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LeIF: A Recombinant *Leishmania* Protein That Induces an IL-12-Mediated Th1 Cytokine Profile[1]

Yasir A. W. Skeiky,[2]* Mary Kennedy,[‡] David Kaufman,[§] Monamaris M. Borges,[¶] Jeffrey A. Guderian,* John K. Scholler,* Pamela J. Ovendale,* Kathleen S. Picha,[‡] Philip J. Morrissey,[‡] Kenneth H. Grabstein,* Antonio Campos-Neto,[†] and Steven G. Reed*[†‖]

We have evaluated the ability of the *Leishmania* protein LeIF to influence the Th1/Th2 cytokine responses and the generation of LeIF-specific T cell clones in the absence of adjuvant. We characterized LeIF-specific T cell responses in *Leishmania major*-infected and uninfected BALB/c mice. These mice develop a strong Th2 response during infection with *L. major*. When lymph node cells from infected BALB/c mice were stimulated in vitro with LeIF, only IFN-γ (and no detectable IL-4) was found in the culture supernatant. In addition, LeIF down-regulated *Leishmania* Ag-specific IL-4 production by lymph node cells from infected BALB/c mice. Subsequently, Th responses were evaluated in naive BALB/c mice following immunization with LeIF. T cell clones derived from mice immunized with LeIF preferentially secreted IFN-γ. Finally, to understand the basis for the preferential Th1 cytokine bias observed with LeIF, the ability of LeIF to influence the early cytokine profile was evaluated in splenocytes of SCID mice. We found that LeIF stimulated fresh spleen cells from naive SCID mice to secrete IFN-γ by IL-12/IL-18-dependent mechanisms. The N-terminal half of the molecule (amino acid residues 1–226) maintained the ability to stimulate IFN-γ from splenocytes of SCID mice. Finally, we also demonstrated that LeIF was able to provide partial protection of BALB/c mice against *L. major*. Thus, our results suggest the potential of LeIF as a Th1-type adjuvant and as a therapeutic and prophylactic vaccine Ag for leishmaniasis when used with other leishmanial Ags. ***The Journal of Immunology,*** **1998, 161: 6171–6179.**

The *Leishmania* are a complex of protozoan parasites that cause a spectrum of clinical diseases, including cutaneous, mucosal, and visceral leishmaniasis. Regardless of clinical presentation, recovery and resistance to reinfection are dependent on the development of Ag-specific T lymphocyte responses that include proliferation, delayed-type hypersensitivity, and the secretion of Th1 cytokines. The separation of $CD4^+$ T cells into subsets based upon cytokine profiles has been valuable for characterizing response patterns in infectious diseases. Th1 cells produce IFN-γ and are involved in cell-mediated immunity, while Th2 cells produce IL-4, IL-5, and IL-10 and are more involved in humoral immunity. In experimental *Leishmania major* infection, a dominant Th1 response is associated with healing, while a predominant Th2 response is associated with disease progression (1, 2).

Of particular interest is the observation that IL-12 can promote the development of Th1 cells (3, 4) and augments cytotoxicity (5–7). IL-12 is produced primarily by phagocytic cells in response to infection with intracellular pathogens or in response to microbial products. Once secreted, IL-12 is a strong stimulator of IFN-γ production by T and NK cells. Several observations indicate that IL-12 is a key cytokine that favors Th1 cell differentiation. This property of IL-12 is directly correlated with host resistance against intracellular pathogens. For example, IL-12 has been implicated in the resolution of leishmaniasis through mechanisms that initiate a Th1 response and protective immunity (4, 8, 9). In the mouse model, when *L. major*-susceptible BALB/c mice are treated with IL-12, they develop a resistant Th1-type response and cure the infection. Conversely, treatment of C3H mice (*L. major*-resistant strain) with anti-IL-12 Ab, which prevents the early production of IFN-γ by NK and T cells, causes these mice to develop a susceptible Th2-type response. As a consequence, the mice develop severe disease (10). Also interesting is the observation that IL-12 can act as an adjuvant to induce Th1 memory cells (11). Thus, BALB/c mice vaccinated with a *L. major* soluble Ag preparation, in the presence of IL-12, are protected from subsequent challenge with this *Leishmania* (11). As expected, these mice developed the Th1 type of response.

The outcome of leishmanial infection thus depends on the early cytokine profile. Parasite molecules that influence early cytokine production may be key determinants of resistance or susceptibility. A protein that stimulates the production of IL-12 could be of significant interest as an immunotherapeutic for leishmaniasis as well as for use as an adjuvant. LeIF was originally described as a leishmanial protein that induced a Th1 response in the PBMC of leishmaniasis patients by an IL-12-mediated mechanism. In the present study, we have further examined the ability of LeIF to 1) modulate the immune responses of BALB/c mice with *Leishmania* infection, 2) specifically favor the expansion of LeIF-specific Th1 clones in the absence of added adjuvant, 3) stimulate the production of IFN-γ by IL-12/IL-18-mediated mechanisms in spleen cells from

*Corixa Corporation and [†]Infectious Disease Research Institute, Seattle, WA 98104; [‡]Immunex Corp., Seattle, WA 98101; [§]Cornell University Medical College, New York, NY 10021; [¶]Instituto Butantan, Sao Paulo, Brazil; and [‖]Department of Pathology, University of Washington, Seattle, WA 98195.

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[2] Address correspondence and reprint requests to Dr. Yasir A. W. Skeiky, Corixa Corporation, 1124 Columbia Street, Seattle, WA 98104. E-mail address: Skeiky@Corixa.Com

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both C3H and BALB/c SCID mice, and 4) protect BALB/c mice against *L. major* infection.

Materials and Methods

Cloning and expression of LmeIf

A cDNA expression library was constructed with poly(A)$^+$ RNA of *L. major* promastigote stage using the ZAP-cDNA unidirectional cloning kit (12). Approximately 500,000 plaque-forming units were screened with a radiolabeled DNA probe comprising nucleotide sequences 258-1188 of the *Leishmania braziliensis* eIF4A homologue (13). Posthybridization washes were at 55°C with 0.5 × SSC containing 0.1% SDS. This resulted in the identification of a clone containing a ~2.7-kb insert (LmeIF). Excision of the pBSK(−) phagemid sequences was conducted according to the manufacturer's protocols. Overlapping clones were generated from the cDNA insert by exonuclease III in combination with primer-directed sequencing by the *Taq* dye terminator system on an Applied Biosystems (Foster City, CA) 373 automated sequencer.

Expression and purification of rLeIF constructs

L. braziliensis eIF protein (LbeIF) was expressed as a 45-kDa β-gal[3] fusion using the pBSK(−) phagemid, as described (13). As a control, r8E (another *L. braziliensis* Ag unrelated to LbeIF) was also expressed as a β-gal fusion using the same vector system. The expressed Ags were purified to homogeneity by preparative SDS-PAGE electrophoresis, followed by excision and electroelution of the rAg, as described (13), and dialyzed in 10 mM Tris, pH 8. Full-length and overlapping *L. major* rLeIF proteins were expressed in *Escherichia coli* with six histidine residues at the amino-terminal portion immediately following the initiator Met residue (N-terminal His-Tag) of the pET plasmid vector (pET-17b) and a T7 RNA polymerase expression system (Novagen, Madison, WI). The cDNA was amplified by PCR from the initial cloned construct in pBSK(−) vector using specific oligonucleotides comprising 5′ and 3′ sequences. The specific oligonucleotide primers used for PCR amplification of the LeIF cDNA were as follows: 1) Full-length sequence [amino acid residues 1–403, 5′ (oligo 1-CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC ***ATG*** GCG CAG AAT GAT AAG ATC GCC and 3′ (oligo 403– CAT GGA ATT CCG C***TT A***CT CGC CAA GGT AGG CAG C]; 2) amino acid residues 1–226, 5′-oligo 1 as above and 3′-oligo 226– CAT GGA ATT C***TTA*** GTC GCG CAT GAA CTT CTT CGT CAG; 3) amino acid residues 196–403, 5′-oligo 196– CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC TTC CGC TTC CTG CCG AAG GAC ATC and 3′-oligo 403 as above; and 4) amino acid residues 129–261, 5′-oligo 129– CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC GAG ACC TTT GTC GGC GGC ACG CGC and 3′-oligo 261-CAT GGA ATT C***TT A***CA GGT CCA TCA GCG TGT CCA GCT T. The 5′- and 3′-oligonucleotides contain *Nde*I and *Eco*RI restriction endonuclease sites (underlined), and primer sequences derived from LeIF sequence are indicated by italics, with the ATG initiator and TAA terminator codons in bold. The PCR products were digested with *Nde*I and *Eco*RI and ligated into the polylinker of pET-17b vector predigested with *Nde*I and *Eco*RI. *E. coli* strain BL21 (DE3) pLysE (Novagen) was used for high level expression.

