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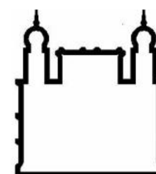

Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

# AVALIAÇÃO DA CAPACIDADE PROTETORA DE ANTÍGENOS RECOMBINANTES CONTRA A LEISHMANIOSE TEGUMENTAR

DIEGO MOURA SANTOS

SALVADOR – BAHIA – BRASIL

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**DIEGO MOURA SANTOS**

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Tese apresentada ao Colegiado do Curso de Pós-graduação em Patologia Humana, como pré-requisito obrigatório para obtenção do grau de Doutor.

**SALVADOR – BAHIA – BRASIL**

**2014**

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DIEGO MOURA SANTOS

Folha de Aprovação

Aos meus pais Daguimar do L. Tanajura Santos e Rita Sueli Moura Santos.

Ao meu irmão Raphaell Moura.

À minha esposa Nathale Prates.

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**RESUMO**

A leishmaniose é uma doença de escala global, que afeta 12 milhões de pessoas e pode causar um espectro de doenças que vai desde a forma cutânea localizada, que tende para a cura espontânea, até a forma visceral que é fatal. Apesar da gravidade da doença, até o momento não existe uma vacina efetiva para prevenir a leishmaniose. Dentre os antígenos promissores para o desenvolvimento de uma vacina, destacam-se as proteínas ribossomais (S4, S6, L3 e L5) e a KMP-11, uma proteína de superfície presente nos membros da família tripanosomatidae. Nosso estudo consistiu em avaliar os efeitos da imunização com estes antígenos frente ao desafio com *L. major* e com *L. braziliensis*, empregando modelos experimentais de infecção. Primeiramente, avaliamos a capacidade protetora dos antígenos ribossomais frente à infecção por *L. major*. Dos quatro antígenos avaliados, apenas L3 ou L5 foram capazes de prevenir o desenvolvimento da lesão e de diminuir a carga parasitária. A vacinação de camundongos com estes antígenos, na presença de CpG, induziu um perfil de resposta Th1, com elevada produção de IFN-γ, baixa produção de IL-10 e presença de anticorpos IgG2a. Em seguida, avaliamos a capacidade protetora dos antígenos L3 e L5 contra o desafio por *L. braziliensis,* na presença da saliva do vetor. A imunização com os antígenos L3 e/ou L5 também induziu uma elevada produção de IFN-γ, resultando em significativa redução na espessura da lesão e menor carga parasitária. Com relação ao antígeno KMP-11, investigamos a sua capacidade protetora utilizando duas estratégias vacinais: a estratégia homóloga que consistiu na imunização de camundongos com um plasmídeo de DNA que codifica KMP11 (DNA KMP-11) e a estratégia heteróloga que consistiu na imunização com nanopartículas de PLGA contendo DNA KMP-11, seguido de um reforço com nanopartículas contendo a proteína KMP-11 sob forma recombinante, na presença de CpG. As nanopartículas protegem o antígeno da degradação enzimática e promovem a liberação controlada deste, além de atuar como um adjuvante. Ambas as estratégias não impediram o desenvolvimento da lesão, após o desafio com *L. braziliensis* e na presença de saliva do vetor. Entretanto, os animais imunizados com a estratégia heteróloga apresentaram uma maior redução da carga parasitária comparado com o grupo imunizado pela estratégia homóloga. Este efeito foi associado com uma maior produção de IFN-γ e de TNF-α no sítio da infecção. Por fim, avaliamos a indução da resposta imune inata em macrófagos estimulados com KMP-11 encapsulados em nanopartículas. Observamos que a estimulação de macrófagos murinos com KMP-11, encapsulada em nanopartículas de PLGA, reduziu a carga parasitária intracelular e aumentou a produção de oxido nítrico, superóxido, TNF-α, IFN-γ, IL-6, IL-1β, IL-18, CCL2/MCP-1, CXCL-1/KC sugerindo a indução de uma potente resposta imune inata. Assim, concluímos que os antígenos L3 e/ou L5 mostraram ser promissores para o desenvolvimento de uma vacina que proteja contra as principais espécies de *Leishmania* e que o encapsulamento de antígenos em nanopartículas é capaz de induzir uma forte resposta imune. Essa estratégia deve ser considerada quanto ao desenvolvimento de vacinas para a leishmaniose.


Palavras-chaves: Leishmaniose cutânea; *Leishmania major*; *Leishmania braziliensis*; Proteínas ribossomais; KMP-11; Macrófagos e Nanopartículas.

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## ABSTRACT

Leishmaniasis is a global disease affecting 12 million people and can cause diseases that range from self-healing localized cutaneous leishmaniasis to fatal visceral leishmaniasis. Despite the severity of the disease, there is no effective vaccine to prevent leishmaniasis. Among the promising antigens for the development of a vaccine, stand out the ribosomal proteins (S4, S6, L3, and L5) and KMP-11, a surface protein, widely found in the members of family Trypanosomatidae. Our study evaluated the effects of immunization with these antigens upon challenge with *L. major* and *L. braziliensis*, employing the experimental models of infection. First, we evaluated the protective ability of ribosomal antigens to infection by *L. major*. Among the four antigens examined only L3 or L5 were able to prevent lesion development and decrease the parasite load. Mice vaccinated with these antigens, plus CpG, developed a Th1-type response with high production of IFN-γ, low production of IL-10 and presence of IgG2a antibodies. Next, we evaluated the protective capacity of L3 and L5 antigens against challenge by *L. braziliensis,* in the presence of sand fly saliva. Vaccination with L3 or L5 also induced a high production of IFN-γ, resulting in significant inhibition of lesion development and lower parasite load. Regarding KMP-11, we investigated its protective capacity using two immunization strategies: the homologous strategy, which consisted in immunizing mice with a plasmid DNA encoding KMP-11(DNA KMP-11) while the heterologous immunization strategy consisted of inoculation of PLGA nanoparticles (NPs) containing DNA KMP-11 followed by a booster inoculation with nanoparticles containing recombinant KMP-11, in the presence of CpG. Nanoparticles protect the antigen from enzymatic degradation and promote controlled release, in addition to acting as an adjuvant. Lesion development was not inhibited following either immunization strategy, after challenge with *L. braziliensis* in the presence of sand fly saliva. However, animals immunized with the heterologous strategy showed a greater reduction in parasite load compared with the group immunized by the homologous strategy. This effect was associated with increased production of IFN-γ e TNF-α at the infection site. Finally, we evaluated the induction of the innate response in macrophages stimulated with KMP-11 encapsulated in NPs. We observed that stimulation of murine macrophages with KMP-11 encapsulated in NPs reduced the parasitic load and increased production of nitric oxide, superoxide, TNF-α, IFN-γ, IL-6, IL-1β, IL-18, CCL2/MCP-1, CXCL-1/KC suggesting the induction of a potent innate immune response. We conclude that the L3 and/or L5 are promising antigens for the development of a vaccine that protects against the main species of *Leishmania* and that encapsulation of antigens into nanoparticles induces strong immune response. This strategy should be considered for the development of vaccines against leishmaniasis.



Key words: Cutaneous leishmaniasis; *Leishmania major*; *Leishmania braziliensis*; Ribosomal proteins; KMP-11; Macrophages and Nanoparticles.

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# INTRODUÇÃO

## 1.1 AS LEISHMANIOSES

As leishmanioses são doenças infecto-parasitárias, não contagiosas, causadas pelo parasita do gênero *Leishmania*. É considerada uma das grandes epidemias do mundo, e depois da malária, é a doença parasitária que mais leva a óbito (revisado em DEN BOER et al., 2011). Dados da Organização Mundial de Saúde demonstram que as leishmanioses são prevalentes em quatro continentes, com mais de 12 milhões de pessoas infectadas e mais de 350 milhões em risco de contrair a doença. Anualmente, são registrados cerca de dois milhões de casos novos, sendo 1,5 milhão de casos de Leishmaniose Tegumentar (LT) e 500.000 casos de Leishmaniose Visceral (LV) (revisado em DEN BOER et al., 2011). Nos últimos anos observou-se que o coeficiente de detecção da LT no Brasil aumentou de 10,45/100.000 para 18,5/100.000 habitantes (BRASIL 2000, 2007). A Região Norte e Nordeste apresentam os maiores números de casos da doença, com 40 e 31%, respectivamente, dos números de casos registrados no Brasil (BRASIL, 2009). Na Bahia, no período de 2010 a 2012, foram registrados 12.805 casos de Leishmaniose Tegumentar Americana (LTA), com uma incidência média de 30,28 casos por 100.000 habitantes (SESAB/SUVISA/DIS/SINAN, 2013).

A LT apresenta um amplo espectro de manifestações clínicas, sendo assim divididas em quatro grupos: leishmaniose cutânea-localizada (LCL), leishmaniose cutânea mucosa (LCM), leishmaniose disseminada (LD) e leishmaniose cutânea-difusa (LCD). No Brasil, a LT tem como principais agentes etiológicos a *L. braziliensis,* e a *L. amazonensis,* enquanto que, no Velho Mundo, as espécies responsáveis por esta manifestação da doença são, principalmente, a *L. tropica,* a *L. major* e a *L. aethiopica.* A LV difere da LT pois consiste em uma infecção generalizada que acomete o sistema retículo endotelial envolvendo o baço, fígado, medula óssea e linfonodo e pode ser fatal se não for tratada (revisado em BITTENCOURT; BARRAL-NETTO, 1995). As manifestações clínicas da leishmaniose dependem de complexas interações que abrangem desde a capacidade infectiva da espécie de *Leishmania* até o estado imunológico do hospedeiro humano (PEARSON; SOUSA, 1996). A cura clínica da leishmaniose nem sempre significa eliminação completa do parasita. Dessa forma, a permanência da *Leishmania* mantém constante uma resposta específica ao antígeno do parasita, dificultando a reinfecção (COUTINHO et al., 2002).

## 1.2 MODELO EXPERIMENTAL MURINO DE LT

O modelo experimental de LT mais amplamente estudado é o da infecção subcutânea de camundongos com *L. major*, no qual se empregam altas doses de parasitas ($10^4$-$10^7$), no sítio subcutâneo (base da cauda ou pata) (revisado em SACKS; NOBEN-TRAUTH, 2002). Neste modelo animal foi estabelecido o paradigma da resposta Th1/Th2 (SCOTT et al., 1988). Segundo este paradigma, camundongos resistentes (C57BL/6) apresentam expansão de células T $CD4^+$ do tipo Th1, que secretam IFN-γ, que leva à ativação de macrófagos e à destruição dos parasitas (GUMY et al., 2004). Uma vez curados da infecção primária, os animais ficam imunes a infecções subsequentes e isto se deve a persistência dos parasitas (SCOTT; FARRELL, 1982). Já camundongos suscetíveis (BALB/c) desenvolvem uma resposta celular do tipo Th2, com produção de IL-4, IL-5 e TGF-ß, citocinas que regulam de forma negativa a ativação dos macrófagos pelo IFN-γ (GUMY et al*.,* 2004). Assim, camundongos BALB/c infectados por *L. major* desenvolvem lesões necróticas extensas que levam os animais a óbito (SCOTT; FARRELL, 1982). No entanto, o modelo de infecção subcutânea não reproduz determinados aspectos da biologia natural da transmissão como a inoculação de baixas doses de parasitas, a presença da saliva do vetor e o sítio de inoculação. A saliva do vetor exerce um papel importante na modulação da hemostasia do hospedeiro, assim como na modulação da inflamação e da resposta imune (TITUS; RIBEIRO, 1990; revisado em OLIVEIRA et al., 2013). Sendo assim, Belkaid e colaboradores (1998) desenvolveram um modelo de infecção intradérmico, no qual cerca de 100 a 1000 parasitas são inoculados, na presença da saliva do vetor, na derme da orelha de camundongos. Neste modelo, o fenótipo de resistência (C57BL/6) e suscetibilidade (BALB/c) foi mantido, mas observou-se que a saliva contribuiu para a formação de lesões mais destrutivas e com maior número de parasitas (BELKAID et al., 1998).

Contrastando com o modelo de *L. major*, há um menor número de trabalhos realizados com *L. braziliensis*, embora essa espécie esteja associada com a LCM. A linhagem de camundongos BALB/c é suscetível à infecção por *L. braziliensis*, embora não desenvolva lesões incuráveis que possam levar ao óbito, como ocorre nas infecções por *L. major* (CHILDS et al., 1984). Como os camundongos BALB/c são mais resistentes a infecção por *L. braziliensis* quando comparada a infecção por *L. major*, pensava-se que este fenótipo estava ligado à capacidade da linhagem BALB/c de produzir níveis mais altos de IFN-γ no primeiro

tipo de infecção, do que no segundo. No entanto, DeKrey e colaboradores (1998) demonstraram que a produção de IFN-γ foi similar na infecção de BALB/c com *L. major* ou com *L. braziliensis*, entretanto, a produção de IL-4 foi menor em animais infectados com *L. braziliensis*. Além disso, camundongos infectados com *L. braziliensis*, quando tratados com anticorpos anti- IFN-γ, não são capazes de controlar a infecção, indicando que um mecanismo dependente de IFN-γ é responsável pela morte da *L. braziliensis* em camundongos BALB/c (DEKREY et al., 1998). A infecção de camundongos BALB/c com *L. braziliensis*, pela via intradérmica (DE MOURA et al., 2005) leva ao desenvolvimento de uma lesão cutânea similar à observada em pacientes com LCL, com a presença de uma lesão localizada, ulcerada, com bordas elevadas e fundo necrótico (LLANOS CUENTAS et al., 1984; JONES et al., 1987) reforçando assim, a importância do sítio da infecção no resultado da doença (BALDWIN et al., 2003). Apesar do desenvolvimento da lesão, o camundongo é capaz de curar a infecção por meio de uma reposta celular mista (Th1 e Th2), caracterizada pela presença de IFN-γ, IL-4 e IL-10. Os parasitas são eliminados do sítio de infecção, mas persistem no linfonodo de drenagem, indicando que a resposta imune não é do tipo esterilizante (DE MOURA et al., 2005). Empregando esse modelo, foi demonstrado que as células T $CD8^+$ estão associadas com a progressão da doença e exacerbação da lesão. Já que camundongos depletados de células T $CD8^+$ apresentaram um menor tamanho da lesão. O mecanismo imunopatológico desencadeado pelas células T $CD8^+$ está relacionado à produção excessiva de perforina e granzima. (NOVAIS et al., 2013). Resultados similares foram observados em pacientes infectados com *L. braziliensis*, nos quais as lesões mais necróticas estavam associadas com maior produção de granzima (FARIA et al., 2009; SANTOS et al., 2013).

## 1.3 ANTÍGENOS CANDIDATOS À VACINA NA LT

Existem evidências que sugerem a possibilidade de se desenvolver uma vacina que previna a leishmaniose: i) após a cura, os seres humanos desenvolvem uma imunidade protetora e de longa duração; ii) algumas pessoas desenvolvem um padrão de resposta imune parcialmente protetor que leva à resolução da lesão (revisado em ALVAR et al., 2013). Desta forma, é factível o desenvolvimento de uma vacina. Na LT humana, o desenvolvimento de uma resposta exacerbada do tipo Th1 não é indicativo de proteção (OLIVEIRA et al., 2011). Por outro lado, já foi demonstrada a importância da IL-10 na proteção, já que a neutralização

do IFN-γ diminui a produção de TNF-α de forma dependente da IL-10 (OLIVEIRA et al., 2014). Evidências para o papel imunopatológico do TNF-α na LT humana têm sido relatadas na literatura, por exemplo, pacientes apresentam um menor nível de TNF-α após tratamento contra a LC e LCM (DA-CRUZ et al., 1996; CARVALHO et al., 2013). Pacientes tratados com antimonial pentavalente combinado com pentoxifilina (inibidor do TNF-α) apresentam uma melhor taxa de cura, além de reduzir o tempo de cicatrização das ulceras cutâneas e mucosas (BÁFICA et al., 2003; MACHADO et al., 2007). Assim, uma vacina contra a LT humana deve induzir uma resposta imune similar àquela observada nos pacientes com a forma subclínica da doença, ou seja, uma resposta do tipo Th1 mais modulada (FOLLADOR et al., 2002; GOMES-SILVA et al., 2007). Em camundongos, a proteção contra *L. major* está associada com a ativação de uma resposta imune mediada por células T $CD4^+$ e $CD8^+$ com predomínio de resposta Th1 (GURUNATHAN et al., 2000; MENDEZ et al., 2002). Esse padrão de resposta reduz a lesão e controla a replicação parasitária (revisado em SACKS; NOBEN-TRAUTH, 2002).

A utilização de antígenos recombinantes no desenvolvimento de vacinas contra leishmaniose vem sendo veemente testada desde 1990, apresentando resultados variáveis (revisado em PALATNIK-DE-SOUSA, 2008). Dentre os muitos antígenos pesquisados, destacamos a GP63 (glicoproteína de 63 kDa) (MAZUMDER et al., 2011), a proteína LACK (homólogo do receptor para quinase C ativada) (HUGENTOBLER et al., 2012) e a vacina Leish-111f/L110 (SAKAI et al., 2010). A imunização de primatas não humanos com a GP63 levou à proteção parcial contra a infecção por *L. major* (OLOBO et al., 1995). No modelo murino, a imunização de camundongos BALB/c com o antígeno GP63 encapsulado em lipossomas juntamente com o adjuvante CpG protegeu contra infecção por *L. major*, através da indução de um perfil resposta Th1 (JAAFARI et al., 2007). A imunização com a proteína LACK, na presença da IL-12, induziu proteção inicial contra infecção por *L. major* (GURUNATHAN et al., 1997), mas não levou ao desenvolvimento de imunidade de longa duração, diferente da imunização com DNA + rIL-12. A imunização com DNA foi capaz de induzir células $TCD4^+$ e $CD8^+$ produtoras de IFN-γ, enquanto a imunização com a proteína LACK + rIL-12 somente induziu células $TCD4^+$ produtoras de IFN-γ (GURUNATHAN et al., 1998). A Leish-111f/L110 é uma poliproteína formada pela fusão de três antígenos (TSA, LmSTI1 e LeIF) que induziu proteção duradoura em camundongos contra infecção por *L.*

*major* (COLER et al., 2002) *e L. amazonensis* (COLER; REED, 2005), fato incomum para os antígenos proteicos. Dois componentes da Leish-111f/L110, os antígenos LmSTI1 (proteína de estresse induzível de *L. major*) e TSA (oxidante tiol específico) também mostraram ser eficazes contra a leishmaniose cutânea no modelo primata (CAMPOS-NETO et al., 2001), entretanto a Leish-111f/L110 não foi capaz de proteger cães naturalmente expostos a infecção por *L. infantum-chagasi* (GRADONI et al., 2005; FOGLIA MANZILLO et al., 2013). Gradoni e colaboradores (2005) demonstraram que 95% dos cães vacinados estavam infectados e os autores correlacionaram esta ausência de proteção com a produção de IL-4 e IL-10 e a baixa indução de resposta imune celular, que pode estar associada com a co-infecção por *Ehrlichia*, já que todos os animais estavam infectados por esta bactéria (GRADONI et al., 2005). Na mesma linha de raciocínio da Leish-111f/L110, existe o KSAC que é uma poliproteína formada pela fusão dos antígenos KMP-11 (proteína de membrana de kinetoplastída de 11 kDa), SMT (esterol 24-c-metiltransferase), A2 (antígeno de amastigota) e CPb (cisteína proteinase b). A imunização de camundongos com o KSAC protegeu contra infecção por *L. major* e por *L. infatum-chagasi* devido à indução de células Th1 multifuncionais produtoras de IFN-γ, TNF-α e IL-2 (GOTO et al., 2011), além de proteger os camundongos contra infecção natural através do vetor infectado por *L. major* (GOMES et al., 2012).

A KMP-11 é expressa tanto na forma promastigota quanto na forma amastigota da *Leishmania* (JARDIM et al., 1995; STEBECK et al., 1995) e vem sendo estudada como possível antígeno vacinal para prevenir tanto a LV, quanto a LT. A imunização de hamsters com plasmídeo que codifica a KMP-11 (KMP-11 DNA) protegeu contra desafio com cepas de *L. donovani* resistentes ou sensíveis ao Glucantime® (BASU et al., 2005). Todos os animais imunizados sobreviveram ao desafio apresentando cargas parasitárias muito reduzidas no fígado e no baço quando comparados com o grupo controle. A imunização levou à uma produção elevada de citocinas pró-inflamatórias e anti-inflamatórias e ao aumento da produção de óxido nítrico por esplenócitos estimulados *in-vitro* (BASU et al., 2005). Utilizando o mesmo modelo experimental, Guha e colaboradores (2013) demonstraram que a imunização com DNA seguida do reforço com o vírus da *Vaccinia*, expressando KMP-11, levou à proteção contra a cepa homóloga de *L. donovani* sensível e também contra uma outra cepa, resistente ao Glucantime®. Resultados similares foram observados no modelo murino

de infecção. A proteção foi associada com maior presença de células T $CD4^+$ e $CD8^+$ produtoras de IFN-γ, IL-2, TNF-α e menor produção de IL-10 (GUHA et al., 2013). Da mesma forma, o tratamento de camundongos com células dendríticas ou macrófagos transfectados com o plasmídeo que codifica a KMP-11 foi capaz de curar camundongos infectados com *L. donovani*, devido a forte resposta imune mediada por células T $CD8^+$ (BASU, 2007a;b).

Após a destruição dos parasitas por mecanismos imunes ou por citólise (revisado em CHANG et al., 2003), postula-se que proteínas intracelulares são liberadas para o meio externo e modulam a resposta imune, podendo levar à progressão ou resolução da doença (revisado em SANTARÉM et al., 2007). Tais proteínas são conhecidas como pan-antígenos (revisado em REQUENA et al., 2000). Ao contrário das proteínas de superfície e das proteínas secretadas, os pan-antígenos são capazes de modular a resposta imune do hospedeiro por não sofrerem pressão seletiva do sistema imune. Portanto, os pan-antígenos podem prover a capacidade imunogênica necessária para o desenvolvimento de vacinas (revisado em REQUENA et al., 2000). Nesse sentido, alguns grupos vem estudando a capacidade protetora das proteínas ribossomais e histonas nucleossomais, consideradas como pan-antígenos.

A imunização de camundongos BALB/c com plasmídeos de DNA que codificam as histonas H2A, H2B, H3 e H4 da *L. infantum-chagasi* levou à proteção contra infecção por *L. major* através da indução de células $TCD4^+$ e $CD8^+$ produtoras de IFN-γ (IBORRA et al., 2004; CARRIÓN et al., 2008). O mesmo foi observado quando os camundongos foram vacinados com células dendríticas pulsadas com as quatro proteínas ribossomais (CARRIÓN et al., 2007). Carneiro e colaboradores (2012) demonstraram que camundongos BALB/c vacinados com as histonas H2A, H2B, H3 e H4 empregando duas estratégias diferentes, homóloga (somente DNA) e heteróloga (DNA mais um reforço com proteínas recombinantes), protegeram contra infecção por *L. braziliensis* na presença da saliva do vetor. O sucesso desta vacinação foi atribuído a uma maior produção da citocina inflamatória, o IFN-γ e uma menor produção de citocinas anti-inflamatórias IL-4 e IL-10 no sítio da infecção (CARNEIRO et al., 2012).

A imunização de camundongos BALB/c com o extrato de proteínas ribossomais da *L. major* (LRP – proteínas ribossomais da *L. major*) mais CpG levou à proteção e esta foi correlacionada com a alta produção de IFN-γ e baixa produção de IL-4 e IL-10. O mesmo foi observado no modelo naturalmente resistente de infecção, o C57BL/6 (IBORRA et al., 2008). Chávez-Fumagalli e colaboradores (2010) demonstraram que a imunização de camundongos BALB/c, com extrato de LRP extraído da *L. infantum-chagasi* associado com a saponina, protegeu contra infecção por *L. infantum-chagasi* e *L. amazonenses,* através da indução de um padrão de resposta Th1, com alta produção de IFN-γ, IL-12, NO e baixa produção de IL-4 e IL-10 (CHAVEZ-FUMAGALLI et al., 2010). Por outro lado, a imunização de camundongos BALB/c com as proteínas LiP0, LiP2a e LiP2b, que são componentes do extrato da LRP, não protegeu contra infecção por *L. major* e os animais imunizados desenvolveram uma resposta mista Th1/Th2, com elevada produção de IL-4 e anticorpos da subclasse IgG1 (IBORRA et al., 2003, 2005, 2007). Outros antígenos que fazem parte da subunidade maior do ribossomo são as proteínas S4, S6, L3 e L5 (RAMIREZ et al., 2013). Os dois últimos antígenos (L3 e L5), associados com o CpG, mostraram ser promissores para o desenvolvimento de uma vacina eficaz contra a LT, causada por *L. amazonensis* e contra a LV, causada por *L. infantum-chagasi.* Os camundongos vacinados e desafiados desenvolveram um padrão de resposta Th1, com elevada produção de IFN-γ e baixa produção de IL-4 e IL-10 (RAMIREZ et al., 2014). As proteínas L3 e L5 são altamente conservadas entre as espécies de *Leishmania*. Entretanto, apresentam uma baixa homologia com as mesmas proteínas humanas (RAMIREZ et al., 2013). Nenhuma resposta humoral ou celular contra as proteínas ribossomais murinas foi detectada nos animais imunizados com estas proteínas (RAMIREZ et al., 2014).

Como descrito anteriormente, vários trabalhos demonstraram que a imunização de camundongos com antígenos e adjuvantes como o CpG foi capaz de conferir proteção contra o desafio por *Leishmania* (JAAFARI et al., 2007; IBORRA et al., 2008; CARNEIRO et al., 2012; RAMIREZ et al., 2014). Os motivos de CpG são regiões ricas em sítios de citosina-guanina (CG) não metilados, que estimulam o sistema imune através do TLR-9, ativando moléculas adaptadoras MyD88, IRAK e TRAF-6 levando ao recrutamento de fatores transcricionais que aumentam a produção de citocinas pró-inflamatórias (IL-1, IL-6, IL-12, IL-18 e TNF-α), comprovando que o CpG é um eficiente indutor de resposta Th1 (GUO et al.,

2007; WU et al., 2011; revisado em AWATE et al., 2013). O CpG também eleva a expressão de moléculas co-estimuladoras como CD40, CD80, CD86, moléculas de MHC-II, além de aumentar o processamento e a apresentação de antígenos pelas células dendríticas (KERKMANN et al., 2003; HUA et al., 2013; CHANDEL et al., 2014), estimular as células B (HERBÁTH et al., 2014) e macrófagos (SATHE; REDDY, 2014). O CpG começa a agir logo após a sua aplicação, aumentando a expressão de genes relacionados com a resposta imune inata e adaptativa (KLASCHIK et al., 2010), e seu período de ação pode durar até algumas semanas (VERTHELYI et al., 2003; ITO et al., 2004).

Além dos motivos CpG, uma outra estratégia capaz de melhorar a resposta imune é a utilização de sistemas de veiculação, que melhoram a apresentação do antígeno para os componentes do sistema imune, como os lipossomos e nanopartículas (ILYINSKII et al., 2014; NEELAND et al., 2014).

