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Curso de Pós Graduação em Patologia

TESE DE DOUTORADO

FOTOINATIVAÇÃO DE *Leishmania* APLICADA À IMUNOPROFILAXIA DE LEISHMANIOSE CUTÂNEA

SAYONARA DE MELO VIANA

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SAYONARA DE MELO VIANA

Orientadora: Profa Dra Camila Indiani de Oliveira.
Tese apresentada ao Curso de Pós-Graduação em Patologia Humana, como pré-requisito obrigatório para obtenção do grau de Doutor.

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SAYONARA DE MELO VIANA

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Pesquisadora
IGM/Fiocruz

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RESUMO

INTRODUÇÃO. A leishmaniose é uma doença global que afeta 12 milhões de pessoas e para a qual não existe uma vacina. A administração de substâncias fotossensibilizadoras e luz torna formas de leishmania inviáveis através da geração de espécies reativas de oxigênio, mas preserva seu uso efetivo para imunização. Os parasitas podem ser fotoinativados através do acúmulo de fotossensibilizadores externos, captados pela da via endocítica, ou pela indução de porfirinas endógenas com o uso delta-aminolevulinato (ALA) em parasitas transgênicos. **OBJETIVOS.** Neste trabalho empregamos a fotoinativação para a geração de parasitas atenuados/inativados, de modo a induzir imunidade contra a leishmaniose cutânea. **MATERIAL E MÉTODOS / RESULTADOS.** Inicialmente, mostramos que a sensibilização exógena de *Leishmania amazonensis* com aminoftalocianina 2 (PC2) e posterior exposição à luz vermelha diminuiu significativamente a viabilidade parasitária e a taxa de infecção de macrófagos. Camundongos inoculados com parasitas fotoinativados por PC2 apresentaram menor carga de doença quando comparados aos controles, inoculados com parasitas viáveis, além de proteção parcial após o desafio. Em seguida, mostramos que uma cepa de *L. amazonensis* geneticamente complementada com os genes que codificam a porfobilinogênio deaminase (PBGD) e a aminolevulinato desidratase (ALAD) acumula uorporfirina 1 (URO1) após exposição ao ácido delta-aminolevulínico (ALA) e URO1 atua como sensibilizador quando exposta à luz. A fotoinativação endógena de *L. amazonensis* com ALA-URO1 também reduziu a viabilidade dos parasitas e a taxa de infecção de macrófagos. Diante desses resultados, testamos o efeito da inoculação de camundongos com parasitas duplamente sensibilizados, empregando PC2 e ALA-URO1, simultaneamente. Nestes ensaios, a fotoinativação foi realizada *in vivo,* após exposição do local da inoculação dos parasitas à luz. Os parasitas não causaram lesão e nem foram detectados por carga parasitária. A imunização induziu uma proteção parcial pois foi capaz atrasar o aparecimento da lesão após o desafio com parasitas vivos. Em seguida, testamos a fotoinativação de *L. braziliensis* e, para isso, geramos uma cepa geneticamente complementada e capaz de expressar ALAD e PBGD. Os parasitas transgênicos também acumularam porfirinas após exposição ao ALA e foram inativados por exposição à luz. Os parasitas fotoinativados foram internalizados por macrófagos murinos em taxas semelhantes aos parasitas controle, embora sua replicação tenha sido menor. Macrófagos infectados com *L. braziliensis* fotoinativada produziram IL-6, TNF e IL-10 e aumentaram a expressão das moléculas co-estimulatórias CD40 e CD86. **CONCLUSÕES.** Estes dados indicam que as linhagens transgênicas de *L. amazonensis e L. braziliensis* podem ser fotoinativadas, permitindo a geração de parasitas atenuados, capazes de induzir proteção parcial em modelos de leishmaniose cutânea.

Palavras-chave: Fotossensibilizador; leishmaniose cutânea; *Leishmania amazonensis*; *Leishmania braziliensis*; fotoinativação; ALA; Uroporfirina I.

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ABSTRACT

INTRODUCTION. Leishmaniasis is a global disease that affects 12 million people and despite its severity, there is no effective vaccine to prevent the onset of disease. The cellular uptake of photosensitizers and light exposure renders leishmania susceptible to photolysis through the generation of reactive oxygen species while preserving their use as vaccines. External photosensitizers are taken up by leishmania through the endocytic pathway while endogenous porphyrins are induced in transgenic parasites with the use of delta-aminolevulinate (ALA). **AIM.** In this work we used photoinactivation for the generation of attenuated / inactivated parasites, aiming to induce immunity against cutaneous leishmaniasis. **MATERIAL AND METHODS / RESULTS.** Initially, we showed that the exogenous photosensitization of *Leishmania amazonensis* with aminophthalocyanine 2 (PC2) and subsequent exposure to red light significantly decreased parasite viability and macrophage infection rates. Mice inoculated with PC2-photoinactivated parasites displayed lower disease burden when compared to controls, inoculated with viable parasites, and partial protection after challenge. Next, we showed that a strain of *L. amazonensis* genetically supplemented with the genes for porphobilinogen deaminase (PBGD) and aminolevulinate dehydratase (ALAD) accumulates uroporphyrin 1 (URO1) when exposed to delta-aminolevulinic acid (ALA). URO1 acts as a photosensitizer when exposed to UVA light; the endogenous photoinactivation of *L. amazonensis* with ALA-URO reduced parasite viability and macrophage infection rates. Mice inoculated with parasites photoinactivated by the endogenous strategy presented no lesions. In view of these results, we tested the effect of the inoculation of mice with doubly photosensitized parasites using both PC2 and ALA-URO1. Photoinactivation was performed *in vivo*, with exposure of parasite inoculation site to light. Parasites did not cause injury and were not detected by limiting dilution. Immunization induced partial protection as it was able to delay the onset of the lesion after challenge with live parasites. Next, we tested *L. braziliensis* photoinactivation through genetic complementation with the genes for ALAD and PBGD. Genetically complemented parasites accumulated porphyrins after incubation with delta-aminolevulinate (ALA) and were photoinactivated upon light exposure. Photoinactivated parasites were internalized by murine macrophages at rates similar to photosensitized control parasites, although their replication was lower. Macrophages infected with photoinactivated *L. braziliensis* produced IL-6, TNF and IL-10 and increased expression of co-stimulatory molecules. **CONCLUSION.** Data indicate that the transgenic lines of *L. amazonensis* and *L. braziliensis* are sensitive to photoinactivation, allowing the generation of attenuated parasites, capable of inducing partial protection in cutaneous leishmaniasis models.

Keywords: Photodynamic therapy; cutaneous leishmaniasis; *Leishmania amazonensis*; *Leishmania braziliensis*; photodynamic vaccination; ALA; Uroporphyrin I.

SUMÁRIO

1. INTRODUÇÃO

1.1 AS LEISHMANIOSES

As leishmanioses são antropozoonoses causadas por protozoários do gênero *Leishmania* e representam a segunda doença parasitária que mais causa óbitos no mundo (revisado em BOER *et al.*, 2011). Segundo estimativas da Organização Mundial de Saúde, as leishmanioses estão presentes em quatro continentes, com cerca de 12 milhões de pessoas infectadas e 2 milhões de novos casos por ano, sendo 1,5 milhão de casos de leishmaniose tegumentar (LT) e 500 mil de leishmaniose visceral (LV) (WHO EXPERT COMITTEE, 2010).

A infecção em mamíferos ocorre através do inóculo de parasitas na pele por fêmeas de flebotomíneos vetores. As manifestações clínicas podem ser localizadas ou generalizadas, de acordo com a espécie de leishmania envolvida. Além disso, a capacidade infectiva do parasita bem como o estado imunológico e nutricional do hospedeiro desempenham importante papel para o desenvolvimento da doença (PEARSON; SOUSA, 1996). Os casos de LV são os mais prováveis de evoluir para óbito caso não sejam tratados, pois resultam da disseminação dos parasitas do local de infecção na pele para o sistema retículo-endotelial de órgãos como fígado, baço, medula óssea e linfonodos (WILSON; JERONIMO; PEARSON, 2005).

A LT apresenta-se sob um amplo espectro de manifestações clínicas, sendo assim dividida em: leishmaniose cutânea localizada (LCL), leishmaniose cutânea mucosa (LCM), leishmaniose disseminada (LD) e leishmaniose cutânea-difusa (LCD). A LT representa importante problema de saúde pública devido à sua alta incidência, assim como pelo risco de o paciente desenvolver deformidades, podendo ser considerada uma doença ocupacional com impacto nos campos social e econômico (BRASIL, 2017). No Brasil, a LT é causada majoritariamente por *Leishmania braziliensis* e *L. amazonensis* (GONTIJO; CARVALHO, 2003). As regiões Norte e Nordeste concentram a maior parte das ocorrências de LT, com 45,7 e 26,8% do número de casos registrados entre 2007 e 2015, respectivamente. Na Bahia, no mesmo período foram registrados 30.126 casos de LT, o que representa 14,9% de todos os casos ocorridos no país (BRASIL, 2016).

Cerca de 90 a 95% dos casos de LT se manifestam como LCL (revisado em SCORZA; CARVALHO; WILSON, 2017), que caracteriza-se pela presença de lesão (lesões) ulcerada(s), de bordas elevadas e com fundo granuloso (GUIMARÃES et al., 2005). Após um período de incubação assintomático a lesão se instala no sítio de entrada do parasita. Na fase inicial da

doença, a pápula ou nódulo é precedida ou acompanhada por linfadenopatia regional, especialmente na infecção por *L. braziliensis* (BARRAL et al., 1995b). A LCL se caracteriza primordialmente pela presença de resposta imunológica celular ativa contra os parasitas. Em regiões endêmicas para *L. braziliensis*, 1 a 10% dos casos de LCL progridem para LCM, a qual é caracterizada por lesões altamente inflamatórias. A LCM é descrita como uma complicação metastática de LCL, causada majoritariamente por *L. braziliensis* (AMATO et al., 2008; MARSDEN, 1986). A LD também é uma forma metastática de LCL, porém menos prevalente que a LCM. Apresenta-se sob a forma de numerosas lesões acneiformes/papulares em duas ou mais regiões anatômicas não contíguas (TURETZ et al., 2002). Finalmente, a LCD, causada por *L. amazonensis*, caracteriza-se pela presença de múltiplas lesões nodulares que podem se unir em placas, cobrindo grandes regiões de pele. As lesões contém amastigotas em abundância e não ulceram, o que é atribuído à anergia da resposta imune celular contra o parasita (BARRAL et al., 1995a).

1.2 MECANISMOS DE PROTEÇÃO NA LEISHMANIOSE CUTÂNEA EXPERIMENTAL

Na leishmaniose experimental, a resposta imunológica inata é mediada primariamente por neutrófilos, macrófagos e células dendríticas, sendo os macrófagos a principal célula hospedeira. Após a fagocitose, ocorre a formação de fagolisossomos e a diferenciação das formas promastigotas em amastigotas, mais resistentes ao ambiente ácido. A explosão respiratória e a formação de espécies reativas de oxigênio (ROS) ocorrem inicialmente após a fagocitose mas as formas amastigotas podem evitar ou resistir ao dano (revisado em CECILIO et al., 2014). A eliminação do parasita depende essencialmente da ativação de uma resposta de células T auxiliares $CD4^+$ do subtipo Th1, produtoras de IFN-γ. Esta citocina induz a expressão de óxido nítrico sintase induzível (iNOS ou NOS2), catalisadora da produção de óxido nítrico (NO) que, conjuntamente com ROS, destroem os parasitas intracelulares (revisado em SCOTT; NOVAIS, 2016).

Grande parte dos estudos de resistência e susceptibilidade em leishmaniose foram desenvolvidos em modelo experimental de infecção por *L. major* (revisado em SACKS; NOBEN-TRAUTH, 2002). Em camundongos C57BL/6 infectados por *L. major* ocorre a diferenciação de células T $CD4^+$ em Th1, a partir da IL-12 produzida principalmente por células dendríticas, o que os torna resistentes à infecção. Por outros lado, camundongos BALB/c desenvolvem a resposta Th2, caracterizada pela produção de IL-4, a qual impede a ativação dos

macrófagos infectados, levando à proliferação do parasita e progressão da doença (revisado em SACKS; NOBEN-TRAUTH, 2002). O TNF também tem um papel importante na indução da produção de NO, atuando em sinergia com o IFN-γ (revisado em MASPI; ABDOLI; GHAFFARIFAR, 2016). No entanto, o paradigma de susceptibilidade *versus* resistência observado na infecção experimental por *L. major* não é completamente aplicável para explicar a doença humana e nem mesmo outros modelos experimentais de leishmanioses (MCMAHON-PRATT; ALEXANDER, 2004).

Na infecção experimental por *L. braziliensis*, tanto os camundongos C57BL/6 quanto os BALB/c são considerados resistentes, pois desenvolvem lesões dérmicas progressivas autolimitadas (CHILDS et al., 1984). A infecção subcutânea de BALB/c com *L. braziliensis* induz a formação de lesões pequenas e nodulares (ROCHA et al., 2007), enquanto a infecção intradérmica produz úlceras semelhantes àquelas observadas em pacientes (MOURA et al., 2005) e em ambos os casos a cura é espontânea. Camundongos BALB/c infectados com *L. braziliensis* produzem menos IL-4 comparado àqueles infectados com *L. major* e o tratamento com anti-IFN-γ leva à formação de lesões rápidas, progressivas e que o animal é incapaz de resolver (DEKREY; LIMA; TITUS, 1998). Camundongos deficientes em IL-12 também desenvolvem lesões grandes, incontroláveis e com parasitas que visceralizam (ROCHA et al., 2007), assim como camundongos deficientes em STAT, a principal molécula de transdução de sinal ativada por meio da ligação entre IL-12 e seu receptor (ROCHA et al., 2007). Assim, o controle de *L. braziliensis* em BALB/c também é dependente da produção de IFN-γ, mas a resposta de células T $CD4^+$ é mista Th1/Th2, com a presença de células secretoras de IFN-γ, IL-4 e IL-10 (MOURA et al., 2005), diferente da resposta polarizada Th2 que se observa após a infecção com *L. major*.

A infecção experimental por *L. amazonensis* leva a lesões progressivas, incuráveis e com alta carga parasitária em grande parte das cepas de camundongos isogênicos (revisado em SOONG, 2012). Quando infectados por *L. amazonensis*, camundongos C57BL/6 podem apresentar lesões progressivas com grande quantidade de parasitas e baixa produção de IFN-γ e proliferação de linfócitos (AFONSO; SCOTT, 1993; MAIOLI et al., 2004). De fato, quando se compara a infecção por *L. amazonensis* e *L. braziliensis* em camundongos C57BL/6 é possível observar que *L. amazonensis* induz a produção de IL-10 e IL-17 enquanto que *L. braziliensis* induz a produção de IFN-γ (XIN et al., 2011). Por outro lado, Velasquez e colaboradores (2016) compararam a infecção por *L. amazonensis* em camundongos C57BL/6 e BALB/c e observaram que os primeiros, mais resistentes à infecção, apresentaram maior

resposta de linfócitos T $CD4^+IFN\text{-}\gamma^+$ no ápice da lesão (VELASQUEZ et al., 2016) enquanto que camundongos BALB/c apresentam presença de TGF-β, IL-10 e inibição de NO, correlacionados com a maior susceptibilidade e lesões progressivas (AFONSO; SCOTT, 1993; WANDERLEY et al., 2006). A resposta mista Th1/Th2 e de baixa amplitude observada na infecção por *L. amazonensis* é particularmente relevante por ser semelhante àquela observada em infecções humanas (SILVEIRA et al., 2009).

De maneira geral, a resolução de uma infecção primária com leishmania confere imunidade de longo prazo à reinfecção, a qual é mediada primariamente por células T $CD4^+$ (LIEW; HALE; HOWARD, 1982). No entanto, a cura não é esterilizante e parasitos permanecem no hospedeiro devido à presença de uma resposta reguladora caracterizada pela presença de IL-10 (BELKAID et al., 2002). Os parasitas persistentes estimulam a população de células T $CD4^+$ Th1 efetoras e específicas, as quais são capazes de responder rapidamente a um novo desafio, impedindo o aparecimento da lesão. Na infecção crônica por *L. major*, células T $CD4^+$ efetoras migram rapidamente para o local da reinfecção produzindo IFN-γ (PETERS et al., 2014). De forma semelhante, também se observa imunidade concomitante em infecção experimental por *L. braziliensis*. O desafio secundário não gera desenvolvimento de lesão após a cura de uma infecção inicial, no entanto, há aumento da população de células T $CD4^+$ $IFN\text{-}\gamma^+$ no linfonodo de drenagem da lesão inicial, indicando a reativação da resposta efetora (FALCÃO et al., 2012).

1.3 VACINAS CONTRA A LEISHMANIOSE

Na leishmaniose humana, os indivíduos infectados por *Leishmania* desenvolvem imunidade à reinfecção após a cura espontânea ou quimioterapêutica, mostrando que é possível induzir imunidade específica anti-leishmania. Na verdade, a vacinação com parasitas virulentos vivos, também conhecida como leishmanização, provou ser eficaz para prevenir a doença. A técnica foi utilizada como uma vacina profilática em Israel e no Irã nas décadas de 1970 e 1980, utilizando parasitas derivados de lesões ativas e crescidos *in vitro* (revisado em KHAMESIPOUR *et al.*, 2006). Entretanto, o fato de o parasita perder virulência após seguidas subculturas *in vitro* comprometeu sua capacidade de causar lesões e, consequentementemente, a eficácia da leishmanização como estratégia profilática. Outros problemas relatados após a leishmanização foram o desenvolvimento de lesões crônicas (KHAMESIPOUR *et al.*, 2012), a falta de padronização e controle de qualidade, além do risco de desenvolvimento de

leishmaniose disseminada em indivíduos imunocomprometidos (revisado em GILLESPIE *et al.*, 2016). Essas dificuldades inviabilizaram o uso da leishmanização com método de imunoprofilaxia e o foco do desenvolvimento de vacinas voltou-se para o uso de subunidades, parasitas mortos ou vivos e atenuados.

As vacinas que utilizam o parasita completo expõem o indivíduo a toda a capacidade antigênica do parasita, incluindo os padrões moleculares, os quais são necessários para a ativação adequada do sistema imunológico. A alternativa mais segura para esse tipo de vacinação seria a utilização de parasitas mantidos *in vitro* e mortos por meio de incubação com timerosal (DE LUCA et al., 1999; MARZOCHI et al., 1998; MENDONCA et al., 1995) , aquecimento e autoclavagem, por exemplo (ARMIJOS et al., 2004; DE LUCA et al., 1999; VÉLEZ et al., 2000).

Estudos pioneiros com voluntários no Brasil mostraram que a imunização com promastigotas mortas levou à conversão do teste de Montenegro para positivo (indicador de geração de hipersensibilidade do tipo tardia, DTH) em pelo menos 74% dos voluntários (MAYRINK et al., 1985; MENDONCA et al., 1995). Entretanto, a imunização rendeu pouca proteção (50%) nos estudos clínicos de fase II subsequentes, o que pôde ser explicado pela baixa incidência de leishmaniose na área estudada naquele período (MAYRINK et al., 1985). Além disso, a imunização com parasitas mortos ou autoclavados induziu a geração de resposta imunológica majoritariamente composta por células T $CD8^+$ (DE LUCA et al., 1999; MENDONCA et al., 1995), enquanto indivíduos com infecção ativa apresentam resposta predominante de células T $CD4^+$. Esse achado evidencia que a imunização com parasitas mortos induz uma resposta imunológica diferente daquela induzida por infecção ativa, o que, novamente, poderia explicar a baixa eficácia observada nos ensaios clínicos. Desta maneira, adjuvantes como BCG (MOMENI et al., 1999), hidróxido de alumínio (alum) (KENNEY et al., 199 9) ou oligonucleotídeos de CpG (CpG-ODN) (VERTHELYI et al., 2002) passaram a ser incorporados à formulação de vacinas mortas, na tentativa de melhorar a imunogenicidade observada. Em um estudo clínico realizado no Equador, a imunização com três cepas locais de *Leishmania* preservadas com fenol mais BCG (utilizado como adjuvante) conferiu alta proteção (72,9%) em crianças (ARMIJOS et al., 1998). No entanto, resultados diferentes foram observados em dois estudos realizados com populações naturalmente expostas a *Leishmania* spp., nos quais a imunização com *L. major* autoclavada + BCG (MOMENI et al., 1999) ou *L. amazonensis* morta por timerosal + BCG (VÉLEZ et al., 2005) não induziu proteção maior que a observada no grupo que recebeu apenas BCG. Além dos resultados contraditórios obtidos em

ensaios clínicos, existem dificuldades para a produção de vacinas mortas de parasita total respeitando bons padrões de fabricação clínica, reforçando a necessidade de busca por alternativas.

Em modelo experimental, a imunização de camundongos BALB/c com *L. major* autoclavada (ALM) mais CpG-ODN induziu proteção de curta duração (OKWOR; LIU; UZONNA, 2009; RHEE et al., 2002) mas esta foi ineficaz frente ao desafio com flebotomíneos infectados (PETERS et al., 2009). Os animais vacinados com ALM+CpG e desafiados com *L. major* montaram uma resposta específica anti-*Leishmania* caracterizada por células T $CD4^+$ produtoras de IFN-γ e TNF-α mas esta resposta foi menor e retardada comparada àquela observada em camundongos curados de infecção prévia com *L. major* (PETERS et al., 2009). Foi sugerido que a resposta imunológica efetora desencadeada pela imunização com parasitas mortos não tem magnitude suficiente para contrabalancear a modulação imunológica gerada pelos produtos do inseto, inoculados durante a picada. Os autores sugerem que qualquer candidato a vacina deverá ser avaliado em modelo experimental envolvendo a transmissão natural da leishmania, ou seja, pela picada do inseto, de modo a avaliar sua eficácia no contexto natural da infecção. Até o momento, nenhuma preparação utilizando parasitas mortos demonstrou eficácia suficiente para ser utilizada amplamente como vacina profilática em humanos (NOAZIN et al., 2009).

As vacinas de subunidades constituem-se de peptídeos, proteínas e componentes não-protéicos dos parasitas ou DNA recombinante, por exemplo. A imunização utilizando proteínas recombinantes geralmente induz uma fraca resposta de células T a qual pode ser fortalecida pela presença de adjuvantes, utilização de coquetéis de proteínas ou por meio de estratégias de *prime-boost* (revisado em DUTHIE et al., 2012). Dentre os muitos antígenos de leishmania, destacamos a glicoproteína gp63 e a proteína LACK, as quais estão entre as mais estudadas em diferentes plataformas de vacinação. A gp63 é uma glicoproteína de 63 kDa expressa na superfície celular de *Leishmania* (HANDMAN; BUTTON; MCMASTER, 1990). A imunização com gp63 encapsulada em lipossomas ou complexos de nanopartículas + CpG-ODN protegeu camundongos BALB/c contra infecção por *L. major*, (FIROUZMAND et al., 2018; JAAFARI et al., 2007) por meio da indução de resposta Th1. A imunização utilizando plasmídeos com DNA codificando gp63 também induziu proteção substancial em camundongos BALB/c contra *L. major* (XU; LIEW, 1995) e, assim, trabalhos subsequentes passaram a utilizar DNA para a imunização. A imunização com DNA codificando gp63 (*prime*) seguido de reforço (*boost*) com proteína recombinante na presença de CpG-ODN induziu

proteção contra *L. donovani* em camundongos (MAZUMDER et al., 2011), assim como a vacina de DNA codificando gp63 e Hsp70 (KAUR; KAUR; JOSHI, 2016). Na LV, a proteção foi associada a uma menor carga parasitária, desenvolvimento de resposta DTH, produção de IFN-γ e IL-2 e supressão na produção de IL-4 e IL-10.

A proteína LACK, homóloga do receptor para quinase C ativada (MOUGNEAU et al., 1995) também se mostrou protetora contra a infecção por *L. major* em BALB/c quando administrada na presença de IL-12 recombinante. A imunização induziu células T $CD4^+$ produtoras de IFN-γ, mas não levou ao desenvolvimento de imunidade de longa duração (HUGENTOBLER et al., 2012). A vacinação com DNA codificando LACK se mostrou parcialmente protetora na infecção experimental com *L. major* (AHMED et al., 2004) ou com *L. chagasi* (GOMES et al., 2007). Já a vacina combinada com DNA codificando LACK, LeIF (fator de iniciação eucariótico) e TSA (antioxidante tiol-específico) conferiu proteção superior contra a infecção por *L. major* comparada àquela obtida com cada antígeno separadamente, induzindo maior razão IFN-γ/IL-4 (MASPI et al., 2018). Uma outra forma de aumentar a imunogenicidade de LACK consistiu em vacinação com DNA seguida de reforço com o vírus vacínia Ankara não-replicante modificado (MVA) expressando a mesma proteína. Essa estratégia induziu proteção contra a infecção por *L. major* (SÁNCHEZ-SAMPEDRO et al., 2013) e contra *L. infantum* (FERNÁNDEZ et al., 2018).

Recentemente, um peptídeo derivado da proteína PEPCK (fosfoenolpiruvato carboxilase glicossomal) foi identificado como imunodominante para a resposta de células T $CD4^+$ na infecção por *L. major*. A imunização com a proteína recombinante + CpG-ODN ou com DNA codificando a mesma proteína +CpG-ODN conferiu ampla proteção contra *L. major* em camundongos C57BL/6, caracterizada por menor lesão, carga parasitária diminuída e o desenvolvimento de células T $CD4^+$ IFN-γ^+ produtoras de IL-2 e TNF-α (MOU et al., 2015). Apesar da imunização com DNA ser mais imunogênica, estas vacinas ainda não foram licenciadas para uso humano, favorecendo ainda o uso de proteínas recombinantes (GILLESPIE et al., 2016).

A Leish-111f é uma poliproteína formada pela fusão de três antígenos de *Leishmania*: o anti-oxidante tiol-específico (TSA, de *L. major*), a proteína induzível por estresse 1 de *L. major* (LmSTI1) e o fator de elongamento e iniciação (LeIF) de *L. braziliensis*. A Leish- 111f apresentou eficácia contra infecção de camundongos com *L. major* e com *L. amazonensis* (COLER et al., 2002) e proteção a longo prazo contra *L. major* em camundongos BALB/c susceptíveis, quando administrada juntamente com a toxina de cólera como adjuvante (SAKAI

et al., 2010). Uma formulação de Leish 111f com o adjuvante MPL-SE tornou-se a primeira vacina a ser investigada em ensaios clínicos e se mostrou segura e bem tolerada em indivíduos com e sem evidência de infecção subclínica prévia em áreas endêmicas para LC e LV (CHAKRAVARTY et al., 2011; VÉLEZ et al., 2010). Os trabalhos citados também evidenciaram a imunogenicidade da vacina, a qual induziu a produção de IFN-γ e resposta DTH em maior magnitude em indivíduos vacinados. Uma segunda proteína de fusão, constituída pela nucleosídeo hidrolase (NH) e esterol 24-c metiltransferase (SMT), protetoras contra a LV experimental, também progrediu para ensaios clínicos do tipo I em formulação com uma nano emulsão (GLA-SE). A vacina LEISH-F3+GLA-SE se mostrou segura e imunogênica em indivíduos de uma área não-endêmica para leishmaniose, e induziu a produção de IFN-γ, IL-2 e TNF-α mas também IL-5 e IL-10 (COLER et al., 2015). No entanto, ambas as proteínas de fusão precisam ser avaliadas em ensaios clínicos subsequentes de modo a demonstrar eficácia em regiões endêmicas.

Diante do exposto acima, a utilização de parasitas atenuados para a imunização surge como mais uma alternativa para a baixa imunogenicidade de parasitas mortos ou de vacinas de subunidade. Parasitas atenuados entregam uma coleção de antígenos a células apresentadoras de antígenos, induzindo uma ativação mais potente e possivelmente imitando o curso natural da infecção, o que pode otimizar a polarização de células T $CD4^+$ para o subtipo Th1 (SALJOUGHIAN; TAHERI; RAFATI, 2014). Abordagens químicas, físicas, bem como genéticas; por meio da deleção de fatores de virulência e/ou essenciais para a sobrevivência da leishmania vem sendo utilizadas para gerar parasitas atenuados. Quando promastigotas são cultivadas de forma axênica e a longo prazo ocorre perda espontânea de virulência (ALI et al., 2013; DE SOUZA et al., 2010; MOREIRA et al., 2012) e a imunização com parasitas atenuados desta maneira induziu proteção contra *L. major* (MITCHELL; HANDMAN; SPITHILL, 1984), mas não contra *L. chagasi* (STREIT et al., 2001). Promastigotas de *L. donovani* irradiadas (raios γ) induziram proteção na LV experimental juntamente com a proliferação de células T e indução de uma resposta Th1 (DATTA et al., 2012; DATTA; ROY; MANNA, 2015). No entanto, apesar de mostrarem efeitos protetores promissores, estes métodos de inativação são inespecíficos e podem gerar uma inativação incompleta, com potencial para a reversão.

Outra forma de atenuação é a genética, por meio da deleção de genes que codificam fatores de virulência ou moléculas essenciais para a sobrevivência intracelular do parasita. Os parasitas gerados ainda são capazes de infectar o hospedeiro e de induzir resposta imunológica mas sem patologia associada (revisado em SALJOUGHIAN; TAHERI; RAFATI, 2014).

Dentre vários estudos nessa área, destacamos os trabalhos pioneiros utilizando *L. major* deficiente em dihidrofolato redutase-timidilato sintase, DHFR-TS (TITUS et al., 1995) ou em lipofosfoglicanos (LIU et al., 2013; UZONNA et al., 2004). A deleção do gene DHFR-TS, essencial para o metabolismo da *Leishmania*, conferiu resistência parcial à infecção com *L. major* virulenta em camundongos BALB/c (TITUS et al., 1995), mas não foi capaz de proteger contra o desafio homólogo em macacos *Rhesus* (AMARAL et al., 2002). A imunização com *L. major* deficiente no gene LPG2 conferiu resistência ao desafio homólogo de forma dependente da geração de células T de memória e de produção de IFN-γ (LIU et al., 2013; UZONNA et al., 2004). Mais recentemente, verificou-se que *L. infantum* deficiente na proteína HSP do tipo 2 (CARRIÓN et al., 2011) induziu proteção contra infecção por *L. major* associada à presença de células T produtoras de IFN-γ (SOLANA et al., 2017). Selvapandian e colaboradores (2009) mostraram que a imunização com *L. donovani* deficiente em Centrina 1 ($LdCen^{-/-}$) garantiu proteção contra desafio homólogo em BALB/c. A proteção foi dependente de IFN-γ, associada a células T $CD4^+$ e $CD8^+$ ativadas, produtoras também de TNF e IL-2 (SELVAPANDIYAN et al., 2009). $LdCen^{-/-}$ também foi capaz de conferir proteção duradoura contra infecção por *L. donovani* em hamsters quando administrado com LJM19, uma proteína presente na saliva do flebotomíneo (FIUZA et al., 2016). Pesquisadores do mesmo grupo desenvolveram *L. donovani* deficiente na proteína p27 ($Ldp27^{-/-}$) e a imunização com $Ldp27^{-/-}$ conferiu proteção duradoura contra desafio com *L. major* e *L. braziliensis* (DEY et al., 2013). Além disso, $LdCen^{-/-}$ e $Ldp27^{-/-}$ induziram resposta predominantemente Th1 após a infecção de células mononucleares derivadas de sangue periférico (PBMCs) de pacientes curados de leishmaniose visceral (AVISHEK et al., 2016), o que indica a indução de resposta similar a observada após a infecção natural.

