Journal of Advanced Zoology
ISSN: 0253-7214
Volume 42 Issue 2 Year 2021 Page 334-339

Vaccination with Leishmania Activated C Kinase (LACK) Antigen Induces Immune Response but Failed to Induce Protection against *Leishmania donovani* Challenge Infection

Sudipta Bhowmick*

**Assistant Professor, Department of Zoology, Dr Kanailal Bhattacharyya College, Howrah, Email: sudipta1239@gmail.com*

Abstract

Visceral leishmaniasis (VL), caused by *Leishmania donovani*, is life-threatening, and development of a safe and effective vaccine has been an essential aim for controlling the disease. In this study, Leishmania Activated C Kinase (LACK) Antigen was purified from leishmania antigens. The liposomal formulation of LACK antigen was tested to induce immune response and protection against *L. donovani* challenge infection. Vaccination with Liposomal LACK induces immune response in BALB/c mice with an elevated level of IFN-γ and IL-10. Surprisingly, vaccination was failed to ensure protection in mice. In conclusion, IL-10 probably played the crucial role in vaccine failure.

Key words: Leishmania donovani, vaccine, LACK antigen, cytokine

Introduction

Leishmaniasis is a vector-transmitted disease distributed worldwide mainly in the tropical and subtropical countries. At least 20 *Leishmania* species can give rise to a wide spectrum of clinical manifestations, ranging from self-healing cutaneous lesions to visceral leishmaniasis (VL), the latter being an invariably fatal disease in the absence of drug treatment. Moreover, *Leishmania* is emerging as important opportunistic pathogen in persons coinfected with human immunodeficiency virus. Therapeutic options for controlling leishmaniasis are limited to a few drugs with inconsistent efficacy and side effects. Thus, there is an urgent need to develop a safe and effective *Leishmania* vaccine to help prevent the two million new cases of leishmaniasis worldwide each year [1].

The development of subunit vaccine based on defined antigens of *Leishmania* is an area of great interest. Unlike DNA vaccines, protein based subunit vaccines appear to be more immunogenic in animal models, nonhuman primates, and humans [2]. A large number of leishmanial antigens have been identified and attempted for vaccination in experimental leishmaniasis, mainly against the cutaneous form. A plethora of novel candidates are being identified with the recent completion of *L. major* genome sequence, but critical in vivo experimental evaluation is still required to establish their utility as protective antigens [3]. Defined antigens of *L. donovani* such as dp72, HASPB, A2 , ORFF and EF-1α have also been evaluated against challenge infection and elicited different degrees of protection [4-6]. However, the number of potential candidates is limited and there is only a little progress in the identification of novel antigens protective against VL. Moreover, proteins identified on their efficacy against cutaneous form when tested against *L. donovani* infection have found to be either unsuccessful or partially protective, suggesting the necessity of identification of new antigens from *L. donovani* [7].

In this study, Leishmania Activated C Kinase (LACK) Antigen was purified from leishmania antigens. The liposomal formulation of LACK antigen was tested to induce immune response and protection against *L donovani* challenge infection.

Methods

Animals and parasites

BALB/c mice were 4–6 weeks old at the onset of the experiments. *L. donovani* strain AG83 (MHOM/IN/1983/AG83) promastigotes were grown at 22°C in Medium 199 supplemented with penicillin G sodium (l00 U/ml), streptomycin sulfate (100 mg/ml) and 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and subcultured in the same medium. [5].

SDS-PAGE and electroelution

The LAg was subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and stained with Coomassie blue. The proteins with molecular mass of 34-kDa were eluted by electrophoresis in running buffer (0.025 M Tris, 0.192 M glycine, 1% SDS) using a Electro-Eluter (model 422; Bio-Rad) at 10 mA for 5 h. After elution, the proteins were dialyzed, lyophilized and resuspended in PBS. Proteins were further visualized by SDS-PAGE and silver staining [6].