## 1.4 NANOPARTÍCULAS

A definição de nanopartículas (NPs) é baseada não somente no tamanho da estrutura, 1 a 1.000 nm, como também nas características físicas, químicas e biológicas que estes materiais apresentam nestas dimensões (Food and Drug Administration - FDA, 2011; European Medicines Agency - EMA, 2012). As NPs podem ser produzidas com diversos tipos de materiais biodegradáveis e biocompatíveis, como por exemplo a albumina, chitosan, lipídeos, ácido glutâmico, polietilenoglicol (PEG) ácido láctico [PLA - poli(ácido láctico)], ácido glicólico [PLG - poli(ácido glicólico) e suas combinações [PLGA - poli(ácido láctico-co-glicólico)] (JONES et al., 1997; ADITYA et al., 2013; UTO et al., 2013; ZHAO et al., 2014). Partículas de PLGA são biodegradáveis, pois quando administradas são clivadas em ácido lático e em ácido glicólico, que são eliminados do corpo pelo ciclo do ácido cítrico, tendo como produtos finais $CO_2$ e água (revisado em SINHA & TREHAN, 2003). Desta forma, as NPs de PLGA, devido a sua segurança e eficácia, vêm sendo muito utilizada em ensaios vacinais, e para desenvolvimento e melhoramento de novas drogas (revisado em SMITH et al., 2013; SHAH et al., 2014) . É importante salientar que as características físico-químicas da NPs influenciam na resposta imune observada. Assim, NPs com cargas positivas e tamanhos entre 300–1000 nm melhoram a entrega do antígeno para as células apresentadoras de antígenos (APCs), em comparação com o antígeno puro; além de diminuir

a quantidade de antígeno administrado para induzir uma potente resposta imune (SINGH et al., 2000). A fagocitose das partículas pelas APCs possibilita que o antígeno seja levado para os linfonodos para processamento e apresentação (KANCHAN & PANDA, 2007; SHIMA et al., 2013). Células dendríticas estimuladas com NP apresentaram a expressão de genes relacionados com o perfil de resposta Th1 tais como IL-1β, IL-2 IL-6, IL-12, TNF-α e IFN-γ. Entre as quimiocinas e seus receptores foi observado um aumento na expressão da CXCL-1, CXCL-2, CCR-7, assim como de moléculas co-estimuladoras (CD40, CD80 e CD86). As NPs induziram uma maior porcentagem de células T $CD8^{+}$ produtoras de IL-2, TNF-α e IFN-γ, comprovando assim o grande potencial das NPs não só de entrega do antígeno, mas também como um bom imunoestimulador (HAMASAKI et al., 2010).

O antígeno quando administrado encapsulado em NPs, pela via subcutânea, permanece no local da aplicação por até 30 dias, diferente da administração do antígeno puro que é rapidamente eliminado do local. Esta maior permanência do antígeno é importante para a indução de uma resposta imune mais robusta e também colabora para uma diminuição do número de reforços da vacina e da quantidade de antígeno administrado (TOITA et al., 2013). Ensaios *in-vitro* demonstraram que logo após a sua aplicação (30 min), as NPs estão co-localizadas nas células dendríticas. Neste mesmo período de tempo, foi observado no modelo experimental que as NPs estavam presentes por toda pele do camundongo e em vários órgãos (rim, fígado, cérebro, pâncreas, baço, coração, intestino e pulmão), sugerindo que teve distribuição das NPs pelo corpo (MA et al., 2012).

As NPs com tamanho variando entre 200-600 nm são preferencialmente fagocitadas pelos macrófagos (KANCHAN; PANDA, 2007; ROBERTS et al., 2013), levando a produção de IFN-γ e aumento na expressão do MHC-I (KANCHAN; PANDA, 2007). Assim, além de atuarem como um sistema de liberação de antígenos, as NPs possuem atividades de adjuvantes pois são capazes de estimular tanto o sistema imune inato, quanto o adaptativo. O recrutamento e ativação de células da imunidade inata, assim como a indução de IL-6 pelas NPs, são mediados pela via do complexo protéico NALP3/inflamassoma nas APCs (SHARP et al., 2009). Diversos tipos de NPs induzem a ativação do inflamassoma pela via do NALP3 (DOSTERT et al., 2008; LUNOV et al., 2011; ZHANG et al., 2012; CAICEDO et al., 2013). Para ativação do inflamassoma por esta via são necessários dois sinais: i) o primeiro sinal leva a produção da forma inativa da IL-1β e IL-18 (pro-IL-1β e pro-IL-18), através do

reconhecimento dos ligantes de TLRs, receptor de IL-1 e receptor de TNF-α. ii) O segundo sinal é desencadeado por uma série de substâncias como toxinas bacterianas, ATP, desestabilização do lisossomo e consequente liberação da catepsina B, efluxo de $K^+$ e a fagocitose de matérias particulados, levando a ativação do complexo multiprotéico do inflamassoma que ativa a caspase-1, levando a produção das formas ativas da IL-1β e IL-18 (revisado em KIM & JO, 2013). Trabalhos na literatura já demonstraram a importância da ativação do inflamassoma na proteção contra diversos tipos de infecções (COSTA et al., 2012; CUNHA; ZAMBONI, 2013; GOMES et al., 2013).

Diante dessas propriedades, as NPs vêm sendo testadas como veículos vacinais em diferentes modelos experimentais, para combater diferentes patologias tais como: hepatite B (CHONG et al., 2005; MISHRA et al., 2011; PAWAR et al., 2013); AIDS (ZHOU et al., 2008; ALINE et al., 2009); tuberculose (BIVAS-BENITA et al., 2009; ANDRADE et al., 2013); malária (MOON et al., 2012) e câncer (DOMINGUEZ; LUSTGARTEN, 2010; MISHRA et al., 2011; ANDRADE et al., 2013; CHEN et al., 2014).

Com relação à leishmaniose, algumas formulações utilizando partículas foram testadas como possíveis vacinas contra LT (Tabela 1). A imunização de camundongos BALB/c com *L. major* autoclavada (ALM), encapsulada em NPs de PLGA, reduziu de forma significativa a lesão dos animais desafiados com *L. major* (TAFAGHODI et al., 2010). A redução da lesão também foi observada quando o adjuvante CpG foi adicionado na formulação e essa proteção foi associada com uma maior indução de resposta Th1 (TAFAGHODI et al., 2011). Como pode ser observado na Tabela 1, todos os trabalhos utilizando NPs como veículo vacinal para a prevenção da LTA foram testados no modelo murino de infecção por *L. major*. Até o momento, nenhum trabalho foi realizado para avaliar a utilização de NPs como veículos de antígenos para a profilaxia da LTA, causada por *L. braziliensis*.

Tabela 1. Antígenos encapsulados em partículas candidatos a vacinas contra LT.

| **Antígeno** | **Hospedeiro** | **Espécie** | **Partícula** | **Comentários** | **Referências** |
|---|---|---|---|---|---|
| HSP65 DNA | BALB/c | Lm | PLGA–MP | Proteção[1] parcial. Redução da carga parasitária em 44,4%. | (COELHO et al., 2006) |
| LmSTI1 | BALB/c | Lm | Lipossoma | Proteção. Não foram detectados parasitas nos animais imunizados. | (BADIEE et al., 2008) |
| ALM | BALB/c | Lm | PLGA-NP | Proteção. A carga parasitária não foi avaliada. | (TAFAGHODI et al., 2010) |
| ALM+CpG | BALB/c | Lm | PLGA-NP | Proteção. A carga parasitária não foi avaliada. | (TAFAGHODI et al., 2011) |
| CPb proteína | C57BL/6 | Lm | SLN | Proteção. Redução da carga parasitária em 50,0% no LNd. | (DOROUD et al., 2011b) |
| CPa/b DNA | BALB/c | Lm | SLN | Proteção. Redução da carga parasitária em 41,4% no LNd. | (DOROUD et al., 2011c) |
| CPa/b/c DNA | BALB/c | Lm | SLN | Proteção. Redução da carga parasitária em 65,4% e 86,3% no LNd e pata, respectivamente. | (DOROUD et al., 2011a) |
| SLA | BALB/c | Lm | Lipossoma | Proteção. Redução significativa da carga parasitária no baço e pata. | (FIROUZMAND et al., 2013) |

[1] – Nesta tabela o termo proteção é definido como redução do tamanho da lesão.

Lm – *Leishmania major*; MP – micropartículas; NP – nanopartículas; SLN – nanopartículas sólidas de lipídios; SLA – Antígeno solúvel de *Leishmania*; LNd – linfonodo drenante.

# 2 JUSTIFICATIVA

Diferente do modelo de camundongo de infecção por *L. major* para testes de vacinas, são poucos os trabalhos na literatura sobre o tema no modelo murino de infecção por *L. braziliensis*. Salay e colaboradores (2007) utilizaram quatro antígenos (LACK, TSA, LmSTI1 e LeIF) da *L. braziliensis* que apresentam um alto grau de homologia com os antígenos da *L. major* e que foram protetores contra este último parasita (revisado em OKWOR; UZONNA, 2009). Entretanto, a imunização de camundongos BALB/c com estes antígenos falhou em induzir proteção contra o desafio por *L. braziliensis* (SALAY et al., 2007). Apesar da importância e gravidade da LT causada por *L. braziliensis*, trabalhos experimentais envolvendo essa espécie são escassos. Assim, justifica-se investigar se os antígenos ribossomais (S4, S6, L3 e/ou L5) e o KMP-11, relatados na literatura como capazes de proteger contra a infecção por *L. infantum-chagasi* e *L. amazonensis* (RAMIREZ et al., 2014), e *L. donovani e L. major* (BHAUMIK et al., 2009) respectivamente, são também capazes de conferir proteção contra a infecção por *L. major* e por *L. braziliensis*.

Além disso, para o desenvolvimento de vacinas eficazes é muito importante o tipo de formulação vacinal, que compreende o antígeno utilizado, o adjuvante e até mesmo o sistema de liberação de antígenos. Portanto, avaliamos também a utilização de NPs de PLGA como um possível sistema de liberação de antígenos para formulações de vacinas contra a LT e sua capacidade como adjuvante.

# 3 HIPÓTESES

**Hipótese I**

A imunização com os antígenos ribossomais (S4, S6, L3 e/ou L5) confere proteção contra a infecção por *L. major* e por *L. braziliensis* e reduz a carga parasitária após o desafio.

**Hipótese II**

A imunização com KMP-11, encapsulado ou não em NPs de PLGA, confere proteção contra  a infecção por *L. braziliensis* e reduz a carga parasitária após o desafio.

**Hipótese III**

NPs de PLGA contendo KMP-11 possuem atividade imunoestimulatória, levando à ativação de macrófagos e à destruição de parasitas.

# 4 OBJETIVOS

## 4.1 OBJETIVO GERAL

Avaliar a resposta imune e a capacidade protetora de antígenos ribossomais e do KMP-11 contra a LT causada por *L. major* e *L. braziliensis*.

## 4.2 OBJETIVOS ESPECÍFICOS

4.2.1 Avaliar a antigenicidade das proteínas S4, S6, L3 ou L5;

4.2.2 Avaliar a capacidade protetora das proteínas S4, S6, L3 ou L5 contra a infecção por *L. major*;

4.2.3 Avaliar a capacidade protetora dos antígenos L3 e/ou L5 contra a infecção por *L. braziliensis* + saliva do vetor;

4.2.4 Analisar a imunogenicidade do antígeno KMP-11 encapsulado ou não em NPs;

4.2.5 Analisar a capacidade protetora do antígeno KMP-11, encapsulado ou não em NPs, contra a infecção por *L. braziliensis* + saliva do vetor.

4.2.6 Investigar a resposta inflamatória de macrófagos estimulados com NPs contendo KMP-11 e infectados com *L. braziliensis*;

4.2.7 Determinar a carga parasitária em macrófagos estimulados com KMP-11 encapsulado em NPs.

# 5. MANUSCRITOS PUBLICADOS

MANUSCRITO I

Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis.

Este artigo foi publicado no periódico Vaccine.

MANUSCRITO II

Towards development of novel immunization strategies against leishmaniasis using PLGA nanoparticles loaded with kinetoplastid membrane protein-11.

Este artigo foi publicado no periódico Internacional Journal of Nanomedicine.

MANUSCRITO III

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

Este artigo foi publicado no periódico Nanomedicine: Nanotechnology, Biology and Medicine.

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# Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis

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ABSTRACT

Four new antigenic proteins located in *Leishmania* ribosomes have been characterized: S4, S6, L3 and L5. Recombinant versions of the four ribosomal proteins from *Leishmania major* were recognized by sera from human and canine patients suffering different clinical forms of leishmaniasis. The prophylactic properties of these proteins were first studied in the experimental model of cutaneous leishmaniasis caused by *L. major* inoculation into BALB/c mice. The administration of two of them, LmL3 or LmL5 combined with CpG-oligodeoxynucleotides (CpG-ODN) was able to protect BALB/c mice against *L. major* infection. Vaccinated mice showed smaller lesions and parasite burden compared to mice inoculated with vaccine diluent or vaccine adjuvant. Protection was correlated with an antigen-specific increased production of IFN-γ paralleled by a decrease of the antigen-specific IL-10 mediated response in protected mice relative to non-protected controls. Further, it was demonstrated that BALB/c mice vaccinated with recombinant LmL3 or LmL5 plus CpG-ODN were also protected against the development of cutaneous lesions following inoculation of *L. braziliensis*. Together, data presented here indicate that LmL3 or LmL5 ribosomal proteins combined with Th1 inducing adjuvants, may be relevant components of a vaccine against cutaneous leishmaniasis caused by distinct species.

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## 1. Introduction

Infection with *Leishmania* protozoan parasites can result in the development of leishmaniasis. Several species cause cutaneous leishmaniasis (CL) including *Leishmania major* and *L. braziliensis* (in the Old or New World, respectively); the latter is also related with development of mucocutaneous leishmaniasis (MCL). Visceral leishmaniasis (VL) is caused by the infection of *L. chagasi* in the New World or *L. infantum* and *L. donovani* in the Old World [1]. In natural and experimental leishmaniasis, effective primary immunity requires the production of IFN-γ by CD4+ T cells and to a minor extend by CD8+ T cells, which mediates nitric-oxide-depending parasite killing by the activation of infected macrophages [2]. Disease progression is related with the induction of humoral and IL-10 mediated responses [3].

A preparation of biochemically purified *Leishmania* ribosomal proteins (LRP) administered with CpG-oligodeoxynucleotides (CpG-ODN) conferred protection against challenge with *L. major* parasites in susceptible BALB/c and resistant C57BL/6 mice. Protection was correlated with a LRP-specific IL-12 dependent production of IFN-γ (in both mouse strains) and a diminished production of both IL-4 and IL-10 in BALB/c mice [4]. Also, BALB/c mice vaccinated with LRP plus CpG-ODN and subsequently infected were able to resist a secondary challenge [5]. A protective response against *L. chagasi* and *L. amazonensis* was observed in BALB/c mice when a LRP-specific IFN-γ mediated response was induced by administration of LRP combined with saponin [6].

In this work, four *L. major* ribosomal antigens have been characterized: LmS4, LmS6, LmL3 and LmL5. Their prophylactic properties were assayed in the *L. major*-BALB/c mouse model of CL. Since immunization of LmL3 or LmL5 combined with CpG-ODN elicited

*Abbreviations:* CpG-ODN, CpG-oligodeoxynucleotides; VL, visceral leishmaniasis; MCL, mucocutaneous leishmaniasis; CL, cutaneous leishmaniasis; LRP, *Leishmania* ribosomal proteins; SLA, soluble *Leishmania* antigens; MRP, mouse ribosomal proteins; CR, coding region; DLN, draining lymph nodes; OPD, ortophenylediaminedihydrochloride.

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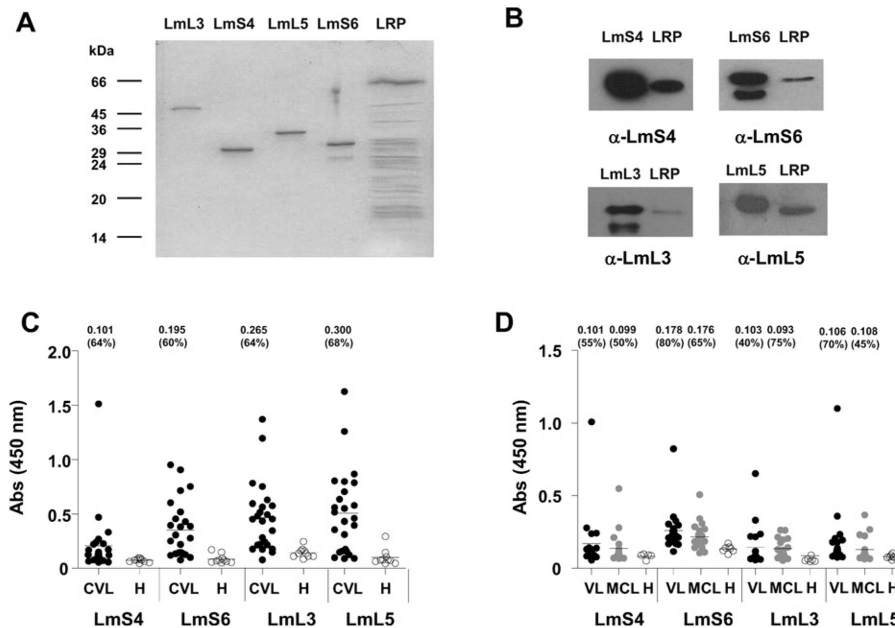



**Fig. 1.** Characterization of the LmS4, LmS6, LmL3 and LmL5 as antigenic components of the *Leishmania* ribosome. (A) One μg of each recombinant protein and 10 μg of *Leishmania major* LRP extracts were electrophoresed on a linear 10–13% gradient SDS-PAGE gel stained with Coomasie blue (B). Gels loaded with each one of the recombinant proteins and the LRP extracts were blotted and individually probed with the sera from mice immunized with LmS4, LmS6 and LmL3 (1/100) or with anti-LmL5 antibody fraction affinity purified from five canine VL sera. Horseradish peroxidase-conjugated anti-mouse (1/2000) or anti-dog IgG (1/2000) antibodies were used as the secondary reagents. (C) Antibody responses of canine VL (CVL, $n = 25$) and healthy animals' sera (H, $n = 8$) against the recombinant proteins. (D) Antibody response of human VL patients ($n = 20$), MCL ($n = 20$) and healthy individuals (H, $n = 8$) against the four recombinant proteins. All sera were tested for IgG reactivity by ELISA (1/200). Horseradish peroxidase-conjugated anti-dog (1/2000) or anti-human IgG (1/2000) antibodies were used as the secondary reagents. The OD value of each individual serum is shown. Bars represent means of each group. The cut-off value for negative and positive samples, calculated by comparison of the reactivity values from each group with the healthy sera using a Receiver-Operating Characteristic (ROC) analysis is indicated. It was defined as the lower O.D. value with a 100% of specificity. In brackets the percentage of positive sera is also indicated.

protective responses against *L. major* infection, their capacity to modulate *L. braziliensis* infection in the same mice strain was tested. Mice vaccinated with both proteins were able to control parasite growth in the site of infection in this New World species experimental model.

## 2. Materials and methods

### 2.1. Mice strains and parasites

Female BALB/c mice (6–8 week old) were purchased from Harlan (BCN, Spain) or were obtained from the Centro de Pesquisa Gonçalo Moniz, FIOCRUZ. Promastigotes of *L. major* strains (WHOM/IR/-/173) or clone V1 (MHOM/IL/80/Friedlin) and from *L. braziliensis* (MHOM/BR/01/BA788), were cultured at 26 °C in Schneider medium (Gibco, NY) supplemented with 10% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

### 2.2. CpG-ODN

Phosphorothioate-modified CpG-ODN (5′-TCAACGTTGA-3′ and 5′-GCTAGACGTTAGCGT-3′) were synthesized by Isogen (The Netherlands) and employed for their capacity to induce Th1 responses in mice when immunized with various leishmanial antigenic preparations [7,8].

### 2.3. Cloning of DNA sequences coding for L. major ribosomal proteins LmS4, LmS6, LmL3 and LmL5

The *L. major* LmS4, LmS6, LmL3 and LmL5 coding regions were obtained from the *L. major* genome database (www.genedb.org/genedb/leish) using the *Saccharomyces cerevisiae* orthologous protein sequences as probes [9]. Coding regions were PCR amplified using specific primers (Supplementary Fig. 1) and the DNA from *L. major* (MHOM/IL/80/Friedlin). Amplified DNAs were cloned into the pQE30 prokaryotic expression vector (Qiagen, Germany). The four clones were double-stranded sequenced in the same plasmid.

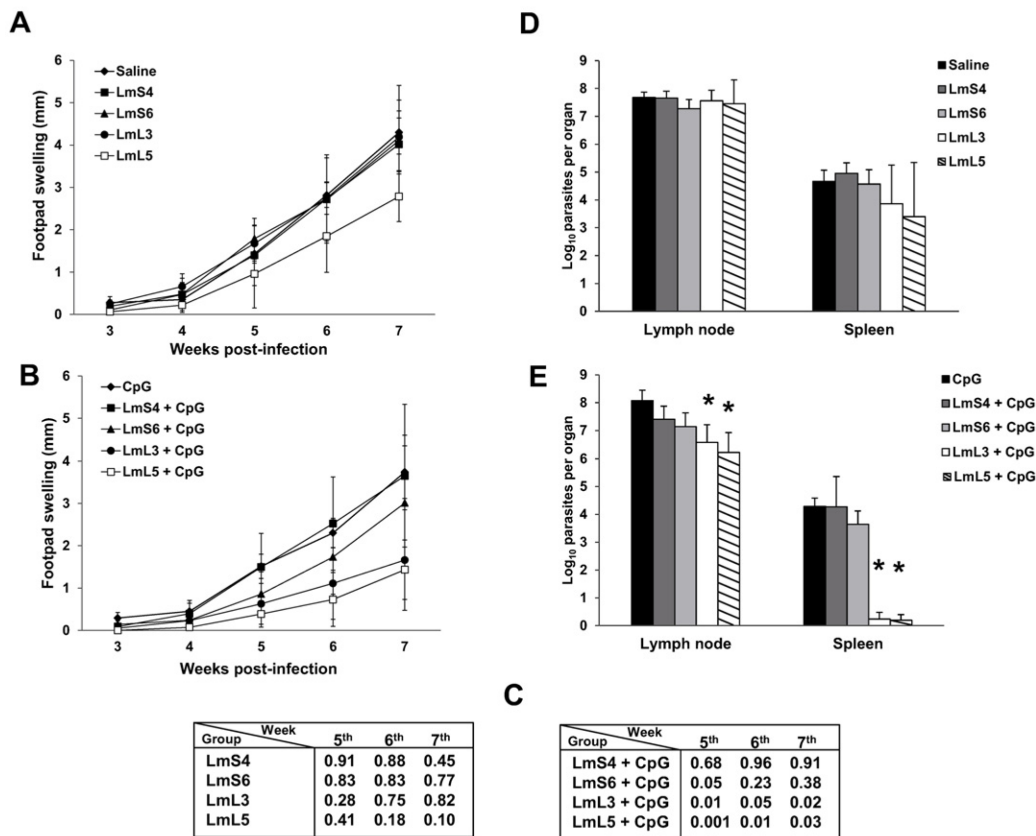
### 2.4. Protein purification

Recombinant proteins were over-expressed in *Escherichia coli*, purified under denaturing conditions onto Ni-nitrilotriacetic-acid-agarose columns (Qiagen) and refolded on the affinity column as described [10]. Polymyxin-agarose columns (Sigma, MO) were employed to remove residual endotoxin content (<10 pg of LPS per 1 μg of recombinant protein, measured by the Quantitative Chromogenic *Limulus* Amebocyte Assay QCL-1000 (BioWhittaker, MD)).

### 2.5. Sera, immunoblotting and ELISA assays

Mice sera were collected at the beginning of the experiment, before challenge with parasites and at the 7th week after challenge with *L. major*. Human VL and MCL sera were obtained from clinical and parasitologically diagnosed Brazilian patients. Canine symptomatic VL sera were collected in the Extremadura region of Spain [11]. Control sera were obtained from healthy individuals.

Soluble *Leishmania* antigens (SLA), mouse ribosomal proteins (MRP) and LRP were prepared as described in [4]. For immunoblotting, recombinant proteins and LRP extracts were electrophoresed and blotted as described in [11]. Anti-LmL3, anti-LmS4 or anti-LmS6 polyclonal sera were obtained from the immunized mice described below. Anti-LmL5 antibodies were obtained by passing canine VL sera through a recombinant LmL5 affinity chromatography column prepared as in [12]. For ELISA, recombinant proteins were used at 1.0 μg per well. Murine, canine and human sera were employed




| Group \ Week | 5th | 6th | 7th |
|---|---|---|---|
| LmS4 | 0.91 | 0.88 | 0.45 |
| LmS6 | 0.83 | 0.83 | 0.77 |
| LmL3 | 0.28 | 0.75 | 0.82 |
| LmL5 | 0.41 | 0.18 | 0.10 |

| Group \ Week | 5th | 6th | 7th |
|---|---|---|---|
| LmS4 + CpG | 0.68 | 0.96 | 0.91 |
| LmS6 + CpG | 0.05 | 0.23 | 0.38 |
| LmL3 + CpG | 0.01 | 0.05 | 0.02 |
| LmL5 + CpG | 0.001 | 0.01 | 0.03 |

**Fig. 2.** Course of *L. major* infection in BALB/c mice vaccinated with ribosomal proteins. Mice ($n = 6$ per group) were vaccinated with the indicated formulations and challenged in the footpad with $5 \times 10^4$ *L. major* promastigotes. Footpad swelling in groups vaccinated with the corresponding proteins without (A) or with (B) adjuvant is shown. Data correspond to the mean ± SD of the difference of thickness between the infected and the uninfected contra-lateral footpads. (C) *P* values (saline *versus* antigen or CpG-ODN *versus* antigen plus CpG-ODN) from data shown in panels A and B. The number of viable parasites (mean + SD) in the draining lymph node on the infected leg (popliteous) and in spleen were determined by limiting dilution at week seven post-challenge in mice vaccinated with the recombinant proteins without (D) or with (E) adjuvant. Serial dilution of each mouse sample was individually performed in triplicates. Comparison were established between each one of the vaccinated groups and their respective control (saline [D] and CpG-ODN [E]) (*$P < 0.05$ significant differences between vaccinated and control mice). Results in each panel are representative of ≥2 independent experiments.

as described in [4,12,13], respectively. Secondary antibodies were purchased from Nordic (Tilburg, The Netherlands). The reciprocal endpoint titre, defined as the inverse of the highest serum dilution factor giving an absorbance >0.15 was determined by serial dilution of the sera.

### 2.6. Immunizations, parasite challenge and parasite quantification

For the *L. major*-BALB/c model, mice ($n = 6$ per group) were independently inoculated in the right hind footpad with 10 μg of each recombinant protein (LmS4, LmS6, LmL3 or LmL5), alone or combined with 25 μg of each CpG-ODN. As control groups, mice were inoculated with 25 μg of each CpG-ODN or with saline. Each group was boosted two and four weeks later with the same dose. Parasite challenge was carried out by subcutaneous inoculation with $5 \times 10^4$ stationary-phase promastigotes of *L. major* (WHOM/IR/-/173) into the left footpad, four weeks after the last immunization. Footpad swelling was measured with a metric caliper (thickness of the left footpad minus thickness of the right footpad). For the *L. braziliensis* model BALB/c mice ($n = 5$ per group) were intradermically (i.d.) inoculated with 10 μg of each recombinant protein (LmL3 or LmL5) combined with 25 μg of each CpG-ODN or with a mixture of the proteins (5 μg each) plus 25 μg of each CpG-ODN in the left ear. Inoculation schedule was the same indicated above. Mice were challenged one month after the last inoculation in the dermis of the right ear with $1 \times 10^5$ stationary-phase promastigotes of *L. braziliensis* in the presence of sand fly saliva, as described [14]. Ear thickness was monitored weekly using a caliper. For parasite load determination the ears (in the case of *L. braziliensis* infection), draining lymph nodes (DLN) and spleen from each mouse were independently processed as described in Ref. [7] and assayed in triplicates by limiting dilution [15].