Entretanto, a atenuação de parasitas por manipulação genética não elimina totalmente a presença de alelos selvagens e estes parasitas não atenuados permanecem capazes de causar doença. Mesmo parasitas deficientes em um gene apresentam um risco de reativação, especialmente quando inoculados em indivíduos imunocomprometidos (SUNDAR; SINGH, 2014). De fato, já foi relatada a ocorrência de mutação compensatória, a qual reverte a capacidade do parasito de expressar o gene deletado, como ocorrido com *L. major* deficiente em $lpg2^-$ (SPATH et al., 2004) que, portanto, voltou a ser virulento. Por isso, torna-se interessante o desenvolvimento de estratégias de atenuação que mantenham a capacidade de infecção da *Leishmania* mas que eliminem a sua capacidade de causar doença.

1.4 TERAPIA FOTODINÂMICA E LEISHMANIOSE

A terapia fotodinâmica (TFD) consiste em um tratamento em duas fases no qual um paciente recebe primeiramente um composto fotossensibilizador (FS) e, em seguida, é exposto a energia luminosa, na presença de oxigênio. O FS (inativo quando protegido da luz) absorve um fóton quando exposto à radiação com comprimento de onda adequado e passa do seu estado fundamental para um estado excitado instável (CASTANO; DEMIDOVA; HAMBLIN, 2004). Durante esse momento a molécula reage com o oxigênio do meio, entre outras moléculas aceptoras de elétrons, através de duas reações fotoquímicas. Na reação do tipo I ocorre a transferência de um elétron para o substrato, levando à formação de ânion superóxido e radicais, enquanto na reação do tipo II, o FS transfere energia para o oxigênio molecular em estado fundamental, gerando o oxigênio singleto ($^1O_2$) (CASTANO; DEMIDOVA; HAMBLIN, 2004; OLEINICK; EVANS, 1998). A geração de ROS via reação do tipo II é muito mais simples que a do tipo I, e acredita-se que a maior parte dos FSs opere desta maneira, produzindo $^1O_2$. A TFD tem aplicação clínica no tratamento de doenças da pele como psoríase (CHEN et al., 2017), queratose actínica (CANAVAN et al., 2017) e também no tratamento de carcinoma (WANG et al., 2015) e de tumores como de bexiga e de pulmão (revisado em DOLMANS; FUKUMURA; JAIN, 2003).

Na leishmaniose, os estudos inicias envolvendo a TFD empregaram o ácido delta-aminolevulínico (ALA) ou o seu derivado éster, o metil-aminolevulinato (MAL) para o tratamento clínico de lesões (ENK et al., 2003, 2015; GARDLO et al., 2003; SOHL et al., 2007), como já vinha sendo feito no tratamento de neoplasias. A exposição celular ao ALA leva ao acúmulo intracelular de protoporfirina IX (ppIX), um potente FS endógeno (STRAKA; RANK; BLOOMER, 1990), induzindo a formação de ROS e, consequentemente, dano celular, quando em contato com a luz UV (revisado por KOŘENÝ; OBORNÍK; LUKEŠ, 2013). Recentemente, Enk e colaboradores (2015) observaram que o tratamento empregando MAL levou à diminuição de lesões causadas por *L. major* e *L. tropica* em pacientes após apenas uma semana, efeito atribuído à rápida destruição de amastigotas. Em estudos com camundongos BALB/c infectados com *L. major* (AKILOV et al., 2007) ou com *L. braziliensis* (SOUZA et al., 2016), a TFD com ALA também induziu um controle significativo da carga parasitária . No entanto, no modelo de *L. major*, a morte dos parasitas foi associada a uma vigorosa inflamação, indicando que o efeito leishmanicida de ALA-PDT é inespecífico e promove dano ao hospedeiro (AKILOV et al., 2007).

Além de ALA, outros FSs também foram avaliados para a fotoinativação de *Leishmania* em estudos experimentais *in vitro* e *in vivo*. O tratamento com azul de metileno e luz LED diminuiu as lesões e a carga parasitária de forma significativa em hamsters infectados com *L. braziliensis* ou com *L. amazonensis* (PELOI et al., 2011; SBEGHEN et al., 2015), mostrando que esse fotossensibilizador pode ser usado de forma terapêutica. A PPA904, uma fenotiazina, controlou a carga parasitária quando administrada como um creme em camundongos infectados por *L. major*, mas também induziu uma irritação cutânea (AKILOV et al., 2009; LATORRE-ESTEVES et al., 2010). Outros FSs como rosa bengala (NAVASCONI et al., 2017), porfirinas de zinco (ANDRADE et al., 2018) e ftalocianinas de zinco (SILVA et al., 2015) também possuem baixo índice de seletividade em relação ao alvo terapêutico, causando danos às células do hospedeiro *in vitro* e, assim, surgiram novos compostos como as ftalocianinas catiônicas de silício (DUTTA et al., 2011) e as aminoftalocianinas (AL-QAHTANI et al., 2016), as quais foram desenhadas para o uso terapêutico pois apresentam atividade cerca de 100 vezes mais eficaz contra parasitas que células hospedeiras. As ftalocianinas de cloreto de alumínio **(**AlPhCl) e a de hidróxido de alumínio (AlPhOH) também foram eficazes no controle da infecção de macrófagos com leishmania, mediando citotoxicidade modesta (NESI-REIS et al., 2018). Por fim, o encapsulamento de FSs em formulações lipossômicas confere maior seletividade e efetividade para o tratamento, representando uma alternativa ao desenvolvimento de novas moléculas (PEREZ et al., 2014; RIBEIRO et al., 2016).

1.5 FOTOINATIVAÇÃO DE LEISHMANIA APLICADA À PROFILAXIA

O ALA e a protoporfirina IX são moléculas produzidas durante a biossíntese do heme, uma molécula essencial para a oxidação fosforilativa e da cadeia transportadora de elétrons; ativação do oxigênio para participação em reações biológicas como a síntese de óxido nítrico e para o transporte de oxigênio molecular e dióxido de carbono entre tecidos (BONKOVSKY et al., 2013). As porfirinas, moléculas intermediárias na via, são FSs constituídos por quatro anéis pirrólicos ligados a um elemento metálico no centro. Os compostos são pigmentados e exibem intensa fluorescência vermelha quando expostos à luz ultravioleta de ondas longas**.** Além das porfirinas, porfirinogênios (porfirinas reduzidas) também são moléculas intermediárias que podem ser rapidamente oxidadas para a forma ativa (STRAKA; RANK; BLOOMER, 1990) . O acúmulo de FSs tais como as porfirinas pode ser induzido tanto visando o tratamento de doenças, como na TFD, ou pode ocorrer devido a deficiências enzimáticas como ocorre nas doenças metabólicas conhecidas como Porfírias (BONKOVSKY et al., 2013).

O heme é sintetizado na maioria das células heterotróficas em uma via que envolve a ação de oito enzimas e inicia-se com a condensação de succinil coenzima A e glicina sob ação da enzima Ácido 5-aminolevulínico sintase (ALA-sintase), originando o ALA (Figura 1). Esta é a reação mais regulada e limitante da via e ocorre no interior da mitocôndria, de onde as moléculas de ALA se deslocam para o citoplasma. Uma vez no citoplasma, a enzima ALA desidratase (ALAD) une duas moléculas de ALA em uma reação de síntese por desidratação, formando porfobilinogênio (PBG), uma unidade do anel pirrólico. O hidroximetilbilano é então formado através da condensação de quatro moléculas de PBG pela enzima PBG desaminase (PBGD) e pode se converter de forma não enzimática em um composto cíclico chamado uroporfirinogênio I ou pode sofrer ação da uroporfirinogênio III sintase (URO III Sintase), formando uroporfirinogênio III (URO III). Este composto sofre descarboxilação através da uroporfirinogênio descarboxilase (UROD) formando coproporfirinogênio III (COPRO III). O COPRO III é transportado de volta para a mitocôndria, onde sofre oxidação mediada por coproporfirinogênio oxidase, formando protoporfirinogênio IX, o qual é convertido em protoporfirina IX na presença de oxigênio. A molécula de heme é então formada a partir da inserção de um átomo de Ferro (Fe^{2+}) no centro do macrociclo da protoporfirina IX, sob ação da ferroquelatase (PONKA, 1999; STRAKA; RANK; BLOOMER, 1990).

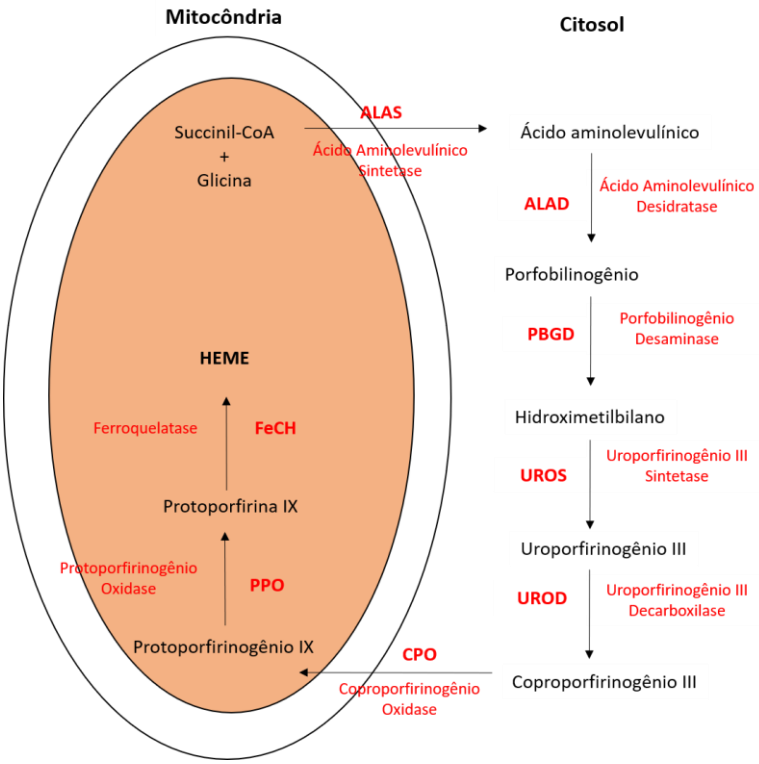

Figura 1. Esquema das enzimas envolvidas na síntese do heme em eucariotos heterotróficos. Fonte: adaptado de Fisher & Lilge, 2015.

Diferentemente de outros eucariotos, sabe-se que *L. amazonensis*, *L. infantum*, *L. major* e *L. tropica* não possuem a via biossintética do heme completa, e há evidência de que as cinco enzimas iniciais da via estejam ausentes ou não-funcionais (DUTTA et al., 2008a; KOŘENÝ; OBORNÍK; LUKEŠ, 2013). Os genes que codificam para as últimas três enzimas (CPO, PPO e FeCH) da via foram descritos em *L. major* através de sequenciamento completo do parasita, mas não há evidência de sua funcionalidade *in vivo* (OPPERDOES; COOMBS, 2007). Diante deste achado e sabendo que as porfirinas, moléculas intermediárias na via do heme, podem atuar como FSs, Sah e colaboradores (2002) geraram uma *L. amazonensis* capaz de expressar as enzimas Ácido Delta-Aminolevulínico Desidratase (ALAD) e Porfobilinogênio Deaminase (PBGD) e Dutta e colaboradores (2008a) também fizeram o mesmo com *L. major*, *L. infantum* e *L. tropica*. Acredita-se que, quando incubados com ALA, estes parasitas geneticamente complementados produzem Hidroximetilbilano o qual, na ausência de Uroporfirinogênio III Sintase (UROS), é oxidado em Uroporfirinogênio I e em Uroporfirina I (URO I), este último um poderoso FS (Figura 2). Como o parasita não possui a enzima Uroporfirinogênio III Sintase (UROS), a URO1 se acumula no interior da célula, tornando o parasita fotossensível, apesar da suposta presença das três últimas enzimas da via (DUTTA et al., 2008a, 2008b). Quando parasitas fotossensíveis pelo acúmulo de URO1 são expostos à luz ultravioleta de ondas longas ou à luz branca, ocorre a formação de oxigênio singleto (O_2^-) entre outros ROS, levando ao dano intracelular imediato e destruição do parasita, levando à sua inativação (DUTTA et al., 2008b; SAH et al., 2002).

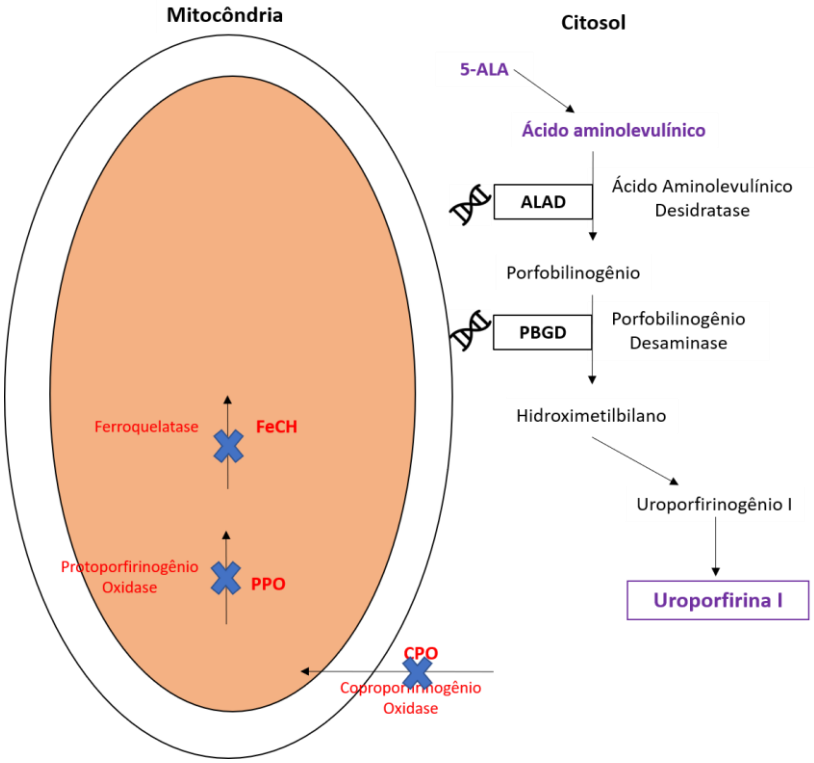

Figura 2. Esquema mostrando a complementação genética de *Leishmania* spp. com ALAD e PBGD e a produção de URO1 por meio do fornecimento de ALA. Fonte: adaptado de Fisher & Lilge, 2015.

Segundo Dutta e colaboradores (2008b), esta estratégia de sensibilização e posterior fotoinativação não induz resistência nos parasitas geneticamente complementados, mesmo após repetidos ciclos de tratamento. Um estudo posterior mostrou que a inoculação de *L. amazonensis* geneticamente complementada em hamsters e fotoinativada por exposição ao ALA e à luz branca induziu proteção contra um desafio com *L. donovani*, na ausência do desenvolvimento de lesões. Apesar de serem extremamente susceptíveis à leishmaniose visceral, os animais imunizados e desafiados não apresentaram caquexia ou hepatoesplenomegalia, e parasitas não foram detectados após 120 dias de infecção. Além disso, a imunização induziu proliferação de linfócitos, resposta DTH específica e níveis aumentados de iNOS, IFN-γ, IL-12 e IgG2, moléculas características de resposta de células T $CD4^+$ Th1. Os hamsters imunizados também apresentaram menor expressão de IL-4, TGF-β e IL-10. Por fim, a transferência de linfócitos T para animais *naive* induziu proteção similar à observada nos a animais imunizados (KUMARI et al., 2009). Esse trabalho mostrou que a vacinação com parasitas fotoinativados pode induzir resposta imunológica específica contra *Leishmania* mesmo na ausência de parasitas persistentes, representando importante alternativa de imunoprofilaxia.

Além da fotoinativação endógena, a *Leishmania* também pode ser fotoinativada utilizando FSs exógenos, tais como as ftalocianinas (PCs). Dutta e colaboradores (2005) observaram que os macrófagos podem ser infectados com promastigotas fotossensibilizadas com AlPhCl de forma que uma exposição subsequente à luz faz com que a fotoinativação ocorra no ambiente intracelular (DUTTA et al., 2005). Pesquisadores do mesmo grupo mostraram que células dendríticas infectadas com *Leishmania* transgênica expressando OVA e fotoinativada com PC15 apresentaram epítopos da proteína e levaram à ativação de células T de forma mais eficiente comparado a células dendríticas expostas a parasitas autoclavados (DUTTA et al., 2011). Este estudo indica que a fotoinativação de *Leishmania* utilizando PCs de nova geração preserva os antígenos do parasita. Dessa forma, a sensibilização de *Leishmania*, seja pela via endógena (parasitas geneticamente complementados e exposição ao ALA para o acúmulo de porfirinas), seja pela via exógena (exposição a PCs), seguida de exposição à luz é uma forma eficiente de inativação dos parasitas**. A hipótese desse trabalho é que a fotoinativação de *Leishmania* pelas vias endógena e/ou exógena pode gerar parasitas competentes para indução de proteção contra LC causada por *L. amazonensis* ou *L. braziliensis*.**

2. JUSTIFICATIVA

O tratamento para a leishmaniose consiste em drogas que apresentam toxicidade significativa e o aumento da incidência de resistência está sendo relatado (YASINZAI et al., 2013), tornando o desenvolvimento de uma vacina eficaz ainda mais urgente. Neste sentido, a fotoinativação é uma estratégia capaz de atenuar os parasitas, permitindo a preservação dos antígenos, tornando-se uma alternativa interessante para o desenvolvimento de vacinas. No presente estudo, avaliamos a fotoinativação de *Leishmania* através de estratégias endógena e exógena. Em seguida, desenvolvemos e caracterizamos uma linhagem de *L. braziliensis* geneticamente complementada capaz de produzir porfirinas endógenas para fotoinativação após o tratamento com ALA, consistindo em mais uma ferramenta a ser explorada para a profilaxia da CL experimental.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Estabelecer um modelo de fotoinativação de *L. amazonensis* e de *L. braziliensis* e avaliar o efeito protetor de promastigotas fotoinativadas *in vitro* e *in vivo*.

3.2 OBJETIVOS ESPECÍFICOS

1. Avaliar a taxa de fotoinativação de *L. amazonensis* com fotossensibilizadores endógenos e exógenos.
2. Quantificar a taxa de infecção de macrófagos com *L. amazonensis* sensibilizada e fotoinativada.
3. Avaliar a segurança do inóculo com *L. amazonensis* fotoinativada e o efeito protetor contra um desafio com parasitas vivos.
4. Desenvolver *L. braziliensis* geneticamente complementada e avaliar a taxa de fotoinativação.
5. Quantificar a taxa de infecção de macrófagos com *L. braziliensis* duplamente sensibilizada e fotoinativada e a produção de radicais livres.
6. Avaliar o perfil de expressão de moléculas de superfície e a produção de citocinas por macrófagos expostos a *L. braziliensis* duplamente sensibilizada e fotoinativada.

4. MANUSCRITO I

Título: Photodynamic Vaccination of BALB/c Mice for Prophylaxis of Cutaneous Leishmaniasis Caused by *Leishmania amazonensis*

Fotossensibilizadores (FS), como porfirinas e ftalocianinas (PC) são excitáveis pela luz e geram oxigênio singleto citotóxico e outras espécies reativas de oxigênio na presença de O_2 atmosférico. A fotoinativação de *Leishmania* por esta estratégia inativa os parasitas mas preserva sua imunogenicidade. A *Leishmania* pode ser fotoinativada após sensibilização com o FS, por meio da captação de PC ou através da geração de URO1 após exposição ao ácido delta-aminolevulínico (ALA). Neste trabalho, a sensibilização por FS e fotoinativação de *Leishmania amazonensis* foram examinadas *in vitro* e *in vivo* para vacinação contra a leishmaniose cutânea (LC). Promastigotas de *L. amazonensis* foram sensibilizadas e fotoinativadas *in vitro* por meio da absorção de PC seguida de exposição à luz vermelha (1-2 J/cm^2) ou por meio do acúmulo de URO1 após o tratamento dos parasitas com ALA e exposição a luz UV. Quando aplicadas individualmente, ambas as estratégias de fotoinativação diminuíram significativamente as atividades de redução de MTT das promastigotas e a sua entrada em macrófagos derivados da medula óssea, além de sua infectividade *in vivo*. Uma combinação de ambas as estratégias foi usada para a inativação completa de *Leishmania*, de forma a obter parasitas inertes, mas imunogênicos para a imunização de camundongos BALB/c. Diferentes locais foram avaliados quanto à eficácia desse método de vacinação fotodinâmica *in vivo*. Inicialmente, o camundongo foi inoculado em diversos locais com promastigotas duplamente sensibilizadas *in vitro* e os animais foram subsequentemente iluminados com luz branca (50 J/cm^2), para a fotoinativação *in situ*. Apenas os parasitas inoculados na derme da orelha foram fotoinativados de forma a não serem detectáveis. Dessa forma, os camundongos foram imunizados uma vez na derme da orelha e desafiados após 3 semanas na base da cauda com *L. amazonensis* virulenta. A ação profilática foi observada em camundongos inoculados com parasitas duplamente fotoinativados, como indicado pelo atraso significativo no estabelecimento da lesão e diminuição substancial na carga parasitária. Dessa forma, a *Leishmania* duplamente sensibilizada e fotoinativada *in situ* se mostrou segura e eficaz quando utilizada como forma de imunização, como indicado pela proteção observada em camundongos BALB/c, inerentemente susceptíveis a LC.

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ORIGINAL RESEARCH
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Photodynamic Vaccination of BALB/c Mice for Prophylaxis of Cutaneous Leishmaniasis Caused by *Leishmania amazonensis*

Sayonara M. Viana[1], Fabiana S. Celes[1], Laura Ramirez[1], Bala Kolli[2], Dennis K. P. Ng[3], Kwang P. Chang[2] and Camila I. de Oliveira[1,4]**

[1] Instituto Gonçalo Muniz (IGM), FIOCRUZ, Salvador, Brazil, [2] Department of Microbiology/Immunology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL, United States, [3] Department of Chemistry, The Chinese University of Hong Kong, Hong Kong, Hong Kong, [4] Instituto Nacional de Ciência e Tecnologia (iii-INCT) - Instituto de Investigação em Imunologia, São Paulo, Brazil

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Edited by:
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Cecilia Parodi, Institute of Experimental Pathology (IPE), Argentina

***Correspondence:**
Camila I. de Oliveira
camila@bahia.fiocruz.br
Kwang P. Chang
kwangpoo.chang@rosalindfranklin.edu

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Background: Photosensitizers (PS), like porphyrins and phthalocyanines (PC) are excitable by light to generate cytotoxic singlet oxygen and other reactive oxygen species in the presence of atmospheric O_2. Photodynamic inactivation of *Leishmania* by this means renders them non-viable, but preserves their effective use as vaccines. *Leishmania* can be photo-inactivated after PS-sensitization by loading via their endocytic uptake of PC or endogenous induction of transgenic mutants with delta-aminolevulinate (ALA) to accumulate cytosolic uroporphyrin I (URO). Here, PS-sensitization and photo-inactivation of *Leishmania amazonensis* was further examined *in vitro* and *in vivo* for vaccination against cutaneous leishmaniasis (CL).

Methods and Results: *Leishmania amazonensis* promastigotes were photodynamically inactivated *in vitro* by PC-loading followed by exposure to red light (1–2 J/cm^2) or ALA-induction of uroporphyrinogenic transfectants to accumulate cytosolic URO followed by longwave UV exposure. When applied individually, both strategies of photodynamic inactivation were found to significantly, albeit incompletely abolish the MTT reduction activities of the promastigotes, their uptake by mouse bone marrow-derived macrophages *in vitro* and their infectivity to mouse ear dermis *in vivo*. Inactivation of *Leishmania* to completion by using a combination of both strategies was thus used for the sake of safety as whole-cell vaccines for immunization of BALB/c mice. Different cutaneous sites were assessed for the efficacy of such photodynamic vaccination *in vivo*. Each site was inoculated first with *in vitro* doubly PS-sensitized promastigotes and then spot-illuminated with white light (50 J/cm^2) for their photo-inactivation *in situ*. Only in ear dermis parasites were photo-inactivated beyond detection. Mice were thus immunized once in the ear and challenged 3 weeks later at the tail base with virulent *L. amazonensis*. Prophylaxis was noted in mice photodynamically vaccinated with doubly photo-inactivated parasites, as indicated by a significant delay in the onset of lesion development and a substantial decrease in the parasite loads.

Conclusion: *Leishmania* doubly PS-sensitized and *in situ* photo-inactivated as described proved to be safe and effective when used for one-time immunization of ear dermis, as indicated by its significant protection of the inherently very susceptible BALB/c mice against CL.

Keywords: *Leishmania*, leishmaniasis, potosensitizer, phthalocyanine, uroporphyrin, photodynamic vaccination, suicidal vaccination, cutaneous leishmaniasis

INTRODUCTION

Cutaneous leishmaniasis (CL) is caused by protozoan parasites in the genus of *Leishmania* and is a wide-spread disease, with estimated 1.5 million new cases per year (WHO, 2010). CL presents a varied spectrum of clinical manifestations that are determined presumably by both the type and magnitude of the human immune responses as well as by the differences of the causative agents (Reithinger et al., 2007). *Leishmania* infection frequently produces no clinical symptom, but sometimes causes a localized lesion, characteristic of simple CL and also more severe diseases, i.e., diffused CL and mucosal leishmaniasis [reviewed in (Bittencourt et al., 1993)]. Clinical management of leishmaniasis has been based solely on treatment of patients by chemotherapy with antiquated and toxic drugs that elicits resistance (Yasinzai et al., 2013), thus making the development of an effective vaccine all the more urgent.

Immunologically competent individuals after recovery from leishmaniasis develop lifelong immunity, indicative of the feasibility to develop an effective prophylactic vaccine. It is possible to elicit protective immunity to human CL by leishmanization, i.e., inoculation of healthy individuals with a low dose of live *Leishmania* (Nadim et al., 1983). Leishmanization is, however, unacceptable because of its association with the development of non-healing lesions, especially in immunocompromised individuals [reviewed in (Palatnik-De-Sousa, 2008)]. Attempts to overcome these difficulties included the use of parasites after attenuation via, for example, long-term *in vitro* cultivation (Daneshvar et al., 2003), genetic modifications (Alexander et al., 1998; Spath et al., 2000; Uzonna et al., 2004; Selvapandiyan et al., 2009; Dey et al., 2013; Bhattacharya et al., 2015) and gamma irradiation (Alexander, 1982). Although such attenuated parasites immunologically protect susceptible animals against experimental challenges, the risk of potential reactivation remains to be a concern for their clinical use, especially among immunocompromised individuals (Sundar and Singh, 2014).

We have explored the principle of photodynamic therapy (PDT) as a new strategy for *Leishmania* inactivation *in vitro* to develop non-viable, but immunologically competent whole cell vaccines and vaccine carriers (Sah et al., 2002; Dutta et al., 2005; Chang and Kolli, 2016). PDT uses photosensitizers (PS) that are excitable by light at a specific wavelength to produce reactive oxygen species (ROS) for the clinical treatment of skin diseases, such as psoriasis (Chen et al., 2017), actinic keratosis (Canavan et al., 2017), carcinoma (Wang et al., 2016) and CL (Enk et al., 2015). Our attention to PDT started with the work on *Leishmania* genetic deficiency in the enzymes of heme biosynthesis. *Leishmania* spp., e.g., *Leishmania amazonensis* were genetically complemented to express the 2nd and 3rd enzymes in this biosynthetic pathway, i.e., delta-aminolevulinate (ALA) dehydratase (ALAD) and porphobilinogen deaminase (PBGD). Upon exposure of these mutants to ALA, uroporphyrin I (URO) accumulates in the cytosol, rendering them light sensitive as a PS to generate cytotoxic singlet oxygen ($^1O_2$) and other ROS (Sah et al., 2002; Dutta et al., 2008). This strategy of photo-inactivation, especially in combination with additional sensitization with exogenous phthalocyanines (PC) irreparably damages all *Leishmania* cells. Significantly, repeated cycles of PDT selected no PDT-resistant mutants (Dutta et al., 2011). These and other properties of PDT argue strongly in favor of its use to generate inactivated parasites for vaccination, especially for eliciting cell-mediated immunity via oxidative and proteolytic processing of vaccines in macrophages and other antigen-presenting cells (APC) for epitope presentation to the immune system. Indeed, vaccination of hamsters with porphyrinogenic *L. amazonensis* followed by *in vivo* ALA treatment and light exposure conferred protection on these susceptible animals against the challenge with virulent *L. donovani* (Kumari et al., 2009). Significantly, this immunity is adaptively transferrable from immunized hamsters to naïve animals.

In the present study, we have evaluated initially both endogenous and exogenous strategies separately for photo-inactivation of *L. amazonensis* based on parasite viability, parasite uptake *in vitro* and lesion development in mice. Only when doubly PS-sensitized with exogenously provided PC together with endogenously generated URO, were promastigotes rendered susceptible to complete photo-inactivation by spot-illumination *in situ*, but only in the ear dermis. Ear dermis was thus the site chosen for immunization of BALB/c mice. This photodynamic vaccination prophylactically protects the highly susceptible strain of mice against challenge infection, as indicted by the delay in lesion development and reduction in parasite loads.

