolvida na fase da glicose, desde a síntese de insulina até o seu uso, pode resultar no fenótipo hiperglicêmico conhecido como diabetes (BRYANT; GOULD, 2020; LETO; SALTIEL, 2012).

No caso do DM1, as ilhotas pancreáticas são destruídas por células T efetoras, como linfócitos T CD8+ e células NK (do inglês, *natural killer*), que reagem a antígenos endógenos destas células reconhecidos como *non-selfie*. Essa perda de tolerância das células T é o resultado da falha na deleção de células T autorreativas no timo e de células T regulatórias incapazes de controlar a resposta de autotolerância. Além disso, a destruição inicial do tecido pancreático gera necrose e, consequentemente, uma resposta inflamatória compensatória associada ao desenvolvimento de resposta humoral autorreativa, intensificando a lesão tecidual. Assim, o DM1 se desenvolve mais comumente na infância, tempo no qual é necessário para que ocorra a geração de autoimunidade e destruição massiva das células beta pancreáticas, tornando o indivíduo incapaz de produzir insulina, necessitando de uma fonte exógena para sobrevivência (ATKINSON et al., 2019; ILONEN; LEMPAINEN; VEIJOLA, 2019; KAUL et al., 2012; NORRIS; JOHNSON; STENE, 2020).

Por outro lado, o DM2 é uma doença multifatorial, complexa e com evolução lenta, caracterizada principalmente por dois desequilíbrios metabólicos, o primeiro é a resistência dos tecidos periféricos à sinalização induzida pela insulina e o segundo corresponde à disfunção das células beta que passam a secretar este hormônio de maneira inadequada (KAUL et al., 2012).

A resistência à insulina antecede o desenvolvimento de hiperglicemia e vem acompanhada de hiperplasia compensatória das células beta, gerando hiperinsulinemia nos estágios iniciais de desenvolvimento da DM2. Neste sentido, a resistência periférica a insulina é caracterizada pela incapacidade dos tecidos alvo em responder a este hormônio, acarretando falha na captação da glicose. No fígado, a produção de novas moléculas de glicose é estimulada em situações de hipoglicemia, mas a disfunção da insulina gera um efeito

semelhante ao não conseguir internalizar moléculas de glicose, fazendo com que hepatócitos iniciem a gliconeogênese mesmo em situações de hiperglicemia (AMA, 2008; TUOMI et al., 2014).

Diversos fatores têm sido associados com a resistência à insulina, como defeitos funcionais na via de sinalização da glicose, no entanto, os fatores primordiais relacionados ao seu desenvolvimento são a obesidade e a inflamação crônica gerada em sua decorrência. O aumento de ácidos graxos livres observado em indivíduos obesos, ocasionado pelo excesso na captação de nutrientes e lipídeos, está associado a uma potente inibição da sinalização intracelular da insulina. Além disso, o acúmulo destes ácidos graxos no interior das células é responsável por desencadear o que é conhecido como estado inflamatório crônico de baixo grau, caracterizado pela produção contínua de citocinas pró-inflamatórias (BROWN; WALKER, 2016; GLASS; OLEFSKY, 2012).

Esse excesso de ácidos graxos em macrófagos e células beta do pâncreas é responsável pelo recrutamento intracelular de proteínas relacionadas ao inflamassoma, acarretando produção de IL-1β, que por sua vez, induz a produção de outras citocinas inflamatórias como TNF-α e IL-6 em um efeito cascata, agindo como indutores independentes de resistência a insulina. Assim, o acúmulo de lipídeos na obesidade pode atuar tanto como indutor direto ou indireto ao gerar inflamação (ABDUL-GHANI; DEFRONZO, 2010; CZECH, 2020; GLASS; OLEFSKY, 2012).

O tecido adiposo também contribui significativamente para o desenvolvimento de resistência à insulina, uma vez que não é apenas um tecido de armazenamento de gordura, mas um órgão endócrino funcional, liberando as chamadas adipocinas, proteínas que regulam o metabolismo energético e inflamatório do organismo. Assim como nos macrófagos e células beta, os adipócitos de indivíduos obesos também produzem quantidades significativas de IL-1β e outras citocinas inflamatórias em razão do excesso de ácidos graxos (ABDUL-GHANI; DEFRONZO, 2010; BROWN; WALKER, 2016; CZECH, 2020).

Essa inflamação, ou estado inflamatório de baixo grau, inicia lentamente o processo de resistência periférica da insulina e a disfunção das células beta, o que reflete a incapacidade destas células em manter as demandas crescentes de insulina a longo prazo. Nos estágios iniciais da resistência insulínica, este

hormônio é secretado em maior quantidade pelas células beta de maneira a compensar sua perda de eficácia, no entanto, o aumento da glicose sérica é imperceptível, pois o excesso de insulina é suficiente para suprir a resistência. No decorrer do tempo, essa compensação gerada pela produção extra de insulina torna-se insuficiente, havendo progressão para hiperglicemia acompanhada de perda da massa total de células beta que são parcialmente destruídas e substituídas por tecido fibrótico, estabelecendo finalmente um quadro irreversível de DM2 (GLASS; OLEFSKY, 2012; KUMAR et al., 2018; TUOMI et al., 2014).

De maneira geral, as complicações do diabetes estão relacionadas ao excesso de glicose circulante que desencadeiam efeitos patogênicos em praticamente todos os sistemas orgânicos. Essa glicotoxicidade desencadeia alterações em diversas vias metabólicas, como a formação de AGE (do inglês, *advanced glycation end-products*), gerados pela ligação de derivados da glicose com grupamentos amino das proteínas. Essa ligação pode provocar diversos efeitos nas proteínas, desde sua alteração conformacional e funcional, até o *crosslink* e aprisionamento proteico. Além disso, macrófagos e linfócitos T com fenótipos inflamatórios apresentam RAGE (do inglês, *receptor of advanced glycation end-products*) e a sinalização do eixo AGE-RAGE desencadeia liberação de citocinas pró-inflamatórias e fatores de crescimento, além de estimular a atividade pró-coagulante e neo-angiogênese, responsáveis pela maioria das manifestações patológicas do diabetes (KUMAR et al., 2018; RAMASAMY; YAN; SCHMIDT, 2011; YAN; RAMASAMY; SCHMIDT, 2009).

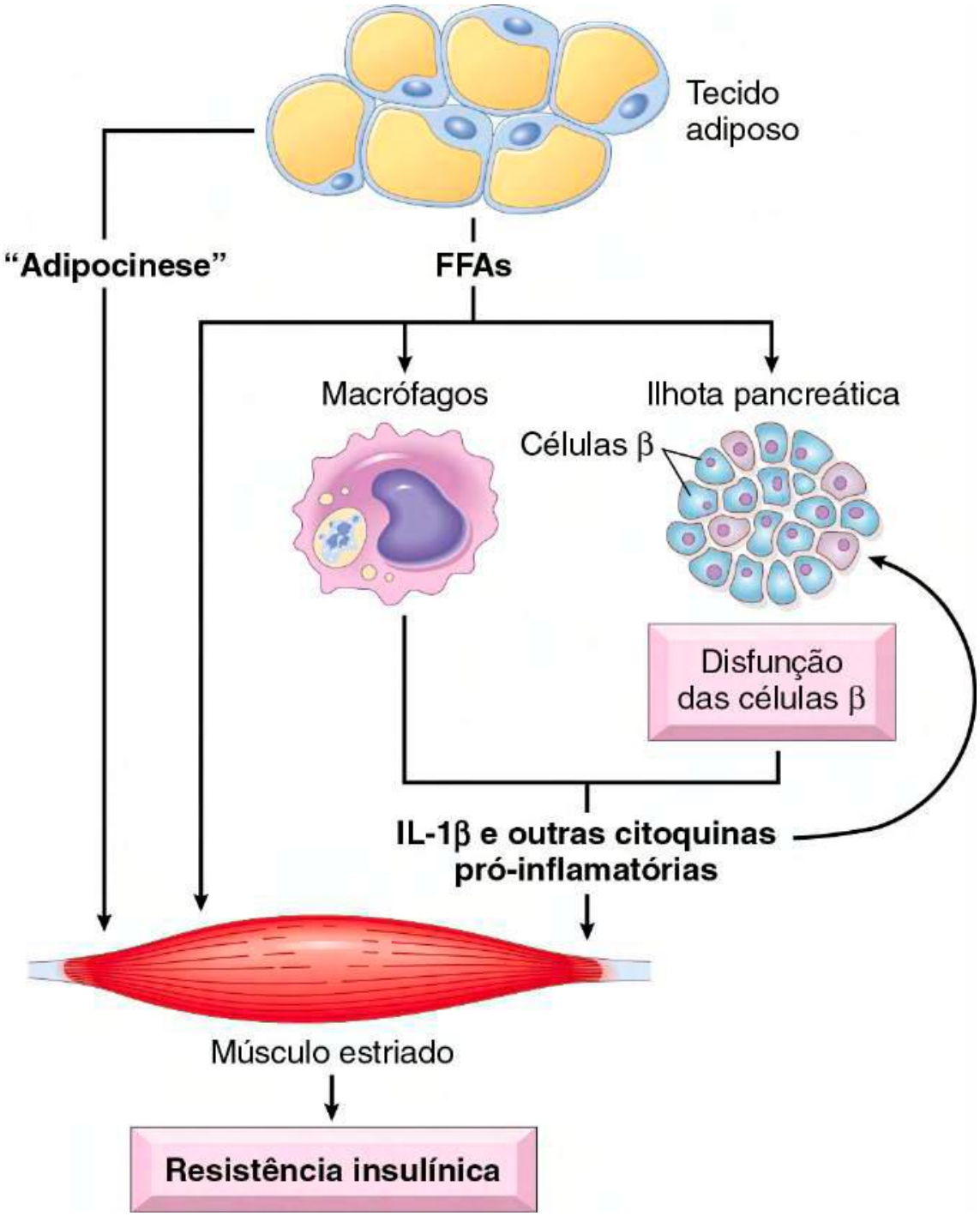

Figura 2. Patogênese do DM2. O acúmulo de ácidos graxo livres (FFA) induz processo inflamatório tanto em macrófagos quanto em células beta pancreáticas pela ativação direta de inflamassoma, resultando em produção de IL-1β e outras citocinas inflamatórias que intensificam a inflamação e geram estresse oxidativo. O excesso de inflamação e a presença excessiva de FFA interferem na sinalização da insulina e reduzem seu efeito sobre o musculo estriado esquelético, impedindo a captação da glicose e, consequentemente, hiperglicemia (Adaptado de: KUMAR; ABBAS; ASTER, 2015).

1.2.2. Disfunção imune no diabetes

Como dito previamente, a ativação anormal de células imunes está relacionada com a imunopatogênese do diabetes, sendo um fator chave no desenvolvimento de hiperglicemia, seja pela destruição de células beta ou indução de resistência a insulina. Entretanto, estas alterações características do diabetes também influenciam a resposta imune frente a outras doenças, especialmente infecciosas, que necessitam de respostas finamente reguladas. Assim, essas desregulações imunológicas, tanto no eixo inato quanto adaptativo, podem predispor indivíduos diabéticos a infecções (DARYABOR et al., 2020).

Os macrófagos têm papel importante no combate a infecções, especialmente na resistência contra *Leishmania*, conforme abordado anteriormente. Durante infecções e lesão tecidual, os macrófagos atuam de maneira pró-inflamatória no início da infecção, para combater os parasitos, e tardiamente mudando para um fenótipo anti-inflamatório, promovendo reparação tecidual. Em condições patológicas, como no diabetes, esse *switch* fenotípico pode não ocorrer de forma adequada, gerando um estado de inflamação crônica (KIM; NAIR, 2019).

Em indivíduos saudáveis, o controle da inflamação tecidual é realizado pela mudança de um ambiente pró-inflamatório para um anti-inflamatório, com alteração do perfil Th1 para Th2/Treg. Esse *switch* fenotípico, principalmente em macrófagos M1 para M2, é mediada pela ativação dos receptores PPAR-γ (do inglês, *peroxisome proliferator-activated receptor gamma*), que atuam como fatores de transcrição, suprimindo a montagem do inflamassoma e inibindo a síntese de citocinas pró-inflamatórias. Entretanto, no DM2 a expressão de PPAR-γ é suprimida em macrófagos residentes na derme, tornando estas células permissivas à ativação de NLRP3 e montagem do inflamassoma de maneira crônica. Além disso, foi demonstrado que em animais PPARγ$^{-/-}$, a ausência deste receptor retardou o processo de cicatrização e aumentou a inflamação tecidual mediada por macrófagos (MIRZA et al., 2015).

Interessantemente, também tem sido demonstrado que espécies de *Leishmania* são capazes de estimular a expressão de PPAR-γ em macrófagos infectados, induzindo um perfil fenotípico M2 e tornando estas células suscetíveis à infecção devido à atenuação da resposta inflamatória, o que poderia ser entendido como benéfico para indivíduos diabéticos. Entretanto,

parasitos do gênero *Leishmania* são capazes de realizar essa modulação em macrófagos no início da infecção, estágio no qual há intenso número de parasitos e a necessidade de macrófagos inflamatórios para controle da carga parasitária e coinfecções adjacentes. A presença de macrófagos M2 logo no início das infecções está associada à falta de controle do patógeno e cronificação da infecção. Portanto, a ativação da inflamação mediada por PPAR-γ induzida por *Leishmania* no início da infecção não deve proporcionar qualquer benefício para estes indivíduos (CHAN; ADAPALA; CHEN, 2012; GALLARDO-SOLER et al., 2008; KIM; NAIR, 2019).

Khanna et al. (KHANNA et al., 2010), demonstraram que macrófagos de camundongos diabéticos apresentam disfunção fagocítica em lesões cutâneas não infecciosas, ao realizem a eferocitose de células mortas. Esses macrófagos desencadearam a indução de mais apoptose no tecido adjacente saudável, intensificando a inflamação e inibindo mediadores anti-inflamatórios e regulatórios como IL-10 e TGFβ, o que acarretou inflamação crônica e lesão tecidual progressiva. Neste sentido, Mirza et al. (MIRZA et al., 2014) avaliaram que animais diabéticos apresentam ambiente pró-inflamatório crônico mediado pela intensa ativação de inflamassoma dependente de NLRP3, promovendo a produção de IL-1β e IL-18 em macrófagos.

O estado hiperglicêmico também altera a resposta gerada por células T e diversos estudos têm verificado disfunções destas células em indivíduos com DM2 (KUMAR et al., 2015; MOURA et al., 2016; RICHARD et al., 2017). Mouro e colaboradores (2016) (MOURA et al., 2016) demonstraram que pacientes diabéticos com lesões cutâneas apresentam número reduzido de linfócitos T naïve e aumento de T efetoras, o que prejudica o estabelecimento de resposta imune a novos agentes infecciosos. Similarmente, Leung et al. (2018) (LEUNG et al., 2018), verificaram que úlceras cutâneas de pacientes com DM2 possuem altos níveis de infiltrado inflamatório do perfil Th1, sem presença significativa de resposta T reguladora para controlar a inflamação, suprimir a angiogênese e reduzir a capacidade de reparação tecidual (DARYABOR et al., 2020; KUMAR et al., 2015, 2014).

Além da resposta imune celular, a hiperglicemia também é capaz de prejudicar a resposta humoral de maneira direta, uma vez que a geração de AGEs também afeta imunoglobulinas (Ig), modificando sua estrutura e atividade.

Tem sido demonstrado que a massa desses anticorpos nos pacientes diabéticos é maior que em indivíduos normoglicêmicos devido ao acúmulo de glicações, levando ao aumento da susceptibilidade a infecções devido à disfuncionalidade da resposta humoral (DARYABOR et al., 2020). Sabe-se também que animais diabéticos falham na produção de anticorpos contra patógenos devido a incapacidade de gerar centros germinativos maduros com linfócitos T e B, causando defeitos na recombinação de troca de classe e na produção de anticorpos com alta afinidade e avidez (FARNSWORTH et al., 2018).

Diante disso, tem sido amplamente demonstrado que indivíduos diabéticos são mais suscetíveis à diversos tipos de infecções, como bactérias (CHANCHAMROEN et al., 2009; JAVID et al., 2016; LIN et al., 2006; RIYAPA et al., 2012; THIMMAPPAIAH JAGADEESH et al., 2017; YANO et al., 2012), vírus (ALRADDADI et al., 2016; CUI et al., 2019; HULME; GALLO; SHORT, 2017; KUMAR et al., 2012; MEMON et al., 2013; VILLAR et al., 2019; WU; MCGOOGAN, 2020; YANG et al., 2006; ZHOU et al., 2020), fungos (AL MUBARAK et al., 2013; CHELLAN et al., 2010; WOLDEMARIAM et al., 2019), bem como diversos tipos de parasitoses, incluindo *Plasmodium falciparum* (DANQUAH; BEDU-ADDO; MOCKENHAUPT, 2010), *Toxoplasma gondii* (LI et al., 2018), *Opisthorchis viverrini* (HTUN et al., 2018), *Strongyloides stercoralis* (MENDONÇA et al., 2006)*, Cryptosporidium parvum* (ALEMU; JEMAL; ZERDO, 2018), *Blastocystis hominis* (MOHTASHAMIPOUR et al., 2015), *Ascaris lumbricoides* (AKINBO et al., 2013; ALEMU; JEMAL; ZERDO, 2018; MACHADO et al., 2018), *Giardia lamblia* (MACHADO et al., 2018) e *Leishmania* (BONYEK-SILVA et al., 2020).

1.3. A diabetes como fator de susceptibilidade para leishmaniose

Apesar do diabetes ser amplamente presente em todas as populações, inclusive em indivíduos que vivem em áreas endêmicas para leishmaniose, são escassos os estudos que investigam a relação e os efeitos que esta doença tem na infecção por parasitos do gênero *Leishmania.*

Mostafavi e colaboradores (89), avaliaram o impacto do diabetes em pacientes com LC de uma região endêmica para *L. tropica* no sudeste do Irã e demonstraram que pacientes concomitantemente com diabetes e LC apresentam lesões maiores em tamanho e número, bem como maior quantidade

de amastigotas no local da lesão. Além disso, estes pacientes apresentaram tempo de cicatrização três vezes maior em comparação com indivíduos com LC sem diabetes. Os autores ainda demonstraram que a associação de LC e diabetes resultou na redução da produção de IFN-γ e TGF-β e aumento de IL-4, indicando que o diabetes prejudica a resposta imune em favor do parasito.

No mesmo sentido, Lago et al. (LAGO et al., 2020), demonstraram que pacientes diabéticos infectados com *L. braziliensis* apresentam lesões atípicas que não respondem ao tratamento convencional com Glucantime em comparação com indivíduos sem diabetes. Estes indivíduos, em geral apresentaram lesões achatadas, superficiais, maiores em tamanho e bordas indefinidas, que foram associadas com a redução no número de linfócitos T CD8+ locais, sugerindo falha no controle da carga parasitária e na destruição de células infectadas.

Bonyek-Silva e colaboradores (80), também demonstraram que pacientes com diabetes infectados com *L. braziliensis* apresentam pior desfecho clínico da LC quando comparados a indivíduos não diabéticos. Foi avaliado que o diabetes leva a níveis sistêmicos mais elevados de leucotrieno B4, IL-6 e TNF-α na LC. Entretanto, apenas leucotrieno B4 se correlacionou com níveis de glicose no sangue e tempo de cicatrização das lesões leishmanióticas em indivíduos diabéticos. As lesões cutâneas de pacientes com leishmaniose e diabetes apresentaram números elevados de neutrófilos e amastigotas. Os macrófagos derivados de monócitos desses indivíduos apresentaram maiores cargas de *L. braziliensis*, produção reduzida de ROS e relação leucotrieno B4/prostaglandina E2 desequilibrada, caracterizando uma inflamação sistêmica impulsionada pelo diabetes em oposição a uma capacidade local reduzida de resolver a infecção e pior desfecho da leishmaniose.

Assim, considerando o delicado equilíbrio imunológico envolvido no combate e resistência à infecção por parasitos do gênero *Leishmania*, a desregulação da resposta inflamatória observada na hiperglicemia crônica pode ser um fator responsável por induzir a suscetibilidade e agravo da leishmaniose. São escassos os trabalhos que investiguem a resposta imunológica em condições de hiperglicemia/diabético frente a infecção por parasitos do gênero *Leishmania.* Portanto, entender melhor como células expostas a um ambiente hiperglicêmico e animais diabéticos se comportam frente a infecção por *L.*

amazonensis e como o parasito subverte a maquinaria celular/imunológica a seu favor, são fundamentais para elucidar melhor porque indivíduos diabéticos são mais suscetíveis a esta infecção, principalmente levando em consideração que o diabetes é uma doença amplamente distribuída entre as populações, incluindo aquelas expostas ao risco para leishmaniose.