### 2.7. Measurement of cytokines

The release of IFN-γ, IL-10 and IL-4 was measured in culture supernatants of splenocytes or DLN cells obtained from the different mice groups, following stimulation with the corresponding recombinant proteins, using commercial ELISA kits (eBioscience, CA) as described [4]. Briefly, spleen or lymph node cells obtained from each mouse were seeded and independently cultured (at $5 \times 10^6$ cells per ml) during 48 h at 37 °C alone or with the next stimuli: recombinant LmL3 (10 μg/ml), recombinant LmL5 (10 μg/ml), SLA (12 μg/ml) or MRP (12 μg/ml). When indicated cells were stimulated with a mixture of the two recombinant proteins (5 μg/ml each one).

### 2.8. Statistical analysis

The Receiver Operating Characteristic (ROC) curves were used to analyze the data obtained with sera samples from patients. Statistical analysis with the vaccinated and infected mice was performed

by a two-tailed Student's *t*-test. Differences were considered significant when $P<0.05$.

## 3. Results

### 3.1. Leishmania ribosomal proteins S4, S6, L3 and L5 are antigenic in canine and human leishmaniasis

The putative *L. major* S4, S6, L3 and L5 ribosomal proteins were identified using as probes the *S. cerevisiae* homologous aminoacid sequences [9] in a BLASTP search (Supplementary Fig. 2A). The degree of sequence identity with yeast and human ribosomal proteins (Supplementary Fig. 2A–B) supports the statement that they are components of ribosomes. In addition, antibodies specific for each protein revealed single bands with the expected molecular weights in a LRP preparation by Western blot (Fig. 1A and B). A high degree of sequence identity was observed for the proteins in different *Leishmania* species (Supplementary Fig. 2B).

The four recombinant proteins were recognized by the sera from dogs affected by VL (Fig. 1C). The percentages of positive sera ranged from 60 to 68% (Fig. 1C). They were also recognized by sera samples from Brazilian patients with VL and MCL, infected by *L. chagasi* and *L. braziliensis*, respectively (Fig. 1D).

### 3.2. Outcome of CL due to L. major following vaccination of BALB/c mice with recombinant ribosomal proteins

Next, we analyzed whether the immunization with the respective recombinant proteins was able to induce protection against *L. major* infection. Different groups of BALB/c mice were independently vaccinated with each recombinant protein in the absence or in the presence of CpG-ODN. Mice groups inoculated with the vaccine diluent (saline) or with the adjuvant alone were established as controls. After the challenge with *L. major* the course of infection was followed-up for 7 weeks (Fig. 2A in the absence and Fig. 2B in the presence of adjuvant). In the absence of adjuvant no significant differences in the footpad swelling between control and vaccinated groups were observed (Fig. 2C). When mice were immunized with the recombinant proteins combined with the adjuvant, the LmL3 plus CpG-ODN or LmL5 plus CpG-ODN groups showed a decrease in their lesion size compared to the control (CpG-ODN vaccinated mice) (Fig. 2B). Differences were significant from week 5 to week 7 (Fig. 2C). LmS6 plus CpG-ODN vaccinated mice showed a delay in the evolution of CL until week 5, but differences were not maintained at the end of the assay (Fig. 2B and C). Mice vaccinated with the proteins without adjuvant had a number of parasites similar to that of the saline control group mice in their DLN and their spleens (Fig. 2D). An approximately 2-log reduction in parasite burden was observed in the DLN cells from mice immunized with LmL3 plus CpG-ODN or LmL5 plus CpG-ODN, relative to the CpG-ODN control group (Fig. 2E). Moreover, spleens from mice immunized with LmL3 plus CpG-ODN or LmL5 plus CpG-ODN were almost free of parasites, contrary to the other vaccinated groups and controls (Fig. 2D and E).

The immune response induced by vaccination with LmL3 and LmL5 was analyzed before challenge. Co-administration of the antigens with the CpG-ODN adjuvant induced a Th1-biased immune response, which was absent in mice immunized with the proteins alone. A significantly higher antigen-specific production of IFN-γ was found after *in vitro* stimulation with the LmL3 ($P=0.0000146$; $P=0.00001468$) (Fig. 3A) and LmL5 ($P=0.000015$; $P=0.00029$) (Fig. 3B) proteins beside an IgG2a dominant antibody response against the vaccine antigen (Fig. 3C and D) when compared with saline and CpG-ODN mice group, respectively. Stimulation with SLA

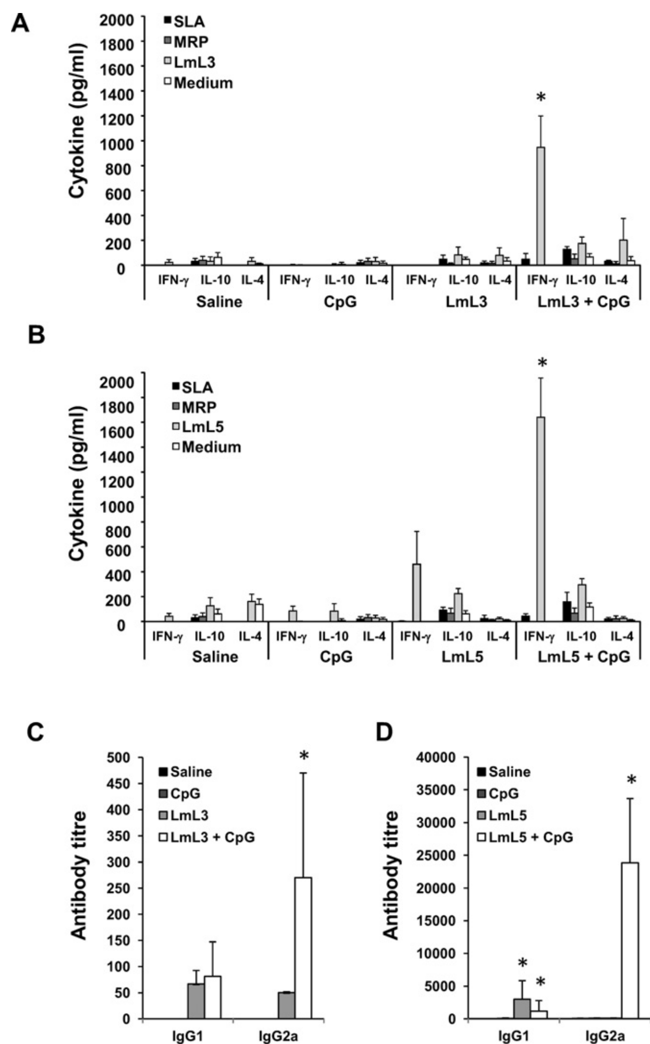



**Fig. 3.** Immune response elicited in mice vaccinated with LmL3 and LmL5. Mice ($n=6$ per group) were vaccinated with LmL3 in the absence and presence of CpG-ODN (A) or LmL5 alone or plus CpG-ODN (B). Splenocytes from each mouse were independently cultured with medium alone or were *in vitro* stimulated with soluble *Leishmania* antigen (SLA), with mouse ribosomal proteins (MRP) and with the corresponding recombinant protein. Splenocytes from control mice, inoculated with saline or CpG-ODN, were stimulated with the same protein preparations in similar conditions. Culture supernatants were collected after 48 h and the levels of IFN-γ, IL-10 and IL-4 were independently measured by sandwich ELISA. Mean + SD are shown. (*$P<0.05$ indicates a statistically difference between vaccinated mice relative to saline and CpG-ODN controls groups). Anti–LmL3 (C) and anti-LmL5 (D) IgG1 and IgG2a antibody titres were individually determined in the indicated mice groups ($n=6$ per group) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (*$P<0.05$ significant differences between vaccinated and control mice). Results in each panel are representative of ≥2 independent experiments.

or MRP did not induce cytokine secretion by spleen cells (Fig. 3A and B).

Immune responses were also studied after challenge. Upon *in vitro* stimulation with the recombinant proteins, LmL3 plus CpG-ODN (Fig. 4A) or LmL5 plus CpG-ODN (Fig. 4B) vaccinated mice displayed a significant increase in the LmL3 or LmL5 driven IFN-γ production ($P=0.00009$ and $P=0.0005$, respectively) paralleled by a decrease in IL-10 secretion ($P=0.009$ and $P=0.00001$, respectively), relative to the CpG-ODN control group. These results are in accordance with the predominant IgG2a antigen-specific antibody response against LmL3 (Fig. 4C) and LmL5 (Fig. 4D), although

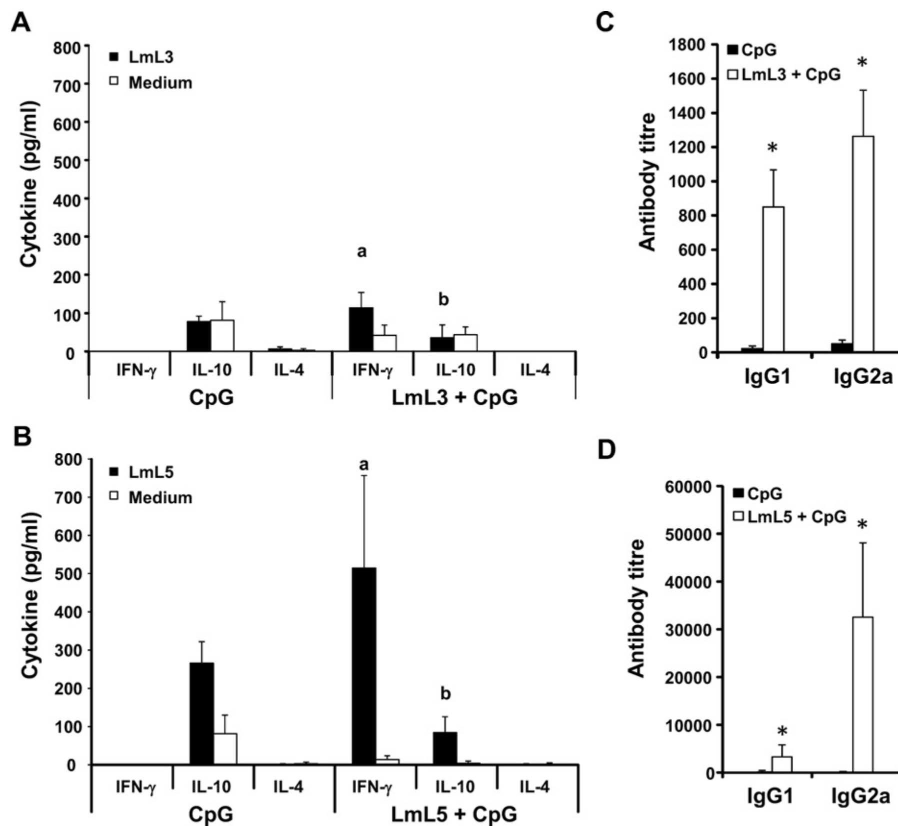



**Fig. 4.** Immune response elicited against LmL3 and LmL5 after *L. major* challenge in the protected mice. Mice ($n = 6$ per group) vaccinated with LmL3 plus CpG-ODN (A) or LmL5 plus CpG-ODN (B) were latter challenged with *L. major*. Splenocytes obtained from each mouse at week 7 after challenge, were independently cultured with medium alone or were *in vitro* stimulated with the corresponding recombinant protein. Control mice were inoculated with CpG-ODN and their splenocytes were stimulated as indicated. Culture supernatants were collected after 48 h and the level of IFN-γ, IL-10 and IL-4 was independently measured by sandwich ELISA. Mean + SD are shown. $P < 0.05$ indicates a statistically increase [a] or decrease [b] in the level of the indicated cytokine between vaccinated and CpG-ODN inoculated mice after infection. Anti-LmL3 (C) and anti-LmL5 (D) IgG1 and IgG2a antibody titres were individually determined in vaccinated and in CpG-ODN control mice ($n = 6$ per group) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (*$P < 0.05$ indicates significant differences between vaccinated and CpG-ODN control mice after infection). Results in each panel are representative of 3 independent experiments.

anti-LmL3 and anti-LmL5 IgG1antibodies were also detected (Fig. 4C and D).

The cellular response against SLA was also analyzed in the protected mice after challenge. Secretion of IFN-γ was significantly higher in LmL3 plus CpG-ODN or LmL5 plus CpG-ODN vaccinated mice, when compared to mice inoculated with CpG-ODN ($P = 0.014$ and $P = 0.017$, respectively) (Fig. 5A). In LmL3 plus CpG-ODN vaccinated mice a decrease in the SLA-dependent IL-10 production was also observed when compared with CpG-ODN immunized mice ($P = 0.009$). An increment in the SLA-specific IgG2a antibodies was

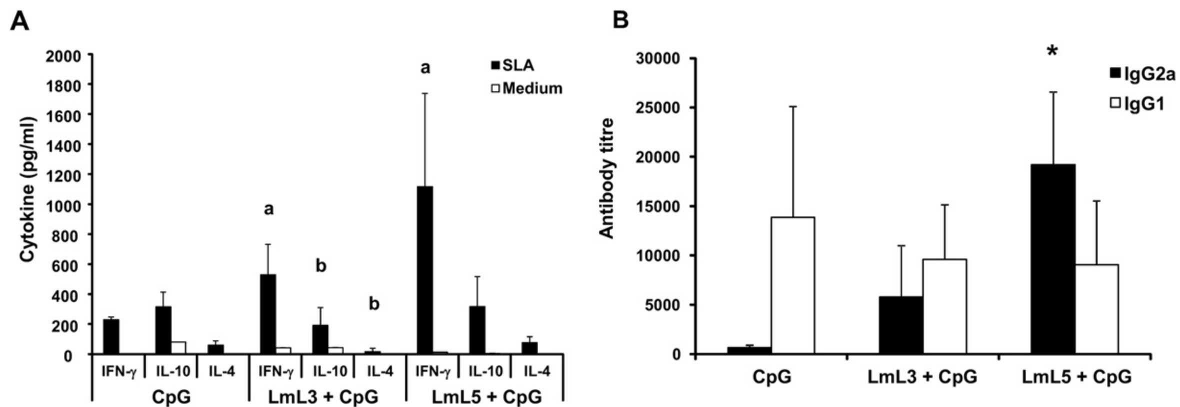



**Fig. 5.** Immune response elicited against soluble leishmanial antigens (SLA) after *L. major* challenge in the protected mice. (A) Mice ($n = 6$ per group) were vaccinated with LmL3 plus CpG-ODN or with LmL5 plus CpG-ODN. Mice were later challenged with *L. major* and splenocytes, obtained from each mouse at week 7 after challenge, were independently cultured with medium alone or were *in vitro* stimulated with SLA. Control mice were immunized with CpG-ODN and their splenocytes were cultured as indicated in the presence or in the absence of SLA. Culture supernatants were collected after 48 h and the levels of IFN-γ, IL-10 and IL-4 were independently measured by sandwich ELISA. Mean + SD are shown. ($P < 0.05$ indicates a statistically increase [a] or decrease [b] in the level of the indicated cytokine between vaccinated and CpG-ODN inoculated mice after infection). (B) Anti-SLA IgG1 and IgG2a antibody titres were individually determined in vaccinated and in CpG-ODN control mice ($n = 6$) by ELISA. Sera were assayed from 1/100 to 1/200,000 and horseradish peroxidase-conjugated anti-mouse IgG1 (1/1000) or IgG2a (1/500) were used as the secondary antibodies. Mean plus SD are shown. (*$P < 0.05$ indicates significant differences between vaccinated and CpG control mice after infection). Results in each panel are representative of 3 independent experiments.

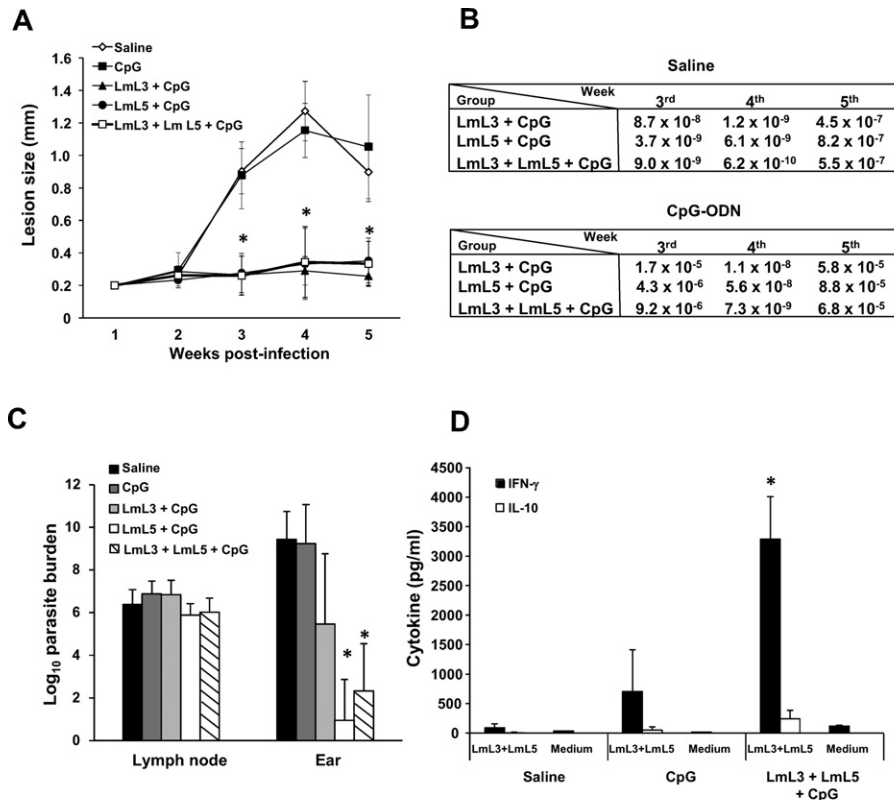



**Fig. 6.** Course of *L. braziliensis* infection in BALB/c mice vaccinated with LmL3 and LmL5. Mice were vaccinated with LmL3 plus CpG-ODN, with LmL5 plus CpG-ODN or with LmL3 plus LmL5 plus CpG-ODN and challenged in the ear with $1 \times 10^5$ *L. braziliensis* promastigotes plus salivary gland sonicate. (A) Course of CL development expressed as the mean ± SD of the ear thickness from five mice (**P* < 0.05 indicates significant differences between vaccinated and saline or CpG-ODN controls). (B) *P* values (saline or CpG-ODN *versus* antigens plus CpG-ODN) from data shown in panel A. (C) Parasite burden in the ear dermis was independently quantified at 5th week five post-infection. Results are expressed as the mean + SD of five ears per group (**P* < 0.05 indicates a significant decrease between mice vaccinated with LmL5 plus CpG-ODN or LmL3 plus LmL5 plus CpG-ODN and control mice inoculated with CpG-ODN or with saline). (D) Production of IFN-γ and IL-10 by lymph node cells following *in vitro* stimulation with a mixture of LmL3 and LmL5 (5 μg each protein) five weeks after challenge. Samples were independently processed and the level of cytokines are expressed as the mean plus SD of five mice per group (**P* < 0.05 indicates a significant increase in IFN-γ levels between rLmL3 plus rLmL5 plus CpG-ODN group *versus* CpG-ODN or saline mice groups). Results in each panel are representative of 2 independent experiments.

observed in LmL3 plus CpG-ODN and LmL5 plus CpG-ODN groups relative to CpG-ODN group, only significant in LmL5 plus CpG-ODN group (*P* = 0.014) (Fig. 5B). The IgG1 response to SLA was similar between the three groups (Fig. 5B).

### *3.3. Vaccination with LmL3 plus CpG-ODN and LmL5 plus CpG-ODN protects BALB/c mice against L. braziliensis challenge*

We also investigated whether immunization with LmL3 or LmL5 was able to confer protection in an experimental model of New World CL. Mice were independently vaccinated with LmL3 or LmL5 combined with CpG-ODN or with a mixed formulation of LmL3 and LmL5 plus CpG-ODN. The outcome of infection with *L. braziliensis* inoculated in the presence of insect vector saliva was evaluated. Ear lesions of vaccinated mice groups were significantly smaller to control groups (saline or CpG-ODN) (Fig. 6A and B). Parasite burden in the ear dermis of the three vaccinated groups was lower than the parasite load of the control groups. Differences with both control groups were significant in the LmL5 plus CpG-ODN (*P* = 0.00016) and in the LmL3 plus LmL5 plus CpG-ODN groups (*P* = 0.005) (Fig. 6C). Similar parasite burden was detected in the DLN of controls and vaccinated mice (Fig. 6C). Finally, to analyze the cellular response elicited against the vaccine antigens, DLN cells from mice immunized with the mixed formulation and both control groups were stimulated with a mixture of the LmL3 and LmL5 recombinant proteins. A significant increase in antigen-specific IFN-γ production was observed in the protected mice relative to both control groups (*P* = 0.015 for saline and *P* = 0.007 for CpG-ODN).

## 4. Discussion

Given that vaccines based on *Leishmania* ribosomal preparations have induced protection against disease development when immunized with Th1 inducing adjuvants [4–6] we have moved toward the identification of protective antigens in the *Leishmania* ribosome.

A few ribosome structural proteins have been described as antigenic in canine or human leishmaniasis, including the P0 [16,17], the L6 [18] and, recently, the L25 and L23a proteins [19]. The four ribosomal proteins identified herein (S4, S6, L3 and L5) are antigenic in different forms of the disease caused by distinct parasite species such as *L. chagasi* and *L. braziliensis* (in humans) and *L. infantum* (in dogs). Although the recombinant proteins were obtained from *L. major* DNA the high degree of sequence conservation existing between *Leishmania* parasite ribosomal orthologues may explain the observed cross-reactivity. Even though it was observed some variability in the recognition between human and canine VL sera (also between VL and MCL human patients) as occur with other parasite antigens [20], our data allow to conclude that the four studied proteins interact with the host immune system, in spite of differences in parasite species and disease forms.

Next, the protective capacities of the characterized antigens were evaluated. Previous reports have identified the prophylactic capacities of three structural proteins of the parasite ribosome: P0 [7], L22 and S19 [21]. Combination of the CpG-ODN adjuvant with the LmL3 or LmL5 proteins induced an immune state that was able to control CL disease due to *L. major* infection in susceptible

BALB/c mice. The immune correlate of protection was the induction of a Th1-like response specific for the recombinant LmL3 or LmL5 proteins. The magnitude of antigen dependent IFN-γ secretion and antigen specific IgG2a titers were higher in the LmL5 plus CpG-ODN vaccinated mice than in mice immunized with LmL3 plus CpG-ODN based vaccine. Antigen specific Th1 responses induced upon vaccination were maintained after infection. Vaccines also controlled the LmL3 and LmL5-driven IL-10 responses induced after infection. However, the presence of IgG1 antibodies specific for LmL3, LmL5 is indicating that the Th2 immune response elicited against them by *L. major* infection was not completely abrogated by the vaccines. The Th2-biased response induced after infection against LmL3 and by LmL5 proteins occurring in human or dogs was also observed in mice vaccinated with the proteins without the adjuvant, since in these mice antibodies elicited against both antigens were of the IgG1 isotype (data not shown). This fact points out the importance of the adjuvant in ribosome-based vaccines. Thus, CpG-ODN motifs were able to redirect toward a protective Th1-like profile the response against LmL3 and LmL5 as also occur with other vaccine formulations like these based on LmSTI1 plus CpG-ODN soluble and particulate preparations [22].

Although the protective effects of the LmL3 or the LmL5 plus CpG-ODN documented here were analyzed in a model that employ a syringe-based challenge the results obtained allowed us to conclude that the protective effect of the immunization of LmL3 and LmL5 based vaccines is similar to that obtained with various parasite proteins assayed for protection under similar conditions [23].

Cross-prophylactic properties of the LmL3 or LmL5 based vaccines were also tested in an experimental model of CL caused by *L. braziliensis* [24]. Many of the proteins known to induce protection against *L. major* or *L. infantum* infection in BALB/c mice, were not able to control the CL caused by *L. braziliensis* [25,26] or only induce partial protection [27]. In this model, BALB/c mice show lesions in the challenge site (ear) that are resolved after induction of a Th1 type immune response that eliminates parasites from ears, maintaining a chronic infection in the DLN [24]. Since co-inoculation of vector saliva and *L. braziliensis* led to a significant exacerbation of both lesion size and parasites load in the mice experimental model [28] we have employed here a syringe-based challenge in which vector saliva and stationary parasites are co-inoculated. Interestingly, we found that ear inflammatory lesions were almost absent in vaccinated mice and a very low number of parasites was detected in the ears 5 weeks after challenge, especially in mice vaccinated with LmL5 plus CpG-ODN or with a combination of both ribosomal proteins and CpG-ODN. Given the antigen specific IFN-γ mediated response was observed in protected mice, it can be suggested that IFN-γ-secreting cells may have migrated to the infected ear early after challenge, promoting parasite killing in the absence of an inflammatory response of a high magnitude. These cells, however, are unable to destroy parasites in the DLNs as also occur in the infected controls in accordance to what it has been previously reported for this experimental model of infection [24]. Since results obtained by our group have shown that immunization with LmL3 or LmL5 ribosomal antigens combined with CpG-ODN also reduced parasite loads in BALB/c mice infected with *L. chagasi* (manuscript in preparation), we conclude that the LmL3 and LmL5 antigens, formulated with Th1 inducing adjuvants should be considered in the development of vaccines against leishmaniasis.

## 5. Conclusions

In this work four new antigenic proteins have been described in *Leishmania* ribosome: S4, S6, L3 and L5. Recombinant proteins obtained from *L. major* were recognized by the sera from individuals infected with different parasite species and suffering different forms of the disease. Two of them, LmL3 or LmL5 were able to protect mice against CL caused by *L. major* and by *L. braziliensis* when administered in the presence of a Th1 inducing adjuvant. In both models, protection was associated with the induction of antigen-specific IFN-γ mediated responses, but also with control of the antigen dependent production of IL-10 in some cases. Altogether, data presented here are indicating that LmL3 and LmL5 may be considered relevant antigens in the formulation of vaccines against leishmaniasis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.12.071.

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ORIGINAL RESEARCH

# Towards development of novel immunization strategies against leishmaniasis using PLGA nanoparticles loaded with kinetoplastid membrane protein-11

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**Background:** Vaccine development has been a priority in the fight against leishmaniases, which are vector-borne diseases caused by *Leishmania* protozoa. Among the different immunization strategies employed to date is inoculation of plasmid DNA coding for parasite antigens, which has a demonstrated ability to induce humoral and cellular immune responses. In this sense, inoculation of plasmid DNA encoding *Leishmania* kinetoplasmid membrane protein-11 (KMP-11) was able to confer protection against visceral leishmaniasis. However, recently the use of antigen delivery systems such as poly(lactic-co-glycolic acid) (PLGA) nanoparticles has also proven effective for eliciting protective immune responses.