MATERIALS AND METHODS

Ethics Statements

Female BALB/c mice, 6–8 weeks of age, were obtained from CPqGM/FIOCRUZ animal facility where they were maintained under pathogen-free conditions. All animal work was conducted according to the Guidelines for Animal Experimentation of the Colégio Brasileiro de Experimentação Animal and of the Conselho Nacional de Controle de Experimentação Animal. The

local Ethics Committee on Animal Care and Utilization (CEUA) approved all procedures involving animals (CEUA-003/2014-IGM/FIOCRUZ).

Parasites

Leishmania amazonensis (MPRO/BR/72/M1845/LV78) clone 12-1 was maintained as promastigotes in Medium 199 (SIGMA) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (penicillin 100 IU/mL and streptomycin 100 μg/mL) (all from Invitrogen). Genetically complemented *L. amazonensis* expressing ALAD and PBGD (Sah et al., 2002) were grown as described above in the presence of G418 (100 μg/mL) (Sigma) and tunicamycin (20 μg/mL) (CalBiochem). Before exposure of the transfectants to ALA for uroporphyrinogenesis, they were grown for one-cycle in drug-free medium to avoid potential cytotoxicity of the carryover drugs to macrophages during *in vitro* and *in vivo* infection.

In Vitro PS-Sensitization and Photo-Inactivation of *Leishmania*

The exogenous PS used for this study included amino-phthalocyanines, e.g., PC2 (Al-Qahtani et al., 2016) and aluminum phthalocyanine chloride (AlPhCl, Sigma) (Dutta et al., 2005). Photosensitizers were dissolved in dimethyl sulfoxide (DMSO) (SIGMA) as 1 mM stock solutions. For exogenous sensitization, *L. amazonensis* promastigotes were grown to late-log phase, washed and resuspended in Hank's Balanced Salt Solution (Invitrogen)/0.01% bovine serum albumin (HBSS-BSA), pH 7.4, in presence of the PC (0.1–10 μM). Cells exposed to diluent (DMSO) equivalent to the highest PC concentration were used as controls. After overnight incubation at 26°C in the dark, PC-sensitized and control cells were washed and illuminated with red light (RL) until the cessation of their flagellar motility (1–2 J/cm^2) as described (Dutta et al., 2011).

For endogenous PS-sensitization, *L. amazonensis* genetically complemented to express ALAD and PBGD (Sah et al., 2002) were exposed to 1 mM ALA (SIGMA) in HBSS-BSA for 24–48 h at 26°C in the dark for accumulation of cytosolic URO (Dutta et al., 2008). URO-loaded *L. amazonensis* were washed, placed in unlidded wells and then exposed to longwave UV (λ_{max} = 365 nm) from the top for 20 min as before. Uroporphyric cells kept in the dark served as controls.

Microscopy, MTT Reduction and Growth Assays

The effect of exogenous (PC) and endogenous (ALA-URO) PS-sensitization with and without photo-inactivation on *L. amazonensis* was examined by phase contrast and fluorescence microscopy for PC and porphyrin using filter sets previously described (Dutta et al., 2008). After incubation for PS-loading (overnight for PC and 24–48 h for ALA-URO), one set of samples were kept in the dark and the other set exposed to light at the excitation wavelengths specific to PC or URO, also as described before (Sah et al., 2002; Dutta et al., 2011). All cell samples were subjected to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay (SIGMA) according to the manufacturer's protocol. Treated and control cells (2×10^5) were also inoculated into Schneider medium containing 20% FBS for growth, as determined by daily enumeration of cell density in a haemacytometer in quintuplicate.

In Vitro Uptake of PS-Sensitized and Photo-Inactivated *L. amazonensis* by Bone Marrow-Derived Macrophages (BMDM)

The macrophages were obtained as previously described (Weischenfeldt and Porse, 2008), resuspended in RPMI 1640 medium (SIGMA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS for seeding onto glass coverslips at 3×10^5 cells/coverslip placed in 24-well plates. Monolayers formed on the coverslips were each infected with 3×10^6 control or experimental cell samples (10:1 parasite/host cell) in RPMI 1640 containing 20% FBS at 35°C, 5% CO_2. After 4 h, monolayers were extensively washed to remove non-internalized parasites, fixed and stained with hematoxylin and eosin. Parasite uptake was determined by microscopic counting of 200 macrophages in quintuplicate for the number of infected cells, non-infected cells and intracellular *Leishmania*.

Inoculation of BALB/c Mouse Ear Dermis with *in Vitro* Singly PS-Sensitized and Photo-Inactivated *L. amazonensis*

Photosensitizers-sensitized promastigotes of *L. amazonensis* with and without photo-inactivation *in vitro* were inoculated into the ear dermis of BALB/c mice, each with ~10^6 cells using a 27.5-gauge needle. Ear thickness was measured periodically by using a digital caliper (Thomas Scientific).

Inoculation of BALB/c Mice at Different Cutaneous Sites with *in Vitro* Doubly PS-Sensitized *Leishmania* for *in Situ* Photo-Inactivation to Select Suitable Site for Immunization

Promastigotes of the mutant *L. amazonensis*, which were doubly sensitized in the dark with PC (AlPhCl, 0.1 μg/ml) and ALA/URO were inoculated into four groups of BALB/c mice at different cutaneous sites: ear dermis, shaved flank or back, footpad and tail base. Each group consisted of four mice, each inoculated subcutaneously in the given location with 10^6 parasites. The use of this cell number was chosen as the most adequate size of inoculation based on prior testing of 10^3 to 10^7 per site. After 24 h, each site received an additional injection of 100 mM ALA (100 μl) to boost uroporphyrinogenesis of the inoculated transfectants *in situ*. After another 36 h, a set of mice was spot-illuminated (individually at the inoculation site) with white light generated from a probe, consisting of heatless fiber optic end-point emitter at 50 J/cm^2 (LumaCare model LC122, MGB Technologies, Inc.); the other set of mice received no spot-illumination. All mice were inspected every other day at the inoculated sites for lesion development. After 3 weeks,

mice were euthanized and tissues surrounding the injection sites were removed and homogenized. The homogenates were subjected to limiting dilution assay in 96 wells for growth to estimate the number of surviving parasites (Dutta et al., 2012).

Ear Dermis Immunization of BALB/c Mice with *in Vitro* Doubly PS-Sensitized *L. amazonensis* for Their *in Situ* Photo-Inactivation Followed by Challenge Infection

The choice of immunization site and dosage was based on the outcome of the experiments described in the preceding section (see section "Results"). Porphyrinogenic transfectants of *L. amazonensis* were doubly PS-sensitized *in vitro* with ALA (1 mM) and AlPhCl (0.1 μM). The PS-sensitized cells were washed and resuspended to 10^9 cells/ml in HBSS-BSA. Controls were similarly prepared, consisting of six different groups: untreated cells exposed to light (*La*+Light), singly PS-sensitized cells without light (*La*+ALA; *La*+AlPhCl), singly PS-sensitized cells with light (*La*+ALA+Light; *La*+AlPhCl+Light) and both PS alone with light (ALA+AlPhCl+Light). There were thus seven groups, each consisting of six BALB/c mice. Each mouse was immunized once in the ear dermis with the experimental or one of the six control cell samples at 10^6 parasites/10 μl HBSS-BSA/mouse. One day later, an additional volume (∼100 μl in total) of 100 mM ALA was injected into the ear dermis. After 36 h, ear dermis was exposed to white light (50 J/cm^2) in five of the seven groups according to the experimental designs indicated. Experimental and control mice were each challenged 3 weeks later with 10^7 stationary-phase *L. amazonensis* promastigotes at the tail base. Lesion size in diameter was measured periodically for a total period of ∼10 weeks post challenge. Parasite loads at the challenge sites were determined by limiting dilution assay of the parasites in the tissues at the end point of the experiment.

Data Analysis

Comparisons between two groups were performed by Mann–Whitney (non-parametric *t*-test) and comparisons among more than two groups were performed by Kruskal–Wallis. Analyses were conducted using Prism (GraphPad, V 5.0) and a p-value ≤ 0.05 was considered significant. The course of disease for mice in all experimental and control groups was plotted individually. Disease burden was calculated in some cases as the diseased area under the curve (AUC). Lesion development was assessed by measuring thickness or lesion diameter, depending on the site of inoculation in ear dermis or tail base. Data are presented as mean ± standard deviation.

RESULTS

PC-Sensitized and Photo-Inactivated *L. amazonensis* Lost Their Viability Substantially, but Remain Susceptible to Endocytosis by Mouse Bone Marrow Macrophages *in Vitro*

Leishmania amazonensis were PC-sensitized and exposed to red light for photo-inactivation, e.g., amino-PC2. Fluorescence microscopy of live promastigotes showed PC2 localization in cytoplasmic vacuoles (**Figure 1A**). Without light exposure, these PC-loaded cells remained, as expected, intact and motile, just like the untreated controls. By MTT reduction assays, PC-loaded (e.g., 0.1–10 μM PC2) cells were shown to lose their viability only after light exposure in contrast to parasites kept in the dark (**Figure 1B**, white bars vs. gray bars). When sensitized with 0.1 μM PC and exposed to light, cells lost flagellar motility; except very few, which failed to grow up on further incubation under the experimental conditions described ($p = 0.0286$; **Figure 1C**). The ED_{50} of the amino-PC for photo-inactivation of these cells falls in between 10–100 nM according to the results with PC2 from both cell viability assays.

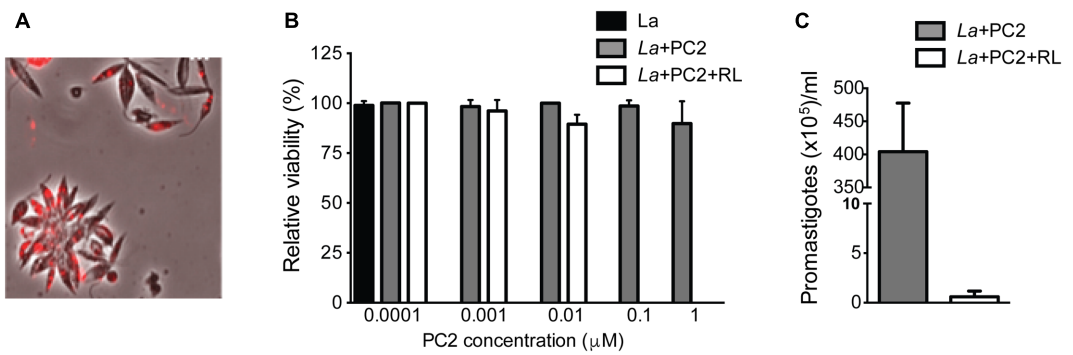

FIGURE 1 | PC2-mediated photo-inactivation of *Leishmania amazonensis*. Promastigotes (La) were incubated in the dark with PC2 for 16 h and exposed to red-light (RL) (=1–2 J/cm^2). **(A)** Merged phase contrast and Cy5 fluorescence images, showing the uptake of PC2 by cells sensitized in the dark overnight with 1 μM PC2. **(B)** MTT reduction activities of cells sensitized with graded concentrations of PC2, as indicated, in the dark (gray bar) and after light exposure (white bar), the values being expressed in % of untreated controls (black bar). **(C)** Disparity in cell density between PC2-sensitized cells with (white bar) and without photo-inactivation (gray bar) after inoculation into culture medium. Similar results were obtained after PS-sensitization with either 0.1 or 1 μM PC2. Data are presented as mean ± SD from a representative set of experiments performed in quadruplicate.

Under the experimental conditions used, PC-loaded and photo-inactivated *L. amazonensis* promastigotes were taken up by BMDM but the uptake was reduced by three–fourfold in comparison to the untreated or PC-loaded controls without light exposure, judging from the rates of uptake (15% vs. 45%) (**Figure 2A**, white vs. gray and black bars) and parasite number/100 cells (50 vs. 150–200, $p < 0.05$) (**Figure 2B**, white vs. gray and black bars). Light microscopy of these samples confirms the observations as described (**Figure 2C**, *La*+PC2+RL vs. *La*+PC2). Thus, under the conditions described, PC-mediated photo-inactivation of *L. amazonensis* reduces viability of the cell population to the extent of no apparent growth when cultured *in vitro* and diminished their uptake by primary macrophages within the time frame of the experiments.

PC-Sensitization and Photo-Inactivation of *L. amazonensis in Vitro* Significantly Reduces, But Does Not Eliminate Its *in Vivo* Disease-Causing Capacity

Lesion development was assessed after inoculating the ear dermis of BALB/c mice with *in vitro* PC-sensitized and photo-inactivated *L. amazonensis* vs. controls not submitted to photoinactivation under otherwise identical experimental conditions. Periodic measurements of the lesions for 9 weeks showed that those produced by the controls developed much more rapidly than those by the photo-inactivated parasites, reaching >2 mm and ∼1 mm in ear thickness at the end point, respectively. This is clearly indicated by mapping the disease burden (AUC) (shown in **Figure 3A**) ($p < 0.01$), confirming that CL produced by the PC-sensitized and photo-inactivated parasites was at least twofold less severe than that produced by the controls (**Figure 3B**, clear vs. gray bar). Therefore, PC-mediated photo-inactivation of *L. amazonensis* diminishes the parasite ability to cause disease *in vivo*.

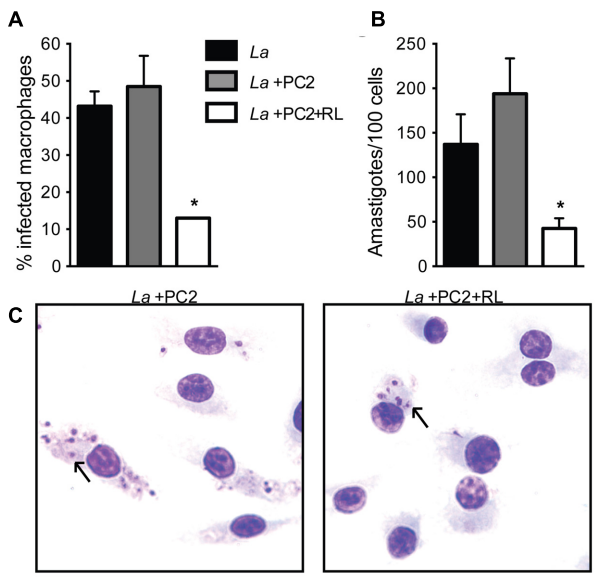

FIGURE 2 | Reduced uptake of PC2-sensitized and photo-inactivated *L. amazonensis* by primary macrophages. Bone marrow-derived macrophages were infected with *L. amazonensis* (La) (black bar) or PC2-sensitized parasites with (white bar) and without (gray bar) exposure to red light (RL) at a host-parasite ratio of 1:10 for 4 h. Cells were processed for microscopy to assess: **(A)** the percentage of infected macrophages; and **(B)** the number of *Leishmania* per 100 macrophages. **(C)** Representative photomicrographs of **(A,B)**. Data are presented as mean ± SD from a representative experiment performed in quadruplicate (Kruskal–Wallis test, $^*p < 0.05$). Arrow, endocytosed *Leishmania*.

ALA-Uroporphyric and Photo-Inactivated *L. amazonensis* Lost Their Viability, but Remain Susceptible to Endocytosis by Mouse Bone Marrow Macrophages *in Vitro*

Genetically complemented *L. amazonensis* exposed to ALA accumulated cytosolic URO, as shown by fluorescence microscopy of live promastigotes (Supplementary Figure 1A). Without UV exposure, ALA-induced uroporphyric cells remained intact and motile, similar to untreated controls, corroborating earlier findings (Sah et al., 2002; Dutta et al., 2008). By MTT reduction assays, the uroporphyric cells were shown to lose their viability after UV exposure in contrast to cells kept in the dark (Supplementary Figure 1B, clear vs. gray bar). Uroporphyric *L. amazonensis* exposed to UV light also became immobilized and reduced to such a small number that few survivors failed to grow within the time frame under the experimental conditions described (Supplementary Figure 1C, $p = 0.002$).

Under the experimental conditions established, photo-inactivated uroporphyric *L. amazonensis* promastigotes were taken up by BMDM macrophages but the uptake was reduced in comparison to no-light controls, judging from rates of uptake (<10% vs. ∼30%) (Supplementary Figure 2A) and from the parasite number per 100 macrophages (∼10 vs. >40, $p > 0.05$) (Supplementary Figure 2B, white vs. gray bar and black bar). Light microscopic examinations of these samples confirm the observations as described (Supplementary Figure 2C, *La*+ALA+UV vs. *La*+ALA). These inactivated parasites produced similar outcome as those after PC-mediated photo-inactivation when inoculated into the mouse ear dermis under the same conditions (data not shown).

Determination of Ear Dermis as the Best Site for Photodynamic Vaccination (PDV) with *in Vitro* Doubly PS-Sensitized *Leishmania* Followed by *in Vivo* Photo-Inactivation

When mice were inoculated with 10^6 of *in vitro* doubly PS-sensitized, but not photo-inactivated *L. amazonensis* ("no light" control groups), lesions developed within the time frame of 3 weeks at all cutaneous sites, except the back (**Figure 4A** lower panel: tail base, foot pad, ear). Back

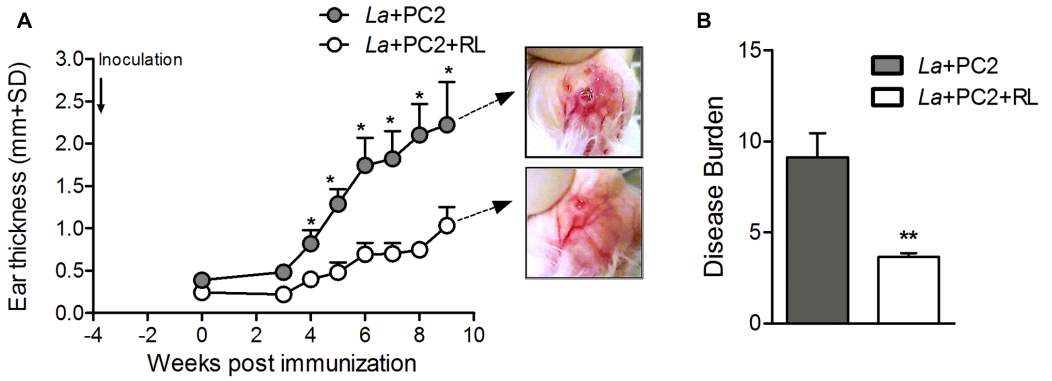

FIGURE 3 | Lesion development in mice inoculated with *in vitro* PC2-sensitized/photo-inactivated *L. amazonensis*. BALB/c mice were inoculated with PC2-sensitized *L. amazonensis* (La) with (open circle/white bar) and without (gray circle/gray bar) prior red light exposure (RL). **(A)** Ear thickness was measured weekly for 9 weeks. Photomicrographs show differential severity in the appearance of the ear lesions at the end point. **(B)** Disease burden calculated by mapping the diseased areas under the curves (AUC) in **(A)**. Data are presented as mean ± SD from control and experimental groups, each consisting of six mice (*$p < 0.05$, **$p < 0.001$, Mann–Whitney test).

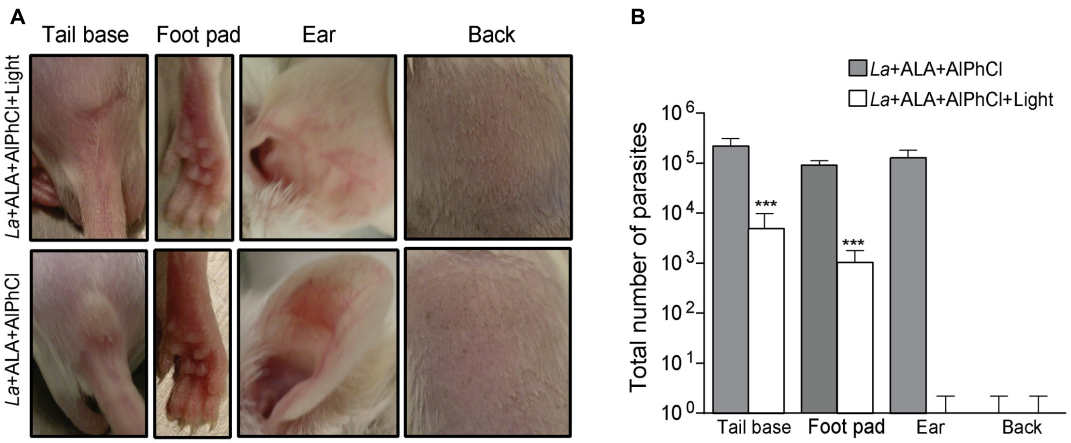

FIGURE 4 | *In situ* photo-inactivation of *in vitro* doubly PS-sensitized *L. amazonensis* abolishes lesion development and parasite loads in the ear dermis of BALB/c mice. Transgenic *L. amazonensis* promastigotes (La) doubly PS-sensitized *in vitro* with URO via ALA (1 mM) treatment and AlPhCl (0.1 μM) were inoculated at 10^6 parasites per site into different cutaneous sites as indicated. After 24 h, each site received an additional ∼100 μL of ALA (100 mM) to boost uroporphyrinogenesis. After another 36 h, the sites were spot-illuminated with white light (50 J/cm^2). **(A)** Representative photographs of ear, tail base, footpad and back of mice 3 weeks after inoculation with experimental samples (*La*+AlPhCl+ALA+Light) (upper panel) and "no-light" controls (*La*+AlPhCl+ALA) (lower panel). **(B)** Limiting dilution of homogenates from tissues of the inoculation sites to estimate the parasite loads at the end points for experimental samples (white bar) and no-light controls (dark bar). Data are presented as mean ± SD for all sites from both control and experimental groups, each consisting of four mice per group. ***$p < 0.001$.

was eliminated from further consideration as a site for photodynamic vaccination, since the absence of lesion in the "no-light" control group raised the uncertainly of whether the inoculated PS-sensitized parasites remained in sufficient number or remained as viable target in this site for subsequent photo-inactivation by *in situ* illumination. In the remaining three inoculation sites (tail base, footpad, and ear), lesions were produced in all the "no-light" control groups, indicative of the retention of the PS-sensitized parasites in these sites. *In situ* spot-illumination of these sites shortly after inoculation to target the PS-sensitized parasites therein for photo-inactivation produced different outcome: lesions still developed, albeit less severe in the tail base and footpad, but not at all in the ear dermis (**Figure 4A** upper panel: tail base, footpad, and ear). Lesion development or the lack of it provided a valid criterion for the efficacy of *in situ* photo-inactivation of the parasites therein, as indicated by quantitative analysis of parasite loads in inoculated sites with and without *in situ* photo-inactivation. In all three inoculation sites (tail base, footpad, and ear) examined at the end point, the parasite load per site was estimated as∼10^5 without *in situ* photo-inactivation (**Figure 4B**, gray bar). *In vivo* photo-inactivation rendered the parasites virtually undetectable in the ear dermis, but only reduced the parasite loads by 1–2 logs in the tail base and foot pad (**Figure 4B**, white bar).

Protection of BALB/c Mice against *L. amazonensis* by Immunization of Their Ear Dermis with *in Vitro* Doubly PS-Sensitized Parasites for *in Vivo* Photo-Inactivation

Groups of BALB/c mice were photodynamically vaccinated once accordingly in the ear dermis with multiple controls and challenged 3 weeks later at the tail base under the conditions as described in Section "Materials and Methods." Periodic measurements of lesion development with time for up to ∼10 weeks (69 days) showed that doubly PS-sensitized parasites followed by *in situ* photo-inactivation conferred the best protection. This was evident in comparison to all the control groups by a significant delay in lesion emergence by at least 14 days (**Figure 5A**, blue square) and a significant reduction of the parasite loads as the lowest of all in the challenge site at the end point (**Figure 5B**, blue bar). Single PS-sensitization of parasites with ALA-URO plus *in situ* photo-inactivation was moderately effective, as indicated by a significant reduction of the parasite loads as the second lowest of all in the challenge site at the end point (**Figure 5B**, red bar). Among the control groups, there were some variations in lesion development and slight differences in the parasite loads at the end point (**Figures 5A,B**), but no significant protection. Together, these data illustrate that immunization of BALB/c mice with doubly sensitized *L. amazonensis* for photo-inactivation by spot illumination *in situ* is safe and effective on account of no parasites recoverable from the site of vaccination and a markedly delayed onset for the emergence of the lesion with much reduced parasite loads against challenge infection.

DISCUSSION

Photodynamic inactivation of microorganisms such as *Leishmania* can be achieved through the intervention of PS, i.e., URO over-produced endogenously or uptake of PC provided exogenously, both being excitable by light to generate $^1O_2$. $^1O_2$ is highly reactive and thus extremely destructive, but too short-lived to cross the plasma membrane of cells, like *Leishmania*, allowing them to maintain structural integrity for extended time before disintegration [reviewed in (Chang and Kolli, 2016)]. Photodynamic inactivation of *Leishmania* has been studied as a new approach for producing non-viable, but immunologically competent whole-cell vaccines for immunization, akin to leishmanization to elicit effective immunity. In this study, we evaluated individually exogenous (PC supplementation) and endogenous (Uroporphyrinogenesis) photodynamic inactivation of *L. amazonensis* based on their viability, uptake by primary macrophages and disease development *in vivo*.

While excitation of all PC by red light is known to generate $^1O_2$ (Cook et al., 1995), the amino-phthalocyanines, e.g., PC2 are 10–40 times more effective to mediate photo-inactivation of *L. tropica* (Al-Qahtani et al., 2016) than other PCs against *L. amazonensis* (Dutta et al., 2011). We extended this finding by showing that the amino-PC also dose-dependently mediates photo-inactivation of *L. amazonensis*, as seen by their loss of

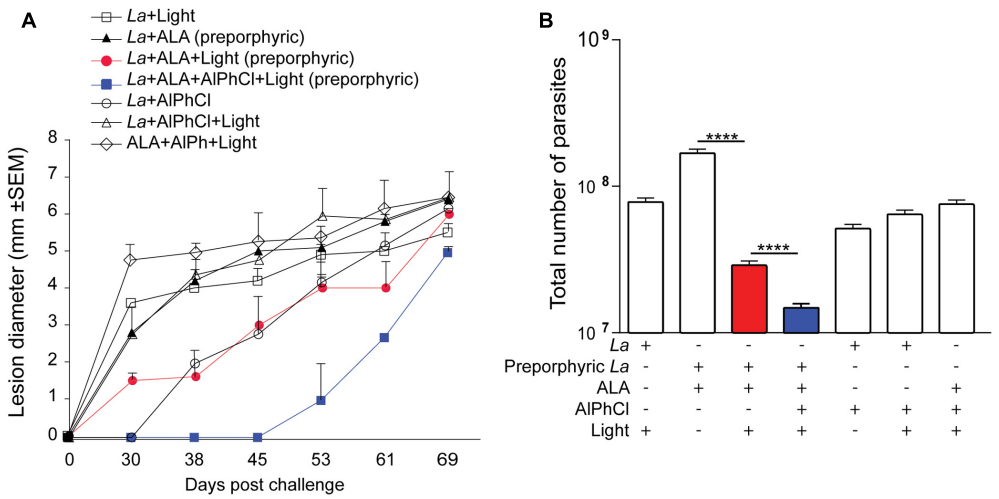

FIGURE 5 | Prophylactic activities of photodynamic vaccination in ear dermis against cutaneous leishmaniasis in BALB/c mouse. Uroporphyrinogenic transfectants of *L. amazonensis* promastigotes (La) were doubly PS-sensitized *in vitro* with URO via induction by ALA (1 mM) (preporphyric) and AlPhCl (0.1 μM) for inoculation at 10^6 cells per site in the ear of mice. After 24 h, their uroporphyrinogenesis was boosted by an additional shot of ALA (100 μL of 100 mM) in the surrounding tissue. After an additional 36 h, vaccination sites were individually illuminated *in situ* with white light (50 J/cm^2) (Light). The experimental group of photodynamic vaccination is designated as *La*+ALA+AlPhCl+Light (Solid blue square) that is controlled by six additional groups as indicated. Mice of all seven groups were each challenged 3 weeks later at the tail base with 10^7 stationary-phase *L. amazonensis* promastigotes. Data are presented as mean ± SD from all groups with six mice per group. **(A)** Lesion size measured periodically for a total period of ∼10 weeks post challenge. **(B)** Parasite loads estimated by limiting dilution assay for the parasites in the tissue homogenates from the challenge sites at the end point of the experiment, day 69. (*La*-ALA) vs. (*La*+ALA+Light), $p < 0.017$. (*La*+ALA+Light) vs. (*La*-ALA+AlPhCl+Light), $p < 0.002$.

cell motility and viability based on microscopic observations and MTT reduction assay, respectively (**Figure 1**). PC-sensitization and photo-inactivation of *L. amazonensis* also reduced its uptake by primary macrophages (**Figure 2**). As shown here, amino-PC-sensitization of *L. amazonensis* is stochastic, leaving a small number of parasites un-sensitized, hence escaping from the fate of photo-inactivation. Stochasticity of single-PS sensitization has been reported previously, including the use of other PC (Dutta et al., 2005, 2008) and ALA induction of cytosolic URO accumulation (Sah et al., 2002; Dutta et al., 2008). This is the case despite amino-PC being much more potent than other PS to mediate *Leishmania* photo-inactivation. The development of lesion, albeit with a delayed onset and significantly reduced disease burden (**Figure 3**) is thus not unexpected after inoculation of BALB/c mice with amino-PC-sensitized and photo-inactivated *L. amazonensis*.

The potential use of single PS-sensitized and photo-inactivated *Leishmania* for PDV raises concern in considering their safety, but not efficacy. The safety is clearly a concern when lesions emerge after inoculation of these inactivated *Leishmania* into mice (**Figure 3**). The reduction of their uptake by primary macrophages *in vitro* (**Figure 2**) also may be taken to indicate diminished "vaccine" loading of the APC when applied *in vivo*. However, after such single-PS photo-inactivation, *Leishmania* transgenically modified to express OVA was shown to effectively deliver this T-cell antigen to macrophages and dendritic cells (DC) for processing and presentation to OVA-epitope specific $CD8^+$ T cells *in vitro* (Dutta et al., 2011, 2012). Interestingly, immunization of Syrian Golden hamsters with single PS-sensitized and photo-inactivated *L. amazonensis in vivo* was shown to protect them against challenge infection with *L. donovani*: the splenic parasite load was drastically reduced by 99%, concomitant with significant increase in the expression of iNOS, IFN-γ, and IL-12 (Kumari et al., 2009). Significantly, this immunity is adaptively transferable with T cells from immunized hamsters to naïve animals, indicating that it requires no stimulation by persisting parasites, as they are unlikely to exist in the T-cell recipients. In contrast, persistence of parasites in small number cannot be ruled out in the hamsters after primary immunization with *Leishmania* subjected to single PS-sensitization/photo-inactivation, which invariably leaves behind few survivors, regardless of the PS used. Thus, while the use of incompletely photo-inactivated *Leishmania* is unacceptable for safety consideration, the results obtained point to the efficacy of PDV as a right path to vaccination.

