Elongation Factor-2, a Th1 Stimulatory Protein of *Leishmania donovani*, Generates Strong IFN-γ and IL-12 Response in Cured *Leishmania*-Infected Patients/Hamsters and Protects Hamsters against *Leishmania* Challenge

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Elongation Factor-2, a Th1 Stimulatory Protein of *Leishmania donovani*, Generates Strong IFN-γ and IL-12 Response in Cured *Leishmania*-Infected Patients/Hamsters and Protects Hamsters against *Leishmania* Challenge

Pramod K. Kushawaha,* Reema Gupta,* Shyam Sundar,† Amogh A. Sahasrabuddhe,‡ and Anuradha Dube*  

In visceral leishmaniasis, Th1 types of immune responses correlate with recovery from and resistance to disease, and resolution of infection results in lifelong immunity against the disease. Leishmanial Ags that elicit proliferative and cytokine responses in PBMCs from cured/exposed/*Leishmania* patients have been characterized through proteomic approaches, and elongation factor-2 is identified as one of the potent immunostimulatory proteins. In this study, we report the cloning and expression of *Leishmania donovani* elongation factor-2 protein (LelF-2) and its immunogenicity in PBMCs of cured/exposed *Leishmania*-infected patients and hamsters (*Mesocricetus auratus*). *Leishmania*-infected cured/exposed patients and hamsters exhibited significantly higher proliferative responses to recombinant LelF-2 (rLelF-2) than those with *L. donovani*-infected hosts. The soluble *L. donovani* Ag stimulated PBMCs of cured/exposed and *Leishmania*-infected patients to produce a mixed Th1/Th2-type cytokine profile, whereas rLelF-2 stimulated the production of IFN-γ, IL-12, and TNF-α but not IL-4 or IL-10. Further, rLelF-2 downregulated LPS-induced IL-10 as well as soluble *L. donovani* Ag-induced IL-4 production by *Leishmania* patient PBMCs. The immunogenicity of rLelF-2 was also checked in hamsters in which rLelF-2 generates strong IL-12– and IFN-γ–mediated Th1 immune response. This was further supported by a remarkable increase in IgG2 Ab level. We further demonstrated that rLelF-2 was able to provide considerable protection (~65%) to hamsters against *L. donovani* challenge. The efficacy was supported by the increased inducible NO synthase mRNA transcript and Th1-type cytokines IFN-γ, IL-12, and TNF-α and downregulation of IL-4, IL-10, and TGF-β. Hence, it is inferred that rLelF-2 elicits a Th1 type of immune response exclusively and confers considerable protection against experimental visceral leishmaniasis. *The Journal of Immunology*, 2011, 187: 000–000.

Visceral leishmaniasis (VL) is a major public health problem in tropical and subtropical countries, such as India, Bangladesh, Nepal, Sudan, and Brazil. The disease is caused by an intracellular protozoan parasite of the *Leishmania donovani*/infantum/L. chagasi complex. VL is characterized by fever, hepatosplenomegaly, cachexia, pancytopenia, and hyper-gammaglobulinemia (1). An estimated 350 million people are at risk for acquiring infection with *Leishmania* parasites worldwide with ~500,000 cases of VL reported each year (2). Two population-based studies of VL incidence in highly endemic subdistricts of Bihar, India, estimate the annual VL incidence as 24.9 (3) and 57.1 (4) per 10,000 population; they suggest that official figures underestimate VL incidence by a factor of eight and four, respectively. Recent epidemics of VL in Sudan and India have resulted in >100,000 deaths (5). With the advent of the HIV epidemics, the disease has emerged as an important opportunistic infection in AIDS patients (6). In India, high incidence has been reported from the states of Bihar, Assam, West Bengal, and Eastern Uttar Pradesh, where resistance and relapse are on the increase. Available chemotherapy for VL is far from satisfactory because antileishmanial drugs are costly with unpleasant side effects. The situation has further worsened with emergence of drug resistance in various regions of endemicity (6). Vaccination would, therefore, be a better option for an effective control strategy for VL.

The characteristic immunological feature of active VL is the absence of parasite-specific cell-mediated immune responses (1). It has been reported that there is an increase in IL-4–positive neutrophils, NK cells, and IL-10 monocytes, whereas the number of IFN-γ–positive, IL-2, and IL-12 eosinophils is significantly decreased. However, serum levels of TNF-α and IL-6 were high in patients with active VL (7–9). In contrast, the cured cases present a characteristic type 1 response with an increase in IFN-γ, IL-2 neutrophils, eosinophils, and NK cells and with an increase in IL-12 monocytes (8–10). Helper and cytotoxic T cells are known to play an integral role in the immune response to this infection,
connecting the innate immune response to the development of efficient adaptive cellular immunity, mainly through IL-2 and IFN-γ production. These two cytokines drive the effector functions of macrophages and trigger a Th1 immune response (9). These findings suggest that any intervention that helps the shift of the immune response from Th2 type toward Th1 type will have a major role in cure and prevention of VL. Therefore, modalities to immunopotentiate the Th1 arm of the immune response could be exploited as vaccine candidates.

In recent years, there has been substantial progress in understanding the immunopathogenesis of leishmaniasis. The search for parasite Ags able to induce an immune response has been predominantly associated with the identification of proteins that may be used for vaccine development. Most studies aimed at identifying Ags from *Leishmania* spp. have searched for molecules with the ability to stimulate Th1-type responses, which are known to be the major defense mechanism against *Leishmania* infection (10–16). In fact, IFN-γ and IL-12 are the main cytokine implicated in the activation of macrophages to the killing of *Leishmania* in the PBMCs of one of the soluble leishmanial protein that induced a Th1 response. Elongation factor-2 (LelF-2) was identified through proteomics as a major role in cure and prevention of VL. Therefore, modalities to immunopotentiate the Th1 arm of the immune response could be exploited as vaccine candidates.

