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Elongation Factor-1α from *Leishmania donovani* in liposomes promotes protective immunity against visceral leishmaniasis via polarized Th1 response

Sudipta Bhowmick*

Abstract

Visceral leishmaniasis (VL) is life-threatening, and development of a safe and effective vaccine has been an essential aim for controlling the disease. In the present study, Elongation factor-1α (EF-1α) of *Leishmania donovani* was purified from soluble leishmanial antigens. EF-1α adjuvanted with cationic liposomes exhibited significant resistance against *L. donovani* in both livers and spleens of BALB/c mice challenged 10 days after last vaccination. Analysis of cytokines in protected mice revealed induction of Th1 cytokines IFN-γ with IL-12 with a down-regulation of Th2 cytokines IL-4 along with immunosuppressive IL-10, hinted toward a Th1 polarized immune response instrumental for protection. Thus, EF-1α, a vaccine candidate antigen from *L. donovani* can be a potential component of future antileishmaniasis vaccines.

Keywords:

Visceral leishmaniasis;
EF-1α (Elongation factor-1α);
IFN-γ;
IL-4.

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1. Introduction

Leishmaniasis, caused by obligate intracellular protozoan parasites, is a significant public health threat affecting 12 million people worldwide. Infection with *Leishmania* spp. through an insect-vector bite can lead to divergent clinical presentations ranging from cutaneous (CL), muco-cutaneous to visceral leishmaniasis (VL). The most serious form of the disease is the fatal visceral one caused by members of the *L. donovani* complex having severe manifestations such as hepatosplenomegaly, fever, pancytopenia, and hypergammaglobulinemia(1). Available chemotherapy is far from satisfactory as the treatments are long, expensive and have adverse side effects (2). This, compounded with the emergence of drug-resistant strains and increased HIV co-infection in developing countries, underscore the need for an effective and safe vaccine against this disease (3).

The efficient induction of a protective immune response to *Leishmania* infection depends upon the development of a Type 1 immune response characterized by the IL12-dependent production of IFN-γ T cells. Disease susceptibility to *L. major* is correlated with IL-4 production from Th2 subsets of $CD4^+$ T cells. However, the precise role of IL-4 in disease progression of *L. donovani* is elusive. Although induction of IL-4 in infected BALB/c and noncuring models has been documented, beneficial roles of IL-4 have also been described for *L. donovani* resistance (4-8). Importantly, recent studies indicate that IL-10 is the major cytokines leading to susceptibility and immunosuppression in VL (9-11). Thus, understanding the complex

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mechanisms underlying protection against VL is required in the rational design of vaccines for this form of leishmaniasis.

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 (3). 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 (12), but critical in vivo experimental evaluation is still required to establish their utility as protective antigens. Defined antigens of *L. donovani* such as dp72, HASPB, A2 and ORFF have also been evaluated against challenge infection and elicited different degrees of protection (3). However, the number of potential candidates is limited and there is only a little progress in the identification of novel antigens protective against VL (3). Moreover, proteins identified on their efficacy against cutaneous form when tested against *L. donovani* infection have found to be either unsuccessful or partially protective (3) suggesting the necessity of identification of new antigens from *L. donovani*.

In this study, 36-kDa polypeptide was purified from soluble leishmanial antigens of *L. donovani*. The polypeptide was previously identified as Elongation factor-1α (EF-1α) of *L. donovani* using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF/TOF) mass spectrometry (13). The protective efficacy of EF-1α adjuvanted with cationic liposomes was tested against *L. donovani* infection and investigated the underlying immune responses associated with protection in murine model.

2. Research Method (12pt)

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) was maintained by serial passage in hamsters and BALB/c mice. The amastigotes were isolated from the spleens of infected animals and allowed to transform into promastigotes at 22°C in Medium 199 supplemented with l00 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich). Freshly transformed promastigotes were subcultured in the same medium at an average density of 2×10^6 cells/ml (5,6).