Entrapment of antigens in liposomes

Cationic liposomes were prepared with distearoyl phosphatidylcholine (DSPC), cholesterol (Sigma-Aldrich) and stearylamine (Fluka, Buchs, Switzerland) at a molar ratio of 7:2:2 as described previously [5. Empty and antigen entrapped liposomes were prepared by the dispersion of lipid film in either 1 ml PBS alone or containing 200 μg/ml antigen respectively. The mixture was vortexed and the suspension sonicated for 30 sec by an ultrasound probe sonicator (Misonix). Liposomes with entrapped antigens were separated from excess free antigen by three successive washing in PBS with ultracentrifugation (105,000 × g, 60 min, 4°C). The encapsulation efficiency was determined by the method of Lowry et al., in the presence of 10%
SDS. The level of encapsulation ranged between 50 to 60% [5].

Immunization of mice and challenge infection

The experimental groups consisted of 4–6 weeks old BALB/c mice. Mice were immunized by intraperitoneal injections of 2.5 μg purified proteins in PBS or incorporated in liposome in a total volume of 200 μl. Animals receiving PBS or empty liposomes served as controls. Mice were boosted two times at 2-week intervals. Ten days after the final immunization rest of the mice were challenged with 2.5×10^7 freshly transformed stationary-phase promastigotes in 200 μl PBS injected intravenously via the tail vein as described earlier. After 3 months of challenge infection, the mice were sacrificed to determine the parasite load in liver and spleen [5]. The course of infection was monitored by the microscopic examination of Giemsa-stained impression smears of liver and spleen. The parasite load was expressed as Leishman-Donovan units and was calculated by the following formula: number of amastigotes per 1000 cell nuclei×organ weight (mg).

Cytokine assays

The spleens were aseptically removed from the immunized and infected BALB/c mice and single cell suspensions were prepared in RPMI 1640 supplemented with 10% FBS, l00 U/ml penicillin
G sodium, 100 mg/ml streptomycin sulfate and 50 mM bmercaptoethanol (Sigma-Aldrich) (complete medium). RBCs were removed by lysis with 0.14 M Tris buffered NH4Cl. The
remaining cells were washed twice with culture medium and viable mononuclear cell number was determined by counting Trypan blue unstained cells in a hemocytometer. Then the cells were cultured in triplicate in a 96 well flat bottom plate (Nunc,Roskilde, Denmark) at a density of 26105 cells/well in a final volume of 200 ml complete medium and stimulated with antigens
(2.5 mg/ml). The cells were incubated for 96 h at 37uC in a humified chamber containing 5% CO2. After 72 h incubation, culture supernatants were collected and the concentration
of IFN-c, IL-4, IL-12p40 and IL-10 (BD Pharmingen, San Diego, CA) were quantitated by ELISA in accordance with the manufacture's instructions [6].

Statistical analysis
One-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test were used for the analysis of parasite burden and antibody response data using Graph Pad Prism version 5.0
(Graphpad Software, v. 5.0, San Diego, CA).

Results

Liposomal LACK was not protective against L. donovani Infection
The LACK antigen entrapped in cationic liposomes was used for vaccination in BALB/c mice. After ten days the mice were challenged against *L. donovani* Ag83 strain intection. It is found that infection with this strain in BALB/c mice results in a progressive infection in the liver and spleen, corresponding with hepato and splenomegaly (Figure 1). Data demonstrated that liposomal LACK antigen vaccinated mice were not significantly protective after 3 months of infection. As expected, control mice injected with PBS or empty liposomes showed high level of parasite burden depicting a progressive infection.

