Intranasal immunization with LACK-DNA promotes protective immunity in hamsters challenged with *Leishmania chagasi*

DANIEL CLAUDIO DE OLIVEIRA GOMES[1,2]*, BEATRIZ LILIAN DA SILVA COSTA SOUZA[1], HERBERT LEONEL DE MATOS GUEDES[1], ULISSES GAZOS LOPES[1] *and* BARTIRA ROSSI-BERGMANN[1]

[1] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21941-902, Rio de Janeiro, RJ, Brazil*
[2] *Núcleo de Doenças Infecciosas, Universidade Federal do Espírito Santo, 29040-091, Vitória, ES, Brazil*

(*Received 18 May 2011; revised 4 July 2011; accepted 21 July 2011; first published online 26 August 2011*)

SUMMARY

LACK (*Leishmania* analogue of the receptor kinase C) is a conserved protein in protozoans of the genus *Leishmania* which is associated with the immunopathogenesis and susceptibility of BALB/c mice to *L. major* infection. Previously, we demonstrated that intranasal immunization with a plasmid carrying the LACK gene of *Leishmania infantum* (LACK-DNA) promotes protective immunity in BALB/c mice against *Leishmania amazonensis* and *Leishmania chagasi*. In the present study, we investigated the protective immunity achieved in hamsters intranasally vaccinated with 2 doses of LACK-DNA (30 μg). Compared with controls (PBS and pCI-neo plasmid), animals vaccinated with LACK-DNA showed significant reduction in parasite loads in the spleen and liver, increased lymphoproliferative response and increased nitric oxide (NO) production by parasite antigen-stimulated splenocytes. Furthermore, hamsters vaccinated with LACK-DNA presented high IgG and IgG2a serum levels when compared to control animals. Our results showed that intranasal vaccination with LACK-DNA promotes protective immune responses in hamsters and demonstrated the broad spectrum of intranasal LACK-DNA efficacy in different host species, confirming previous results in murine cutaneous and visceral leishmaniasis.

Key words: *Leishmania chagasi*, hamster, LACK-DNA, mucosal vaccine.

INTRODUCTION

Visceral leishmaniasis (VL) caused by *Leishmania (Leishmania) chagasi* [syn *Leishmania (Leishmania) infantum*] is one of the most important emergent diseases in both tropical and subtropical areas. The disease can be particularly virulent, attaining a level of 98% mortality in certain non-treated human cases (Romero *et al*. 2010).

Despite the recent advances in new anti-leishmanial compounds (Oliveira *et al*. 2011), the first-line therapy for all disease forms is still based on painful multiple injections of pentavalent anti-monials which invariably produce serious toxicity. The problem is further aggravated by the surge of antimonial resistance in some areas where the disease is endemic (Kulshrestha *et al*. 2011), urging the development of effective anti-*Leishmania* vaccines.

Several vaccination strategies using the parenteral route have been tested with limited success in experimental leishmaniasis including whole parasites (killed or irradiated promastigotes) (Okwor and Uzonna, 2008), soluble extracts (Holzmuller *et al*. 2005), synthetic peptides (Bertholet *et al*. 2009), crude and pure surface antigens (Pinheiro *et al*. 2007), recombinant antigens (Mizbani *et al*. 2009), amastigote antigens (Fernandes *et al*. 2008), immuno-stimulating complexes (Tewary *et al*. 2004) and plasmid DNA (Basu *et al*. 2005). On the other hand, mucosal vaccination is emerging as a potential administration route for eliciting both antigen-specific mucosal and systemic immunity, and has been widely used as an efficient and non-invasive route not only for inducing tolerance to Th2 response-associated allergens and other disease-promoting antigens (Czerkinsky *et al*. 1997; Takagi *et al*. 2005), but also for eliciting active systemic immunity against the parasites (Arakawa *et al*. 2005; Chabot *et al*. 2005).

Previously, our laboratory reported the efficacy of both oral and intranasal (i.n.) vaccination with particulated leishmanial antigens and plasmid en-conding the cytoplasmic LACK protein from *L. chagasi* (LACK-DNA) in protecting BALB/c mice against *L. amazonensis* and *L. chagasi* (Pinto *et al*. 2004; Gomes *et al*. 2007). LACK-DNA intranasal (i.n.) vaccination induced the expansion of mixed Th1 and Th2 systemic responses associated with high production of IFN-γ and IL-4, a decrease of IL-10 and TNF-α and the induction of a long-lasting protective immunity (Gomes *et al*. 2007).