2. OBJETIVOS

2.1. Objetivo geral

Investigar o efeito e as alterações na resposta desencadeadas pela exposição a um ambiente hiperglicêmico/diabético frente a infecção experimental por *Leishmania amazonensis.*

2.2. Objetivos específicos

- Investigar se PBMC de indivíduos diabéticos são mais suscetíveis a infecção experimental por *L. amazonensis* em comparação com células de indivíduos saudáveis;
- Determinar o perfil de estresse oxidativo gerado por PBMC de indivíduos diabéticos e saudáveis frente a infecção com *L. amazonensis;*
- Avaliar a modulação na produção de mediadores envolvidos no metabolismo da glicose e sinalização inflamatória em PBMCs diabéticos e saudáveis na leishmaniose experimental;
- Comparar capacidade fagocítica e leishmanicida de macrófagos diferenciados de células THP-1 estimuladas ou não com meio hiperglicêmico frente a infecção por *L. amazonensis*;
- Avaliar a produção de moléculas microbicidas e radicais livres (óxido nítrico, ânion superóxido e espécies reativas de oxigênio totais) frente a infecção destas células com *L. amazonensis*, bem a atividade de enzimas relacionadas ao sequestro de radicais livres;
- Investigar o efeito do estímulo hiperglicêmico na infecção com *L. amazonensis* em células THP-1 diferenciadas sobre a transcrição de genes relacionados a resposta imune, como MyD88, CD40, CCL4 e CXCL2, bem como a produção de TNF-α;
- Determinar se o estímulo hiperglicêmico e/ou a infecção por *L. amazonensis* induzem alterações na presença de mediadores relacionados ao fenótipo de monócitos pelo padrão de expressão de CD14 e CD16 e de macrófagos M1 (HLA-DR, CD80 e CD86) e M2 (Arginase e CD206);
- Analisar se o diabetes experimental em camundongos C57BL/6 altera o curso da infecção e agravam a lesão induzida por *L. amazonensis;*

- Caracterizar o perfil de resposta imunológica (Th1, Th2 e Th17) e a produção de óxido nítrico/arginase em camundongos C57BL/6 saudáveis e diabéticos, frente a infecção por *L. amazonensis;*
- Investigar a capacidade de macrófagos peritoneais de camundongos saudáveis e diabéticos em processar e apresentar antígenos de *Leishmania amazonensis*, bem como promover a ativação/proliferação de linfócitos esplênicos autólogos e a produção de IFN-γ.

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4. PRODUÇÃO CIENTÍFICA

4.1. Artigo 01: Impairment of effector molecules response in diabetes induces susceptibility to *Leishmania amazonensis* infection

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Impairment of effector molecules response in diabetes induces susceptibility to *Leishmania amazonensis* infection

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ABSTRACT

Type 2 Diabetes is a chronic disease resulting from insulin dysfunction that triggers a low-grade inflammatory state and immune impairment. Leishmaniasis is an infectious disease characterized by chronic inflammation resulted from the parasite's immunomodulation ability. Thus, due to the delicate immune balance required in the combat and resistance to *Leishmania* infection and the chronic deregulation of the inflammatory response observed in type 2 diabetes, we evaluated the response of PBMC from diabetic patients to *in vitro Leishmania amazonensis* infection. For that, peripheral blood was collected from 25 diabetic patients and 25 healthy controls matched for age for cells extraction and subsequent experimental infection for 2 or 24 h and analyzed for phagocytic and leishmanicidal capacity by optical microscopy, oxidative stress by GSSG generation, labeling of intracellular mediators by enzyme-Linked immunosorbent assay, and cytokines measurement with cytometric beads array technique. We found that the diabetic group had a higher percentage of infected cells and a greater number of amastigotes per cell. Also, even inducing NF-kB phosphorylation and increasing TNF production after infection, cells from diabetic patients were unable to downregulate NRF2 and generate oxidative stress, which may be associated with the exacerbated levels of IL-6 observed. PBMC of diabetic individuals are more susceptible to infection by *L. amazonensis* and fail to control the infection over time due to the inability to generate effector microbicidal molecules.

1. Introduction

Diabetes is a chronic disease resulting from dysfunction of the body insulin production or proper use leading to hyperglycemia, which failure to control over time cause several lesions, mainly in the nervous system and blood vessels [1]. Diabetes can be subdivided into categories according to the disease etiology, as follows: type 1 - of autoimmune origin resulting from the destruction of pancreatic beta cells that cause the body to be unable to produce insulin, and type 2 - derives from peripheral resistance to insulin, excessive secretion of glucagon, and failure to secrete insulin by pancreatic beta cells, corresponding to approximately 90% of all diabetes cases with an incidence growing worldwide over the last 30 years [2].

Hyperglycemia caused by diabetes is responsible for triggering a low-grade inflammatory state, with continuous production of several mediators secreted by immune cells and adipocytes that play a central role in the disease pathogenesis, besides altering the physiological response in other tissues. In this sense, changes in the immune system triggered by

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the hyperglycemic state may have serious consequences in the response against pathogens. This is the case of the continuous production of cytokines such as TNF-α and IL-6, despite their important pro-inflammatory properties against infections, they are intrinsically related to body desensitization to infectious stimuli and affect the metabolism of glucose and lipids [3]. Also, it has been described that diabetic individuals are more susceptible to infection, as hyperglycemia significantly reduces the phagocytic and microbicidal capacity of macrophages and neutrophils [4,5].

Leishmaniasis is a disease caused by protozoa of the Leishmania genus, characterized by chronic skin inflammation, mucous membranes, or internal organs. According to the World Health Organization (WHO), it is among the six most important infectious diseases for public health affecting more than one million people every year [6].

The immunopathogenesis of leishmaniasis results from both host and parasite factors, the immune response triggered during infection may determine the clinical form of the disease and the susceptibility/resistance profile of the individual. However, differences between species or even distinct strains of the same species may present particular characteristics that alter the virulence, tropism, and pathogenicity of the parasite. The polarization of Th1/Th2 response patterns and the unbalanced production of cytokines such as TNF-α and IL-4 are directly related to the pathogenesis of cutaneous leishmaniasis that leads to chronic persistent infection [7]. In addition to the polarization of lymphocytes, the response triggered by monocytes/macrophages is fundamental for the immunopathogenesis and clinical evolution of leishmaniasis and requires a balance between pro- and anti-inflammatory activities [8].

Considering the delicate immune balance involved in the combat and resistance to infection by parasites of the Leishmania genus, the chronic deregulation of the inflammatory response in type 2 diabetes, and the concomitant occurrence of both diseases in the population, our objective was to assess the response and susceptibility of mononuclear cells of diabetic patients to in vitro *Leishmania (Leishmania) amazonensis* infection.

2. Patients and methods

2.1. Subjects and design

This is an experimental study that used peripheral blood mononuclear cells (PBMC) from diabetic patients and healthy controls to investigate the response to *Leishmania (Leishmania) amazonensis* infection. For this, peripheral blood was collected from 25 diabetic patients and 25 healthy controls for PBMC isolation, and as inclusion criteria, it was established age between 50 and 70 years old for both groups and present glucose hemoglobin (HbA1c) levels above 6.4% for the diabetic group and below 5% for the healthy control group, and as exclusion criteria, it was established the presence of comorbidities, acute and chronic infections, immunosuppression and pregnancy. This study was submitted to and approved by the Human Research Ethics Committee of the State University of Londrina (CAAE n° 56533816.8.0000.5231 – Op n° 175 - Process 6974.2016.66).

2.2. Samples collection and PBMC isolation

All patients who agreed to participate in the study signed the Free and Informed Consent Form and donated peripheral blood collected by venipuncture in heparinized and without anticoagulant tubes. The tubes containing heparinized blood were processed for PBMC isolation following the Histopaque®-1077L (Sigma-Aldrich, USA) density gradient separation method. Briefly, the blood was centrifuged for 7 min at 24 °C, and 2000 rpm, the buffy coat was extracted with a sterile disposable Pasteur pipette and diluted in PBS in the ratio of 1:2. The diluted buffy coat was added carefully over the FICOLL-HISTOPAQUE reagent in a conical tube to obtain two phases and then centrifuged for 30 min at 20 °C, and 1400 rpm, without brake and acceleration. The separate cell layer was collected, washed with sterile PBS, and centrifuged for 10 min at 4 °C, and 1500 rpm, the cells were then resuspended in red cell lysis buffer (NH_3Cl_2) for 5 min at 4 °C and washed with PBS as described above. The cell pellet was resuspended in RPMI 1640 culture medium, counted in a Neubauer chamber, and seeded in 24-well plates, with or without 16mm round coverslips, in a total concentration of 5×10^6 (considering the ratio 1:10 monocyte/lymphocyte) containing 10% autologous serum inactivated for 30 min at 56 °C. The cells were incubated at 37 °C with 5% CO_2 for 24 h for adhesion, however, all the seeded cells were kept in the culture throughout the experiment to better mimic the immune response presented in living organisms.

2.3. Maintenance of L. amazonensis strains and experimental infection

Promastigotes forms of *L. amazonensis* (MHOM/BR/1989/166MJO) were maintained in culture medium 199 (GIBCO, Invitrogen, New York, USA) pH 7.18–7.22 supplemented with 10%-fetal bovine serum (FBS) (GIBCO, Invitrogen, New York, USA), 10 mM-HEPES buffer, 0.1%-human urine, 0.1%-L-glutamine, 10 U/mL-penicillin and 10 μg/mL-streptomycin (Invitrogen-GIBCO) and 10%-sodium bicarbonate as described for Miranda-Sapla, et al [9]. The cell culture was maintained at 25 °C in a 25 cm^2 culture flask and all experiments used promastigote forms at the stationary growth phase (5-day culture). The cells plated as described in the previous topic were infected with promastigote forms of *L. amazonensis* (5×10^6), taking into account the 10:1 ratio parasite/monocytes, and evaluated at 2- or 24 h post-infection.

2.4. Phagocytic and leishmanicidal assay

After 2 or 24 h of infection, cells seeded in 24-well plates containing round coverslips were washed in sterile PBS to remove non-internalized parasites, fixed in methanol for 20 min, stained by the May-Grunwald-Giemsa method (Laborclin, Brazil), and 200 sequential cells were analyzed under an optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Japan) (1000x magnification), to determine the percentage of infected cells and the number of amastigotes per cell at the respective times of infection. The culture supernatant was aliquoted and stored at -80°C to measure the levels of cytokines and nitric oxide.

2.5. Nitrite quantification as an estimate of nitric oxide (NO) production

NO levels were determined based on the Griess method, using the aliquoted supernatant as described above. Briefly, 60 μL of supernatant was added to 60 μL of Griess reagent (1% sulfanilamide and 0.1% of N-(1-Naphthyl) ethylenediamine in orthophosphoric acid (H_3PO_4) 5%), incubated for 10 min protected from light at room temperature, and read in a plate reader (Thermo Scientific, Multiskan GO, USA) at 550 nm. Serial dilutions of $NaNO_2$ were used for quantification.

2.6. Determination of oxidative stress by GSH and GSSG

To determine the intensity of oxidative stress, reduced (GSH) and oxidized (GSSG) glutathione measurement was performed. The PBMC of diabetic patients and healthy controls were plated as described above and after 24 h of infection, GSH and GSSG quantification was performed according to the method described by Rahman *et al.* [10]. The concentrations were determined based on a standard curve constructed with serial dilutions of GSH.

2.6. Cytokine measurement

The Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine kit (BD Biosciences, USA, 551809) was used following the manufacturer's instructions to measure levels of TNF, IFN-γ, IL-10, IL-6, IL-4, and IL-2 in the aliquoted supernatant as described above. The values were

expressed in pg/mL and the detection limits were 2.6 (IL-2 and IL-4), 2.8 (IL-10 and TNF), 3.0 (IL-6), and 7.1 pg/ml (IFN-γ).

2.7. Protein extraction and Enzyme-Linked Immunosorbent Assay

The cells were plated and infected with *L. amazonensis* for 24 h as previously described, after this period, the wells were washed with sterile PBS to remove non-adherent cells and non-internalized parasites. The adhered cells were incubated with RIPA lysis buffer (150 mM sodium chloride, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 50 mM Tris, pH 8.0) overnight at -80 °C. The cell lysate was collected and centrifuged at 13.000 x g for 20 min at 4 °C, the supernatant was transferred to a new tube and the total protein concentration was quantified in NanoVue Plus (Biochrom, USA). The protein concentration of all samples was normalized to 20 μg/mL and a 100 μL aliquot was added in a 96-well ELISA plate for adsorption of proteins overnight at 4 °C, followed by incubation for 1 h with blocking buffer (ELISA/ELISPOT, eBioscience™, USA). The wells were washed 3x with wash buffer (PBS in 0.5% Tween 20) and incubated with primary antibody anti-human AKT, phosphoAKT, NF-kB, phosphoNF-kB, PI3K, NOS2, arginase-1, and NRF2 (Santa Cruz Biotech, USA) for 2 h at room temperature. The wells were washed to remove unbound antibodies, followed by the addition of universal biotinylated secondary antibody (LSAB2 System-HRP, Dako, USA), 1 h incubation, washing, and addition of streptavidin-HRP (LSAB2 System-HRP, Dako, USA) for 1 h. After the incubation time, the wells were washed 5 times and 100 μL of TMB Substrate Solution (eBioscience™, USA) was added, followed by incubation for 30 min and 100 μL of stop solution (1 N sulfuric acid) was added. The plate reading was performed in a microplate reader at 450 nm (GloMax, Promega, USA).

2.8. Statistical analysis

Statistical analyses were performed using Graphpad Prism 8 (GraphPad Software, USA). Data were submitted to the Shapiro-Wilk and Levene tests and those with normal distribution and homogeneity of the variances were evaluated according to the One-Way ANOVA-test with posthoc Tukey. Data without normal distribution were transformed by natural logarithm to show normality. Statistical significance was set at $p<0.05$ and the p-values were categorized through *($p\leq0.05$); **($p\leq0.01$); ***($p\leq0.001$); ****($p\leq0.0001$) for all analyses.

3. Results

3.1. PBMC of diabetic patients are more susceptible to L. amazonensis infection

We challenged the cells with the parasite to investigate the susceptibility of the PBMC of diabetic patients to infection by *L. amazonensis*. We found that for the periods of 2 and 24 h of infection, the percentage of infected cells ($p=0.0202$, and $p<0.0001$, respectively) and the number of amastigotes per cell (both time points $p<0.0001$) were higher in the diabetic group than in the healthy control. Also, the control group was able to significantly reduce the percentage of infected cells by 20% ($p=0.0025$) and the number of amastigote per cell (4.8±0.6 to 2.9±0.3, $p=0.0070$) between 2 and 24 h of infection, which did not occur in the diabetic group (Fig. 1).

3.2. L. amazonensis controls NO production and oxidative stress in PBMC of diabetic patients

As described above, diabetic patients have higher baseline inflammatory state than healthy individuals [11], therefore, we assessed the baseline levels of NO in PBMC of diabetic patients and healthy controls without infection and after 24 h of infection with *L. amazonensis*. We found that the healthy controls cells did not alter the NO production against infection, remaining at levels similar to the uninfected group. In contrast, diabetic patient cells had higher baseline levels of NO than healthy controls; however, after 24 h of infection, the NO levels significantly decreased in the diabetic group ($p=0.0282$), matching the baseline level of the control group ($p=0.9531$). These results indicate the parasite's ability to regulate the NO production in cells of diabetic patients and that cells of healthy individuals can control the infection independently of this mediator (Fig. 2**A**).

In the same sense, we measured the levels of reduced glutathione and oxidized glutathione to assess oxidative stress after 24 h of infection with *L. amazonensis*. We found that the diabetic group presented higher GSH levels after infection (500.2±180.0 to 1253.0±292.9, $p=0.0095$), which did not occur in the control group (1181.0±133.1 to 1071.0±102.4, $p=0.9752$). In addition, the diabetic group had lower basal GSH levels than the control group ($p=0.0349$), but after the infection, they were able to increase to levels similar to the control, indicating the activation of an antioxidant signaling pathway. In contrast, GSSG levels remained similar in the diabetic group (496.0±39.4 to 478.8±50.0, $p=0.9986$), but increased in the control group (727.2±36.27 to 1000.0±96.9, $p=0.0221$) after infection, indicating that despite recovering their antioxidant machinery for self-protection, diabetic cells were unable to induce oxidative stress when infected, which can explain their susceptibility to infection (Fig. 2**B**).

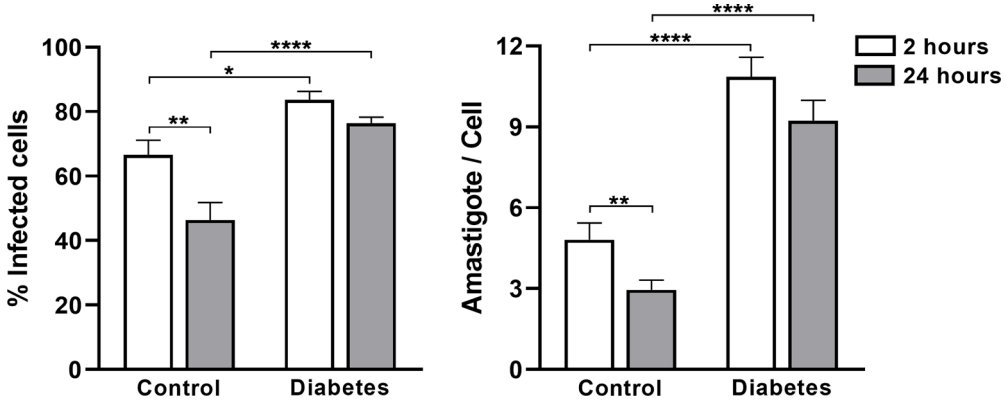

Fig. 1. *L. amazonensis*-infected health and diabetic cells. PBMC from diabetic and health (control) individuals were incubated for 2 and 24 h with *L. amazonensis* and assessed for the percentage of infected cells and the number of amastigotes per cell. The values represent the mean±SEM of 25 different patients each group performed in quadruplicate. * ($p\leq0.05$); ** ($p\leq0.01$); *** ($p\leq0.001$); **** ($p\leq0.0001$).

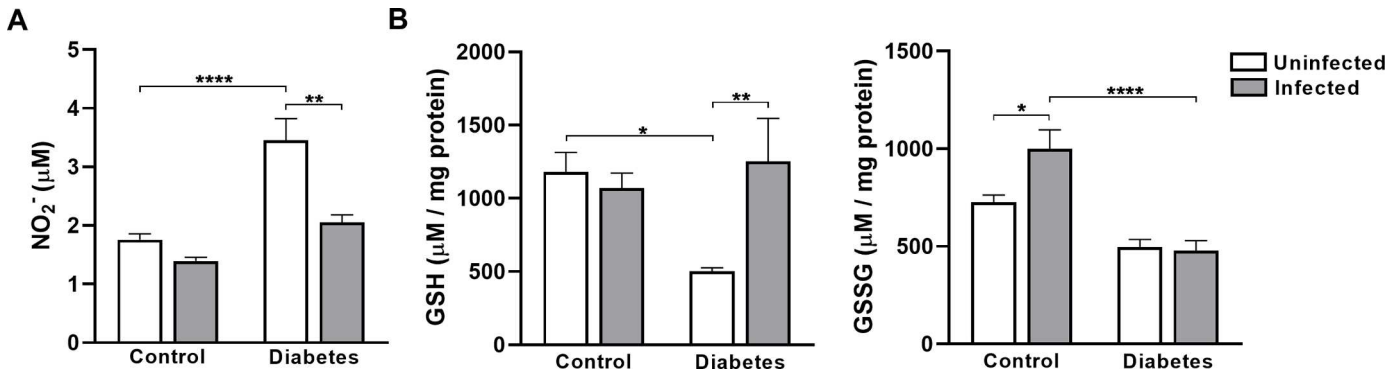

Fig. 2. Quantification of NO and oxidative stress production in *L. amazonensis*-infected health and diabetic PBMC. The Griess method was performed for nitrite levels dosage in PBMC culture supernatant (a), and GSH and GSSG levels were analyzed in PBMC lysates (b). The values represent the mean±SEM of 25 different patients each group performed in quadruplicate, white columns represent uninfected and grey columns represent 24 h of infection with *L. amazonensis*. * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

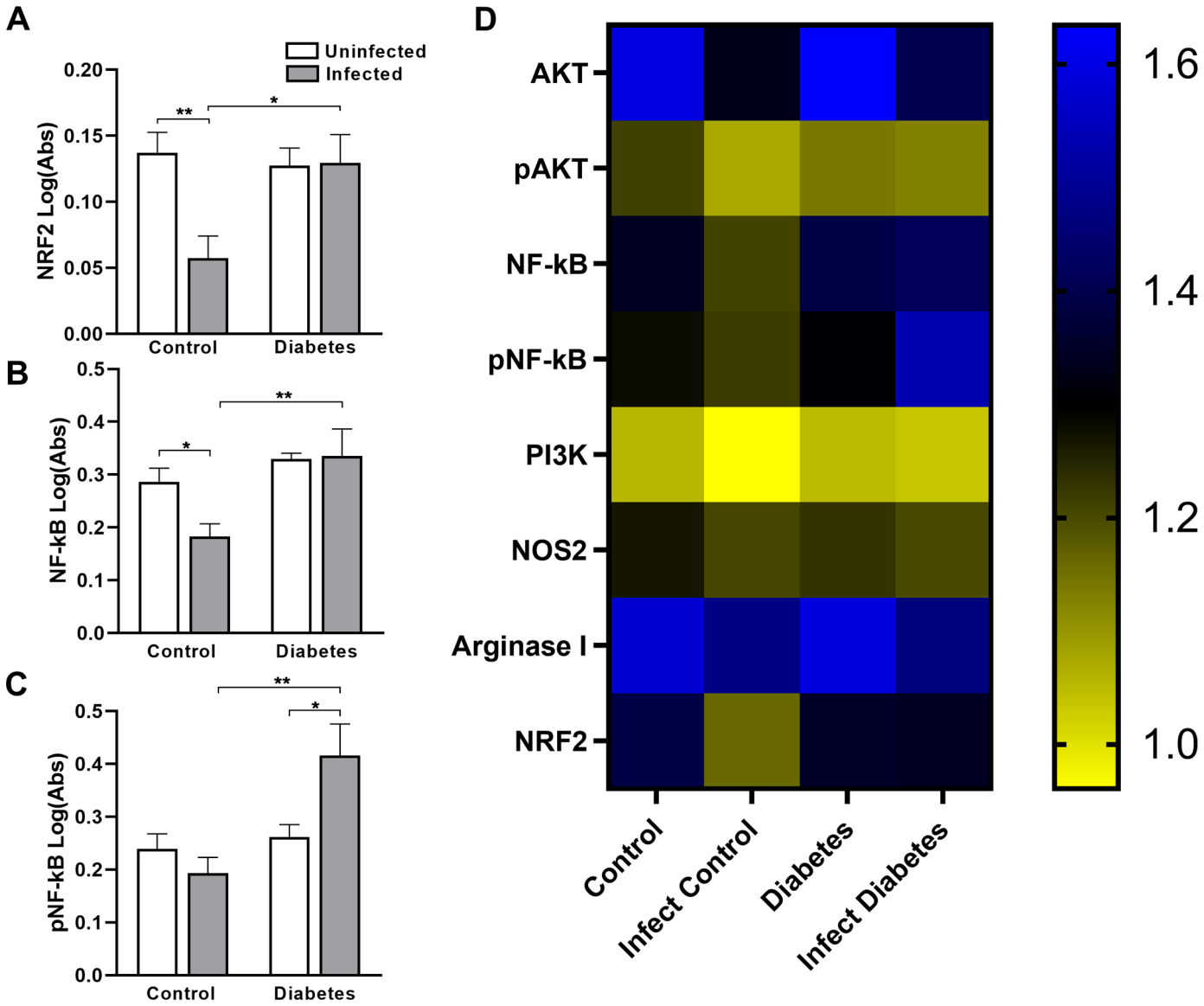

Fig. 3. Evaluation of intracellular signaling mediators involved in diabetes and leishmaniasis immunopathogenesis. ELISA measurement of the levels of NRF2 (a), NF-kB (b), and pNF-kB (c) presented as the natural logarithm of the absorbance, white columns represent uninfected and grey columns represent 24 h of *L. amazonensis* infection. Heat map of direct absorbance of all evaluated mediators ranging from more (blue) to less (yellow) antigen detection after 24 h of culture with or without *L. amazonensis* infection (d). The values represent the mean±SEM of 25 different individuals each group performed in quadruplicate. * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

3.3. PBMC from diabetic patients increases NF-kB phosphorylation but fail to down-regulate NRF2 in L. amazonensis infection

To investigate the signaling pathways involved in *L. amazonensis*-infected PBMC, we assessed some mediators associated with *Leishmania* infection and diabetes. We found significantly lower NRF2 levels in the control group after 24 h of infection with *L. amazonensis* ($p=0.0021$), which also differed significantly from the infected diabetic group ($p=0.0300$). However, in the diabetic group, NRF2 levels remained similar even after 24 h of infection ($p>0.9999$) (Fig. 3**A**).