In keeping with the synergism of two different photosensitizers in combination to enhance PDT efficacy against cancer (Schneider-Yin et al., 2009; Villanueva et al., 2010; Gyenge et al., 2013; Acedo et al., 2014), we previously showed that *L. amazonensis* doubly sensitized with URO+PC *in vitro* was fully susceptible to *in vivo* photo-inactivation in BALB/c X C57BL/6 mice, producing no lesion and no detectable parasites 8 weeks after inoculation with 10^6 cells per site into their ear dermis (Dutta et al., 2012). Here, we were able to duplicate this finding in BALB/c mice and found it specific to the ear dermis, but not footpad or tail base. Translucency of ear due to its thinness may facilitate the efficiency of *in situ* illumination with white light, especially the short wavelength of the spectrum, i.e., ∼400 nm optimal for excitation of URO. Ear dermis was thus chosen as the site for PDV against challenge infection in the tail base. This choice is based not solely on the consideration of safety, but on that of efficacy. *Leishmania* subjected to double PS-sensitization alone without photo-inactivation *in vitro* was found to persist in comparable abundance in all three cutaneous sites (ear dermis, footpad, and tail base), but only those in the ear dermis were photo-inactivated beyond detection by *in situ* illumination (**Figure 4**). Thus, at this site there is an optimal amount of "vaccines" made available to APC via photo-inactivation of *Leishmania* therein and, more importantly, little or no immunosuppression by live parasites expected due to their virtual absence. By both accounts, PDV appears to create a microenvironment more favorably to elicit immunity in the ear dermis than tail base and foot pad. This immunity produced by PDV of the ear dermis is manifested against challenge infection by significantly delaying the onset of lesion development and by the marked reduction of parasite load seen in the challenging site at the end point of day 69 (**Figure 5**). This level of protection is significant, considering that most mouse lineages, including C3H, C57BL/6, BDA, and CBA all fail to heal or control *L. amazonensis* infection (Soong et al., 1997; Cortes et al., 2010), to which a susceptible phenotype of mixed Th1–Th2 response is often developed (Afonso and Scott, 1993; Mcmahon-Pratt and Alexander, 2004). BALB/c mice are especially susceptible to the infection by this species, presenting progressive development of non-healing necrotic lesion (Pereira and Alves, 2008). More complete protection by PDV is expected by optimizing the experimental conditions, e.g., reduction of the parasite dose for challenge infection, increasing the frequency of immunization more than once and/or adjustment of the time intervals between inoculation of doubly PS-sensitized *Leishmania* and *in situ* photo-inactivation. Optimization of this interval is expected to maximize photo-inactivation of doubly PS-sensitized *Leishmania*, thereby minimizing their migration from ear dermis to the draining lymph nodes. Minimization of this migration reduces the parasite population that escapes photo-inactivation by spot-illumination of the inoculation site, thereby reducing their immunosuppressive activities in the lymph nodes and facilitating immune clearance. The importance of immune clearance has been discussed previously and cannot be over-emphasized, considering its apparent necessity for post chemotherapeutic cure of leishmaniasis, since no drug is expected to reach every parasite in the patients, regardless of dosages used and treatment duration (Chang, 2014).

The current working hypothesis is that macrophages and DC take up *in vitro* doubly PS-sensitized *Leishmania* for subsequent cytolysis to release antigens therein after *in vivo* photo-inactivation, while the host APC remains viable and functional (Dutta et al., 2012). Therefore, antigen presentation and ensuing cellular-based immune response can be induced effectively, in keeping with the development of life long immunity known to occur after spontaneous or chemotherapeutic cure of human leishmaniasis. In summary, results presented demonstrate that PS-sensitization and photo-inactivation of *L. amazonensis*

based on a combination of endogenous and exogenous strategies renders them non-viable, but immunologically competent against CL. The results presented remain to be a proof-of-principle, pending further investigation to reduce the complexity of PS-sensitization and photo-inactivation. Work is underway toward the simplification of these steps to facilitate the standardization and scale-up production of such photodynamically inactivated vaccines.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SV, CdO, and KC. Performed the experiments: SV, FC, and LR. Analyzed the data: SV, FC, BK, CdO, and KC. Contributed reagents/materials/analysis tools: DN, BK, and KC. Wrote the manuscript: SV, BK, KC, and CdO.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00165/full#supplementary-material

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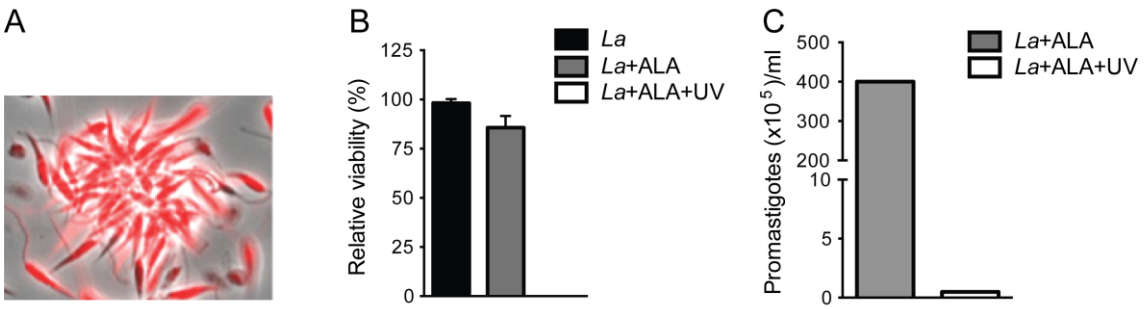
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

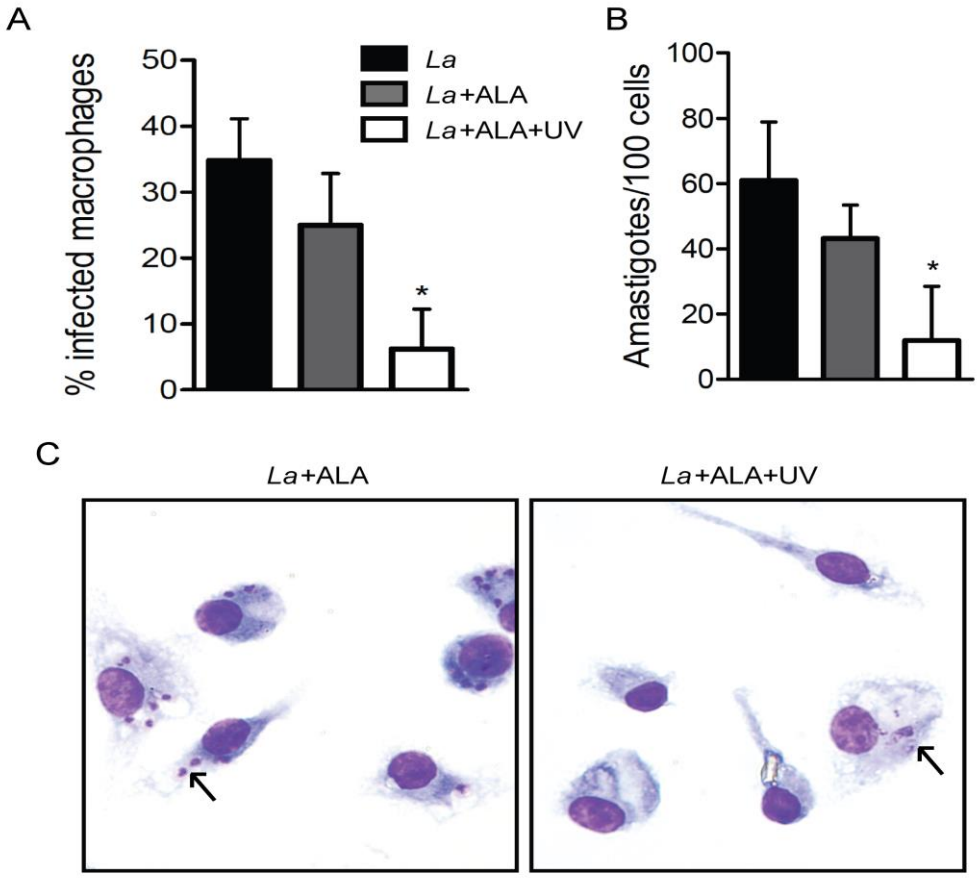
Photodynamic vaccination of BALB/c mice for prophylaxis of cutaneous leishmaniasis caused by *Leishmania amazonensis*

Sayonara M. Viana[1], Fabiana S. Celes[1], Laura Ramirez[1], Bala Kolli[2], Dennis Ng[3], Kwang Poo Chang[2,*], Camila I. de Oliveira[1,4*]

Supplemental Material



Supplemental Figure 1 – Viability of transgenic *L. amazonensis* induced with ALA for uroporphyrin accumulation followed by light exposure. *L. amazonensis* (**La**) genetically complemented to express ALAD and PBGD were incubated in the dark with ALA (1 mM) (+**ALA**) for 24 h and exposed to longwave UV light for 20 min (+**UV**). **[A]** A merged image captured first under phase contrast and then under porphyrin filter for fluorescence, showing cytosolic URO accumulation throughout the cells. **[B]** MTT reduction activities of untreated cells (**black bar**), those treated with ALA alone (**gray bar**) and in combination with UV light (**blank**). **[C]** Disparity between *La*+ALA and *La*+ALA+UV in cell density after incubation in culture medium for 7 days. Data are presented as mean ± S.D. from a representative experiment set performed in quadruplicate.



Supplemental Figure 2 – **Reduced uptake of uroporphyric and photo-inactivated *L. amazonensis* by primary macrophages.** Bone marrow-derived macrophages were infected at a host to parasite ratio of 1:10 for 4h with untreated *L. amazonensis* (**black bar**), those ALA-sensitized alone (**gray bar**) and in combination with exposure to UV light (**blank bar**). Cells were processed for microscopy to determine: **[A]** the percentage of infected macrophages; and **[B]** the number of *Leishmania* per 100 macrophages. **[C]** Representative photomicrographs of cultures shown in A and B. Data are presented as mean ± S.D from a representative experiment set performed in quadruplicate (Kruskal-Wallis test, *$p<0.05$). Arrow, Endocytosed *Leishmania*.

5. MANUSCRITO II

Título: Photodynamic inactivation of *Leishmania braziliensis* doubly sensitized with porphyrin and diamino-phthalocyanine for loading primary macrophages to activate vaccination-favorable effector functions in vitro

Fotossensibilizadores, como as porfirinas, são excitáveis pela luz e geram oxigênio singleto citotóxico entre outras espécies reativas de oxigênio na presença de O_2 atmosférico. A fotoinativação de *Leishmania* por esse meio torna os parasitas inviáveis, preservando sua antigenicidade. Neste trabalho geramos *Leishmania braziliensis* transgênica capaz de acumular porfirinas citosólicas a partir da exposição ao ácido delta-aminolevulínico (ALA). Inicialmente, a *L. braziliensis* foi transfectada com os genes que codificam porfobilinogênio desaminase (PBGD) e aminolevulinato desidratase (ALAD) e a expressão destas enzimas foi confirmada de maneira funcional por meio do acúmulo de porfirinas no citoplasma após exposição ao ácido delta-aminolevulínico (ALA). A análise de citometria de fluxo mostrou uma porcentagem variável de células positivas para porfirinas entre os diferentes clones gerados, enquanto os ensaios de redução de MTT confirmaram a viabilidade diminuída de *L. braziliensis* geneticamente complementada após sensibilização e fotoinativação com luz branca. Após a fotoinativação, a *L. braziliensis* foi fagocitada em taxa semelhante àquela observada com parasitos não fotoinativados, mas a capacidade de se replicar dentro de macrófagos murinos derivados da medula óssea foi prejudicada. A fagocitose de parasitas duplamente sensibilizados com porfirinas e amino-ftalocianina (PC2) e, posteriormente, fotoinativados aumentou a produção de superóxido, óxido nítrico, TNF e IL-6 e a expressão de moléculas co-estimulatórias, comparado à infecção com parasitas controle. Em conjunto, estes dados sugerem que a infecção por *L. braziliensis* sensibilizada com porfirinas endógenas e com PC2 induz a ativação de macrófagos e representa uma ferramenta potencial para a vacinação contra a leishmaniose cutânea.

Photodynamic inactivation of *Leishmania braziliensis* doubly sensitized with porphyrin and diamino-phthalocyanine for loading primary macrophages to activate vaccination-favorable effector functions *in vitro*

Rohit Sharma[1], Sayonara M. Viana[1], Dennis K. P. Ng[2], Bala Kolli[3], Kwang Poo Chang[3], Camila I. de Oliveira[1,4]*

[1]Instituto Gonçalo Moniz, FIOCRUZ, Salvador, BA, Brazil; [2]Chinese University of Hong Kong, Sha Tin, Hong Kong; [3]Rosalind Franklin University of Medicine and Science, Chicago, USA; [4]INCT - Instituto de Investigação em Imunologia

Abstract

Photosensitizers, like porphyrins are excitable by light to generate cytotoxic singlet oxygen and other reactive oxygen species in the presence of atmospheric O_2. Photodynamic inactivation of *Leishmania* by this means renders parasites non-viable while preserving their antigenicity. Here, we have generated transgenic *Leishmania braziliensis* for photo-inactivation with delta-aminolevulinate (ALA) to accumulate cytosolic porphyrins. *L. braziliensis* was transfected and expression of the two enzymes [porphobilinogen deaminase (PBGD) and aminolevulinate dehydratase (ALAD)] was functionally confirmed by accumulation of porphyrins in the cytoplasm upon exposure to delta-aminolevulinate (ALA). Flow cytometry analysis showed a variable percentage of porphyrin-fluorescent cells among the different *L. braziliensis* clones generated whereas MTT reduction assays confirmed diminished viability of genetically complemented *L. braziliensis* following porphyrin sensitization and photo-inactivation with white light. Porphyrin-sensitized and photo-inactivated *L. braziliensis* showed phagocytosis rate close to non-photoinactivated parasites, but impaired ability to replicate inside bone-marrow derived murine macrophages. Uptake of doubly sensitized with porphyrin and amino-phthalocyanine (PC2) and photoinactivated parasites increased macrophage production of superoxide, nitric oxide, TNF and IL-6 as well as expression of co-stimulatory markers. Taken together, data suggests that the infection with porphyrinogenic and PC2-sensitized *L. braziliensis* induces macrophage activation and represents a potential tool for vaccination against cutaneous leishmaniasis.

Introduction

Leishmaniasis are neglected tropical diseases (NTDs) caused by protozoan pathogen of *Leishmania spp*. Human leishmaniasis alone has a prevalence of 14 million cases and an estimated 350 million people are at risk of infection worldwide. Nearly 1.3 million new cases and 20,000-30,000 deaths occur annually [1], second only to malaria [2]. In Brazil, *Leishmania braziliensis* is the leading cause of cutaneous leishmaniasis (CL), a disease characterized by the development of skin ulcers. Infection by *L. braziliensis* may metastasize into mucosal sites, originating mucocutaneous leishmaniasis (MCL), a debilitating form of leishmaniasis associated with extensive tissue destruction [3].

The control of *Leishmania* infection relies mainly on chemotherapy with pentavalent antimonials, which have toxic side effects [4] or prove ineffective due to emergence of resistant strains [5]. Thus, the development of an effective vaccine remains a crucial area of research. Individuals infected once with *Leishmania* become resistant to re-infection after recovery, driving efforts to develop prophylactic vaccines. Indeed, there are numerous attempts at developing a successful vaccine against leishmaniasis including DNA vaccines, killed parasite vaccines and recombinant protein vaccines (rev. in [6]). Vaccination with live attenuated parasites is an appealing approach as it closely mimics natural infection and may lead to the development of similarly effective immune responses, without the inherent danger associated with live virulent or disease-causing infection. *Leishmania* attenuation has been accomplished by long-term *in vitro* culture known to select for avirulence [7] and genetic modifications [8-10] or gamma irradiation [11] for producing live, but non-replicative *Leishmania*. Although live attenuated vaccines have shown substantial potential to protect animal models against challenges, their potential reversion to virulence is a concern, precluding human application.

We have been exploring Photodynamic therapy or treatment (PDT) of *Leishmania* as a strategy of their inactivation for use as whole-cell vaccines or vaccine carriers [12,13]. Photodynamic therapy (PDT) involves the use of photosensitizers that are photoactivable with light of specific wavelength to produce reactive oxygen species (ROS). We genetically complemented *L. amazonensis* parasites for the expression of delta-aminolevulinate dehydratase (ALAD) and porphobilinogen deaminase (PBGD). Exposure of these genetically modified parasites to δ-aminolevulinic acid (ALA) leads to their cytosolic accumulation of uroporphyrin I (URO1) - a PS excitable with light of 405 nm in wavelength to produce cytotoxic singlet oxygen and other ROS for their inactivation [14,15]. Moreover, repeated cycles of ALA-treatment selected no PDT-resistant mutants [13,16,17]. These and other properties of such PDT

strongly argue in favor of its use to generate non-viable parasites for vaccination purposes. Vaccination of hamsters with porphyrinogenic *L. amazonensis* followed by ALA treatment and light exposure *in vivo* conferred protection against the challenge with virulent *L. donovani* and the immunity is adaptively transferrable from immunized hamsters to naïve animals [18]. Combinational approaches using both exogenously supplemented PS and endogenous accumulation of URO1 have shown synergistic enhancement in the photo-inactivation and photolysis of *L. amazonensis* [19]. More importantly, immunization with doubly sensitized *L. amazonensis* confers protection of BALB/c mice against a homologous challenge, as seen by a significant reduction in parasites burden as well as a significant delay in lesion development [20].

In this study, we generated porphyrinogenic mutant clones of *L. braziliensis*, rendering them similarly ALA-inducible to accumulate porphyrins for UVA-inactivation. Uptake of such photo-inactivated parasites by bone marrow derived macrophages increased their oxidative burst, immunity-enhancing cytokine production and expression of co-stimulatory markers, especially after additional amino-phthalocyanine (PC2)-mediated photo-inactivation.

Methods

Ethics statements

Female BALB/c mice, 6–8 weeks of age, were obtained from IGM/FIOCRUZ animal facility where they were maintained under pathogen-free conditions. All animal work was conducted according to the Guidelines for Animal Experimentation of the Colégio Brasileiro de Experimentação Animal and of the Conselho Nacional de Controle de Experimentação Animal. The local Ethics Committee on Animal Care and Utilization (CEUA) approved all procedures involving animals (CEUA-003/2014-IGM/FIOCRUZ).

Cells

Leishmania braziliensis promastigotes (MHOM/BR/00/BA788) [21] were maintained in Schneider's insect medium (SIGMA) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen) or in Medium 199 (SIGMA) supplemented with 20% heat-inactivated fetal calf serum (FCS), Hepes (40mM, pH 7.4**),** Adenine (0.1mM), Hemin (5 µg/ml), Biotin (1µg/ml) and antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL) (all from Invitrogen). Porphyrinogenic *L. amazonensis* were grown under similar conditions with 20 ug/ml tunicamycin and 100 ug/ml G418 {Sah, 2002 #2459}.

Primary macrophages were obtained from mouse bone marrow as previously described [22]. Cells were resuspended and maintained in RPMI 1640 medium (SIGMA) supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin and 10% FBS.

Construction of recombinant expression vectors

pX-NEO-*alad* [14] and pXG-HYG-*pbgd* were used or constructed for transfection of *L. braziliensis* (MHOM/BR/00/BA788) to express mammalian ALAD and PBGD. For the construction of pXG-HYG-*pbgd*, p6.5-*pbgd* [14] was *BamHI*-digested to remove the entire *pbgd* coding sequence, which was gel-purified and subcloned at the *BamHI* sites of the pXG-HYG expression vector. The correct plasmid orientation was verified by restriction mapping and used to transform *E. coli* (DH5α).

Transfection and selection of genetically complemented *L. braziliensis*

Mid-log phase promastigotes were harvested by centrifugation, resuspended in Tb-BSF buffer [23] at a density of 10^8 cells/ml. A mixture of 0.4 ml of the cell suspension and pX-NEO-*alad* (5 μg) was placed in a pre-chilled 2mm electroporation cuvette (SIGMA) for electroporation using a Bio-Rad Gene Pulser [24]. Electroporated cells were kept in an ice-bath for exactly 10 minutes and diluted with 10 ml culture medium for shock recovery at 26°C for 24 h. An ALAD-positive clone of the transfectants was subjected to a second round of transfection with pXG-HYG-*pbgd* as described above. Doubly transfected cells were drug-selected and cloned by limiting dilution with culture medium supplemented with G418 (50 μg/ml) and/or HYG (50 μg/ml) in 96-well tissue culture plates. The cultures monitored for 2-4 weeks. Emerging parasites in the wells of the highest dilutions were taken for further expansion in selective medium with increasing drug pressures to G418 (100 ug/mL) and Hygromycin (HYG) (100 μg/ml).

ALA-induced uroporphyrinogenesis of genetically complemented *L. braziliensis* clones

Transfectant clones were grown in Schneider's medium plus G418 (100 ug/mL) and (HYG) (100 μg/ml) (SIGMA). Cells of 12 independent clones were harvested, washed and resuspended to $5x10^7$ cells/ml for exposure in the dark for 48h at 26° C to 1.6 mM delta-aminolevulinate (ALA) (SIGMA) in RPMI 1640 medium (Gibco)/0.01% bovine serum albumin (RPMI-BSA), pH 7.4. ALA-exposed cells were washed and examined by phase

contrast and fluorescence microscopy for integrity and porphyrin fluorescence using an Olympus FluoView confocal microscope equipped with a krypton/argon-mixed gas laser. Specimens were illuminated with the 488 nm excitation line. The specific fluorescent emission of the porphyrin was collected by a photomultiplier tube after passing through a 605 nm bandpass emission filter. Parental wild-type *L. braziliensis* was used as negative control and *L. amazonensis* porphyrinogenic mutants simultaneously and similarly exposed to ALA were used as a positive control. All cells of the 12 clones so processed were assessed for their porphyrinogenesis by flow cytometry (BD-LSR Fortessa) at an excitation wavelength of 405 nm and emission wavelength of 610 nm. Data from 30,000 events were acquired to determine the % of Porphyrin-positive population for each clone. The specificity of fluorescence settings was defined by using non-fluorescent negative controls (wild-type parental *L. braziliensis*) and fluorescent positive uroporphyric cells. Data were analyzed using FlowJo software (Tree Star, Version 10.2).

Photo-inactivation of porphyric cells by UVA exposure and their viability assessed by MTT reduction assay

Porphyric cell populations of the 12 clones obtained after ALA treatments as described above were each suspended to $5x10^7$ cells/ml in RPMI-BSA and placed in 500 µl/well in 24-well culture plates in two sets. One set was unlidded and exposed to UVA (longwave UV, λ_{max}=365 nm) from the top for 30 min. The 2nd set was kept covered in the dark. Cell viability of all samples was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (SIGMA) according to the manufacturer's protocol. Wild-type parental *L. braziliensis* was included as a control.

***In vitro* uptake of ALA-sensitized and photo-inactivated *L. braziliensis* by bone marrow-derived macrophages (BMDM)**

The macrophages suspended in culture medium were seeded onto glass coverslips placed in 24-well plates at $3x10^5$ cells/500 µl RPMI 1640 /coverslip. Monolayers formed on the coverslips were washed to remove non-adherent cells. Each received experimental or control *Leishmania* at $3x10^6$/500µl RPMI 1640 containing 10% FBS (10:1 parasite/host cell ratio). The plates were covered and incubated at 35 °C, 5% CO_2. After incubation for 6 and 24 hours, coverslips with monolayers were extensively washed to remove non-internalized *Leishmania*, methanol-fixed and stained with hematoxylin and eosin. The extent of uptake was

microscopically determined in quintuplicate samples by scanning 200 macrophages in each for enumeration of cells with and without *Leishmania*, and the total number of intracellular *Leishmania*.

Exogenous sensitization *L. braziliensis* with diamino-phthalocyanine (PC2) for their photo-inactivation with red light

L. braziliensis and its porphyrinogenic clones were sensitized with PC2 under the conditions, as previously described [16]. Promastigotes were grown to late-log phase, washed and resuspended at $5x10^7$ cells/ml in RPMI-BSA in presence of PC2 at graded concentrations of 0.001 to 5 μM. Cells exposed to the concentration of DMSO equivalent to that of the highest PC2 concentration were used to serve as the solvent control. After overnight incubation at 26°C in the dark, PC2-sensitized and unsensitized control cells were washed and resuspended to its original cell density for exposure to red light (RL) from the bottom of the culture plates for 60 min until the cessation of their flagellar motility (1-2 J/cm^2) [17]. The viability of all cell samples was assessed by MTT reduction assay, as described in the preceding section.

Double photo-sensitization and -inactivation of porphyrinogenic *L. braziliensis*

L. braziliensis promastigotes of the porphyrinogenic Clone #4 were subjected to double photo-sensitization and -inactivation at $5x10^7$/ml in RPMI-BSA, pH 7.4. They were first cytosolically sensitized with porphyrins by ALA exposure and then endosomally PC2 (1 uM)-sensitized, as described above. These doubly sensitized cells were washed and resuspended in RPMI-BSA to their original cell density for photo-inactivation under above-described conditions, i.e. exposure first with lid off to UVA (λmax=365 nm) from the top for 30 min and then to red light (λmax=~600 nm) from the bottom for 60 min until flagellar immobilization (1-2 J/cm^2).

Effector responses of bone marrow-derived macrophages (BMDM) to doubly photo -sensitized and -inactivated *L. braziliensis*

Bone marrow derived macrophages obtained were seeded in 24-well culture plates at a density of 10^6 cells per well. The seeded macrophages were incubated with URO1-PC2 double sensitized *L. braziliensis* using two different strategies. In Strategy #1, seeded macrophages were incubated (for 6 and 24h) with doubly-sensitized *L. braziliensis* illuminated with UVA and red light, as described above (**Mac+ [*Lb*+2x PS+L]**). In Strategy #2, seeded macrophages

were first incubated with the doubly-sensitized *L. braziliensis* for 6h and then infected macrophages were exposed to longwave UV and red light. After illumination, cells were cultured for another 18h **([Mac+*Lb*+2x PS] +L)**.

Quantification of nitric oxide, superoxide and cytokines in culture supernatants of Lb-exposed BMDM

The culture supernatants were collected for these assays from *Lb*-exposed BMDM according to Strategy #1 and Strategy #2. Prior to sample collections, BMDM were further incubated for 24h with 0.5 mM hydroxylamine hydrochloride (Acros Organics) and primed with LPS (40 ng/mL) and IFN-γ (10 ng/mL) (all from Invivogen) for 24h before use for determining superoxide and NO, respectively. NO and Superoxide in the culture supernatants were quantified using Griess reagent [25]. Cytokine levels in the culture supernatants were determined by ELISA following manufacturer's instructions (eBioscience).

Flow cytometric analysis of BMDM for MHC II and co-stimulating molecules after exposure to double PS-sensitized and photo-inactivated *L. braziliensis*

BMDM *Lb*-exposed according to both strategies for 24h were stained with fluorochrome-labeled antibodies for CD40, CD86 and MHC II. FITC, PE and PerCP isotype controls were included in all experiments. Initially, cells were blocked at 4 °C with rat anti-mouse CD16/32 (5 mg/ml; BD Pharmingen) for 10 min and then stained with anti-mouse antibodies for 30 min (1:200 dilution each molecule) at 4 °C. The cells were then stained with Fixable Viability Dye (eBioscience/ThermoFisher), which stain dead cells. Cells were fixed in PBS with 2% paraformaldehyde for 10 min and finally were washed with wash buffer and kept at 4 °C in the dark until acquisition. Data were acquired in a Fortessa flow cytometer (BD Biosciences, USA), for analysis by using FlowJo software (Tree Star, Version 10.2).

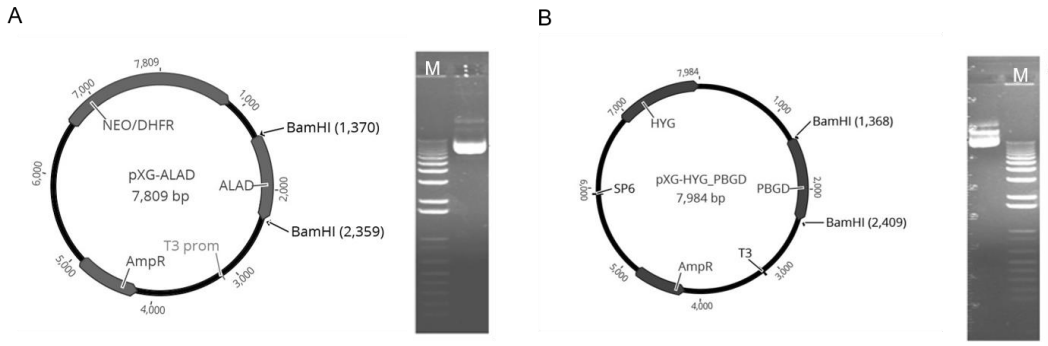
Statistical analysis

Comparisons between two groups were performed by using Mann-Whitney (non-parametric t-test) and among more than two groups by using Kruskal Wallis. Analyses were conducted using Prism (GraphPad, V 5.0) and a p value ≤ 0.05 was considered significant. Data are presented as mean± standard deviation.

Results

Genetic complementation of BA788 L. braziliensis with plasmids to express ALAD and PBGD

Promastigotes of *L. braziliensis* were subjected to two consecutive rounds of electroporation to obtain double transfectants expressing both ALAD and PBGD. The first round used pX-NEO-ALAD (Supplemental Figure 1A) yielding two independent single transfectants, both being grown stably after selection for G418-resistance. The single-transfectants were subjected to a second round of electroporation with the newly constructed pXG-HYG-PBGD (7.9 kb) (Supplemental Figure 1B), thereby producing *L. braziliensis* double transfectants on hygromycin selection to express both ALAD and PBGD. The double transfectants were cloned by the serial dilution method in 96 well plates giving 12 independent clones, all passageable repeatedly with stable growth under the selectable drug pressures as mentioned above.



Supplemental Figure 1. Plasmid maps of PBGD and ALAD expression constructs used for genetic complementation of *L. braziliensis*. pX-NEO-alad [A] and pXG-HGY-pbgd [B], ~ 8 kb each, were constructed for sequential transfection of promastigotes to express ALAD and PBDG. Both migrated in 0.8% agarose gel as circular DNA expected. Lane M, 1-kb DNA marker.