The consequence of leishmanial infection depends on the early cytokine profile, and, as such, parasite molecule/s that induces early cytokine production may be the key determinants of resistance or susceptibility. Therefore, a protein that stimulates the production of IL-12 and IFN-γ could be of major interest as a vaccine candidate for leishmaniasis as well as for its use as an adjuvant. *L. donovani* elongation factor-2 (LelF-2) was identified through proteomics as one of the soluble leishmanial protein that induced a Th1 response in the PBMCs of *Leishmania*-infected cured/endemic patients (19). In the current study, cloning, expression, purification, and molecular characterization of LelF-2 were carried out. We further examined the ability of recombinant LelF-2 (rLelF-2) to: 1) stimulate the immune responses in leishmania infected cured/endemic contact individuals’ PBMCs; 2) modulate the immune response in hamsters; and 3) protect naive hamsters against *L. donovani* challenge.

## Materials and Methods

### Animal

Laboratory-bred male golden hamsters (*Mesocricetus auratus*, 45–50 g) from the Institute’s animal house facility were used as experimental host. They were housed in a climatically controlled room and fed with standard rodent food pellet (Lipton India, Mumbai, India) and water ad libitum. The usage of hamsters was approved by the Institute’s Animal Ethical Committee (protocol number 24/05/Para/IAEC dated September 15, 2005).

### Parasites

The *L. donovani* clinical strain was procured from a patient admitted to the Kala-azar Medical Research Centre, Institute of Medical Sciences, Benaras Hindu University, Varanasi, India, and was cultured in vitro as described elsewhere (16). Promastigotes were grown in RPMI 1640 medium at 26°C (*Sigma-Aldrich*) in 75-cm² culture flasks (Nunc) (20). The strain has also been maintained in hamsters through serial passage (i.e., from amastigote to promastigote) (20).

### Cell line

Mouse macrophage cell line J774A.1 was procured from the Tissue Culture Facility of the Institute of Medical Sciences and maintained in RPMI 1640 through serial passage in 75-cm² culture flasks (Nunc) at 37°C and 5% CO₂. The confluent cells were harvested using cell scraper for the estimation of NO production.

### Preparation of soluble *L. donovani* promastigote Ag

Soluble *L. donovani* Ag (SLD) promastigote was prepared as per method described by Gupta et al. (19). Briefly, log-phase promastigotes (10⁷) were harvested from 3 to 4 d of culture and washed four times in cold PBS, resuspended in PBS containing protease inhibitors mixture (Sigma-Aldrich) and subjected to ultracentrifigation at 40,000 × g for 30 min. The protein content of the supernatant was estimated (21) and stored at −70°C.

### Cloning, expression, and purification of rLelF-2

*L. donovani* genomic DNA was isolated from 10⁶ promastigotes, washed, suspended in NET buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, and 1 mM EDTA), and incubated with proteinase K (1 mg/ml; Invitrogen Life Technologies) and 0.5% SDS at 50°C for 4 h. Nucleic acids were extracted by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Genomic DNA was spooled and subjected to RNase (100 μg/ml) treatment. LelF-2 gene was amplified using Taq Polymerase (Takara) lacking a 5′- to 3′- exonuclease activity, PCR was performed using rLelF-2–specific primers (based on the *L. infantum*–*LelF-2* gene sequence): forward, 5′-GAATTCGAGAAGGGCACTGTGGCGATC-3′; and reverse, 5′-CTCGAGTTCACACTTATCCAGAACGTGGTC-3′ (EcoRI and XhoI sites are underlined) in a Thermocycler (Bio-Rad) under conditions at one cycle of 95°C for 4 min, 30 cycles of 95°C for 1 min, 50°C for 1.30 min, 72°C for 2 min, and finally one cycle of 72°C for 10 min. Amplified PCR product was electrophoresed in agarose gel and eluted by using Gen Elute Columns (Qiagen). Eluted product was ligated in pTZ57R/T (TA) cloning vector (Fermentas) and transformed into competent *Escherichia coli* DH5α cells. The transformants were screened for the presence of recombinant plasmids by transforming the rLelF-2 insert by gene-specific PCR under similar conditions as previously mentioned. Isolated positive clones were sequenced from Delhi University (New Delhi) and submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore/EU929069; accession number EU920069.1). rLelF-2 was further subcloned at the EcoRI and XhoI site in bacterial expression vector pET28a (Novagen). The expression of rLelF-2 was checked in bacterial cells by transforming the rLelF-2 plus pET28a construct in *E. coli* Rosetta strain. The transformed cells were inoculated into 5 ml Luria–Bertani medium and allowed to grow at 37°C in a shaker at 220 rpm. Cultures in logarithmic phase (at OD₆₀₀ of ~0.5–0.6) were induced for 3 h with 1.0 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at 37°C. After induction, 1 ml cells were lysed in 100 μl sample buffer (50 mM Tris- HCL [pH 8], 10% glycerol, 10% SDS, and 0.05% bromophenol blue, with 100 mM DTT), and whole cell lysates (WCL) were analyzed by 12% SDS-PAGE (22). Uninduced control culture was analyzed in parallel. The overexpression of rLelF-2 was visualized by staining the gel with Coomassie brilliant blue R-250 (Sigma-Aldrich).

For purification, 200 ml Luria–Bertani medium containing 34 μg/ml chloramphenicol and 35 μg/ml kanamycin were inoculated with *E. coli* rosetta strain transformed with pET28a plus rLelF-2 and grown to an OD₆₀₀ of ~0.6. Recombinant protein expression was induced by addition of 1 mM IPTG, and the culture was incubated for an additional 3 to 4 h. The rLelF-2 was purified by affinity chromatography using Ni²⁺-chelating resin to bind the His-tag fusion peptide derived from the pET28a vector. The cell pellet was resuspended in 4 ml lysis buffer (50 mM Tris-HCL [pH 8], 300 mM NaCl, and 20 mM imidazole) containing 1:200 dilution of protease mixture inhibitor (Sigma-Aldrich) and 1% Triton X-100, incubated for 30 min on ice with 1 mg/ml lysozyme (Sigma-Aldrich), and the suspension was sonicated for 10 × 20 s (with 30-s intervals between each pulse) on ice. The sonicated cells were centrifuged at 15,000 × g for 30 min, and the supernatant was incubated at 4°C for 1 h with the 2 ml Ni-NTA Superflow resin (Qiagen, Hilden, Germany) previously equilibrated with lysis buffer. After washing with buffer (50 mM Tris-HCL [pH 8] and 300 mM NaCl) containing different concentrations of imidazole (i.e., 20, 50, and 100 mM), the purified rLelF-2 was eluted with elution buffer (50 mM Tris-HCL, 100 mM NaCl, and 250 mM imidazole [pH 7.5]). The eluted fractions were analyzed by 12% SDS-PAGE for purity and stained. The protein content of the fractions was estimated by the Bradford method using BSA as standard.