In addition, NF-kB levels in the control group decreased after 24 h of infection ($p=0.0165$), which did not occur in the diabetic group ($p=0.9994$) (Fig. 3**B**). Interestingly, the levels of phosphorylated NF-kB remain similar in the control group after 24 h of infection ($p=0.7120$), but increased in the diabetic group ($p=0.0457$) after 24 h of infection (Fig. 3**C**). This indicates that the reduction in NF-kB in the control group did not occur due to phosphorylation, but perhaps because of the result of parasite control over the inflammatory response in these cells. Furthermore, the levels of Akt, pAkt, PI3K, NOS2 and arginase I differed statistically after infection, but not between groups, indicating that these mediators play a role in *L. amazonensis* infection, but are not related to the different responses observed between healthy and diabetic individuals (Fig. 3**D**).

3.4. L. amazonensis infection triggers different cytokine responses in PBMC of healthy and diabetic individuals

To investigate the immunomodulation triggered by *L. amazonensis* infection in PBMC of diabetic and healthy patients, we measured the main cytokines of the Th1 and Th2 response patterns in non-infected cells and at 2- and 24 h post-infection. We found that the TNF levels in the control group did not show significant changes after infection at the evaluated times (2 h 211.9±49.8 to 24 h 150.9±32.6, $p=0.9985$), remaining similar to the baseline level (98.7±36.6). The diabetic group, on the other hand, showed intense TNF production in the first 2 h after infection comparing with the baseline level (1175.0±120.6 vs 203.1±37.5, $p<0.0001$), which significantly decreased after 24 h (565.4±110.3, $p<0.0001$) but remained higher than the control levels ($p>0.05$) (Fig. 4**A**).

Regarding IL-10 levels, the control group showed no difference between baseline level and infection times (8.0±2.4, 17.3±6.1, and 14.6±4.5, respectively, $p>0.05$), whereas the diabetic group indicated lower production of this cytokine from 2 to 24 h of infection (60.0±10.7 to 34.5±6.1, $p=0.0335$); however, none of the two times differed from the baseline level (46.8±6.8, both $p>0.05$). In addition, both baseline and 2 and 24 h IL-10 levels in the diabetic group were significantly higher than in the control group ($p=0.0422$, 0.0037, and 0.0325, respectively) (Fig. 4**B**). IL-6 levels did not show significant intra-group differences, with baseline levels and times of infection remaining similar. However, there was a significant difference when comparing the control versus diabetic groups both at baseline (1712.0±617.1 vs 13766.0±1580.0, $p<0.0001$), and in 2 (2452.0±676.7 vs 17146.0±1453.0, $p<0.0001$) and 24 h (2780.0±792.2 vs 17066.0±1308.0, $p<0.0001$) of infection (Fig. 4**C**). Besides that, a ratio between TNF and IL-10 means indicates that the diabetic group presents a higher inflammatory response, while the health group showed a more balanced response between pro- and anti-inflammatory mediators (Fig. 4**D**). The levels of IFN-γ, IL-2, and IL-4 remain below the detection limit of the kit (data not shown).

4. Discussion

In this study, we demonstrated that PBMC of diabetic patients are more susceptible to infection by *L. amazonensis*, with a higher percentage of infected cells and internalized parasites per cell in relation to healthy controls, which suggest that immunological changes and metabolic disorders triggered by diabetes predispose the body to infections. Besides, we also found that the cells of diabetic patients were

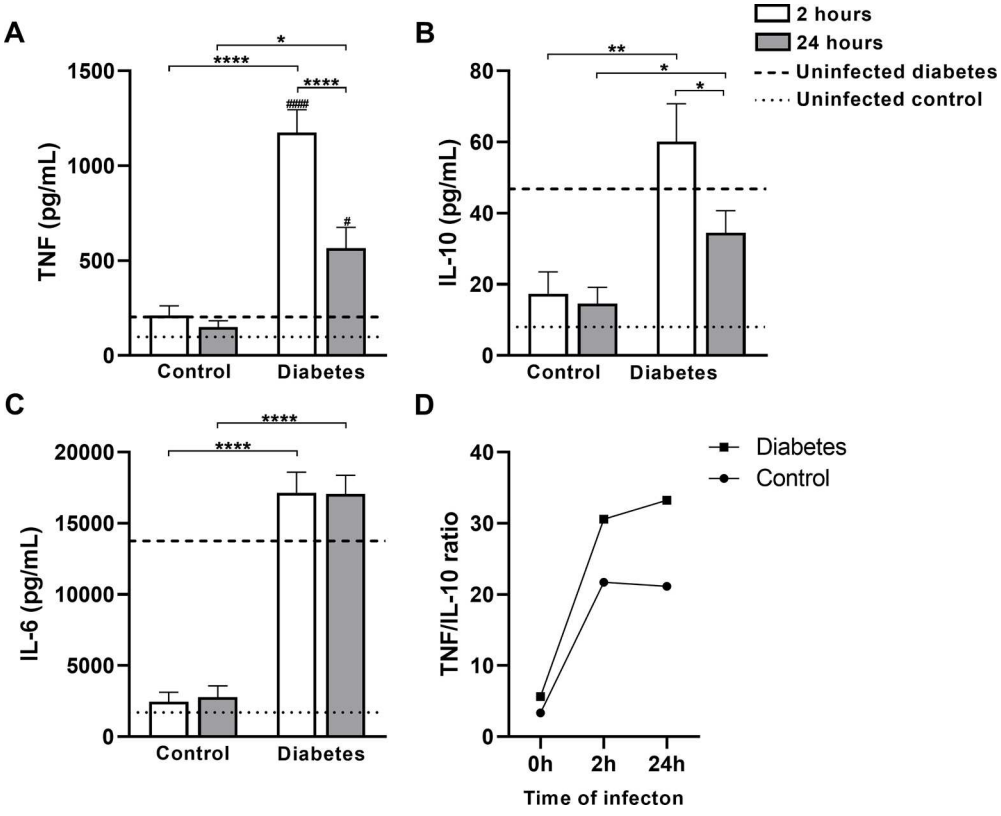

Fig. 4. Differences between cytokines production by *L. amazonensis*-infected PBMC from diabetic and healthy individuals. TNF (A), IL-10 (B), and IL-6 (C) levels in the supernatant of PBMC culture of diabetic and healthy individuals without, with 2, or 24 h of *L. amazonensis* infection. Ratio calculation between TNF and IL-10 means in uninfected, 2, and 24 h of infection (D). Pointed lines represent the uninfected control and dashed lines uninfected diabetes group. White columns represent 2 h and grey columns 24 h of *L. amazonensis* infection. The values represent the mean±SEM of 25 different patients each group performed in quadruplicate. Significant statistical differences are demonstrated as * ($p\leq0.05$); ** ($p\leq0.01$); *** ($p\leq0.001$); **** ($p\leq0.0001$); or with $^{\#}$($p\leq0.05$) and $^{\#\#\#\#}$ ($p<0,0001$) for differences compared to uninfected groups.

not able to reduce the parasitic load over the time of infection, as occurred in healthy cells. Therefore, in addition to being predisposed to infection, these cells are also unable to eliminate phagocyte parasites. Our results are in accordance with a recent study by Bonyek-Silva, *et al.* [12] with monocyte-derived macrophages from patients with cutaneous leishmaniasis and diabetes challenged with *in vitro L. braziliensis*. The authors also found higher percentage of infected cells number of parasites per cell in relation to cells of patients with cutaneous leishmaniasis without diabetes.

Furthermore, hyperglycemic status and diabetes have been widely described in the literature as susceptible factors for various infections [13–16]. Since PBMC from diabetic patients were more susceptible to infection and failed to eliminate internalized parasites comparing with healthy PBMC, we verified possible differences between the groups regarding the production of microbicidal molecules, such as NO and oxidative stress, and found a higher baseline NO production in the diabetic group than in the healthy control. However, along the infection time, the NO levels decreased and equaled the control, which indicate that the higher baseline inflammatory state found in diabetic individuals does not favor the organism in the face of infection.

In addition, GSH is an important antioxidant agent present in cells and marker of oxidative stress, thus its lower levels and oxidized form (GSSG) may indirectly indicate the production of reactive oxygen species (ROS) [10]. We found that the PBMC of diabetic patients was unable to increase oxidative stress in the face of *L. amazonensis* infection likethe healthy control group was. Besides, the control group showed lower NFR2 production after infection, which did not occur in the diabetic group, maintaining the same level after infection. NRF2 is a transcription factor that coordinates the activation of several cytoprotective mechanisms both under physiological conditions and stress. Activation of NRF2 generally induces the production of components of antioxidant systems, such as GSH and thioredoxin (TXN), in addition to enzymes involved in NADPH regeneration, ROS, and xenobiotics detoxification, maintaining redox homeostasis of cells [17]. Although the diabetic group induces higher GSH production after infection, probably due to the NRF2 response maintenance, the GSSG levels remain the same, indicating that these cells activate their antioxidant mechanisms, but do not generate real oxidative stress production.

Similarly, monocyte-derived macrophages from diabetic patients did not show higher ROS production after infection with *L. braziliensis*; however, cells from non-diabetic individuals managed to increase the production of these microbicidal molecules and reduce the parasite load [12]. In the same sense, PBMC and granulocytes from healthy individuals maintained the ability to produce ROS by LPS-induced oxidative burst in a hyperglycemic environment reduced [4]. Despite being well described that oxidative stress occurs continuously in diabetic individuals and is directly related to the vascular changes of the disease [18], surprisingly, data referring to its role in infections in diabetic individuals are scarce.

The fact that only the PBMC of healthy individuals were able to recognize the infection, induce oxidative stress to combat the parasite, and decrease the antioxidant activity by reducing NRF2 may indicate that PBMC of diabetic patients are less responsive and consequently more susceptible to infections.

When activated, NF-kB is a transcription factor that induces the production of inflammatory mediators responsible for directing the immune response to a Th1 pro-inflammatory pattern type. The main cytokines induced by the activation of NF-kB include TNF-α and IL-6, which are involved in insulin resistance and the low-grade chronic inflammatory state observed in diabetic patients [11]. Thus, we investigated the levels of cytokines characteristic to the Th1 and Th2 response patterns to check for differences between the PBMC-induced immune response of diabetic individuals and healthy controls against *L. amazonensis* infection. We fond that the control group was unable to alter the production of TNF, however, the diabetic group significantly increased the production of this mediator in 2 h after infection, whilw in 24 h, the levels of TNF reduced closer to the baseline level.

TNF-α is one of the most important inflammatory mediators involved in the immune response against infections, but it has also been linked to the immunopathogenesis of several diseases of non-infectious origin, such as diabetes, for being mainly produced by adipocytes inducing tissue-specific inflammation by generating oxidative stress and activating transcription factors for pro-inflammatory genes. The systematic increase in TNF-α induces insulin resistance by impairing its signaling pathways, culminating in a hyperglycemic state and type 2 diabetes [19]. It has been shown that mice fed with hyperglycemic diets have higher baseline levels of TNF-α and IL-6 [20], and diabetic rats stimulated with LPS significantly increase TNF-α production in the first few hours, then decreasing over time [21]. Also, the systematic increase in TNF-α levels induces the generation of inflammatory monocytes associated with tissue resistance to insulin [22].

In leishmaniasis, the role of TNF-α is still quite controversial, it has been shown to be a key cytokine in fighting infection mainly due to its role in the induction of NO by macrophages [23,24]. In contrast, it has also been described that excess of TNF-α is associated with the appearance of lesions and worsening of the infection [25,26]. Thus, the higher TNF levels observed in the diabetic group in our study may demonstrate a deleterious effect on the response against *L. amazonensis* infection, corroborating the data presented by Bonyek-Silva et al [12], who found higher levels of systemic TNF-α and more amastigotes at the lesion sites in patients with cutaneous leishmaniasis and diabetes comparing with patients with cutaneous leishmaniasis only. Also, in a long-term high glucose condition, BMDM cells were capable of responding to LPS stimulus, producing IL-1β and TNF-α, but shows an impaired microbicidal capacity [27]. In addition, peritoneal macrophages from alloxan-induced diabetic rats and RAW 264.7 macrophages cultured in a hyperglycemic medium showed impaired ROS production upon LPS stimulation [28]. Thus, the results found in our data indicate that the diabetic PBMC were unable to control infection due to incapacity to produce oxidative stress even in the presence of TNF.

IL-6 is a multifunctional cytokine that acts to induce adaptive immune response and differentiation of B lymphocytes against infections, however, continued IL-6 secretion contributes to chronic low-grade inflammation in diabetic patients [29]. Additionally, IL-6 has been reported to play an important role in the pathogenesis of leishmaniasis, an *in vitro* study found that pretreatment with IL-6 significantly inhibited the production of IFN-γ and TNF-α by macrophages after infection with *L. amazonensis* in a time and dose-dependent manner. Besides, this pretreatment with IL-6 was also able to down-modulate the oxidative stress-mediated by TNF-α in these macrophages, impairing the leishmanicidal capacity of the cells [30]. In addition, it has been demonstrated that IL-6 suppresses oxidative stress by activating the NRF2 pathway [31]. Thus, the excess of IL-6 produced by PBMC of diabetic patients in our study, both at baseline and in infected cells, may have a direct relationship with the deficient antileishmania response observed in this group in relation to healthy individuals.

5. Conclusion

In conclusion, this study found that PBMC of diabetic individuals is more susceptible to *L. amazonensis* infection and has a reduced capacity to eliminate the parasite due to the lack of generation of oxidative burst caused by inability to inhibit NRF2 activity, probably resulting from high levels of IL-6 produced by these cells. In addition, *L. amazonensis* can immunomodulate the production of TNF and IL-10 in PBMC of diabetic patients throughout the infection, which may be directly related to the failure of diabetic cells to respond against the infection.

CRediT authorship contribution statement

Taylon Felipe Silva: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing

– original draft, Writing – review & editing. **Manoela Daiele Gonçalves:** Data curation, Writing – review & editing. **Virgínia Márcia Concato:** Data curation, Writing – review & editing. **Bruna Taciane da Silva Bortoleti:** Data curation, Writing – review & editing. **Fernanda Tomiotto-Pellissier:** Data curation, Writing – review & editing. **Raquel Arruda Sanfelice:** Data curation, Writing – review & editing. **Ana Carolina Jacob Rodrigues:** Data curation, Writing – review & editing. **Mariana Barbosa Detoni:** Data curation, Writing – review & editing. **Andréa Name Colado Simão:** Validation, Visualization, Writing – review & editing. **Luiz Antonio Custodio:** Validation, Visualization, Writing – review & editing. **Tânia Longo Mazzuco:** Validation, Visualization, Writing – review & editing. **Idessania Nazareth da Costa:** Validation, Visualization, Writing – review & editing. **Milena Menegazzo Miranda-Sapla:** Validation, Visualization, Writing – review & editing. **Wander Rogério Pavanelli:** Validation, Visualization, Writing – review & editing. **Ivete Conchon-Costa:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

Competing of interests

The authors confirm that there are no conflicts of interest in this work.

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4.2. Artigo 02: Hyperglycemia and diabetes favors *Leishmania amazonensis* infection impairing pro-inflammatory response, inhibiting oxidative stress and suppressing antigenic presentation.

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Hyperglycemia and diabetes favor *Leishmania amazonensis* infection impairing pro-inflammatory response, inhibiting oxidative stress and suppressing antigenic presentation

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ABSTRACT

Leishmaniasis is a group of infectious diseases caused by protozoa of the *Leishmania* genus and its immunopathogenesis results from host and parasite factors that trigger an unbalanced immune response during the infection. Diabetes is a chronic disease resulting from dysfunction of the body's production of insulin or the ability to use it properly, leading to hyperglycemia whose chronic out-of-control causes several tissue injuries and impairs the immune system. Thus, the objective of this work was to evaluate the THP-1-derived macrophages exposed to a hyperglycemic environment and C57BL/6 diabetic mice against *Leishmania amazonensis* infection and how these conditions alter the immune response to the parasite. We also performed an in vitro hyperglycemic stimulus model with THP-1 cells and infected them with the same species of parasite for several times. In addition, we induced experimental diabetes with streptozotocin (STZ) in C57BL/6 mice and evaluated cutaneous infection in the footpad for 5 weeks. We observed that diabetes and hyperglycemia impair the leishmanicidal capacity of macrophages derived from THP-1 cells, mainly by increasing the activity of the free radical scavenging enzymes SOD and catalase, respectively, in addition to reversing the resistance profile that C57BL/6 mice present against infection by L. *amazonensis*, inducing more exacerbated lesions compared to non-diabetic animals. In addition, the hyperglycemic stimulus favored the increase of markers related to the phenotype of M2 macrophages in a parasite-mediated manner. In the same sense, the induction of experimental diabetes in C57BL/6 mice resulted in a failure in the production of nitric oxide (NO) in the face of infection, in addition to generating a Th2/Th17-like response in these animals compared to control animals that generated a response similarly to the Th1 profile. We also observed that macrophages from diabetic animals failed to process and present Leishmania antigens during infection, being unable to activate and induce proliferation of antigen-specific lymphocytes. Together, these data demonstrate that diabetes and hyperglycemia can impair the cellular immune response, mainly of macrophages, against infection by parasites of the genus Leishmania.

Key-words: Leishmaniasis; Diabetes; Glucose; Macrophage phenotype; Antigen presentation.

1. Introduction

Leishmaniasis is a group of infectious diseases caused by protozoa of the *Leishmania* genus, which are transmitted to animals and humans through the blood meal of infected female sandflies. The main clinical forms of the disease are cutaneous leishmaniasis (CL), visceral leishmaniasis, and mucocutaneous leishmaniasis. CL is the most common form and is among the six most important infectious diseases for public health, affecting more than one million people each year (WHO, 2021).

The leishmaniasis immunopathogenesis results from host and parasite characteristics, once immune response triggered during the infection can determine the clinical form of the disease. However, differences between species or even different strains of the same species can present particular characteristics which alter the virulence, tropism, and pathogenicity of the parasite. The host's polarization of Th1 and Th2 response patterns is directly related to the pathogenesis of CL that leads to persistent chronic infection (SOONG; HENARD; MELBY, 2012).