**Methods:** In the present work, we tested two immunization strategies with the goal of obtaining protection, in terms of lesion development and parasite load, against cutaneous leishmaniasis caused by *L. braziliensis*. One strategy involved immunization with plasmid DNA encoding *L. infantum chagasi* KMP-11. Alternatively, mice were primed with PLGA nanoparticles loaded with the recombinant plasmid DNA and boosted using PLGA nanoparticles loaded with recombinant KMP-11.

**Results:** Both immunization strategies elicited detectable cellular immune responses with the presence of both proinflammatory and anti-inflammatory cytokines; mice receiving the recombinant PLGA nanoparticle formulations also demonstrated anti-KMP-11 IgG1 and IgG2a. Mice were then challenged with *L. braziliensis*, in the presence of sand fly saliva. Lesion development was not inhibited following either immunization strategy. However, immunization with PLGA nanoparticles resulted in a more prominent reduction in parasite load at the infection site when compared with immunization using plasmid DNA alone. This effect was associated with a local increase in interferon-gamma and in tumor necrosis factor-alpha. Both immunization strategies also resulted in a lower parasite load in the draining lymph nodes, albeit not significantly.

**Conclusion:** Our results encourage the pursuit of immunization strategies employing nanobased delivery systems for vaccine development against cutaneous leishmaniasis caused by *L. braziliensis* infection.

**Keywords:** vaccine, nanoparticle, Leishmania, kinetoplastid membrane protein-11

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## Introduction

Leishmaniasis is a group of diseases caused by infection with unicellular protozoan parasites of the genus *Leishmania*, which are transmitted through the bite of an infected sand fly. Disease burden remains important, involving 88 countries and 350 million people at risk, with 500,000 new cases of visceral leishmaniasis and 1–1.5 million cases

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of cutaneous leishmaniasis per year.[1] Multiple *Leishmania* species are known to cause disease; *L. braziliensis* inoculation into the skin leads to the development of an ulcer with elevated borders and a necrotic center. A chronic inflammatory response develops despite the paucity of parasites. In 1%–5% of patients, mucocutaneous leishmaniasis may develop, and in this case, extensive tissue destruction is observed.[2] Species such as *L. infantum chagasi* multiply in the spleen and liver, causing visceral leishmaniasis. The disease may present with acute, subacute, or chronic evolution, but most infected individuals remain completely asymptomatic.[3] The visceral form of leishmaniasis is associated with an estimated incidence of 59,000 deaths annually.[4] The feasibility of preventing *Leishmania* infection through vaccination is supported by the fact that individuals who recover from a primary infection are resistant to overt clinical manifestations upon reinfection. In general, the key mediator of resistance to leishmaniasis is cellular immunity, whereby interferon-gamma (IFN-γ) produced by CD4+ Th1 cells promotes the oxidative burst in phagocytes that host the intracellular pathogen, promoting parasite killing.[5,6]

Vaccination against leishmaniasis has been pursued using different strategies, ranging from inoculation of virulent parasites (leishmanization) to immunization with killed parasite preparations, live attenuated parasites, or with recombinant proteins or plasmid DNA coding for defined *Leishmania* antigens.[7,8] DNA vaccines encode a potent adjuvant, in the form of unmethylated dinucleotides, which are able to activate antigen-presenting cells through Toll-like receptor 9, stimulating the system towards a Th1-type response. DNA vaccination has also been tested in heterologous prime-boost vaccination regimes[9] in which the immune system is primed with DNA and boosted with a different formulation of the corresponding antigen. This strategy proved effective in models of visceral[10–12] and cutaneous leishmaniasis.[13–15] However, the adjuvant effect can also be achieved by encapsulation of antigens into biodegradable and biocompatible particles.[16] In this sense, immunization with antigen-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles induced potent immune responses in different experimental models of disease.[17–19] Similar and encouraging results have been described regarding encapsulation of *Leishmania* antigens and the development of leishmaniasis.[20,21]

The 11 kDa kinetoplastid membrane protein (KMP-11)[22] is a promising vaccine candidate against leishmaniasis because it is a strong inducer of IFN-γ production by cells from cured patients[23] and it is highly conserved among the trypanosomatids.[24] DNA immunization with KMP-11 was able to confer protection against visceral leishmaniasis caused by *L. donovani*[25] and against cutaneous leishmaniasis caused by *L. major*, when used in combination with interleukin (IL)-12.[26] Given that there are few published studies regarding vaccine development against *L. braziliensis*,[27] a species prevalent in Brazil and South America, we evaluated the ability of KMP-11 to confer protection against cutaneous leishmaniasis caused by *L. braziliensis*, employing two strategies. One strategy involved immunization with a naked plasmid DNA coding for KMP-11, whereas a parallel strategy comprised priming with PLGA nanoparticles loaded with a plasmid DNA encoding KMP-11 followed by PLGA nanoparticles loaded with the recombinant KMP-11 protein.

## Materials and methods

### Mice

Female BALB/c mice (6–8 weeks of age) were obtained from the animal facility at Centro de Pesquisas Gonçalo Moniz, FIOCRUZ. All mice were maintained under pathogen-free conditions. The local Ethics Committee on Animal Care and Utilization approved all procedures involving animals.

### Plasmid and recombinant protein purification

The DNA insert containing the coding region of *Leishmania* KMP-11 was obtained after BamHI/SmaI digestion of the pQE-KMP-11 plasmid[28] and was subcloned in the BamHI/EcoRV sites of the pcDNA3 eukaryotic expression plasmid. Plasmid DNA (pcDNA3 and pcDNA3-KMP-11) was purified using an Endofree Plasmid Giga Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The recombinant plasmid, pQE30-KMP-11,[28] was transformed into *Escherichia coli*. Recombinant protein expression was performed as described previously.[29] Nonrecombinant pcDNA3, pcDNA3-KMP-11, and recombinant KMP-11 protein were encapsulated into PLGA nanoparticles, as described below.

### Preparation and characterization of KMP-11 nanoparticles

Nanoparticles were prepared by employing a solvent evaporation process using a Total Recirculation One-Machine System, which has been used previously for the encapsulation of DNA into PLGA particles.[30] Briefly, 100 mg of PLGA Resomer 503 copolymer (4% w/v, polylactic:glycolic acid ratio 50:50, molecular weight 34 kDa, carrying uncapped

hydroxyl and carboxyl, Boehringer Ingelheim, Ingelheim, Germany) and 10 mg of cationic lipid 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOTAP, chloride salt, Avanti Polar Lipids Inc, Alabaster, AL) were dissolved in 2.5 mL dichloromethane and injected through a needle (inner diameter 0.17 mm) under a turbulent regime (50 mL/minute) onto a Pluronic F68® solution (500 µL 6% w/v, Sigma-Aldrich, St Louis, MO) containing 2 mg of either recombinant KMP-11 protein, non-recombinant pcDNA3, or recombinant pcDNA3-KMP-11. This $W_1$/O emulsion was forced to circulate through the system for 4 minutes in order to homogenize the emulsion droplet size. The preformed emulsion was injected into the outer water ($W_2$) phase, ie, 15 mL of polyvinyl alcohol 0.5% w/v (87% hydrolyzed, molecular weight 115,000; BDH Prolabo; VWR International, Radnor, PA) under a constant pump flow. The turbulent injection resulted in formation of a double emulsion ($W_1$/O/$W_2$) that was homogenized by circulation through the system for 8 minutes. The final emulsion was magnetically stirred to allow solvent evaporation and particle formation. The resulting particles (recombinant KMP-11-loaded, nonrecombinant pcDNA3-loaded, pcDNA3-KMP-11-loaded nanoparticles) or unloaded nanoparticles were centrifuged (9300 × g), washed, freeze-dried, lyophilized and stored at –20°C. Nanoparticle size and zeta potential were determined, respectively, by photon correlation spectroscopy and laser Doppler velocimetry using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK) after dilution of the samples in distilled water or KCl (1 mM). All measurements were performed in triplicate. The recombinant KMP-11 protein content of the recombinant KMP-11-loaded nanoparticles was determined using the Micro BCA protein assay (Pierce, Rockford, IL) following the manufacturer's instructions. The colorimetric reaction was measured in a spectrophotometer at 562 nm and compared with the absorbance obtained with nonencapsulated recombinant KMP-11. For this purpose, control calibration curves (1.5–50 µg/mL) were prepared using recombinant KMP-11 dissolved in NaOH 0.1 N. The amount of plasmidial pcDNA3-KMP-11 DNA or nonrecombinant pcDNA3 loaded into the nanoparticles was estimated using a fluorimetric assay (PicoGreen® dsDNA quantitation kit; Molecular Probes, Eugene, OR), following the manufacturer's instructions. The amount of encapsulated recombinant KMP-11 in the nanoparticles was 3.5 ± 0.5 per mg of recombinant KMP-11-loaded particles; nonrecombinant pcDNA3-loaded nanoparticles contained 7.2 ± 0.7 µg of pcDNA3 per mg of particles, and pcDNA3-KMP-11-loaded nanoparticles contained 6.4 ± 1.2 µg of pcDNA3-KMP-11 DNA per mg of particles.

## Immunization with KMP-11 plasmid DNA or recombinant KMP-11-loaded PLGA nanoparticles

BALB/c mice (in groups of six) received 100 µg of nonrecombinant pcDNA3 or pcDNA3-KMP-11 in saline into the right quadriceps on days 0, 14, and 28. Alternatively, mice were primed with pcDNA3-KMP-11-loaded nanoparticles (containing 30 µg of pcDNA3-KMP-11), injected into the left ear dermis, and were boosted 21 days later with recombinant KMP-11-loaded nanoparticles (containing 10 µg of recombinant KMP-11) in the presence of 25 µg of each CpG oligodeoxynucleotide (5′-TCAGCGTTGA-3′ and 5′-GCTAGCGTTAGCGT-3′) (E-OLIGOS).[31] Control mice were primed with nonrecombinant pcDNA3-loaded nanoparticles (containing 30 µg of pcDNA3), also injected in the left ear dermis, and were boosted with unloaded (empty) nanoparticles + CpG. Samples of immune sera were collected 2 weeks after the last immunization.

## Cytokine detection in mice immunized with KMP-11 plasmid DNA or with recombinant KMP-11-loaded nanoparticles

BALB/c mice were immunized as described above. Two weeks after the last immunization, the mice were euthanized, and single-cell suspensions of lymph nodes draining the immunization site (popliteal for DNA-injected mice and retroaxillary for PLGA nanoparticle-injected mice) were prepared aseptically. Briefly, the draining lymph nodes were homogenized in RPMI 1640 medium and the cells were resuspended in RPMI medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal calf serum (all from Invitrogen, Carlsbad, CA) and 0.05 M β-mercaptoethanol. Cell suspensions were stimulated with recombinant KMP-11 (10 µg/mL) for 48 hours. Culture supernatants were harvested and the presence of cytokines was assayed using a Th1/Th2 cytokine cytometric bead array (BD Biosciences, Franklin Lakes, NJ), which detects murine IL-2, IL-4, IL-5, IFN-γ, and tumor necrosis factor-alpha (TNF-α), following the manufacturer's instructions. Data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry, San Jose, CA) and CBA analysis software (Becton-Dickinson, Franklin Lakes, NJ).

## Humoral immune response in mice immunized with KMP-11 plasmid DNA or with recombinant KMP-11-loaded nanoparticles

Enzyme-linked immunosorbent assay microplates were coated overnight at 4°C with recombinant KMP-11 (1 μg/mL) in coating buffer ($NaHCO_3$ 0.45 M, $Na_2CO_3$ 0.02 M, pH 9.6). After washing with phosphate-buffered saline-Tween, the wells were blocked with phosphate-buffered saline-Tween plus 5% dried skim milk for one hour at 37°C. The wells were incubated overnight with sera (diluted 1:100) from mice immunized with pcDNA3-KMP-11 only or with pcDNA3-KMP-11-loaded nanoparticles followed by recombinant KMP-11-loaded nanoparticles, in the presence of CpG. After further washings, wells were incubated with alkaline phosphatase-conjugated antimouse IgG antibody (Promega, Madison, WI) diluted (1:2500) in phosphate-buffered saline-Tween, for one hour at 37°C. Following another washing cycle, wells were developed with p-nitrophenylphosphate in sodium carbonate buffer at pH 9.6 with 1 mg/mL of $MgCl_2$. The absorbance was recorded at 405 nm. Serum IgG subclasses were determined using antimouse IgG1 or IgG2a alkaline phosphatase conjugates (Sigma-Aldrich).

## Challenge with *L. braziliensis* and sand fly saliva

*L. braziliensis* promastigotes (strain MHOM/BR/01/BA788[32]) were grown in Schneider medium (Sigma-Aldrich) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated fetal calf serum (all from Invitrogen). Stationary-phase promastigotes were used in all experiments. Adult *Lutzomyia intermedia* sand flies were captured in Corte de Pedra, Bahia, and used for dissection of salivary glands. Preparation of salivary gland sonicate was conducted as described elsewhere.[33] The level of lipopolysaccharide contamination of salivary gland sonicate preparations was determined using a commercially available *Limulus* amebocyte lysate chromogenic kit (QCL-1000, Lonza Biologics, Newington, NH); the lipopolysaccharide concentration was $<0.1$ ng/mL. Two weeks after the last immunization, all groups of mice were challenged in the dermis of the right ear with *L. braziliensis* promastigotes + salivary gland sonicate, as described earlier.[34] The progress of infection was monitored weekly, for 10 weeks, by measuring of ear swelling with a digital caliper (Thomas Scientific, Swedesboro, NJ). Parasite load in the infected ear and in its draining lymph nodes was determined as described below.

## Parasite load estimate

Parasite load was determined using a quantitative limiting-dilution assay as described elsewhere.[32] Briefly, infected ears and lymph nodes draining the infection site were aseptically excised at five weeks following infection with *L. braziliensis* + salivary gland sonicate and homogenized in Schneider medium (Sigma-Aldrich). The homogenates were serially diluted in Schneider medium supplemented as before and seeded into 96-well plates containing biphasic blood agar (Novy-Nicolle-McNeal) medium. The number of viable parasites was determined from the highest dilution at which the promastigote could be grown out after up to 2 weeks of incubation at 25°C.

## Evaluation of cellular immune response after challenge by flow cytometry

Five weeks following infection with *L. braziliensis* + salivary gland sonicate, the mice were euthanized, and single-cell suspensions of lymph nodes draining the infection site were prepared as described above. Cells were activated in the presence of anti-CD3 10 μg/mL and anti-CD28 10 μg/mL or with Con A 5 μg/mL (Amersham Biosciences, Piscataway, NJ), and were later incubated with Brefeldin A 10 μg/mL (Sigma-Aldrich). Cells were blocked with anti-Fc receptor antibody (2.4G2) and were double-stained simultaneously with antimouse surface CD4 (H129.19) conjugated to FITC. For intracellular staining of cytokines, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) and incubated with the anticytokine antibodies conjugated to PE:IFN-γ (XMG1.2), IL-4 (BVD4-1D11), and IL-10 (JES5-16E3). The isotype controls used were rat IgG2b (A95-1) and rat IgG2a (R35-95). Data were collected and analyzed using CELLQuest software and a FACSort flow cytometer (Becton-Dickinson). The steady-state frequencies of cytokine positive cells were determined using lymph node cells from control mice.

## Cytokine expression at challenge site

Five weeks following infection with *L. braziliensis* + salivary gland sonicate, the mice were euthanized, infected ears were excised and placed into RLT buffer, and total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's instructions. Ear tissue was mechanically lysed with ceramic beads in a MagNALyzer® instrument (Roche Molecular Systems, Pleasanton, CA), according to the manufacturer's instructions. The resulting tissue lysates were then employed in downstream total RNA extraction. The resulting RNA was resuspended in 20 μL

of water and stored at −80°C until use. cDNA synthesis was performed after reverse transcription (Im Prom-II™ reverse transcription system; Promega) of RNA. Real-time polymerase chain reaction was performed in triplicate on the ABI Prism 7500 (Applied Biosystems, Foster City, CA); thermal cycle conditions consisted of a two-minute initial incubation at 50°C followed by ten-minute denaturation at 95°C, and 50 cycles at 95°C for 15 seconds and 60°C for one minute each. Each sample and the negative control were analyzed in triplicate for each run. The comparative method was used to analyze gene expression. Cytokine cycle threshold ($C_t$) values were normalized to GAPDH expression, as determined by $\Delta C_t = C_{t\,(cytokine)} - C_{t\,(GAPDH)}$. Fold change was determined by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_{t\,(experimental)} - \Delta C_{t\,(control)}$.[35] The primers employed herein are described elsewhere.[33]

## Statistical analysis

Data are presented as the mean ± the standard error. The significance of the results was calculated using nonparametric statistical tests, ie, the two-sided Mann-Whitney test for comparisons between two groups. Analyses were conducted using Prism software (version 5.0; GraphPad Software, Inc, San Diego, CA). Differences were considered statistically significant at $P \leq 0.05$.

# Results

## Cellular immune response after immunization with plasmid DNA ± recombinant PLGA nanoparticles

Initially we investigated the anti-KMP-11 cellular immune response induced by immunization using the different strategies. As shown in Figure 1, in vitro stimulation of draining lymph node cells from mice immunized with pcDNA3-KMP-11 induced a significantly higher production of IL-2 (Figure 1A), IFN-γ (Figure 1B), TNF-α (Figure 1C), IL-4 (Figure 1D), and IL-5 (Figure 1E), when compared with control mice.

The recombinant nanoparticles used herein had a mean size of 300–450 nm, irrespective of the type of encapsulated antigen (DNA or protein). The mean zeta potential values were between 20 mV and 30 mV, indicating a positive charge at pH 7.4, and independently of the nanoparticle load (recombinant KMP-11 or pcDNA3-KMP-11). Recombinant KMP-11 content per mg of recombinant particle was 3.5 ± 0.5 µg. Regarding plasmid DNA content, one mg of recombinant particles contained 7.2 ± 0.7 µg and 6.4 ± 1.2ug of wild-type and pcDNA3-KMP-11, respectively. When mice were immunized with the recombinant nanoparticle formulations, IL-2 (Figure 2A) and IFN-γ (Figure 2B) production was also increased. However, in this case, TNF-α levels were significantly higher (Figure 2C), whereas IL-4 (Figure 2D) and IL-5 (Figure 2E) production was similar to that detected in control mice.

## Humoral immune response on immunization with plasmid DNA or PLGA nanoparticles

We also probed for the anti-KMP-11 humoral immune response induced by immunization with the different strategies. We did not detect anti-KMP-11 antibodies in mice immunized with either pcDNA3-KMP-11 or with nonrecombinant pcDNA3 (data not shown). However, mice inoculated with pcDNA3-KMP-11-/recombinant KMP-11-loaded nanoparticles + CpG developed a strong and antigen-specific humoral immune response (Figure 3A). IgG1 and IgG2a subclasses (Figure 3B) were detected in immune sera which could be associated with the presence of both IL-4/IL-5 and IFN-γ/TNF-α, as seen upon restimulation of draining lymph node cells (Figure 2).

## Outcome of *L. braziliensis* infection in mice immunized with plasmid DNA or PLGA formulations

Next, we investigated the outcome of infection with *L. braziliensis*, in the presence of sand fly saliva. Immunization with pcDNA3-KMP-11 did not alter the course of clinical disease upon a live challenge (Figure 4A), with both immunized and control mice displaying the same outcome followed by spontaneous healing. However, mice inoculated with pcDNA3-KMP-11 had a significantly lower ($P < 0.05$) parasite load at the ear dermis 5 weeks after infection when compared with control mice (Figure 4B). A similar finding was observed in draining lymph nodes (Figure 4C), although the difference was not significant.

Interestingly, immunization with pcDNA3-KMP-11-/recombinant KMP-11-loaded nanoparticles + CpG also did not prevent development of disease (Figure 5A) when compared with control animals. Of note, the ear thickness of mice immunized with recombinant nanoparticles was slightly smaller when compared with controls (Figure 5A) at 5 weeks after infection. Similar to results obtained upon DNA immunization (Figure 4B), immunization with the recombinant formulations also significantly ($P < 0.05$) decreased parasite load in the ear dermis (Figure 5B). Interestingly, parasite load in draining lymph nodes was also lower when compared with control mice (Figure 5C).

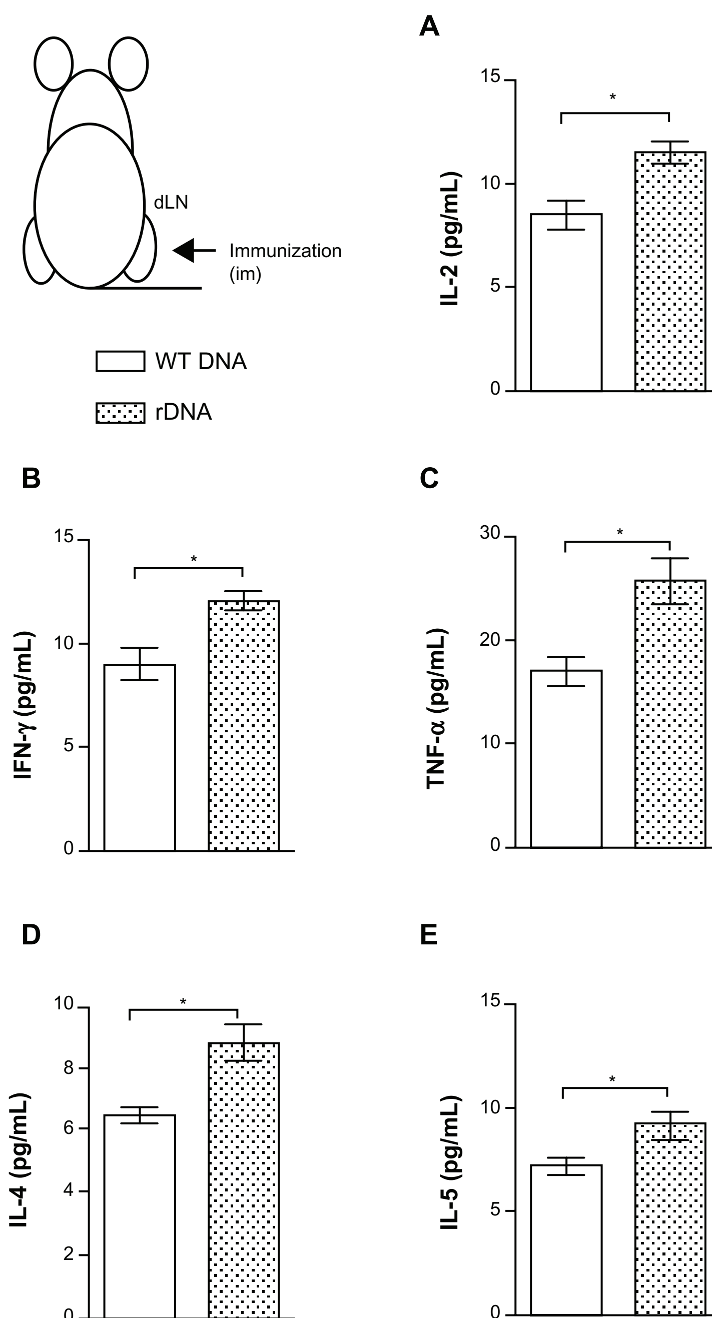


**Figure 1** Cytokine production in mice immunized with a plasmid DNA encoding KMP-11.

**Notes:** BALB/c mice were immunized with nonrecombinant pcDNA3 (open bars) or with pcDNA3-KMP-11 (closed bars), as described. Two weeks after the last immunization, draining lymph nodes were collected and the cells were restimulated with recombinant KMP-11. The presence of cytokines in culture supernatants was determined by flow cytometry, using a Th1–Th2 cytometric bead array. Data are presented as the mean ± standard error and are from two independent experiments, each performed with six mice per group. $^{*}P < 0.05$.

**Abbreviations:** IFN, interferon; IL, interleukin; KMP-11, kinetoplastid membrane protein-11; TNF, tumor necrosis factor.

## Cytokine production in situ after *L. braziliensis* + salivary gland sonicate challenge

Because both immunization strategies significantly reduced parasite load at the infection site, we probed for cytokine expression therein 5 weeks after infection. Remarkably, mice immunized with pcDNA3-KMP-11 showed upregulation in both IFN-γ and IL-10 expression (Figure 6A) at the infection site; IFN-γ expression was upregulated by approximately 5-fold in comparison with control animals, whereas this increase was about 15-fold for IL-10. TNF-α expression was not detected in mice immunized with pcDNA3-KMP-11. On the other hand, mice immunized with pcDNA3-KMP-11-/recombinant

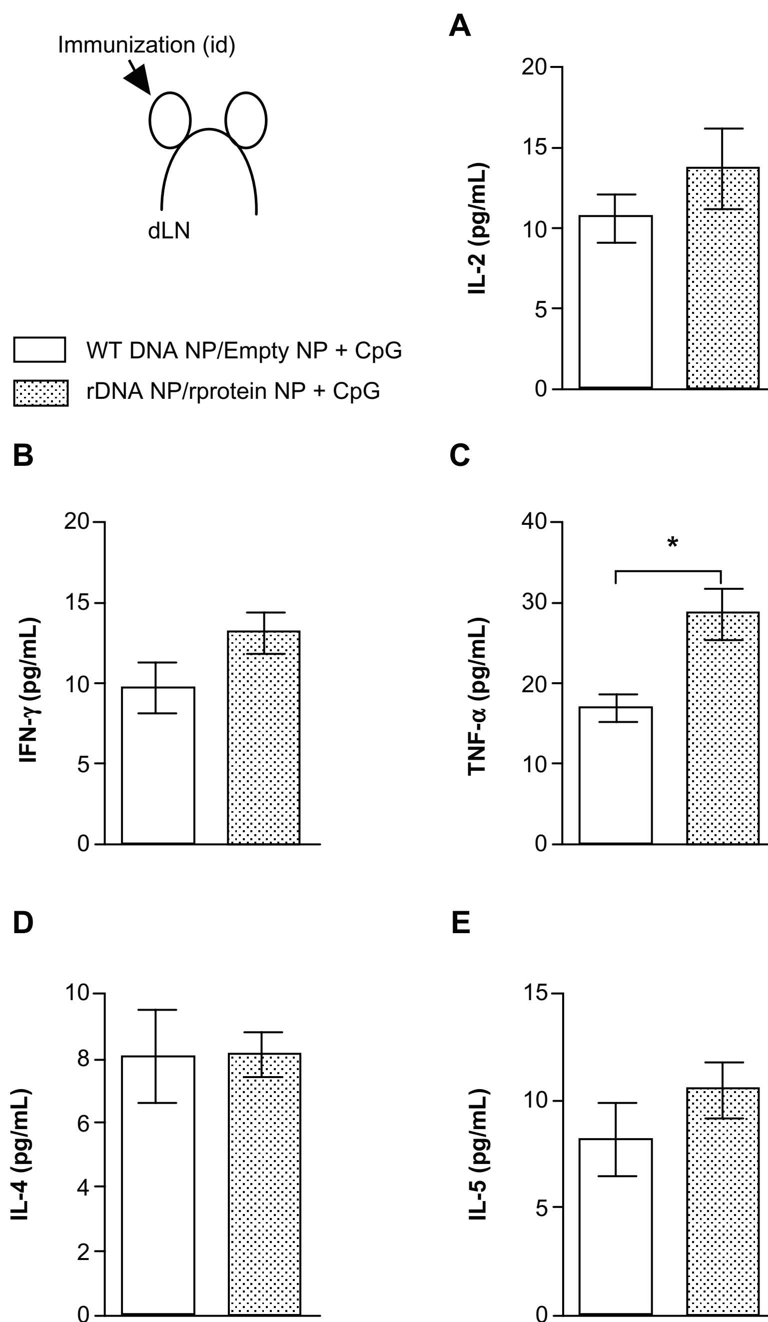


**Figure 2** Cytokine production in mice immunized with KMP-11-loaded nanoparticles.
**Notes:** Control BALB/c mice were immunized with nonrecombinant pcDNA3-loaded nanoparticles followed by unloaded (empty) nanoparticles + CpG (open bars). Experimental BALB/c mice were immunized with pcDNA3-KMP-11-loaded nanoparticles followed by recombinant KMP-11-loaded nanoparticles + CpG (closed bars). Two weeks after the last immunization, draining lymph nodes were collected and cells were restimulated with recombinant KMP-11. The presence of cytokines in culture supernatants was determined by flow cytometry, using a Th1–Th2 cytometric bead array. Data are presented as the mean ± standard error and are from two independent experiments, each performed with six mice per group. *$P < 0.05$.
**Abbreviations:** IFN, interferon; IL, interleukin; KMP-11, kinetoplastid membrane protein-11; TNF, tumor necrosis factor.