Preparation of Leishmania *antigens*

SLA was prepared also from *L. donovani* promastigotes. Briefly, stationary-phase promastigotes, harvested after the third or fourth passage, were washed four times in cold 20mM phosphate buffered saline (PBS), pH 7.2 and resuspended at a concentration of 1.0 g of cell pellet in 50 ml of cold 5mM Tris-HCl buffer (pH 7.6), containing 5 μg of leupeptin/ml, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride and 1mM iodoacetamide (Sigma-Aldrich) (lysis buffer). The suspension was vortexed and centrifuged at 2310 × *g* for 10min. The pellet was resuspended in10 ml of lysis buffer and sonicated for 3 min by ultrasound probe sonicator (Misonix). The suspension thus obtained was solubilised with 1% (w/v) octyl-β-D-glucopyranoside (Sigma-Aldrich) in the lysis buffer with overnight incubation at 4°C, and was finally ultracentifuged for 1 h at 100,000 × *g*. The supernatant containing SLA was then dialyzed against 2mM PBS and stored at -20°C until use. The amount of protein obtained from 1.0 g cell pellet, as assayed by the method Lowry et al. (14), was approximately 2 mg (5).

SDS-PAGE and electroelution

The SLA was subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and stained with Coomassie blue. The proteins with molecular mass of 36-kDa was 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 (5). Proteins were further visualized by SDS-PAGE and silver staining. The proteins were quantified by Lowry's method (14).

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 mg 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 last booster, serum samples were collected, and spleens were removed aseptically for the analysis of cellular responses after immunization. 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 (5,6). After 3 months of challenge infection, the mice were sacrificed to determine the parasite load in liver and spleen. 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) (15).

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 µg/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 cellswere cultured in triplicate in a 96 well flat bottom plate (Nunc, Roskilde, Denmark) at a density of 2×10^5 cells/well in a final volume of 200 µl complete medium and stimulated with antigens (2.5 µg/ml). After 72 h incubation, culture supernatants were collected and the concentration of IFN-γ IL-4, IL-12p40 and IL-10 (BD Pharmingen, San Diego, CA) were quantitated by ELISA in accordance with the manufacture's instructions (5,6).

Statistical Analysis

Data comparisons were tested for significance by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-test or unpaired, two-tailed Student's t test. Results with $p < 0.05$ were considered to be statistically significant. All of the analyses were done using GraphPad Prism (version 4.0) software.

3. Results and Analysis

3.1. Protection afforded by EF-1α adjuvanted with cationic liposomes

To assess the protective ability of EF-1α, BALB/c mice were immunized intraperitoneally with EF-1α alone and entrapped in cationic liposomes. The vaccination was repeated twice at 2-week intervals and the mice were challenged intravenously with *L. donovani* promastigotes 10 days after the last immunization. Control mice were injected with the PBS and empty liposomes. Infection with the Indian strain, *L. donovani* AG83 in BALB/c mice results in a progressive infection in the liver and spleen, corresponding with hepato and splenomegaly (5-8). Our data demonstrated that mice immunized with EF-1α alone, were resistant to hepatic infection at partial level at 90 days after challenge infection (47%, Fig. 1A). Again, mice receiving liposomal EF-1α acquired higher resistance (76% at 90 days), significantly higher than controls ($p < 0.001$). In BALB/c mice persistence of *L. donovani* in the spleen causes concomitant development of considerable organ-specific pathology similar to that seen in the human kala-azar. It was, therefore, more important to evaluate the impact of vaccination in this organ. In spleen, immunization with liposomal EF-1α demonstrated 79% protection at 90 days after parasite challenge (Fig. 1B). The reduction in parasitic load was statistically higher than controls ($p < 0.001$).