Concerning lymphocyte polarization, the response triggered by monocytes/macrophages is essential for the immunopathogenesis and clinical evolution of leishmaniasis and requires a balance between pro and anti-inflammatory profiles. Macrophages have a dualistic impact on leishmaniasis since they provide a safe place for parasites to survive and proliferate, however they also can trigger an inflammatory response and oxidative stress that can control parasite replication. Therefore, these cells are the essentials to disease progression, and their interaction with parasites can establish the success or failure of the infection. (TOMIOTTO-PELLISSIER et al., 2018).

It should be noted that *Leishmania* spp. can actively manipulate their hosts and subvert microbicidal mechanisms by modifying/delaying the development of the response. These escape mechanisms are still being characterized, but they differ according to the parasite species and the host's genetic background (ACUÑA et al., 2017; AOKI et al., 2019a).

Diabetes Mellitus (DM) is a chronic disease resulting from the dysfunction of the body's insulin production or the ability to use it properly, leading to hyperglycemia whose chronic lack of control causes several injuries, especially

in the nervous system and blood vessels (KAUL et al., 2013). DM can be subdivided into categories according to the etiology of the disease, being type 1 (DM1), characterized as autoimmune and results from the destruction of pancreatic beta cells that make the body unable to produce insulin, and type 2 (DM2), derives from peripheral insulin resistance, excessive glucagon secretion and failure of insulin secretion by pancreatic beta cells, accounting for approximately 90% of all diabetes cases, with a rising incidence worldwide in the last 30 years (MEKALA; BERTONI, 2020)

Hyperglycemia caused by diabetes is responsible for inducing a low-grade inflammatory state, with the continuous production of several mediators secreted by immune cells and adipocytes, which play a central role in the pathogenesis of the disease, altering the physiological response in other tissues. In this sense, changes in the immune system triggered by the hyperglycemic state can have serious consequences in the response to pathogens, being intrinsically related to body desensitization to infectious stimuli (LONTCHI-YIMAGOU et al., 2013). Furthermore, diabetic individuals have been described as more susceptible to infection once hyperglycemia significantly reduces the microbicidal capacity of macrophages and neutrophils (GIRI et al., 2018; OTTO et al., 2008).

Our research group previously demonstrated that human peripheral blood mononuclear cells (PBMC) from diabetic individuals are more susceptible to *L. amazonensis* infection than healthy individuals, with a higher number of infected cells and parasites, mainly due to the inability to generate oxidative stress after infection. In addition, the parasite can effectively modulate the production of proteins responsible for blocking free radicals such as nuclear factor erythroid 2-related factor 2 (Nrf2) (SILVA et al., 2021).

Considering the immunological balance involved in combating and resisting infection by parasites of the *Leishmania* genus, dysregulation of the inflammatory response observed in chronic hyperglycemia may be a factor that induces susceptibility and aggravation of leishmaniasis in these individuals. Thus, a better understanding of how cells exposed to a hyperglycemic environment behave against *Leishmania* infection and how the parasite controls the cellular machinery in their favor are essential to better elucidate why diabetic individuals are more susceptible to this infection.

2. Material and methods

2.1 Culture of *Leishmania (Leishmania) amazonensis*

Promastigotes of *L. amazonensis* (MHOM/BR/1989/166MJO) and *L. amazonensis* expressing an enhanced green fluorescent protein (eGFP) addressed to the glycosome (MHOM/BR/1973/M2269) were maintained in M199 culture medium (Gibco, Invitrogen, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1 M HEPES buffer, 1% human urine, 1% L-glutamine, streptomycin and penicillin (Gibco), 0,5% Hemin, and 10% sodium bicarbonate. For parasite selection, was added 10 µg/mL of geneticin-G418 (Sigma, USA) in the eGFP strain culture. The parasites were incubated in a B.O.D. at 24 °C in a 25 cm² culture flask. All experiments used promastigotes in the stationary growth phase (five-day culture).

2.2 Promastigote proliferation assay

To evaluate the effect of D-glucose on *L. amazonensis,* $1x10^6$ of promastigotes were seeded in 24-well plates with several concentrations of D-glucose (10, 20, 30, 50, 100, 200, and 400 mM) and incubated for 24, 48, and 72 h in a B.O.D. at 24 °C. For all experiments, control group was cultured with a normoglycemic medium with 5 mM of D-glucose. After the respective incubation times, aliquots of 200 µL of the cultures were added to a black 96-well plate with the addition of 1 µL of resazurin (Sigma Aldrich, USA) to a final concentration of 10 µM and incubated at 24 ºC for 2 h. Samples were read in a GloMax® Plate Reader fluorimeter (Promega™, USA) with excitation (ex) 563 nm and emission (em) 587 nm. Data were shown as arbitrary units of resofurin fluorescence.

2.3 THP-1 cell culture and hyperglycemic stimulus

THP-1 cell line was cultured in RPMI-1640 medium containing 1% L-glutamine, 10% sodium bicarbonate, 10% FBS, and 1% streptomycin and penicillin (Gibco) at 37 °C and 5% CO_2 in a 75 cm² culture flask for 7 days in presence of 5 mM (control groups) or 30 mM (hyperglycemic groups) of D-glucose. After 7 days of culture with the respective D-glucose concentrations, cells were seeded in well plates with 5 ng/mL of Phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) for 48h to differentiate into macrophages, followed by 5 days

with a fresh medium. Then fully differentiated cells were used for the following experiments.

2.4 Cytotoxicity assay

THP-1 cells ($1x10^4$) were seeded in 96-well plates as described in 2.3. with several concentrations of D-glucose (10, 20, 30, 40, 60, 80, 100, 200, and 400 mM). 5 mM of D-glucose was used as the control group and dH_2O as vehicle at the same volume used in 400 mM concentration. Posteriorly, the medium with D-glucose was removed, and cells were incubated for 3 h with fresh RPMI-1640 medium containing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to a final concentration of 50 µg/mL. The MTT product (formazan crystals) was diluted with 100 µL of DMSO (dimethyl sulfoxide) and read in a spectrophotometer (Thermo Scientific, Multiskan GO) at 540 nm. The percentage of viable cells was calculated based on the absorbance of the control group.

2.5 *In vitro* experimental infection

THP-1 cells ($1x10^6$) were seeded in 6-well plates as described in Section 2.3. After differentiation, cells were infected with *L. amazonensis* promastigote forms ($1x10^7$) (MOI, multiplicity of infection of 10) for 2 h. After this, cells were washed with sterile phosphate-buffered saline (PBS) to remove non-internalized parasites, and cells were incubated for 24, 48, and 72 h at 37 ºC with 5% CO_2. The supernatants were collected for downstream applications and the cells used for infection assessment.

2.6 DNA extraction and parasite load quantification by real-time PCR

Cells described in the previous topic were collected and homogenized in 500 µL lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K (Invitrogen, CA)) and incubated at 37 °C overnight. After incubation, 105 µL of NaCl (6 M) was added and the samples were centrifuged for 5 min at 14000 rpm. The recovered supernatant was transferred to a new tube and DNA was precipitated with twice the volume of absolute ethanol, washed two times with 75% ethanol, left to dry at room temperature, and resuspended in 30 µL of TE buffer (10 mM Tris HCl pH 8.0 and 1 mM EDTA).

For extraction of DNA from animal tissues, footpads were mechanically homogenized (Tissue-tearor, BioSpec) in TELT buffer (50 mM de Tris-HCl pH 8, EDTA 62,5 mM, Triton-X 4% e LiCl2 2.5 M) with proteinase K (2 mg/mL) and incubated at 56 ºC for 2 h. Organic phase extraction was performed with buffer-containing phenol-chloroform-isoamyl alcohol (25:24:1, respectively). The recovered aqueous phase was added twice the volume of absolute ethanol, precipitated DNA was washed with 70 ° ethanol and left to dry at room temperature. DNA was eluted in 50 µL of TE buffer and stored in a freezer at −20 °C until use.

DNA concentrations were quantified using NanoVue Plus (Biochrom, Holliston, USA) and qPCR was performed with GoTaq® qPCR MasterMix (Promega, USA) normalized with 100 ng of total genomic DNA (gDNA). Parasite quantification was performed using primers for the *Leishmania* AAP3 gene, as fallow: forward 5'-GGCGGCGGTATTATCTCGAT-3') and reverse 5'-ACCACGAGGTAGATGACAGACA-3') (TELLEVIK et al., 2014). The samples were amplified on a StepOne Plus thermal cycler (Applied Biosystems, USA) under the following PCR conditions: an initial step of 2 min at 95 ºC and 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC, followed by a dissociation step 55 °C to 99 ºC with an increase in temperature of 0.5 ºC / sec. The results were calculated based on a standard curve constructed with DNA extracted from culture samples of *L. amazonensis* promastigote forms.

2.7 Flow cytometry infection analysis

For evaluation of experimental infection by flow cytometry, cells cultured as described in 2.5 were collected after 48 h of infection with *L. amazonensis* eGFP. For that, 200 µL of trypsin 0.25% was added to each well for 5 min followed by the addition of 400 µL of FBS. Cells collected were centrifuged at 1500 rpm for 5 min, and the pellet was resuspended in 50 µL of Live Cell Imaging Solution (Invitrogen, USA) with 2 µg/mL of propidium iodide (PI) (Sigma, USA) e anti-human CD11b antibody conjugated with phycoerythrin (PE) (dilution 1:200) (Invitrogen, USA). After 20 min incubation at 37 ºC, cells were washed 3 times with sterile PBS and analyzed in a BD Accuri C6 flow cytometer (BD Biosciences, USA) with the acquisition of 10,000 events $CD11b^{+}/PI^{-}$. The infection analysis

was performed considering the percentage of $eGFP^+$ cells and the intensity of infection was determined based on eGFP fluorescence intensity.

2.8 Promastigote recovery assay

Cells ($1x10^5$) were seeded in 24-well plates and infected with *L. amazonensis* promastigote forms ($1x10^6$) (MOI of 10), for 2 h. Then, were washed with sterile PBS to remove non-internalized parasites and incubated for 48 h at 37 ºC with 5% CO_2. After infection, RPMI-1640 medium was replaced for 199 medium, and cells were incubated in a B.O.D. at 24 °C for rupture and release of parasites. The number of promastigote forms in the medium was counted in Neubauer's chamber at 24, 48, and 72 h after medium change.

2.9 Nitrite quantification as an estimate of nitric oxide (NO) production

NO levels were determined based on the Griess method using the aliquoted supernatant. Briefly, 60 µL of supernatant was added to 60 µL of Griess reagent (1% sulfanilamide and 0.1% of N-(1-Naphthyl) ethylenediamine in orthophosphoric acid (H_3PO_4) 5%), incubated for 10 min protected from light at room temperature, and read in a plate reader (Thermo Scientific, Multiskan GO, USA) at 550 nm. Serial dilutions of $NaNO_2$ were used for quantification. The quantification of NO in mice serum samples was performed as previously described (MIRANDA et al., 2015).

2.10 Quantification of superoxide anion

To determine the levels of superoxide anion produced by THP-1 cells infected or not *L. amazonensis*, cells ($1x10^4$) were seeded in 96-well plate and infected with promastigote forms ($1x10^5$), as described in 2.5. After 48 h of infection, cells were incubated for 2 h with fresh RPMI-1640 medium containing 100 µg/mL of nitroblue tetrazolium (NBT). The medium was removed, and cells lysed with 50 µL of KOH (2 M) followed by the addition of 50 µL of DMSO. The reading was performed at 600 nm and data were expressed as optical density (OD).

2.11 Production of reactive oxygen species (ROS)

ROS generation was evaluated by the conversion of nonfluorescent 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular free radicals. Cells were plated in a black 96-well microplate and cultured proceeded as described in 2.10. After incubation, cells were washed with PBS, and H_2DCFDA (10 M) solution (Sigma Aldrich, USA) was added for 45 min in the dark at 37 °C. The samples were read at Ex 488 nm and Em 530 nm on a GloMax® plate reader (Promega TM, USA). Hydrogen peroxide (H_2O_2) was used as a positive control. Data were expressed as arbitrary units of DCF fluorescence.

2.12 N-acetyl-β-D-Glucosaminidase (NAG) enzymatic activity assay

For determination of NAG activity, THP-1 cells were seeded ($1x10^5$), differentiated, and infected in a 24-well plate as described in 2.5. After infection, the medium was removed and cells were washed 3 times with sterile PBS, followed by the addition of 100 µL of PBS and 3 sequential cycles of freezing at -80°C for 10 min and thawing at 37 °C for 5 min. Posteriorly, 50 µL of cell lysate was incubated for 30 min at room temperature with 50 µL of 4-Nitrophenyl N-acetyl-β-D-glucosaminide (3 mM) in hexadecyl trimethyl ammonium bromide buffer (HTAB) (15 mM, pH 4,7). After incubation, 50 µL of stop solution (glycine 200 mM, pH>10) was added and read at 540 nm. The concentrations were determined based on the absorbance of a known standard control of 4-nitrophenol, considering that 1 unit of NAG hydrolyzes 1 µM of 4-Nitrophenyl N-acetyl-β-D-glucosaminide to 4-nitrophenol and N-acetyl-β-D-glucosaminide per minute. Data were normalized by milligrams (mg) of protein/mL.

2.13 Superoxide dismutase (SOD) activity assay

For determination of SOD activity, THP-1 cells ($1x10^5$) were seeded, differentiated, and infected in a 24-well plate as described in 2.12. Subsequently, the total cell lysate was collected, and SOD activity was quantified as described by Marklund (MARKLUND; MARKLUND, 1974). Total protein was determined from cell lysate using the method proposed by Lowry (LOWRY et al., 1951), and the data were normalized by mg protein/mL.

2.14 Catalase activity assay

Catalase activity was determined based on the method prosed by Hadwan (HADWAN, 2016) following the same experimental protocol as described in 2.12. Data were normalized by mg of protein/mL.

2.15 Arginase activity assay

Arginase activity was determined based on the hydrolysis of L-arginine by arginase-generating urea that reacts with α-Isonitrosopropiophenone producing a colored compound. Briefly, 50 µL of THP-1 cells lysate or supernatant from mechanically homogenized footpads from mice was incubated with 50 µL of arginase activation buffer (20 mM of $MnCl_2$ in Tris-HCl 50 mM pH 7,5), for 10 min at 56 ºC. Next, 100 µL of L-arginine (500 mM) (Sigma, USA) was added to the reaction and incubated for 1 h at 37 ºC. To stop the reaction, 300 µL of stop solution (H_2SO_4: H_3PO_4: dH_2O [1:3:7 vol/vol]) was added, followed by the addition of 30 µL of α-Isonitrosopropiophenone solution (4.5%) (Sigma, USA) in DMSO and incubation for 1 h at 100 ºC. The samples were transferred to a 96-well plate and read at 540 nm. The concentrations were determined based on a standard curve constructed with serial dilutions of urea, considering that 1 unit of arginase catalyzes 1 µM of urea/min. Data were normalized by mg of protein/ml or mg of tissue.

2.16 RNA extraction

THP-1 cells were cultured as described in 2.3 and infected as described in 2.5. After 48 h of infection, cells were washed with sterile PBS to complete remove medium, 500 µL of TRIzol reagent (Invitrogen, USA) was added and the cell lysate was collected and frozen at -80 ° C overnight. Next, 100 µL of chloroform was added to the samples followed by centrifugation for 15 min at 4 ºC, 12,000 rpm. The upper aqueous phase was collected, added to 500 µL of isopropanol, and left to precipitate overnight at -20 ºC. The samples were centrifuged for 15 min at 4 ºC, 12,000 rpm, and the formed pellet was washed 3 times, once with 70º ethanol and twice with absolute ethanol. Thereafter, the precipitated RNA was dried at room temperature for 10 min, resuspended in 30

µL of TE buffer, quantified using NanoVue Plus (GE Life Science, USA), and concentrations adjusted to 100 ng/µL of RNA.

2.17 Reverse transcription and gene expression

To analyze gene expression, 2 µg of total RNA extracted as described above were reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied biosystems, USA) following the manufacturer's instructions. Next, qPCR was performed with GoTaq® qPCR MasterMix (Promega, USA) normalized with 100 ng of cDNA, using primers for human Myeloid differentiation primary response 88 (MyD88) forward: 5'-GGCTGCTCTCAACATGCGA-3') and reverse: 5'-CTGTGTCCGCACGTTCAAGA-3'), human CD40 forward: 5'-TTGGGGTCAAGCAGATTGCTA-3') and reverse: 5'-GCAGATGACACATTGGAGAAGA-3'), human CCL4 forward: 5'-ACTTTGAGACGAGCAGCCAGTG-3') and reverse: 5'-TTTCTGGACCCACTCCTCACTG-3'), human CXCL2 forward: 5'-GGCAGAAAGCTTGTCTCAACCC-3') and reverse: 5'-CCAGTCGCCTGTGTACATGGA-3'), and human β-actin forward: 5'-CTCGACACCAGGGCGTTATG-3') and reverse: 5'-CCACTCCATGCTCGATAGGAT-3'). The samples were amplified on a StepOne Plus thermal cycler (Applied Biosystems, USA) under the following PCR conditions: an initial step of 2 min at 95 °C and 40 cycles of 15 s at 95 °C, 1 min at 60 °C, followed by a dissociation step 55 ºC to 99 ºC with an increase in temperature of 0.5 ºC/sec. The results were calculated as $2^{-(\Delta\Delta Ct)}$ and normalized for biological significance $\left\{2^{-(\Delta\Delta Ct)} \geq 1 = \frac{2^{-(\Delta\Delta Ct)}}{1}\right\}$ or $\left\{2^{-(\Delta\Delta Ct)} < 1 = \frac{-1}{2^{-(\Delta\Delta Ct)}}\right\}$.

2.18 Cytokine measurement

The Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences, USA, 551809) was used to measure levels of TNF, IFN-γ, IL-17A IL-10, IL-6, IL-4, and IL-2 in the supernatant of mechanically homogenized footpads and in plasma samples from mice collected as described below. Also, for human TNF-α detection BD OptEIA™ Human TNF-α ELISA Set II kit (BD biosciences, USA) was used.

2.19 Immunophenotyping and labeling of surface receptors

To detect the presence of cellular surface markers, $1x10^5$ of THP-1 cells were seeded in 24 well plates and cultures/infected as described in 2.3 and 2.5 topics. After 48 h of infection, cells were harvested and resuspended in 50 µL of Live Cell Imaging Solution (Invitrogen, USA) with conjugated primary antibodies anti-HLA-DR-FITC, CD80-PECy5, CD86-PE, CD209-PerCP-Cy5.5 diluted 1:50, 1:200, 1:100, and 1:200 respectively. For Arginase and CD206, unconjugated antibodies were used (both diluted 1:200), following incubation with anti-IgG-FITC secondary antibody. For detection of monocyte markers, THP-1 cells were cultures in 75 cm³ flaks for 7 days in normoglycemic or hyperglycemic medium as described before. Next, $1x10^5$ of cells were seeded in 24 well plates and infected with $1x10^6$ of *L. amazonensis.* After 48 h of infection, cells were collected and labeled with anti-CD14-Alexa488 and CD16-PE-Cy7 antibodies. For all labelling, after 20 min of incubation at 37 ºC, cells were washed twice in PBS and resuspended in 200 µL of Live Cell Imaging Solution and 10,000 events of singlets acquired in a BD Accuri C6 flow cytometer (BD Biosciences, USA).

2.20 *Experimental* diabetes and *Leishmania amazonensis* infection *in vivo*

C57BL/6 mice weighing approximately 25–30 g and aged 10-15 weeks, were kept under sterile conditions, and used according to protocols approved by the Institutional Animal Care and Committee. This study was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina, CEUA n° 010.2021. Mice received intraperitoneally a single dose of 150 mg/kg of streptozotocin (STZ) (Sigma, USA) for the induction of experimental diabetes. The control group animals received the same volume of 50 mM sodium citrate vehicle buffer. All animals will receive ad libitum water with 30% sucrose in the first 24 h to avoid death from hypoglycemia caused by STZ. The glycemic level of the animals was measured weekly with an Accu-Chek Active glucometer (ROCHE, Brazil) and those with venous blood glucose greater than 250 mg/dL after 3 weeks were considered diabetic. The animals were divided into 4 groups ($n \geq 5$): uninfected control, infected control, uninfected diabetic, and infected diabetic, containing between five and seven animals each. Infected groups

received subcutaneously $5x10^6$ promastigote forms of *L. amazonensis* in both footpads and uninfected groups received the same volume of 199 medium.

2.21 Analysis of edema and evolution of infection

Paw edema was measured weekly using a digital caliper (Starrett 799). At each time point, both paws were measured, and the data were plotted as the mean between the right and left paws of each animal. Final data are expressed as the mean edema of animals in the group.

After five weeks of infection, animals (n≥5) were euthanized in a CO_2 chamber. Peritoneal macrophages were harvested as described by Miranda-Sapla, et al (MIRANDA-SAPLA et al., 2019), blood was collected by cardiac puncture in a vacuum heparinized tube, spleen, and the footpads were collected, weighed, and processed for the assays.

2.22 Quantification of anti-*Leishmania* IgG

The levels of IgG anti-Leishmania were determined by an ELISA assay *in house* (Enzyme-linked immunosorbent assay). Briefly, *L. amazonensis* soluble antigen was extracted as previously described (GOMES-SILVA et al., 2008) and 2 µg/well were adsorbed in ELISA microtiter plate overnight at 4 ºC. Then, serum samples were diluted 1:20 and added in triplicate. Horseradish peroxidase labelled goat anti-mouse IgG was used as detector system (eBiosciences, USA). The results were expressed as ELISA index (EI), obtained by mean of sample absorbance divided by mean of negative controls (n≥5) absorbance.