* Corresponding author: Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21941-902, Rio de Janeiro, RJ, Brazil. Tel/Fax: 55 (27) 33357210 and 55 (27) 33357207. E-mail: dgomes@ndi.ufes.br

Parasitology (2011), **138**, 1892–1897. © Cambridge University Press 2011
doi:10.1017/S0031182011001417

https://doi.org/10.1017/S0031182011001417 Published online by Cambridge University Press

Although human VL is characterized by a progressive disease, mouse models are able to control the infection and do not reflect the human disease symptoms (Melby *et al*. 2001*a*). Unlike mice, Syrian hamster (*Mesocricetus auratus*) reproduce clinicopathological features of human VL, with uncontrolled disease (Garg and Dube, 2006), making it a better animal model to study *L. donovani* and *L. chagasi* infections. Hamsters also provide excellent models for vaccine studies and they have been used for selection of vaccine candidates prior to tests in dogs (Garg and Dube, 2006).

In this study, we evaluated the intranasal LACK-DNA vaccine in an extremely sensitive animal model of VL, to ascertain its protective efficacy and potential application to humans.

MATERIALS AND METHODS

Animals, parasites and antigens

Female Syrian golden hamsters (*Mesocricetus auratus*) (4–6 weeks old), were reared in our departmental facility. Their use in this study was approved by the Ethics Committee on the Use of Experimental Animals at the Universidade Federal do Rio de Janeiro under the number IBCCF118. *L. chagasi* strain MHOM/BR/1974/M2682 was continuously maintained by repeated passage in hamsters and expanded in DMEM medium, pH 6.8 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoetanol and 20 μg/ml gentamicin (DMEM 20% SFB) at 25 °C. The *L. chagasi* antigen (LcAg) was prepared from late-log-phase promastigotes by centrifugation, washing 3 times in PBS and disruption by 5 rounds of freezing-thawing. The protein content was estimated by the Lowry method (Lowry *et al*. 1995). The recombinant LACK protein (rLACK) was obtained as described by Coelho *et al*. (2003). The expression vector pCI-neo (Promega) encoding the LACK gene (LACK-DNA) was prepared as described previously, and was a kind gift from Dr Vicente Larraga (Gonzalez-Aseguinolaza *et al*. 1999).

Vaccination and infection

Hamsters (5/group) were vaccinated intranasally by instillation with 30 mg of LACK-DNA with a fine tip attached to a micropipette, and boosted 7 days later with the same vaccine dosage. The controls received PBS or empty pCI-neo. One week after booster, the animals were challenged using the intracardiac (i.c.) route with 10^7 log-phase *Leishmania chagasi* promastigotes.

Determination of the parasite burden

Thirty days after infection, hamster spleens and livers were isolated for parasite burden determination by limiting-dilution assay. The whole organs were individually weighed and homogenized in a tissue grinder with DMEM medium containing 20% heat-inactivated fetal calf serum (HIFCS). Serial dilutions were done in quadruplicate followed by culture (15 days/25 °C). The original number of parasites in each organ was calculated from the reciprocal of the highest dilution containing promastigotes.

Cutaneous hypersensitivity reaction

Twenty-four hours after infection, vaccinated and non-vaccinated hamsters were injected in the hind footpad with 20 μg of LcAg in 20 μl of PBS. Footpad swelling was measured with a dial caliper, and the results were expressed as the difference between the thickness of the injected and pre-injected footpads.

Nitric oxide production and parasite-specific antibodies

On day 30 of infection, splenocytes were re-stimulated *in vitro* with LcAg (50 μg/ml) for 48 h and the nitric oxide (NO) production measured in the supernatants using the Griess method (Green *et al*. 1982). Briefly, 50 μl of cell culture supernatants were mixed with 50 μl of Griess reagent (1% sulphanilamide and 0·1% N-1-naphthylethylenediamine dihydrochloride in 2·5% *o*-phosphoric acid) and incubated at room temperature for 10 min. The nitrite concentrations were determined at 540-nm wavelength using a standard sodium nitrite curve. The parasite-specific IgG, IgG_1 and IgG_2 levels were measured in the sera of individual hamsters by direct ELISA using LcAg-coated microplates as described by Gomes *et al*. (2007).