### Production of polyclonal Abs against rLelF-2 and Western blot analysis

The purified rLelF-2 protein was used for raising Abs in New Zealand white rabbit. Rabbit was first immunized using 50 μg rLelF-2 in Freund’s complete adjuvant. After 15 d, the rabbit was given three booster doses of 25 μg rLelF-2 each in IFA at 2 wk intervals, and blood was collected for serum 8 d after the last immunization. Ab titer was determined by ELISA.
and found to be 1–64,000. For immunoblotting experiments, purified rLeIF-2 protein and SLD were resolved on 12% SDS-PAGE and transferred on to nitrocellulose membrane using a semidry blot apparatus (Amersham Biosciences) (23). After overnight blocking in 5% BSA, the membrane was incubated with antiseraum to rLeIF-2 protein at a dilution of 1:2000 for 120 min at room temperature (RT). The membrane was washed three times with PBS containing 0.5% Tween 20 and then incubated with goat anti-rabbit IgG HRP conjugate (Bangalore Genie) at a dilution of 1:10,000 for 1 h at RT. Blot was developed using diamino benzidine plus imidazole plus H2O2 (Sigma-Aldrich).

**Patients and isolation of PBMCs**

The study groups for human samples have been summarized in Table I, and a brief description of the same is given below:

1) Seven treated cured patients (four males and three females, age ranging from 5–40 y) from hyperendemic areas of Bihar. All of the patients had received a complete course of amphotericin B and had recovered from VL. Samples were collected from 2 mo to 1 y after the completion of treatment. Diagnosis was established in all cases by demonstration of parasites in splenic aspirates and found negative at the time of study.
2) Five endemic household contacts (two males and three females, age range 15–45 y) who neither showed clinical symptoms nor received any treatment for Kala-azar. They belonged to the family of infected/cured patients.
3) Five infected patients (three males and two females, age range 5–40 y) showing clinical symptoms of Kala-azar.
4) Five normal healthy donors (three males and two females, age range 25–30 y) from nonendemic areas, without any history of leishmaniasis, served as negative control.

The study was approved by the ethics committee of the Kala-azar Medical Research Centre, Muzaffarpurm, India (protocol number EC-KAMRC/Vaccine/VL/2007-01).

Heparinized venous blood (10 ml each) was collected from all of the study subjects, and PBMCs were isolated from blood by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077; Sigma-Aldrich) as described by Garg et al. (16). A final suspension of 1 × 10⁶ cells/ml was made in complete RPMI 1640 medium after determining cell viability by trypan blue staining method. These were used for various immunological assays.

**Treatment of L. donovani-infected hamsters and isolation of mononuclear cells (lymph node cells)**

Approximately 20 hamsters, infected with 10⁶ amastigotes intracardially, were assessed 1 mo later for parasitic burden by spleen biopsy through a small incision in the upper left quarter of the abdomen, a small piece of splenic tissue was cut, and dab smears were made on slides. The incised portion was stitched with nylon suturing thread. Following biopsy, an adequate amount of antibiotic powder (Neosporin; GlaxoSmithKline) was applied on the stitched portion and finally sealed with tincture of benzoin. In addition, neosporin sulfate (100 mg/kg body weight) was also given orally the day before and the day after the biopsy for healing. The smears were fixed in methanol, stained with Giemsa, and the number of amastigotes/1000 cell nuclei was counted. The animals harboring >25–30 amastigotes/100 macrophage cell nuclei were then treated with anti-leishmanial drug Miltefosine (Zentaris) at 40 mg/kg body weight daily for 5 d. The animals were reassessed for complete cure by spleen biopsy performed on day 30 posttreatment. Mononuclear cells were separated from lymph nodes of cured, infected, as well as normal hamsters following the protocol of Garg et al. (16), and a suspension of 10⁶ cells/ml was made in complete RPMI 1640 medium. These cells were employed for lymphoproliferative assay and the estimation of NO production.

**Assessment of immunogenicity of rLeIF-2 in naive hamsters**

Two groups of animals each containing 16 hamsters were immunized intradermally (i.d.) on the back with rLeIF-2 (50 μg) and PBS, respectively. After 15 d, a booster dose of half of the amount of Ag and PBS each was given to the hamsters of both the experimental groups. Fifteen and 30 d later, the animals were sacrificed, and their spleens were isolated to extract RNA to assess the level of cytokines by quantitative real-time PCR (qRT-PCR).

**Vaccination with rLeIF-2 in hamsters**

For immunization, a total of 50 Syrian golden hamsters (40–45 g) were divided into the following three groups, each containing 14–17 animals, wherein group 1 served as control and groups 2 and 3 as the main experimental groups: group 1, unvaccinated and unchallenged (only PBS, normal control); group 2, unvaccinated and challenged (infected control); and group 3, rLeIF-2 vaccinated (vaccinated groups). The hamsters of group 3 were injected i.d. with 100 μg rLeIF-2 in 100 μl PBS. Fourteen days later, a booster dose of 50 μg rLeIF-2 plus 100 μl PBS was given i.d. to group 3. Day 21 after vaccination, each animal of groups 2 and 3 was challenged intracardially with 10⁴ late log-phase promastigotes of the clinical strain of L. donovani. On day 60 postchallenge (p.c.), three to five hamsters per group were necropsied for the assessment of parasitological and immunological (cytokines by real-time PCR and Ab level by ELISA) progression of VL. The impression smears/touch blots of different organs, namely spleen, liver, and bone marrow (femur bone), of experimental animals were made, and the criteria for the assessment of parasitic burden was based on the counting of the number of amastigotes per 1000 cell nuclei in each organ. The percentage of inhibition of parasite multiplica-

tion was calculated in comparison with the unvaccinated control using the following formula: percentage of inhibition = (number of parasite count from infected control – number of parasites from the vaccinated group/ number of parasite count from infected control) × 100.