Delta-aminolevulinate (ALA)-induction of the double transfectants for neogenesis of cytosolic porphyrin, indicative of ALAD and PBGD co-expression in all clones.

All 12 independently obtained clones of the double transfectants were responsive to ALA without exception, producing intense porphyrin-specific fluorescence when excited with UVA, but not visible with white light (Fig.1A left panel vs right panel). Notably, all ALA-exposed clones remained intact and motile, like untreated controls when kept in the dark. Co-

migration of the fluorescence with sedimented cells after centrifugation is indicative of its cell-association, just like the similarly treated double transfectants of *L. amazonensis* (Figure 1A, 1-12 vs C2). Confocal microscopy of these live promastigotes showed porphyrin fluorescence throughout the cells including flagella, indicative of its cytosolic localization, (Figure 1B), consistent with previously reported findings in such genetically complemented *Leishmania spp.* [14,26]. The fluorescence observed is due to the accumulation of intermediate porphyrins and possibly uroporphyrin I (URO1) as the product, since wild type *Leishmania* have no URO1-utilizing enzyme immediately downstream of PBGD. Indeed, at least ALAD and PBGD are absent in the wildtype *L. braziliensis*, as indicated by the absence of any porphyrin fluorescence after their exposure to ALA and UVA light (Fig. 1A, C1, right panel).

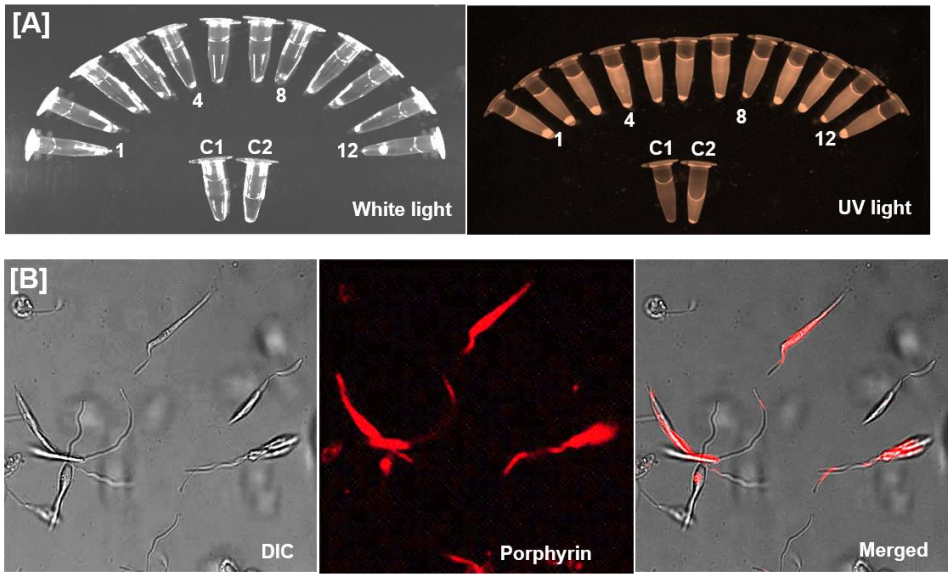


Figure 1. Aminolevulinate (ALA)-mediated uroporphyrinogenesis of genetically complemented *L. braziliensis* clones. Twelve clones of *L. braziliensis* promastigotes transfected to express PBGD and ALAD were exposed in the dark to ALA (1 mM) for 48 h. [A] Cell samples were centrifuged, showing co-sedimentation of porphyrin fluorescence with cell pellets by illumination with UVA (right panel), but not white light (left panel). Wild type *L. braziliensis* (C1) and porphyrinogenic *L. amazonensis* (C2) were similarly processed as negative control and positive control, respectively. [B] Microscopic images of porphyric *L. braziliensis*, showing cytoplasmic porphyrin accumulation. DIC, Differential interference; Porphyrin, Filter set for URO1 fluorescence.

Clonal variations in porphyrinogenesis was noted among the 12 clones, as shown by flow cytometric analysis for URO1 fluorescence in a time course study from 24 to 72h after ALA-exposure. Judging from the percentage of porphyric cell populations and their mean fluorescence intensity (MFI), porphyrinogenesis of all clones was detectable at a low range at

24h (Figure 2A) and peaked 48 hours after ALA-exposure (Figure 2B). After further incubation to 72h, the percentage of porphyric cells remained similar, while porphyrin MFI decreased sharply in all 12 clones (Figure 2C, right panel). This is consistent with the release of URO1 from the porphyric cells, as noted before [14] and from the fluorescence in the supernatants (Figure 1A, right panel). Clones 2 and 8-10 were substantially lower than the rest in the peak levels of porphyrin. According to this kinetics analysis, clones # 4-6 and 11-12 are clearly more porphyrinogenic than the others and clone #4 was chosen for further phenotypic analysis.

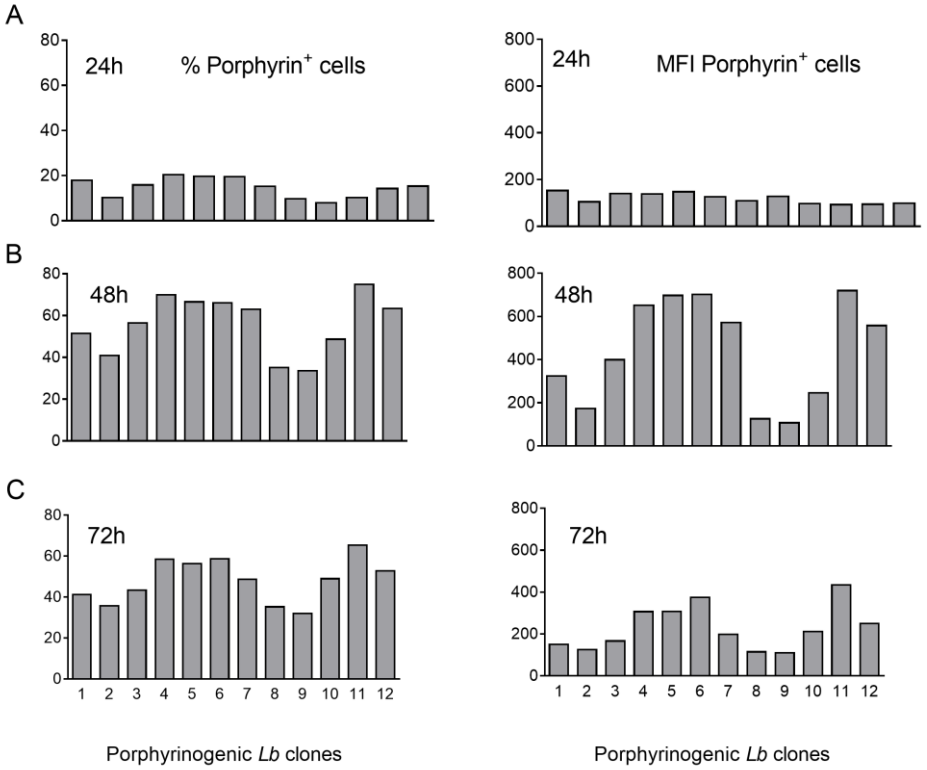


Figure 2. Porphyrinogenesis in transgenic *L. braziliensis* clones. Twelve independent *L. braziliensis* clones expressing PBGD and ALAD were exposed to 1.6 mM ALA and the resulting uroporphyrin (URO1) accumulation was evaluated by flow cytometry. Individual bars show clonal variations in % porphyric cells (left panel) and fluorescent intensity of porphyrin (right panel) during ALA-induced porphyrinogenesis for 24h [A], 48h [B] and 72h [C].

Porphyrinogenesis of all 12 clones renders them sensitive to inactivation with UVA

By MTT reduction assays, we observed a significant decrease in the viability of all twelve *L. braziliensis* clones of porphyrinogenic cells after UVA exposure in contrast to their counterpart kept in the dark (Figure 3, white bar vs. black bar). In contrast, the wild-type parental *L. braziliensis* line remained viable, independent of UVA exposure (Figure 3, WT *Lb*

white bar vs. black bar). Clones 4 and 11 were more susceptible to UVA inactivation, consistent with the heightened level of their uroporphyrinogenesis in response to ALA (see Fig. 2).

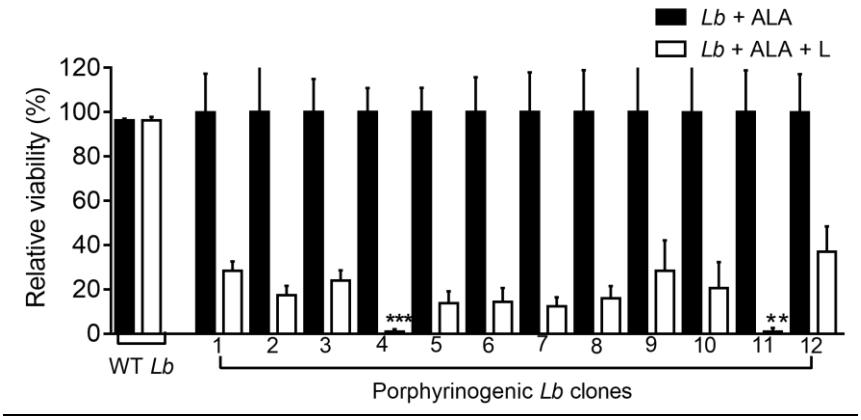


Figure 3. Susceptibility of *L. braziliensis* porphyric clones to photo-inactivation. Cells were first exposed to 1.6 mM ALA in the dark for 48 h. Cells were then split into two sets: one was kept in the dark (Black bar, *Lb*+ALA) and the other was exposed to longwave UV for 30 min (white bar, *Lb*+ALA+L). Cell viability was assessed by MTT reduction assays. Photo-inactivation of UVA-exposed cells for each clone was expressed as % of the corresponding dark control. Negative controls: wild-type *L. braziliensis* (WT *Lb*) similarly exposed to ALA with (white bar) and without (black bar) UVA-exposure. Data are presented as mean ± S.D from a representative experiment performed in quadruplicate. *** $p < 0.001$.

Porphyric and photo-inactivated L. braziliensis remain susceptible to endocytosis by mouse bone marrow macrophages in vitro.

Subsequent experiments were performed with clone #4 because it displayed high porphyrinogenic capacity (Figure 2) as well as a low level of dark toxicity (i.e. loss of viability in the presence of ALA only) (Figure 3). Initially, we determined parasite uptake after 6h of co-incubation with BMDM and under the experimental conditions used, porphyric and photo-inactivated *L. braziliensis* were taken up by BMDM at approximate rates (~40% in Mac+[*Lb*+ALA+L]) compared to parasites kept in the dark (~60% in Mac+[*Lb*+ALA]) (Figure 4A, white vs. black bars). At 24h, however, the number of BMDM containing *Lb* was significantly lower in cultures incubated with porphyric and photo-inactivated *L. braziliensis* (~20% in Mac+[*Lb*+ALA+L]) compared to BMDM cultured with parasites kept in the dark (~50% in Mac+[*Lb*+ALA]) (Figure 4A, white vs. black bars). Comparing the two time-points, the percentage of *Lb*-containing BMDM was significantly lower at 24h (~20% in Mac+[*Lb*+ALA+L]) compared to 6h (~40% in Mac+[*Lb*+ALA+L]) (Figure 4A). Additionally, the average number of internalized parasites was reduced by 2-fold to <200 in

Mac+[*Lb*+ALA+L] from ~400 in Mac+[*Lb*+ALA], at 6h, and by 8-fold to ~50 in Mac+[*Lb*+ALA+L], at 24h (Figure 4B, white vs. black bars). Again, a comparison of the two time points showed that the number of intracellular parasites is significantly lower at 24h when BMDM were cultured with porphyric and photo-inactivated *L. braziliensis*. Representative photomicrographs illustrate a visible reduction in the number of intracellular parasites in Mac+[*Lb*+ALA+L] compared to Mac+[*Lb*+ALA] at 24h (Figure 4C). These data confirm that porphyric and photo-inactivated *L. braziliensis* remain susceptible to internalization by BMDM but become more susceptible to killing.

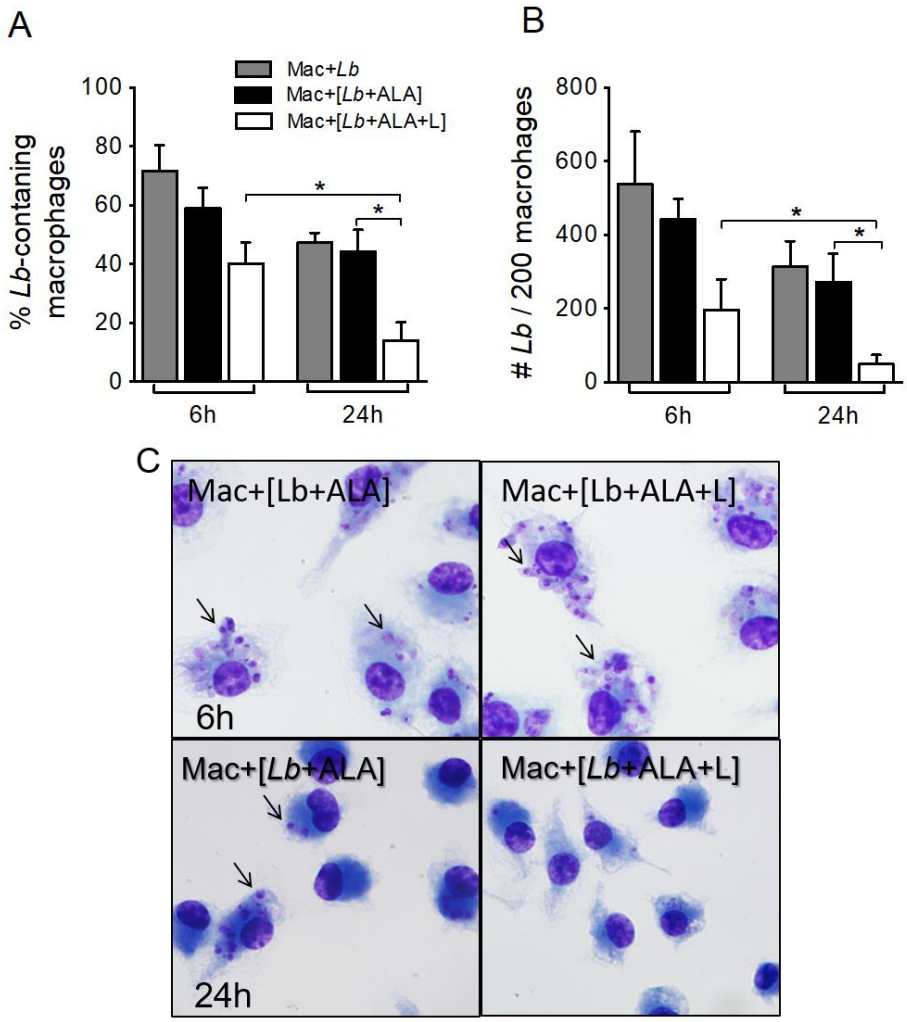


Figure 4. Endocytosis of photo-inactivated porphyric *L. braziliensis* by primary macrophages. Bone marrow-derived macrophages (BMDM) were co-cultured with wildtype *L. braziliensis* (*Lb*) (gray bar), and its uroporphyric transfectants with (white bar) and without (black bar) photo-inactivation by exposure to UVA light (L) for 30 min at a host-parasite ratio of 1:10. Cells were processed at 6 and 24 h for microscopy to assess: [A] the percentage of BMDM containing *L. braziliensis*; and [B] the number of *Leishmania* per 200 macrophages. Data are presented as mean ±. S.D from a representative experiment performed in quadruplicate, *p<0.05. (C) Representative photomicrographs of (A, B).

Survival of L. braziliensis is compromised upon double sensitization and photo-inactivation

We have previously shown that the combination of two sensitizers enhances the efficacy of *L. amazonensis* photo-inactivation leading to inhibition of lesion development *in vivo* [19,20]. We initially established exogenous sensitization of *L. braziliensis* with PC2 [16] as the photosensitizer. By MTT reduction assays, PC2-loaded parasites (0.001-5 µM PC2) were shown to lose their viability only after light exposure in contrast to *L. braziliensis* kept in the dark (Figure 5, white vs. black bars), confirming the susceptibility of *L. braziliensis* to exogenous sensitization and subsequent photo-inactivation.

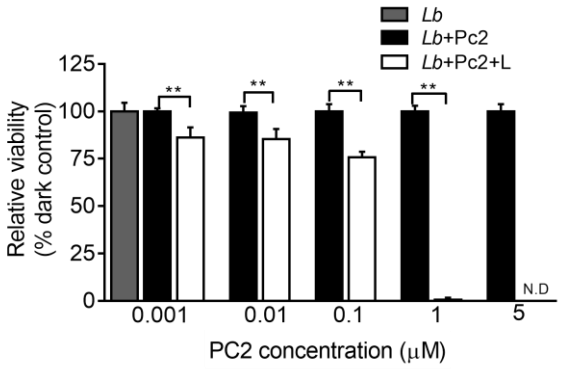
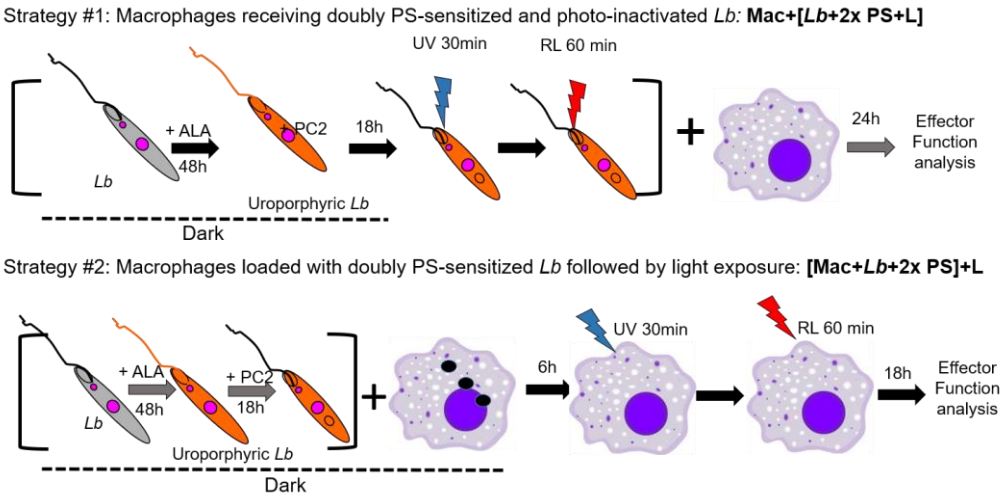


Figure 5. PC2-mediated photo-inactivation of *L. braziliensis*. Promastigotes (*Lb*) were incubated in the dark with PC2 for 18h (dark bar, *Lb*+PC2) and exposed to red-light (L) for 60 min. The viability of WT *L. braziliensis* cells (gray bar), those sensitized with graded concentrations of PC2 in the dark (black bar) and the latter group after light exposure (white bar) was assessed by their MTT reduction activities, the values being expressed in % of dark controls. Data are presented as mean ± S.D from a representative experiment performed in quadruplicate. ** $p<0.01$. *** $p<0.001$.

Following these findings, we thus evaluated the outcome of exposure of double-sensitized and photo-inactivated *L. braziliensis* to BMDM. As indicated above, double sensitization consisted of exposing promastigotes to PC2 (exogenous photosensitizer) plus ALA (generation of URO1, an endogenous photosensitizer). Moreover, this evaluation was performed using two strategies (Supplemental Figure 2): In the first strategy, **Mac+ [*Lb*+2x PS+L],** promastigotes were sensitized endogenously (ALA) and exogenously (PC2) and then illuminated to achieve photo-inactivation. Thus photo-inactivated *L. braziliensis* were cultured with BMDM for effector functions analyses (Supplemental Figure 2, Strategy #1, upper panel). In the second strategy, **[Mac+*Lb*+2x PS] +L,** *L. braziliensis* promastigotes were sensitized with ALA and PC2 and were then co-cultured with BMDM for 6h. After parasite uptake, BMDM were illuminated to achieve parasite photo-inactivation. In this second strategy, parasites were

sensitized at the promastigote stage, but photo-inactivation occurred within the host cell (Supplemental Figure 2, Strategy #2, lower panel).



Supplemental Figure 2. Two strategies for photo-inactivation of ALA/PC2 doubly sensitized-*Leishmania* before and after loading macrophages. Lb, *L. braziliensis* transfected to express PBGD and ALAD; ALA, delta-aminolevulinate for 1st PS-sensitization by inducing cytosolic accumulation of Porphyrin, PC2, diamino-phthalocyanine for 2nd PS-sensitization via its endosomal uptake; UV, Longwave UV (365 nm wavelength) to excite porphyric Lb; and RL, red light (~600 nm wavelength) to excite PC2 in the endosomes of Lb.

Initially, we determined parasite uptake of double-sensitized and photo-inactivated *L. braziliensis* by BMDM. Following strategy #1 (Supplemental Figure 2), *L. braziliensis* promastigotes were ALA and PC2-sensitized and then illuminated. Co-culture of photo-inactivated parasites and BMDM for 24h induced a 10-fold reduction in the percentage of *Lb*-containing macrophages, ~5% in Mac+[*Lb*+ALA+PC2+L] (white bar) from ~50% in Mac+[*Lb*+ALA+PC2] (black bar) (Figure 6A). Similarly, the number of intracellular parasites was also reduced upon co-culture of BMDM with doubly-sensitized and photo-inactivated *L. braziliensis* (Mac+[*Lb*+ALA+PC2+L]) (Figure 6B) (white bar) compared to parasites kept in the dark Mac+[*Lb*+ALA+PC2] (black bar).

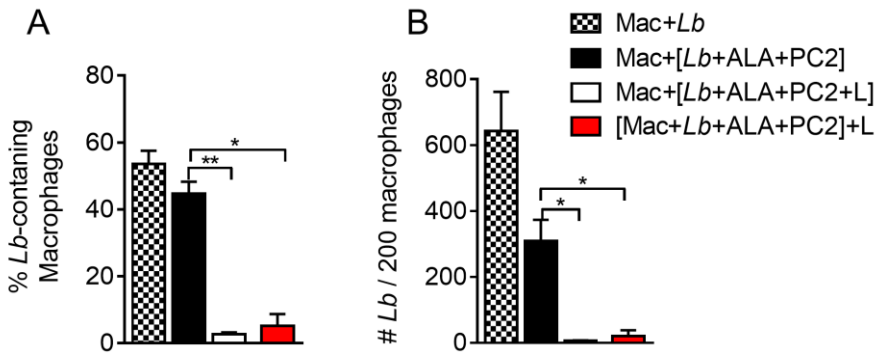
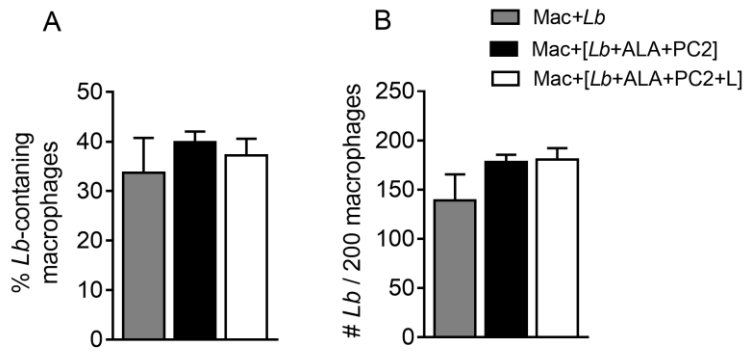


Figure 6. Uptake of doubly-sensitized and photo-inactivated *L. braziliensis* by primary macrophages. Bone marrow-derived macrophages (BMDM) were exposed at a host-parasite ratio of 1:10 to URO1/PC2 doubly photo-sensitized parasites with (white bar, Mac+[Lb+ALA+Pc2+L]) and without (black bar, Mac+[Lb+ALA+PC2]) exposure to UVA/red light. Alternatively, parasites were doubly sensitized and then cultured with macrophages, which were exposed to UVA/red light (red bar, [Mac+Lb+ALA+Pc2] +L). Macrophages infected with untreated *Lb* served as controls (checkered bar, Mac+Lb). Cells were processed after 24h for microscopy to assess: [A] the percentage of Lb-containing macrophages and [B] the number of parasites per 200 macrophages. Data are presented as mean ±. S.D from a representative experiment performed in quadruplicate, *p<0.05; **p<0.01

Equivalent results were obtained when photo-inactivation occurred after parasite internalization by BMDM: both the percentage of *Lb*-containing macrophages (Figure 6A, [Mac+Lb+ALA+PC2]+L, red bar) and the number of intracellular amastigotes (Figure 6B, [Mac+Lb+ALA+PC2]+L, red bar) were significantly reduced compared to parasites kept in the dark (Figure 6A and B, (Mac+[*Lb*+ALA+PC2], black bars). These results confirm that like single sensitization with ALA only (Figure 4) double sensitization with ALA and PC2 does not disturb parasite uptake by BMDM but impairs parasite survival within the host cell, regardless of the stage at which photo-inactivation occurs (before or after parasite internalization).

Importantly, this outcome was unrelated to the ability of BMDM to uptake photo-inactivated *L. braziliensis* since co-cultures performed for 6h showed that the percentage of *Lb*-containing macrophages (Supplemental Figure 3A) and the number of internalized parasites (Supplemental Figure 3B) was similar comparing doubly-sensitized and photo-inactivated *L. braziliensis* (Mac+[*Lb*+ALA+PC2+L], white bars) vs. parasites kept in the dark (Mac+[*Lb*+ALA+PC2], black bars).



Supplemental Figure 3. Uptake of doubly sensitized and photo-inactivated *L. braziliensis* by primary macrophages. Bone marrow-derived macrophages (BMDM) were exposed, at a host-parasite ratio of 1:10 for 6h, to *L. braziliensis* (Lb) (gray bar), URO1/PC2 doubly sensitized transfectants with (white bar, Mac+[Lb+ALA+Pc2+L) and without (black bar, Mac+[Lb+ALA+PC2) exposure to red and UVA light. Cells were processed for microscopy to assess: [A] the percentage of Lb containing macrophages and [B] the number of *Leishmania* per 200 macrophages. Data are presented as mean ±. S.D from a representative experiment performed in quadruplicate.

Culture with double sensitized and photo-inactivated L. braziliensis upregulates BMDM innate responses

Nitric oxide and superoxide are key effector molecules in the microbicidal arsenal of macrophages. Herein, NO production was increased by 3-fold when BMDM were cultured with doubly sensitized *L. braziliensis* and then exposed to light (Figure 7A, [Mac+Lb+ALA+PC2] +L, red bar) compared to parasites left in the dark (Figure 7A, Mac+[*Lb*+ALA+PC2], black bar). Regarding superoxide production, however, significant increases were observed either when photo-inactivation occurred at the promastigote stage (Figure 7A, Mac+[*Lb*+ALA+PC2+L], white bars) or after internalization by BMDM (Figure 7B, [Mac+Lb+ALA+PC2] +L, red bar). These results corroborate the significantly decreased numbers of intracellular parasites observed earlier, again employing either strategy (Figure 6).

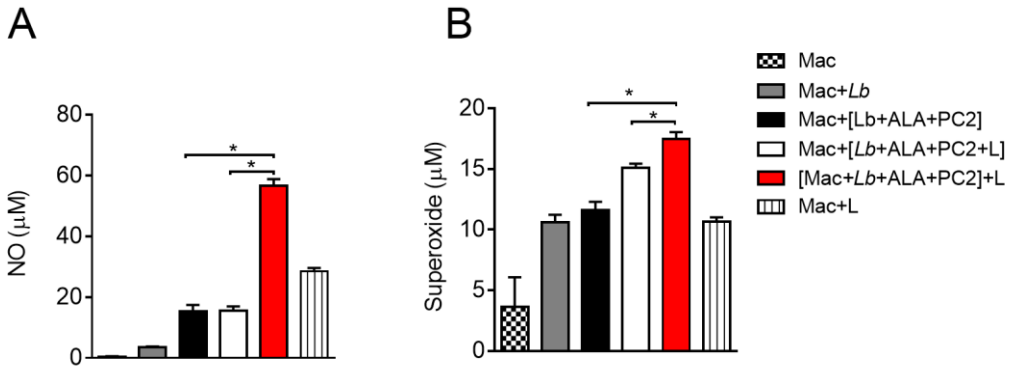


Figure 7. Respiratory burst of macrophages up-regulated after loading with doubly-sensitized and photo-inactivated *L. braziliensis*. Bone marrow-derived macrophages (BMDM) were infected at a host-parasite ratio of 1:10 with URO1/PC2 doubly sensitized parasites with (white bar, Mac+[Lb+ALA+Pc2+L]) and without (black bar, Mac+[Lb+ALA+PC2]) exposure to UVA/red light. Alternatively, parasites were doubly sensitized and delivered to macrophages, which were then exposed to UVA/red light (red bar, [Mac+Lb+ALA+Pc2] +L). Macrophages infected with untreated Lb (checkered bar, Mac+Lb) or exposed to light only (striped bar, Mac+L) served as controls. [A] Nitric oxide and [B] superoxide levels were quantified in culture supernatants 24 hours after priming of BMDM with LPS+IFN-γ. Data are presented as mean ±. S.D from a representative experiment performed in quadruplicate, *p<0.05

Next, we probed for the expression of CD40 and CD86, two co-stimulatory molecules involved in priming cellular immune responses. As before, *L. braziliensis* were sensitized and photo-inactivated prior to co-culture with BMDM or macrophages were co-cultured with sensitized parasites and later illuminated and the percentage of CD40 and CD86 cells was evaluated by flow cytometry. We observed a 2-fold increase in CD40 population upon co-culture of BMDM with doubly sensitized and photoinactivated *L. braziliensis* (~50%, Mac+[*Lb*+ALA+PC2+L], white bars) compared to parasites left in the dark (<20%, Mac+[*Lb*+ALA+PC2], black bar) (Figure 8B). Photo-inactivation of *L. braziliensis* after BMDM uptake induced a similar outcome ([Mac+Lb+ALA+PC2] +L, red bar, Figure 8B). Again, using either photo-inactivation strategy, we also observed a significant increase in the percentage of CD86 population for BMDM co-cultured with photoinactivated parasites (~30%, Mac+[*Lb*+ALA+PC2+L], white bar and [Mac+*Lb*+ALA+PC2+L], red bar compared to parasites left in the dark (~20%, [Mac+Lb+ALA+PC2]+L, black bar) (Figure 8C). These results indicate that BMDM upregulate co-stimulatory properties upon contact with photo-inactivated *L. braziliensis*, suggesting an increased capacity of antigen presentation.