The recombinant (His-Tag) Ags were purified from the insoluble inclusion body of 500 ml of IPTG-induced batch cultures by affinity chromatography using the one-step QIAexpress Ni-NTA agarose matrix (Qiagen, Chatsworth, CA) in the presence of 8 M urea. Briefly, 20 ml of an overnight saturated culture of BL21 containing the pET construct was added into 500 ml of 2xYT media containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, grown at 37°C with shaking. The bacterial cultures were inducd with 2 mM IPTG at an OD 560 of 0.3 and grown for an additional 3 h (OD = 1.3–1.9). Cells were harvested from 500-ml batch cultures by centrifugation and resuspended in 20 ml of binding buffer (0.1 M sodium phosphate, pH 8; 10 mM Tris-HCl, pH 8) containing 2 mM PMSF and 20 μg/ml leupeptin. *E. coli* was lysed by adding 15 mg of lysozyme and rocking for 30 min at 4°C following sonication (4 × 30 s), then spun at 12 *k* rpm for 30 min to pellet the inclusion bodies.

The inclusion bodies were washed three times in 1% 3-[(3-cholamidoproply)dimethyl ammonio]-1-propanesulfonic acid in 10 mM Tris-HCl (pH 8). This step greatly reduced the level of contaminating LPS. The inclusion body was finally solubilized in 20 ml of binding buffer containing 8 M urea, or 8 M urea was added directly into the soluble supernatant. Recombinant Ags with His-Tag residues were batch bound to Ni-NTA agarose resin (5 ml of resin per 500-ml inductions) by rocking at room temperature for 1 h, and the complex was passed over a column. The flow through was passed twice over the same column, and the column was washed three times with 30 ml each of wash buffer (0.1 M sodium phosphate and 10 mM Tris-HCl, pH 6.3) also containing 8 M urea. Bound protein was eluted with 30 ml of 100 mM imidazole in wash buffer, and 5-ml fractions were collected. Fractions containing the recombinant Ag were pooled, dialyzed against 10 mM Tris-HCl (pH 8) bound one more time to the Ni-NTA matrix, eluted, and dialyzed in 10 mM Tris-HCl (pH 7.8). The yield of recombinant protein varies from 25 to 150 mg/L of induced bacterial culture with >98% purity. Endotoxin levels were typically <10 EU/mg protein (i.e., <1 ng LPS/mg).

Mice

Female BALB/cByJ and BALB/cByJmn-*scid*/J mice were from The Jackson Laboratory (Bar Harbor, ME) and were age matched (4–6 wk) within each experiment.

T cell proliferation, cloning, and cytokine production

Female BALB/c mice (three per group) were immunized with recombinant 8E or LeIF in PBS. Each mouse received 70 μg of the indicated Ag in a final volume of 200 μl administered s.c. and distributed over three sites on the shaved flank. Inguinal, brachial, axillary, and periaortic lymph nodes were removed on day 10. For proliferation, the lymph node cells were cultured for 72 h at a density of 4 × 10^5/well in 96-well flat-bottom plates in the presence of various concentrations of the indicated Ag. The cultures were pulsed with [3H]thymidine for the final 16 h of culture and were harvested onto filters. The incorporation of radioactivity was determined using a Matrix 96 Direct beta counter (Packard Instrument, Meriden, CT). For T cell cloning, the lymph node cells were first cultured for 10 days at 6 × 10^6/well in a 2-ml vol in the presence of 0.5 μg/ml 8E or 10 μg/ml LeIF. The short-term T cell lines were then cloned by limiting dilution in the presence of 8E or LeIF (0.5 or 10 μg/ml, respectively), irradiated BALB/c splenocytes (2.5 × 10^5/well), and IL-2 (10 ng/ml). Three weeks later, the resulting clones were resuspended in a total volume of 300 μl and were transferred into wells containing immobilized anti-CD3 (0.5 μg/well of a 48-well plate). Supernatants were collected 48 h later. The levels of IFN-γ, IL-10, and IL-4 were determined by sandwich ELISA, using Ab pairs and procedures available from PharMingen (San Diego, CA). All determinations of cytokine levels were derived by testing serial dilutions of the supernatants. Standard curves were generated using recombinant mouse cytokines available from Immunex, Seattle, WA (IL-4), or Genzyme (Cambridge, MA) (IL-10 and IFN-γ). The ELISAs for IFN-γ and IL-10 were sensitive to 100 pg/ml of the appropriate cytokine, and the ELISA for IL-4 was sensitive to 20 pg/ml. Hamster anti-CD3 (500A2, gift of Dr. J. P. Allison, University of California, Berkeley, CA) was purified at Immunex. Since IL-10 production always correlated with IL-4 production, but IL-4 production did not always correlate with IL-10 production, the results for the Th profiles are shown in terms of IL-4 vs IFN-γ production only.

SCID splenocyte assays and RT-PCR cytokine analysis

Spleens from C3H/HeJ SCID mice were removed aseptically, and single cell suspension was prepared in complete RPMI following lysis of RBC. One hundred microliters of cells (2 × 10^5 cells) were plated per well in a 96-well flat-bottom microtiter plate. Cultures were stimulated with the indicated concentrations of rLeIF with or without polymyxin B or proteinase K. Pretreatment of LeIF with polymyxin B (10 μg/ml) or proteinase K (0.1 mg/ml) was performed at 37°C for 30 min in a final volume of 200 μl. Following the incubation period, proteinase K was inactivated by the addition of 1 mM PMSF. As control, rLeIF alone was incubated at 37°C for 15 min before assay. Supernatants from the cultures were harvested at 72 h and assayed for IFN-γ. For cytokine inhibition assays, splenocyte cultures were incubated with 10 μg/ml of LeIF in the presence or absence of goat anti-mouse IL-12 and IL-18 neutralizing Abs or the appropriate isotype control (R&D Systems, Minneapolis, MN; catalog AB-419-NA, AF422, AB-108-C, respectively).

For cytokine RT-PCR analysis, 0.5 ml of splenocyte culture of C3H SCID mice (5 × 10^6 cells) was plated in a 24-well microtiter plate and stimulated with rLeIF (10 μg/ml) or LPS (1 μg/ml). Cells were harvested 24 h later and lysed directly by adding 0.5 ml of ultrapure TRIzol (total RNA isolation reagent; Life Technologies, Gaithersburg, MD). RNA was isolated as suggested by the manufacturer and resuspended in 50 μl of RNase-free water. One hundred nanograms of total RNA were used per RT-PCR reaction. β-actin was used as control and for standardization by performing 30 PCR cycles (annealing temperature 60°C). The conditions used for the cytokine PCR were: IFN-γ (55°C annealing and 35 cycles),

[3] Abbreviations used in this paper: β-gal, β-galactosidase; IPTG, isopropyl β-D-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; SLA, soluble *Leishmania* antigen.