**Immunological assays**

Assessment of lymphocyte proliferative responses (lymphocyte transformation test) in cured/exposed patients and hamsters. Lymphocyte sus-
pension (1 × 10⁶ cells/ml) of cured/exposed patients and normal, infected (30 d postimmunization [p.i.]), and cured hamsters was cultured in 96-well flat-bottom tissue culture plates (Nunc). This assay was carried out as per protocol described by Garg et al. (16) with some modifications, wherein XTT (Roche Diagnostics) was used instead of [³H]Thymidine. About 100 μl predetermined concentration (10 μg/ml) of mitogens (PHA for patient’s PBMCs and Con A for hamster’s lymphocytes), as well as rLeIF-2 and SLD (10 μg/ml each), were added to the wells in triplicate. Wells with stimulants served as blank controls. Cultures were incubated at 37°C in a CO₂ incubator with 5% CO₂ for 3 d in the case of the mitogens and for 5 d in the case of the Ags. Eighteen hours prior to termination of culture, 50 μl XTT (Roche Diagnostics) was added to 100 μl supernatants of each well and absorbance measured at 480 nm with 650 nm as reference wave-

length.

**Estimation of NO activity in macrophages of hamsters and cell lines.** Isolated lymphocytes from all three study groups of hamsters (nearly, Table I. Aggregate clinical data of VL patients and controls

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<td>3/2</td>
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*Duration of illness (based on clinical symptoms such as fever and hepatosplenomegaly) 1–3 wk. These patients were confirmed by diagnostic test (i.e., rk39).*

*Taking individual patients in consideration from hyperendemic areas of Bihar, the posttreatment period after which blood samples were collected ranged between 2 and 12 mo. All of the patients had received complete course of amphotericin B and had recovered from VL. Diagnosis was established in all cases by demonstration of parasites in splenic aspirates and found negative at the time of study.*

*Endemic household contacts belonged to the group who neither showed clinical symptoms nor received any treatment for Kala-azar. They belonged to the family of infected/cured patients.*

*Individual from nonendemic areas, without any history of leishmaniasis, served as negative control.*
normal, infected [30 p.i.], and cured) were suspended in culture medium, plated at 10^5 cells/well, and stimulated for 3 d in case of mitogen (LPS) and 5 d in case of Ags (rLeIF-2, SLD) at 10 µg/mL. The presence of NO was assessed using Griess reagent (Sigma-Aldrich) in the culture supernatants of peritoneal macrophages of cured hamsters (16) as well as macrophage cell lines (J774 A.1) after the exposure with supernatant of stimulated lymphocytes. The supernatants (100 µl) collected from macrophage cultures 24 h after incubation was mixed with an equal volume of Griess reagent and left for 10 min at room temperature. The absorbance of the reaction was measured at 540 nm in an ELISA reader (24). The nitrite concentration in the macrophages’ culture supernatant samples was extrapolated from the standard curve plotted with sodium nitrite.

**Assessment of cytokine levels—IFN-γ/INF-α/IL-12/IL-10/IL-4—in lymphocytes of cured/infected patients.** Culture of PBMCs (1 × 10^6 cells/ml) from human patients was set up in 96-well culture plates, and rLeIF-2 was added at a concentration of 10 µg/ml in triplicate wells. The level of IFN-γ, TNF-α, IL-12, IL-10, and IL-4, was estimated by ELISA kit (OptEIA set; BD Pharmingen) after 5 d of incubation with Ag using supernatants. The results were expressed as picograms of cytokine/ml, based on the standard curves of the respective cytokine provided in the kit. The lower detection limits for various cytokines were as follows: 4.7 pg/ml for IFN-γ, 7.8 pg/ml for IL-12/p40, 7.0 pg/ml for IL-10, and 7.8 pg/ml for TNF-α and IL-4.

**Quantification of mRNA cytokines and inducible NO synthase in hamsters by real-time PCR.** qRT-PCR was performed to assess the expression of mRNAs for various cytokines and inducible NO synthase (iNOS) in splenic tissues. Splenic tissues were taken from each of the three randomly chosen animals. Total RNA was isolated using TRI reagent (Sigma-Aldrich) and quantified by using Gene-quant (Bio-Rad). One microgram total RNA was used for the synthesis of cDNA using a first-strand cDNA synthesis kit (Fermentas). For real-time PCR, primers were designed using Beacon Designer software (Bio-Rad) on the basis of cytokines and iNOS mRNA sequences available on PubMed (25) (Table II). qRT-PCR was conducted as per the protocol described earlier (25) by using 12.5 µl SYBR Green PCR master mix (Bio-Rad), 1 µg cDNA, and primers at a final concentration of 300 nM in a final volume of 25 µl. PCR was conducted under the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s per cycle using the iQ5 multicolor real-time PCR system (Bio-Rad). cDNAs from normal hamsters were used as comparator samples for quantification of those corresponding to test samples, whereas in vaccination studies, cDNAs from infected hamsters were used as comparator samples. All quantifications were normalized to the housekeeping gene HPRT. A no-template control cDNA was included to eliminate contaminations or nonspecific reactions. The cycle threshold (CT) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). Differences in gene expression were calculated by the comparative ΔΔCT method (26). This method compares test samples to a comparator sample and uses results obtained with a uniformly expressed control gene (HPRT) to correct for differences in the amounts of RNA present in the two samples being compared with generate a ΔCT value. Results are expressed as the degrees of difference between ΔCT values of test and comparator samples.