Lymphoproliferation assay

Hamster splenocytes were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich, USA). The cell concentration was adjusted to 5×10^5 cells/well in 96-well culture plate in the presence of Concanavalin A (10 μg/ml, Sigma–Aldrich), LaAg (50 μg/ml) or rLACK (5 μg/ml). Cultures were incubated at 37 °C in a CO_2 incubator with 5% CO_2 for 3 days followed by 3H-thymidine addition (1 μCi) for a further 18 h. The radioactivity was measured in a liquid scintillation counter (Beckman, USA) and the results were expressed as counts per minute (cpm).

Statistics

Data were statistically analysed using the GraphPad Prism software. Means of normally distributed variables were compared by ANOVA analysis simple factorial test and by one-way ANOVA-Tukey's honestly significant difference (Tukey's HSD)

https://doi.org/10.1017/S0031182011001417 Published online by Cambridge University Press

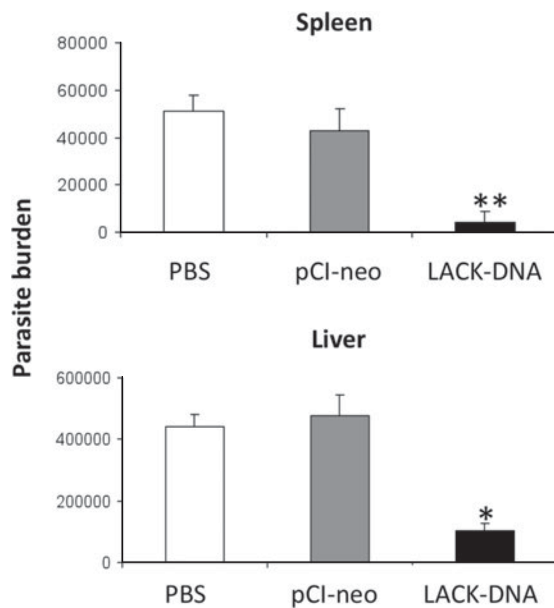

Fig. 1. Parasite burden in pre-vaccinated infected hamsters. Golden Syrian hamsters were intranasally vaccinated twice with 30μg of LACK-DNA and challenged by the intracardiac route with 1×10^7 *L. chagasi* late-log-phase promastigotes 1 week after booster. Controls received empty pCI-neo vector or PBS. The parasite burden in individual organs was measured on day 30 of infection by limiting-dilution culture. Results are represented as arithmetic means ± S.D. from 3 independent experiments. *$P < 0{\cdot}05$, ** $P < 0{\cdot}01$ as compared with both control groups.

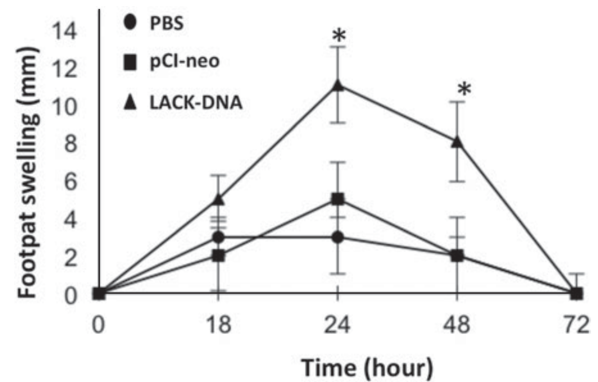

Fig. 2. Delayed-type hypersensitivity in hamsters pre-vaccinated with LACK-DNA. Hamsters were intranasally vaccinated and i.c.-infected as described for Fig. 1, and 24 h after infection, they were s.c.-challenged in the footpad with 25 μg of LaAg and the footpad swelling was recorded at the indicated times. The data are presented as means ± S.D. of 3 independent experiments. *$P < 0{\cdot}05$ as compared with both control groups.

post-hoc method, and were considered significantly different when $P < 0{\cdot}05$.