2.23 Myeloperoxidase (MPO) activity

Neutrophil recruitment to the footpad was evaluated through quantification of the MPO enzymatic activity in the supernatant from mechanically homogenized footpads from mice. Briefly, weighed paws were homogenized in 200 µL of 50 mM K2HPO4 bufer (pH 6.0) containing 0.5% HTAB and centrifuged at 10.000 rpm for 2 min at 4º C. 15 µL of recovered supernatant was incubated for 20 min with 200 µL of 50 mM K2HPO4 bufer (pH 6.0), containing 0.0167% *ortho*-dianisidine dihydrochloride and 0.05% H_2O_2. The absorbance was determined at 450 nm (Multiskan GO microplate spectrophotometer, ThermoScientifc, Vantaa,

Finland), and MPO activity of samples was compared to a standard curve of neutrophils and presented as neutrophils/mg of tissue.

2.24 Antigen presentation assay

To evaluate the antigen presentation capacity, peritoneal macrophages ($1x10^5$) obtained from diabetic and healthy infected mice were seeded in 24-well plate for 3 h for adhesion following by 48 h of *L. amazonensis* infection. At the same time, the spleens collected from the same mice were dissociated by passing through a 70 µm cell strainer. Mononuclear cells were isolated from the single cell suspension using density gradient method with Histopaque®-1077 (Sigma, USA) as described by Silva et al (SILVA et al., 2021) and 10^8 of cells were seeded in 47 mm petri dishes. After 48 h of incubation (37 ºC with 5% CO_2), non-adherent cells were considered lymphocytes and collected for the assay, and the remaining adhered cells were discarded. Lymphocytes were labeled with carboxyfluorescein diacetate succinimidyl ester (10 µM) (CFSE) as described by Lim et al (LIM; BERGER; SU, 2016), and added in a 1:1 ratio to the culture of peritoneal macrophages previously seeded to recognize the *Leishmania* antigens being presented by macrophages. To improve recognition, the culture plates were centrifuged at 800 rpm for 5 min to increase cell-cell contact. The co-culture was incubated at 37 ºC for 72 h and the evaluation of successful antigenic presentation was assessed by lymphocyte proliferation, measuring the geometric dilution of CFSE fluorescence by flow cytometry and by the production of IFN-γ quantified in the culture supernatant.

2.25 Statistical analysis

Data were expressed as a mean ± standard error mean, and three independent experiments were performed, each with quadruplicate datasets. Data were analyzed using GraphPad Prism 8 statistical software (GraphPad Software, USA). Data were submitted to the Shapiro-Wilk and Levene tests, and those with normal distribution and homogeneity of the variances were evaluated according to the one-way ANOVA-test with post hoc Tukey. Data without normal distribution were transformed by natural logarithm to show normality. Statistical

significance was set at $p<0.05$ and the p-values were categorized through *($p<0.05$); **($p\leq0.01$); ***($p\leq0.001$); ****($p\leq0.0001$) for all analyses.

3. Results

3.1 Hyperglycemic stimulus predispose THP-1 cells to *L. amazonensis* infection

First, we evaluated high levels of D-glucose affect the proliferation of promastigote forms of *L. amazonensis*, and interestingly we found that even 400 mM of D-glucose did not increase or decrease the parasite proliferation/viability (**Fig. 1A**). To investigate whether a hyperglycemic environment increases susceptibility to L. *amazonensis* infection, we performed an *in vitro* model of hyperglycemic stimulus using THP-1-derived macrophages (**Fig. 1B**). Second, we validated the hyperglycemic stimulus model by testing several concentrations of D-glucose to rule out the possibility of bias induced by stimulus-induced reduction in cell viability, and we verified that THP-1 cells showed a reduction in cell viability starting only at 100 mM, meaning that the concentration of 30 mM used during the stimulus is not able to reduce the viability of the cells during the next experiments (**Fig. 1C**).

Next, we evaluated if THP-1-derived macrophages exposed to a hyperglicemic environment have altered ability to phagocytize (2 h) and eliminate leishmania parasites over time (24-72 h). When we quantified the parasite load by qPCR, interestingly, there was no difference between control and HG cells after the phagocytosis period (2 h) (**Fig. 1D**) and 24 h (**Fig. 1E**) of infected macrophages culture; however, from 48 h, HG cells showed double the parasite load compared to control ($p<0.0001$), and this difference remains at 72 h, indicating that HG stimulus does not change the cellular phagocytic capacity of cells, but impairs parasite clearance over time (**Fig. 1E**). Considering that the difference between the groups was observed after 48 h, we performed the following experiments only at this point of analysis.

We also investigated the infection rate in these cells by flow cytometry using a strain of *L. amazonensis* expressing eGFP and found a higher number of infected cells ($eGFP^+$ cells) and higher eGFP fluorescence intensity (number of parasites per cell) in the HG group compared to the control (both $p<0.0001$) (**Fig.**

1F-G and **Fig. S1**). To confirm the effect found in parasite load and by flow cytometry, we carried out the recovery of viable parasites after 48 h of infection by incubating them in favorable conditions to the differentiation of viable intracellular amastigotes into free promastigotes. Similarly, we found 3.5 times higher number of parasites in HG than in the control group ($p<0.0001$) (**Fig. 1H**).

3.2 *L. amazonensis* controls microbicidal mediators and oxidative stress in hyperglycemic-stimulated THP-1-derived macrophages

Also, we evaluated the production of free radicals and other microbicidal molecules responsible for the death of the intracellular parasite. As expected, we found that NO levels remain the same in the control group ($p>0.05$), since *L. amazonensis* can actively control its production. Moreover, even though the HG group had a higher basal level than the control ($p<0.0001$), infection was able to reduce it to levels similar to those observed in the control ($p>0.05$) (**Fig. 2A**).

Regarding superoxide anion levels, HG showed a higher baseline level than the control group ($p<0.05$), however, after infection, these levels were significantly reduced ($p<0.01$). On the other hand, the control group, which had lower basal levels of superoxide anion, increased significantly after infection ($p<0.05$) (Fig. 2B). Similarly, the control group was able to increase total ROS production and NAG activity after infection ($p<0.0001$) while the HG group failed, maintaining levels similar to baseline ($p>0.05$) (**Fig. 2C-D**).

Considering the difference between groups regarding oxidative stress, we evaluated the enzymatic activity of two important free radical scavengers, superoxide dismutase (SOD), and catalase. It was found that control and HG groups has the same basal levels of SOD activity, however, after infection the control group reduces ($p<0.0001$) while the HG group increases ($p<0.01$) this enzyme activity (**Fig. 2E**). Concerning catalase, the control group maintained the same levels even after infection ($p>0.05$), while HG significantly increased the enzymatic activity when infected with *L. amazonensis* ($p<0.01$) (**Fig. 2F**).

3.3 Hyperglycemic stimulus alters immune mediators triggered by *L. amazonensis* infection

Considering that macrophages are important immune cells and have a key function against *Leishmania*, we assessed if hyperglycemia would be able to alter the expression immune mediators in these cells during *L. amazonensis* infection. First, we found that the HG group had a significant increase in arginase enzymatic activity after infection ($p<0.0001$), which did not occur in the control group ($p>0.05$), indicating a possible parasite manipulation of cellular machinery (**Fig. 3A**). In contrast, control cells significantly increased CXCL2, CCL4, and MyD88 expression by 2.2 ($p<0.01$), 11.4 ($p<0.001$), and 3-fold ($p<0.001$) after infection, respectively (**Fig. 3B, C**, and **E**, respectively). The HG group showed significantly lower basal levels of CXCL2 expression than the control group ($p<0.0001$) and failed to increase the expression of both chemokines and MyD88 after infection ($p>0.05$). On the other hand, CD40 expression remained the same in control group, but significantly decrease in the HG group after infection ($p<0,05$) (**Fig. 3D**). Regarding cytokines, the basal levels of TNF-α were equal between the groups ($p>0.05$), however, after infection, only the control group managed to increase it ($p<0.0001$) (**Fig. 3F**).

3.4 Hyperglycemia changes the monocyte phenotype to an intermediate pattern

Taking into account that hyperglycemic stimulus influences the response triggered by *L. amazonensis* infection we investigated whether hyperglycemia would be able to alter the phenotype of monocytes, which are the precursor cells of the macrophages used and changes in their phenotype could explain the different responses observed so far. For this, we assessed the CD14 and CD16 expression pattern and found that the control and HG have distinct phenotypic patterns (**Fig. 4A-B**). While the control showed a classic phenotype ($CD14^{+}CD16^{-}$), the HG presented an intermediate phenotype ($CD14^{+}CD16^{+}$). Interestingly, in this case *L. amazonensis* infection had no effect on the observed phenotypic differences ($p>0.05$). In the analysis, 85.5% of cell population from control and 45.5% from HG were CD14alexa488^{+}/CD16-PECy7^{-}. In contrast, only 6.6% of

control were CD14-Alexa488^{+}/CD16-PECy7^{+}, while this was the majority of the population in HG group, corresponding to 54% of the cells (**Fig. 4C**).

3.5 *L. amazonensis* infection increase M2 related markers in hyperglycemic-stimulated macrophages.

Since our previous result showed a change in monocyte phenotype after hyperglycemic stimulus and also an increase in arginase activity in the HG group after infection and higher expression of CXCL2 and CCL4 in control group after infection and considering that the former is an important marker of the M2 macrophage phenotype and the last ones markers of M1 macrophages, we decided to investigate if hyperglycemic stimulus alters the presence of M1 and M2 macrophage phenotype markers during *L. amazonensis* infection. We observed that both uninfected groups have similar levels of HLA-DR, CD80, CD86, Arginase, and CD206 ($p>0.05$). However, after infection control group increased M1 related markers (**Fig. 5 A, B, and C**) while HG group increased M2 markers (**Fig. 5 D-E**) represented by an increase of 29.2% ($p<0.0001$), 12.2% ($p<0.0001$), and 24.9% ($p<0.0001$) of HLA-DR, CD80, and CD86 positive cells after infection, respectively, in control group, while M2 markers, CD206 and arginase, did not change after the infection in this group. In other hand, for the HG group, arginase and CD206 positive cells increased 33% ($p<0.0001$) and 10.6% ($p<0.01$), respectively, and M1 markers showed no changes.

3.6 Diabetes aggravate *L. amazonensis*-induced edema in C57BL/6 mice and increases parasite load

To investigate if diabetes could induce susceptibility to *L. amazonensis* infection, we performed an *in vivo* model of STZ-induced diabetes in C57BL/6 mice, which is known to be a strain partially resistant to *L. amazonensis* infection. After infection, we observed that diabetic animals showed a higher footpad edema than non-diabetic animals. Diabetic mice showed a statistical tendency to have greater edema from the first week post-infection, however, it was only after the third ($p<0.01$) and fourth week ($p<0.0001$) that it was possible to observe a statistical difference between the groups (**Fig. 6A**). Similarly, the parasite load in

the paws tissue after 4 weeks of infection was higher in the diabetic than the control group ($p<0.05$) (**Fig. 6B**).

On the other hand, diabetic mice showed a higher neutrophil infiltrate after 5 weeks post-infection than non-diabetic mice ($p<0.01$), which did not demonstrate any increase in neutrophil infiltrate (MPO activity) after the infection time analyzed ($p>0.05$) (**Fig. 6C**).

3.7 Diabetic mice fail to increase nitric oxide production and have higher neutrophil infiltrate during *L. amazonensis* infection

Next, we investigated the systemic and local levels of nitric oxide in *L. amazonensis*-infected healthy and diabetic C57BL/6 mice and observed that non-diabetic mice were able to increase the production nitric oxide after infection both locally ($p<0.05$) (**Fig. 7A**) and systemically ($p<0.0001$) (**Fig. 7B**) which did no occurred in diabetic mice ($p>0.05$). Interestingly, we also found that non-diabetic mice also presented higher levels of arginase activity than diabetic mice ($p<0.0001$), but did not change even after infection in both groups ($p<0.05$) (**Fig. 7C**).

We also analyzed the Person correlation between nitric oxide and arginase levels measured in the lesion site and found that uninfected non-diabetic group present a negative correlation ($r= -0.8320$ and $p<0.05$), while uninfected diabetic animals showed a positive correlation ($r= 0.9098$ and $p<0.05$). On the other hand, when we evaluate the same groups after infection, non-diabetic mice did not showed correlation between the two mediators evaluated ($r= 0.07275$ and $p>0.05$) while diabetic mice increased the correlation power ($r= 0.9495$ and $p<0.05$) (**Fig. 7E**).

3.8 *L. amazonensis* infection triggers different cytokine responses in diabetic and non-diabetic C57BL/6 mice

We analyzed the profile of released cytokines by diabetic and non-diabetic mice during *L. amazonensis* infection and we found a distinct pattern between these two groups. After infection, non-diabetic mice showed higher local production of TNF ($p<0.05$), while diabetic animals showed higher levels of IL-17A ($p<0.05$) (**Fig. 8A**). At the systemic level, diabetic mice showed increased

levels of IL-17A ($p<0.01$), IL-10 ($p<0.05$), and IL-4 ($p<0.01$) after the infection time analyzed, while non-diabetic animals only showed an increase in IL-6 levels ($p<0.05$). Interestingly, non-diabetic animal showed a higher basal level of IL-2 which significantly reduced after infection ($p<0.01$). IL-4 levels in the paws and IL-2 levels in the serum remained below the detection limit of the technique and were not showed (**Fig. 8B**).

3.9 Diabetes impairs IgG production and presentation of *Leishmania* antigens by macrophages

We measured the total anti-leishmania IgG present in serum samples of diabetic and non-diabetic mice infected with *L. amazonensis* for 5 weeks and found that diabetic mice had almost 1.5 times less IgG raised against the parasite that non-diabetic animals ($p<0.01$) (**Fig. 9A**).

In the same way, we noted that macrophages stimulated by hyperglycemic-stimulated macrophages fail to increase important receptors related to antigen presentation, such as HLA-DR, CD80, and CD86 after *L. amazonensis* infection. Therefore, we investigated if peritoneal macrophages from non-diabetic and diabetic mice were able to process and present leishmania antigens after infection with viable and heat killed *L. amazonensis* in a co-culture model with autologous splenic lymphocytes. As expected, we found that macrophages from non-diabetic mice efficiently induced lymphocyte proliferation as well as IFN-γ production ($p<0.0001$) compared to uninfected co-culture. In contrast, macrophages from diabetic mice failed to induce lymphocyte proliferation, demonstrating a similar result to that observed in uninfected group. However, interestingly, diabetic group managed to increase IFN-γ production ($p<0.0001$) even without inducing lymphocyte proliferation, but in a much less intense way than healthy group ($p<0.0001$) (**Fig. 9B-C**).

Infection with heat-killed *L. amazonensis* resulted in a 3-fold less exacerbated IFN-γ response in both groups (**Fig. 9C**); however, in this scenario, the diabetic group was able to induce lymphocyte proliferation, indicating that *L. amazonensis* can effectively inhibit antigenic presentation in diabetic macrophages (**Fig. 9B**).

4. Discussion

Several studies have demonstrated the effect of hyperglycemia and diabetes on infection by several pathogens (CHANCHAMROEN et al., 2009; DANQUAH; BEDU-ADDO; MOCKENHAUPT, 2010; HTUN et al., 2018; JAVID et al., 2016; KUMAR et al., 2012; LI et al., 2018; LIN et al., 2006; YANG et al., 2006; YANO et al., 2012), however, there are few studies that assess the infection by parasites of the genus *Leishmania* in this context (BONYEK-SILVA et al., 2020; LAGO et al., 2020; SILVA et al., 2021). Therefore, in this work we demonstrate that hyperglycemic stimulus disables macrophages derived from THP-1 cells and impairs their ability to eliminate *L. amazonensis* by increasing antioxidant activity of the cells, reducing their ability to generate free radicals responsible for acting directly on the parasites. Besides that, hyperglycemic altered the production of immune mediators an promoted an increase in markers related to M2 macrophages in a *Leishmania*-mediated manner, in addition to inhibiting the increase in receptors related to antigen-presenting and markers of M1 macrophages.

Similarly, the development of experimental diabetes in C57BL/6 mice increased the susceptibility to *L. amazonensis* infection worsening paw edema and impairing NO production, besides that diabetic animal showed higher neutrophil infiltrate and a cytokine profile that resembles Th2/Th17 with higher level of IL-4, IL-10, and IL-17, while non-diabetic mice induced a local Th1 related pattern with increased TNF production. In the same way, diabetic mice failed to induce lymphocyte activation and proliferation, showing that diabetes impairs the anti-*Leishmania* immune response and might be a susceptibility factor for leishmaniasis. These data corroborate with our previous work in which we demonstrated that PBMC from diabetic patients are more susceptible to infection by *L. amazonensis* and have a decreased capacity to eliminate the parasite over time when compared to PBMC from healthy individuals (SILVA et al., 2021).

The use of THP-1 monocytic lineage cells become an attractive model for *in vitro* investigation of the effect of hyperglycemic stimulus on the cellular ability against *L. amazonensis* infection, since they have been widely used to investigate monocyte/macrophage functions, mechanisms, signaling pathways, and nutrient and drug transport. Furthermore, Miao and colleagues (MIAO et al., 2007)

demonstrated that culture of THP-1 cells in hyperglycemic medium induces a transcriptomic and epigenetic behavior profile very similar to PBMC from diabetic individuals, making THP-1 a useful *in vitro* model for studying the effect of hyperglycemia on the immune response.

The reduced ability to eliminate parasites observed in our cells exposed to a hyperglycemic environment may be related to the activation of antioxidant mechanisms. The production of microbicidal molecules such as nitric oxide and free radicals is known as crucial in the control and elimination of parasites of the genus *Leishmania* (OLEKHNOVITCH; BOUSSO, 2015). We verify that infection of macrophages exposed to a hyperglycemic environment with *L. amazonensis* resulted in lower levels of ROS and superoxide anion, probably by activation of free radical scavenger enzymes SOD and catalase, these enzymes are important to the antioxidant effect critical for parasite survival (MITTRA et al., 2017).

Mittra et al (MITTRA et al., 2017) demonstrated that SOD promotes *Leishmania* virulence by protecting parasites against mitochondria-generated oxidative stress and by initiating ROS-mediated signaling mechanisms which are required for the differentiation of promastigotes into amastigotes within cells. Additionally, the blockade of parasite SOD resulted in the generation of aberrant strains unable to proliferate, and the blockade of host cell SOD resulted in unsuccessful infections with intense parasite elimination (OLIVEIRA et al., 2019). Bonyek-Silva et al. (BONYEK-SILVA et al., 2020) also showed that macrophages from diabetic patients are incapable of producing reactive oxygen species when infected by *L. braziliensis*. In addition, diabetic individuals diagnosed with leishmaniasis showed a higher number of parasites per cell in the lesion site than individuals with leishmaniasis without diabetes.

We demonstrated in a previous study that PBMC from diabetic patients fail to generate oxidative stress in response to *L. amazonensis* infection possibly by activation of NRF2 induced by the parasite (SILVA et al., 2021). NFR2 is a nuclear transcription factor responsible for the activation of a multitude number of antioxidant enzymes responsible for controlling oxidative stress. Activation of NFR2 by parasites of the *Leishmania* genus has been widely discussed (VIVARINI; LOPES, 2020a). In murine peritoneal macrophages and human macrophages lineages, *L. amazonensis* infection leads to the increase of SOD1 expression in a PKR/Nrf2 axis-dependent way. The parasite is also able to

explore the PI3K/Akt pathway to induce translocation of NFR2 into the nucleus (VIVARINI et al., 2017; VIVARINI; LOPES, 2020b). Therefore, a hyperglycemic environment probably makes the cell more susceptible to parasite-mediated NFR2 activation, which culminates in the activation of antioxidant responses such as SOD and catalase (GHANIM et al., 2021; LIN et al., 2019).

In our findings, infection by *L. amazonensis* in THP-1 derived macrophages resulted in increased of arginase enzyme activity. It is well-known that arginase is an essential enzyme for the survival and proliferation of *Leishmania* parasites for two main factors: first by catalyzing the conversion of L-arginine into polyamines essential for parasite metabolism; and second because it competes with iNOS for the substrate and consequently reduces the host cell's ability to produce nitric NO (AOKI et al., 2019b; PESSENDA; DA SILVA, 2020). In fact, Tomiotto-Pellissier et al. (TOMIOTTO-PELLISSIER et al., 2021) demonstrated that BALB/c mice that present a susceptibility profile to infection by *L. amazonensis* exhibit intense arginase production, in contrast to that observed in C57BL/6 mice with a resistance profile to this parasite. Thus, the intense arginase activation observed in HG cells, besides indicating a susceptibility profile, may also represent the subvert of the cellular machinery by the parasite.

Interestingly, we observed the same effect of NO in our THP-1-derived macrophages to that observed in our previous study with PBMC from diabetic patients. As expected, PBMC from healthy individuals and THP-1 cell cultivated in normoglycemic medium did not increase NO level after infection, since parasite can actively regulate it production. On the other hand, PBMC from diabetic patients and THP-1 cultivated in a hyperglycemic condition showed a higher basal level of NO which decreased after infection to levels similar to those found in healthy/normoglycemic controls, indicating that besides diabetes or hyperglycemia induces higher basal levels of NO, *L. amazonensis* infection can effectively downregulate it (SILVA et al., 2021).