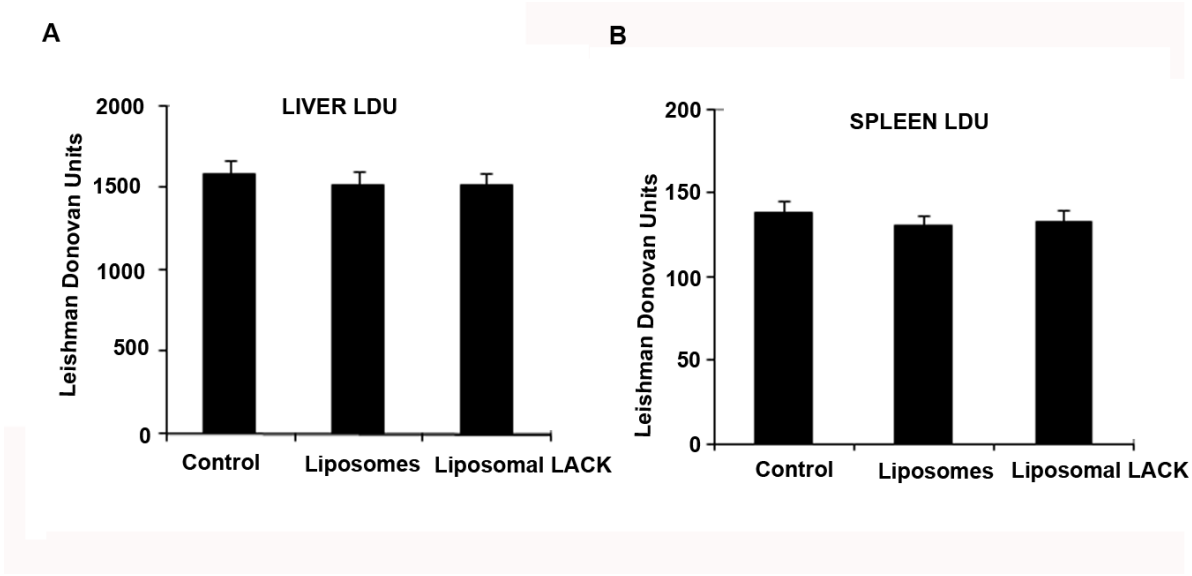

Figure 1: Parasite burden following *L. donovani* challenge in immunized BALB/c mice. Liver (A) and spleen (B) parasite burden of mice immunized with control, Liposomes and Liposomal LACK. Results represent mean ± S.E. (n=5).

Vaccination with liposomal induces IFN-γ and IL-10 production
IFN-γ and IL-10 are currently thought to be essential cytokines governing the fate of the parasite challenge. The levels of IFN-γ and IL-10 produced by splenocytes after in vitro restimulation with specific antigens were determiuned. Liposomal LACK vaccination induced lower but significant levels of IFN-γ (Figure 2). In addition, significant levels of IL-10 were released by splenocyctes from liposomal LACK. In response to the crude LAg, both IFN-γ and IL-10 levels were elevated in liposomal LACK vaccinated mice, indicating that crude antigens may mimic the parasite challenge and predict the responses after challenge infection. Again, liposomal LACK vaccinated mice showed a lower IFN-γ:IL-10 ratio1.06. Thus, IL-10 might have a regulatory role in vaccine failure and IFN-γ:IL-10 provides a further predictive correlate of vaccine-mediated protection from VL.