**Results and discussion**

**Table II. Sequences of forward and reverse primers of hamster cytokines used for quantitative real-time RT-PCR**

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<th>Primer Sequence</th>
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<td>HGPRT forward</td>
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</tr>
<tr>
<td>2</td>
<td>TNF-α forward</td>
<td>5’-TCTTCCCTCCCTGGTGTTG-3’</td>
</tr>
<tr>
<td>3</td>
<td>IFN-γ forward</td>
<td>5’-GCTCTGCGTTGGAGAAGTTAG-3’</td>
</tr>
<tr>
<td>4</td>
<td>IL-12 forward</td>
<td>5’-TATGTGTTGAGGTGGACTG-3’</td>
</tr>
<tr>
<td>5</td>
<td>IL-12 reverse</td>
<td>5’-TTGGCGGAGTGGATTG-3’</td>
</tr>
<tr>
<td>6</td>
<td>TGF-β forward</td>
<td>5’-AGCGAAGAGAAGACCTGGTG-3’</td>
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<td>7</td>
<td>TGF-β reverse</td>
<td>5’-GGTGGCTGTTGGAGATGG-3’</td>
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<tr>
<td>8</td>
<td>iNOS forward</td>
<td>5’-CCAGCACCGACCATGAAAGG-3’</td>
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<tr>
<td>9</td>
<td>iNOS reverse</td>
<td>5’-AGAAGTCGAGAAGAGGACATC-3’</td>
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</table>

**Measurement of Ab response in hamsters.** The level of IgG Ab and its isotypes in sera samples of hamsters of different experimental groups was measured as per protocol by Samant et al. (26) with slight modifications.Briefly, 96-well ELISA plates (Nunc) were coated with rLeIF-2 (0.2 µg/µl/well) overnight at 4°C and blocked with 1.5% BSA at RT for 1 h. Sera was used at a dilution of 1/100 for IgG, IgG1, and IgG2 and kept for 2 h at RT. Biotin-conjugated mouse anti-Armenian and Syrian hamster IgG, IgG1, and biotinylated anti-Syrian hamster IgG2 (BD Pharmingen) were added for 1 h at RT/1000 dilutions and were further incubated with peroxidase-conjugated streptavidin at 1/1000 (BD Pharmingen) for 1 h. Finally, the substrate O-phenylenediamine dihydrochloride (Sigma-Aldrich) was added, and the plate was read at 492 nm.

**Statistical analysis**

Results were expressed as mean ± SD. Two sets of experiments were performed, and the results were analyzed by Student t test and one-way ANOVA test followed by Dunnets or Tukey’s posttest, whichever was appropriate in each case, using the GraphPad Prism software program (GraphPad). The upper level of significance was chosen as p < 0.001 (highly significant).

**Results**

Partial rLeIF-2 was cloned, sequenced, and expressed in E. coli Rosetta strain

The rLeIF-2 gene of *L. donovani* was successfully amplified, T/A cloned (Fig. 1A), and sequenced, which was 99% homologous with *L. infantum* rLeIF-2 (Table III). It was further subcloned in bacterial expression vector pET28a (Fig. 1B). The expression revealed that the size of the expressed protein was ~70 kDa (Fig. 1C), and the rLeIF-2 was eluted at 250 mM imidazole concentration (Fig. 1C). Immunoblots of lysates from *L. donovani* promastigote were performed with the polyclonal anti-r-LeIF-2 Ab, which detected one dominant protein species of ~95 kDa (Fig. 1D).

rLeIF-2 induced significant lymphoproliferative and NO responses in normal/infected/cured hamsters

The cellular responses of lymph node cells of cured hamsters were assessed by XTT (Roche Diagnostics) against the mitogen (i.e., Con A as well as SLD and rLeIF-2). The responses were compared with that of normal as well as *L. donovani*-infected groups that served as controls. The normal control as well as cured *Leishmania*-infected group had shown significantly higher proliferative responses against Con A as compared with the *L. donovani*-infected group (Fig. 2A). The proliferative response of PBMCs against rLeIF-2 was significantly higher in cured/infected hamsters (mean OD 0.419 ± 0.0263 and 0.891 ± 0.090) than normal healthy controls (mean OD 0.256 ± 0.125). The difference was statistically significant (p < 0.001).

NO-mediated macrophage effector mechanism is known to be critical in the control of parasite replication in the animal model; hence, its production in peritoneal macrophages of cured hamsters as well as macrophage cell line J774 A.1 was studied after 24 h of incubation in the presence of rLeIF-2 and SLD. For comparison, NO production in mitogenic (LPS)-stimulated and unstimulated cells served as positive and negative controls, respectively (Fig. 2B). NO production was recorded to be higher against rLeIF-2 (p < 0.001). We observed similar responses using murine cell line (J774 A.1) (Fig. 2C).

rLeIF-2 stimulates PBMCs from Leishmania patients to proliferate and express a predominant Th1 cytokine profile

We further validated the cellular responses (lymphocyte transformation test [LTT] and cytokine levels) of rLeIF-2 in PBMCs of cured patients, endemic and nonendemic controls, and *L. dono-\*\vani*-infected donors (Table I). Individual donors in each study group were found to elicit different responses. Lymphoprolifera-
generation of Th1 responses and IFN-γ, the development of cell-mediated immunity (CMI), including the profile against SLD, wherein high levels of IL-10/IL-4 and very cured/endemic contacts generated a mixed Th1/Th2 cytokine (ranging from 9.45–36.34 and 17.45–65.46 pg/ml). PBMCs of from 12.3–40.23 and 15.2–60.5 pg/ml) and infected patients (650–2740 and 727–2390 pg/ml, respectively) patients followed by endemic contacts (ranging 773–2545 and 350–1890 pg/ml) consistently higher than those observed with SLD (102–325–1565 and 290–1670 pg/ml, respectively). On the contrary, a very low level of IL-10 and IL-4 cytokines against rLelF-2 was detected in supernatants of cured (8.29–25.55 and 10.45–55.52 pg/ml) PBMCs from all of the cured and active VL patients proliferated in response to rLeIF-2 with mean OD values of 1.25 ± 0.0681 and 0.42 ± 0.125, which was higher than SLD (mean OD values of 0.574 ± 0.156) and 0.493 ± 0.134), respectively (p < 0.001) (Fig. 3A). The results demonstrate that rLelf-2 is a potent T cell Ag recognized by a majority of L. donovani-infected/cured/endemic individuals in different stages or manifestations of infection.