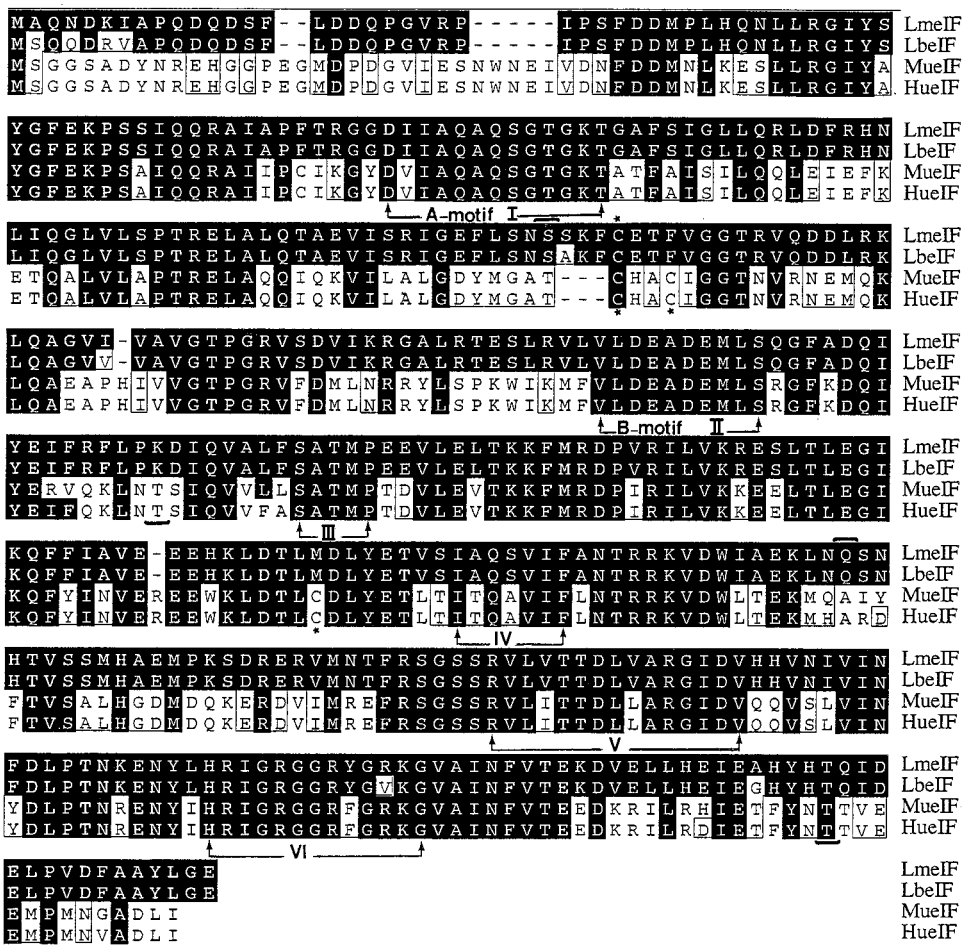

FIGURE 1. Comparison of the predicted amino acid sequences of *L. major* eIF4A (LmeIF), with the homologous proteins from *L. braziliensis* (LbeIF), mouse (MeIF) and human (HeIF). Positions of identical residues to LmeIF are shaded black. Boxed sequences represent identity between the mouse and human proteins that are distinct from the *Leishmania* homologue or conservative substitutions. Regions of similarity, with conserved elements found in RNA helicases, are indicated (I–VI). I and II (DEAD) represent specialized versions of the A and B motifs described in other ATP-binding proteins. Cysteine residues are indicated by *, and potential *N*-linked glycosylation sites are underlined.

IL-12 p35 and p40 (55°C annealing and 35 cycles), and IL-18 (55°C annealing, 40 cycles). The nucleotide sequences for the sense and antisense oligonucleotide primers, respectively, are: β-actin, TGTGATGGTGG GAATGGGTCAG and TTTGATGTCACGCACGATTTCC (product size 514 bp); IFN-γ, TACTGCCACGGCACAGTCATTGAA and GCAGC GACTCCTTTTCCGCTTCCT (product size 405 bp); IL-12p40, ATGGC CATGTGGGAGCTGGAG and TTTGGTGCTTCACACTTCAGG (product size 335 bp); IL-12p35, GCCGGCTATCCAGACAATTA and CTACCAAGGCACAGGGTCAT (product size 463 bp); and IL-18, ACTGTACAACCGCAGTAATACGG and AGTGAACATTACAGATT TATCCC.

Infection studies

L. major (Friedlan strain) promastigotes were cultured at 26°C in M199 (Life Technologies) containing 10% FCS (HyClone, Logan, UT). For in vitro responses, BALB/c mice were infected with 2×10^5 stationary phase *L. major* promastigotes in each hind footpad. At 10 and 28 days postinfection, animals were sacrificed and popliteal lymph nodes were removed. Single cell suspensions were prepared from the nodes, and plated at density of either 2×10^5 cells/well (96-well flat-bottom plates) for analysis of proliferative responses or 2×10^6 cells/well (24-well plate) for cytokine analyses. Cells were pulsed with 1 [mu]Ci of [^{3}H]thymidine after 72 h of culture, and incorporation of radioactivity was determined approximately 16 h later. Levels of cytokines (IFN-γ and IL-4) secreted into the culture supernatants after 72 h of culture were measured by ELISA.

Protection experiment

Female BALB/c mice were preimmunized once with 50 μg of rLeIF (13), followed by s.c. infection with *L. major* (2×10^5 stationary phase; metacyclic stage) 2 days later and subsequent daily boosts with 10 μg LeIF (i.p.) for 3 wk. A control group was immunized with saline. Disease progression was monitored by caliper measurement of footpad swelling. Animals were sacrificed when signs of footpad ulceration in the control group became apparent, at approximately 6 wk postinfection.

Anti-LeIF Ab response

rLeIF was diluted in coating buffer (15 mM Na_2HCO_3/28 mM NaH_2CO_3, pH 9.6) and plated onto Corning Easy Wash ELISA plates (Corning Glass Works, Corning, NY) at 1 μg/well and incubated overnight at 4°C. Plates were then blocked at room temperature with PBS containing 1% BSA for 1 h. BALB/c mice were infected with 2×10^5 stationary phase *L. major* promastigotes in each hind footpad and were used as source of infection sera at 8 wk postinfection. A total of 100 μl of sera diluted in PBS containing 0.1% BSA and 0.1% Tween 20 was added and incubated at room temperature for 30 min. Following removal of unbound Abs (five washes with PBS containing 0.1% Tween 20), bound Abs were detected with goat anti-mouse IgG horseradish peroxidase-conjugated secondary Ab (Southern Biotechnology, Birmingham, AL). Plates were developed using TMB (3,3′,5,5′-tetramethylbenzidine; Kirkegaard & Perry, Gaithersburg, MD) and read at 500 nm.

Results

Cloning, characterization, and expression of the L. major *homologue of eIF4A (LmeIF)*

We previously reported on the cloning and immunologic evaluation of recombinant *L. braziliensis* eIF4A homologue (LbeIF) as an Ag that preferentially stimulates a Th1 cytokine profile in patient PBMC by an IL-12-mediated mechanism (13). Because of the advantages of using *L. major* for murine studies, we decided to use the *L. major* homologue of eIF4A in experiments involving *L. major* infection. Therefore, we cloned the *L. major* homologue (LmeIF) and compared its protein sequence with those of LbeIF as well as with the homologous proteins from mice and humans.