KMP-11-loaded nanoparticles + CpG showed a more moderate upregulation in IFN-γ and TNF-α expression (Figure 6B) at the infection site. In contrast with mice immunized using pcDNA3-KMP-11, challenge infection with parasites induced downregulation in IL-10 expression in the mice receiving recombinant PLGA nanoparticles (Figure 6B).

Upon challenge with *L. braziliensis* + salivary gland sonicate, mice immunized with pcDNA3-KMP-11 or with the recombinant nanoparticle formulations also displayed a lower parasite load within the draining lymph nodes (Figure 4C and 5C, respectively). Therefore, we also evaluated the frequency of cytokine-secreting cells therein. After infection, mice immunized

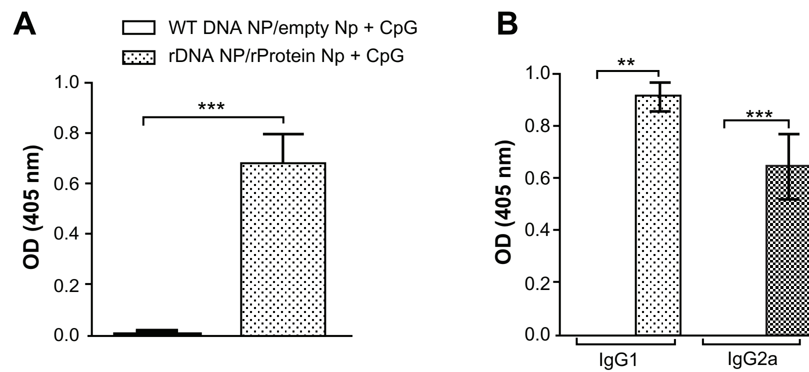


**Figure 3** Humoral immune response in mice immunized with KMP-11-loaded nanoparticles.
**Notes:** Control BALB/c mice were immunized with nonrecombinant pcDNA3-loaded nanoparticles followed by unloaded nanoparticles + CpG (open bars). Experimental BALB/c mice were immunized with pcDNA3-KMP-11-loaded nanoparticles followed by recombinant KMP-11-loaded nanoparticles + CpG (closed bars). (**A**) Presence of anti-KMP-11 IgG antibodies was determined by enzyme-linked immunosorbent assay. IgG subclasses present were determined by enzyme-linked immunosorbent assay using IgG1 and IgG2a conjugates (**B**).Data are presented as the mean ± standard error and are from two independent experiments. $*P < 0.01$.
**Abbreviation:** KMP-11, kinetoplastid membrane protein-11.

with pcDNA3-KMP-11 displayed a lower percentage of CD4+ IFN-γ+ (Figure 7A), CD4+ IL-4+ (Figure 7B), and CD4+ IL-10+ (Figure 7C) cells when compared with controls.

Cells from mice immunized with pcDNA3-KMP-11-/recombinant KMP-11-loaded nanoparticles + CpG also displayed a decreased frequency of CD4+ IFN-γ+ T cells (Figure 8A), after infection. In these animals, the percentage of CD4+ IL-4+ (Figure 8B) and CD4+ IL-10+ cells (Figure 8C) was similar (Figure 7B) or slightly higher (Figure 7C) when compared with controls. Similar results

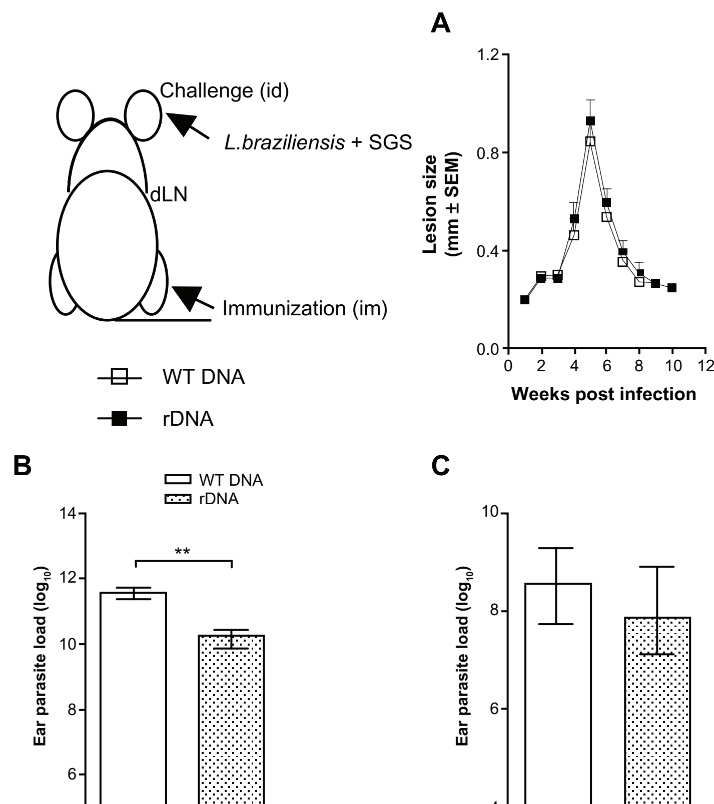


**Figure 4** Lesion development and parasite load in mice immunized with a plasmid DNA encoding KMP-11, following a live challenge with parasites.
**Notes:** BALB/c mice were immunized with nonrecombinant pcDNA3 (open bars) or with pcDNA3-KMP-11 (closed bars), as described. Two weeks after the last immunization, mice were infected in the ear dermis with *Leishmania braziliensis* + salivary gland sonicate. The course of lesion development was monitored weekly (**A**), parasite load in the ear (**B**), and in draining lymph nodes (**C**) was determined 5 weeks following infection. Data are presented as the mean ± standard error and are from two independent experiments. $**P < 0.01$.
**Abbreviations:** KMP-11, kinetoplastid membrane protein-11; SGS, salivary gland sonicate.

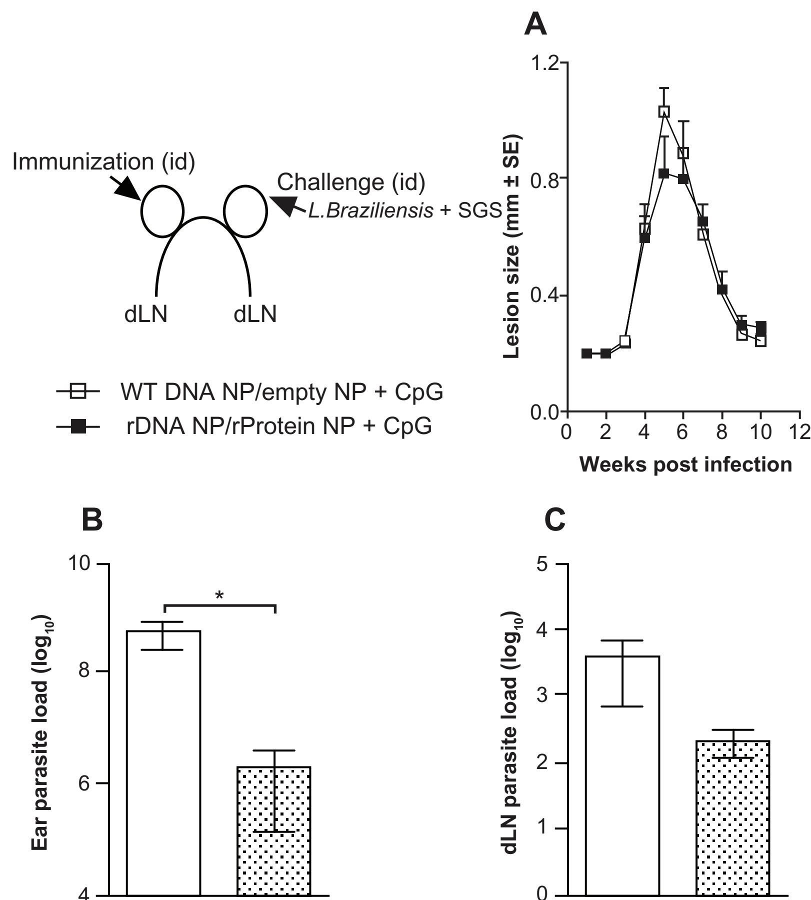


**Figure 5** Lesion development and parasite load in mice immunized with KMP-11-loaded nanoparticles, following a live challenge with parasites.

**Notes:** Control BALB/c mice were immunized with nonrecombinant pcDNA3-loaded nanoparticles followed by unloaded nanoparticles + CpG (open bars). Experimental BALB/c mice were immunized with pcDNA3-KMP-11-loaded nanoparticles followed by recombinant KMP-11-loaded nanoparticles + CpG (closed bars). Two weeks after the last immunization, mice were infected in the ear dermis with *Leishmania braziliensis* + salivary gland sonicate. The course of lesion development was monitored weekly (**A**). Parasite load in the ear (**B**) and in draining lymph nodes (**C**) was determined 5 weeks following infection. Data are presented as the mean ± standard error and are from two experiments. *$P < 0.05$.

**Abbreviations:** KMP-11, kinetoplastid membrane protein-11; SGS, salivary gland sonicate.

were found for cytokine-secreting CD8+ T cells (data not shown).

## Discussion

In the present work, we evaluated two immunization strategies for their potential to elicit protective immune responses in an experimental model of infection. One strategy consisted of immunization with plasmid DNA and the other involved use of PLGA nanoparticles loaded with plasmid DNA or with the respective recombinant protein. We hypothesized that encapsulation would protect the antigen from degradation and, in parallel, that a heterologous prime-boost strategy would enhance the immune response.

Herein, immunization of BALB/c mice with a plasmid DNA coding for *L. infantum chagasi* KMP-11 elicited a mixed Th1/Th2-type immune response. However, immunization with the recombinant nanoparticle formulations, in the presence of CpG, induced a significant increase in TNF-α upon restimulation in vitro. Indeed, nanoparticles formulated with DOTAP, the cationic lipid used here, promote a proinflammatory response, with presence of IL-2, IFN-γ, TNF-α,[36] and oligodeoxynucleotides, such as CpG motifs, are able to trigger plasmacytoid dendritic cells, resulting in TNF-α production.[37] Use of DOTAP in our formulations and of CpG in our immunization scheme may therefore explain the elevated TNF-α levels in immunized mice. We also detected the presence of IgG1 and IgG2a antibodies (Figure 3), suggesting participation of both IL-4 and IFN-γ in antibody isotype switching, even though levels of these cytokines were not significantly increased in mice immunized with PLGA nanoparticles (Figure 2).

Following immunization, the mice were challenged with live parasites in the presence of sand fly saliva, mimicking the context of natural infection with

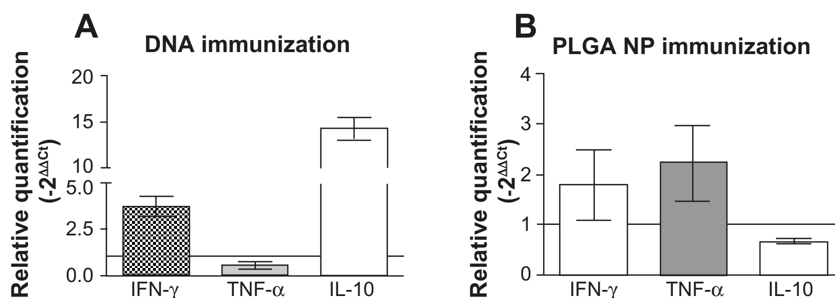


**Figure 6** Cytokine expression at the ear dermis following a live challenge with parasites. BALB/c mice were immunized with pcDNA3-KMP-11 (**A**) or with pcDNA3-KMP-11-loaded nanoparticles followed by recombinant KMP-11-loaded nanoparticles + CpG (**B**).

**Notes:** Two weeks after the last immunization, the mice were infected with *Leishmania braziliensis* + salivary gland sonicate. Relative quantification of IFN-γ, TNF-α, and IL-10 at the infection site was determined 5 weeks after infection, in relation to a housekeeping gene, by real-time polymerase chain reaction (see materials and methods section). Data (mean ± standard error) are presented as the fold increase in gene expression of immunized mice over control mice and are from two independent experiments.

**Abbreviations:** KMP-11, kinetoplastid membrane protein-11; PLGA, poly(lactic-co-glycolic acid); NP, nanoparticles: IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; IL-10, interleukin-10.

*Leishmania* spp. Upon challenge, neither immunization strategy prevented lesion development. Air pouch stimulation with *L. braziliensis* + *L. intermedia* saliva enhances CXCL10, CCL2, TNF-α, and IL-10 expression,[33] confirming the immunomodulatory role of saliva from *L. intermedia*. Although we did not probe for the protective capacity of our immunization strategies in the absence of sand fly saliva, we may speculate that salivary molecules at the time of parasite challenge may have modulated the microenvironment, favoring lesion development.

Despite the inability of the present immunization strategies to prevent disease manifestation, a significant reduction in parasite load was detected at the challenge site. Mice immunized with either DNA alone or with recombinant PLGA nanoparticles displayed increased IFN-γ expression at the infection site. Moreover, mice immunized with recombinant PLGA nanoparticles + CpG also showed elevated TNF-α. IFN-γ and TNF-α act in concert to activate inducible nitric oxide synthase for the production of nitric oxide, and TNF-α stimulates macrophages to produce nitric oxide.[6] We can suggest that, in the group immunized with recombinant PLGA nanoparticles, upregulation of IFN-γ and TNF-α combined with downregulation of IL-10, may explain the greater parasite killing at the challenge site. In mice immunized with DNA alone, upregulation of IFN-γ expression was also observed but was accompanied by a strong elevation of IL-10 expression. Mononuclear cells from patients with leishmaniasis produced high levels of IL-10 upon stimulation with recombinant KMP-11,[38] whereas addition of recombinant KMP-11 to cells previously stimulated with soluble *Leishmania* antigen decreased IFN-γ secretion.[39] We could speculate that the immune response induced in DNA-immunized mice may have been more prone to modulation exerted by parasite-derived KMP-11 compared with the response elicited by immunization with PLGA nanoparticles.

Parasite load in the draining lymph nodes was also lower following immunization with either DNA alone or with recombinant PLGA nanoparticles, although differences between the experimental and control groups

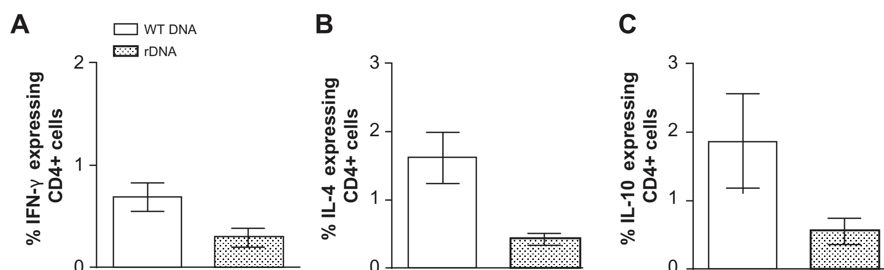


**Figure 7** Intracellular cytokine production by CD4+ and CD8+ T cells in mice immunized with a plasmid DNA encoding KMP-11, following a live challenge with parasites.

**Notes:** BALB/c mice were immunized with nonrecombinant pcDNA3 (open bars) or with pcDNA3-KMP-11 (closed bars), as described. Two weeks after the last immunization, mice were infected with *Leishmania braziliensis* + salivary gland sonicate. Five weeks after infection, draining lymph node cells were restimulated in vitro. Data (mean ± standard error) represent the percentages of CD4+ cells secreting IFN-γ, (**A**), IL-4 (**B**), or IL-10 (**C**) and are from two independent experiments.

**Abbreviations:** KMP-11, kinetoplastid membrane protein-11; IFN-γ, interferon gamma; tumor IL-10, interleukin-10; IL-4, interleukin-4; WT DNA, nonrecombinant pcDNA3; rDNA, pcDNA3-KMP-11.

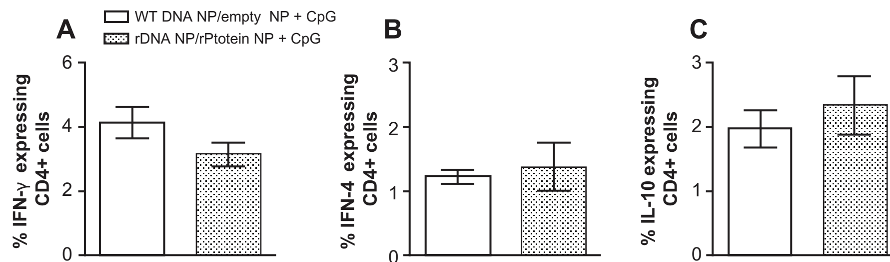


**Figure 8** Intracellular cytokine production by CD4+ and CD8+ T cells in mice immunized with the recombinant nanoparticle formulations, following a live challenge with parasites.
**Notes:** Control BALB/c mice were immunized with non-recombinant pcDNA3-loaded nanoparticles followed by unloaded nanoparticles + CpG (open bars). Experimental BALB/c mice were immunized with pcDNA3-KMP-11-loaded nanoparticles followed by recombinant KMP-11-loaded nanoparticles + CpG (closed bars). Two weeks after the last immunization, mice were infected with *Leishmania braziliensis* + salivary gland sonicate. Five weeks after infection, draining lymph node cells were restimulated in vitro. Data (mean ± standard error) represent the percentages of CD4+ cells secreting IFN-γ, (**A**), IL-4 (**B**), or IL-10 (**C**) and are from two independent experiments.
**Abbreviations:** KMP-11, kinetoplastid membrane protein-11; IFN-γ, interferon gamma; tumor IL-10, interleukin-10; IL-4, interleukin-4; WT DNA, nonrecombinant pcDNA3; rDNA, pcDNA3-KMP-11.

were not significant. One could consider that migration of the effector T cell population (CD4+ IFN-γ+) to the infection site, with parasite killing, explains the lower frequency of cytokine-secreting CD4+ cells in mice immunized with plasmid DNA or with PLGA nanoparticles, when compared with the respective controls. Of note, parasites persist in draining lymph nodes of BALB/c mice inoculated with *L. braziliensis*,[32] despite resolution of dermal lesions and parasite clearance from the infection site. Parasite persistence in cutaneous leishmaniasis has been associated with the presence of regulatory T cells.[40] Therefore, another possibility concerns the presence of regulatory T cells within draining lymph nodes preventing parasite clearance. In the draining lymph nodes, these regulatory T cells could counteract the presence of effector cells. Indeed, in mice immunized with PLGA nanoparticles, the frequency of CD4+ IL-10+ T cells was elevated in draining lymph nodes.

A stronger immune response is elicited when antigen is associated with particles, compared with soluble antigen alone.[41] In the case of leishmaniasis, immunization CpG and PLGA nanospheres loaded with autoclaved *L. major* was able to decrease *L. major* infection and this effect was associated with increased IFN-γ and decreased IL-4 production.[21] Doroud et al showed that immunization with solid lipid nanoparticles loaded with plasmid DNA coding for *Leishmania* cysteine proteinase conferred protection against *L. major*,[42] and was associated with increased IFN-γ levels before challenge and an elevated ratio of IFN-γ/IL-5 after challenge. In addition to the choice of antigen and experimental model, several variables such as particle chemistry, size, and surface charge, affect the ensuing immune response,[43,44] and may explain the different outcomes observed in terms of immunity against leishmaniasis. Of note, we performed experiments in which mice were immunized with naked DNA coding for KMP-11 and were boosted with recombinant KMP-11 + CpG. Following this strategy, mice did not develop a strong humoral immune response, nor was parasite load decreased following a challenge with live parasites (data not shown). Therefore, we can suggest that antigen encapsulation enhanced efficacy of the immune response, possibly by protecting the antigen from rapid degradation and or by ascertaining uptake by antigen-presenting cells, as seen in the present results.

Antigens that have proven effective against *L. major*, such as LACK, LbSTI1, LeIF, and TSA, have not induced similar responses when tested against *L. braziliensis*.[45] Vaccination with soluble *L. major* promastigote exogenous antigens conferred protection against *L. donovani* but also failed to induce a similar response against *L. braziliensis*.[46] Since the current findings highlight the need to probe actively for antigens and strategies capable of preventing cutaneous leishmaniasis caused by *L. braziliensis*, we believe recombinant nanoparticles comprise a platform tailored for such discoveries.

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## Disclosure

The authors report no conflicts of interest in this work.

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Research Article

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

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**Abstract**

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

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

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The use of particles as a tool for vaccine delivery was based on the favored uptake of particulate, rather than soluble, forms of antigen (review in Storni et al.[1]) and the ability of antigen-loaded particles to act as depots, leading to the slow release of antigen and thereby prolonging antigen availability to the immune system (review in Panyam and Labhasetwar[2]). Antigen-loaded particles are also efficiently taken up by antigen presenting cells (APCs) and are subsequently delivered to secondary lymphoid organs[3] where priming of the adaptive immune response occurs. Among the polymers used for in the production of particles, able to induce protective immune responses is the biodegradable and biocompatible polyester poly(lactide-co-glycolide acid) (PLGA), which has been used in humans as reabsorbable suture material and in delivery systems for the controlled release of drugs.[4]

Immunization with antigen-loaded PLGA particles induces protective immune responses in different experimental models.[5–7] In the field of leishmaniasis, a disease caused by *Leishmania* parasites that infect and multiply within macrophages, immunization with cysteine proteinase entrapped in solid lipid nanoparticles (SLNs)[8,9] or with autoclaved *Leishmania* antigen encapsulated in PLGA nanoparticles[10] conferred protection against disease. Recently, we evaluated the capacity of nanoparticles loaded with the 11-kDa kinetoplastid membrane protein (KMP-11)[11] to confer protection against cutaneous leishmaniasis caused by *Leishmania braziliensis*.[12] A promising vaccine candidate, KMP-11 is highly conserved among trypanosomatids,[13] and KMP-11 stimulation of cells obtained from cured leishmaniasis patients induces IFN-γ production.[14] Vaccination with KMP-11 also prevented disease development in different experimental models of leishmaniasis.[15–17] We demonstrated that mice inoculated with PLGA nanoparticles loaded with plasmid DNA coding for KMP-11 or loaded with KMP-11 recombinant protein developed a cellular immune response. Following challenge with live *L. braziliensis* parasites, immunized mice displayed a significant reduction in the parasite load, an outcome associated with increased levels of IFN-γ and TNF-α.[12]

Given this capacity of KMP-11-loaded PLGA nanoparticles to induce an effective adaptive immune response in vivo, we hypothesized that recombinant nanoparticles also stimulate innate responses in macrophages, the host cell in which *Leishmania* replication occurs. This paper describes the effects of cell stimulation with KMP-11-loaded nanoparticles on infection with *L. braziliensis* parasites—distinguished from other etiological agents of leishmaniasis by its chronicity, latency, and tendency to metastasize in the human host[18] and the mechanisms that are associated with parasite killing.

## Methods

### *Preparation and characterization of KMP-11-loaded nanoparticles*

Nanoparticles (NPs) were prepared employing a solvent evaporation process using Total Recirculation One Machine System (TROMS®).[19] Initially, several formulation parameters were assayed in order to achieve high antigen (recombinant KMP-11) loading and monodisperse particles (< 500 nm). The effect of the different formulations and particle characteristics were studied by factorial design. Factors and levels studied were PLGA polymer type [Resomer® 503H co-polymer (PL/GA ratio 50:50, MW 34 kDa carrying uncapped hydroxyl and carboxyl) or Resomer® 756, PL/GA 75:25, MW 98 kDa, Boehringer Ingelheim], which differs in molecular weight, organic solvent (Dicloromethane, DCM, or ethyl acetate, EA, Panreac, Spain), stabilizer (Pluronic F68® alone or Pluronic F68+ cationic lipid DOTAP [(1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (Avanti Polar Lipids, Inc.)] and antigen amount (2 or 4 mg of rKMP11). The study of formulation factors was conducted with rKMP-11 since previous studies indicated that DNA encapsulation in PLGA particles containing the cationic lipid DOTAP in the organic phase was close to 100%.[20] Different formulations of the nanoparticles loaded with rKMP-11 were developed, enabling optimization of nanoparticles with desired particle size, polydispersity and encapsulation efficiency. Size, size distribution and encapsulation efficiency were evaluated as the responses (Supplemental Material, Table 1). Nanoparticle size and polydispersion were analyzed by photon correlation spectroscopy (Malvern Instruments, UK). The amount of rKMP-11 (rProtein) loaded onto nanoparticles was determined by the Micro BCA Protein Assay (Pierce).

Statistical analysis revealed that among parameters studied, the type of PLGA polymer significantly affected particle size and choice of stabilizer significantly affected the encapsulation efficiency. Other factors (solvent and amount of antigen) did not have any significant effect on the parameters evaluated (Supplemental Material, Fig. 1). Based on these results, nanoparticle formulation was conducted as described.[12] Briefly, 100 mg of lactic and glycolic acid (PLGA) Resomer 503 co-polymer (4% wt/vol) and 10 mg of DOTAP were dissolved in dicloromethane (DCM) and injected onto a Pluronic F68® solution (500 μL 6% wt/vol) containing 2 mg of recombinant KMP-11 (rProtein), wild-type plasmid DNA (pcDNA3) (WT DNA) or pcDNA3 coding for KMP-11 (rDNA). This $W_1$/O emulsion circulated through the system to homogenize the emulsion droplet size. The preformed emulsion was injected into the outer water ($W_2$) phase, 15 mL of polyvinylalcohol (PVA, 87% hydrolized, molecular weight 115 000, BDH, UK) (0.5% wt/vol), under a constant pump flow. The resulting double emulsion ($W_1$/O/$W_2$) was homogenized by circulation through the system. After solvent evaporation under magnetic stirring, the nanoparticles were purified by centrifugation, lyophilized and stored at −20°C. The amount of plasmid DNA (rDNA or WT) loaded onto nanoparticles was determined by a fluorimetric assay (PicoGreen® dsDNA Quantitation Kit, Molecular Probes).[12] To estimate integrity, nanoparticles loaded with plasmid DNA (rDNA or WT DNA) were dissolved with DMSO, and DNA was ethanol precipitated at −80°C. The samples were analyzed by agarose gel electrophoresis. Nanoparticles loaded with KMP-11 (rProtein) were dissolved in methylene chloride. The organic solvent was evaporated, and the residue was resuspended in electrophoresis sample buffer. The samples were subjected to SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250.