On the other hand, in our murine model of diabetes we observed that infection with *L. amazonensis* resulted in failed capacity to generate NO while non-diabetic mice successfully induced NO production after infection. However, regarding arginase, interestingly, non-diabetic mice showed higher activity when compared to diabetic animals, but in both cases the infection did not alter the activity of this enzyme. When we did correlation analysis between NO and

arginase both measured in paws, non-diabetic animals showed a negative correlation before the infection and no correlation after infection and the diabetic group showed a positive correlation before and after infection. These data could indicate that non-diabetic animal can regulate the production of NO in an arginase-independent manner, while diabetic animals keep these two mediators linked, and, as their effects are essentially opposite, they end up being overruled (OLEKHNOVITCH; BOUSSO, 2015; PESSENDA; SILVA, 2020).

Arginase is also an important marker of M2 phenotype macrophages, characterized by their anti-inflammatory profile (TOMIOTTO-PELLISSIER et al., 2018). Our results demonstrated that hyperglycemic stimulation alone does not alter the phenotype markers of THP-1-derived macrophages differentiated with low-dose PMA. However, the infection with *L. amazonensis* resulted in an increase in markers related to M1 profile in normoglycemic macrophages and M2 in hyperglycemic macrophages, indicating that the exposure to high glucose levels makes macrophages more susceptible to the control exerted by the parasite.

It is known that usually resting macrophages exhibit a neutral profile considered as naive or M0, however, the microenvironment in which these cells are found provides different signals that activates them and leads to the development of functionally distinct phenotypes, termed classical activation or M1 (pro-inflammatory) and alternative activation or M2 (anti-inflammatory). The activation of M1 macrophages by IFN-γ produced by Th1 lymphocytes, triggers pro-inflammatory signaling and TNF-α, being crucial for the elimination of intracellular parasites by eliciting the oxidative burst (LOCATI; CURTALE; MANTOVANI, 2020; SHAPOURI-MOGHADDAM et al., 2018).

In addition, M1 cells can increase the production of ROS, including superoxide anion, hydrogen peroxide, hydroxyl radicals, and nitric oxide which exhibit high microbicidal capacity. However, it has been described that anti-inflammatory M2 macrophages have a key role in parasite dissemination and infection success by negatively regulating the pro-inflammatory responses through the production of IL-4 and TGF-β, making these cells permissive to infection since they are unable to generate oxidative stress and increasing the metabolic capacity of internalized amastigotes by enhancing the activity of the enzyme arginase I (3).

In the same sense, *L. amazonensis* infection in hyperglycemic stimulated macrophages inhibited the increase in expression of mediators responsible for intracellular signaling, such as MyD88, and the chemokines CCL4 and CXCL2 responsible for the recruitment of immune cells with leishmanicidal potential (MENZIES; MACPHAIL; HENRIQUEZ, 2016). However, the role of these mediators in *Leishmania* infection is dualistic, MyD88 activation via TLR9 has been associated with susceptibility to infection (SAUTER et al., 2019), while its activation via TLR4 induces a resistance profile. MyD88-dependent signaling pathways are essential for the development of an IL-12-mediated Th1-type protective response, since MyD88 knockout mice develop a non-protective Th2 response (MURAILLE et al., 2003; VARGAS-INCHAUSTEGUI et al., 2009).

CCL4 and CXCL2, respectively termed MIP-1β and MIP2-alpha, are inflammatory cell recruitment chemokines specifically monocytes, T lymphocytes and polymorphonuclear cells. Both chemokines are characteristically produced by M1-type macrophages. In our data, infection by *L. amazonensis* in macrophages exposed to a hyperglycemic environment led to a reduction in the expression of these chemokines, promoting the generation of macrophages unable to induce inflammation (TOMIOTTO-PELLISSIER et al., 2018) In the same sense, these chemokines have important role in the development of cellular immune response and their absence could result in defect in the recruitment of immune cells and an ineffective response against the parasite (MENZIES; MACPHAIL; HENRIQUEZ, 2016).

We also investigated if hyperglycemic stimulus and infection with *L. amazonensis* were able to alter the original monocytic phenotype of THP-1 cells before differentiation into macrophages. Interestingly, we found that hyperglycemic stimulus altered the phenotype of these monocytes from a classical profile (CD14+/CD16-) to an intermediate ($CD14^{hi}$/CD16+), however, the infection with *Leishmania* did not influence the observed profiles. It has been described that classical monocyte can differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells, in addition to performing an integral role in the formation of inflammation and its resolution in tissues (MENEZES et al., 2016).

In contrast, intermediate monocytes express higher levels of antigen-presentation-related molecules (LEE et al., 2017; WONG et al., 2011), and the

numbers of $CD14^{hi}$/CD16+ monocytes are increased in the blood of patients with systemic infections, which implies that they must play an important role in the initial defense against pathogens (KAPELLOS et al., 2019). However, their exact role is still unclear, since it has been described that this subpopulation of cells is responsible for producing large amounts of IL-10 as a result of TLR4 activation (SKRZECZYŃSKA-MONCZNIK et al., 2008).

In this context, the phenotype of these monocytes could have an impact on differentiation of macrophages, although the expression of M1 and M2 markers showed no difference between the control and HG before infection, indicating that these cells possess a similar phenotype, the differences observed after *L. amazonensis* infection may have been influenced by the different phenotypes of monocytes observed (DAIGNEAULT et al., 2010; KAPELLOS et al., 2019).

Similarly to our *in vitro* results, experimental infection with *L. amazonensis* in STZ-induced diabetic C57BL/6 mice showed an increased edema of paws in comparison with non-diabetic mice. C57BL/6 mice are known to be partially resistant to *L. amazonensis* infection and develop a self-healing tissue lesion (TOMIOTTO-PELLISSIER et al., 2021). Our results could indicate that diabetes subvert that resistance profile making this mouse strain more susceptible to infection. We also observed higher MPO activity in the paws of infected diabetic mice, which is an indicative of neutrophil infiltrate in the tissue. Bonyek-Silva *et* (BONYEK-SILVA et al., 2020) *al*. demonstrated that patients with diabetes have more neutrophil infiltrate and amastigotes of *L. braziliensis* in lesion site than individuals without diabetes associated with longer healing time. The role of neutrophil in leishmaniasis immunopathogenesis is dualistic, they can either kill the parasites and help to control parasite load or serve as a parasite reservoir, favoring their survival and proliferation. However, in this context late neutrophil infiltrate could be detrimental to host once exacerbate tissue inflammation and injury (PASSELLI; BILLION; TACCHINI-COTTIER, 2021).

We also investigated the cytokine production by STZ-induced diabetes in C57BL/6 mice upon experimental leishmaniasis and observed a distinct pattern, while non-diabetic mice showed an increased production of locally TNF and systemic IL-6, diabetic mice demonstrated local production of IL-17A and systemic production of IL-17A, IL-10, and IL-4. While non-diabetic animals

showed a Th1-like phenotype, diabetic mice showed a Th2/Th17 phenotype. It has been described that C57BL/6 mouse present a mixed response pattern with an early Th1 response followed by a late Th2, which confers resistance of this strain of mice to *L. amazonensis* (SCOTT; NOVAIS, 2016). The Th2/Th17 response observed in diabetic mice could be implicated in the increased edema size and higher susceptibility observed in this group after infection, since Th2 response has been involved in attenuation of the immune response against the parasite (SCOTT; NOVAIS, 2016) and IL-17A is a major neutrophil recruiter, explaining the excess of neutrophil infiltrate observed in diabetic animals (ALLEN; SUTHERLAND; RÜCKERL, 2015; XIAO et al., 2017). This Th2/Th17 has already been described in asthma models, acting synergistically to exacerbate the immunopathogenesis of the disease (CHOY et al., 2015).

Since, theoretically, intermediate monocytes should have increased phagocytic capacity and functions related to antigenic presentation, and macrophages derived from these monocytes in our results showed a reduction in the expression of molecules related to antigenic presentation, we evaluated whether peritoneal macrophages of mice with STZ-induced diabetes would be able to phagocyte *Leishmania* and present antigens to autologous splenic lymphocytes, inducing their proliferation and production of IFN-γ. As a result, we found that macrophages derived from healthy mice are effectively able to phagocyte the parasites and process/present their antigens, inducing the activation of lymphocytes, while macrophages from diabetic mice fail in this function. Interestingly, when macrophages of diabetic mice were infected with heat-killed *L. amazonensis*, lymphocyte activation occurred, which indicates that the parasites are able to exert greater control in cells exposed to a hyperglycemic environment.

It has been widely described in the literature that parasites of the genus Leishmania are able to modulate infected macrophages and inhibit antigenic presentation via MHC class I and II (PODINOVSKAIA; DESCOTEAUX, 2015) and in this context, probably the hyperglycemic/diabetic environment intensifies this regulation. Among the main mechanisms of inhibition of antigenic presentation, we can highlight the change in the fluidity of the plasma membrane of cells. Increased membrane fluidity severely compromises the stability of expression with peptide-MHC complex in lipid raft regions, thus nullifying T-cell-

mediated signaling in the infected host. Parasites trigger these changes mainly by extinguishing the host's cholesterol, which acts as a membrane fluidity stabilizing material (PRADHAN et al., 2021).

In addition, *Leishmania* subvert the lysosomal pathways of the host in order to hinder the antigen processing/presentation machinery. By means of SNARE proteins, lysosomes merge with phagosomes, generating phagolysosomes with intense degradation activity. Phagolysosomes contribute to host immunity by linking the destruction of phagocytic agents within these organelles with the processing of antigens for presentation in MHC class I or II molecules to T cells. However, *Leishmania* can prevent immune recognition by inhibiting phagolysosome biogenesis. Gp63 metalloprotease of the cell surface of parasites cleaves a subset of SNAREs, including VAMP8, whose inactivation prevents the NADPH oxidase complex from mounting in phagosomes, thereby altering their pH and degradation activity. Consequently, the presentation of *Leishmania* antigens in MHC molecules is inhibited, resulting in reduced activation of T cells (MATHEOUD et al., 2013).

5. Conclusion

In conclusion, *in vitro* hyperglycemic stimulus compromises the leishmanicidal capacity of THP-1 cell-derived macrophages to generate oxidative stress by increasing the activity of free radical scavenging enzymes. Furthermore, glucose excess makes these cells more susceptible to cell subversion by *Leishmania*, which induces the expression of M2 related markers in macrophages, unable to eliminate parasites. In this way, the hyperglycemic environment is also able to alter the phenotype of monocytes to the intermediate profile, providing distinct responses to infection. Moreover, experimental diabetes induction with STZ in C57BL/6 mice generated abrogate that resistant profile of this mice strain to *L. amazonensis,* impairing their capacity to generate NO and inducing a Th2/Th17 response pattern with high neutrophil infiltrate at lesion site. Besides that, macrophages from diabetic mice were unable to process and present antigens after infection with viable *L. amazonensis* parasites, resulting in failure in activation and induction of *Leishmania*-specific lymphocyte proliferation. Together, these data demonstrate that hyperglycemia and diabetes impair the

cellular immune response, particularly that of macrophages, against infection with *Leishmania* parasites.

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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Figure legends

Figure 1. Effect of glucose on *Leishmania amazonensis* proliferation and infection. (A) Evaluation of proliferation of *L. amazonensis* promastigote forms exposed to different concentrations of D-glucose measured by resazurin assay at different time points. (B) Flow chart of the hyperglycemic stimulus model used in THP-1 cells. (C) Effect of D-glucose on THP-1 cells to validate the hyperglycemic model. Water was used as the vehicle group in the same proportion as the volume of 400 mM D-glucose. (D-E) Parasite load in infected macrophages at different time points. (F-G) Evaluation of 48 h of infection by flow cytometry. The gate strategy used to isolate the specific cell population (**Supl. Fig. 1**). (H) Number of promastigotes recovered from the cells after 48 h of infection. White columns are for control cells and black columns are for HG cells (right graphs). *(p≤0.05); **(p≤0.01); ***(p≤0.001); ****(p≤0.0001).

Figure 2. Oxidative stress and enzymatic activity during *L. amazonensis* infection on THP-1-derived macrophages under hyperglycemic condition. (A) Nitrite levels were measured in the culture supernatant by Griess method as an estimative of nitric oxide production after 48 h of infection. (B) Superoxide anion quantified in macrophages after 48 h of infection through NBT reduction. Data are presented as optical density (OD) $x10^{-1}$. (C) Total reactive oxygen species (ROS) produced by THP-1-infected cells after 48 h of *L. amazonensis* infection. Data were normalized by exclusion of cells autofluorescence and expressed as arbitrary units of fluorescence. (D, E, and F) Enzymatic activity of N-acetyl-β-D-Glucosaminidase (NAG), superoxide dismutase (SOD), and catalase, respectively, were measured in whole-cell lysate and expressed as enzyme units per milliliter. White columns represent uninfected cells, while black columns represent infected cells. *(p≤0.05); **(p≤0.01); ***(p≤0.001); ****(p≤0.0001).

Figure 3. Expression of immune signaling mediators during *L. amazonensis* infection in THP-1-derived macrophages exposed to a hyperglycemic environment. (A) Enzymatic activity of arginase measured in whole-cell lysate after 48 h of infection and expressed as enzyme units per milliliter. (B-E) Gene expression of CXCL2, CCL4, CD40, and MyD88 presented as $2^{-(\Delta\Delta Ct)}$ normalized for biological significance as described in 2.17. (F) TNF-α levels were quantified in culture supernatant after 48 h of infection. White columns represent uninfected cells, while black columns represent infected cells. *(p≤0.05); **(p≤0.01); ***(p≤0.001); ****(p≤0.0001).

Figure 4. Immunophenotyping of monocytes. Detection of the CD14 and CD16 expression pattern in monocytes exposed or not to a hyperglycemic environment w/ or

w/o *L. amazonensis* infection for 48 h. All histograms (A-B) and zebra plot (C) are a representative of at least three independent experiments. Red lines represent control group, and black lines HG groups.

Figure 5. Immunophenotyping of macrophage. Detection of HLA-DR, CD80, CD86, Arginase, and CD206 in macrophages exposed to a hyperglycemic environment and infected with *L. amazonensis* for 48 h. All zebra plots are a representative of at least three independent experiments and the gates represent the positive population of the M1 and M2 markers evaluated.

Figure 6. Paws edema of *L. amazonensis*-infected diabetic and non-diabetic mice and neutrophils infiltrate. (A) C57BL/6 mice (n≥5) were infected in the left footpad with $5x10^6$ of promastigote forms of *L. amazonensis* and paws edema was measured weekly. Black circles represent non-diabetic mice while squares represent the diabetic group. (B) Parasite load quantified by real time PCR in paw tissue of infected mice. (C) MPO activity quantified in the homogenized footpad supernatant as an estimate of neutrophils infiltrate at lesion site expressed as number of neutrophils per mg of tissue. **(p<0.05), **(p<0.01) and ****(p<0.0001).

Figure 7. Nitric oxide and arginase axis. Evaluation of nitric oxide in serum (A) and paws (B), and arginase in paws (C) after 5 weeks of infection. (D) Person correlation between nitric oxide and arginase levels measured in paws. Dashed lines represent an estimate of the strength of the correlation and computed R value. White columns represent uninfected cells, while black columns represent infected cells. The values represent the mean±SEM of at least 5 animals per group. *(p<0.05); **(p≤0.01); ***(p≤0.001); ****(p≤0.0001).

Figure 8. Cytokines profile from diabetic and non-diabetic mice during experimental leishmaniasis. (A) paws and (B) serum Th1/Th2/Th17 cytokines production by *L. amazonensis*-infected C57BL/6 mice after 5 weeks of infection. The values represent the mean±SEM of at least 5 animals per group. White columns represent uninfected cells, while black columns represent infected cells. *(p<0.05); **(p≤0.01); ***(p≤0.001); ****(p≤0.0001).

Figure 9. Anti-leishmania IgG and lymphocyte proliferation and activation-induced by macrophages from non-diabetic and diabetic mice. (A) total anti-leishmania IgG production quantified in serum samples of diabetic and non-diabetic mice infected with

L. amazonensis for 5 weeks. Data are expressed as ELISA index calculated as the ratio between the absorbance values of samples from infected and uninfected mice (anti-leishmania IgG negative). (B) Offset histograms showing geometric dilution of CFSE fluorescence of splenic lymphocytes co-cultured with peritoneal macrophages infected with *L. amazonensis* (viable or heat killed). Blue histograms represent controls for the technique, while red histograms represent lymphocytes from healthy mice and grey from diabetic mice. Histograms represent the mean of cells collected from 5 animals and seeded in culture well plates in quadruplicate. (C) IFN-γ production measured in the culture supernatant of the macrophage-lymphocyte co-culture after 72 h. White columns represent controls of the technique, black columns represent macrophages infected with viable parasites, and the gray columns represent macrophages infected with heat-killed *L. amazonensis*. # symbol represent the statistical difference of the group with uninfected control. ##(p≤0.01); ####(p≤0.0001); ****(p≤0.0001).

Supplementary figure 1. Gate strategy for assess cell infection. Representative histogram of *Leishmania* fluorescence within infected cells, the first 3 windows represent the gate strategy used to isolate the analyzed cell population and the histogram represents the comparison of eGFP fluorescence intensity in control (black line) vs HG (red line) cells.

Supplementary figure 2. Immunophenotyping of macrophages. Detection of HLA-DR, CD80, CD86, Arginase, and CD206 in macrophages exposed to a hyperglycemic environment and infected with L. *amazonensis* for 48 h. Mean and SEM of at least three independent experiments made in triplicate. White columns represent uninfected while black columns represent *L. amazonensis* infection (MOI 1:10). **(p<0.01), ****(p<0.0001).

Figure 1

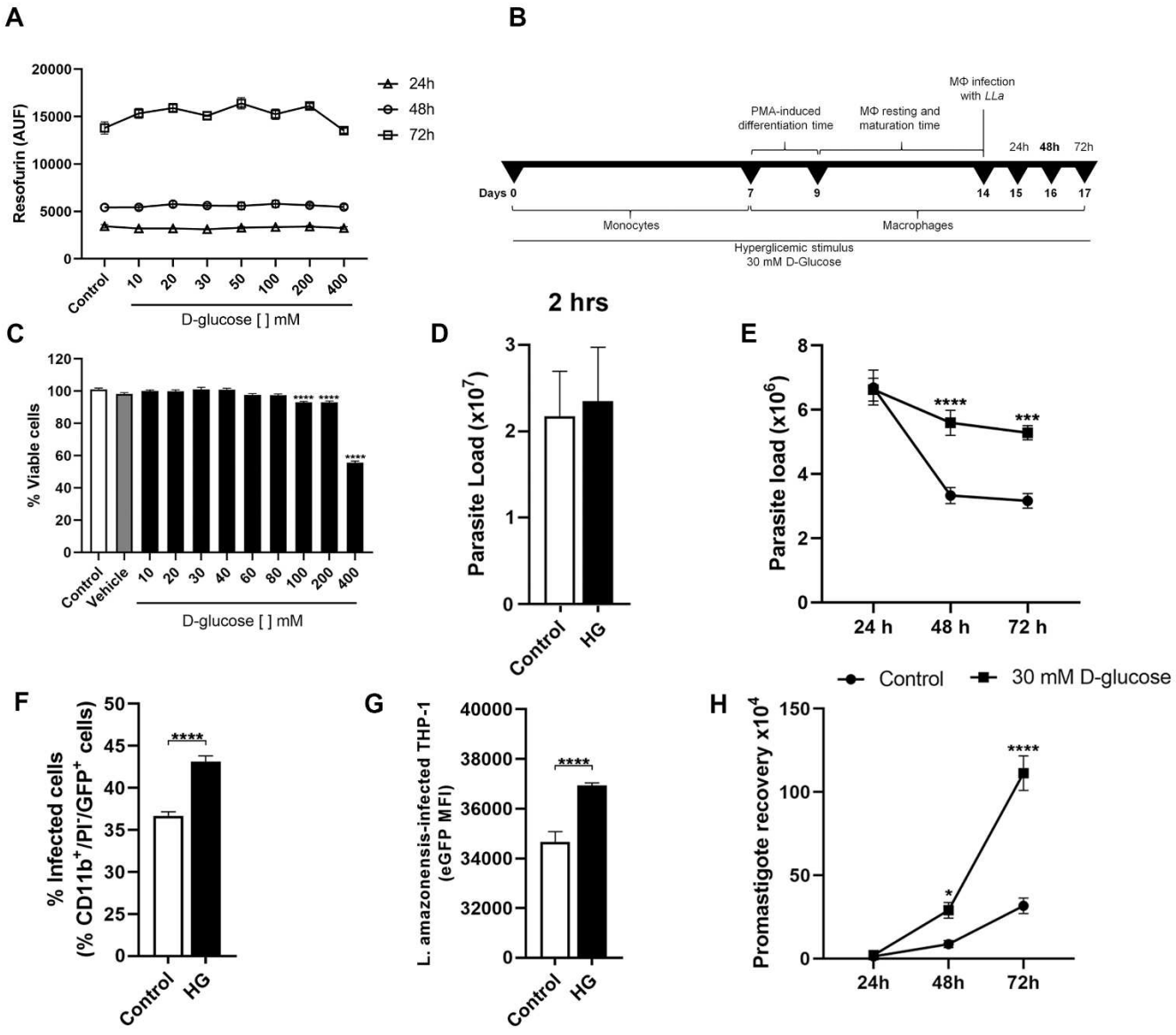

A
Resofurin (AUF)
24h
48h
72h
Control
D-glucose [ ] mM
B
MΦ infection with LLa
PMA-induced differentiation time
MΦ resting and maturation time
24h 48h 72h
Days 0 7 9 14 15 16 17
Monocytes
Macrophages
Hyperglicemic stimulus 30 mM D-Glucose
C
% Viable cells
Control
Vehicle
D-glucose [ ] mM
D
2 hrs
Parasite Load (x10^7)
Control
HG
E
Parasite load (x10^6)
24 h
48 h
72 h
Control
30 mM D-glucose
F
% Infected cells (% CD11b+/PI-/GFP+ cells)
Control
HG
G
L. amazonensis-infected THP-1 (eGFP MFI)
Control
HG
H
Promastigote recovery x10^4
24h
48h
72h