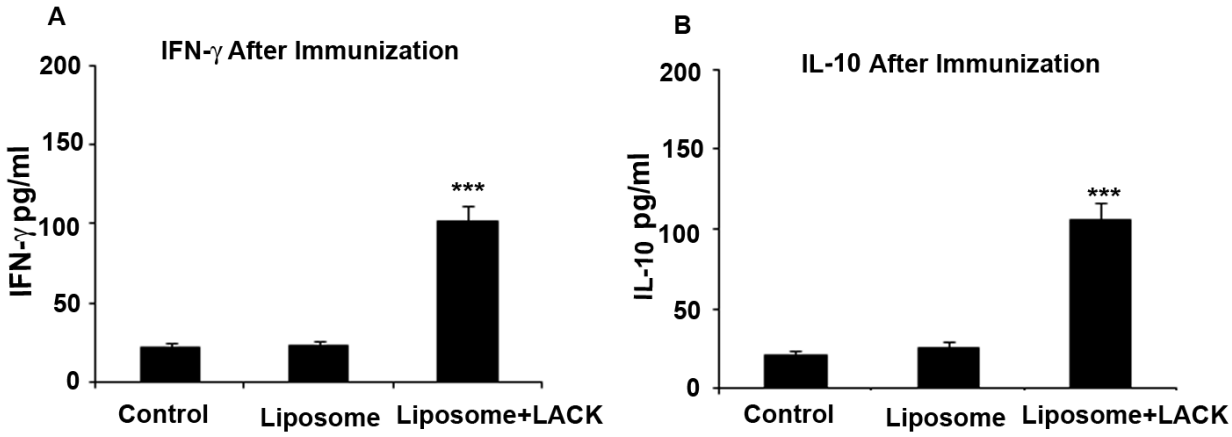

Figure 2: After immunization levels of IFN-γ (A) and IL-10 (B) in the splenic culture of mice 72 h after specific antigen stimulation. Results represent mean ± S.E. (n=5). *** P < 0.0001 in comparison to control groups.

Challenge infection further polarizes IFN-γ and IL-10 production in mice vaccinated with liposomal LACK

Antigen specific IFN-γ and IL-10 levels were measured in culture supernatants of splenocytes isolated from vaccinated mice after 3 months of infection. Comparabler levels of IFN-γ, Th1 cytokine were observed in liposomal LACK vaccinated mice (Figure 3) with the controls. Again, a significant increase in the level of IL-10 was demonstrated by the liposomal LACK immunized mice suggesting IL-10 may provide a key to vaccine failure.

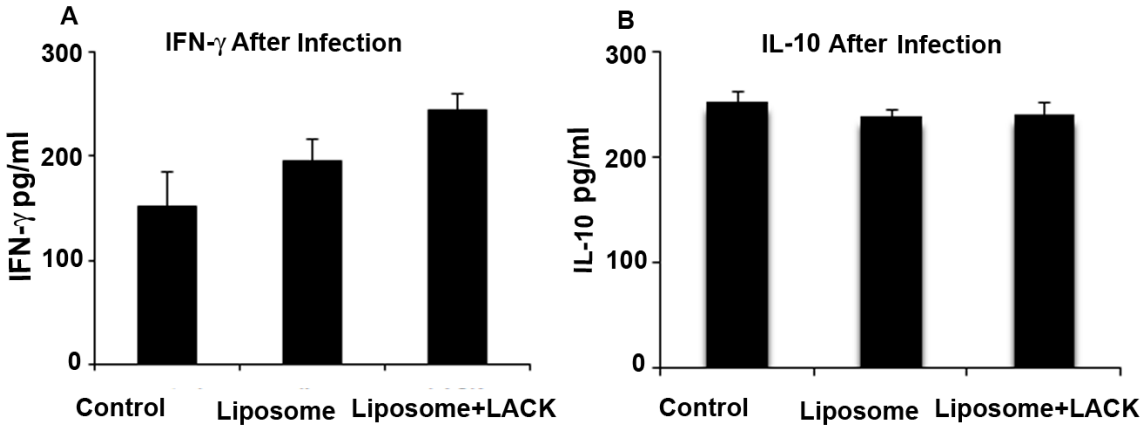

Figure 3: After challenge infection levels of IFN-γ (A) and IL-10 (B) in the splenic culture of mice 72 h after specific antigen stimulation. Results represent mean ± S.E. (n=5).

DISCUSSION

Over the past years major efforts have been dedicated to develop novel vaccine candidates against leishmaniasis and evaluate them in a variety of animal models. These efforts were fueled by the publication of the *L. major* and *L. infantum* genome and renewed interest in the field of leishmaniasis prophylaxis led to the identification of a plethora of putative candidates for vaccine development [3]. The vaccine components identified from genome however, requires critical in vivo experimental evaluation to establish their utility as candidate antigens [7].