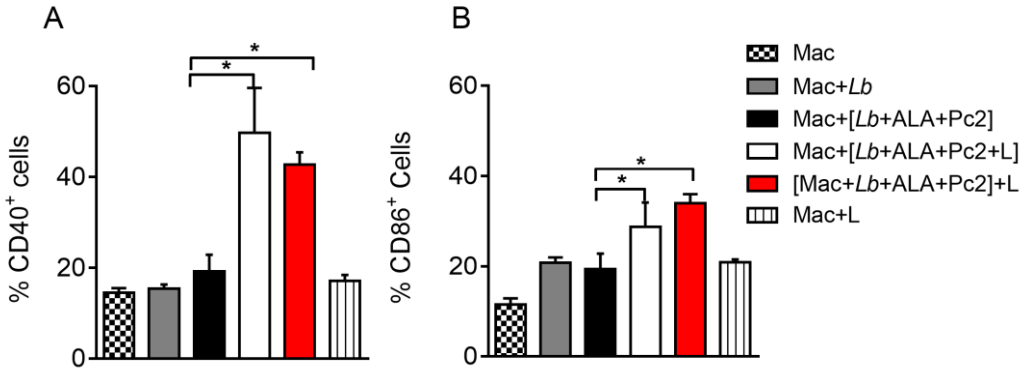


Figure 8. Expression of co-stimulatory molecules up-regulated in primary macrophages after loading with doubly-sensitized and photo-inactivated *L. braziliensis*. Bone marrow-derived macrophages (BMDM) were exposed at a host-parasite ratio of 1:10 to URO1/PC2 doubly sensitized parasites with (**white bar**, **Mac+[*Lb*+ALA+Pc2+L**]) and without (**black bar, Mac+[*Lb*+ALA+PC2])** exposure to red and UVA light. Alternatively, parasites were doubly photo-sensitized and used to infect macrophages followed by exposure to UVA/red light (**red bar, [Mac+*Lb*+ALA+Pc2] +L**). Macrophages infected with untreated *Lb* (**checkered bar, Mac+*Lb***) or exposed to light only (**striped bar**, **Mac+L**) served as controls. Bar graphs showing the percentage of cells expressing **[A]** CD40 and **[B]** CD86, as determined after incubation for 24h by flow cytometry. Data are presented as mean ±. S.D from a representative experiment performed in quadruplicate, *p<0.05.

Lastly, we investigated the cytokine production induced by the two strategies of *L. braziliensis* photo-inactivation. Culture of BMDM with doubly sensitized and photo-inactivated parasites did not alter TNF production (Figure 9A, Mac+[*Lb*+ALA+PC2+L], white bars white bar) but increase IL-6 secretion by ~3-fold (Figure 9B, white bar) while decreasing IL-10 production by 2-fold (Figure 9C, white bar). On the other hand, photo-inactivation of doubly sensitized *L. braziliensis* after uptake by BMDM modulated all three cytokines evaluated (Figure 9, [Mac+Lb+ALA+PC2] +L, red bars). These results show that, in this strategy, BMDM can clear the infection more efficiently, while upregulating co-stimulatory and cytokine production functions.

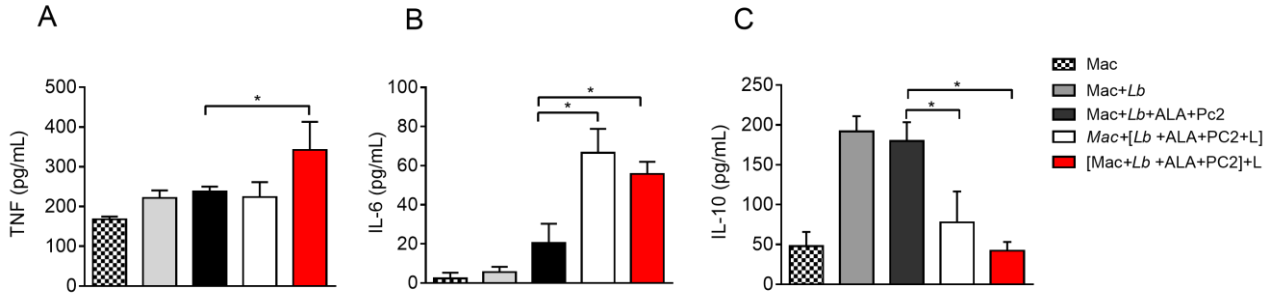


Figure 9. Production of immunity-favorable cytokines by primary macrophages after loading with doubly-sensitized and photo-inactivated *L. braziliensis*. Bone marrow-derived macrophages (BMDM) were exposed at a host-parasite ratio of 1:10 with to URO1/PC2 doubly photo-sensitized parasites with **(white bar, Mac+[Lb+ALA+Pc2+L])** and without (**black bar, Mac+[Lb+ALA+PC2])** exposure to red and UVA light. Alternatively, parasites were doubly photo-sensitized and delivered to macrophages, which were then exposed to UVA/red light (**red bar, [Mac+Lb+ALA+Pc2] +L**). Macrophages infected with untreated Lb (**checkered bar, Mac+Lb**) served as controls. The presence of TNF [A], IL-6 [B] and IL-10 [C] into culture supernatants after incubation for 24 h was determined by ELISA. Data are presented as mean ±. S.D from a representative experiment performed in quadruplicate, *p<0.05

Discussion

Vaccination providing lasting immunity is fundamental to the control of leishmaniasis. An immunization strategy involving live parasites would enable the presentation of the entire antigenic spectrum to the immune system; however, immunosuppression and parasite persistence currently limit this approach. To overcome this limitation, physical, chemical and genetic attenuation have been pursued to generate a safe yet efficient vaccine for leishmaniasis. In the present study, we developed a genetically complemented *L. braziliensis* cell line that could be endogenously sensitized and photoinactivated upon illumination. We show that these transgenic parasites become inactivated upon light exposure but remain capable of infecting macrophages. Within the host cell, parasites are readily susceptible to the oxidative burst while the macrophage upregulates cytokine production and co-stimulatory markers.

Photosensitizers are light-excitable ring compounds that produce cytotoxic ROS in response to illumination. Photodynamic vaccination refers to the use of PS to sensitize *Leishmania*, thusly rendering them photoinactivated through light exposure and allowing such parasites to then be employed as whole-cell vaccines. The photodynamic inactivation of parasites can be accomplished in two ways, either through the uptake of PS, such as phthalocyanines [27], or through the endogenous production of PS, such as URO1, via a

transgenic approach [15]. To pursue the latter, we initially generated genetically complemented *L. braziliensis* expressing ALAD and PBGD by two consecutive transfections. Upon ALA exposure, we successfully induced uroporphyrinogenesis in all the resulting clones. The percentage of uroporphyric cells seen herein was not as high as that described for *L. amazonensis* [14]. The ability to transition from a porphyric to an aporphyric state is not a stable trait in *L. amazonensis*, suggesting that genetically complemented *L. braziliensis* may behave similarly and have a reduced capacity to uptake ALA as opposed to an increased ability to efflux URO1, as was reported earlier [26]. We thus selected one genetically complemented *L. braziliensis* clone for further characterization.

We functionally demonstrated that the sensitization of *L. braziliensis* with ALA induced URO1 accumulation that, upon excitation by white or UVA light, resulted in parasite inactivation. Photoinactivation rates were consistent with experimentation performed in *L. amazonensis* [15,26]. The ability of photoinactivated *L. braziliensis* promastigotes to infect bone marrow-derived macrophages and subsequently differentiate into amastigotes remained uncompromised. The infectivity of attenuated uroporphyrinogenic *L. braziliensis* exposed to light was comparable to that of wild-type parasites. However, after internalization and differentiation into amastigotes, the attenuated parasites were unable to persist as long as wild-type counterparts. Similar results have been reported upon the attenuation of *L. chagasi* with amotosalen followed by low doses of UVA radiation [28] and upon the deletion of centrin and p27 in *L. donovani* [29].

Immunization of Syrian hamsters with *L. amazonensis* submitted to single photo-inactivation, that is using one photosensitizer only, conferred protection against heterologous infection [18], indicating that photodynamic vaccination against leishmaniasis can be readily achievable. Nonetheless, in the mouse model, we have shown that the use of a single photosensitizer such as endogenous ALA may not induce complete photo-inactivation of the light-exposed parasite population and dermal lesions develop, raising safety concerns [20]. To address both safety and efficacy issues, we evaluated the outcome of double sensitization, using endogenous ALA-URO1 and exogenous PC2 as photosensizers. It has been shown that PC2 is 10-40 times more effective at mediating photo-inactivation of *L. tropica* [16] and, in our hands, it was also effective for photo-inactivation of *L. amazonensis* promastigotes and *L. braziliensis*. Therefore, PC2 was employed as the external photosensitizer besides ALA-URO that acted as the endogenous photosensitizer. Additionally, we also investigated the outcome of macrophage exposure to light, after internalization of doubly photosensitized *L. braziliensis*. The rationale

for such strategy is that photo-inactivation inactivates intracellular *Leishmania* leading to the release of antigens within the host macrophage, without compromising the antigen presenting cell (APC) itself [19]. That is because the host cell is not sensitized at the time of light exposure and oxygen radicals produced during that step are limited to the parasite and do not cause oxidative damage to the host cell. Indeed, double sensitization allowed efficient parasite uptake by BMDM but compromised its replication and this outcome was regardless photoinactivation occurring before or after parasite internalization.

We speculate that a vaccination strategy involving internalization of photo-inactivated *L. braziliensis* by BMDM would result in the release of antigens into phagolysosomes and the cytosol of viable macrophages, thusly enabling downstream events related to the development of cellular immunity. Accordingly, using Strategy #2, we found a significant increase in the production of reactive oxygen and nitrogen species, which may account for parasite killing, but, at the same time, the host cell upregulated cytokine production and co-stimulatory effectors such as CD40 and CD86. CD86 is a co-stimulatory molecule expressed on activated APCs that binds CD28 expressed on the T cell. CD40 is also expressed on APCs and binds CD40L expressed in activated T cells, delivering signals important to enhance expression of CD80 and CD86, for example, on the APCs. We also observed TNF production which, together with IFN-γ, activates the macrophage to kill intracellular parasites [30]. TNF induces both NO and SO production by the macrophage and these mediators can be accounted for the *L. braziliensis* killing we observed following photo-inactivation, using both strategies. Moreover, IL-10 levels were decreased in cultures of BMDM exposed to photo-inactivated *L. braziliensis*. In *L. major* experimental infection, IL-10 produced by infected macrophages prevented parasite killing and pre-treatment of cultures with recombinant IL-10 enhances intracellular parasite survival [31]. Collectively, our results suggest that BMDM exposure to photo-inactivated *L. braziliensis* enhanced parasite killing by the infected cell, advocating for efficacy of the double sensitization and photo-inactivation strategies evaluated here. More importantly, we also show that these strategies enhance the antigen presenting capacity of BMDM exposed to photo-inactivated *L. braziliensis* which, we speculate, shall induce the development of protective cellular responses, in vivo.

Immunization with attenuated parasites obtained by the targeted deletion of genes coding for lipophosphoglycan [32], biopetrin transporter [33], HSP70 [34], ALO [35] and centrin [36] was shown to induce long-lasting protective immunity in experimental models. Nonetheless, while attenuated vaccines achieved by genetic deletion offer an interesting approach for

immunization, parasites may revert to their virulent form, and parasite persistence may induce disease reactivation in immunosuppressed individuals. Moreover, whole genome sequencing of *L. donovani* centrin-deficient parasites showed that in addition to knocking out the centrin gene, additional deletions were also observed [37]. While such deletions did not impact the attenuation of the centrin-deficient parasites, this emphasizes the need for comprehensive characterization of attenuated parasite lines. In conclusion, our generation of transgenic mutants of *L. braziliensis* demonstrated a potential for applicability in photodynamic vaccination against cutaneous leishmaniasis caused by *L. braziliensis*.

Acknowledgments

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6. DISCUSSÃO

Apesar de ainda não existir uma vacina eficaz contra a leishmaniose, indivíduos curados não voltam a desenvolver a doença, evidenciando a indução de imunidade duradoura após a cura clínica (revisado em GILLESPIE *et al.*, 2016). Assim, diversos esforços vêm sendo empreendidos pela comunidade científica para o desenvolvimento de vacinas para a leishmaniose, tais como a utilização de parasitas mortos, proteínas recombinantes ou plasmídeos de DNA que codificam proteínas parasitárias bem como parasitas atenuados, os quais mimetizam a infecção natural, sem indução de doença (revisado em REZVAN; MOAFI, 2015).

A inativação da leishmania pode ser obtida por meio da exposição do parasita a fotossensibilizadores (FSs), os quais se acumulam no seu interior. A consequente exposição dos parasitas fotossensibilizados a uma fonte de luz induz a geração de ROS tais como superóxido e oxigênio singleto ($^1O_2$), induzindo assim dano por oxidação. O alcance do $^1O_2$ é muito curto (20 Å) (JUZENAS; MOAN, 2006), provavelmente por ser altamente reativo e destrutivo, e especula-se que uma vez gerado o $^1O_2$ age no interior da célula, preservando a estrutura e as moléculas de superfície por horas após a fotoinativação do parasita (revisado em CHANG; KOLLI, 2016). Não se conhecem enzimas capazes de detoxificar $^1O_2$, tornando o dano oxidativo mediado por esta molécula letal (revisado em VATANSEVER et al., 2013). No presente estudo, induzimos a atenuação de leishmania a partir de sua exposição a FSs seguido da fotoinativação por exposição à luz. Neste sentido, hipotetizamos que a imunização de camundongos com parasitas fotoinativados induz imunidade contra um desafio subsequente com parasitas virulentos, conferindo proteção contra a LT.

As ftalocianinas (PCs) são FSs de segunda geração, conhecidas por induzir a produção de $^1O_2$ após a exposição à luz vermelha (COOK et al., 1995). A PC2 é uma aminoftalocianina descrita como 10 a 40 vezes mais eficaz para a fotoinativação de *L. tropica* (AL-QAHTANI et al., 2016) quando comparada a ftalocianinas ligadas a grupos anilina ou piridil usadas inicialmente na fotoinativação de *L. amazonensis* (DUTTA et al., 2011). Os resultados obtidos no presente trabalho mostram que a PC2 induz um fenótipo tóxico dose-dependente em *L. amazonensis*, evidenciado pela perda de mobilidade e de viabilidade celular, conforme avaliado pelo ensaio de MTT. Também mostramos que a fotoinativação de *L. amazonensis* mediada por PC2 reduziu a sua captação por macrófagos em cerca de 5 vezes quando comparado a parasitas controle apenas sensibilizados com PC2, mesmo em tempos iniciais de infecção (4h). Isso

sugere que a fotoinativação utilizando a dose de PC e/ou de iluminação empregadas em nossos experimentos dificulta a entrada dos parasitas na célula hospedeira. O achado de que uma fração de macrófagos permanece infectada com sucesso, apesar da fotoinativação dos parasitas, pode ser atribuída ao modo estocástico de sensibilização por PC2. Ou seja, uma pequena porcentagem de parasitas não absorve o sensibilizador e, portanto, não é fotoinativada, permanecendo viável e infectiva (DUTTA et al., 2005, 2011).

Uma vez determinada a dose com a qual *L. amazonensis* pode ser sensibilizada e fotoinativada de maneira exógena, avaliamos a fotoinativação de *L. amazonensis* por via endógena. Nesta estratégia, utilizamos parasitas geneticamente complementados com ALAD e PBGD (SAH et al., 2002), as quais estão ausentes em parasitas selvagens. A presença destas enzimas leva à produção de URO, a qual se acumula no citosol e funciona como um FS, semelhante a PC2 (DUTTA et al., 2008a; SAH et al., 2002). A exposição de *L. amazonensis* uroporfírica à luz UV de ondas longas gera oxigênio singleto (DUTTA et al., 2008a; SAH et al., 2002), causando dano irreparável a estes parasitas. Em nossos experimentos, a fotoinativação endógena de *L. amazonensis* induzida por URO reduziu a quantidade de células infectadas em cerca de 2,5 vezes, mas assim como a fotoinativação exógena com PC2, aproximadamente 10% dos macrófagos permaneceram infectados.

Em seguida, avaliamos o desfecho da inoculação de parasitas fotoinativados em camundongos BALB/c. Observamos que os animais inoculados com *L. amazonensis* fotoinativada de maneira exógena utilizando PC2 apresentaram lesões mais tardiamente comparado àqueles inoculados com *L. amazonensis* apenas fotossensibilizada. Esse resultado pode ser explicado por nossos ensaios *in vitro*, os quais mostraram redução na quantidade de macrófagos infectados com os parasitas fotoinativados.

Dutta e colaboradores (2011) mostraram que células dendríticas infectadas com *L. amazonensis* transgênica para a expressão de OVA e fotoinativada com uma ftalocianina foram capazes de ativar linfócitos T $CD8^+$ epítopo-específicos *in vitro*. Esse trabalho evidenciou o potencial da utilização de parasitas atenuados por fotoinativação para a imunoprofilaxia. Um outro estudo mostrou que a imunização de hamsters com *L. amazonensis* fotoinativada conferiu proteção contra a infecção heteróloga com *L. donovani* (KUMARI et al., 2009). Neste estudo, a imunização induziu uma resposta imune capaz de reduzir a carga parasitária em 99% e, em paralelo, aumentou a expressão de iNOS, IFN-γ e IL-12 nos animais desafiados. Além disso, a transferência de células T obtidas de hamsters imunizados conferiu proteção a animais *naïve*,

os quais foram infectados posteriormente, mostrando que a resposta celular induzida nos animais imunizados pode ser transferida de maneira a permanecer protetora.

Dada a natureza estocástica da fotossensibilização simples, parasitas podem permanecer viáveis mesmo após a exposição a luz, e, portanto, o risco de desenvolvimento de doença permanece. Para minimizar este risco, testamos a combinação de FSs com o objetivo de aumentar a eficácia da fotoinativação, pois dois FSs podem atuar em sinergia como mostrado na terapia fotodinâmica aplicada a linhagens celulares tumorais e em modelo de melanoma em camundongos (ACEDO et al., 2014; SCHNEIDER-YIN et al., 2009; VILLANUEVA et al., 2010). Mesmo quando a aplicação de FSs combinados não aumenta a mortalidade celular, ela pode reduzir a toxicidade inespecífica induzida por cada molécula, e também amplificar a resposta imunológica contra o tumor (GYENGE et al., 2012). Com relação a *Leishmania*, Dutta e colaboradores (2012) observaram que *L. amazonensis* fotossensibilizada com AlPhCl e com URO1 *in vitro* tornou-se totalmente sensível à fotoinativação *in vivo* pois camundongos BALB/c inoculados na derme da orelha não apresentaram lesões, mesmo 8 semanas após a infecção (DUTTA; WAKI; CHANG, 2012). O acúmulo de AlPhCl e URO1 pela *Leishmania* ocorre em diferentes sítios subcelulares; o URO1 se forma no citosol e é depois restrito a vacúolos no citoplasma e vesículas endo- ou exocíticas (DUTTA et al., 2008a), enquanto que AlPhCl é internalizada e aparentemente se liga a membranas intracelulares, graças à sua natureza hidrofóbica (DUTTA; WAKI; CHANG, 2012). Uma vez que esses FSs são excitados em diferentes comprimentos de onda, a exposição à luz branca (consistindo em todo o espectro visível) pode resultar na produção simultânea de $^1O_2$ em diferentes compartimentos do parasita, resultando em maior eficácia da fotoinativação.

No presente trabalho replicamos esta estratégia, inoculando *L. amazonensis* fotossensibilizada com ALA e AlPhCl na pata, base da cauda, dorso e derme da orelha. Observamos que promastigotas duplamente fotossensibilizadas, mas não fotoinativadas *in vitro* foram detectáveis nos diversos locais inoculados de forma comparável, com exceção daquelas inoculadas na região dorsal do animal. A derme da orelha foi o único local onde os parasitas foram fotoinativados de forma satisfatória após exposição à luz, pois não foi possível detectar parasitas após três semanas. Embora não possamos descartar a possibilidade de migração dos parasitas inoculados para os gânglios linfáticos regionais, propomos que a translucidez da orelha seja particularmente adequada para a iluminação, aumentando assim o sucesso da fotoinativação, diferentemente do ocorrido na base da cauda ou da pata. Podemos também especular que a fotoinativação dos parasitas inoculados na orelha permitiu a indução de uma

resposta imune efetora com pouca ou nenhuma imunossupressão pois observamos atraso no desenvolvimento da lesão e redução significativa na carga parasitária. O nível de proteção observado foi significativo, considerando que a maior parte das linhagens de camundongos, incluindo C3H, C57BL/6, BDA e CBA são suscetíveis à infecção por *L. amazonensis*, desenvolvendo lesões progressivas (CÔRTES et al., 2010; SOONG, 2012) e uma resposta mista Th1-Th2 após a infecção (AFONSO; SCOTT, 1993; MCMAHON-PRATT; ALEXANDER, 2004). Os camundongos BALB/c são particularmente suscetíveis a essa infecção e apresentam lesões necróticas com crescimento progressivo (PEREIRA; ALVES, 2008).

Estes achados constituem forte evidência de que a fotoinativação é uma estratégia viável para a geração de parasitas imunogênicos e seguros. Acreditamos que os níveis de proteção possam ser melhorados a partir da otimização dos procedimentos experimentais tais como a redução da dose de parasitas no desafio, o aumento da frequência de imunizações e ajuste do intervalo entre o inóculo de parasitas fotossensibilizados e a iluminação *in vivo*. A otimização do intervalo é necessária sobretudo para eliminar parasitas persistentes, os quais podem induzir uma resposta supressora, contrabalanceando a resposta protetora e dificultando a eliminação completa de parasitas após a infecção (ROSSI; FASEL, 2017). Em suma, os resultados apresentados na primeira parte dessa tese demonstram que a fotoinativação de *L. amazonensis* usando FSs endógenos ou exógenos reduz sua viabilidade e capacidade de infecção em macrófagos. Além disso, a fotoinativação de *L. amazonensis* por meio da combinação de FSs reduz a viabilidade do parasita, mas o mesmo permanece capaz de induzir uma resposta celular parcialmente protetora, em modelo experimental.

Na segunda parte do trabalho, partimos para avaliar se *L. braziliensis* poderia ser fotossensibilizada e inativada da mesma maneira, sobretudo porque *L. braziliensis* causa as formas mais complicadas da LT é responsável pela maioria dos casos no Brasil. Adicionalmente, o modelo de infecção intradérmica de camundongos BALB/c com *L. braziliensis* é autolimitado, gerando lesões ulceradas que apresentam cura espontânea e resposta imunológica mista Th1/Th2, semelhante ao que ocorre em pacientes com LCL (DE MOURA et al., 2005).

Inicialmente, geramos uma linhagem de *L. braziliensis* complementada com os genes ALAD e PBGD, de forma a torná-la fotossensível pela geração e acúmulo de porfirinas intermediárias da via do heme e, posteriormente, suscetível a fotoinativação. Após duas transfecções consecutivas, os genes ALAD e PBGD foram inseridos em *L. braziliensis* e os 12 clones resultantes cresceram de forma estável sob pressão seletiva, por várias passagens. A

expressão dos genes inseridos foi verificada funcionalmente através de intensa fluorescência vermelha característica da presença de porfirinas tanto nas células quanto nos sobrenadantes após a incubação com ALA e exposição à luz UV de ondas longas. Parasitas *selvagem* ou transfectados apenas com ALAD não apresentaram fluorescência quando incubados com ALA. Além disso, o fato de o acúmulo de porfirinas apenas ocorrer em *L. braziliensis* geneticamente complementada quando as promastigotas foram incubadas com ALA confirma que essa espécie também é naturalmente deficiente na enzima ALA Sintase. Em nosso modelo com a *L. braziliensis* geneticamente complementada não pudemos determinar qual porfirina da via é a responsável pela coloração vermelha e pela fototoxicidade observada. No entanto, Dutta e colaboradores (2008a) verificaram que *L. amazonensis*, *L. infantum*, *L. major* e *L. tropica* complementadas com ALAD e PBGD produzem apenas URO1 após a incubação com ALA, logo são deficientes em pelo menos 5 enzimas da via de biossíntese do heme.

Em nosso modelo, todos os clones testados apresentaram fluorescência em proporções variadas após exposição ao ALA, mas com valores menores ao descrito anteriormente para *L. amazonensis*. É sabido que promastigotas de *L. amazonensis* contam com a geração de "porfirinossomas" que realizam a detoxificação celular de URO1 (DUTTA et al., 2008b). Acreditamos que um mecanismo similar pode ser responsável pela fluorescência de porfirinas observada no sobrenadante de culturas de *L. braziliensis* após a incubação com ALA. A capacidade reduzida de captar ALA, bem como a rápida mobilização de porfirinas podem ser responsáveis pela população fluorescente menor, observada em *L. braziliensis* quando comparado a *L. amazonensis*. Semelhante aos modelos de Porfíria desenvolvidos anteriormente (DUTTA et al., 2008a; SAH et al., 2002), os clones de *L. braziliensis* apresentaram maior população fluorescente e intensidade de fluorescência após 48h de incubação com ALA. É interessante observar a clara heterogeneidade tanto na geração de fluorescência quando na taxa de inativação entre os 12 clones gerados. Após a exposição à luz UVA, a taxa de fotoinativação variou entre os clones de acordo com a proporção celular que havia se mostrado fluorescente para porfirinas, confirmando a atividade fotossensibilizadora das moléculas geradas e que a fluorescência pode ser interpretada como um indicador da susceptibilidade à fotoinativação. Assim, demonstramos funcionalmente a atividade de ALAD e PBGD após a transfecção em *L. braziliensis*, tornando-a capaz de gerar porfirinas intracelulares utilizando ALA exógeno, e a subsequente fotoinativação após a exposição a luz branca ou UVA.

A complementação genética de *L. braziliensis* não alterou sua capacidade de infectar macrófagos murinos e de transformar-se na forma amastigota nos períodos estudados. Na

verdade, mesmo após sensibilização com ALA ou após dupla sensibilização com ALA e Pc2 e exposição à luz, os parasitas foram internalizados em taxa semelhante àqueles não fotoinativados. Entretanto, após 24h houve redução significativa da taxa de infecção e do número de amastigotas nas células. Resultados similares foram observados em macrófagos humanos ou murinos infectados com *L. donovani* deficiente em p27 e centrina (BHATTACHARYA et al., 2015; DEY et al., 2010; SELVAPANDIYAN et al., 2009). Apesar da taxa de infecção diminuída, a infecção com essas linhagens atenuadas induziu a regulação positiva de citocinas de perfil Th1 e de moléculas co-estimulatórias de superfície em macrófagos murinos (BHATTACHARYA et al., 2015). Em nosso modelo, cerca de 10% das amastigotas porfirinogênicas não foram eliminadas dos macrófagos após 24h, corroborando os achados anteriores os quais mostraram que alguns parasitas são capazes de sobreviver à fotólise devido à absorção reduzida de ALA e/ou ao efluxo excessivo de porfirinas formadas. Entretanto, no modelo de *L. amazonensis* esses sobreviventes se mostraram tão sensíveis a uma nova indução de uroporfirina quanto clones de parasitas parentais nunca expostos a ALA (CHANG; KOLLI, 2016), o que permitiria sua posterior fotoinativação.

Em seguida, avaliamos a fotoinativação exógena de promastigotas de *L. braziliensis* com PC2, que a 1uM induziu aproximadamente 100% de fotoinativação após exposição à luz vermelha, se caracterizando como promissor para a dupla fotoinativação empregando também ALA-URO1. A taxa de infecção de macrófagos com *L. braziliensis* duplamente fotoinativada caiu até 4 vezes após 24h, quando comparado às células infectadas com parasitas fotoinativados apenas por meio de ALA-URO1. A partir daí avaliamos a infecção de macrófagos com parasitas previamente fotoinativados e a iluminação de macrófagos infectados com *L. braziliensis* fotossensibilizada. Desta maneira, pudemos comparar os efeitos da infecção com parasitas fotoinativados *versus* a infecção com parasitas sensibilizados e submetidos à fotoinativação posteriormente (após a internalização pelo macrófago). Como mostrado na primeira etapa desta tese, a fotoinativação *in vivo*, ou seja, após a inoculação dos parasitas fotossensibilizados em animais conferiu proteção contra *L. amazonensis* e contra *L. donovani* (KUMARI et al., 2009).

De fato, a iluminação de macrófagos murinos infectados com *L. braziliensis* duplamente fotossensibilizada induziu maior produção de NO e de superóxido, moléculas microbicidas responsáveis pela destruição do parasita. Ao mesmo tempo, observamos aumento na expressão de CD40 e de CD86, moléculas co-estimulatórias expressas na superfície de células apresentadoras de antígeno e importantes para a iniciação e sustentação da resposta imunológica adaptativa. CD86 liga-se a CD28 presente na superfície de linfócitos T fornecendo o sinal

secundário para sua ativação (KHAN et al., 2012). Já o CD40 liga-se ao CD40L presente em células T ativadas e se mostrou crucial para a indução de imunidade contra *L. major*, por ser responsável pela amplificação da secreção de IL-12p70 e diferenciação de células Th1 (CAMPBELL et al., 1996). Além disso, CD40 fornece sinais para a ativação de macrófagos e amplificação da expressão de CD80 e CD86 em APCs (CAUX et al., 1994). A infecção por *Leishmania* é capaz de diminuir a expressão de moléculas co-estimulatórias, fazendo com que as células T se tornem anérgicas (CAMPBELL et al., 1996). Dessa forma, especulamos que a infecção com *L. braziliensis* fotoinativada pode ser capaz de subverter a imunossupressão desencadeada pela infecção, aumentando a capacidade de apresentação de antígenos em macrófagos, *in vivo*.

Paralelamente, observamos a indução de TNF nos macrófagos infectados, que juntamente com IFN-γ ativa os macrófagos para destruir os parasitas intracelulares através da indução de NO e SO. Esses mediadores podem ser responsáveis pela morte de *L. braziliensis* que observamos após a fotoinativação, usando ambas as estratégias. Além disso, a infecção com parasitas fotoinativados inibiu a produção de IL-10 em até 4 vezes, dependendo da estratégia de fotoinativação utilizada. Sabe-se que IL-10 é uma importante citocina regulatória e o pré-tratamento de culturas de macrófagos com IL-10 recombinante aumenta a sobrevivência intracelular de *L. major* (KANE; MOSSER, 2001). Nossos resultados sugerem que a exposição de macrófagos a *L. braziliensis* fotoinativada aumentou a atividade efetora da célula hospedeira contra o parasita, corroborando a eficácia das estratégias de dupla fotossensibilização utilizadas aqui.