Figure 1 shows a comparison of the predicted protein sequence of LmeIF with the homologous sequences from *L. braziliensis*

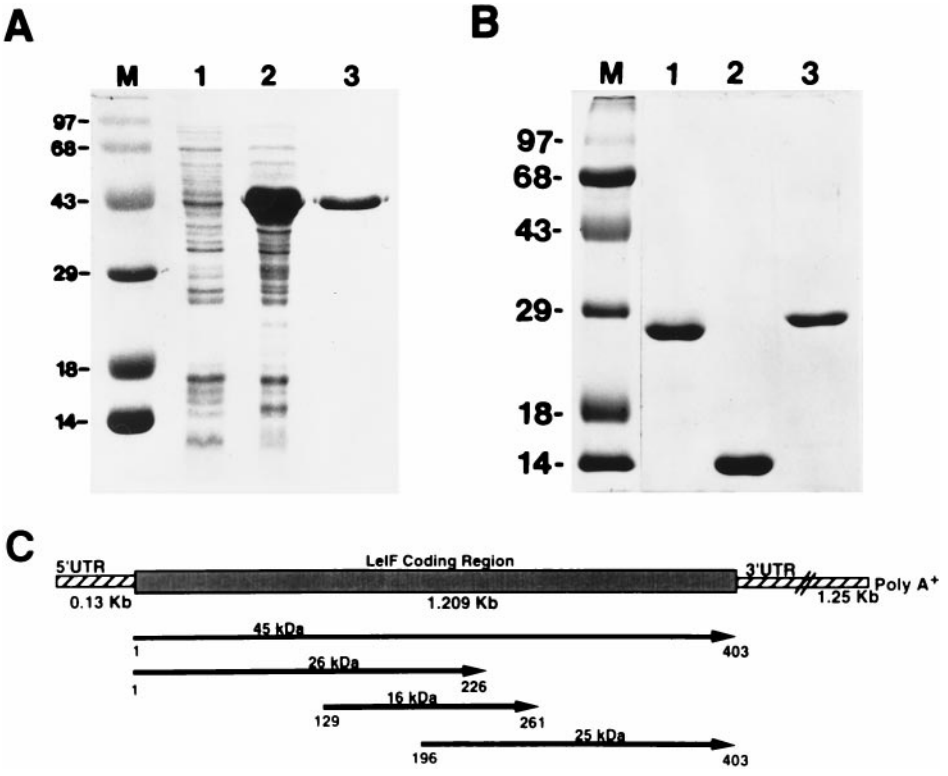

FIGURE 2. Expression and purification of rLmeIF. Coomassie blue-stained 12% SDS-PAGE of *A. E. coli* lysates before (*lane 1*) and after (*lane 2*) induction with IPTG to express rLeIF with six amino-terminal histidine tag residues. rLmeIF was purified from the inclusion body by affinity chromatography on Ni-NTA column (*lane 3*). *B*, Overlapping LeIF deletions. The recombinant clones were designed to encode the N-terminal half (26 kDa, residues 1–226, *lane 1*), the middle portion (16 kDa, residues 129–261, *lane 2*), and the C-terminal half (25 kDa, residues 196–403) of LeIF (*B*), with six His-tag residues and the proteins purified over NiNTA resin. Protein m.w. markers (lane M) are indicated to the *left*. *C*, Schematic representation of the full-length cDNA clone of *L. major* LeIF comprising a 0.13-kb sequence of 5′ untranslated (5′UTR) segment, an open reading frame of 1.209 kb coding for 403-amino acid-long protein, and a 1.25 kb of 3′ UTR terminating with a stretch of poly(A) tail. The arrows below show the location and sizes of both the full-length and overlapping fragments of the LeIF constructs.

(LbeIF, 13) mice (MeIF, 14) and humans (HeIF, 15) showing that LmeIF has the highest sequence homology to *L. braziliensis* eIF protein with 99.8% total homology (98.3% identity, 1.5% conservative substitution). Both LmeIF and LbeIF are of identical length, with only seven amino acid residue substitutions (six being conservative) over their entire lengths (Fig. 1). In contrast, LmeIF shows ~50% identity with the eIF proteins of mice and humans, with the N-terminal half representing the most variable portion between the eIF proteins of *Leishmania* and those of mice and humans. In fact, it was necessary to introduce gaps in the sequences to allow for maximum alignment between the *Leishmania* proteins and the mammalian homologues. Despite these differences, all four proteins have a series of conserved motifs arranged in identical order characteristic of the DEAD box family of RNA helicases (16–19). Two of these conserved sequences represent specialized versions of the A and B motifs previously described in other ATP-binding proteins. The four-amino acid sequence Asp-Glu-Ala-Asp (DEAD) is part of the specialized version of the B motif. Motif I (Gly-Thr-Gly-Lys-Thr) corresponds to the A site of the nucleoside triphosphate (NTP)-binding motif and is found in most nucleotide-binding proteins, including ATPases, kinases, and DNA and RNA helicases. Motif II corresponds to the B site of the NTP-binding motif, which interacts through the invariant D residue with the Mg^{2+} moiety of Mg-ATP (20).

Full-length and overlapping portions of rLmeIF were expressed as a nonfusion protein in *E. coli*, with six histidine residues at the amino terminus for ease of purification. The sizes of the expressed proteins (Fig. 2) correlated well with their predicted m.w. The yield of purified rLmeIF was in the 50–100-mg/L range. The N-terminal sequence of all preparations was confirmed by directly sequencing the purified protein with a Procise 494 sequencer (Perkin-Elmer (Norwalk, CT)/Applied Biosystems Division). Western blot analyses of rLmeIF with a rabbit antisera made against the previously described *L. braziliensis* eIF protein (13) revealed strong specific reactivity (not shown). Hereafter, LmeIF will be referred to generically as LeIF for *Leishmania* eIF protein.

Immune responses to LeIF by L. major*-infected and LeIF-immunized BALB/c mice*

LeIF was originally described as an Ag that elicited an exclusive Th1 response in patient PBMC (13). We therefore examined the cellular immune responses to LeIF in BALB/c mice with *Leishmania* infections. These mice develop a predominant Th2 profile by 7 to 10 days following infection with *L. major*, subsequently resulting in fulminant progressive lesions. Mice were infected with *L. major* in the footpad, and the draining lymph nodes were obtained either 10 or 28 days later. Cells isolated from infected animals responded to rLeIF in a dose-dependent manner by eliciting proliferative responses (Fig. 3*A*). In parallel, culture supernatants of Ag-stimulated lymph node cells were also analyzed for the production of IL-4 and IFN-γ. When cells from infected mice were stimulated in vitro with soluble *Leishmania* Ag (SLA), high levels of IL-4 and very little IFN-γ were detected, as previously described (11). However, the same cultures when stimulated with rLeIF produced high levels of IFN-γ and no IL-4 (Fig. 3*B*). By comparison, rLmSTI1 (a recently described immunogenic *L. major* Ag (12)) yielded a mixed cytokine profile (not shown). To show that the effects of LeIF on lymph node cells isolated from *L. major*-infected animals did not result from non-Ag-specific mechanisms, we performed similar experiments, but using lymphocytes from BALB/c mice infected with a nonrelated pathogen, *Mycobacterium tuberculosis*. We found that while these cells responded to purified protein derivative by proliferating and producing IFN-γ, LeIF did not stimulate a measurable proliferative response or IFN-γ production (not shown).