RESULTS

Parasite burden

To investigate whether the protective effect of intranasal vaccination with LACK-DNA seen in mice could be reproduced in a susceptible model of VL, Syrian Golden hamsters were vaccinated with PBS, pCI-neo or LACK-DNA and challenged with *L. chagasi* promastigotes 1 week after the booster. LACK-DNA vaccinated hamsters exhibited significant parasite burden reduction in both liver and spleen when compared to PBS or pCI-neo control groups (Fig. 1). These findings were compatible with their healthier clinical signs and agility and contrary to prostrated animals receiving empty plasmid or PBS. On the other hand, no significant differences in spleen and liver parasite burden between pCI-neo and PBS groups were observed (Fig. 1).

Delayed-type hypersensitivity test

The acquisition of protective immunity following *Leishmania* infection is associated with the development of both positive DTH reaction and antigen-specific cell proliferation. The specific DTH response was evaluated by LcAg injection into the footpad 1 day after *L. chagasi* infection, followed by swelling measurement during 72 h. LACK-DNA – vaccinated hamsters showed the highest level of DTH 24 and 48 h after antigen challenge when compared to control groups (Fig. 2). No significant difference was observed between PBS or pCI-neo vaccinated animals upon LcAg challenge (Fig. 2).

Splenocyte proliferation and nitric oxide production

Splenocytes from LACK-DNA hamsters exhibited significantly higher proliferation upon *in vitro* re-stimulation with either LcAg or rLACK, when compared to PBS and pCI-neo control groups (Fig. 3A). Moreover, no significant increase in proliferation was observed in PBS and pCI-neo cells after antigen recall (Fig. 3A), compatible with the observed parasite burden and DTH results.

To speculate on the effective mechanism involved in the protection followed by LACK-DNA intranasal immunization, NO production on day 30 of infection was measured in the splenocyte supernatants stimulated with LcAg or rLACK. As seen in Fig. 3B, cells from LACK-DNA-vaccinated hamsters produced more NO than PBS and pCI-neo controls ($P < 0{\cdot}01$).

Humoral immune response

To understand whether the differential degree of protection obtained by intranasal LACK-DNA immunization correlated with a specific pattern of immunoglobulin production, we evaluated the leishmanial antigen-specific IgG and its isotype (IgG1 and IgG2) levels in the sera of control and vaccinated groups (Fig. 4). Sera from LACK-DNA hamsters

https://doi.org/10.1017/S0031182011001417 Published online by Cambridge University Press

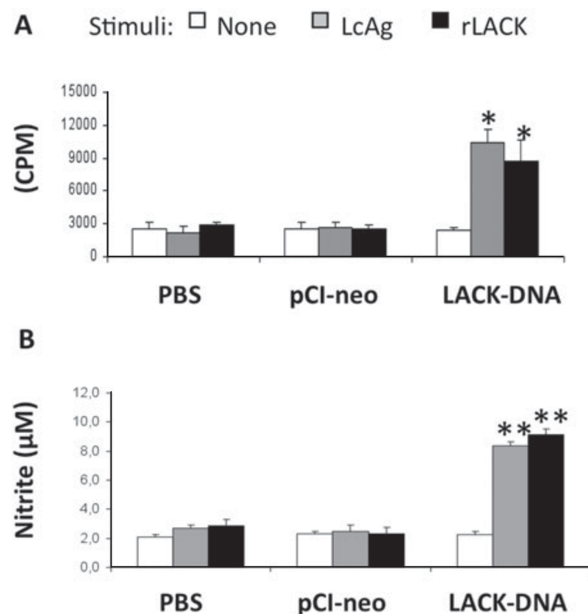

Fig. 3. Parasite-specific lymphoproliferative responses and NO production. Animals were intranasally vaccinated and i.c.-challenged as described in Fig. 1. On day 30 of infection, their splenocytes were isolated. (A) The parasite-induced lymphoproliferative responses were determined by 3H-thymidine incorporation after *in vitro* re-stimulation with LcAg (50μg/ml), rLACK or none. The rate of ^{3}H-incorporation was expressed as cpm with means ± S.D. from 3 independent experiments. *$P<0{\cdot}05$ as compared with both control groups. (B) Nitric oxide production was determined in the splenocyte supernatants after *in vitro* re-stimulation with the same antigens as in (A). Means ± S.D. from 3 independent experiments. *$P<0{\cdot}05$, ** $P<0{\cdot}01$ as compared with both control groups.

