# A Recombinant *Leishmania* Antigen that Stimulates Human Peripheral Blood Mononuclear Cells to Express a Th1-Type Cytokine Profile and to Produce Interleukin 12

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## Summary

*Leishmania braziliensis* causes cutaneous and mucosal leishmaniasis in humans. Most patients with cutaneous leishmaniasis heal spontaneously and may therefore have developed protective immunity. There appears to be a mixed cytokine profile associated with active cutaneous or mucosal disease, and a dominant T helper (Th)1-type response associated with healing. Leishmanial antigens that elicit these potent proliferative and cytokine responses from peripheral blood mononuclear cells (PBMC) are now being identified. Herein, we report on the cloning and expression of a *L. braziliensis* gene homologous to the eukaryotic ribosomal protein eIF4A (LeIF) and patient PBMC responses to rLeIF. Patients with mucosal and self-healing cutaneous disease had significantly higher proliferative responses than those with cutaneous lesions. Whereas the parasite lysate stimulated patient PBMC to produce a mixed Th1/Th2-type cytokine profile, LeIF stimulated the production of interferon $\gamma$ (IFN-$\gamma$), interleukin 2 (IL-2), and tumor necrosis factor $\alpha$ but not IL-4 or IL-10. Recombinant LeIF (rLeIF) downregulated both IL-10 mRNA in the "resting" PBMC of leishmaniasis patients and LPS-induced IL-10 production by patient PBMC. rLeIF also stimulated the production of IL-12 in cultured PBMC from both patients and uninfected individuals. The production of IFN-$\gamma$ by patient PBMC stimulated with either rLeIF or parasite lysate was IL-12 dependent, whereas anti-IFN-$\gamma$ monoclonal antibody only partially blocked the LeIF-induced production of IL-12. In vitro production of both IFN-$\gamma$ and IL-12 was abrogated by exogenous human recombinant IL-10. Therefore, we have identified a recombinant leishmanial antigen that elicits IL-12 production and Th1-type responses in patients as well as IL-12 production in normal human PBMC.

*L*eishmania are obligate intracellular protozoan parasites of macrophages that cause a spectrum of human diseases, including self-healing skin lesions, diffuse cutaneous and mucosal manifestations, or severe visceral disease (1). *Leishmania braziliensis* commonly causes localized cutaneous leishmaniasis (CL)[1]. Most patients with CL heal spontaneously (2). However, a chronic mucosal leishmaniasis (ML) develops in some infected individuals, with severe and progressive destruction of the nasal, oral, and/or pharyngeal mucous membranes (3, 4). Patients with CL generally have positive delayed hypersensitivity and in vitro proliferative responses to leish-

manial Ag during both active and cured infection. These responses are often exaggerated in ML patients (5, 6).

The induction of Th1 effector cells capable of activating macrophages to a microbicidal state is necessary for eliminating these intramacrophage parasites. In experimental leishmaniasis, immunological interventions to direct CD4+ T cell response must be administered before the establishment of Th1 or Th2 effector subsets. IL-12, which facilitates the development of Th1 responses by stimulating the production of IFN-$\gamma$ while downregulating the production of IL-4, has been demonstrated to cure susceptible BALB/c mice against *L. major* infection (7, 8), whereas neutralization of IL-12 made resistant mice susceptible to infection (8). Although neutralization of IFN-$\gamma$ abrogates the natural resistance of C3H/HeN mice to infection with *L. major* (9), by itself, IFN-$\gamma$ did not confer protection to susceptible mice (10).

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[1] *Abbreviations used in this paper:* CL, cutaneous leishmaniasis; LeIF, *L. braziliensis* homologue of the eukaryotic initiation factor 4A; ML, mucosal leishmaniasis; rAg, recombinant antigen; rLeIF, recombinant LeIF.