We also measured the anti-LeIF Ab titers in sera from BALB/c mice at 28 days postinfection (Fig. 3*C*). Only low levels of anti-LeIF Ab were detected. Subclass analysis of the LeIF Ab response showed only measurable levels of IgM, but little, if any, of IgG1, IgG2A, IgG2B, IgG3, or IgE (not shown). In contrast, the same mice develop extremely high LmSTI1-specific titers (>1:200,000)

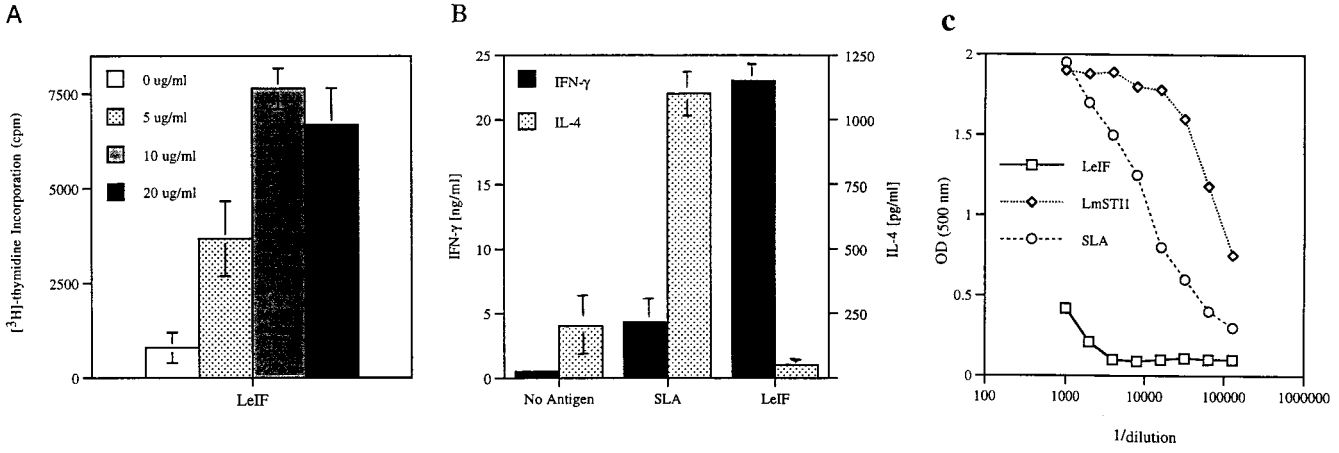

FIGURE 3. Analysis of the in vitro responses of draining lymph node cells from *L. major*-infected BALB/c mice against rLeIF. Draining popliteal lymph node cells (2×10^6/ml) isolated at 10 (*A*) and 28 (*B*) days of infection were restimulated in vitro with varying concentrations of LeIF (proliferative assay (*A*)) or with 10 μg/ml each of rLeIF or SLA (for cytokine assay (*B*) of supernatant levels for IFN-γ and IL-4). *C*, Analysis of anti-LeIF Ab titers in serum of *L. major*-infected BALB/c mice. *L. major* infection sera from BALB/c mice (28 days postinfection) were analyzed and titrated for the presence of anti-rLeIF or rLmSTI1-specific Ab and compared with total promastigote lysate (SLA). Bound Abs were detected with horseradish peroxidase-conjugated goat anti-mouse IgG secondary Ab.

and high Ab titers to SLA, demonstrating that LeIF induces relatively weak B cell responses during *L. major* infection.

Finally, to directly show that LeIF contains T cell epitope(s), BALB/c mice were immunized with rLeIF alone, and lymph node cells harvested 10 days later were restimulated in vitro with varying concentrations of the same Ag. Figure 4 shows that immunized animals responded to rLeIF by strong in vitro proliferation in a dose-dependent fashion (*A*) as well as the secretion of IFN-γ (*B*), but with little or no IL-4 detected. In contrast, control animals showed only marginal proliferative response and IFN-γ production (Fig. 4, *A* and *B*).

rLeIF down-regulates the in vitro production of SLA-induced IL-4 from draining lymph nodes of infected BALB/c mice

We evaluated the ability of rLeIF to down-regulate the production of IL-4 by lymphocytes of *L. major*-infected mice. Mice were infected for 28 days with *L. major*, followed by culturing of lymph node cells with SLA. This resulted in the production of IL-4, but not IFN-γ. The addition of various concentrations of rLeIF and a fixed amount of SLA to the lymph node cell cultures caused a nearly complete abrogation of the SLA-induced IL-4 secretion in a dose-dependent manner (Fig. 5). It was also observed that SLA had no effect on LeIF-induced IFN-γ production.

LeIF favors the development of Th1 clones from BALB/c mice immunized in the absence of adjuvant

Our first report on LeIF also demonstrated that it can stimulate the production of IL-12 from patient and normal human PBMC (13). Because IL-12 is a key cytokine that favors the development of Th1 responses, we tested the ability of LeIF to induce a LeIF-specific Th1 profile in naive BALB/c mice in the absence of added adjuvant. In this set of experiments, the *L. braziliensis* eIF homologue (LbeIF) was used as the immunizing Ag (13). Animals were primed with either rLbeIF or r8E (another recombinant leishmanial

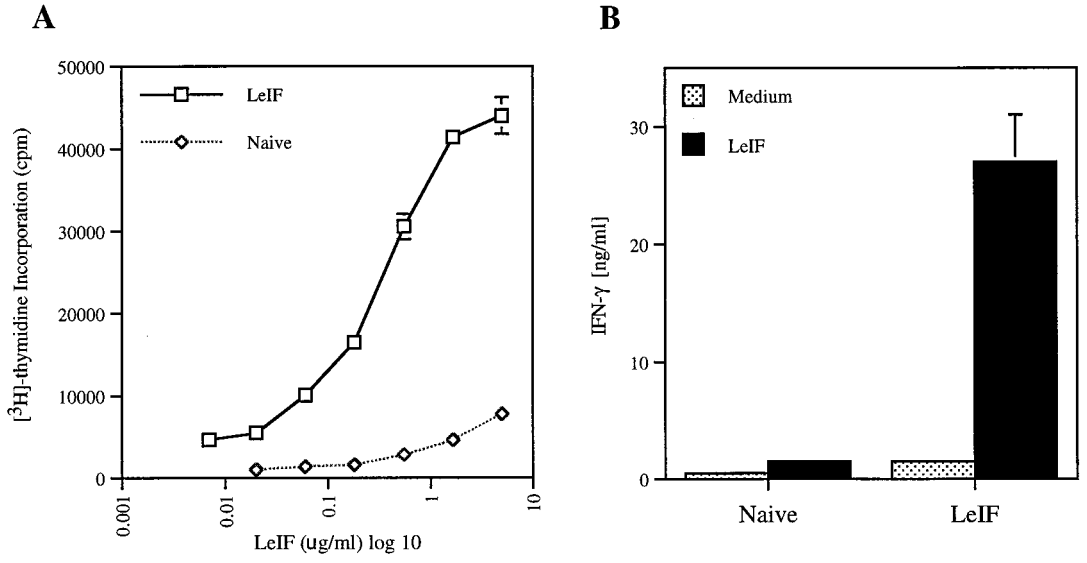

FIGURE 4. Analysis of the in vitro response of draining lymph node cells from LeIF-immunized BALB/c mice. Lymph node cells isolated from LeIF-immunized mice in the absence of adjuvant were tested for their ability to respond to LeIF by in vitro proliferation and cytokine production. *A*, Dose-dependent proliferative response of lymph node cells from LeIF-immunized and control mice using varying concentrations of rLeIF. *B*, Culture supernatants of lymph node cells stimulated in vitro with 2.5 μg/ml LeIF were assayed for the presence of soluble IFN-γ.