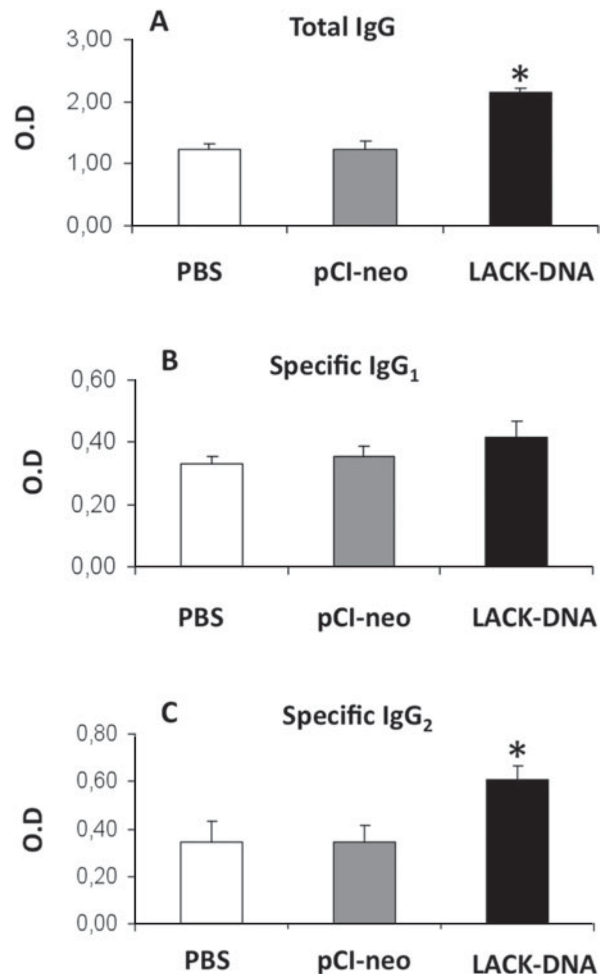

Fig 4. Pattern of antibody production during infection. Hamsters were vaccinated and infected as described in Fig. 1. On day 30 of infection, their sera were collected and assayed by ELISA for (A) total IgG, (B) IgG_1 and (C) IgG_2. Arithmetic means ± S.D. *$P<0{\cdot}05$ as compared with both control groups.

presented a substantial increase in parasite-specific IgG and IgG_2 levels as compared with control groups (Fig. 4A and C). Furthermore, no significant differences in IgG and IgG_2 production were observed between control groups (PBS and pCI-neo) (Fig. 4A and C), also in the IgG_1 levels between controls (PBS and pCI-neo) and LACK-DNA vaccinated group (Fig. 4B).

DISCUSSION

Previous studies from our laboratory have hinted towards the prophylactic potential of LACK-DNA intranasal administration to induce a significant immune response in experimental models of cutaneous and visceral murine leishmaniasis (Pinto *et al.* 2004; Gomes *et al.* 2007). Syrian golden hamsters (*Mesocricetus auratus*) have proven to be the most susceptible and appropriate experimental model of rodents, reproducing with more fidelity the clinical and pathological features of human VL, including the continued parasitaemia, cachexy, hepatosplenomegaly and death (Bhowmick *et al.* 2007).

The parenteral use of LACK-DNA vaccine was not able to protect mice against challenge with *L. donovani* (Melby *et al.* 2001*b*) and *L. chagasi* (Marques-da-Silva *et al.* 2005), unlike the protection achieved with intranasal vaccination against *L. chagasi* (Gomes *et al.* 2007). In those studies, Melby *et al.* (2001*b*) and Marques da Silva *et al.* (2005) tested vaccination efficacy between 4 and 12 weeks post-immunization, and demonstrated that long-term protection cannot be achieved. The present study evaluated, for the first time, the LACK-DNA vaccine in hamsters. Similar to observations in mice, hamsters doubly vaccinated with 30 μg of LACK-DNA (i.n.) showed an impressive reduction of parasite load in both the spleen and liver. Both our previous (mice) and present (hamster) studies evaluated vaccine efficacy at 1 week after booster. We recently investigated in mice the possibility that not only effector but also long-term memory immunity was established after i.n. vaccination. In that mouse study, both LACK mRNA tissue expression and

https://doi.org/10.1017/S0031182011001417 Published online by Cambridge University Press

protective immunity lasted for at least 12 weeks post-vaccination (*manuscript submitted*). Although the vaccine memory duration has not been yet evaluated in hamsters, it is worth noting that even a short-term memory vaccine can be of use, for instance when taken just prior to a visitation to an endemic area. This is particularly true if the vaccine is potentially self-administrable as is the case of an intranasal vaccine.