Figure 2

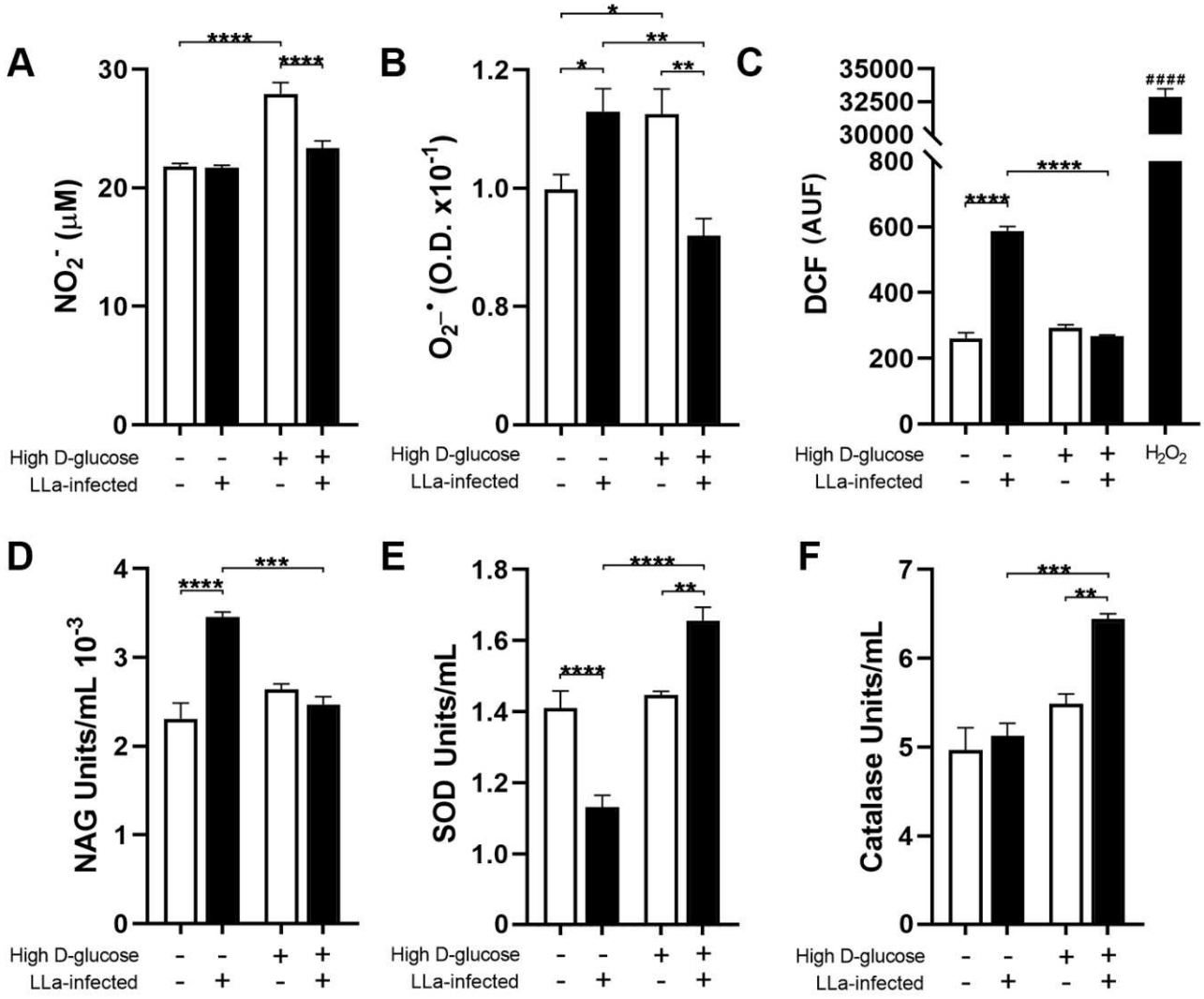

A
B
C
D
E
F
NO_2^- (μM)
$O_2^{-\bullet}$ (O.D. x10^{-1})
DCF (AUF)
NAG Units/mL 10^{-3}
SOD Units/mL
Catalase Units/mL
High D-glucose
LLa-infected
H_2O_2
****
***
**
*
####

Figure 3

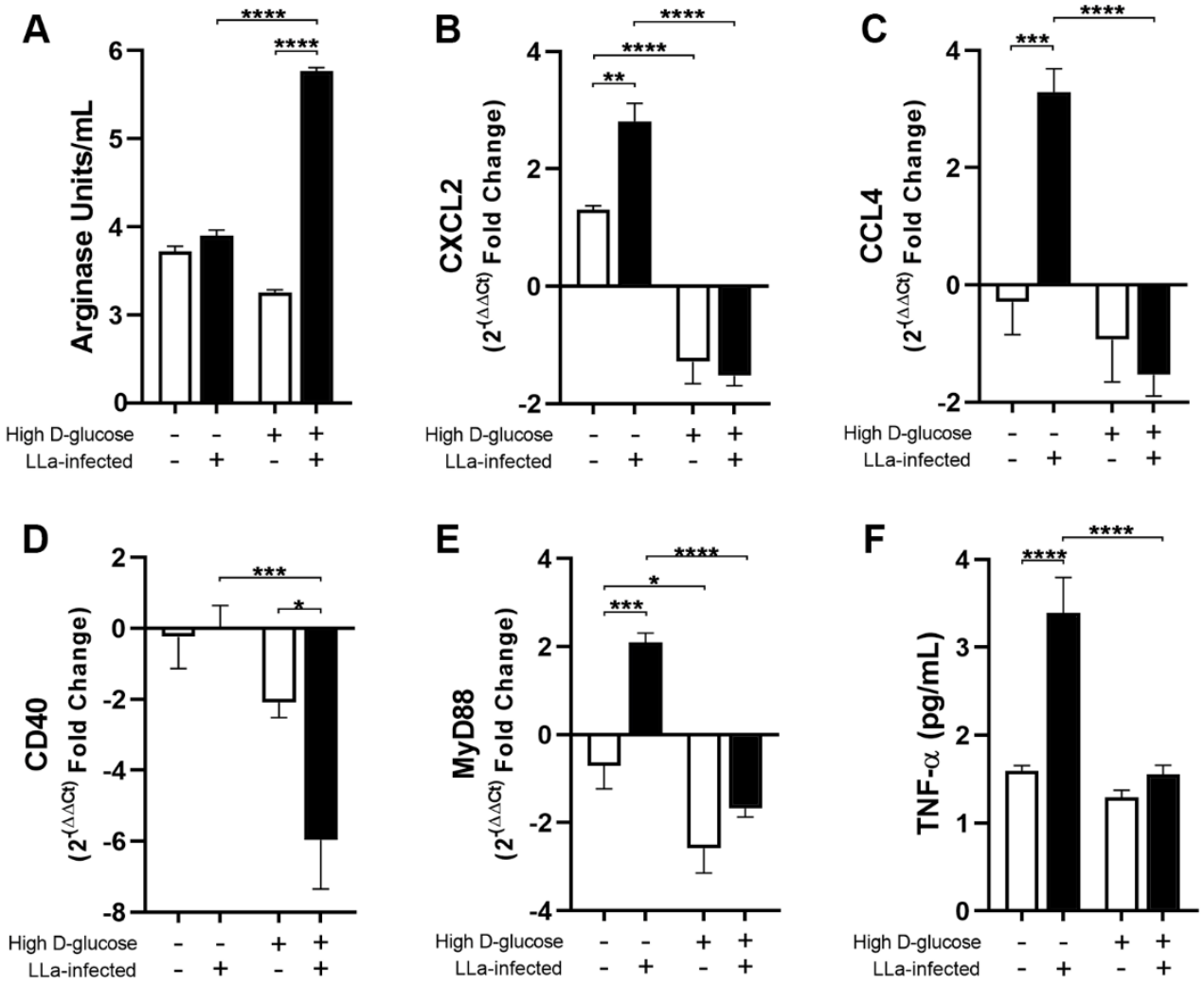

A
Arginase Units/mL
B
CXCL2
(2^(-ΔΔCt)) Fold Change
C
CCL4
(2^(-ΔΔCt)) Fold Change
D
CD40
(2^(-ΔΔCt)) Fold Change
E
MyD88
(2^(-ΔΔCt)) Fold Change
F
TNF-α (pg/mL)
High D-glucose
LLa-infected

Figure 4

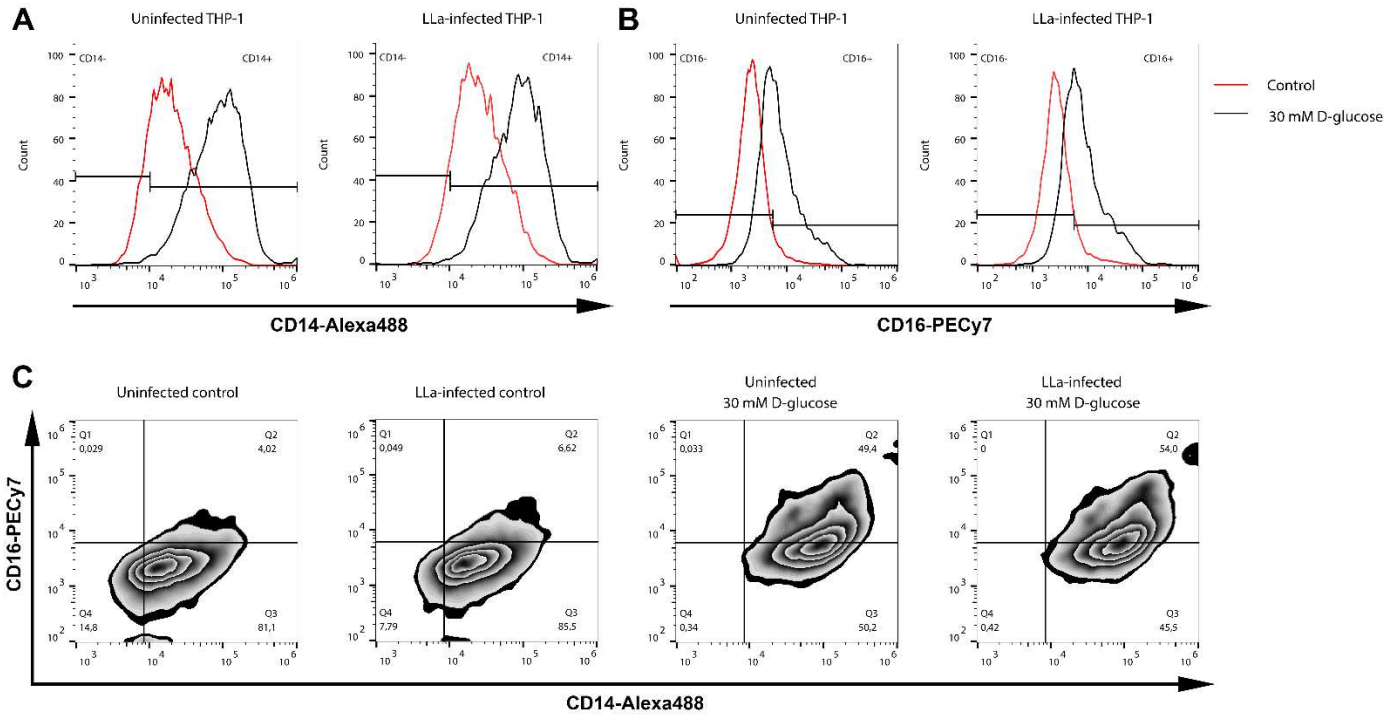

A
Uninfected THP-1
LLa-infected THP-1
CD14-
CD14+
Count
CD14-Alexa488
B
Uninfected THP-1
LLa-infected THP-1
CD16-
CD16+
Count
CD16-PECy7
Control
30 mM D-glucose
C
Uninfected control
LLa-infected control
Uninfected
30 mM D-glucose
LLa-infected
30 mM D-glucose
Q1 0,029
Q2 4,02
Q4 14,8
Q3 81,1
Q1 0,049
Q2 6,62
Q4 7,79
Q3 85,5
Q1 0,033
Q2 49,4
Q4 0,34
Q3 50,2
Q1 0
Q2 54,0
Q4 0,42
Q3 45,5
CD16-PECy7
CD14-Alexa488

Figure 5

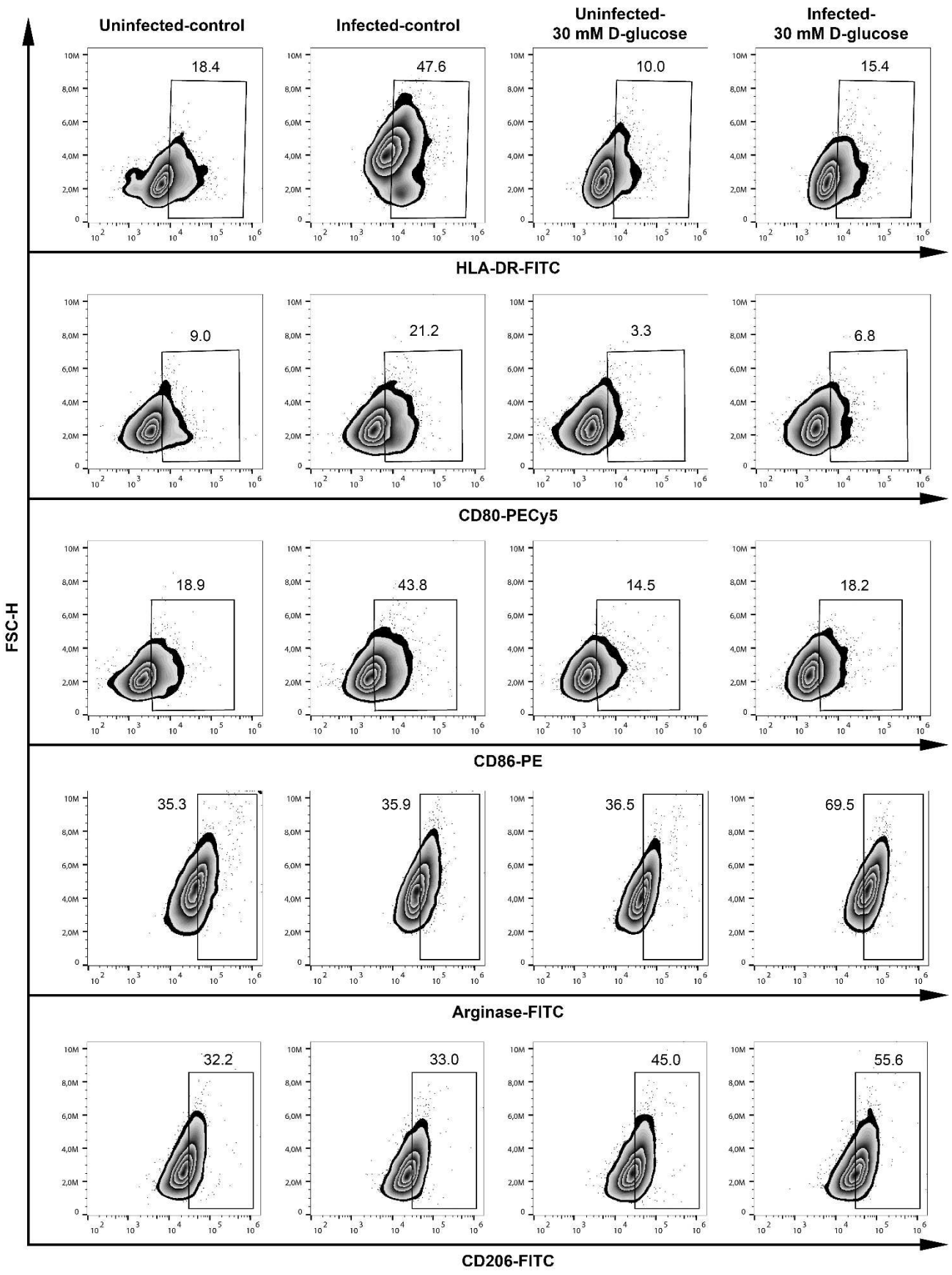

Uninfected-control
Infected-control
Uninfected-
30 mM D-glucose
Infected-
30 mM D-glucose
18.4
47.6
10.0
15.4
HLA-DR-FITC
9.0
21.2
3.3
6.8
CD80-PECy5
18.9
43.8
14.5
18.2
CD86-PE
35.3
35.9
36.5
69.5
Arginase-FITC
32.2
33.0
45.0
55.6
CD206-FITC
FSC-H

Figure 6

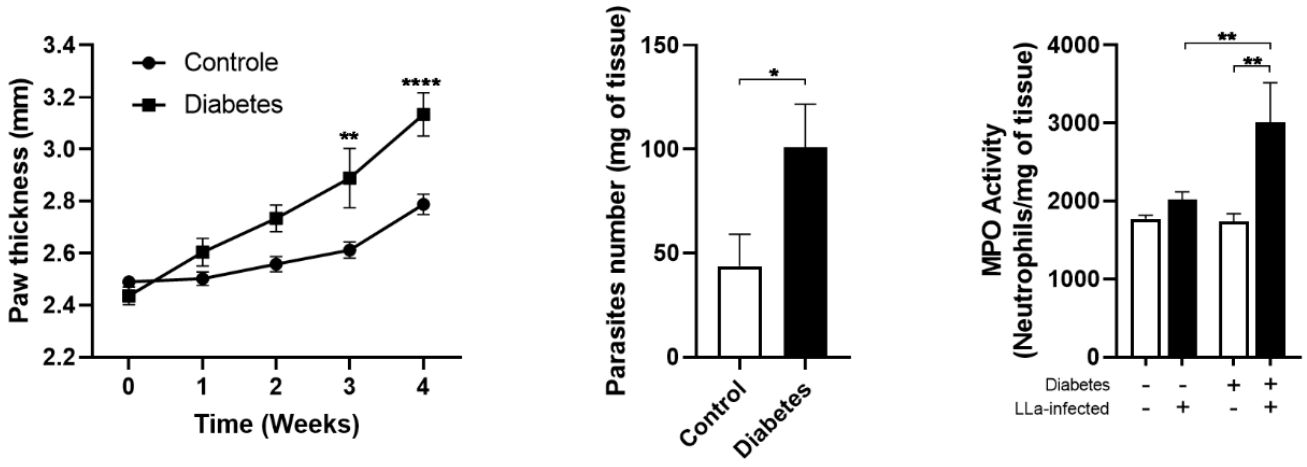

Paw thickness (mm)
3.4
3.2
3.0
2.8
2.6
2.4
2.2
Controle
Diabetes
**
****
0
1
2
3
4
Time (Weeks)
Parasites number (mg of tissue)
150
100
50
0
*
Control
Diabetes
MPO Activity
(Neutrophils/mg of tissue)
4000
3000
2000
1000
0
**
**
Diabetes
LLa-infected