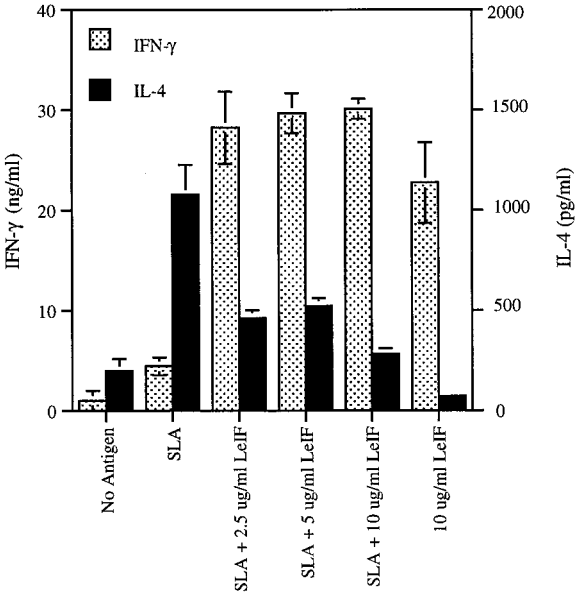

FIGURE 5. Abrogation of the SLA-induced IL-4 secretion by LeIF. Lymph node cells were obtained from BALB/c mice infected with *L. major* (4 wk postinfection) and were stimulated with SLA (10 μg/ml) alone or in the presence of various concentrations of LeIF. Cells were cultured for 3 days, and supernatants were collected and assayed for the production of IL-4 and IFN-γ by ELISA.

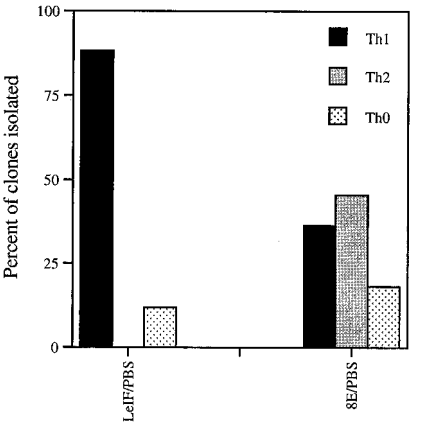

FIGURE 6. Profile of T cell clones isolated from rLeIF- vs r8E-primed BALB/c mice. Mice were immunized s.c. with 70 μg of the respective Ags without adjuvant. Ten days later, their lymph node cells were restimulated in vitro under limiting dilution with the same Ag, irradiated APCs, and IL-2. The resulting clones were restimulated with anti-CD3 mAb, and supernatant cytokine patterns of the Th1 (IFN-γ), Th2 (IL-4), or Th0 (IFN-γ and IL-4) were determined by ELISA. The result is presented as the percentage of clones expressing a Th1, Th2, or Th0 cytokine profile.

Ag that stimulates patient PBMC to produce high levels of IL-10; Y. A. W. Skeiky et al., unpublished observation) in PBS. Both Ags contain the same 4-kDa β-gal N-terminal fusion sequence. Ten days after priming, lymph node cells were restimulated in vitro with the homologous Ags, cloned by limiting dilution, and assayed for IFN-γ and IL-4. It was found that ~90% of the T cell clones isolated from LeIF-primed mice were Th1, producing IFN-γ and no IL-4, while the remaining 10% were Th0, producing both IFN-γ and IL-4 (Fig. 6). In contrast, clones from mice primed with 8E had a mixed cytokine profile.

rLeIF stimulates the production of IFN-γ, but not proliferation in splenocytes from SCID mice

The stimulation of a predominantly Th1 response in naive and *L. major*-infected mice by a protein Ag without the use of added adjuvant is a novel finding and suggests that the ability of LeIF to drive a specific Th1 response may be mediated by an adjuvant activity of LeIF itself. We found that, when added to splenocytes of BALB/c mice, LeIF stimulated the production of IFN-γ (not shown). To examine the activity of LeIF on spleen cells in the absence of T cells, the above experiments were repeated using splenocytes from SCID mice of two different genetic backgrounds, both *Leishmania* resistant (C3H) and *Leishmania* susceptible (BALB/c). Briefly, cells from spleens of 6-wk-old female SCID mice were cultured at 2×10^6 and stimulated with 10 μg/ml with rLeIF or SLA. At 12, 24, and 72 h, supernatants from the induced cultures were harvested, and the levels of IFN-γ were measured. The results revealed that LeIF stimulated the production of high levels (ng range) of IFN-γ by splenocytes of SCID mice of both genetic backgrounds (Fig. 7*A*). In contrast, SLA did not stimulate the production of detectable IFN-γ. The production of IFN-γ by SCID splenocytes following stimulation with rLeIF did not result in NK cell proliferation and was found to depend on the presence of APCs (M. M. Borges et al., manuscript in preparation).

To exclude the possibility that the effects of LeIF may be mediated by endotoxin contamination or bacterial DNA sequences containing CpG motifs (known inducers of IL-12), rLeIF was pretreated with proteinase K or polymyxin B before assay. Treatment with proteinase K abrogated the IFN-γ-inducing activity of LeIF, but polymyxin B had no significant effect on its biologic activity (Fig. 7*B*). Therefore, the effects of LeIF are mediated by properties inherent to the protein. Several other recombinant proteins of *Leishmania* and bacterial origins expressed and purified using protocols similar to those used for rLeIF were found not to stimulate IFN-γ production from SCID splenocytes (not shown).

The amino-terminal half of LeIF induces the production of IFN-γ in splenocytes from SCID mice by IL-12/IL-18-mediated mechanisms

We initiated the mapping of the active region(s) by constructing three overlapping LeIF deletions. The recombinant clones were designed to encode the N-terminal half (amino acid residues 1–226), the middle portion (residues 129–261), and the C-terminal half (residues 196–403) of LeIF (Fig. 2*B*). Purified proteins were subsequently evaluated for their ability to stimulate IFN-γ production in splenocytes from C3H SCID mice. As shown in Figure 8*A*, the N-terminal half of LeIF retained the ability to induce IFN-γ with levels that were generally higher than observed with the full-length molecule, while the middle fragment was only marginally active and the C-terminal half did not stimulate the production of detectable levels of IFN-γ.

To understand the mechanism(s) by which LeIF stimulates the production of IFN-γ, we performed inhibition assays using anti-cytokine Abs. Addition of anti-IL-12 Ab inhibited the production of IFN-γ by splenocyte cultures of C3H SCID mice (Fig. 8*B*). A similar result was obtained when splenocytes from CB17 SCID mice were used (not shown). We also evaluated the possibility that the production of IFN-γ by LeIF-stimulated splenocytes could be mediated by IL-18. As shown in Fig. 8*B*, the addition of neutralizing anti-IL-18 Ab in cultures stimulated with LeIF down-regulated the production of IFN-γ by ~45%. In contrast, the isotype control did not affect the levels of IFN-γ production. These results suggest that, in SCID splenocytes, LeIF mediates its effects by