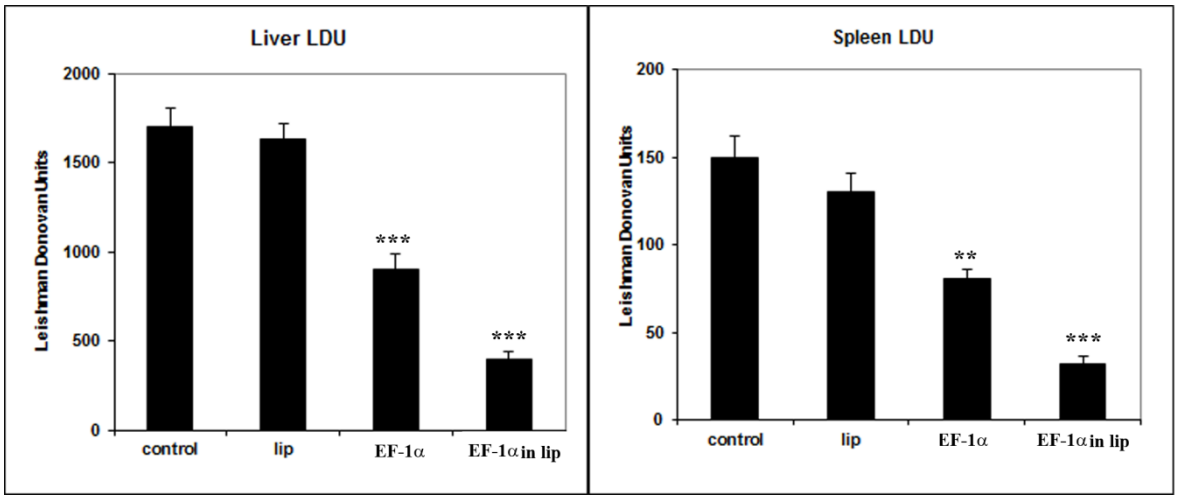

Figure1. Parasite burdens in BALB/c mice vaccinated with EF-1α alone or entrapped in cationic liposomes after challenge infection. *, $p < 0.05$; **, $p < 0.01$; ***, $p< 0.001$ vs PBS and Lip control groups using one-way ANOVA followed by Tukey's multiple comparisons post-test.

Development of an effective vaccine has been an essential aim for the control of VL. A major impediment to develop vaccine against this disease lies in identifying relevant antigens able to stimulate protective immunity. In this study, we purified EF-1α from and which was identified previously using MALDI-TOF/TOF mass spectrometry (13). Vaccination with EF-1α in cationic liposomal formulation exhibited significant resistance in both the visceral organs liver and spleen of BALB/c mice challenged with L. donovani 10 days after last vaccination.

Previously, EF-1α from *L. donovani* identified as a Src-homology 2 domain containing protein tyrosine phosphatase-1 (SHP-1) binding protein, property not shared by the human homologue, causes macrophage deactivation through activation of SHP-1 in infected cells (16). Despite a high degree of homology with human counterpart, amino acid substitutions along with absence of a hairpin-loop of 12 amino acids was observed in *Leishmania* EF-1α (17). Deletion of this hairpin-loop offered an exposed region on the main body of *Leishmania* EF-1α that may be involved in protein synthesis (18). Since EF-1α is involved in virulence as it is essential for pathogen's survival (18), it could be a promising vaccine candidate. Furthermore, presence of EF-1α in both the amastigote and the promastigote forms of the parasite and high degree of conservation among *Leishmania* spp, suggesting its candidature as potential antigen (17). EF-1α had been recognized as an immunoreactive protein with mediterranean visceral leishmaniasis patients' sera (19). The other subunit, EF-1β of EF-1 complex is also suggested as an authentic virulence factors in *Leishmania* due to its plausible role in parasite survival and persistence (20). EF-1γ was identified as a novel antigenic target for VL vaccine using a two-step library screening procedure first by reactivity with patients' sera followed by their ability to induce T cell proliferation and IFN-γ responses in immune mice (21). Thus, these observations further strengthen the rationale for selection of EF-1α as a potential vaccine candidate and its further evaluation for protective efficacy in *Leishmania* model.