### In vitro release studies

Nanoparticles (5 mg) loaded with rProtein or with rDNA were dispersed in 1 mL of PBS and maintained under agitation at 37°C. At predetermined intervals, the samples were centrifuged at 27.100 × *g* for 20 min, and the amount of rDNA or rProtein released from the particles into the supernatants was determined. The release data were expressed as the cumulative percentage of rProtein or rDNA of the initial content of the particles versus time.

### Mice

Female BALB/c mice (6-8 weeks of age) were obtained from the CPqGM/FIOCRUZ Animal Facility. All mice were maintained under pathogen-free conditions. All animal procedures were approved by the local Ethics Committee on Animal Care and Utilization (CEUA—CPqGM/FIOCRUZ- L-065-8).

### Parasite culture

*L. braziliensis* promastigotes (strain MHOM/BR/01/BA788)[21] were grown in Schneider medium (Sigma-Aldrich) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (all from Invitrogen). Stationary-phase promastigotes were used in all experiments.

### Infection of PLGA-stimulated macrophages with L. braziliensis

BALB/c mice were injected i.p. with 3% thioglycolate. Five days after injection, peritoneal lavage was performed using 8 mL RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen). To obtain monolayers, cells ($6 \times 10^5$cells/mL) were place into glass coverslips within the wells of a 24-well plate and were left to adhere for 2 h, at 37°C and 5% $CO_2$. Nonadherent cells were removed by gentle and extensive washing with PBS; purity was routinely above 99% (data not shown). Remaining cells ($3 \times 10^5$cells/mL) were stimulated with plasmid DNA-loaded nanoparticles (carrying the equivalent of 10 μg/mL of encapsulated rDNA or 10 μg/mL of encapsulated WT DNA), rProtein-loaded nanoparticles (carrying the equivalent of 3 μg/mL of encapsulated recombinant KMP-11) or with unloaded (empty) nanoparticles (847 μg/mL of polymer) in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen). After 4 h of stimulation, the cells received $3 \times 10^6$ cells/mL of stationary-phase *L. braziliensis* promastigotes and were incubated at 37°C in complete RPMI medium. After 24 h of infection, the glass coverslips containing the infected macrophages were washed and stained with H&E, and the intracellular amastigotes were counted by light microscopy. The results are shown as the percentage of infected cells per 100 macrophages. The number of intracellular amastigotes was counted in 100 macrophages. Alternatively, the infected macrophages were washed extensively, and the medium was replaced with 0.5 mL of Schneider medium (Sigma) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 26°C for an additional 5 days, and the number of viable promastigotes was determined with a hemocytometer. In some experiments, the macrophages were pretreated for 24 h with Z-WEHD-FMK (R&D systems) (100 μM) to block caspase-1 activation and were later stimulated with the recombinant nanoparticles as above. Subsequently, the cells were infected with *L. braziliensis* as described and assayed for the percentage of infected macrophages and for the number of intracellular amastigotes.

Table 1
Characterization of nanoparticles formulated with rProtein, WT DNA or rDNA.

| Nanoparticle formulation | Size (nm) | Zeta potential (mV) | Molecule loading (μg/mg NP) | % EE[a] |
|---|---|---|---|---|
| Empty | 315 ± 35 | 30 ± 10 | _ | _ |
| rKMP11 (rProtein) | 370 ± 32 | 23 ± 7 | 3.5 ± 0.5 | 43.7 ± 6.3 |
| pcDNA3 (WT DNA) | 373 ± 15 | 23 ± 9 | 7.2 ± 0.7 | 90.3 ± 8.2 |
| pcDNA3-KMP11 DNA (rDNA) | 443 ± 75 | 28 ± 6 | 6.4 ± 1.2 | 79.5 ± 14.5 |

Nanoparticles were produced by the solvent evaporation method. Results represent the mean ± SD of two independent preparations.
[a] EE, encapsulation efficiency.

### Production of reactive species

Macrophages ($3 \times 10^6$ cells/mL) were obtained and stimulated with plasmid DNA-loaded nanoparticles (carrying the equivalent of 10 μg/mL of encapsulated rDNA or 10 μg/mL of encapsulated WT DNA), rProtein-loaded nanoparticles (carrying the equivalent of 3 μg/mL of encapsulated recombinant KMP-11) or with unloaded (empty) nanoparticles (847 μg/mL of polymer) in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen), in the presence of LPS (5 ng/mL) for 24 h. The Griess method was used to measure nitric oxide (NO) production via its stable reaction product, nitrite ($NO_2^-$).[22] For the detection of intracellular reactive oxygen species (ROS), macrophages were also stimulated as described above. Following incubation, 10 μM of the oxidative fluorescent dye probe DHE (dihydroethidium) (Invitrogen) was added to the cultures and cells were further incubated for 30 min at 37°C. The data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry) and FlowJo software (Tree Star).

### Cytokine and chemokine production

Macrophages ($3 \times 10^6$ cells/mL) were obtained and stimulated as above for 4 h. The supernatants were collected, and the production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was evaluated using an inflammatory Cytometric Bead Array (BD Biosciences) following the manufacturer's instructions. The data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry) and FCAP Array™ CBA Analysis Software (BD Biosciences). CXCL1/KC was detected by sandwich ELISA (R&D Systems) according to the manufacturer's instructions. For the detection of caspase-1 activity, as

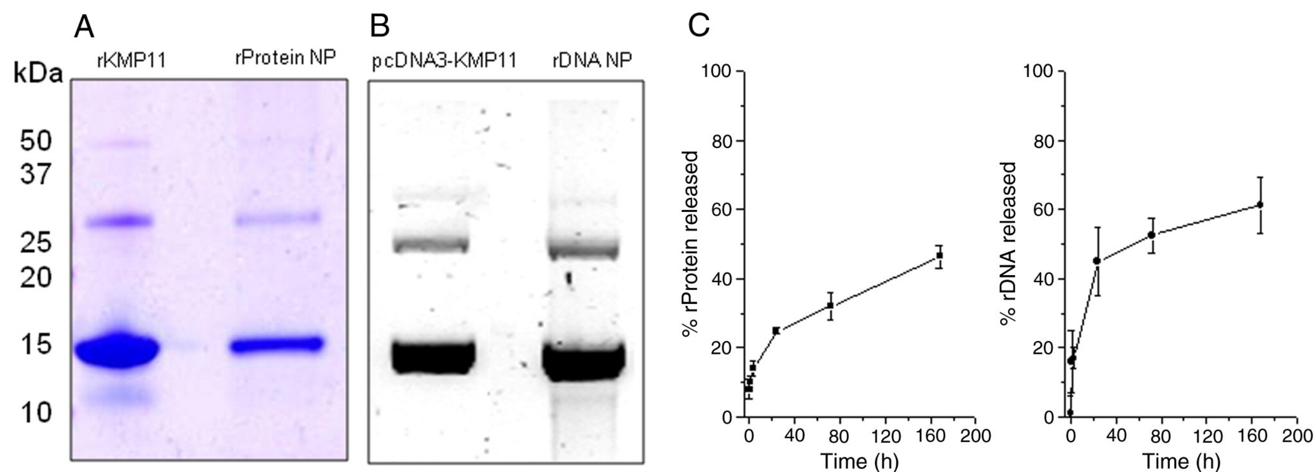



Figure 1. Study of rProtein and rDNA integrity after loading in NP. **(A)** SDS-PAGE of KMP-11 and rProtein nanoparticles extracted with 0.1N NaOH. **(B)** Agarose gel of rDNA alone and rDNA extracted from recombinant nanoparticles (rDNA NPs) and stained with ethidium bromide. **(C)** In vitro release of rProtein and rDNA from nanoparticles over time.

well as the presence of IL-1β and IL-18, experiments were performed in the presence of LPS (5 ng/mL). After 24 h, the caspase-1 activity was measured by a colorimetric assay (R&D Systems) according to the manufacturer's instructions. The supernatants were collected and analyzed by ELISA for the presence of mature IL-1β (E-Bioscience) and IL-18 (Medical & Biological Laboratories). For inhibitory assays, the macrophages were pretreated for 24 h with Z-WEHD-FMK (R&D systems) (100 μM) to block caspase activation. The supernatants were collected and assayed for the presence of mature IL-1β as above. Alternatively, the supernatants were collected, frozen at −20°C and employed in macrophage and neutrophil chemotaxis assays.

*Chemotaxis assay*

Macrophages were obtained as described above, while neutrophils were obtained following stimulation with 3% thioglycolate for 7 h. Briefly, peritoneal exudate neutrophils obtained 7 h after 3% thioglycolate solution injection were incubated at 37°C/5% $CO_2$ for 1 h in 250-mL flasks (Costar). Nonadherent cells were stained with anti-Ly-6G to assess purity. Data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry) and FlowJo software (Tree Star). According to this protocol, purity of Ly-6G$^+$ cells was routinely above 93% (data not shown). The cells (polymorphonuclear cells or macrophages) were resuspended in complete RPMI 1640 medium ($10^5$ cells/well) and added to the upper compartment of a Chemo TX System (Neuro Probe). The supernatants of macrophages stimulated with rDNA- or rProtein-loaded nanoparticles or with unloaded (empty) nanoparticles, as described earlier, were added to the bottom compartment. The plates were incubated for 90 min (macrophages)[23] or 60 min (polymorphonuclear cells)[24] and migration in the presence of culture medium (random chemotaxis) was used as a negative control. Chemotaxis indices were calculated as the ratio of the number of cells that migrated in the presence of the stimulus to the number of cells that migrated following stimulation with culture medium alone.[25]

*Statistical analysis*

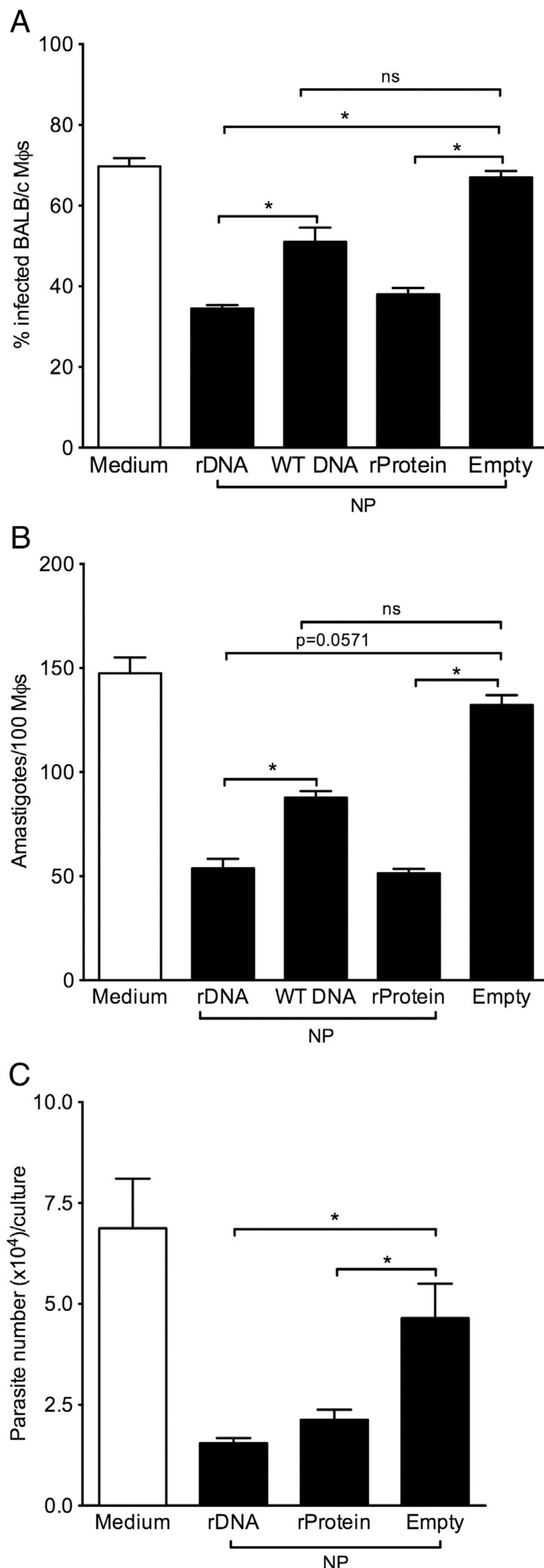
The data are presented as the mean ± SEM. The significance of the results was calculated using the following nonparametric statistical tests: two-sided Mann–Whitney for comparisons between two groups and Kruskal–Wallis followed by Dunn's multiple comparison test for comparisons between three or more groups. The analyses were conducted using GraphPad Prism 5.0 software. Differences were considered statistically significant when $P \leq 0.05$.

## Results

*Characterization of KMP-11-loaded PLGA nanoparticles*

The nanoparticles prepared with rProtein or plasmid DNA (WT or rDNA) displayed a mean size of 300-450 nm, irrespective of the type of encapsulated antigen (WT, rDNA or rProtein) (Table 1). The mean zeta potential values were between 20 and 30 mV, indicating a positive charge at pH 7.4, which was also independent of the nanoparticle load (rDNA or rProtein). Antigen content varied from 3.5 to 7.2 μg/mg NP and encapsulation efficiency was above 43%. The presence of DOTAP was necessary for the optimal (70%-90%) encapsulation efficiency of DNA.[20] We maintained the cationic lipid in the formulation of rProtein-loaded nanoparticles, despite the decreased encapsulation of recombinant KMP-11 by approximately 50%, to allow comparisons among the different formulations. Moreover, the modulation of multiple activation pathways with DOTAP and other cationic lipids has been previously reported.[26]

The electrophoretic mobility of recombinant protein extracted from rProtein nanoparticles showed a similar profile to that obtained with recombinant KMP-11 alone (Figure 1, *A*). The absence of bands of lower molecular weight also indicates the maintenance of protein integrity after encapsulation. The mobility of naked pcDNA3-KMP-11 was similar to that observed with plasmid DNA extracted from rDNA-loaded nanoparticles and the presence of supercoiled DNA within rDNA nanoparticles




indicated structural integrity (Figure 1, *B*). We also determined the cumulative release of rDNA and rProtein from recombinant nanoparticles. The particles presented a low burst effect: 15% each of rProtein and rDNA (Figure 1, *C*) were released during the first 3 h, corresponding with the fraction located near the particle surface. Subsequently, we observed a phase of sustained release with an accumulation of ~50% (rProtein) and ~65% (rDNA) released by the time of the final observation at day 7.

*KMP11-loaded PLGA nanoparticles induce parasite killing in infected macrophages*

Macrophage stimulation with rDNA- and rProtein-loaded nanoparticles or with empty nanoparticles did not alter cell viability, indicating that formulations are not toxic (Supplemental Material, Fig. 2). To probe for microbicidal effects, the cells were incubated with rDNA-, WT DNA-, or rProtein-loaded nanoparticles or with unloaded (empty) nanoparticles and were subsequently infected with *L. braziliensis*. Stimulation with rDNA-loaded nanoparticles significantly decreased the number of infected macrophages when compared with WT DNA-loaded or control (empty) nanoparticles (Figure 2, *A*). Similarly, stimulation with rProtein-loaded nanoparticles also significantly decreased the parasite load when compared with control (empty) nanoparticles (Figure 2, *A*). Importantly, stimulation with recombinant KMP-11 alone failed to decrease the parasite load (Supplemental Material, Fig. 3). The average number of infected macrophages in unstimulated cultures (medium) was significantly higher [69.7 ± 4.1 (mean ± SEM)] than that of cultures stimulated with rDNA-loaded nanoparticles [34.5 ±1.7(mean ± SEM)] or with rProtein-loaded nanoparticles [(38 ±3.1) (mean ± SEM)]. Macrophage treatment with rDNA- or rProtein-loaded nanoparticles also significantly decreased the number of amastigotes per infected cell, compared to empty nanoparticles (Figure 2, *B*). Furthermore, macrophage stimulation with rDNA- or rProtein-loaded nanoparticles significantly inhibited the in vitro differentiation of *L. braziliensis* promastigotes compared to control (empty) nanoparticles (Figure 2, *C*).

*KMP-11-loaded PLGA nanoparticles induce nitric oxide and superoxide production*

Stimulation with rDNA- and WT DNA-loaded nanoparticles significantly increased nitric oxide (NO) production compared with control (empty) nanoparticles (Figure 3, *A*). Production of NO was lower upon treatment with rProtein-loaded or with empty nanoparticle stimulation. We detected increased superoxide levels in the cells treated with either rDNA- or rProtein-loaded nanoparticles. In these conditions, the mean fluorescence

Figure 2. Pre-stimulation of macrophages with KMP11-loaded nanoparticles (NP) promotes L. braziliensis killing. Thyoglycolate-elicited macrophages were stimulated with rDNA-, WT DNA-, or rProtein-loaded nanoparticles or with control (empty) nanoparticles. The control cultures were left unstimulated (medium). The macrophages were infected with *L. braziliensis* and assessed for the percentage of infected cells **(A)** and for the number of amastigotes per infected macrophage **(B)** using light microscopy or culture in Schneider medium **(C)**. The data are from two independent experiments and are shown as the mean ± SEM. *$P < 0.05$.

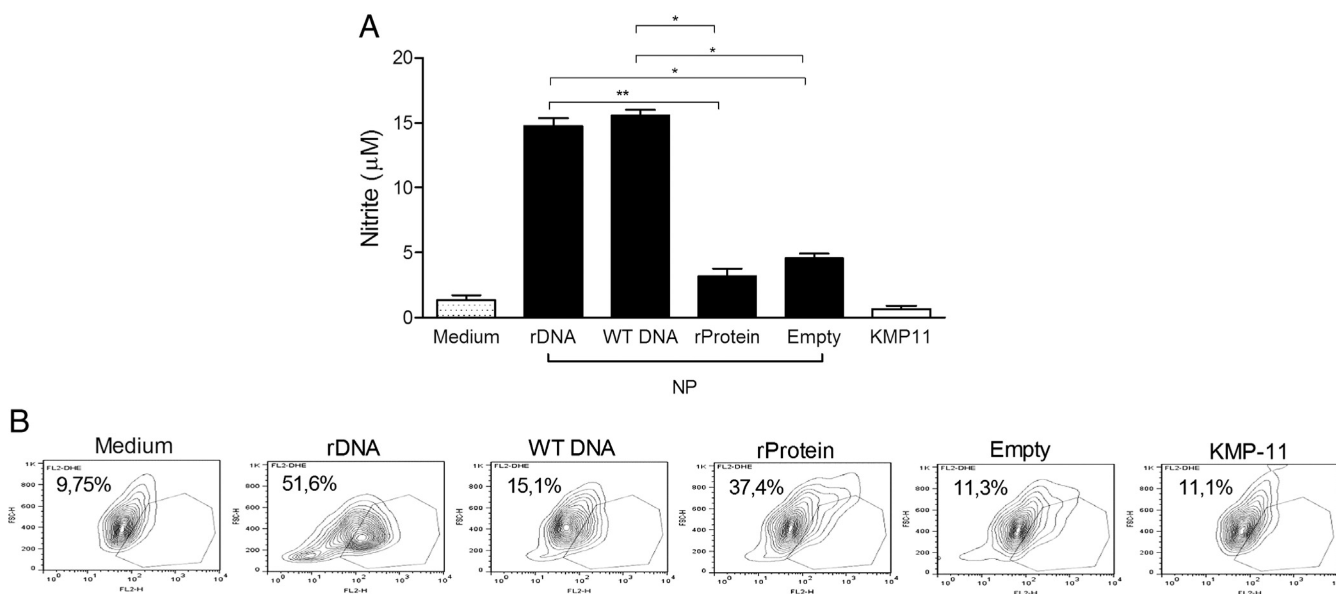



Figure 3. NO and superoxide production following macrophage stimulation with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA-, WT DNA or rProtein-loaded nanoparticles or with control (empty) nanoparticles in the presence of LPS. The control cultures were left unstimulated (medium). **(A)** After 24 h, the supernatants were assayed for nitrite production. The data are from a single experiment representative of two independent experiments and are shown as the mean ± SEM. $^{**}P < 0.01$. **(B)** The cells were incubated with 10 µM DHE and analyzed for superoxide production by flow cytometry. The data are shown as the percentage of $DHE^+$ cells. The results shown are from one experiment representative of two independent experiments.

intensity, as detected by flow cytometry, was higher than that of the control cultures (Figure 3, *B*), including cells stimulated with WT DNA-loaded and empty nanoparticles. Therefore, we may associate parasite killing upon stimulation with KMP-11-loaded nanoparticles with induction of the oxidative burst.

### *KMP-11-loaded PLGA nanoparticles promote cytokine secretion and cell recruitment*

Next, we evaluated the cytokine production upon exposure to KMP-11-loaded nanoparticles. Macrophages incubated with rDNA- or rProtein-loaded nanoparticles produced significantly more TNF-α (Figure 4, *A*) and IL-6 (Figure 4, *B*) than those incubated with control (empty) nanoparticles. The production of IL-10 did not change significantly (Figure 4, *C*). When used at concentrations (3 µg/mL) similar to those of rProtein-loaded nanoparticles, recombinant KMP-11 alone failed to alter the production of TNF-α and IL-6 (Supplemental Material, Fig. 4). Macrophage stimulation with rDNA- or rProtein-loaded nanoparticles also significantly augmented CCL2/MCP-1 (Figure 5, *A*) and CXCL1/KC (Figure 5, *B*) secretion, when compared with control (empty) nanoparticles.

The biological effects of CCL2/MCP-1 and CXCL1/KC on macrophage and neutrophil recruitment were evaluated in a chemotaxis assay. The supernatants from cells stimulated with rProtein-loaded nanoparticles promoted macrophage recruitment compared with control supernatants (Figure 5, *C*), indicating the presence of bioactive CCL2/MCP-1 (Figure 5, *A*). In parallel, the supernatants from cells stimulated with either rDNA NP- or rProtein-loaded nanoparticles also significantly increased neutrophil recruitment in comparison with the control supernatants (Figure 5, *D*), confirming the presence of bioactive CXCL1/KC (Figure 5, *B*).

### *KMP-11-loaded PLGA nanoparticles trigger inflammasome activation*

The uptake of poly(lactide-co-glycolide) (PLG) and polystyrene microparticles promotes inflammasome activation[27]; therefore, we also analyzed caspase-1 activation and the secretion of IL-1β and IL-18. Stimulation with either rDNA- or rProtein-loaded nanoparticles significantly increased caspase-1 activation in the presence of LPS (Figure 6, *A*) in comparison with control (empty) nanoparticles. Additionally, the secretion of mature IL-1β (Figure 6, *B*, open bars) was also elevated when compared with control (empty) nanoparticles, indicative of caspase-1 activation (Figure 6, *B*, open bars). On the contrary, the presence of the caspase-1 inhibitor Z-WEHD-FMK (Figure 6, *B*, closed bars) significantly blocked the secretion of mature IL-1β by cells stimulated with either rDNA- or rProtein-loaded nanoparticles (Figure 6, *B*, closed bars). Moreover, only rDNA-loaded nanoparticles led to increased IL-18 production when compared with control (empty) nanoparticles (Figure 6, *C*).

Because pre-stimulation with recombinant nanoparticles induced parasite killing (Figure 2) and inflammasome assembly (Figure 6, *A-C*), we next determined whether this pathway is involved in nanoparticle-induced *L. braziliensis* killing. The macrophages were pretreated with Z-WEHD-FMK, stimulated with recombinant nanoparticles and infected with *L. braziliensis*. Notably, pretreatment with the caspase-1 inhibitor significantly augmented the number of infected cells (Figure 6, *D*) and

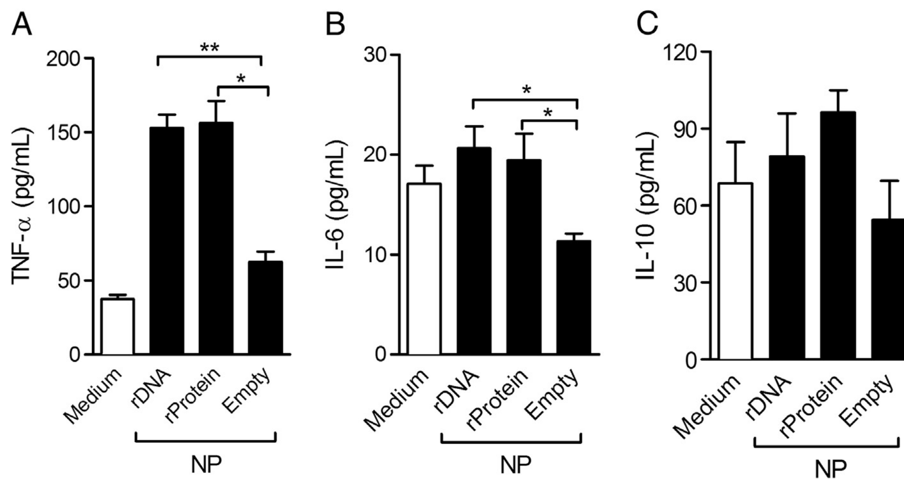



Figure 4. Cytokine secretion following macrophage stimulation with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles. The control cultures were left unstimulated (medium). The presence of secreted TNF-α **(A)**, IL-6 **(B)** and IL-10 **(C)** were detected in culture supernatants using a Cytometric Bead Array. The data are from a single experiment representative of three independent experiments and are shown as the mean ± SEM. $*P < 0.05$ and $**P < 0.01$.

amastigotes (Figure 6, *E*), despite pre-stimulation with rDNA- or rProtein-loaded nanoparticles. This effect was not observed in cells stimulated with unloaded (empty) nanoparticles.

## Discussion

PLGA is a biodegradable and biocompatible polymer used for the preparation of microparticles and nanoparticles. The uptake of antigen-loaded PLGA particles by dendritic cells enhanced antigen delivery,[28] prolonged MHC class-I presentation[29] and promoted a Th1 immune response.[30] We showed that immunization with PLGA nanoparticles loaded with KMP-11, a *Leishmania* vaccine candidate,[15,17] elicited a cellular immune response in mice and led to a lower parasite load following challenge with *L. braziliensis* parasites.[12] Given these results, we studied the effects of PLGA nanoparticles loaded with KMP-11 on the innate immune response, focusing on the macrophage given its dual role as host and effector cell during *Leishmania* infection.

The stimulation of macrophages with recombinant nanoparticles followed by *L. braziliensis* infection decreased parasite load and significantly increased NO production, a hallmark of microbicidal function. Immunostimulatory CpG motifs, present in the parasite gene insert coding for KMP-11 and in plasmid DNA, can enhance phagocytic activity when presented with other stimulatory elements[31] and may account for the elevated NO production, an effect not observed with recombinant protein, for example. Differently from NO, superoxide production was higher only in cells stimulated with rDNA and with rProtein-loaded nanoparticles. Since superoxide is important for experimental *L. braziliensis* elimination,[32] this finding may explain the reduced parasite load observed in these conditions. Internalization of silver nanoparticles by monocytes leads to leakage of cathepsins into the cytoplasm, resulting in production of ROS[33] and PLGA nanoparticles co-localize to endosomal compartments upon incubation with J774 macrophages.[34] In agreement, we also noted the accumulation of fluorescently labeled nanoparticles within stimulated macrophages (data not shown) suggesting that superoxide production may have resulted from internalization of the recombinant formulations.