The determination of cytokine profiles in PBMC from leishmaniasis patients with different clinical presentations as well as the identification of defined Ag that induce and elicit cell-mediated immune responses is important to our understanding of the nature of immune responsiveness during infection, as well as the potential identification of vaccine molecules. The elucidation of cytokines associated with Th1 and/or Th2 responses by defined *L. braziliensis* Ag in patients with leishmanial infections has not been previously described. Because the course of human infection with *L. braziliensis* is variable, ranging from self-healing infection to chronic disease, it is an excellent system in which to study immunoregulatory aspects of leishmaniasis, including the effects of parasite Ag on host responses. In the present study, *Leishmania*-specific PBMC responses in patients representing a spectrum of *L. braziliensis* infection were analyzed. A leishmanial Ag, *L. braziliensis* homologue of the eukaryotic initiation factor 4A (LeIF), capable of stimulating strong Th1-type responses in leishmaniasis patients' PBMC as well as IL-12 production in patient and nonpatient cells, was identified.

## Materials and Methods

*Parasites.* L. braziliensis (MHOM/BR/75/M2903), L. guyanensis (MHOM/BR/75/M4147), L. amazonensis (IFLA/BR/67/PH8), L. chagasi (MHOM/BR/82/BA-2, C1 and MHOM/BR/84/Jonas), L. donovani (MHOM/Et/67/HU3), L. infantum (IPT-1), L. major (LTM p-2), L. tropica (1063C), Trypanosoma cruzi (MHOM/CH/00/Tulahuen C2), and T. brucei (TREU 667) were used (11). Promastigotes of *Leishmania* and epimastigotes of *T. cruzi* were cultured in axenic media. *L. chagasi* and *L. amazonensis* amastigotes were obtained from spleens of Syrian hamster and footpads of BALB/c ByJ mice, respectively.

*Library Construction and Isolation of Genomic Clones.* A genomic expression library with sheared DNA from *L. braziliensis* (MHOM/BR/75/M2903) was constructed in bacteriophage ZAPII (Stratagene, La Jolla, CA). The expression library was screened with *Escherichia coli* adsorbed sera from patient with ML due to infection with *L. braziliensis*. A plaque containing an immunoreactive recombinant antigen (rAg) (LeIF.1) was purified, the pBSK(−) phagemid excised, nested deletions generated using Exonuclease III for single-stranded template preparations and sequencing (12). Clone pLeIF.1 lacked the first 48 amino acid residues (144 nucleotides) of the full-length sequence. The insert was subsequently used to isolate the full-length genomic sequence.

*Expression and Purification of Recombinant LeIF (rLeIF).* The 45-kD rAg of the LeIF.1 genomic clone was purified from 500 ml of isopropyl β D-thiogalactoside (IPTG)-induced cultures (12). Inclusion bodies were isolated and sequentially washed in TNE (50 mM Tris, pH 8.0, 100 mM NaCl, and 10 mM EDTA) containing 2, 4, and 8 M urea. Fractions containing solubilized rAg (usually the 8 M cut) were pooled, dialyzed against Tris-buffered saline (TBS), and concentrated by precipitation with 30% ammonium sulfate. Purification to homogeneity was accomplished by preparative SDS-PAGE electrophoresis, followed by excision and electroelution of the rAg as described. All Ag used in our studies had negligible (<10 pg/ml) endotoxin in a Limulus amebocyte assay.

*Production of Rabbit Antiserum against rLeIF.* An adult rabbit (New Zealand White; R & R Rabbitry, Stanwood, WA) was immunized with 200 µg s.c. of purified rLeIF in IFA (GIBCO BRL, Gaithersburg, MD) with 100 µg of muramyl dipeptide (adjuvant peptide; Calbiochem-Novabiochem Corp., La Jolla, CA), followed

by a s.c. boost 4 wk later with 100 µg of the rAg in IFA alone. After 3 wk, the rabbit was boosted with 25 µg i.v. of rLeIF in saline, and serum collected 1 wk later.

*Cell Lysate.* Parasite and mammalian cell lysates were prepared by freeze-thaw lysis of pellets in SDS sample buffer without glycerol or β-ME. Insoluble material was separated from the supernatant by centrifugation at 10,000 rpm. Protein concentrations were determined using a bicinchoninic acid protein assay kit (BCA; Pierce Chemical Co., Rockford, IL).