3.2. Liposomal EF-1α immunization elicit cellular responses

Surrogate markers for immunity to leishmaniasis are thought to be antigen-induced delayed-type hypersensitivity (DTH) responses in vivo and splenocyte proliferation and production of cytokines following stimulation of antigen in vitro. These recall responses were evaluated 10 days after the last vaccination. Fig. 2 shows that the mice receiving free EF-1α displayed a positive DTH response, whereas liposomal EF-1α vaccinated mice showed a higher response. Immunized mice challenged with L. donovani 10 days after last vaccination, were re-evaluated 90 days after infection for DTH response. Higher DTH response was observed in liposomal EF-1α immunized mice (Fig. 2).

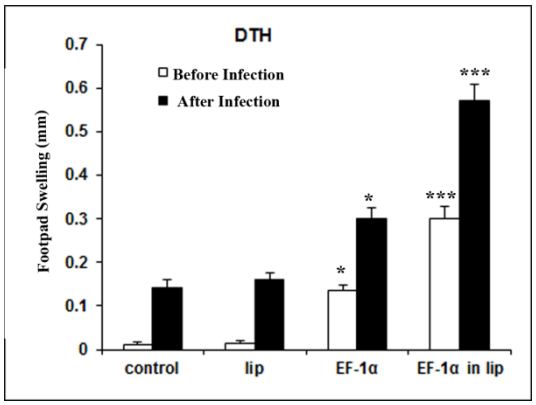

Figure 2. DTH response in mice vaccinated with EF-1α alone or entrapped in cationic liposomes. *, p < *0.05;* **, p < *0.01;* ***, p< *0.001 vs PBS and Lip control groups.*

Splenocytes isolated from the mice that received liposomal EF-1α immunization exhibited stronger stimulation of both IFN-γ and IL-4 than the mice vaccinated with PBS (Fig. 3A, 3C) ($p < 0.001$). However, IFN-γ level was higher with an IFN-γ:IL-4 ratio of 4.10, suggesting a Th1 bias and further providing a predictive correlate for protection. Again, levels of another type 1 cytokines IL-12 was measured in the splenocytes supernatants of liposomal EF-1α vaccinated mice. A higher level of IL-12 (Fig. 3B) ($p< 0.001$) was observed. Importantly, IL-10 was not produced by any of the immunized groups (3D).

Immunized mice challenged with *L. donovani* 10 days after last vaccination, were re-evaluated 90 days after infection for cytokine production to understand the underlying immune mechanism associated with protection. Antigen-specific proliferation of IFN-γ levels (Fig. 3A) measured in liposomal EF-1α vaccinated

mice was again higher. In addition to higher IFN-γ levels, a level of IL-12 (3B) was also enhanced in liposomal EF-1α immunized mice, suggesting a skewing towards Th1 response after challenge infection. With infection, in PBS and liposome immunized mice higher levels of IL-4 (Fig. 3C, IL-10 (Fig. 3D) were observed. These responses are associated with progressive infection in control mice. In contrast, the levels of IL-4 and IL-10 were inhibited in liposomal EF-1α immunized group to a greater extent. Thus the polarization towards Th1 response after challenge infection, consistent with the prechallenge observation correlates with the protection.