Several vaccine candidates have been identified and evaluated against different Leishmania spp. However, in spite of homology between the different species and a certain degree of cross protection, the need for identification of new immunostimulatory molecules from individual species is still required. In this study, we have purified and evaluated the protective efficacy of LACK protein in the BALB/c mice model against *L. donovani* challenge infection. In the present study, we have used cationic liposomes as an adjuvant using DSPC in formulation. Our result demonstrated that liposomal LACK protein failed to elicit protection with an elevated level of IL-10. Studies indicate that LACK antigen provides significant protection against *L.*

major [8-12], while failed to protect against *L amazonensis* [13] and *L. mexicana* [14]. Although the intranasal delivery of DNA encoding the LACK found to be protective [15,16], the intramuscular immunization failed to protect against *L. chagasi* [17].The LACK DNA vaccine also found to be protective against *L. infantum* when live viral vectors were used in the booster dose [18]. Further, a LACK DNA vaccine, although induced a robust IFN-γ but not IL-4 production, failed to protect against *L. donovani* [18].
The most important finding is the high prechallenge antigen specific IL-10 production by the liposomal LACK immunized mice may associate with failure of protection in these mice. Studies with IL-10 knockout [19] or transgenic mice [20] have demonstrated an association of IL-10 production with susceptibility to *L. donovani*. IL-10 is a pleiotropic Th1 suppressive cytokine able to block macrophage activation, thus preventing antigen-specific T stimulation and limiting IFN-γ production [21]. IL-10 seems to be also able to directly promote parasite survival through its influence on the macrophage activation state [21]. IL-10 can be produced by many cell types including B cells, macrophages, DCs, Th2 cells and both natural and antigen induced (Tr1) regulatory T cells [22]. Study with *L. major* LV39 strain indicated that failure of LACK antigen vaccination is associated with an antigen-driven, IL-10 secreting Tr1 like cell population [23]. Tr1 cells secrete high levels of IL-10, but little or no IFNγ. Tr1 cells are also capable of suppressing both Th1 and Th2 responses [24].

Acknowledgments

I thank my guide Dr. Nahid Ali, emeritus scientist IICB, Jadavpur, India for her guidance during this study. I thank Mr. Shahfiaz Khan for his support.

References

1. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis
2. Das, A. & N. Ali, 2012. Vaccine Development Against *Leishmania donovani*. Front. Immunol., 3:99.
3. Ivens, A.C., C.S. Peacock CS, E.A. Worthey EA, et al., 2005 The genome of the kinetoplastid parasite, *Leishmania major*. Science , 309 (5733): 436–442.
4. Sabu, R.A., S. Bhowmick, R. Chhajer R, S. Ejazi, N. Didwania N, M. Asad, A. Bhattacharyya A, U. Sinha U & N. Ali, 2018. Liposomal elongation factor-1α triggers effector CD4 and CD8 T cells for induction of long-lasting protective immunity against visceral leishmaniasis. Front Immunol, 9:18.
5. Bhowmick, S., R. Ravindran & N. Ali, 2007. Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1response. Vaccine , 25 (35):6544–6556.
6. Bhowmick, S. & N. Ali, 2009. Identification of novel *Leishmania donovani* antigens that help define correlates of vaccine-mediated protection in visceral leishmaniasis. PLoS ONE, 4(6):e5820.
7. Kumari, S., A. Kumar, M. Samant, N. Singh & A. Dube, 2008. Discovery of novel vaccine candidates and drug targets against visceral leishmaniasis using proteomics and transcriptomics. Curr. Drug. Targets., 9(11):938-47.
8. Salehi-Sangani, G., M. Mohebali, V. Jajarmi, A. Khamesipour, M. Bandehpour, M. Mahmoudi & H. Zahedi-Zavaram, 2019. Immunization against Leishmania major infection in BALB/c mice using a subunit-based DNA vaccine derived from TSA, LmSTI1, KMP11, and LACK predominant antigens. Iran. J. Basic. Med. Sci., 22(12):1493-1501.
9. Fernández, L., E. Carrillo, L. Sánchez-Sampedro, C. Sánchez, A.V. Ibarra-Meneses, M.A. Jimenez, V.D.A. Almeida, M. Esteban & J. Moreno, 2018. Antigenicity of Leishmania-Activated C-Kinase Antigen (LACK) in Human Peripheral Blood Mononuclear Cells, and Protective Effect of Prime-Boost Vaccination With pCI-neo-LACK Plus Attenuated LACK-Expressing Vaccinia Viruses in Hamsters. Front. Immunol, 9:843.
10. Ghaffarifar, F., O. Jorjani, Z. Sharifi, A. Dalimi, Z.M. Hassan, F. Tabatabaie, F. Khoshzaban & H.Z. Hezarjaribi, 2013. Enhancement of immune response induced by DNA vaccine cocktail expressing complete LACK and TSA genes against *Leishmania major*. APMIS, 121(4):290-298.
11. Gurunathan, S., D.L. Sacks, D.R. Brown, S.L. Reiner, H. Charest, N. Glaichenhaus & R.A. Seder, 1997. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. J. Exp. Med., 186(7):1137-1147.
12. Méndez, S., S. Gurunathan, S. Kamhawi, Y. Belkaid, M.A. Moga, Y.A. Skeiky, A. Campos-Neto, S. Reed, R.A. Seder & D. Sacks, 2001. The potency and durability of DNA- and protein-based vaccines