A imunização com parasitas atenuados por meio da deleção de genes codificando para lipofosfoglicano (SPATH, 2000), transportador de biopterina (PAPADOPOULOU et al., 2002), HSP70 (SOLANA et al., 2017), centrina (SELVAPANDIYAN et al., 2009) e p27 (DEY et al., 2013) induziram proteção a longo prazo em modelos experimentais de leishmaniose. No entanto, a imunidade gerada por essas vacinas é baseada na infecção natural e persistência de *Leishmania*, e sabe-se que indivíduos curados de uma infecção inicial podem sofrer reativação da doença quando tornam-se imunossuprimidos (MORTAZAVI; SALEHI; KAMYAB, 2014; SOUZA et al., 2017; VAN GRIENSVEN et al., 2014). Em nosso trabalho, a *L. amazonensis* fotossensibilizada infecta a célula hospedeira por um período limitado e é eliminada em seguida pela própria célula quando previamente fotoinativada, ou através da fotoinativação *in situ*, oferecendo menor chance de persistência que linhagens atenuadas. Além disso, parasitas atenuados através da deleção de um grupo de genes podem sofrer reversão para a forma

virulenta, ou a deleção pode levar a rearranjos genômicos inesperados, como observado recentemente através do sequenciamento de genoma de *L. donovani* deficiente em centrina (GANNAVARAM et al., 2017). Nesse estudo foi observado que outros genes também foram deletados e, apesar de isso não impactar na atenuação ou imunogenicidade do parasita, enfatiza-se a necessidade de ampla caracterização de linhagens atenuadas de parasitas para fins de imunização. Nesse trabalho, empregamos um sistema de expressão protéica episssomal para a produção das enzimas ALAD e PBGD. Uma vez estabelecida a expressão das moléculas de interesse, o sistema não tem efeitos inesperados no organismo transfectado (ROBERTS, 2011), o que confere estabilidade para seu emprego como imunização.

Em suma, o presente estudo mostrou que *L. braziliensis* e *L. amazonensis* podem ser fotoinativadas por FSs que atuam via endógena ou exógena, e que há sinergismo nos efeitos induzidos. Também observamos que é possível induzir a porfirinogênese em *L. braziliensis* duplamente complementada com ALAD e PBGD. Mesmo fotoinativados, os parasitas são fagocitados por macrófagos, conferindo proteção em modelo de CL causada por *L. amazonensis* e induzindo a ativação de macrófagos infectados por *L. braziliensis*. A fotoinativação após a infecção com parasitas fotossensibilizados induziu proteção parcial *in vivo* e maior perfil de ativação de funções efetoras e de apresentação de antígenos de macrófagos *in vitro*, sugerindo a possibilidade de utilização dessa estratégia para imunoprofilaxia

7. CONCLUSÃO

A fotoinativação de *L. amazonensis* e de *L. braziliensis* fotossensibilizadas de maneira endógena ou exógena reduziu a viabilidade dos parasitas e sua capacidade de infecção. A imunização de camundongos com *L. amazonensis* duplamente fotoinativada reduziu a carga de doença de maneira significativa após o desafio com parasitos vivos. Já a infecção de macrófagos com *L. braziliensis* duplamente fotoinativada levou à ativação de suas funções efetoras. Assim, propomos o uso da dupla fotossensibilização e inativação de leishmania como estratégia de imunização para a leishmaniose tegumentar.

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Parasites & Vectors

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New "light" for one-world approach toward safe and effective control of animal diseases and insect vectors from leishmaniac perspectives

Kwang Poo Chang*, Bala K. Kolli and the New Light Group

Abstract

Light is known to excite photosensitizers (PS) to produce cytotoxic reactive oxygen species (ROS) in the presence of oxygen. This modality is attractive for designing control measures against animal diseases and pests. Many PS have a proven safety record. Also, the ROS cytotoxicity selects no resistant mutants, unlike other drugs and pesticides. Photodynamic therapy (PDT) refers to the use of PS as light activable tumoricides, microbicides and pesticides in medicine and agriculture.

Here we describe "photodynamic vaccination" (PDV) that uses PDT-inactivation of parasites, i.e. *Leishmania* as whole-cell vaccines against leishmaniasis, and as a universal carrier to deliver transgenic add-on vaccines against other infectious and malignant diseases. The efficacy of *Leishmania* for vaccine delivery makes use of their inherent attributes to parasitize antigen (vaccine)-presenting cells. Inactivation of *Leishmania* by PDT provides safety for their use. This is accomplished in two different ways: (i) chemical engineering of PS to enhance their uptake, e.g. Si-phthalocyanines; and (ii) transgenic approach to render *Leishmania* inducible for porphyrinogenesis. Three different schemes of *Leishmania*-based PDV are presented diagrammatically to depict the cellular events resulting in cell-mediated immunity, as seen experimentally against leishmaniasis and *Leishmania*-delivered antigen in vitro and in vivo. Safety *versus* efficacy evaluations are under way for PDT-inactivated *Leishmania*, including those further processed to facilitate their storage and transport. *Leishmania* transfected to express cancer and viral vaccine candidates are being prepared accordingly for experimental trials.

We have begun to examine PS-mediated photodynamic insecticides (PDI). Mosquito cells take up rose bengal/cyanosine, rendering them light-sensitive to undergo disintegration in vitro, thereby providing a cellular basis for the larvicidal activity seen by the same treatments. Ineffectiveness of phthalocyanines and porphyrins for PDI underscores its requirement for different PS. Differential uptake of PS by insect *versus* other cells to account for this difference is under study.

The ongoing work is patterned after the one-world approach by enlisting the participation of experts in medicinal chemistry, cell/molecular biology, immunology, parasitology, entomology, cancer research, tropical medicine and veterinary medicine. The availability of multidisciplinary expertise is indispensable for implementation of the necessary studies to move the project toward product development.

Keywords: Photosensitizers, *Leishmania*, Mosquito, Photodynamic therapy, Photodynamic vaccination, Photodynamic insecticide

* Correspondence: kwangpoo.chang@rosalindfranklin.edu
Department of Microbiology/Immunology, Chicago Medical School/Rosalind Franklin University of Medicine and Science, 3333 Green Bay Rd, North Chicago, IL 60064, USA

Background

Photosensitizers (PS)

These are ring compounds whose soluble form is light-excitable to produce cytotoxic reactive oxygen species (ROS) [1]. Naturally occurring PS include tetrapyrroles, e.g. corrins, chlorins and porphyrins – intermediates in the biosynthesis of vitamin B12, chlorophyll and heme [2]. The stoichiometry of these intermediates is stringently regulated by necessity to minimize their phototoxicity. Many plants produce PS as secondary metabolites for self-protection, e.g. psoralen and hypericin [3]. Other PS are chemically synthesized: the fluorescein analogues, rose bengal and cyanosine, and phthalocyanines (PC). Natural and synthetic PS include Food and Drug Administration (FDA)-approved drugs, cosmetic, food and fabric dyes.

PDT-generated singlet oxygen ($^1O_2$) and -cell susceptibility

PDT has been used to eliminate tumors, pathogens and pests with cytotoxic ROS that is produced by illumination of targets treated with PS, e.g. porphyrins, PC and rose bengal, at their respective excitation wavelengths [4]. PDT initially generates singlet oxygen ($^1O_2$) and/or hydroxyl radicals, leading to the production of additional ROS, including peroxides and superoxides. $^1O_2$ is highly reactive and destructive, but too short-lived (2–3 μs) to cross the cell membrane. $^1O_2$ is produced by plants during photosynthesis, but not by non-photosynthetic mammals, insects and *Leishmania*. Cells from the latter group are thus most susceptible to oxidative damage by $^1O_2$ because they lack mechanisms of detoxification. $^1O_2$ has the potential for strategic deployment to inflict maximal destruction of specific cell types with minimal collateral damage.

PDT, especially using $^1O_2$ generating PS for non-photosynthetic cells, is unlikely to select for resistance, since neither light nor PS alone is cytotoxic. Their use in combination produces ROS inactivating multiple targets, minimizing the likelihood of selecting resistant traits. In support of this concept, no resistant *Leishmania* were selected after six consecutive cycles of PDT, i.e. induced uroporphyrinogensis plus light (see below) [5, 6]. Few survivors emerged after each PDT cycle as aporphyric cells, resulting from reduced uptake of the inducer and/or heightened efflux of uroporphyrin I (URO). These phenotypes are not stable traits, since populations from the survivors after each of the six PDT cycles remain equally sensitive to the same PDT. Total inactivation of *Leishmania* by PDT is achievable when using two different PS, i.e. URO and PC (see below).

Cellular uptake and subcellular targeting of PS for effective PDT

The effectiveness of PDT is a function of light intensity delivered at a wavelength specific to the PS and its quantum yield [4]. Under physiological conditions, PDT is critically dependent on the uptake of PS by the target cells. The best example to illustrate this is the all-or-none phototoxicity of the $^1O_2$ generating URO, depending on its presence in the cytosol or in the extracellular milieu [5, 6]. URO is highly water-soluble, but not taken up by cells, like *Leishmania*. These cells are thus light-insensitive and remain fully viable, as indicated by their active motility when bathed in URO-containing milieu [5]. This changes dramatically for uroporphyrinogenic *Leishmania*, which are transgenically modified to express the 2nd and 3rd enzymes in the heme biosynthetic pathway, rendering them inducible with the product of the 1st enzyme in this pathway, i.e. delta-aminolevulinate (ALA) for cytosolic accumulation of URO [5–7]. During ALA-induced uroporphyrinogenesis, these mutants cease flagellar motility abruptly when examined under dim light for microscopy as URO begins to form in the cytosol [5, 6]. Clearly, intracellular delivery of PS even in a minute amount is sufficient to sensitize cells to photoinactivation.

Cellular uptake of PS varies with their chemical structures. PC have been chemically modified to enhance such bioavailability. Modifications of their coordinating metals, side-chains and/or axial ligands increase cationicity for affinity to the negatively charged cell surface and solubility for persistence in the milieu [8, 9]. Figure 1 shows some PS, which are localized to different subcellular sites of *Leishmania*. ALA-induced cytosolic accumulation of URO was discussed earlier (Fig. 1B, B'). The hydrophobic/lipophilic hypericin (A, A') and aluminum phthalocyanine (Al-PC) (C, C') are taken up rapidly. These PS become associated immediately with and remain bound constantly to cellular membranes with undiminished fluorescence, but are transferrable from sensitized cells to the membranes of untreated cells [10]. How these phenomena are related to the expected turnover of cellular membranes is a question of interest for investigation. In contrast, the amino-PC [9] is endocytosed by *Leishmania* into their endosome-lysosome vacuolar system [11]. Other Si- or Zn-PC analogues [8] are either not taken up at all by *Leishmania* or are taken into the endosome-lysosome system or mitochondria [12].

Illumination of the PC-sensitized *Leishmania* with red light (~600 nm excitation wavelength) at low fluence (1–2 J cm^2) generates enough $^1O_2$ to inactivate them [11, 12]. The inactivated cells lose their flagellar motility and viability, but remain intact structurally for hours before disintegration. In many instances, *Leishmania* differ from mammalian cells in their response to different PS for PDT. Elucidation of these differential mechanisms is of interest for optimizing the utility of PS for targeted PDT.

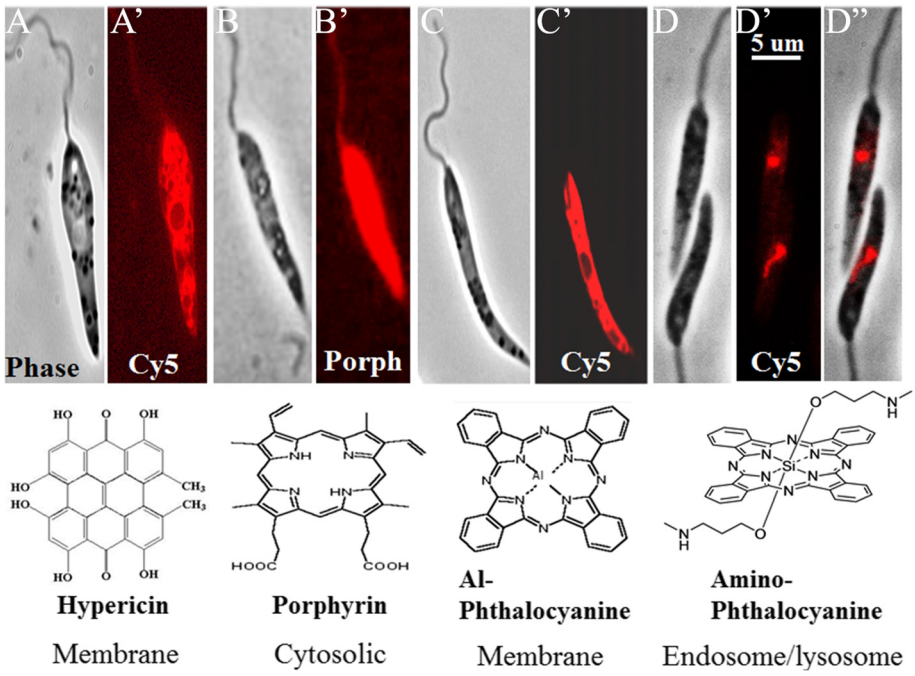

Fig. 1 Photosensitization of *Leishmania* promastigotes with different photosensitizers. A-D, Phase contrast; A'-D", Fluorescence images taken under Cy5 or porphyrin filters. Under each image are the name, structure and cellular localization of the photosensitizer used. Cells were exposed to each photosensitizer overnight in the dark and imaged under live conditions as previously described [6, 7, 10–12]

PDT in clinical use: PDT of cutaneous leishmaniasis (CL) and post-treatment immune clearance of infection

PDT is an accepted clinical regimen for treating solid tumors and skin diseases, and for removing diseased tissues [4]. PDT begins with PS-sensitization of the target tissues with a PS or an inducer of endogenous PS, i.e. ALA to transiently up-regulate cellular porphyrin biosynthesis. The sensitized target is then illuminated to generate ROS for its destruction. Clinical PDT is thus limited to superficial and localized targets, e.g. solid tumors accessible to PS-sensitization and to the subsequent photo-inactivation by illumination from an external light source. Targets several centimeters below the skin are still PDT-treatable by using PC excitable with deep-penetrating red light.

PDT has been explored for treating infectious diseases of the skin [1], including cutaneous leishmaniasis (CL). Various PS have been assessed for PDT of experimental and clinical CL using different light sources: LED, laser and sun light (see [11]). PDT has the potential to shorten the often protracted duration of simple CL before spontaneous healing. The ultimate cure of all infectious diseases is thought to depend on post-therapeutic immune clearance, since no drug is expected to reach all individual pathogens in any given infection, regardless of the dosages used and the frequency of applications. The "post-PDT immune clearance" of CL foretells the potential of photodynamic vaccination (PDV) for both immuno-prophylaxis and -therapy.

Photodynamic vaccination (PDV)

Prophylactic vaccination is the best preventive measure against infectious diseases, especially zoonosis, which cannot be controlled readily because of its persistence in animal reservoirs (Cf. [13]). Here we describe PDV using PDT-inactivation of *Leishmania* for vaccination. The evolution of *Leishmania* for intra-antigen-presenting cells (APC) parasitism and their sensitivity to PDT via PS accumulation are exploited for developing strategies to optimize the efficacy and safety of PDV.

PDT-inactivation of *Leishmania* for vaccination against leishmaniasis

Background

Lasting immunity after cure of leishmaniasis and "leishmanization" Development of effective prophylactic vaccines for this disease has long been considered as feasible from the lasting or life-long immunity seen after spontaneous healing of simple CL and after chemotherapeutic cure of visceral leishmaniasis (VL) (Cf. [14]). Infection of healthy individuals with lesion-derived live parasites in a hidden place is the crudest form of vaccination for simple CL. This is known as "leishmanization" [15] and has been practiced for millennia in the endemic

sites of the Middle East and Central Asia. The vaccinees develop lasting immunity after self-healing and are thus immune for life from the potentially facial disfiguring CL. The lasting immunity results from a T cell-mediated response to *Leishmania* naturally occurring vaccines, adjuvants and other immune-stimulating factors. The residence of *Leishmania* in APC makes these molecules readily available for processing and presentation, accounting very likely for the effective elicitation of cell-mediated immunity and the post-therapeutic immune clearance.

***Leishmania* vaccine availability, efficacy and safety**

Vaccines are still under development for both human and canine leishmaniasis. "Leishmanization" is effective, but unacceptable unless accomplished without a full-blown leishmaniasis. The extensive literature on the use of cultured *Leishmania* as the vaccine sources has been exhaustively reviewed recently (see Supplemental Table 1 in [16]). Live vaccines using avirulent strains, drug-crippled parasites and genetically attenuated mutants have been examined in experimental animal models. Most extensively studied are inanimate vaccines from the following materials: (1) whole-cells of cultured *Leishmania* killed or inactivated by chemical or physical means, e.g. formalinization, heating/autoclaving and irradiation; (2) soluble or insoluble fractions of cultured *Leishmania* or their secretory products; and (3) recombinant products of immunologically active *Leishmania* antigens. Prophylactic efficacy has been shown for most of them against experimental leishmaniasis in animal models, but few have reached the stages of clinical trials. Of note from these trials are the findings that inanimate vaccines from categories (2) and (3) are safe and immunogenic [17–19], but are only partially effective at best against human and canine leishmaniasis. The only whole-cell vaccine examined in category (1) is ineffective, but proven safe, i.e. autoclaved promastigotes at a dose of ~200 ug (100–400 ug) ([20]; F. Modabber, personal communication). This dosage is equivalent to $\sim 4 \times 10^7$ promastigotes, comparable to the number used as leishmanin (up to 2×10^7 promastigotes/dose in phenol or merthiolate) in Montenegro skin test for delayed type hypersensitivity (DTH) [21]. These chemically or physically inactivated promastigotes have been injected into several hundred thousands of people. The continuing use of leishmanin test for DTH attests to the safety of whole-cell *Leishmania* when inactivated appropriately.

Here we exploit PDT as a new modality of *Leishmania* inactivation for assessing the safety and efficacy of their use for vaccination.

Three schemes of PDT-inactivated *Leishmaina* for vaccination

The application of PDT in two steps (PS-sensitization followed by photo-inactivation) offers three different ways to inactivate *Leishmania* for vaccination, as depicted diagramatically in Fig. 2.

Scheme 1 uses the uroporphyrinogenic *Leishmania* transfectants [5–7], which have the wildtype efficiency for entry into APC and differentiation/replication in their phagolysosomes [22] (**Events 1–4**). The 1st PDT step is the addition of ALA to the infected APC, resulting in porphyrinogenesis of both the intra-phagolysosomal *Leishmania* transfectants and their host APC (**Event 5**). The latter become aporphyric shortly afterward, since they possess a complete heme biosynthetic pathway, thereby rapidly exhausting the excessive porphyrins produced; In contrast, the transgenic *Leishmania* produce URO, which persists and accumulates in their cytosol because of their deficient heme biosynthesis pathway, lacking the downstream URO-utilizing enzymes (**Event 6**). Light-exposure of these infected APC excites URO in the uroporphyric *Leishmania* for their selective inactivation (**Event 7**) and eventual lysis to release antigens into the phagolysosomes and cytosol of the viable host APC (**Event 8**).

Scheme 2 is similar to Scheme 1, except that the uroporphyrinogenic *Leishmania* are doubly pre-PS-sensitized for the 1st PDT step with ALA for URO accumulation in the cytosol and Si-PC for uptake into endosomes [11, 12]. These doubly PS-sensitized *Leishmania* infect APC in the dark, as described for Scheme 1 (**Events 1–4**). Subsequent light-exposure of these infected cells for the step 2 PDT produces the same outcome (**Event 6**), also as described for Scheme 1, except that the changes in the protocol reduce the events to 6 from 8 in Scheme 1.

Scheme 3 is similar to Schemes 1–2, except that uroporphyrinogenic *Leishmania* are doubly PS-sensitized and photo-inactivated to complete both PDT steps as described for Scheme 2 before use for loading APC (**Event 1**). The changes of the protocol simplify the events to 4 from 6 to 8 for schemes 1–2. This scheme of APC-loading involves no replicative cycle of *Leishmania* in the host APC (**Events 2–4**).

Cell-mediated immunity depicted for PDT vaccination

Diagrammatic illustration Figure 3 depicts the elicitation of cell-mediated immunity by all three PDV schemes based on experimental evidence described in the subsequent paragraphs. PDT selectively inactivates intracellular *Leishmania*, resulting in the eventual release of their contents into the viable host APC (**Event 1**). The materials released from photolysed *Leishmania* are expected to include antigenic vaccines and other putative immune stimulating factors, as depicted in the foregoing sections. Several pertinent issues are of interest to mention here. APC in schemes 1–2 remain unscathed and viable after PDT [22]. This is expected, since these host APC are not PS-sensitized at the time of illumination, and since the $^1O_2$ produced is limited to

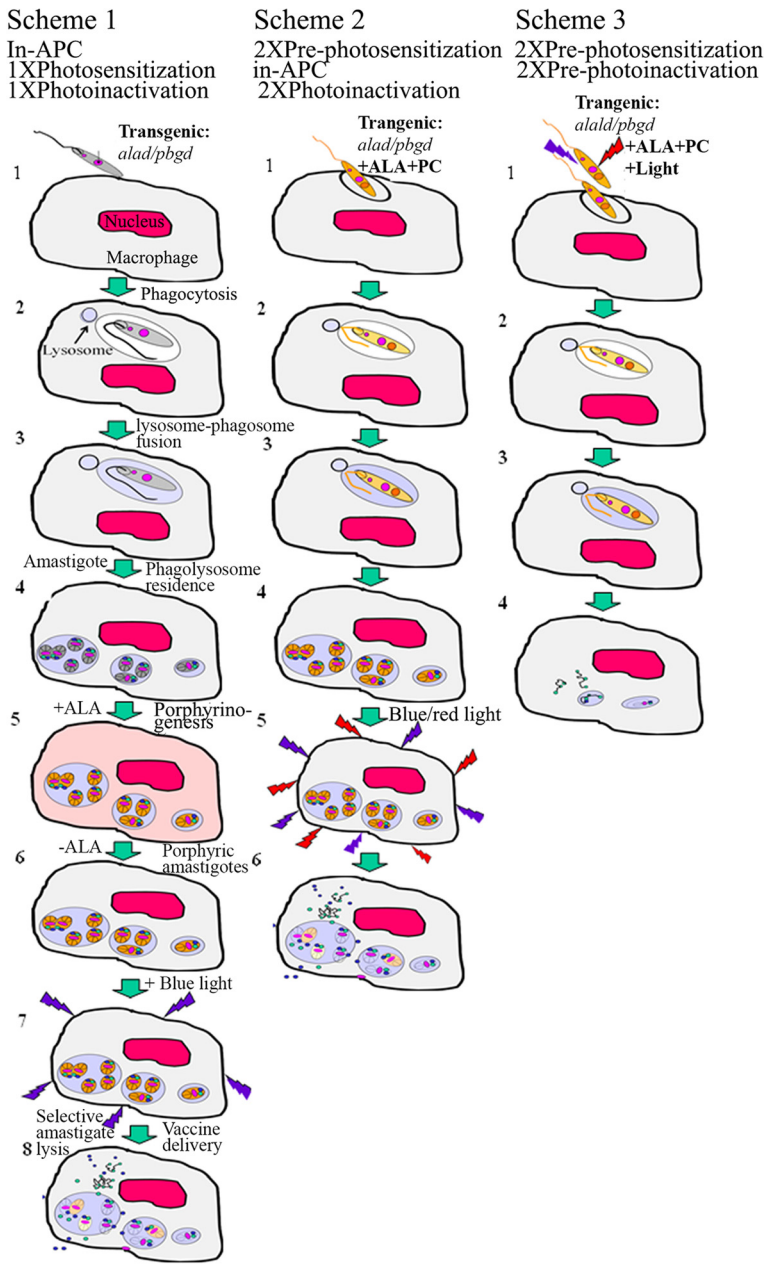

Fig. 2 Diagramatic illustration depicting three different schemes of *Leishmania*-based photodynamic vaccination in vitro. Transgenic:*alad/pbgd*, Porphyrinogenic *Leishmania* transfected with two mammalian cDNAs encoding the 2nd and 3rd enzymes in heme biosynthetic pathway, rendering them susceptible to delta-aminolevulinate (ALA)-induced neogenesis of uroporphyrin (URO); PC, Si-phthalocyanine photosensitizer [6, 11, 12]; *Light*, Illumination; *Blue* and *red lightening symbols*, *Blue* (400–500 nm wavelength) and *red* (~600 nm wavelength) for excitation of URO and PC, respectively. Scheme 1: In-antigen presenting cell (APC) single PS-sensitization/photo-inactivation [22]. 1–2, Phagocytosis of porphyrinogenic, but untreated *Leishmania* by APC; 3, Fusion of *Leishmania*-containing phagosome with lysosome; 4, *Leishmania* differentiation into amastigotes and their replication in the phagolysosomes; 5, Exposure of the parasitized APC to ALA, resulting in porphyrinogenesis of both APC and phagolysosomal amastigotes; 6, Removal of ALA, resulting in disappearance of porphyrins from APC and persistence of URO in amastigotes; 7–8, Illumination of these APC resulting in selective lysis of URO-loaded amastigotes, releasing vaccines into phagolysosomes and cytosol. Scheme 2: Same as Scheme 1, except that porphyrinogenic *Leishmania* are doubly PS-sensitized with ALA and PC in the dark before use for infecting APC [35]. 1–4, as described for Scheme 1, except that the *Leishmania* are pre-loaded with URO and PC, hence no further ALA treatment; 5–6, Illumination of the infected cells with blue and red light to excite URO and PC, lysing amastigotes with singlet oxygen and other ROS generated for releasing vaccines in APC. Scheme 3: Same as Schemes 1–2, except that *Leishmania* are pre-PS-sensitized and pre-photo-inactivated before use for vaccine delivery to APC [12]. 1–4, Uptake of oxidatively photo-inactivated *Leishmania* by APC, lysosome-phagosome fusion and their lysis to release vaccines as described

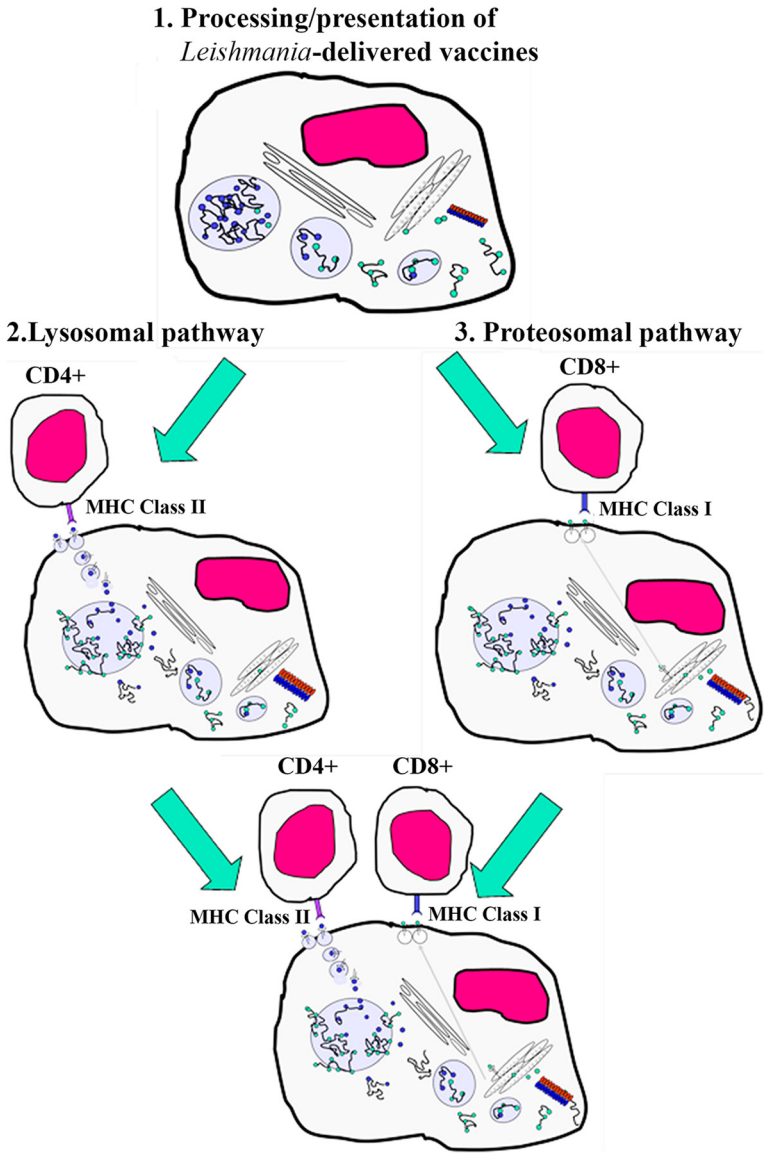

Fig. 3 Diagrammatic depiction of processing and presentation of *Leishmania*-delivered vaccines by antigen-presenting cells. 1, *Leishmania*-released vaccines represented as dots and lines in phagolysosomes and cytosol and relevant organelles: RER, Golgi and proteasome stacks; 2, Antigen presentation by lysosomal pathway via MHC Class II for activation of CD4+ T cells; 3, Antigen presentation by proteosomal pathway via MHC Class I for activation of CD8+ T cells; *Bottom*, Combination of both pathways shown in 2 and 3

the PS-sensitized *Leishmania*, as these ROS are too short-lived to cross multiple membranes to cause oxidative damage to the host APC. The endogenous antioxidants of APC are expected to protect themselves from other ROS generated secondarily from PDT. In addition, PDT may contribute positively to the APC functions in two ways: (1) Antigen processing by PDT-generated $^1O_2$ and/or other ROS via oxidative modifications of the APC proteases involved and/or the *Leishmania*-released antigens as their substrates, e.g. $^1O_2$ oxidation of their aromatic amino acid residues [23]; and (2) PDT-activation of ROS signal pathways favourable for the elicitation of immunity [24]. Clearly, the selective PDT-inactivation of intracellular *Leishmania* relieves their host APC of immunosuppression caused by the infection [22]. The subsequent processing of *Leishmania* vaccine antigens is predicted to follow the conventional lysosomal pathway (**Event 2**) and/or proteosomal pathway (**Event 3**) for co-presentation with MHC Class II and Class I molecules to activate CD4+ and CD8+ T cells, respectively (**Events 2–3**). The latter pathway is envisioned to proceed via cross presentation of *Leishmania* antigens, which are translocated from phagolysosomes to the cytosol. Other *Leishmaina*-derived factors

may further participate in the step of co-stimulation (not shown).