To assess the Th1/Th2 stimulatory potential of the rLeIF-2, we further studied the cytokine levels, namely IFN-γ, TNF-α, and IL-12, as well as IL-10 and IL-4, in PBMCs from cured/infected patients as well as in endemic contacts against rLeIF-2. The levels of IFN-γ and TNF-α was observed to be higher in the supernatants of cured patients with a range of 773–2545 and 350–1890 pg/ml, respectively, as compared with endemic contacts (506–1745 and 200–1465 pg/ml, respectively) and infected patients (325–1565 and 290–1670 pg/ml, respectively). On the contrary, a very low level of IL-10 and IL-4 cytokines against rLeIF-2 was detected in supernatants of cured (8.29–25.55 and 10.45–55.52 pg/ml, respectively) patients followed by endemic contacts (ranging from 12.3–40.23 and 15.2–60.5 pg/ml) and infected patients (ranging 9.45–36.34 and 17.45–65.46 pg/ml). PBMCs of cured/endemic contacts generated a mixed Th1/Th2 cytokine profile against SLD, wherein high levels of IL-10/IL-4 and very low levels of IFN-γ and IL-12p40 were noticed in response to SLD in infected patients (Fig. 3B, 3D–F).

IL-12 has been shown to play a pivotal immunoregulatory role in the development of cell-mediated immunity (CMI), including the generation of Th1 responses and IFN-γ production in intracellular bacterial or parasitic infections (24). Because rLeIF-2 stimulated a dominant Th1 cytokine profile and downregulated Th2 responses, we explored a possible role for IL-12 in responses of PBMCs to rLeIF-2. rLeIF-2 stimulated IL-12p40 production from cured and infected patient PBMCs with values (650–2740 and 727–2390 pg/ml) consistently higher than those observed with SLD (102–515 and 8–30 pg/ml), respectively. PBMCs from uninfected individuals also produced IL-12p40 when cultured with rLeIF-2, although no IL-12p40 was detected in response to SLD. This may suggest a role for IFN-γ in the SLD-induced IL-12p40 level observed in PBMCs of Leishmania patients, which produced higher IFN-γ than normal PBMCs after Ag stimulation (Fig. 3C).

Taken together, the above results demonstrate that rLeIF-2 elicits a predominant Th1 cytokine profile from the PBMCs of five infected patients while downregulating the expression of IL-10 mRNA. We have further demonstrated the effect of rLeIF-2 in downregulating IL-10 production. PBMCs from infected patients were cultured in the presence of LPS with or without rLeIF-2. LPS-stimulated PBMCs from all the individuals tested secreted IL-10, with values ranging from 204 to 546 pg/ml. However, in the presence of rLeIF-2, the production of LPS-induced IL-10 secretion was reduced by ~50% (Fig. 4A). Because IFN-γ can inhibit IL-10 production by activated macrophages (23), and rLeIF-2 stimulated patient PBMCs to secrete IFN-γ, the secreted IFN-γ may be responsible for partially inhibiting the production of LPS-induced IL-10 (Fig. 5A).

We further evaluated the ability of rLeIF-2 to downregulate the production of IL-4 by PBMCs of L. donovani-infected patients. The addition of rLeIF-2 and SLD to the PBMC cultures lead to the downregulation of SLD-induced IL-4 secretion (Fig. 5B).

**Immunization with rLeIF-2 favors the development of Th1-type cellular responses in naive hamsters**

To assess the immunogenicity of rLeIF-2 in modulating Th1/Th2 type of immune response, we further studied the status of iNOS transcript and cytokine levels, namely IFN-γ, a, and IL-12, as well as IL-10, IL-4, and TGF-β in naive hamsters at different time intervals p.i. The level of Th1 cytokines in immunized hamsters was witnessed to be significantly upregulated by ~1.5–2-fold (p < 0.001) on as early as day 15 p.i. However, the level of iNOS transcript was observed to be moderately upregulated by ~1.5-fold on day 30 p.i. The level of Th2 cytokines, in contrast, was found to be downregulated on day 30 p.i. (p < 0.5) (Fig. 4).

**Immunization with rLeIF-2 modulates Leishmania-specific IgG and its isotypes in naive hamsters**

To assess the Ab level in the serum of immunized animals, we further estimated the level of IgG and its isotypes. We observed significantly higher IgG2 response by ~8-fold (mean OD, 0.72 ± 0.05) on day 30 p.i. (p < 0.001) as compared with the unimmunized animals (mean OD, 0.0938 ± 0.002). However, there was no
apparent difference in the IgG1 response between the immunized and unimmunized animals (mean OD, 0.0192 ± 0.0032 versus 0.0292 ± 0.04; p < 0.05) on day 30 p.i. (Fig. 6).

Vaccination with rLelF-2 protect hamsters against L. donovani challenges

The hamsters vaccinated with rLelF-2 were found to resist the challenge infection of L. donovani when assessed on days 60 and 90 p.c. A significant parasite inhibition was observed in hamsters, as evidenced by decrease in parasite load in Giemsa-stained splenic smears of spleen, liver, and bone marrow on day 90 as compared with unvaccinated and Leishmania-infected control. The reduction in parasite load was well correlated with body, spleen, and liver weights of vaccinated hamsters as compared with the infected controls (Fig. 7). The rLelF-2—vaccinated hamsters were noticed to be healthy and survived until day 120 p.c. until the experiment was terminated. The unvaccinated infected controls, in contrast, could survive only up to 2 to 3 mo p.c.