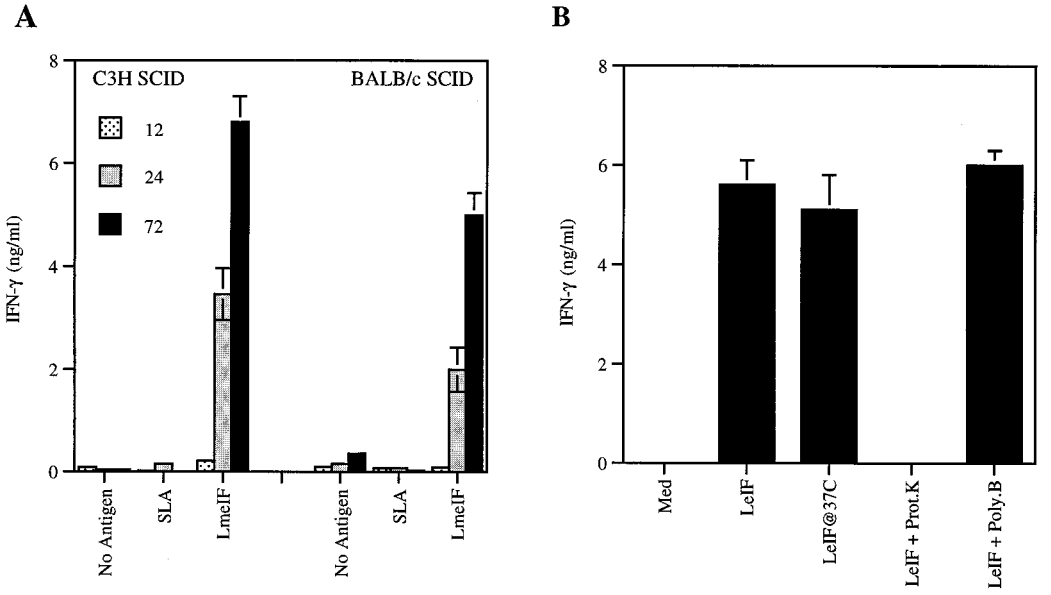

FIGURE 7. LeIF stimulates the production of IFN-γ by splenocytes from naive C3H and BALB/c SCID mice. *A*, Splenocytes from SCID mice of both BALB/c and C3H background were cultured at 2×10^6 and stimulated with 10 μg/ml of the indicated Ag. Supernatants were harvested at 12, 24, and 72 h and assayed for the production of IFN-γ. *B*, 10 μg/ml of the N-terminal fragment of LeIF was assayed on splenocyte cultures directly (LeIF) or following preincubation at 37°C (LeIF@37°C) for 15 min. rLeIF was also pretreated with proteinase K (LeIF + Prot. K) or polymyxin B (LeIF + Poly. B) at 37°C before being assayed on splenocyte cultures.

stimulating the production of both IL-12 and IL-18 in the absence of exogenous IFN-γ.

To complement the effects of adding anti-cytokine Abs on the production of IFN-γ, we directly analyzed the mRNA cytokine profile of LeIF-stimulated splenocyte cultures. Total RNA was isolated at 24-h post-LeIF (10 μg/ml) or LPS (1 μg/ml) treatment, and the expression of IFN-γ, IL-12 p35 and p40, and IL-18 mRNA was evaluated using primer-specific RT-PCR (Fig. 8*C*). The results show that LeIF up-regulated the expression of higher levels of IFN-γ mRNA than did LPS, in agreement with the production of IFN-γ protein (not shown). In addition, both LeIF and LPS stimulated the expression of high levels of IL-12 p35 mRNA, but had little or no effect on the basal level of IL-12 p40 mRNA. For IL-18 mRNA, we had variable and inconsistent levels of PCR products, which we attributed to low expression (or rapid turnover) and the need to perform 40 cycles before detectable levels could be visualized (not shown).

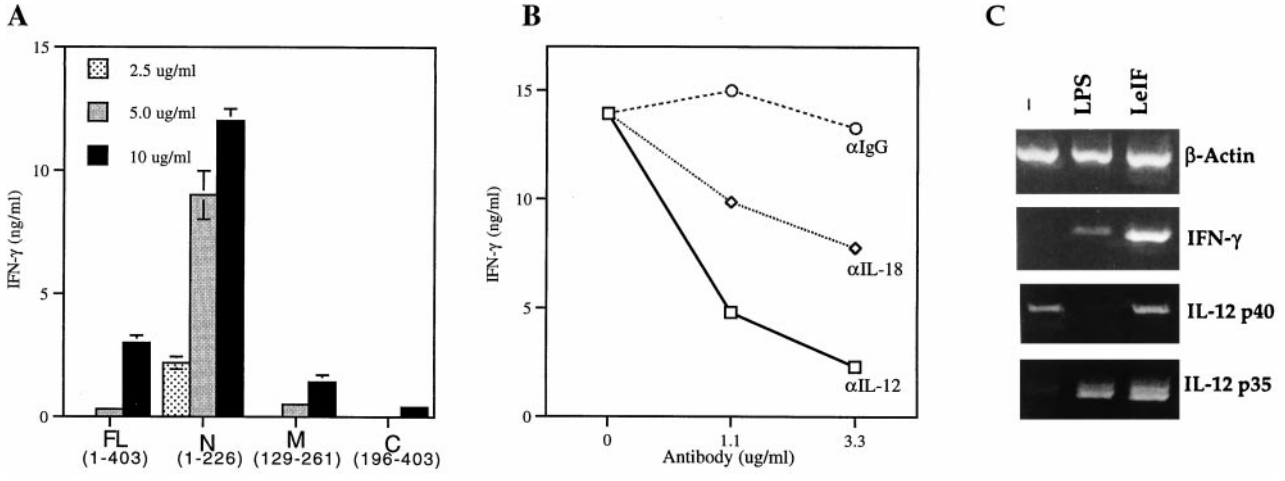

FIGURE 8. *A*, Mapping of the adjuvant epitope of LeIF. The full-length (FL) molecule (amino acid residues 1–403) and three overlapping LeIF recombinants (amino-terminal half, N; middle portion, M; and the C-terminal half, C) comprising amino acid residues 1–226, 129–261, and 196–403, respectively, were assayed at 2.5, 5, and 10 μg/ml in splenocyte cultures from C3H SCID mice. *B*, The production of IFN-γ by LeIF-stimulated SCID splenocyte is mediated by IL-12 and IL-18. Supernatant cultures of C3H SCID splenocytes stimulated with 10 μg/ml LeIF in the presence of 1.1 and 3.3 μg/ml of the neutralizing Abs (anti-IL-12 and anti-IL-18) or isotype control (IgG) were harvested at 24 h and assayed for the production of IFN-γ. *C*, Cytokine RT-PCR analysis of splenocyte culture of C3H SCID mice following stimulation with rLeIF (10 μg/ml) or LPS (1 μg/ml). Cells were harvested 24 h later, and 100 ng of total RNA was used per RT-PCR reaction using sequence-specific oligonucleotide primers flanking intron sequences. β-actin was used as internal standard for the evaluation of the mRNA expression levels of IFN-γ and IL-12 p35 and p40.

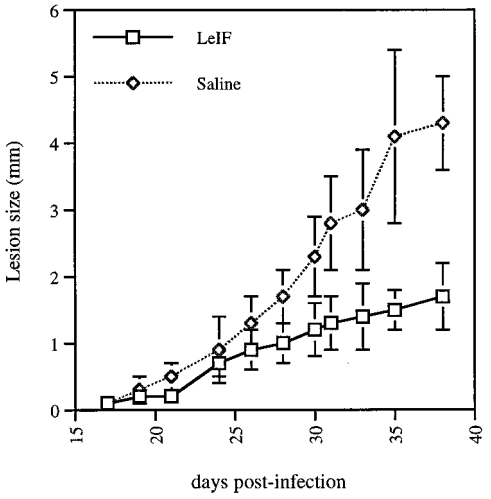

FIGURE 9. Protection of BALB/c mice against *L. major* infection with rLeIF. BALB/c mice were immunized i.p. with 50 μg of rLeIF, followed by s.c. infection with *L. major* 2 days later and subsequent daily boosts with 10 μg LeIF (i.p.) for 3 wk. A control group was immunized similarly, except that saline was used. Similar results were obtained in two experiments.