The vaccine success in humans and animal models has often been related to delayed-type hypersensitivity (DTH) and antigen-specific T-cell responsiveness (Mazumdar *et al*. 2005; Bhowmick *et al*. 2007). Although those parameters do not always correspond to protection efficiency (Velez *et al*. 2005), strong antigen-specific cellular immune responses such as the DTH and the lymphoproliferative response are related to VL healing (Garg *et al*. 2006). Notably, LACK-DNA-vaccinated hamsters showed a significant DTH response at 24–48 h when compared to control groups. Hamsters infected with *L. donovani* by the subcutaneous route do not mount a DTH response, unlike intradermally-infected animals that display a healing-associated DTH response (Gifawesen and Farrell, 1989). The induction of DTH following s.c. antigen challenge confirmed the peripheral cellular immunity promoted by the i.n. LACK-DNA. Moreover, splenocytes from these animals exhibited enhanced lymphoproliferative responses after LcAg or rLACK *in vitro* antigen recall, demonstrating the correlation between the cellular immune response and resistance to *L. chagasi* infection.

Active VL has been related to decreased cellular immune responses associated with high antibody production prior to parasite detection (Bhowmick *et al*. 2007). Like mice, hamsters show changes of IgG class to IgG_1 or IgG_2 influenced by IL-4 or IFN-γ production, respectively. Thus, the level of antibody response appears to be comparable with the extent of protection outcome observed in animals vaccinated prior to infection (Bhattacharjee *et al*. 2009). Intranasal LACK-DNA vaccination induced an increase in IgG and IgG_2 levels, but not of IgG_1, suggesting that in these animals the cellular immune response was skewed to Th1, and correlated with the observed specific cellular immune response and lower parasitism. In conformity with these findings, we found elevated NO production in hamsters vaccinated with LACK-DNA while the control animals (PBS and pCI-neo) had baseline production, even under parasite antigen stimulation, suggesting nitrite-mediated induction of a macrophage mediator mechanism towards intracellular parasite control. The absence of NO production is associated with progressive disease in hamsters (Melby *et al*. 2001*b*), while the induction of NO by LACK-DNA vaccination reverts the progressive disease as a mechanism of disease control. The NO production suggests IFN-γ activation and/or inhibition of IL-10 and TGF-β production which results in Th1 response involved in hamster protection.

Our study clearly demonstrates a high degree of protection accomplished with intranasal LACK-DNA in an extreme VL susceptibility model promoting systemic immunity that effectively protects hamsters against VL caused by *L. chagasi*. Besides its effectiveness and potential broad-spectrum efficacy against leishmaniasis, this vaccine offers a number of advantages over parenteral vaccines (Arakawa *et al*. 2005; Chabot *et al*. 2005). Since no needles are used, compliance is increased and blood transmissible diseases are avoided.

REFERENCES

Arakawa, T., Komesu, A., Otsuki, H., Sattabongkot, J., Udomsangpetch, R., Matsumoto, Y., Tsuji, N., Wu, Y., Torii, M. and Tsuboi, T. (2005). Nasal immunization with a malaria transmission-blocking vaccine candidate, Pfs25, induces complete protective immunity in mice against field isolates of *Plasmodium falciparum*. *Infection and Immunity* **73**, 7375–7380. doi: 10.1128/IAI.73.11.7375-7380.2005.

Basu, R., Bhaumik, S., Basu, J. M., Naskar, K., De, T. and Roy, S. (2005). Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *The Journal of Immunology* **174**, 7160–7171.

Bertholet, S., Goto, Y., Carter, L., Bhatia, A., Howard, R. F., Carter, D., Coler, R. N., Vedvick, T. S. and Reed, S. G. (2009). Optimized subunit vaccine protects against experimental leishmaniasis. *Vaccine* **27**, 7036–45. doi: 10.1016/j.vaccine.2009.09.066.