FIGURE 2. Cellular responses of rLelF-2 of L. donovani in hamsters. A, LTT response of mononuclear cells (lymph nodes) from normal, L. donovani-infected (30 d p.c.) and treated hamsters in response to Con A, SLD, and rLelF-2 at 10 μg/ml each. Proliferation was represented as mean OD of stimulated culture — mean OD of unstimulated control. Each bar represents the pooled data (mean ± SD value) of six hamsters, and the data represent the means of triplicate wells ± the SD. NO production (μM) by peritoneal macrophages of hamsters (n = 5) (B) and J774A.1 cell line (C). The peritoneal macrophages as well as J774 A.1 cells were primed with the supernatants of stimulated lymphocytes (3 d with mitogen and 5 d with Ags) of normal, infected, and cured hamsters in response to rLelF-2, SLD, and LPS, respectively, at 10 μg/ml each. The estimation of NO production was done using Greiss reagent in supernatants collected from macrophage cultures 24 h after incubation, and the absorbance of the reaction product was measured at 540 nm. Significance values indicate the difference between the SLD and rLelF-2 stimulation. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 3. Cellular responses. LTT (A) Th1 and Th2 cytokine production (B–F) in PBMCs from individuals of cured VL patients (7), infected individuals (5), and endemic controls (5) in response to rLelf-2 and SLD Ags. Each data point represents one individual. Values are given as concentration in picograms per milliliter. Significance values indicate the difference between the SLD and rLelF-2 stimulation of PBMCs. *p < 0.05, ***p < 0.001.
rLeIF-2 vaccination generates Th1-type cytokine profile as determined by qRT-PCR

Because considerable protective efficacy was observed in rLeIF-2–vaccinated hamsters, the expression of Th1 and Th2 mRNA cytokines was further evaluated by qRT-PCR on day 60 p.c. The iNOS transcript was found to be significantly upregulated by ∼2-fold (\(p < 0.001\)) in the vaccinated group. The expression of TNF-\(\alpha\) as well as IFN-\(\gamma\) was ∼2-fold higher (\(p < 0.01\)) in the hamsters of vaccinated group in comparison with that of the \(L.\) donovani-infected group. The case was similar with IL-12, which was also significantly (∼1.5-fold in vaccinated hamsters. In contrast, the expression of IL-10 was found to be significantly upregulated in the infected group but was downregulated (\(p < 0.01\)) in the vaccinated hamsters. IL-4 and TGF-\(\beta\), which were found to be associated with progressive VL, significantly downregulated by ∼1- and ∼2-fold (\(p < 0.01\) and \(p < 0.001\)) respectively, in vaccinated hamsters (Fig. 8).

rLeIF-2 vaccination generates IgG2-type antileishmanial Ab response

The antileishmanial IgG and IgG1 were observed to be elevated by 1- to 2-fold in infected group in comparison with the rLeIF-2–vaccinated group, in which their level was very low. By day 60 p.c.,
the rLelF-2–vaccinated group showed a significant elevation of IgG2 level by 3- to 4-fold over the infected group (Fig. 9). As a measure of CMI, the elevation of IgG2 was indicator of the development of effective immune responses.

Discussion
LelF-2 has been identified as Th1 stimulatory protein from a subfraction of SLD through proteomics belonging to the molecular mass range of 68–97.4 kDa (19, 27). This protein though, is involved in protein synthesis, has not been characterized in Leishmania species. The proteins of a similar family, EL-1α, EL-1B, and EL-4a, have been reported in various Leishmania species (14, 28, 29). Of these, EL-1α, cloned and characterized in L. donovani (29), has been attributed to have an essential role in survival and thus can serve as a good drug target (30). The other two, EL-1B (28) and EL-4a (14), have been characterized biochemically and immunologically, respectively, in L. major. In this study, we are reporting the cloning and characterization of the partial sequence (1932 bp) of EL-2, which has 99% homology with L. infantum LelF-2. The complete sequence of this gene is 2538 bp. Immunoblot study of L. donovani promastigote lysates with the polyclonal anti–rLelF-2 Ab revealed one dominant protein of ~95 kDa.

FIGURE 6. Ab levels (OD value) in hamsters (six to eight) immunized with rLelF-2 on day 15 and 30 p.i. Each bar represents the pooled data (mean ± SD value) of three replicates. Significance values indicate the difference between the immunized group and normal group. ***p < 0.001.

FIGURE 7. Body weight (A), spleen weight (B), and liver weight (C) in grams. Parasite burden (number of amastigotes per 100 cell nuclei) in the spleen (D), liver (E), and bone marrow (F) on days 60 and 90 p.c. Significance values indicate the difference between the vaccinated groups and infected group. *p < 0.05, **p < 0.01, ***p < 0.001.
Because we have reported earlier that a T cell response develops when cells from cured individuals are stimulated with SLD and its subfractions (16, 31), we first tested whether rLeIF-2 alone could induce cellular immune response in Leishmania-infected and cured hamsters and then further validated its immunogenicity in endemic nonimmune donors (household contacts without any clinical symptoms) and in immune patients of VL that were cured either with amphotericin B or miltefosine. There has been reservation in accepting human Th1 and Th2 to the rodent Th1 and Th2 subsets probably due to the existence of dichotomy in the human system, which is not as clear as in rodent particularly murine cells. Besides, the infection pattern also does not simulate the human profile, as it is self-limiting in murine VL. In contrast, the systemic infection of the hamster with L. donovani is very similar to human Kala-azar, as it results in a relentlessly increasing visceral parasite burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia, and, ultimately, death (25). Hence, analysis of cellular immune response of the rLelF-2 was carried out using lymphocytes as well as macrophages of cured hamsters to correlate the observations made with the human lymphocytes. We have earlier observed that the cellular responses to Leishmania membrane as well as soluble Ags were similar in endemic controls and cured patients of VL as well as in hamsters, indicating that the results so obtained with the hamster could be translated into humans (16, 31).