PLGA microspheres loaded with *Histoplasma capsulatum* antigen and $LTB(_4)$ induced TNF-α and NO production by bone marrow-derived macrophages.[35] TNF-α is a pleiotropic inflammatory cytokine that synergizes with IFN-γ to induce cell activation and *Leishmania* killing,[36,37] dependent on induction of NO[38] and, in the case of *L. braziliensis*, of superoxide.[32] In this study, stimulation of macrophages with rDNA- or rProtein-loaded nanoparticles induced production of TNF-α, IL-6 and superoxide, and we propose that these mediators ultimately synergized toward *L. braziliensis* killing. Because KMP-11 alone, not loaded into nanoparticles, promoted the secretion of TNF-α, IL-6 and CCL2/MCP-1 only when employed at high concentrations (≥ 15 μg/mL) (Supplemental Material, Fig. 3), we suggest that the entrapment of KMP-11 in PLGA nanoparticles significantly enhances the ability of KMP-11 to induce parasite killing.

Chemokines are major players that regulate the sequential steps of leukocyte rolling, firm adherence, and transmigration to sites of inflammation. CXCL1 is a dominant chemokine in murine inflammatory responses[39] and is critical for neutrophil recruitment.[40] CCL2 induces chemotaxis, the respiratory burst in human monocytes[41] and stimulates the elimination of *Leishmania* parasites..[42] CCL2 and CXCL1 were detected in the supernatants of cells stimulated with KMP-11-loaded nanoparticles, suggesting that CCL2 may have synergized with superoxide in the killing of *L. braziliensis*. Following immunization with KMP-11-loaded nanoparticles, we observed an intense inflammatory reaction at the inoculation site (D.M Santos, unpublished), indicating in vivo effects of cellular recruitment and inflammation.

Inflammasomes are multiprotein complexes containing one or more nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that assemble in response to danger signals. Among these, the Nalp3 inflammasome is activated by “danger-

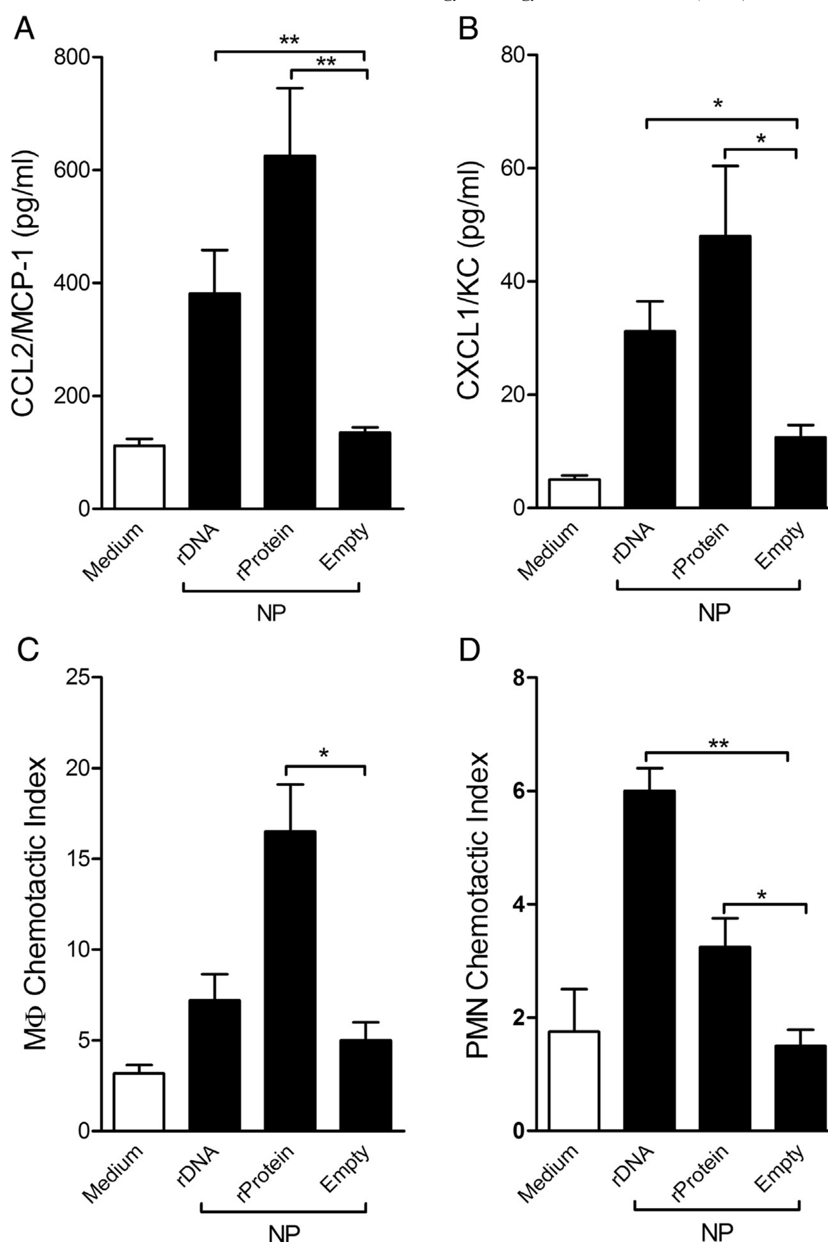



Figure 5. Chemokine production and cell chemotaxis following macrophage stimulation with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles. Control cultures were left unstimulated (medium). CCL2/MCP-1 **(A)** and CXCL1/KC **(B)** were detected using a Cytometric Bead Array and an ELISA, respectively. The culture supernatants were assayed for macrophage **(C)** and neutrophil **(D)** chemotaxis. The data are from a single experiment representative of two independent experiments and are shown as the mean ± SEM. *$P < 0.05$ and ** $P < 0.01$.

associated molecular patterns" or DAMPs (non-microbial/non-pathogenic), such as ATP,[43] uric acid crystals[44] and aluminum salt particles.[45] Stimulation of Nalp3 regulates caspase-1 activity, leading to the cleavage of pro-IL-1β and pro-IL-18 into the bioactive cytokines IL-1β and IL-18. Indeed, PLGA microparticle uptake by DCs promotes IL-1β secretion and caspase-1 activation[27] and carbon nanotubes also exert this effect in human monocytes.[46] Lastly, phagocytosis of particulate structures leads to lysosomal rupture, release of cathepsin B into the cytoplasm and inflammasome activation.[47] Here, co-stimulation with KMP-11-loaded nanoparticles and LPS activated caspase-1, leading to secretion of IL-1β and IL-18, indicative of inflammasome triggering. We may suggest that nanoparticle internalization led to superoxide production and inflammasome triggering, culminating in parasite killing. Of note, IL-18 secretion was detected only upon stimulation with rDNA-loaded nanoparticles. Two recent studies showed that, in response to intracytoplasmic nucleic acids, the AIM2 inflammasome regulates the caspase-1-dependent production of bioactive IL-1β and IL-18.[48,49] Mice deficient in *Aim2* and *Asc* showed reduced IL-18 concentrations when challenged with mCMV.[50] We then propose that stimulation with rDNA-loaded

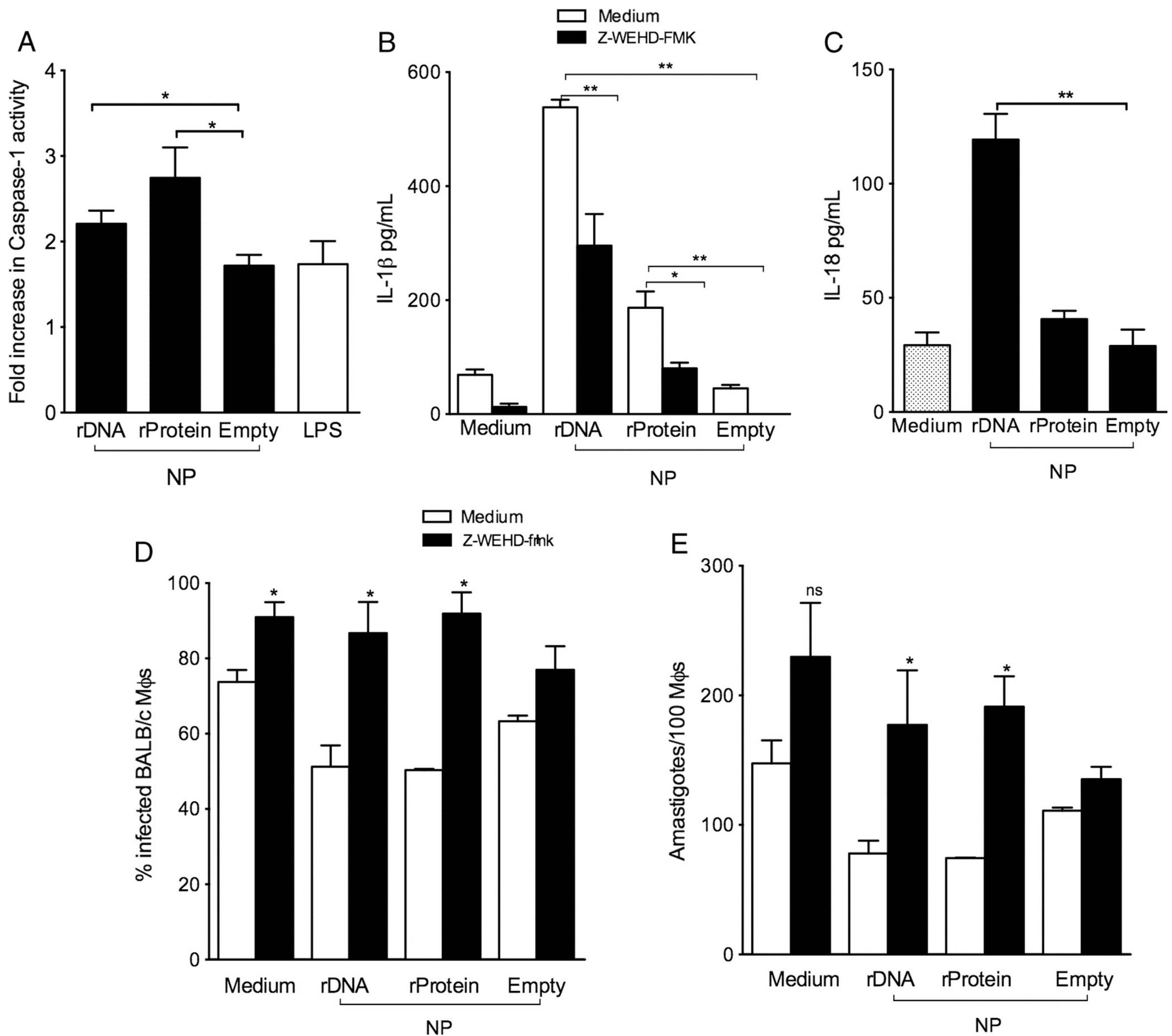



Figure 6. Activation of the inflammasome following stimulation of macrophages with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles in the presence of LPS. Caspase-1 activity **(A)** was detected using a colorimetric assay. The data are represented as the fold increase in the caspase-1 activity over that of cells incubated in medium only. **(B)** Cells were stimulated as described in the presence (closed bars) or absence (open bars) of Z-WEHD-FMK. Secreted IL-1β was detected using an ELISA. **(C)** The cells were stimulated as described above, and IL-18 was detected using an ELISA. **(D)** Cells were treated with Z-WEHD-FMK (closed bars) or were untreated (open bars) and were subsequently stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles. The cells were infected with *L. braziliensis* and assessed for the percentage of infected macrophages and for the number of amastigotes per infected macrophage **(E)** using light microscopy. The data are shown as the mean ± SEM and originate from two (A and C-E) or three **(B)** independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$.

nanoparticles may also trigger the AIM2 inflammasome, in parallel with the Nalp3 sensor.

PLGA nanoparticles loaded with a *Leishmania* protein (KMP-11) promote a powerful innate immune response in macrophages, characterized by the secretion of pro-inflammatory cytokines and chemokines, superoxide production and also inflammasome triggering. Collectively, these effects lead to cell activation and *L. braziliensis* killing. These results build on our previous findings in which immunization with KMP-11-loaded nanoparticles induced an immune response in mice and a reduction in parasite load, following a challenge with live parasites.[12] Based on this evidence, we propose that the current formulations can be further pursued as delivery vehicles in the development of vaccines against cutaneous leishmaniasis caused by *L. braziliensis*.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2013.04.003.

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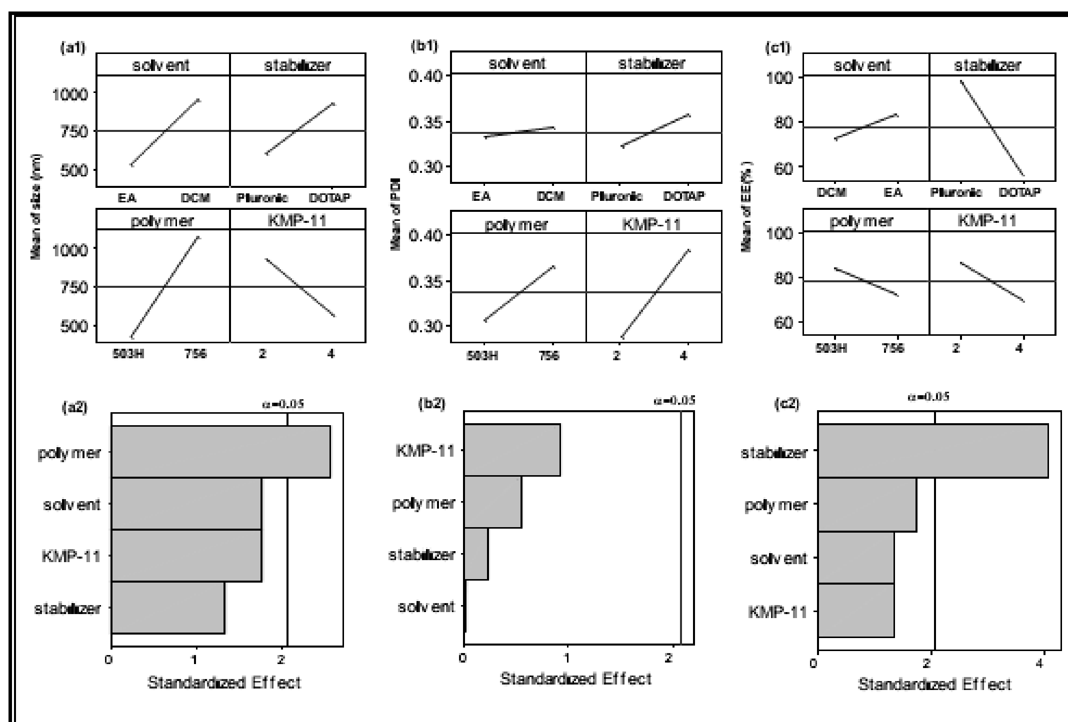
**Supplemental Material Table 1**. Run parameters for two-level four-factorial experimental design and experimental responses obtained for the different formulations.

| Experiment # | Factors | | | | Results | | |
|---|---|---|---|---|---|---|---|
| | PLGA | Organic Solvent | Stabilizer | Ag* (mg) | Mean size (nm) ± SD | PDI‡ | EE (%)§ |
| 1 | 756 | EA | Pluronic+ DOTAP | 4 | 1345.2 ± 955.8 | 0.5 ± 0.5 | 25.6 ± 16.2 |
| 2 | 756 | DCM | Pluronic | 4 | 450.8 ± 46.5 | 0.4±0.2 | 88.3 ± 48.9 |
| 3 | 503H | DCM | Pluronic | 2 | 507.3 ± 66.5 | 0.4± 0.2 | 95.2± 15.6 |
| 4 | 756 | EA | Pluronic | 4 | 216.1 ± 47.5 | 0.5 ± 0.6 | 91.5± 5.74 |
| 5 | 503H | DCM | Pluronic | 4 | 704.0 ± 192.3 | 0.3 ± 0.1 | 64.7 ± 9.8 |
| 6 | 756 | DCM | Pluronic+ DOTAP | 4 | 554.1 ± 43.5 | 0.3 ± 0.2 | 70.0 ± 36.2 |
| 7 | 503H | DCM | Pluronic+ DOTAP | 2 | 428.7 ± 91.2 | 0.2 ± 0.0 | 42.8 ± 11.1 |
| 8 | 756 | EA | Pluronic | 2 | 1942.0 ± 91.9 | 0.5 ± 0.6 | 87.6± 31.9 |
| 9 | 756 | DCM | Pluronic | 2 | 451.7 ± 12 | 0.2 ± 0.0 | 92.4 ± 20.2 |
| 10 | 503H | DCM | Pluronic+ DOTAP | 4 | 409.3± 100.5 | 0.3±0.2 | 47.2 ± 8.1 |
| 11 | 756 | DCM | Pluronic+ DOTAP | 2 | 705.3± 98.6 | 0.2±0.1 | 19.1± 13.3 |
| 12 | 503H | EA | Pluronic+ DOTAP | 4 | 531.6 ± 404.8 | 0.5 ± 0.4 | 86.2 ± 29.1 |
| 13 | 503H | EA | Pluronic | 4 | 256.2 ± 32.2 | 0.1 ± 0.0 | 83.6± 5.9 |
| 14 | 503H | EA | Pluronic+ DOTAP | 2 | 323.1± 25.3 | 0.3±0.2 | 71.5± 7.1 |
| 15 | 756 | EA | Pluronic+ DOTAP | 2 | 2469.9 ± 1017.3 | 0.2 ± 0.0 | 90.8± 5.6 |
| 16 | 503H | EA | Pluronic | 2 | 241.4 ± 3.6 | 0.1 ± 0.0 | 83.4± 47.5 |

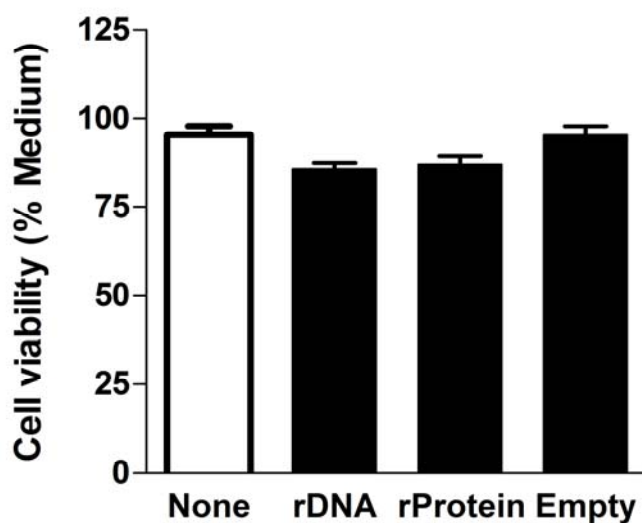
*Antigen

‡Polydispersion

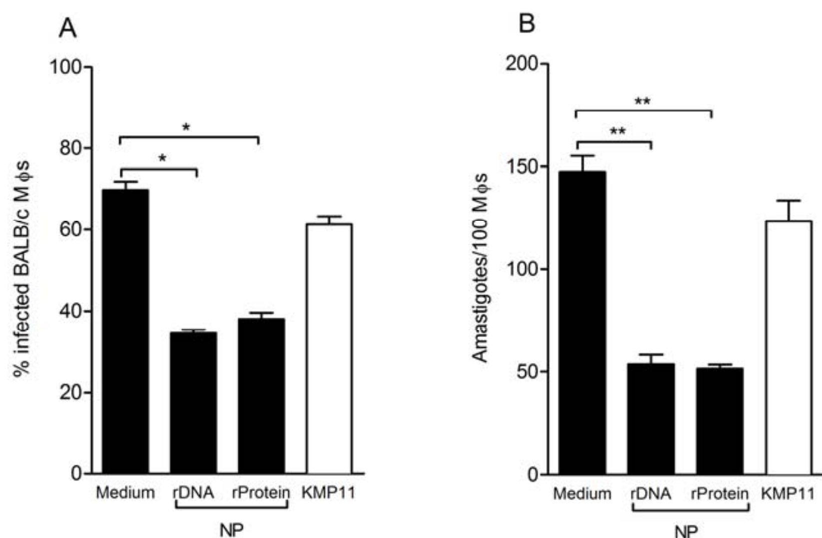
§Encapsulation Efficiency



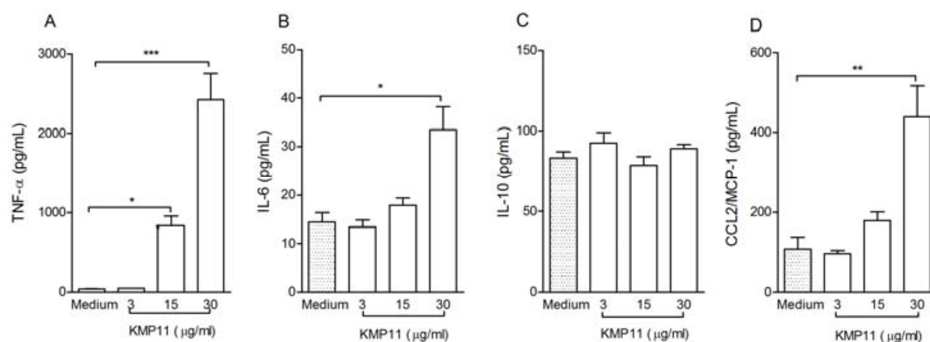

**Supplemental Material Figure 1**. Effect of variables (a1,b1,c1) and pareto chart of standardized effects (a2,b2,c2) of polymer type (503H, 756), organic solvent (DCM, EA), amount of KMP-11 (2, 4 mg) and stabilizer (pluronic, pluronic and DOTAP) on the mean particles size (a1,a2), polydispersion PDI (b1,b2) and encapsulation efficiency EE(%) (c1,c2).




**Supplemental Material Figure 2**. Macrophage viability following stimulation with KMP-11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA-, WT DNA-, rProtein-loaded nanoparticles or with control (empty) nanoparticles. The control cultures were left unstimulated (none). Viability was evaluated by MTT assay, performed 24 hours following nanoparticle stimulation. The data are from two independent experiments and are shown as the mean + SEM.




**Supplemental Material Figure 3.** Outcome of infection following macrophage stimulation with KMP11-loaded nanoparticles (NP) or recombinant KMP-11 alone. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with recombinant KMP-11 alone (3 µg/ml). The control cultures were left unstimulated (medium). The cells were infected with L. braziliensis and were assessed for the percentage of infected macrophages (A) and for the number of amastigotes per infected macrophage (B) using light microscopy. The data are from two independent experiments and are shown as the mean + SEM. *, p<0.05 and **, p<0.01.




**Supplemental Material Figure 4.** Cytokine production following macrophage stimulation with KMP-11 recombinant protein. Thyoglycolate-elicited macrophages were stimulated with increasing concentrations of recombinant KMP11 alone. Secreted TNF-α (A), IL-6 (B), IL-10 (C) and CCL2/MCP-1 (D) were detected using a Cytometric Bead Array. The data are shown as the mean + SEM and originate from a single experiment. *, p<0.05; **p<0.01 and ***p<0.001.

# 6. DISCUSSÃO

A leishmaniose é uma das doenças parasitárias que mais mata no mundo (revisado em DEN BOER et al., 2011) e até o momento não se tem uma vacina eficaz para prevenir a doença. Entretanto, o desenvolvimento de uma vacina contra a leishmaniose não é impossível, já que a cura da infecção confere proteção imunológica duradoura (revisado em ALVAR et al., 2013). Desta forma, a comunidade científica vem se esforçando para desenvolver e testar estratégias de uma vacina empregando proteínas recombinantes (revisado em KHAMESIPOUR et al., 2006), sistemas baseados em nanotecnologia (revisado em BADIEE et al., 2013) e até mesmo proteínas presentes na saliva do vetor (revisado em BRODSKYN et al., 2003).

Na 1ª geração de vacinas para a leishmaniose temos como exemplo a leishmanização que consiste na inoculação de parasitas vivos (KHAMESIPOUR et al., 2005). Essa estratégia foi muito utilizada no passado, mas, devido ao desenvolvimento de complicações tais como lesões que não cicatrizavam, está quase em desuso (revisado em KEDZIERSKI et al., 2006; revisado em KHAMESIPOUR et al., 2006). Problemas também foram relatados com o uso de parasitas atenuados que continuavam causando doenças (revisado em OKWOR; UZONNA, 2009; SPATH et al., 2004). Sendo assim, a maior parte dos estudos que visam desenvolver uma vacina, faz o uso de estratégias de 2ª ou 3ª geração. As vacinas de 2ª geração, caracterizadas pelo uso de antígenos recombinantes ou purificados são consideradas mais seguras, entretanto, apresentam uma baixa imunogenicidade e, assim, necessitam um bom adjuvante (BHOWMICK; ALI, 2008). Dentre os diversos adjuvantes utilizados, podemos destacar os oligodeoxinucleotídeos, como os motivos de CpG (IBORRA et al., 2008; ALAVIZADEH et al., 2012). O CpG é conhecido como um potente indutor de resposta Th1, que leva à ativação tanto da imunidade inata quanto da adaptativa (KUMAGAI et al., 2008; BODE et al., 2011). Já as vacinas de DNA são administradas, muitas vezes, na ausência de adjuvantes pois o plasmídeo de DNA contém numerosos motivos de CpG inseridos e as moléculas de DNA são reconhecidas por sensores citosólicos, levando à indução de uma resposta imune pró-inflamatória (revisado em KUMAGAI et al., 2008; revisado em COBAN et al., 2008, 2013).

Trabalhos na literatura mostraram que a imunização de camundongos BALB/c e C57BL/6 com extrato de proteínas ribossomais, na ausência de adjuvante, não protegeu contra a infecção por *Leishmania* (IBORRA et al., 2008; CHAVEZ-FUMAGALLI et al., 2010). Esse efeito foi associado à alta produção de IL-4, IL-10 e à baixa produção de IFN-γ. Entretanto, a imunização com extrato de proteínas ribossomais, na presença do CpG, levou ao desenvolvimento de uma resposta Th1 e à proteção contra infecção por *L. major* (IBORRA et al., 2008). O mesmo foi observado quando camundongos BALB/c foram vacinados dessa maneira e foram posteriormente desafiados com *L. infantum-chagasi* ou com *L. amazonensis* (CHAVEZ-FUMAGALLI et al., 2010). No extrato de proteínas ribossomais estão presentes quatro antígenos (S4, S6, L3 e L5) que foram reconhecidos pelo soro de pacientes com LT e LV e pelo soro de cães com LV. Como estes antígenos demonstraram ser antigênicos, avaliamos a capacidade protetora destas proteínas, frente a infecção por *L. major* e por *L. braziliensis* (Manuscrito I). Para isto, camundongos BALB/c foram imunizados com cada um dos antígenos, separadamente, na presença ou não do adjuvante CpG e foram desafiados com *L. major*. A vacinação com os antígenos L3 ou L5, na presença do CpG, induziu um perfil de resposta Th1, com elevada produção de IFN-γ, baixa produção de IL-10 e presença de anticorpos IgG2a. Nestes animais, observamos um menor tamanho da lesão em comparação com o grupo controle. Com relação à carga parasitária, vimos que somente os animais imunizados com L3+CpG ou L5+CpG apresentaram uma diminuição significativa no número de parasitas no linfonodo e no baço. Macrófagos, quando ativados por IFN-γ, controlam a infecção por *Leishmania* através da indução de espécies reativas de oxigênio (LIEW et al., 1990). Logo, a proteção induzida pelos antígenos L3 ou L5 pode ser associada com a indução, antígeno-específica, de IFN-γ e ao controle na produção de citocinas associadas com a susceptibilidade, como a IL-4 e a IL-10 (CHATELAIN et al., 1992; KANE; MOSSER, 2001). A IL-4 foi relacionada com um maior desenvolvimento da lesão e maior carga parasitária na infecção por *Leishmania* (KOPF et al., 1996; RADWANSKA et al., 2007).