against *Leishmania major* evaluated using low-dose, intradermal challenge. J. Immunol., 166 (8):5122-128.
13. Coelho, E.A., C.A. Tavares, F.A. Carvalho, K.F. Chaves, K.N. Teixeira, R.C. Rodrigues, H. Charest, G. Matlashewski, R.T. Gazzinelli & A.P. Fernandes, 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. Infect. Immun., 71(7):3988-3994.
14. Dumonteil, E., R.S. Maria Jesus, E.O. Javier & G.M. Maria del Rosario GM. DNA vaccines induce partial protection against *Leishmania mexicana*. Vaccine, 21(17-18):2161-2168.
15. Gomes, D.C., E.F. Pinto, L.D. de Melo, W.P. Lima, V. Larraga, U.G. Lopes & Rossi-B. Bergmann, 2007. Intranasal delivery of naked DNA encoding the LACK antigen leads to protective immunity against visceral leishmaniasis in mice. Vaccine, 25(12):2168-2172.
16. Dondji, B., E. Pérez-Jimenez, K. Goldsmith-Pestana, M. Esteban & D. McMahon-Pratt,2005. Heterologous prime-boost vaccination with the LACK antigen protects against murine visceral leishmaniasis. Infect. Immun., 73(8):5286-5289.
17. Marques-da-Silva, E.A., E.A. Coelho, D.C. Gomes, M.C. Vilela, C.Z. Masioli, C.A. Tavares, A.P. Fernandes, L.C. Afonso & S.A. Rezende, 2005. Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-gamma production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. Parasitol. Res. 98:67-74.
18. Melby, P.C., J. Yang, W. Zhao, L.E. Perez & J. Cheng, 2001. *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infect. Immun., 69(8):4719-4725.
19. Murphy, M.L., U. Wille, E.N. Villegas, C.A. Hunter & J.P. Farrell, 2001. IL-10 mediates susceptibility to *Leishmania donovani* infection. Eur. J. Immunol., 31(10):2848-56.
20. Murray, H.W., C.M. Lu, S. Mauze, S. Freeman, A.L. Moreira, G. Kaplan & R.L. Coffman, 2002. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect. Immun., 70(11):6284-6293.
21. Moore, K.W., R. de Waal Malefyt, R.L. Coffman & A. O'Garra, 2001, Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol., 19:683-765.
22. Nylén, S. & D. Sacks, 2007. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trend.s Immunol., 28 (9):378-384.
23. Stober, C.B., U.G. Lange, M.T. Roberts, A. Alcami & J.M. Blackwell, 2005. IL-10 from regulatory T cells determines vaccine efficacy in murine *Leishmania major* infection. J. Immunol.,175:2517-2524.
24. Mills, K.H, 2004. Regulatory T cells: friend or foe in immunity to infection? Nat. Rev. Immunol., 4(11):841-855.