In the case of leishmanial infections, macrophages become activated by IFN-γ derived from parasite-specific T cells and are able to destroy intracellular parasites through the production of several mediators, principal among which is NO (32). It is well documented that IFN-γ induces production of NO in phagocytic cells (principally macrophages) that harbor Leishmania parasite, which leads to destruction of the parasite (22). In the absence of

**FIGURE 8.** Splenic iNOS and cytokine mRNA expression profile analysis of normal and immunized hamsters on day 60 p.c. by qRT-PCR. Significance values indicate the difference between the vaccinated group and infected group. **p < 0.01, ***p < 0.001.

**FIGURE 9.** Leishmania-specific IgG and its isotypes IgG1 and IgG2 in rLeIF-2–vaccinated hamsters in comparison with the uninimmunized infected hamsters on day 60 p.c. Significance values indicate the difference between the vaccinated group and infected group. **p < 0.01, ***p < 0.001.
cytokine reagents against hamsters, in this study, we have observed the effect of rLelF-2 on NO production by peritoneal macrophages of cured hamster and also validated the same effect in the J774A.1 cell line. rLelF-2 gave significantly higher cellular responses, namely lymphoproliferative as well as NO release, against all of the cured hamsters in comparison with normal and infected ones. It is well established that recovery from Leishmania infection relies on induction of the Th1 response (33, 34) with production of IFN-γ and IL-12 and enhanced expression of iNOS (31, 35).

Some of the rAgS have previously been shown to induce lymphocyte proliferation and IFN-γ production in subjects cured of VL and in patients with cutaneous or mucosal leishmaniasis (36, 37). Although lymphocyte proliferation has been widely used to analyze T cell function, the documentation that the CD4+ population is heterogeneous regarding to the cytokine profile secreted indicates that cytokines should be measured to determine if an immune response could be protective or deleterious. In the current study, whereas rLelF-2 induces strong T cell proliferation in subjects with endemic control and cured patients of VL, the IFN-γ production mediated by this protein was significantly higher than that observed in healthy controls. Thus, individuals who control parasite burden successfully either following treatment in the case of patients or due to adequate immunity, as in endemic contacts, exhibit good T cell reactivity to the Leishmania Ag. The presence of a positive immune response in all of the five endemic contacts suggests that the frequency of subclinical infection in an endemic area such as Bihar is high, as has been reported in other areas of the world (38–40). The majority of people infected with L. chagasi or L. donovani have been shown to clear their infection spontaneously and develop protective immunity, characterized by lymphocyte proliferation and secretion of IFN-γ in response to Ag in vitro.

It has been observed that SLD-stimulated PBMCs from L. donovani-infected individuals elicit a mixed Th1- and Th2-like immune response; in this study, we noticed that rLelF-2 shifted this pattern toward an exclusive Th1 (IFN-γ and IL-12) cytokine profile. In addition, rLelF-2 stimulated cured/patient and normal PBMCs to produce IL-12 and IFN-γ, whereas SLD did not stimulate the production of IL-12 and IFN-γ in normal individuals and elicited a very low level of both cytokines in infected patients. Finally, both rLelF-2 and SLD stimulated PBMCs from cured and infected patients to secrete TNF-α. The observation that rLelF-2 stimulated T cells from all cured/infected patients to proliferate and produce IFN-γ suggests that PBMCs’ response to rLelF-2 may be associated with protective immunity. Thus, rLelF-2 appears to have distinctive immunological properties. Cytokines appear to be essential mediators of immunity to Leishmania (13), wherein IFN-γ and TNF-α synergize to induce leishmanicidal activity in macrophages (41–43). A study using human monocytes has demonstrated that the addition of TNF-α (5–10 ng) can induce IL-10 production (44). In our study, TNF-α and IL-12 but not IL-10 were produced by cured/patient PBMCs stimulated with rLelF-2. This may be due to the relatively lower level of either TNF-α or IL-12 produced by patient PBMCs, as well as the ability of IFN-γ, produced in high amounts by patient PBMCs, to inhibit the production of IL-10 (45). Further, it was observed that rLelF-2 downregulated the levels of IL-10 in patient PBMCs as well as in LPS-stimulated PBMCs. In normal (nonendemic) contacts, we were not able to detect IL-4 and IL-10 cytokines (data not shown). Similarly, IL-12 and IFN-γ were not detected in normal samples in response to SLD (data not shown). The use of patient PBMCs, rather than purified cell populations, may be more relevant to the in vivo situation because the dominant cytokine pattern is dependent on the interplay of modulatory cytokines. It, therefore, appears that certain Leishmania Ag may be able to elicit a dominant Th1 cytokine profile as well as inhibit the production of Th2 cytokines (IL-10) by mechanisms that are not fully understood, because IL-12 plays a central role in the initiation and maintenance of Th1 responses in humans and mice (46–49) and is a potent inducer of IFN-γ (36), which may inhibit the production of IL-10 (45). Our results have demonstrated that a single component of Leishmania is capable of stimulating the production of IL-12 and IFN-γ.

This study represents the first report of cloning, expression, and purification of recombinant L. donovani protein rLelF-2 that stimulates the production of the Th1 type of cytokines (IL-12 and IFN-γ) in cured/patient and normal PBMCs of VL. We further assessed the prophylactic potential of rLelF-2 and observed that it provided resistance to Leishmania challenge to the tune of 65%, which was further supported by the surge in iNOS, IFN-γ, TNF-α, and IL-12 mRNA levels along with significant downregulation of IL-10, IL-4, and TGF-β. A rise in the level of Leishmania-specific IgG2 was also observed that was indicative of enhanced CMI. Thus, the ability of rLelF-2 to protect hamsters moderately against L. donovani challenges generating a T cell response suggests that immunization with rLelF-2 in combination with other potential Th1 stimulatory proteins (as a mixture vaccine) may provide absolute prophylactic/therapeutic and lifelong protection against VL.

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Disclosures

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References