Figure 7

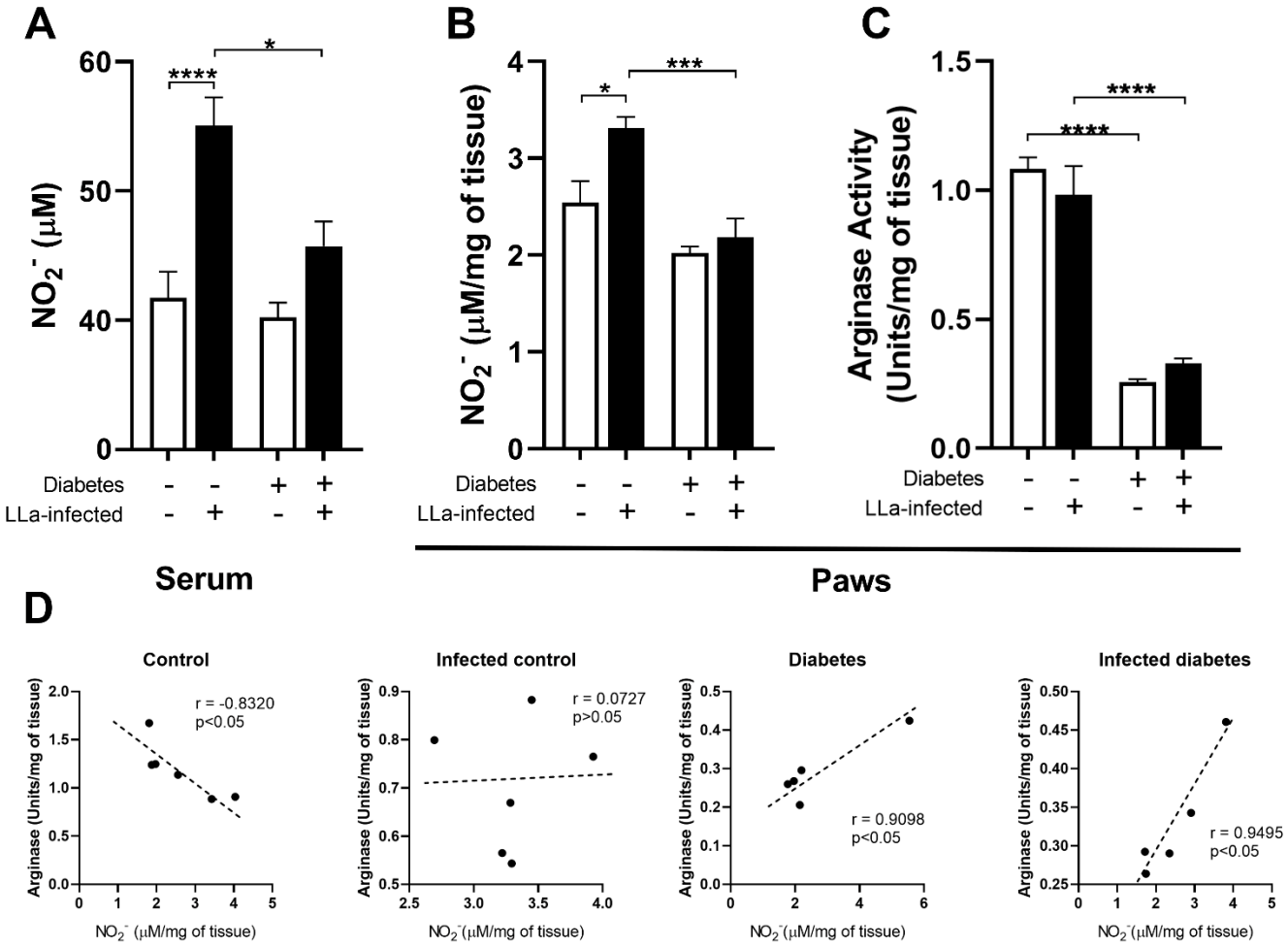

A
B
C
NO_2^- (μM)
NO_2^- (μM/mg of tissue)
Arginase Activity (Units/mg of tissue)
Diabetes
LLa-infected
Serum
Paws
D
Control
Infected control
Diabetes
Infected diabetes
r = -0.8320
p<0.05
r = 0.0727
p>0.05
r = 0.9098
p<0.05
r = 0.9495
p<0.05
Arginase (Units/mg of tissue)
NO_2^- (μM/mg of tissue)

Figure 8

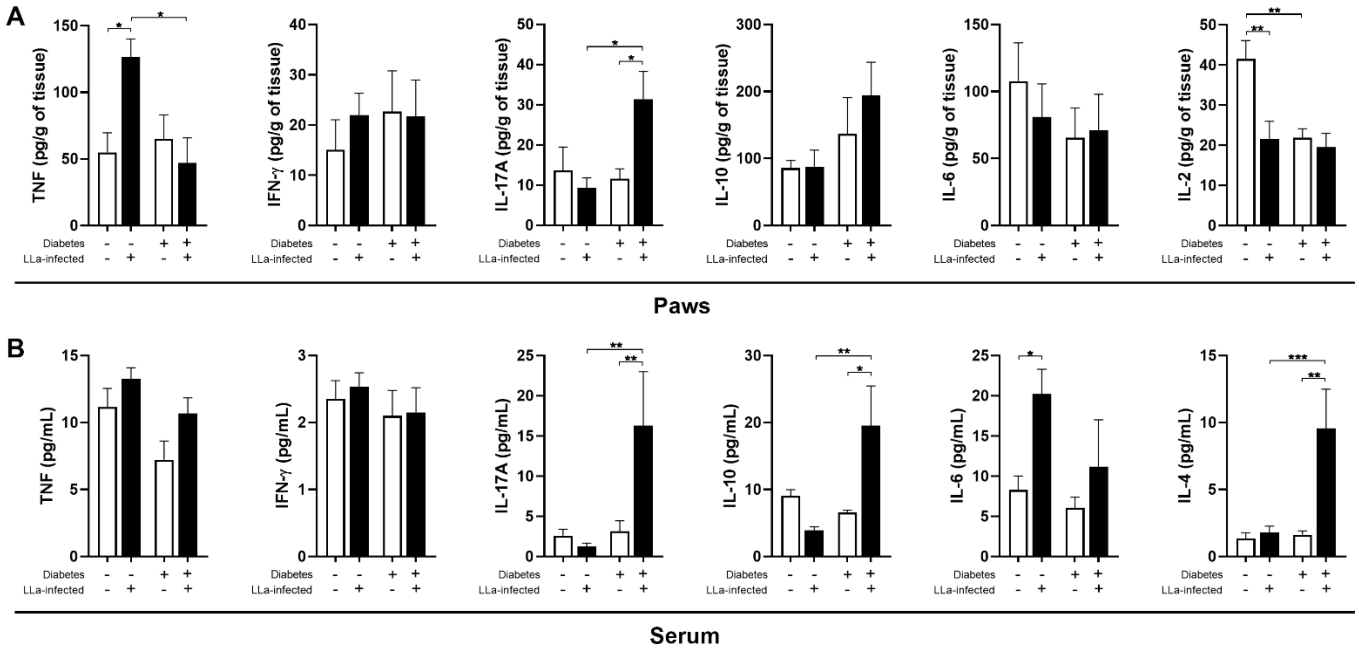
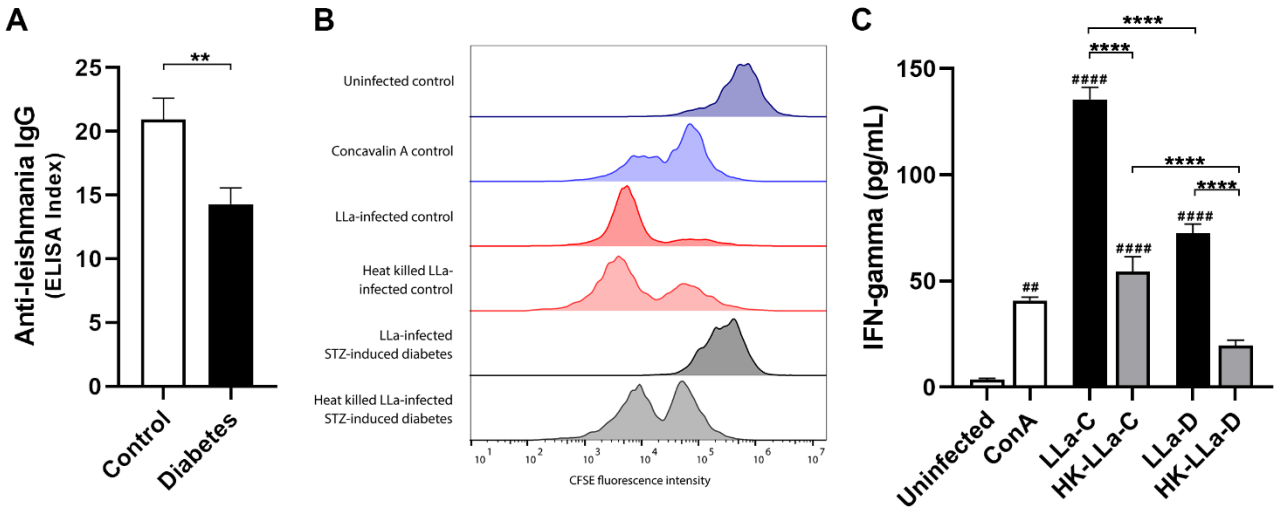
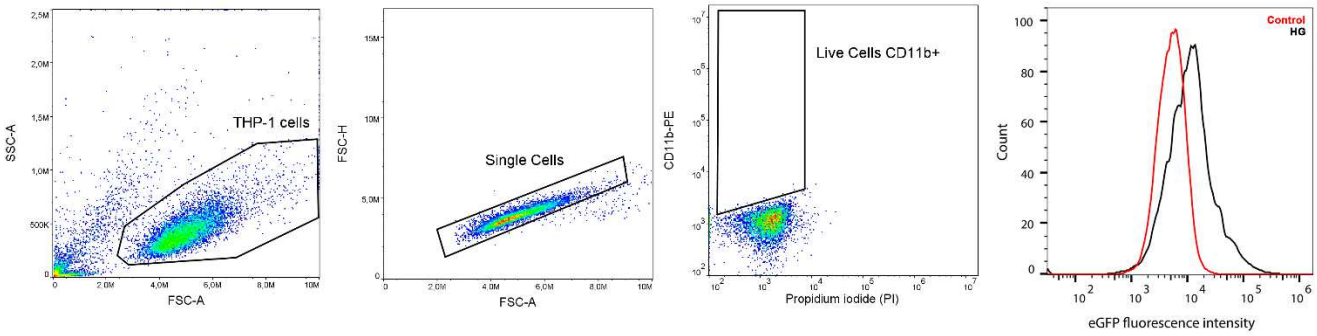

A
TNF (pg/g of tissue)
IFN-γ (pg/g of tissue)
IL-17A (pg/g of tissue)
IL-10 (pg/g of tissue)
IL-6 (pg/g of tissue)
IL-2 (pg/g of tissue)
Diabetes
LLa-infected
Paws
B
TNF (pg/mL)
IFN-γ (pg/mL)
IL-17A (pg/mL)
IL-10 (pg/mL)
IL-6 (pg/mL)
IL-4 (pg/mL)
Diabetes
LLa-infected
Serum

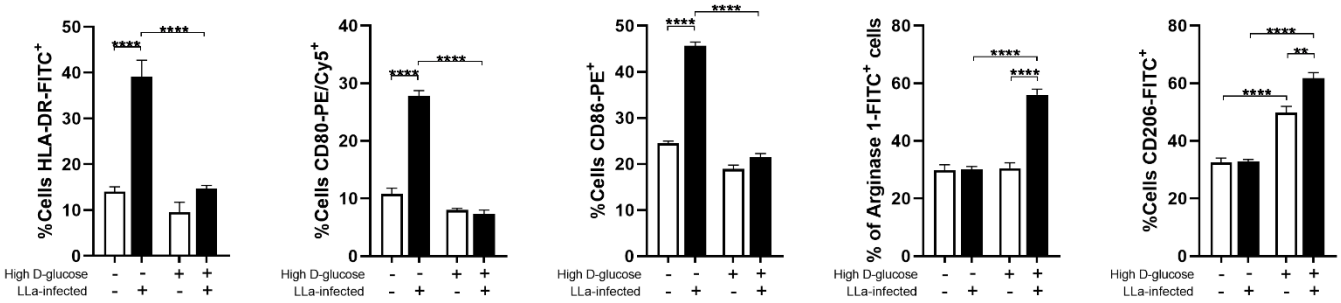
Figure 9


A
Anti-leishmania IgG
(ELISA Index)
25
20
15
10
5
0
**
Control
Diabetes
B
Uninfected control
Concavalin A control
LLa-infected control
Heat killed LLa-infected control
LLa-infected STZ-induced diabetes
Heat killed LLa-infected STZ-induced diabetes
CFSE fluorescence intensity
C
IFN-gamma (pg/mL)
150
100
50
0
****
****
****
****
####
####
####
##
Uninfected
ConA
LLa-C
HK-LLa-C
LLa-D
HK-LLa-D

Supplementary figure 1



Supplementary figure 2



5. CONCLUSÕES

Em conclusão, este trabalho verificou que PBMC de indivíduos diabéticos são mais suscetíveis à infecção por *L. amazonensis* e tem capacidade reduzida de eliminar os parasitos devido à falta de geração de estresse oxidativo causada pela incapacidade de inibir a atividade de NRF2, provavelmente resultante de níveis elevados de IL-6 produzida por essas células. Além disso, *L. amazonensis* pode imunomodular a produção de TNF e IL-10 em PBMC de pacientes diabéticos ao longo da infecção, o que pode estar diretamente relacionado à falha das células diabéticas em responder contra a infecção. Similarmente, o estímulo hiperglicêmico *in vitro* compromete a capacidade leishmanicida de macrófagos derivados de células THP-1 de gerar estresse oxidativo, aumentando a atividade de enzimas sequestradoras de radicais livres. Além disso, o excesso de glicose torna essas células mais suscetíveis à subversão celular por *L. amazonensis*, ao impedir a produção de mediadores relacionados a indução de resposta imune eficaz contra o parasito, além de induzir a expressão de marcadores relacionados a macrófagos M2, incapazes de eliminar parasitas. Dessa forma, o ambiente hiperglicêmico também é capaz de alterar o fenótipo dos monócitos para o perfil intermediário, proporcionando respostas distintas à infecção. Além disso, a indução experimental de diabetes com STZ aumentou a susceptibilidade a infecção em camundongos C57BL/6 que apresentam perfil de resistência a *L. amazonensis*, prejudicando sua capacidade de gerar NO e induzindo um padrão de resposta Th2/Th17 com alto infiltrado de neutrófilos no local da lesão. Além disso, macrófagos de camundongos diabéticos foram incapazes de processar e apresentar antígenos após infecção por parasitas viáveis de L. amazonensis, resultando em falha na ativação e indução da proliferação de linfócitos específicos de Leishmania. Juntos, esses dados demonstram que a hiperglicemia e o diabetes prejudicam a resposta imune celular, particularmente a dos macrófagos, contra a infecção pelo parasita Leishmania amazonensis.

ANEXOS

Anexo A – Parecer de aprovação do Comitê de Ética em Pesquisas Envolvendo Seres Humanos da Universidade Estadual de Londrina (CEP-UEL)

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação da resposta de PBMC de pacientes diabéticos na infecção por Leishmania (Leishmania) amazonensis in vitro

Pesquisador: Ivete Conchon Costa

Área Temática:

Versão: 3

CAAE: 56533816.8.0000.5231

Instituição Proponente: Programa de Pós Graduação em Patologia Experimental

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.890.439

Apresentação do Projeto:

A Leishmaniose Tegumentar Americana (LTA) é uma doença causada por protozoários do gênero Leishmania, caracteriza-se por apresentar inflamações crônicas de pele e mucosas. Sabe-se que o estado inflamatório tem relação com o diabetes, por ser uma síndrome de etiologia múltipla decorrente da secreção deficiente de insulina e/ou resistência ao seu efeito sobre o transporte de glicose para a maioria das células; esta doença atinge 8,3% da população mundial. A hiperglicemia favorece o estado inflamatório, devido ao aumento da produção de espécies reativas de oxigênio, importantes na eliminação de patógenos intracelulares, como a Leishmania. Além disso, sabe-se que a alteração metabólica decorrente do diabetes pode aumentar a liberação do hormônio de crescimento e do fator de crescimento semelhante à insulina (IGF-I). O IGF-I é produzido no fígado, tecido adiposo e particularmente em macrófagos, principais células envolvidas na defesa contra Leishmania. Existem estudos associando o IGF-I na infecção por várias espécies de Leishmania a um aumento expressivo do tamanho da lesão em camundongos, assim como a quantidade de parasitos presentes na lesão. Sabendo-se que as duas doenças são endêmicas em várias partes do mundo e que ambas alteram o estado imune do paciente, é importante que se conheça a resposta imune de pacientes com diabetes frente à infecção por Leishmania, uma vez que estes possuem níveis alterados do hormônio IGF-I. Portanto avaliaremos a resposta imune de

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células periféricas circulantes de pacientes com diabetes na infecção com formas promastigotas de Leishmania (L.) amazonensis in vitro. Estudaremos a atividade fagocítica e leishmanicida das células monucleadas, o perfil (Th1, Th2, Th17) de resposta imunológica assim como a concentração de NO no sobrenadante de cultura de PBMCs, a expressão do RNAm da iNOS e IGF-1R, vias de ativação envolvidas na resposta imune das células e também investigar se existe uma associação entre os parâmetros acima e o estado inflamatório e metabólico dos indivíduos cujas células PBMC foram avaliadas.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar a resposta imune de células periféricas circulantes de pacientes com diabetes na infecção com formas promastigotas de Leishmania (L.) amazonensis in vitro.

Objetivo Secundário:

Avaliar as culturas de células PBMC humanas de indivíduos diabéticos e saudáveis infectadas com formas promastigostas de Leishmania (L) amazonensis em relação aos seguintes parâmetros:• Atividade fagocítica das células monucleadas• Atividade leishmanicida das células monucleadas .

• O perfil (Th1, Th2, Th17) de resposta imunológica nas culturas de PBMC• Dosar a concentração de NO no sobrenadante de cultura de PBMC

• Expressão gênica do IGF-1R e CCR5 nas culturas de PBMC das células• Vias de ativação da resposta imune nas culturas de PBMC

•Investigar se existe uma associação entre os parâmetros acima e o estado inflamatório e metabólico dos indivíduos cujas células PBMC foram avaliadas.

Avaliação dos Riscos e Benefícios:

Riscos:

O sangue periférico será coletado na sala de coleta do Hospital das Clínicas, havendo, portanto, risco mínimo para o sujeito de pesquisa apenas o desconforto causado pela venopunção. Caso ocorra algum tipo de desconforto para o participante, o mesmo será prontamente atendido e amparado pelo pesquisador.

Benefícios:

Sabendo-se que a intensidade da lesão causada por Leishmania é determinada pela resposta imune do hospedeiro e que pacientes com diabetes descompensado são naturalmente imunossuprimidos e que ambas as patogenias são comuns em nossa região, esperamos com este

projeto conhecer a resposta desses pacientes frente a uma infecção por Leishmania e, deste modo, poder dar subsídios que facilitem tomadas de decisões quanto a medidas de tratamento e acompanhamento desses pacientes. Além disso, no futuro pretendemos avaliar a atividade de imunomoduladores com objetivo de melhorar a resposta imune de diabéticos frente a este patógeno.Estes dados serão importantes para a comunidade científica envolvida tanto nos estudos de diabetes como na leishmaniose e outros agentes infecciosos, portanto estes dados serão publicados em revista científica indexada para conhecimento da comunidade científica em geral.

Comentários e Considerações sobre a Pesquisa:

Trata-se de uma ementa ao projeto original aprovado, com parecer registrado por meio da CAAE 56533816.8.0000.5231. Foram solicitadas as seguintes alterações:

* exclusão do grupo de pacientes contendo diabetes gestacional, com o objetivo de evitar que a gestação seja um fator que interfira na pesquisa.

* número de indivíduos participantes por grupo (de 10 para 100 participantes por grupo). A quantidade de participantes conforme informações registradas no projeto básico não permitiria a identificação da possível frequência genética para os receptores do hormônio IGF-1 e CCR5 nos grupos de pacientes com diabetes do tipo 1, 2 e indivíduos saudáveis.

Considerações sobre os Termos de apresentação obrigatória:

TCLE- Ok

Autorizações - OK

Recomendações:

não há

Conclusões ou Pendências e Lista de Inadequações:

não há

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_841069_E1.pdf	10/12/2016 13:01:32		Aceito
TCLE / Termos de Assentimento /	TCLE.doc	30/08/2016 16:13:26	Ivete Conchon Costa	Aceito

Justificativa de Ausência	TCLE.doc	30/08/2016 16:13:26	Ivete Conchon Costa	Aceito
Declaração de Instituição e Infraestrutura	ParecerDiabetes.pdf	25/05/2016 20:43:25	Ivete Conchon Costa	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO1.pdf	01/04/2016 11:55:28	Ivete Conchon Costa	Aceito
Folha de Rosto	FolhaDeRostoPreenchida.pdf	31/03/2016 21:41:03	Ivete Conchon Costa	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

LONDRINA, 13 de Janeiro de 2017

Assinado por:
Rosana Lopes
(Coordenador)

Anexo B – Parecer de aprovação do Comitê de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA-UEL)

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 051/2021

Londrina, 17 de maio de 2021.

Prezado(a) professor(a),

Certificamos que o projeto intitulado: **"Avaliação do perfil de suscetibilidade de camundongos C57BL/6 com diabetes induzida por estreptozotocina e alimentação hipercalórica frente a infecção por *Leishmania amazonensis*."** protocolo CEUA n° **010.2021** sob a responsabilidade de **Ivete Conchon-Costa**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) em **17/05/2021**.

Este projeto tem por objetivo avaliar o perfil de suscetibilidade e resistência e a resposta imunológica de camundongos C57BL/6 com diabetes induzida por estreptozotocina (STZ) e dieta hipercalórica frente à infecção por *Leishmania amazonensis*. **Grau de invasividade: GI3.**

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	01/06/2021 a 31/05/2023
Espécie/ linhagem/ raça	Camundongo heterogênico/ C57BL/6
N° de animais	**32**
Peso/ Idade	30 g/ 10-15 semanas
Sexo	Machos
Origem	Biotério da Fiocruz de Curitiba-PR
Amostras a serem coletadas	Sangue, fígado, baço, rins, pâncreas, membros posteriores, cérebro, linfonodos poplíteos, linfonodos do estomago, testículos e intestino.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Em cumprimento às exigências do CONCEA, em até 30 dias da finalização do projeto de pesquisa ou extensão envolvendo o uso de animais (verificar período de vigência expresso neste ofício), é necessário encaminhar relatório da descrição de uso de animais para ceua@uel.br, conforme modelo disponível no site da CEUA: http://www.uel.br/comites/ceua/pages/relatorio-de-projetos.php.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente,

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Londrina
ceua@uel.br / (43) 3371-5454

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr. (a) Ivete Conchon-Costa
Responsável pelo projeto
C/C para a Chefia do Departamento de Ciências Patológicas/ CCB
C/C para a Direção do Centro de Ciências Biológicas/CCB