Bhattacharjee, S., Gupta, G., Bhattacharya, P., Adhikari, A., Majumdar, S. B. and Majumdar, S. (2009). Anti-iL-10 mAb protection against experimental visceral leishmaniasis via induction of Th1 cytokines and nitric oxide. *Indian Journal Experimental Biology* **47**, 489–497.

Bhowmick, S., Ravindran, R. and Ali, N. (2007). Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1 response. *Vaccine* **29**, 6544–6556. doi:10.1016/j.vaccine.2007.05.042.

Chabot, S., Brewer, A., Lowell, G., Plante, M., Cyr, S., Burt, D. S. and Ward, B. J. (2005). A novel intranasal Protollin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice. *Vaccine* **23**, 1374–1383. doi: 10.1016/j.vaccine.2004.09.010.

Coelho, E. A., Tavares, C. A., Carvalho, F. A., Chaves, K. F., Teixeira, K. N., Rodrigues, R. C., Charest, H., Matlashewski, G., Gazzinelli, R. T. and Fernandes, A. P. (2003). Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. *Infection and Immunity* **71**, 3988–3994. doi: 10.1128/IAI.71.7.3988-3994.2003.

Czerkinsky, C., Anjuere, F., McGhee, J. R., George-Chandy, A., Holmgren, J., Kieny, M. P., Fujiyashi, K., Mestecky, J. F., Pierrefite-Carle, V., Rask, C. and Sun, J. B. (1997). Mucosal immunity and tolerance: relevance to vaccine development. *Immunological Reviews* **170**, 197–222.

Fernandes, A. P., Costa, M. M., Coelho, E. A., Michalick, M. S., de Freitas, E., Melo, M. N., Luiz Tafuri, W., Resende, D. de M., Hermont, V., Abrantes, C. De. F and Gazzinelli, R. T. (2008). Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. *Vaccine* **29**, 5888–5895. doi: 10.1016/j.vaccine.2008.05.095.

Garg, R. and Dube, A. (2006). Animal models for vaccine studies for visceral leishmaniasis. *Indian Journal of Medical Research*. **123**, 439–454.

Garg, R., Gupta, S. K., Tripathi, P., Hajela, K., Sundar, S., Naik, S. and Dube, A. (2006). *Leishmania donovani*: identification of stimulatory soluble antigenic proteins using cured human and hamster lymphocytes for their prophylactic potential against visceral leishmaniasis. *Vaccine* **24**, 2900–2909. doi: 10.1016/j.vaccine.2005.12.053.

Gifawesen, C. and Farrell, J. P. (1989). Comparison of T-cell responses in self-limiting versus progressive visceral *Leishmania donovani* infections in golden hamsters. *Infection and Immunity* **57**, 3091–3096.

Gomes, D. C. O., Pinto, E. F., de Melo, L. D., Lima, W. P., Larraga, V., Lopes, U. G. and Rossi-Bergmann, B. (2007). Intranasal delivery of naked DNA encoding the LACK antigen leads to protective immunity against visceral leishmaniasis in mice. *Vaccine* **25**, 2168–2172. doi:10.1016/j.vaccine.2006.11.060.

Gonzalez-Aseguinolaza, G., Taladriz, S., Marquet, A. and Larraga, V. (1999). Molecular cloning, cell localization and binding affinity to DNA replication proteins of the p36/LACK protective antigen from *Leishmania infantum*. *European Journal of Biochemistry* **259**, 909–916.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1962). Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Analytical Biochemistry* **126**, 131–138.

Holzmuller, P., Cavaleyra, M., Moreaux, J., Kovacic, R., Vincendeau, P., Papierok, G. and Lemesre, J. L. (2005). Lymphocytes of dogs immunised with purified excreted-secreted antigens of *Leishmania infantum* co-incubated with *Leishmania* infected macrophages produce IFN gamma resulting in nitric oxide-mediated amastigote apoptosis. *Veterinary Immunology and Immunopathology* **106**, 247–257. doi:10.1016/j.vetimm.2005.03·001.