Experimental evidence The cell-mediated immunity depicted (Figs. 2 and 3) is based on the experimental outcome from the PDV schemes carried out in different experimental models, as briefly summarized below:

Scheme 1 was applied to immunization of Syrian Golden hamsters, eliciting a Th1 response for prophylaxis against Indian kala-azar produced by challenges with virulent *Leishmania donovani* [25]. The vaccination produces lasting immunity, as shown by the analysis of hepatosplenomegaly, parasite loads and cytokine profiles. Significantly, the immunity is adoptively transferable by splenic T cells from immunized animals to naïve hamsters, indicating that the immunity is cell-mediated and requires no antigen stimulation from persistent parasites, if any, at least in the recipients.

Scheme 2 was used for immunization of BALB/c mice against CL produced by challenges with *Leishmania amazonensis*. The observed prophylactic protection is significant, albeit incomplete, as indicated by comparing immunized mice *versus* the control groups. Immunization delayed the emergence of lesions for several weeks and significantly reduced the lesion size and their parasite loads by 10-fold *versus* the controls (Unpublished data. See legend to Fig. 4, Experimental-in-brief). The vaccination is considered effective, considering that BALB/c mice are known to bias toward Th2 with extreme levels of genetic susceptibility to cutaneous leishmaniasis.

Scheme 3 PDV used PDT-inactivated *Leishmania,* which were transfected to express ovalbumin (OVA) as a marker antigen or surrogate vaccine [12]. The cell-mediated immune responses to OVA delivered by PDT-inactivated transfectants were examined in in vitro and in vivo mouse models. APC loaded with the PDT-inactivated *Leishmania* were shown to deliver OVA, which was effectively processed for MHC Class I presentation of its specific peptide for activation of CD8+ T cell line [12]. In the in vivo studies, BL57 mice were immunized three times, each with ~10^6 PDT-inactivated OVA-*Leishmania*. Splenic T cells of these immunized mice were activated in response to CD4+ and CD8+ T cell-specific OVA peptides that increased proportionally with the number of immunizations (Unpublished data. See legend to Fig. 4, Experimental-in-brief). Most significantly, T cell activation is 6-fold higher with OVA delivered by PDT-inactivated *Leishmania* than that delivered by conventional means.

The safety of *Leishmania* PDT-inactivation for vaccination increases in the order of Schemes 1 to 3. *Leishmania* were singly and doubly PDT-inactivated for Scheme 1 and Schemes 2–3, respectively. They were completely inactivated by both PDT steps of PS-sensitization followed by double photo-inactivation before loading APC in Scheme 3 (see Fig. 4 and text for further discussion).

PDT-inactivation of *Leishmania* for vaccine delivery against other infectious and malignant diseases

The utility of PDT-inactivated *Leishmania* for delivery of add-on vaccines against other diseases is feasible, as indicated by the favourable outcome of the immune responses seen in vitro *and* in vivo to OVA delivered by this means. The successful delivery of OVA is significant, considering its expression at minuscule amount against a background of *Leishmania* proteins in overwhelming quantity and diversity in ~10^6 cells used for the delivery. This is taken to indicate that *Leishmania* creates no antigen-overload for vaccine delivery at least for OVA as a well-known T cell antigen.

Leishmania are naturally endowed with favourable attributes, making these parasites highly deployable as a universal vaccine carrier [22]. Many *Leishmania* species can be cultured safely as promastigotes in serum-free, chemically defined media [26] and scaled up for expansion [27]. The biosynthetic machineries of *Leishmania* are capable of high capacity transcription, translation and correct post-translational modification of foreign proteins. A number of efficient vectors are available for their abundant expression episomally or chromosomally as add-on vaccines in *Leishmania* - a favourable milieu of adjuvanticity and antigenicity conducive to elicit cell-mediated immunity.

Efficient delivery of add-on vaccines by *Leishmania* is due to their surface coat, consisting of unique lipid-saccharide-protein complexes [28]. In natural infection, they are known to protect *Leishmania* against the lytic humeral factors abundant in the animal body fluids and to target them to the phagolysosomes of APC. This mode of parasitism is further facilitated by the secretory products of *Leishmania*, e.g. nucleoside diphosphate kinase [29]. Full deployment of these molecular attributes by *Leishmania* is expected to protect the payload of add-on vaccines for homing to APC when using non-sensitized or PS-sensitized *Leishmania* for vaccine delivery according to Schemes 1–2 (Fig. 2). Notably, *Leishmania* PDT-inactivated according to Scheme 3 are no longer viable, but remain OVA-delivery competent. The integrity of their surface coat may account for this, since it is unaffected by the $^1O_2$, which is generated in and limited to the cytosol of PDT-inactivated *Leishmania*.

Uroporphyrinogenic *Leishmania* are being evaluated for their ability to serve as a carrier of candidate vaccines for trials against other infectious and malignant diseases [30–33]. PDV with PDT-inactivated *Leishmania* transfectants will follow Schemes 1–3 (Fig. 2) to obtain safety and efficacy data. In vitro vaccination of DCs will

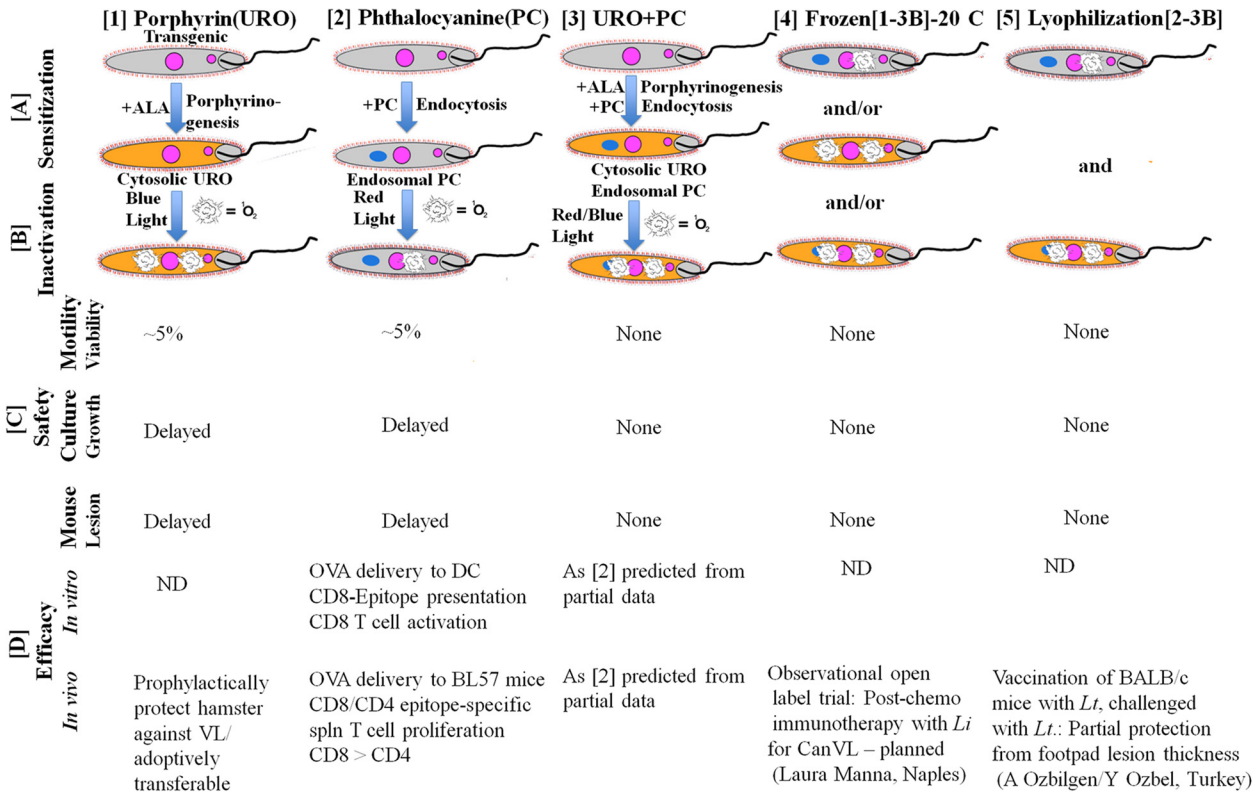

Fig. 4 Safety and efficacy evaluations of PDT-inactivated *Leishmania* prepared under different conditions for immuno-prophylaxis and therapy. *Horizontal*: **[1]** and **[2]**, Single PS-sensitization with ALA (+ALA) for uroporphyrin (URO) or Si-PC-loading (PC) followed by single photo-inactivation with blue or red light illumination for generation of cytotoxic $^1O_2$ (Symbol as shown), respectively [6, 7, 10–12]; **[3]**, Double PS-sensitization/double photo-inactivation using a combination of **[1]** and **[2]** conditions [35]; **[4–5]**, Singly or doubly PDT-inactivated *Leishmania* from **[1-3B]** stored frozen at −20 °C and lyophilized, respectively. See legend to Fig. 2 for other abbreviations used. *Vertical*: [A–B], PS-sensitization/photo-inactivation of *Leishmania* under the conditions as described in **[1–5]**. [C], Safety evaluations of the samples examined by microscopic observation for promastigote flagellar motility, for growth after inoculation into culture medium for 2 weeks, and lesion development after injection to BALB/c mouse ear dermis or tail base for ~60 days; [D], Efficacy evaluation in vitro and/or in vivo briefly summarized from published, on-going or planned studies. See text for **[1D]** and **[2D]** efficacy. Experimental-in-brief: see [25] for [1D in vivo]; see [12] for [2D in vitro]; [**2D in vivo**]: Groups of BL57 mice (~30 gm, 15/group) were immunized i.d. with 10^6 photo-inactivated OVA transfectants/10 ul PBS/ear for 3 times 1-week apart. Control groups were simultaneously and similarly immunized with un-treated, PC-sensitized, light-exposed, freeze-thawed OVA-transfectants, and 1 ug OVA. Splenic cells were collected from four mice from each group 2-weeks after 1–3 immunization for in vitro activation with OVA CD4- and CD8-specific peptides for ~4 days. Proliferation of CFSE-labeled lymphocytes was assayed flow cytometrically, providing the results briefly described in the text; [**3D in vivo**]: Female BLAB/c mice (~30 g) were immunized exactly as described for [**2D in vivo**], except that doubly PS-sensitized *Leishmania* were used. Controls included 6 groups using untreated, single PS-sensitized, light-exposed samples. Day 3 after immunization, photo-inactivation of *Leishmania* was carried out *in situ* at ~5 J/cm^2 using LumaCare LC-122 white light probe. Mice were each challenged at the tailbase with 10^7 parasites. Lesion size was measured weekly in all groups. Experiments were terminated after ~10 weeks when parasite loads were determined by limiting dilution method. Preliminary results obtained were briefly described in the text. *Abbreviations*: CanVL, canine leishmaniasis; *Li, Leishmania infantum; Lt, Leishmania tropica*

be pursued, as described [33, 34]. This presents a new approach by using a eukaryotic vehicle for safe and effective vaccine delivery.

Safety *versus* efficacy evaluation of five *Leishmania* PDT-inactivation formats

Figure 4 summaries the available data of *Leishmania*, which are PS-sensitized [A] and photo-inactivated [B] with or without additional treatments in different ways [1]–[5] for assessing their safety [C] and efficacy [D]. The safety is assessed after PDT inactivation of *Leishmania* by three different ways: microscopy for flagellar motility, cultivation for growth (2 weeks) and inoculation of mouse ear or tail base for lesion development (~2 months). Not all preparations were assessed by all criteria mentioned and the assessments for some samples are on-going or planned. The available results are briefly discussed below:

1. Single PDT of *Leishmania* by ALA-induced uropoprhyrinogensis [1] or PC-loading [2] alone inactivated ~95 % of these cells, as determined by the criteria described [C]. Interestingly, PDV based on protocol [1] elicited adoptively transferable cell-mediated

immunity and produced no visible pathology of the vaccination sites in hamster [25].
2. Double-PDT of *Leishmania* with a combination of Protocols 1-2 [3] resulted in no viable cells, as assessed by all three criteria [C], indicative of a complete inactivation [35]. Immunization of BALB/c mice according to [3] is protective, although incomplete due to their inherent sensitivity to CL, as already discussed.

Products [4] and [5] prepared by freezing and lyophilization of PDT-inactivated *Leishmania* [1–3B], respectively, were undertaken to facilitate their storage and transport and to increase their safety at the expense of their efficacy. Although still on-going, lyophilized samples [5] appear to have some prophylactic activities against CL challenges after immunization of BALB/c mice.

From the available data, the double-PDT inactivation of *Leishmania* by method [3] provides the best vaccination format for use with optimal safety and efficacy. The other regimens are being optimized for further safety *versus* efficacy evaluation.

Photodynamic insecticides (PDI)

Background

History PDT to control insect pests was first mentioned in the early 1900's (see [36]). From 1980's to1990's, The American Chemical Society published several symposium volumes on "Light-activated pesticides" [37–39]. Since then, follow-up publications have been limited and were summarized in the reviews [36, 40, 41]. Different dyes were used in experimental and/or field trials as PDI against various insects, mainly mosquito larvae and Mediterranean fruit flies. Industrial interests (PhotoDye International, Inc) included aerial spray of dye mixtures (xanthenes) or "SureDye®" (Red Dye #28 and Yellow Dye #8) (http://www.cdpr.ca.gov/docs/emon/pubs/ehapreps/suredye.htm) in attempt to control the latter pest. The work in the past decades showed some effectiveness of PDI, but this area of research has not gained attention.

Preamble PDI has the potential as an effective measure to control disease-transmitting vectors and other harmful insects. Development of resistance by insect pests to insecticides is a recurrent scenario [42], calling attention to different approaches, like PDT, which is unlikely to elicit resistance. The potential of PDI to control different insect pests are briefly discussed below.

Phytophagous insects cause substantial losses in crops and livestock despite the use of genetically modified (GM) insect-resistant plants [43]. Phloem/xylem sap-feeding insects cause additional damage by transmitting plant diseases. These vectors are PDT-targetable, since they engorge voluminous plant saps amenable to PS-loading and are translucent to light for photo-inactivation. The use of $^1O_2$-generating PS for PDT has the potential to discriminate these and other phytophagous insects for selective killing, sparing their photosynthetic and $^1O_2$-resistant host plants.

Many animal biting insects feed on blood and transmit serious diseases, accounting for substantial morbidity and mortality of domestic animal and human populations worldwide. Application of PDI to control such insect vectors is highly desirable, e.g. *Anopheles* mosquitoes, which transmit malaria and *Aedes* spp., which transmit Chikungunya, Dengue and Zika fever, causing epidemics in the tropical/subtropical world today. The only new non-PDI approach to control these vectors is to release GM mosquitoes based on *Wolbachia*- or male-induced infertility [44, 45]. For PDT of female mosquitoes and other blood feeders (phototropic and day-light active species), PS is deliverable via the bloodstream of susceptible hosts or the use of suitable baits to sensitize the insects for sun light inactivation. The larval stages of all mosquitoes (and also black flies) are aquatic and thus are receptive to water-soluble PS for PDT [46, 47].

PS-sensitization of all insects is possible by direct spraying for their uptake via surface contact and/or systematically via the hosts, as used for the current insecticides. Direct incorporation of PS into the drinking and food sources of insects will deliver them into the digestive tracts for sensitization of cells therein. In either case, accessibility of PS-sensitized cells to light is necessary to generate cytotoxic ROS for target destruction. Nocturnal and darkness-loving insects are less amenable to PDT unless a light-emitter is provided with the PS for their excitation.

Summarized below are some observations from our preliminary studies of few insects on their uptake of selected PS and susceptibility to PDI.

Screening of PS for their PDI against selected insects

Exposure of the 4th instar mosquito larvae (*Culex pipiens quinquefasciatus*) and adult sand flies (*Phlebotomus dubosqi*) [48] to rose bengal (RB) and cyanosine (CY) overnight resulted in the accumulation of these red dyes that are visible in the gut of the larvae (Fig. 5a) and of both female and male flies (Fig. 5b, c). Their uptake of the other PS examined is less clear, including aluminumphthalocyanine (Al-PC), protoporphyrin IX (PROTO) and Nile blue sulfate (NB). Only RB- and CY-sensitized larvae lost their viability after light exposure based on their mobility (not shown). The sand fly response to the PDI is inconclusive due to a high mortality of the control group, pending further investigation. This is also true for PDI of the plant-sucking insects, e.g. aphids, suggestive of a need

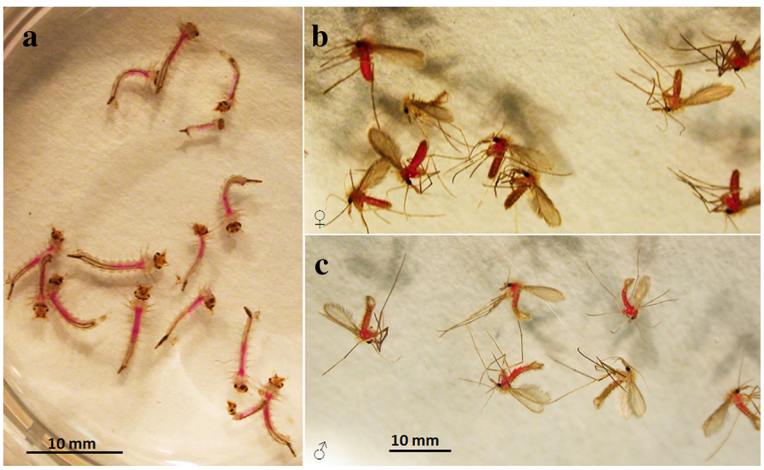

Fig. 5 Uptake of rose bengal by selected insects and their photo-inactivation. **a** *Culex pipiens quinquefasciatus* 4th instar larvae exposed to rose Bengal (10 ug/ml) (~20 larvae/5 ml water/well in 6-well plate) in dark for ~24 hours followed by exposure to white light for ~6 h at ~2500 lux; **b**-**c** *Phlebotomus duboscqi* female (**b**) and male (**c**) adult flies (~20 flies/screened paper cup) fed with 5 % sucrose solution and 500 ul of 5 mg/ml rose bengal in a cotton ball for ~20 h in the dark followed by exposure to ~2500 lux of white light for 3 h. Duplicate samples were prepared and kept in the dark as controls. Rose bengal is taken up by the flies of both sexes. Phototoxicity is evident for the mosquito larvae, but inconclusive for the flies. The tests were done in Petr Volf's lab

to use membrane-feeding techniques instead of using cut or potted plants [49, 50].

Our observations as described are preliminary, but represent the first study of PDI on sand flies, showing their uptake of PS used. The mosquito larvicidal activities of the PDI seen are consistent with the results of an early work (see [36]) and the reports using marigold alpha-terthienyl as the PS and different mosquito species [46, 47].

Uptake of PS by mosquito cells in vitro

Since the uptake of PS by mammalian and *Leishmania* cells is a prerequisite for their sensitization for PDT, we have begun to assess this with insect cells, e.g. *Aedes albopictus* clone C6/36 (ATCC CRL-1660). Figure 6 shows the uptake of RB and CY by these mosquito cells, rendering them sensitive to photo-inactivation. Untreated cells ([1]-None) are adherent (1A-DIF) and

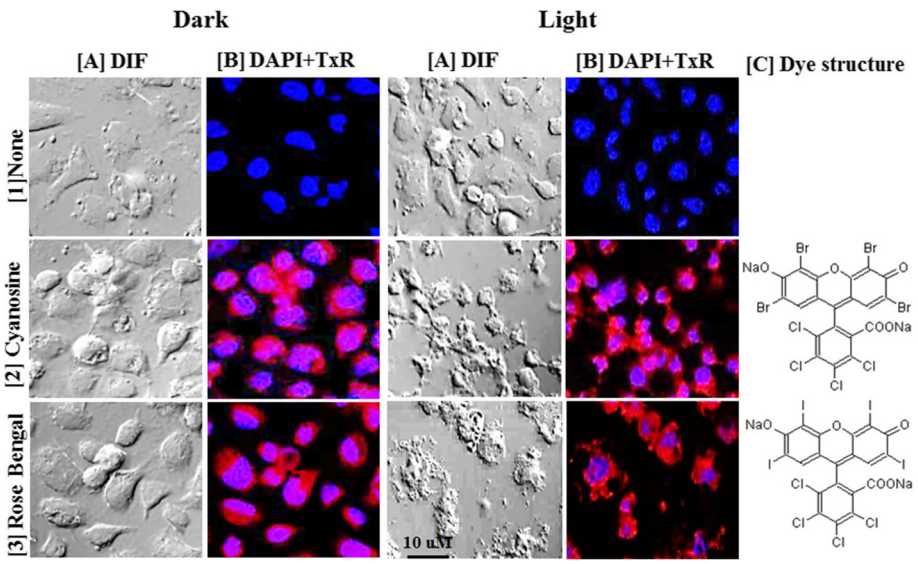

Fig. 6 Uptake of cyanosine and rose bengal by mosquito cells of the C6/36 line and their photosensitivity in vitro. The insect cells were exposed to both dyes overnight and illuminated with white light under conditions similar to those described for mammalian and *Leishmania* cells [10–12, 22]. Images were captured first under differential interference (DIF) [A] and then under the filter sets for DAPI and Texas red [B]. [C] Chemical structure of cyanosione and rose Bengal. Uptake of both dyes by the cells after incubation in the dark overnight (*Dark*, A2-3, B2-3) and cellular disintegration after light exposure for 4 h (*Light*, A2-3, B2-3) in contrast to the untreated controls (Dark and Light, A1, B1). Work done by Shin-Hong Shiao

non-fluorescent (1B DAPI + TxR), irrespective of illumination (1 Dark and Light). Cells exposed to CY [2] and RB [3] show cytoplasmic fluorescence (2B, 3B DAPI-txR), indicative of dye uptake. Sensitized cells remain adherent and intact (Dark, 2A, 3A-DIF), but become disintegrated after light-exposure (Light, 2A, 3A-DIF). These results are consistent with the larvicidal activities of RB and CY observed, providing a cellular basis for their PDT activities. Notably, the mosquito cells were not sensitized for PDT with the following PS: Al-PC [10], PC3-4 [11], NB and a porphyrin analogue [51]. Insect cells are thus similar to other cells in their requirement of PS uptake for susceptibility to PDT, but require different PS for PDI.

The preliminary data point to the feasibility of screening additional PS for PDT of cells from different insects, both harmful and beneficial, and from other life forms in their environments. Such in vitro screening of PS for activities has the potential to identify PDI, which discriminate harmful pests from beneficial insects and other friendly organisms for selective killing of the former. Of further interest is to elucidate the mechanisms of differential PS-uptake by cells of different origin, providing clues for designing PS with specificity for PDI targeting.

Conclusions

PDT-inactivation of *Leishmania* offers the versatility and flexibility to balance safety *versus* efficacy for vaccination against leishmaniasis and as potential carriers of vaccines against other infectious and malignant diseases (PDV). The development of this new approach will benefit from governmental and public acceptance and support. The ingenuity of the new leadership [52] is needed for novel regulation that will ensure the safety of vaccines with no barrier to disrupt innovation. The advocacy groups also call attention to rectify the existing barriers between science and cures, e.g. fasterCures (http://www.fastercures.org/). Development of vaccines including PDV will further benefit from effective measures against the anti-vaccination movement [53].

PDI represents an alternative approach to control insect pests. It is still in its early infancy of development despite the idea first emerged almost 100 years ago. Many PS for PDI are innocuous compounds, which have long been used among our everyday household products. Their application as PDI is not expected to select for resistance in contrast to the chemical pesticides in current use. PDI has the potential to complement the GM approaches in the field of agriculture and medicine. It will be particularly suitable for development in places where the population is sensitive to GM organisms.

The lynchpin between PDV and PDI is the PS for light excitation to generate cytotoxic ROS. The expertise in medicinal chemistry is essential for synthesis and design of novel PS. This depends on the input of biologists to elucidate the mechanisms of their cellular/molecular activities. New PS need to be assessed by expert clinicians, veterinarians, entomologists, cancer researchers, microbiologists and immunologists, hence the consortium of collaborators enlisted.

Abbreviations

ALA, delta-aminolevulinate; Al-PC, aluminum phthalycyanine; APC, antigen-presenting cells; CL, cutaneous leishmaniasis; CY, cyanosine; DIF, differential interference; DTH, delayed type hypersensitivity; GMO, genetically modified organisms; NB, Nile blue; OVA, ovalbumin; PC, phthalocyanines; PDI, photodynamic insecticide; PDT, photodynamic therapy; PDV, photodynamic vaccination; PROTO, protoporphyrin IX; PS, photosensitizer; RB, rose bengal; ROS, reactive oxygen species; URO, uroporphyrin I; VL, visceral leishmaniasis

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Collaboration group

Ramesh B. Batchu, PhD, Director, Division of Surgical Oncology & Developmental, Therapeutics, Associate Professor, The Michael and Marian Ilitch Department of Surgery, Wayne State University, 4646 John R Road, Detroit, MI 48201, USA. rameshbabu.batchu@gmail.com
Hui-Wen (Winni) Chen, DVM, PhD (陳慧文), Assistant Professor, Department of Veterinary Medicine, National Taiwan University, 1 Sec. 4 Roosevelt Rd., Taipei, 10617 Taiwan. winnichen@ntu.edu.tw
Larry Ming C. Chow, ScD (周銘祥), Associate Head and Professor, Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Hong Kong. larry.chow@polyu.edu.hk
Robert Elliott, MD/Jonathan F. Head, PhD, Elliott Mastology Center, 541 Shadows Lane, Baton Rouge, LA 70806, USA. relliott@eehbreastca.com
Chia-Kwung Fan PhD (范家堃), Professor and Chairman, Department of Molecular Parasitology and Tropical Diseases, School of Medicine and Center for International Tropical Medicine, College of Medicine, 250 Wu-Xing Street, Taipei Medical University, Taipei, Taiwan. tedfan@tmu.edu.tw
Chen-Hsiung Hung, PhD (洪政雄), Investigator and Vice Director, Institute of Chemistry, Academia Sinica, 128, Sec. 2, Academia Road,, Nankang, Taipei, Taiwan 11529. chhung@gate.sinica.edu.tw
Dar-Der Ji, PhD (稚達德), Associate Professor, Department of Tropical Medicine/International Health Programs, National Yang-Ming University, Beitou, Taipei 112, Taiwan. ddji@ym.edu.tw
Zhao-Rong Lun, PhD (伦照荣), Professor & Director, Center for Parasitic Organisms, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, P.R. China. lsslzr@mail.sysu.edu.cn
Laura Manna, PhD, DVM, School of Veterinary Medicine and Animal Productions, University of Naples, Federico II, Naples, Italy. laumanna@unina.it
Yoshitsugu Matsumoto, DVM, PhD, Professor, Laboratory of Molecular Immunology, School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Tokyo, Japan 113-8657 aysmatsu@mail.ecc.u-tokyo.ac.jp
Dennis KP Ng, DPhil (吳基培), Professor, Department of Chemistry, Chinese University of Hong Kong, Sha Tin, N. T., Hong Kong. dkpn@cuhk.edu.hk
Camila I. de Oliveira PhD/Sayonara Melo, Centro de Pesquisas Gonçalo Moniz. FIOCRUZ, R. Waldemar, Falcão, 121 Candeal, Salvador- BA -40296-710, Brazil. camila@bahia.fiocruz.br
Yusuf Ozbel, MD, Professor, Ege University Faculty of Medicine, Department of Parasitology, 35100 Bornova, Izmir,TURKEY. yusuf.ozbel@gmail.com
Ahmet Özbilgin. PhD, Professor, Celal Bayar Üniversitesi, Tip Fakültesi Parazitoloji A.D, Dekanlik Binasi Uncubozköy, 45030, Manisa, Türkiye. a.ozbilgin@yahoo.com
Joseph Reynolds, PhD, Department of Microbiology/Immunology, Chicago Medical School/RFUMS, N Chicago, IL 60064, USA. joseph.reynolds@rosalindfranklin.edu
Chizu Sanjoba, PhD, Laboratory of Molecular Immunology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1Yayoi, Bunkyo-ku, Tokyo 113-8657 Japan. asanjoba@mail.ecc.u-tokyo.ac.jp

Shin-Hong Shiao, PhD (蕭信宏), Assistant Professor, Department of Parasitology, Institute of Microbiology, School of Medicine, National Taiwan University, Taipei, Taiwan. shshiao@ntu.edu.tw
Nang-Yao Shih, PhD (施能耀), Associate Investigator, National Institute of Cancer Research, National Health Research Institutes, Tainan, Taiwan. jshih@nhri.org.tw
Chi-Wei Tsai, PhD (蔡志偉), Associate Professor, Department of Entomology, College of Natural Resources and Agriculture, National Taiwan University, 1 Sec. 4 Roosevelt Rd., Taipei, 10617 Taiwan. chiwei@ntu.edu.tw
Maria da Graça H. Vicente, PhD, Distinguished Professor, Department of Chemistry, 249 Chemistry and Materials Building, Highland Rd, Louisiana State University, Baton Rouge, LA 70803, USA. vicente@lsu.edu
Petr Volf, PhD, Professor, Department of Parasitology, Faculty of Science, Charles University Vinicna 7, 128 44 Praha 2, Czech Republic. volf@cesnet.cz
Yueh-Lung Wu, PhD (吳岳隆), Assistant Professor, Department of Entomology, College of Natural Resources and Agriculture, National Taiwan University, 1 Sec. 4 Roosevelt Rd., Taipei, 10617 Taiwan. runwu@ntu.edu.tw
Chao-Lan Yu, PhD (郁兆蘭), Professor, Department of Biomedical Sciences, Chang Gung University, No. 259, Wenhua 1st Rd., Guishan Dist., Taoyuan City 33302, Taiwan. clyu@mail.cgu.edu.tw
Xiao-Nong Zhou, PhD (周晓农),Professor and Director, National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, 207 Rui Jin Er Road, Shanghai 200025, P.R. China. zhouxn1@chinacdc.cn; ipdzhouxn@sh163.net

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The authors declare that they have no competing interests.

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