Protective efficacy of LeIF in L. major*-infected BALB/c mice*

The ability of LeIF to skew a T cell response toward a Th1 profile by an IL-12-mediated mechanism suggested that priming with LeIF may provide protection in *L. major*-infected BALB/c mice. We therefore performed experiments similar to those previously reported by Heinzel et al. using IL-12 (3). BALB/c mice were immunized i.p. with 50 μg of LeIF, followed by s.c. infection with *L. major* 2 days later, and subsequently boosted with 10 μg LeIF (i.p.) daily for 3 wk. The results show that LeIF-immunized mice had significant reduction in lesion size as compared with saline-treated controls (Fig. 9). Animals were sacrificed when signs of footpad ulceration in the control group became apparent, at approximately 6 wk postinfection. These data demonstrated that LeIF had potent immunoregulatory activity in vivo, which resulted in a therapeutic effect during *L. major* infection.

Discussion

We have characterized the *L. major* ribosomal initiation factor 4A (eIF4A) (LeIF) and showed that LeIF is a protein that preferentially stimulates a Th1-type cell-mediated immune response. Infection of BALB/c mice with *L. major* is commonly used as a model system for cell-mediated immune regulation. These mice are widely accepted as developing a predominant Th2 profile by 7 to 10 days following infection, thereby resulting in disease progression. However, in the present study, we showed that even at 28 days postinfection (by which time a clear Th2 pattern was established in terms of disease progression), draining lymph nodes from *L. major*-infected BALB/c mice contain LeIF-specific T cells that are exclusively IFN-γ producers. These results established the ability of rLeIF to stimulate IFN-γ-producing cells that are present in infected BALB/c mice. Thus, in contrast to the widely accepted belief, infected BALB/c mice have Th1 T cell responses that can be detected following stimulation with defined recombinant Ags, although the response to lysate is Th2. Specifically, we have shown that LeIF is a selective activator of Th1 cytokines, a property not shared with any other *Leishmania* Ag described to date. The predominant Th2 profile elicited by SLA, which contains small amounts of LeIF, suggests that SLA has other components that stimulate the production of cytokines that down-regulate IFN-γ production or that LeIF is present in insufficient amounts.

IFN-γ is the cytokine most associated with resistance to leishmanial infection, whereas IL-4 production accompanies disease progression. IL-12 is a strong stimulator of IFN-γ by T and NK cells and is a key cytokine that favors the Th1 cell differentiation. This property of IL-12 is directly correlated with host resistance against intracellular pathogens. For example, IL-12 has been implicated in the resolution of leishmaniasis through mechanisms that initiate a Th1 response and protective immunity (4, 8–11). Our present study demonstrates that LeIF may mimic the effects of IL-12 by down-regulating IL-4 secretion in lymph node cell cultures of BALB/c mice infected with *L. major*. In support of our finding that LeIF can inhibit IL-4 production after Th2 commitment, a recent study also demonstrated that, in contrast to previously held beliefs, IFN-γ can indeed abrogate the antagonistic effect of IL-4 by permitting the conversion of Th2 populations to IFN-γ producers by IL-12 (21). This result is particularly important in predicting the usefulness of LeIF as an immunotherapeutic and is consistent with our previous report that LeIF can stimulate the production of IL-12 (13). In addition, LeIF-specific T cell clones derived from BALB/c mice immunized with rLeIF in the absence of adjuvant were predominantly (~90%) of the Th1 type. The latter observation can be most likely attributed to the superimposed activity of LeIF to act on APC and to stimulate the production of Th1-modulating cytokines (e.g., IL-12).

Using splenocytes from naive SCID mice, we also showed that LeIF stimulated the expression of IL-12 mRNA in the absence of exogenous IFN-γ. In addition, the production of IFN-γ by LeIF-stimulated SCID splenocytes was mediated by IL-12/IL-18-dependent mechanisms. RT-PCR cytokine analysis of LeIF-stimulated splenocyte cultures revealed that LeIF up-regulated the expression of high levels of IL-12 p35 mRNA, but had little, if any, effect on the basal level of p40. Since the addition of LeIF to splenocyte cultures resulted in high levels of IFN-γ production, the up-regulation of IL-12 p35 mRNA is most likely due to the transcriptional activation of the p35 promoter by IFN-γ. In support of our data, Ma et al. had shown previously that IFN-γ can up-regulate the expression of p35 (22). C3H/HeJ SCID mice are hyporesponsive to LPS, and splenocytes from these mice do not produce IFN-γ in response to 100 pg/ml LPS (not shown). Our preparations of rLeIF contain $<$5 ng/mg of endotoxin; therefore, when assayed at the highest concentration of 10 μg/ml (200 μl final volume), the amount of endotoxin (10 pg/ml) is insufficient to stimulate splenocytes of C3H SCID mice to produce IFN-γ. Finally, the production of IFN-γ by LeIF was sensitive to proteinase K, but was not inhibited by polymyxin B. The same amount of polymyxin B inhibited the IFN-γ-induced production by high concentrations (1 μg/ml) of LPS (not shown).

The ability of LeIF to stimulate splenocytes from SCID mice to produce IFN-γ in the absence of a proliferative response is a novel finding described to date for a polypeptide and suggests that in this T cell-independent pathway, LeIF acts by stimulating IL-12 and IL-18 production that would subsequently stimulate NK cells to produce IFN-γ. Since LeIF requires the presence of IFN-γ to stimulate the production of IL-12 by purified APCs (23), it may be that the production of IL-18 occurs at an earlier time (before IL-12) and acts on NK cells to produce IFN-γ. This initial source of IFN-γ could then act with LeIF to activate p35 gene transcription and boost the formation of biologically active IL-12 p70 heterodimer with the basal levels of the p40 chain in the system. IL-12 and IL-18 would then act alone or synergistically on NK cells to trigger the production of higher levels of IFN-γ and IL-12 p35 expression.

It therefore appears that LeIF may adjuvant its own T cell response, and this may explain the reasons for a predominant bias toward the generation of LeIF-specific Th1 clones.

The ability of LeIF to skew a T cell response toward a Th1 profile by an IL-12-mediated mechanism suggested that immunization with LeIF alone may provide protection against *L. major* in BALB/c mice. In particular, we were interested in finding out whether LeIF can mimic the protective effect seen with rIL-12 following infection of BALB/c mice with *L. major* (3). We used a slightly modified protocol by immunizing (i.p. injection) 2 days before infection, followed by daily LeIF treatment. We found that indeed LeIF-immunized mice were partially protected.

In addition to showing that LeIF does contain immunomodulatory properties of a Th1-type adjuvant, we also showed that LeIF contains T cell epitope(s). LeIF-specific proliferative responses and IFN-γ production were readily detected following stimulation of lymph node cells derived from rLeIF-immunized mice, but not from control animals. In earlier studies, LeIF was also shown to stimulate PBMC from *Leishmania*-infected individuals to proliferate and secrete IFN-γ (13).

The IL-12/IFN-γ-inducing region of LeIF was localized to the N-terminal half (amino acid residues 1–226) of the molecule. As shown in Fig. 1, the N-terminal half represents the most variable portion between the eIF proteins of *Leishmania* and those of mice and humans. In fact, it was necessary to introduce gaps in the sequences to allow for maximum alignment between LeIF and the mammalian homologue. In addition, LeIF has a single cysteine residue, whereas the homologous proteins from mice and humans each contain three cysteines, suggesting that the latter have different folding properties. It therefore appears that LeIF interacts with surface molecules on professional APCs following IFN-γ activation to elicit its biologic effects.

It is perhaps not surprising that such potent IL-12-inducing molecules would be found in an obligate macrophage parasite that, in the vast majority of cases, induces nonpathologic infections. In natural infections, *Leishmania* is very effective at inducing protective immune responses. This may be to the advantage of the parasite, i.e., nonfatal chronic infections that can be the source of new transmission. Our results demonstrate that *Leishmania*, and perhaps other macrophage pathogens, may be a source of new adjuvant proteins and suggest the potential of LeIF as a component of an immunotherapeutic approach for leishmaniasis.

Acknowledgments

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