Kulshrestha, A., Singh, R., Kumar, D., Negi, N. S. and Salotra, P. (2011). Antimony resistant clinical isolates of *Leishmania donovani* are susceptible to paromomycin and sitamaquine. *Antimicrobial Agents and Chemotherapy* **1**, 0: AAC.00812-10v1.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with Folin-phenol reagent. *The Journal of Biological Chemistry* **193**, 265–275.

Marques-da-Silva, E. A., Coelho, E. A., Gomes, D. C., Vilela, M. C., Masioli, C. Z., Tavares, C. A., Fernandes, A. P., Afonso, L. C. and Rezende, S. A. (2005). Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-gamma production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. *Parasitology Research* **98**, 67–74. doi: 10.1007/s00436-005-0008-8.

Mazumdar, T., Anam, K. and Ali, N. (2005). Influence of phospholipid composition on the adjuvanticity and protective efficacy of liposome-encapsulated *Leishmania donovani* antigens. *The Journal of Parasitology* **91**, 269–274. doi: 10.1645/GE-356R1.

Melby, P. C., Chandrasekar, B., Zhao, W. and Coe, J. E. (2001*a*). The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-Like cytokine response. *The Journal of Immunolology* **166**, 1912–1920.

Melby, P. C., Yang, J., Zhao, W., Perez, L. E. and Cheng, J. (2001*b*). *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Infection and Immunity* **69**, 4719–4725. doi: 10.1128/IAI.69.8.4719-4725.2001.

Mizbani, A., Taheri, T., Zahedifard, F., Taslimi, Y., Azizi, H., Azadmanesh, K., Papadopoulou, B. and Rafati, S. (2009). Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. *Vaccine* **28**, 53–62. doi: 10.1016/j.vaccine.2009.09.114.

Okwor, I. and Uzonna, J. (2008). Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. *Immunological Research* **41**, 123–136. doi: 10.1007/s12026-008-8016-2.

Oliveira, L. F., Schubach, A. O., Martins, M. M., Passos, S. L., Oliveira, R. V., Marzochi, M. C. and Andrade, C. A. (2011). Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta Tropica* **118**, 87–96. doi: 10.1016/j.actatropica.2011.02·007.

Pinheiro, R. O., Pinto, E. F., de Matos Guedes, H. L., Filho, O. A., de Mattos, K. A., Saraiva, E. M., de Mendonça, S. C. and Rossi-Bergmann, B. (2007). Protection against cutaneous leishmaniasis by intranasal vaccination with lipophosphoglycan. *Vaccine* **25**, 2716–22. doi: 10.1016/j.vaccine.2006.05.093.

Pinto, E. F., Pinheiro, O. R., Rayol, A., Larraga, V. and Rossi-Bergmann, B. (2004). Intranasal vaccination agaist cutaneous leishmaniasis using a particulated leishmanial antigen or LACK-DNA. *Infection and Immunity* **72**, 4521–4527. doi: 10.1128/IAI.72.8.4521-4527.2004.

Romero, G. A. and Boelaert, M. (2010). Control of visceral leishmaniasis in Latin America-a systematic review. *PLoS Negleted Tropical* Disease **19**, e584. doi: 10.1371/journal.pntd.0000584.

Takagi, H., Hiroi, T., Yang, L. J., Tada, Y., Yuki, Y., Takamura, K., Ishimitsu, R., Kawauchi, H., Kiyono, H. and Takaiwa, F. A. (2005). Rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proceedings of the National Academy of Sciences, USA* **102**, 17525–17530. doi: 10.1073/pnas.0503428102.

Tewary, P., Sukumaran, B., Saxena, S. and Madhubala, R. (2004). Immunostimulatory oligodeoxynucleotides are potent enhancers of protective immunity in mice immunized with recombinant ORFF leishmanial antigen. *Vaccine* **13**, 3053–3060. doi:10.1016/j.vaccine.2004.02.007.

Vélez, I. D., Gilchrist, K., Arbelaez, M. P., Rojas, C. A., Puerta, J. A., Antunes, C. M., Zicker, F. and Modabber, F. (2005). Failure of a killed *Leishmania amazonensis* vaccine against American cutaneous leishmaniasis in Colombia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**, 593–598. doi: 10.1016/j.trstmh.2005.04.002.

https://doi.org/10.1017/S0031182011001417 Published online by Cambridge University Press