Trabalhos prévios também demonstraram a indução de proteção por outras proteínas ribossomais como a P0 (IBORRA et al., 2005), L22 e S19 (STOBER et al., 2006) contra infecção por *L. major*. Este padrão de resposta, elevada produção de IFN-γ e baixa produção de IL-10, definido como “protetor” também foi obtido em ensaios de imunização com outras proteínas tais como LmSTI1 (BADIEE et al., 2008), Leish-111f/L110 (SAKAI et al., 2010),

LACK (KEDZIERSKA et al., 2012) e KSAC (GOMES et al., 2012). Vale salientar que a modulação na produção de IL-10 está associada com presença ou ausência de proteção (STOBER et al., 2005). Por exemplo, a imunização com a Leish-111f/L110 + CpG só conferiu proteção quando os animais receberam três imunizações, pois a IL-10 limitou a geração de células Th1 multifuncionais. Contudo, a inibição da IL-10 no momento da imunização, por meio da administração de anti-IL-10, elevou a magnitude e a qualidade da resposta Th1, reduzindo o número de imunizações (DARRAH et al., 2010). Uma vez que a proteção contra a infecção por *L. major* foi relacionado com a ausência de células T secretoras de IL-10, sugere-se que estas células podem influenciar não só o desfecho da infecção, mas também a eficácia da vacinação (SCHWARZ et al., 2013).

Em seguida, investigamos se os antígenos L3 e L5 seriam capazes de proteger contra a infecção por *L. braziliensis*. Desta forma, os camundongos foram imunizados com as proteínas L3, L5 ou L3+L5, na presença do CpG, e foram infectados com *L. braziliensis* na presença da saliva do vetor, mimetizando o contexto da infecção natural com *Leishmania* (BELKAID et al., 1998). Mesmo na presença da saliva, os camundongos imunizados apresentaram apenas um leve edema no sítio de inoculação. Trabalhos na literatura mostram que moléculas presentes na saliva do vetor exacerbam a infecção (SAMUELSON et al., 1991; TITUS, 1996; BELKAID et al., 1998; DONNELLY et al., 1998). A saliva inibe a ativação do macrófago pelo IFN-γ e a produção de NO, logo aumentando a viabilidade do parasita (HALL; TITUS, 1995). Recentemente, foi mostrado também que a saliva induz a apoptose de neutrófilos e diminui a produção de espécies reativas do oxigênio, aumentando a viabilidade do parasita dentro do neutrófilo (PRATES et al., 2011). Portanto, para o teste de novos candidatos vacinais contra a infecção por *Leishmania* é imprescindível a utilização da saliva no momento da infecção.

Com relação à carga parasitária no sítio da infecção, esta foi significativamente menor nos grupos imunizados com L5 ou L3+L5. Uma vez que a imunização com essas proteínas induziu a produção de IFN-γ, isso sugere que as células produtoras desta citocina migraram para o sítio de infecção, promovendo a destruição do parasita. Resultado similar foi obtido após a imunização com plasmídeos de DNA que codificam histonas nucleossomais seguido do desafio com *L. braziliensis,* na presença da saliva (CARNEIRO et al., 2012). Assim, a imunização com os antígenos ribossomais L3 e L5 conferiu proteção contra a infecção por *L.*

*major* e *L. braziliensis* (Manuscrito I) assim como protegeram contra a infecção por *L. infantum-chagasi* e por *L. amazonensis* (RAMIREZ et al., 2014). O desenvolvimento de uma única vacina que proteja contra as diferentes espécies de *Leishmania*, causadoras de uma doença espectral seria o ideal pois muitas espécies ocorrem nas mesmas regiões (revisado em HANDMAN, 2001). Este trabalho, juntamente com o de Ramirez e colaboradores (2014) apontam para essa possibilidade.

Na busca por outros candidatos a vacina, avaliamos a capacidade protetora do KMP-11. Já foi demonstrado que o KMP-11 induz a proliferação de células T (JARDIM et al., 1991) e a produção de IFN-γ por CMSP (Células mononucleares do sangue periférico) de pacientes curados de LV (KURTZHALS et al., 1994). A imunização com KMP-11 também protegeu hamsters (BASU et al., 2005) e camundongos da infecção por *L. donovani* e *L. major* (BHAUMIK et al., 2009). Sendo assim, avaliamos a capacidade protetora do KMP-11 contra a infecção por *L. braziliensis* (Manuscrito II). Neste estudo, testamos dois esquemas de imunização. O esquema homólogo consistiu de uma imunização inicial ("*priming*") seguida de um reforço "*boost*" e em ambos o antígeno foi inoculado na mesma formulação. No esquema heterólogo, o reforço foi realizado com o antígeno formulado de maneira distinta. Foi mostrado que o esquema heterólogo pode aumentar de 10 a 100 vezes a frequência de células T comparado com a estratégia homóloga (AURISICCHIO; CILIBERTO, 2011) e pode induzir mais rapidamente uma maior população de células T $CD8^+$ de memória (MASOPUST et al., 2006; PHAM et al., 2010). Antígenos tais como LACK (MELBY et al., 2001; MARQUES-DA-SILVA et al., 2005) e TRYP (RODRÍGUEZ-CORTÉS et al., 2007) não conferiram proteção contra a LV pela estratégia homóloga mas, sim, pela estratégia heteróloga (DONDJI et al., 2005; CARSON et al., 2009).

Em nosso trabalho (Manuscrito II), observamos que a imunização de camundongos com plasmídeo de DNA que codifica KMP-11 (rDNA – estratégia homóloga) levou à uma produção mista de citocinas (IL-2, IFN-γ, TNF-α, IL-4 e IL-5), sugerindo um padrão de resposta Th1/Th2. Resultados similares foram descritos anteriormente (BHAUMIK et al., 2009). Entretanto, a imunização com o plasmídeo de DNA que codifica KMP-11 (rDNA), encapsulado em NPs, seguida do reforço com a proteína recombinante, também encapsulada em NPs, e na presença de CpG (rDNA NP/rProteína NP + CpG – estratégia heteróloga) induziu um aumento significativo na produção de TNF-α. Nesse trabalho, utilizamos o

DOTAP, um lipídeo catiônico para o encapsulamento do plasmídeo de DNA nas NPs, e esse lipídeo induz a produção de citocinas (IL-2, IFN-γ e TNF-α) e quimiocinas pró-inflamatórias (MCP-1/CCL-2, MIP-1β/CCL4 ) (KEDMI et al., 2010; LONEZ et al., 2012). Além do mais, também usamos o CpG nas imunizações, conhecido por induzir produção de citocinas pró-inflamatórias como IFN-γ e TNF-α (KLINMAN et al., 1996; ZHU; MARSHALL, 2001). Esses componentes podem explicar a elevação na produção de TNF-α, observada após a imunização heteróloga (rDNA NP/rProteína NP + CpG).

Com relação à resposta imune humoral, observamos que animais imunizados com rDNA (estratégia homóloga) não apresentaram níveis detectáveis de anticorpos anti-KMP-11. Esse resultado foi diferente do relatado em outros trabalhos, nos quais hamsters e camundongos BALB/c imunizados com o plasmídeo de rDNA (BASU et al., 2005; BHAUMIK et al., 2009) desenvolveram resposta humoral. Todavia, nestes trabalhos a detecção da resposta humoral foi realizada após o desafio, o que pode levar à participação do KMP-11 do parasita na indução de resposta humoral. Já nos animais imunizados com rDNA NP/rProteína NP + CpG (estratégia heteróloga) observamos uma forte resposta imune humoral, com presença de IgG1 e IgG2a, sugerindo a participação tanto de IL-4 e IFN-γ na mudança de isotipos de anticorpos. As NPs têm um papel chave na indução da resposta imune humoral e celular, pois elas protegem os antígenos da degradação, melhoram a sua distribuição e facilitam a fagocitose (FREDRIKSEN; GRIP, 2012; HARDY et al., 2013). Além do que, a administração de antígenos proteicos encapsulados em NPs aumenta consideravelmente a produção de anticorpos, comparado com a administração do antígeno puro (DIWAN et al., 2002; TAFAGHODI et al., 2010), o que está de acordo com os nossos resultados.

Após a imunização com as diferentes formulações do KMP-11 (rDNA – estratégia homóloga e rDNA NP/rProteína NP + CpG – estratégia heteróloga), os camundongos foram infectados com *L. braziliensis,* na presença da saliva do vetor. Diferentemente do encontrado com as proteínas L3 e L5 (Manuscrito I), nenhuma das estratégias de imunização com KMP-11 preveniu o desenvolvimento da lesão. Apesar disso, observamos uma redução significativa da carga parasitária no sítio de infecção. Camundongos imunizados com rDNA NP/rProteína NP + CpG (estratégia heteróloga) apresentaram maior expressão de IFN-γ e TNF-α no sítio da infecção e menor expressão de IL-10. Esse resultado está de acordo com outros trabalhos que

também demonstram uma potente resposta imune celular em camundongos imunizados com antígenos de *Leishmania* encapsulados em NPs (DOROUD et al., 2011a, 2011b; TAFAGHODI et al., 2011). Tanto IFN-γ quanto TNF-α agem em conjunto para ativar a oxido nítrico sintetase, induzindo a produção de oxido nítrico (SKERRETT; MARTIN, 1996). Então, podemos sugerir que nos animais imunizados com rDNA NP/rProteína NP + CpG (estratégia heteróloga) houve maior redução na carga parasitária devido à maior expressão de IFN-γ e de TNF-α. No entanto, nos animais imunizados com o rDNA (estratégia homóloga) observamos maior expressão de IFN-γ e de IL-10 e esta última pode explicar a menor redução da carga parasitária. A IL-10 diminui a ação do IFN-γ pois reduz a produção de oxido nítrico por macrófagos ativados (VIETH et al., 1994; VOULDOUKIS et al., 1997). O pré-tratamento de macrófagos com IL-10, antes da adição do IFN-γ, inibe a ativação elevando o número de parasitas (KANE & MOSSER, 2001). Além disso, CMSP de pacientes com LT estimuladas com KMP-11 produzem altos níveis de IL-10 (DE CARVALHO et al., 2003) e a adição de KMP-11 às células pré-estimuladas com antígeno solúvel de *Leishmania* diminuiu a produção de IFN-γ (CARVALHO et al., 2005). Desta forma, podemos especular que a resposta imune induzida nos camundongos imunizados com rDNA (estratégia homóloga) pode ter sido modulada. Por outro lado, nos camundongos imunizados com rDNA NP/rProteína NP + CpG (estratégia heteróloga) a resposta imune pode ter sido positivamente modulada pela presença da NP e do CpG (LEE et al., 2010; DE TITTA et al., 2013).

Em síntese, observamos que as imunizações com rDNA (estratégia homóloga) ou com rDNA NP/rProteína NP + CpG (estratégia heteróloga) induziram uma resposta que resultou no controle da replicação parasitária após a infecção por *L. braziliensis* + saliva, mas incapaz de evitar a progressão da lesão (Manuscrito II). Nos animais imunizados, a redução da carga parasitária no local da infecção foi de 2 (rDNA -estratégia homóloga) e 3 logs (rDNA NP/rProteína NP + CpG estratégia heteróloga). Por outro lado, a imunização com os antígenos L3 e/ou L5 (Manuscrito I) induziu uma resposta capaz de controlar a replicação parasitária e capaz de inibir o desenvolvimento da lesão. Nesses animais, houve redução da carga parasitária em aproximadamente 4 (L3+CpG), 8 (L5+CpG) e 7 (L3+L5+CpG) logs. Possivelmente, a indução de uma resposta celular mais robusta, induzida pelas imunizações com os antígenos ribossomais (L3 e/ou L5) traduzida pela elevada produção de IFN-γ pode explicar essas diferenças. Outros trabalhos mostraram que a imunização com os antígenos

LmSP (RAFATI et al., 2006), a Leish-111f/L110 (SAKAI et al., 2010), o KSAC (GOMES et al., 2012) e as histonas (CARRIÓN et al., 2008) foi capaz de induzir uma resposta que controla a carga parasitária e o desenvolvimento da lesão devido à elevada produção de IFN-γ. Do mesmo modo, o encapsulamento dos antígenos CPa/b/c gerou uma resposta capaz de diminuir a carga parasitária de animais infectados por *L. major* em 86,3%, também devido a uma elevada produção de IFN-γ, enquanto a redução observada após a imunização com os antígenos não encapsulados ficou em 38% (DOROUD et al., 2011c).

Apesar das imunizações com rDNA (estratégia homóloga) e rDNA NP/rProteína NP + CpG (estratégia heteróloga) não terem impedido o desenvolvimento da lesão, observamos redução do número de parasitas na orelha infectada. Dados similares foram encontrados em outro estudo realizado em nosso laboratório, no qual hamsters imunizados com rDNA e desafiados com *L. infantum-chagasi* também apresentaram redução na carga parasitária (DA SILVA et al., 2011). Nossos resultados diferem, contudo, do estudo de Bhaumik et al. (2009), no qual camundongos BALB/c apresentaram redução do tamanho da lesão em aproximadamente 72% quando imunizados com rDNA e infectados com *L. major*. Em paralelo, houve redução de mais de 93% quando os animais foram imunizados com rDNA + IL-12. É possível que esta discrepância seja fruto de diferenças biológicas entre as espécies de parasitas *L. major* e *L. braziliensis* (revisado em SMITH et al., 2007).

No manuscrito II, demonstramos que a imunização de camundongos com o antígeno KMP-11 encapsulado em NPs de PLGA induziu uma resposta imune celular levando a diminuição da carga parasitária após infecção com *L. braziliensis*. Associado a esse efeito, observamos também um aumento de TNF-α e IFN-γ. As partículas de PLGA exercem um efeito adjuvante (SHARP et al., 2009) e esse efeito pode ser aumentado quando as NPs são combinadas com adjuvantes como o alum (RAGHUVANSHI et al., 2001), o CpG (DE TITTA et al., 2013) e a Poly(I:C) (LEE et al., 2010). Assim, decidimos avaliar o efeito das formulações de KMP-11 contendo NPs sobre a resposta imune inata de macrófagos, já que estas células são tanto infectadas quanto efetoras durante a infecção por *Leishmania* (Manuscrito III).

A estimulação de macrófagos com plasmídeo de DNA que codifica KMP-11 encapsulado em NPs (rDNA NP), KMP-11 recombinante encapsulada em NPs (rProteína NP)

ou com a NP vazia não diminuiu a viabilidade celular, indicando que estas formulações são seguras. Resultados similares foram descritos por Grabowski e colaboradores (2013). A estimulação de macrófagos com rDNA NP e rProteína NP seguida da infecção por *L. braziliensis* diminuiu significativamente a carga parasitária nas células infectadas e esse efeito pode ser associado com uma maior produção de óxido nítrico. Os motivos de CpG, presentes no plasmídeo, podem ter induzido a produção de óxido nítrico (HARTMANN & KRIEG, 1999). Além disso, o encapsulamento do plasmídeo de DNA em partículas aumenta a fagocitose destas, melhorando assim a ativação celular (FUKUHARA et al., 2007). Diferente do óxido nítrico, a produção de superóxido foi maior nos grupos estimulados tanto com rDNA NP quanto com rProteína NP. Entretanto, a NP vazia não foi capaz de induzir a produção de superóxido, o que está de acordo com a literatura (XIONG et al., 2013), assim como o antígeno puro de KMP-11.

A fagocitose das NPs é facilitada pelo tamanho e partículas de até 500 nm, com carga positiva e que contenham DOTAP, são mais propensas a serem fagocitadas (FOGED et al., 2005; THURN et al., 2007). Observamos que as NPs de PLGA são facilmente fagocitadas pelos macrófagos no tempo de quatro horas de incubação (mais de 93,0% - dados não mostrados). Dados similares foram relatados pela literatura (NICOLETE et al., 2011; MA et al., 2012). A estimulação de macrófagos com rDNA NP e rProteína NP também levou à produção de TNF-α, IL-6, CCL2/MCP-1 e CXCL-1/KC. O TNF-α em conjunto com o IFN-γ induz a ativação celular e a morte da *Leishmania*, através da produção de óxido nítrico (ASSREUY et al., 1994) e espécies reativas do oxigênio (KHOURI et al., 2010; NOVAIS et al., 2014). As quimiocinas são fundamentais para determinar o desfecho da leishmaniose (OGHUMU et al., 2010), pois algumas espécies de *Leishmania* podem modular o padrão de citocinas/quimiocinas do hospedeiro, recrutando células que favoreçam a persistência do parasita (KATZMAN; FOWELL, 2008) ou tornando a sua entrada mais "silenciosa" (MATTE; OLIVIER, 2002; JI et al., 2003). A CXCL-1/KC é responsável pela quimiotaxia de neutrófilos e já foi demonstrado que os neutrófilos possuem um papel importante no controle da infecção por *L. braziliensis* (NOVAIS et al., 2009). A CCL2/MCP-1 é responsável por induzir a quimiotaxia, a explosão respiratória em monócitos humanos (ROLLINS et al., 1991) e a eliminação do parasita (RITTER; MOLL, 2000). Desta forma, a CCL2/MCP-1 pode contribuir tanto para o recrutamento quanto a ativação dos macrófagos, tendo como

consequência a destruição do parasita. Contrapondo o exposto acima, a estimulação de macrófagos com a proteína de KMP-11 somente foi capaz de induzir a produção de TNF-α, IL-6 e CCL-2/MCP-1 quando em altas concentrações (≥ 15 μg/mL). Diante disso, podemos inferir que o encapsulamento do KMP-11 em NPs de PLGA potencializa a ação da KMP-11 em induzir a morte do parasita.

O inflamassoma é um complexo multiprotéico intracelular que tem como componente os receptores do tipo NOD (NLRs). Estes receptores são responsáveis por detectar padrões moleculares associados a patógenos (PAMPS) e padrões moleculares associados ao perigo (DAMPs). O inflamassoma NLRP3 é um complexo multiprotéico composto pelo NLRP3, ASC e caspase-1 (revisado em KIM; JO, 2013). Para ativar o inflamassoma NLRP3 são necessários dois sinais: o primeiro sinal é induzido pelos ligantes de Toll (TLR), receptor de IL-1 e de TNF, o que leva à produção da pró-IL-1β e pró-IL-18. O segundo sinal leva à oligomerização do complexo do inflamassoma, causando a ativação da caspase-1 e, consequentemente, a ativação e secreção da IL-1β e IL-18, nas suas formas bioativas. Este segundo sinal pode ser o ATP (MARIATHASAN et al., 2006), cristais de ácido úrico (MARTINON et al., 2006), asbestos, sílica (DOSTERT et al., 2008) e partículas (EISENBARTH et al., 2008). Já foi demonstrado que micro/nanopartículas de PLGA fagocitadas por células dendríticas ou macrófagos promovem a secreção da IL-1β e a ativação da caspase-1 (DEMENTO et al., 2009; SHARP et al., 2009). A fagocitose de materiais particulados induz a ruptura do lisossomo, a liberação da catepsina B no citoplasma e a ativação do inflamassoma (HORNUNG et al., 2008; YANG; CHOI, 2013). Em nosso trabalho, observamos que a estimulação de macrófagos com rDNA NP e rProteína NP aumentou de modo significativo a ativação da caspase-1 e a produção da IL-1β na presença do LPS, indicando a ativação do inflamassoma. Em paralelo, a adição do inibidor da caspase-1 (Z-WEHD-FMK) bloqueou a produção da IL-1β nos grupos estimulados com rDNA NP e rProteína NP. Por outro lado, a produção da IL-18 só foi detectada no grupo estimulado com rDNA NP. Podemos inferir que a presença do rDNA no citoplasma celular pode ter ativado tanto o inflamassoma NALP3 quanto o AIM2 pois esse último está relacionado com o reconhecimento de DNA intracitoplasmático, levando à ativação da caspase-1 e à produção da forma ativa de IL-1β e IL-18 (FERNANDES-ALNEMRI et al., 2009; HORNUNG et al., 2009). Em paralelo, observamos que macrófagos tratados com o inibidor da caspase-1 e

estimulados com rDNA NP e rProteína NP apresentaram um aumento da carga parasitária, sugerindo assim a participação do inflamassoma na morte da *Leishmania*, o que está de acordo com os dados recentes de Lima-Junior e colaboradores (2013). Nesse trabalho, os autores mostraram que o NALP3 inflamassoma é ativado em resposta à infecção por *Leishmania* e possui um papel importante na restrição da replicação do parasita em ensaios com macrófagos e animais infectados com *L. amazonensis*, *L. infatum-chagasi* e *L. braziliensis* (LIMA-JUNIOR *et al*., 2013). A ativação do inflamossoma foi relacionada com o controle da replicação de vários parasitas como bactérias (FUKUHARA et al., 2007; MCNEELA et al., 2010) , vírus (ALLEN et al., 2009; ERMLER et al., 2014) e protozoários (ZHOU et al., 2012).

No manuscrito III, mostramos que as NPs de PLGA encapsuladas com KMP-11 (rDNA NP e rProteína NP) induziram uma forte resposta imune inata em macrófagos, com produção de citocinas e quimiocinas pró-inflamatórias, produção de superóxido e também ativação do inflamassoma. Em conjunto, estes efeitos levaram à ativação celular e consequente destruição da *L. braziliensis* (manuscrito III). Além da indução da resposta imune inata, observamos também a indução da imunidade adaptativa (Manuscrito II), uma vez que a imunização de camundongos com KMP-11 encapsulados em NPs levou à redução da carga parasitária em animais desafiados com *L. braziliensis*.

# 7. CONCLUSÃO

A imunização de camundongos BALB/c com os antígenos ribossomais L3 ou L5, associados com o adjuvante CpG, foi capaz de proteger contra a LT causada pela *L. major* e *L. braziliensis*, levando a uma redução significativa na carga parasitária e até mesmo prevenção da lesão. Em ambos os modelos, a proteção induzida foi associada com uma maior produção de IFN-γ. Por outro lado, a imunização com KMP-11 não foi capaz de prevenir o desenvolvimento da doença em camundongos BALB/c infectados com *L. braziliensis*. Entretanto, as duas estratégias de imunização que utilizamos (plasmídeo de DNA somente – rDNA, e antígeno encapsulado em NPs + CpG - rDNA NP/rProteína NP + CpG) levaram à redução da carga parasitária no local da infecção. Além disso, as NPs demonstraram características de adjuvantes, já que houve ativação da resposta imune inata, através da produção de citocinas e quimiocinas pró-inflamatórias, produção de superóxido e ativação do inflamassoma. Desta forma, propomos que a formulação de antígenos em nanopartículas deve ser explorada quanto ao desenvolvimento de vacinas contra leishmaniose cutânea causada por *L. braziliensis*.

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# APÊNDICE

Artigos produzidos durante o período do doutorado e que não entraram no corpo da tese.

Chemotherapeutic Potential of 17-AAG against Cutaneous Leishmaniasis caused by Leishmania braziliensis. **D M Santos**, A L O A Petersen, F S Celes, V M Borges, P S T Veras and C I de Oliveira. **Submetido na PLOS Neglected Tropical Diseases**.

L Ramirez, L Corvo, M C Duarte, M A Chávez-Fumagalli, D G Valadares, **D M Santos**, C I de Oliveira, M R Escutia, C Alonso, P Bonay, C AP Tavares, E AF Coelho and M Soto. Cross-protective effect of a combined L5 plus L3 Leishmania major ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis. **Parasites & vectors**, v. 7, n. 1, p. 3, 2014.

M W Carneiro; **D M Santos**; K F Fukutani; J Clarencio; J C Miranda; C Brodskyn; A Barral; M Barral-Netto; M Soto and C I de Oliveira. Vaccination with L. infantum chagasi nucleosomal histones confers protection against new world cutaneous leishmaniasis caused by Leishmania braziliensis. **PLoS One**, v. 7, n. 12, p. e52296, 2012.

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# Vaccination with *L. infantum chagasi* Nucleosomal Histones Confers Protection against New World Cutaneous Leishmaniasis Caused by *Leishmania braziliensis*

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## Abstract

***Background:*** Nucleosomal histones are intracellular proteins that are highly conserved among *Leishmania* species. After parasite destruction or spontaneous lysis, exposure to these proteins elicits a strong host immune response. In the present study, we analyzed the protective capability of *Leishmania infantum chagasi* nucleosomal histones against *L. braziliensis* infection using different immunization strategies.

***Methodology/Principal Findings:*** BALB/c mice were immunized with either a plasmid DNA cocktail (DNA) containing four *Leishmania* nucleosomal histones or with the DNA cocktail followed by the corresponding recombinant proteins plus CpG (DNA/Protein). Mice were later challenged with *L. braziliensis*, in the presence of sand fly saliva. Lesion development, parasite load and the cellular immune response were analyzed five weeks after challenge. Immunization with either DNA alone or with DNA/Protein was able to inhibit lesion development. This finding was highlighted by the absence of infected macrophages in tissue sections. Further, parasite load at the infection site and in the draining lymph nodes was also significantly lower in vaccinated animals. This outcome was associated with increased expression of IFN-γ and down regulation of IL-4 at the infection site.

***Conclusion:*** The data presented here demonstrate the potential use of *L. infantum chagasi* nucleosomal histones as targets for the development of vaccines against infection with *L. braziliensis*, as shown by the significant inhibition of disease development following a live challenge.

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## Introduction

Leishmaniasis is an infectious disease with significant economic impact in several countries. Over three hundred million people are exposed to the parasites, with 12 million infected worldwide, predominantly in tropical and subtropical countries (World Health Organization page: http://www.who.int/emc/diseases/leish/leisdis1.html). Leishmaniasis can be caused by different species of *Leishmania spp.* protozoans that infect macrophages in the human host. The treatments available for all forms of leishmaniasis are toxic, and drug resistance is on the rise, further increasing the need for vaccine development [1].

Numerous attempts have been made to find a protective antigen against leishmaniasis and several candidates have been tested for this purpose [2,3], including histones. Histones are structural proteins found in the nucleus, where they play an important role in the organization and function of chromatin. There are five main classes of histones; four of them (H2A, H2B, H3 and H4) form the nucleosomal core unit of chromatin, whereas H1 joins to linker DNA. The percentage of similarity between *Leishmania* nucleosome forming histones and their mammal counterparts ranges from 49% (for the H2B) to 63% (for the H3) [4]. Differences are mainly located in the aminoacid sequences of the nucleosome-exposed tails of the four histones [5]. So far, no cross reactivity was found between *Leishmania* histones and their mammalian counterparts. Antibodies specific for parasite histones, obtained from dogs with visceral leishmaniasis, react against *Leishmania* H2A [6], H3 [7], H2B and H4 [8] but do not recognize mammalian histones. Antibodies in sera from patients with cutaneous or mucocutaneous leishmaniasisis also recognize parasite histone H1 but not the human counterpart [9]. Regarding the T cell immunogenicity of parasite histones, recombinant versions of H2B [10] or H2A [11] induced IFN-γ secretion upon stimulation of PBMCs obtained from cutaneous leishmaniasis patients. The T cell response was

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# Cross-protective effect of a combined L5 plus L3 Leishmania major ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis

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