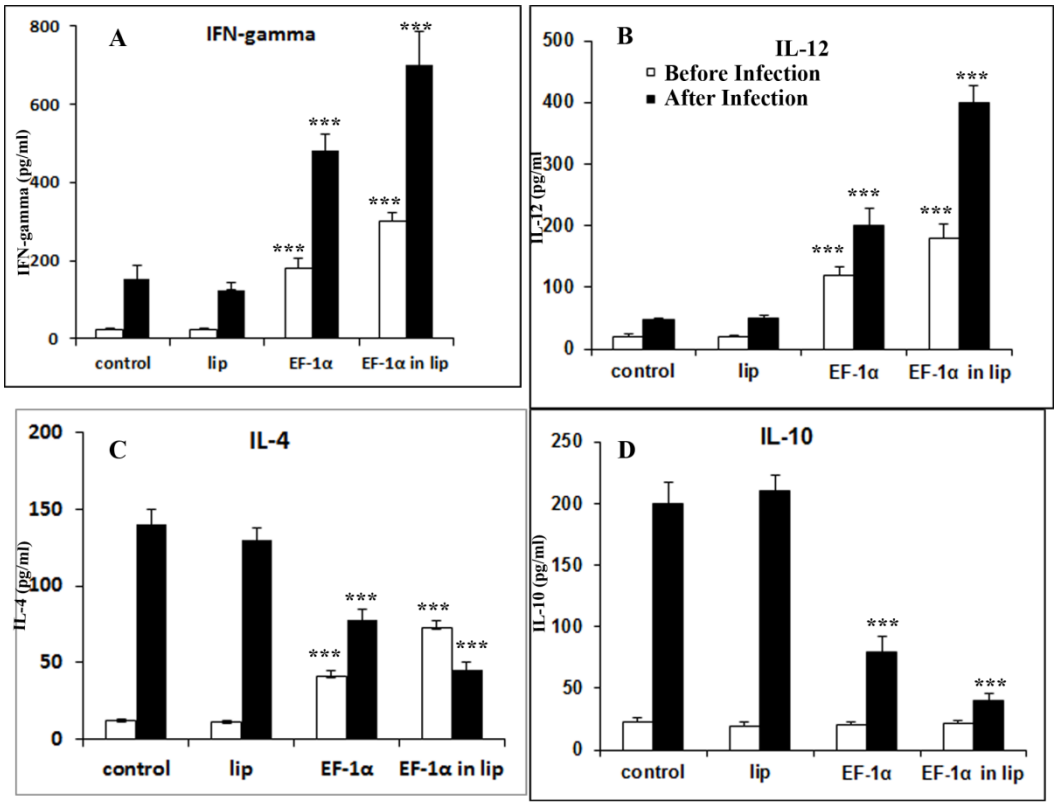

Figure 3. Cytokine responses in mice vaccinated with EF-1α alone or entrapped in cationic liposomes. *, p < *0.05; **, p < 0.01; ***, p< 0.001 vs PBS and Lip control groups.*

IFN-γ, a signature cytokine of Th1 type response that has a crucial role in macrophage activation for parasiticidal killing activity (4), was found to be induced in liposomal EF-1α immunized animals. Along with IFN-γ, the level of other Th1 cytokines IL-12 was also elevated in protected liposomal EF-1α immunized group. IL-12 is known to be involved not only in activation of NK cell but also development of Th1 response and granuloma formation (4-8). Induction of IL-4, a signature Th2 cytokine, in infected BALB/c and noncuring models (5,6) has been reported. Commensurate with these findings elevated level of IL-4 was detected in control infected mice although IL-4 levels decreased remarkably in liposomal EF-1α vaccinated mice. But the interesting finding is that liposomal EF-1α immnized mice significantly produced IL-4 following immunization before challenge infection. This early though low level of prechallenge IL-4 may be beneficial as well as correlate with protection against VL (5-8). IL-10, rather than being a cytokine of the Th2 regime, is a pleiotropic cytokine able to block T-cell proliferation, and secretion of and macrophage responsiveness to activating Th1 cell-associated cytokines. The use of IL-10 knockout and transgenic mice has confirmed an association of IL-10 with susceptibility in VL(9-11). Thus, the decreased level of IL-10 in liposomal EF-1α vaccinated mice after infection correlated with protection.

4. Conclusion

Based on these considerations, this study stated that vaccination with EF-1α induced significant protection in BALB/c mice against challenge with *L. donovani* when cationic liposomes was used as an adjuvant. Our results also indicate that induction of Th1 response with elevated levels of IFN-γ, IL-12 and an inhibition of IL-4, IL-10 levels can tilt the immune system toward protection against VL.

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