*Immunoblot Analysis.* 5–10 µg of parasite or cell extracts or 0.5–1.0 µg of rAg were separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Reactivities of the antisera were assessed as previously described (12) using $^{125}$I-protein A, followed by autoradiography.

*Patients.* Peripheral blood was obtained from individuals living in an area endemic for *L. braziliensis* (Corte de Pedra, Bahia, Brazil) in which epidemiological, clinical, and immunological studies of leishmaniasis have been performed for over a decade (3). Diagnosis was made by clinical findings associated with at least one of the following: parasite isolation, leishmanial skin test, or positive serology. An epidemic in the field area without access to glucantime enabled us to detect patients who had healed spontaneously (self-healing).

*PBMC Culture, Proliferation, and Cytokine Assays.* Peripheral blood from *Leishmania* infected or uninfected individuals were collected and PBMC isolated from whole blood by density centrifugation through Ficoll (Pharmacia Biotech Inc., Piscataway, NJ). For in vitro proliferation assays, 2–4 × 10$^5$ cells/well were cultured in complete medium (RPMI 1640 supplemented with gentamycin, 2-ME, L-glutamine, and 10% screened pooled A+ human serum; Trimar, Hollywood, CA) in 96-well flat bottom plates with or without 10 µg/ml of the indicated Ag or 5 µg/ml PHA (Sigma Immunochemicals, St. Louis, MO) for 5 d. The cells were then pulsed with 1 µCi of [$^3$H]thymidine for the final 18 h of culture. Data are represented as mean cpm of triplicate cultures and the stimulation index (SI) defined as mean cpm of cultures with Ag/mean cpm of cultures without Ag. For determination of cytokine production, PBMC were cultured at 1–2 × 10$^6$ cells/ml in medium containing endotoxin-free FCS (Cellect Gold; ICN Biomedicals, Inc., Costa Mesa, CA) with or without leishmanial Ag and in the presence or absence of human (Hu) rIL-10 (10 ng/ml, DNAX, Palo Alto, CA), anti–IFN-γ (10 µg/ml; Chemicon, Temucula, CA), or anti–IL-12 (Wistar Institute). Supernatants and cells were harvested and analyzed for secreted cytokine or cytokine mRNAs after 24 (for IL-12) or 72 (for IL-10 and IFN-γ) h of culture as described below.

*Cytokine ELISA.* Supernatants were assayed for IFN-γ, TNF-α, IL-4, and IL-10. IFN-γ was quantitated by a double sandwich ELISA using mouse anti–Hu IFN-γ mAb (Chemicon) and polyclonal rabbit anti–human IFN-γ serum. Hu rIFN-γ (Genentech Inc., San Francisco, CA) was used to generate a standard curve. IL-4 was quantitated in supernatants by a double sandwich ELISA using a mouse anti–human IL-4 mAb (M1) and a polyclonal rabbit anti–human IL-4 sera (P3). Hu rIL-4 (Immunex Corp., Seattle, WA) was used to generate a standard curve ranging from 50 pg/ml to 1 ng/ml. IL-10 was measured using a rat anti–human IL-10 mAb (Cat.# 18551D; PharMingen,) to capture secreted IL-10 and a biotinylated rat anti–human IL-10 mAb (Cat.# 18562D; PharMingen) for detection of bound IL-10 with streptavidin-conjugated horse radish peroxidase and ABTS as substrate. A standard curve was obtained using Hu rIL-10 (kindly provided by DNAX Research Institute, Palo Alto, CA), ranging from 30 pg to 2 ng/ml. IL-12 p40 was measured in cell-free supernatant by RIA (detection limit of 10

pg/ml) using the mAb pairs C11.79/C8.6 as described (13). Biologically active IL-12 p70 heterodimer (detection limit 1 pg/ml) was measured as described (14).

*Cytokine PCR.* For cytokine analysis, 0.5–1 ml of PBMC (1–2 × $10^6$ cells/ml) were cultured with or without Ag for 48 and 72 h. Supernatants and cells were harvested and analyzed for cytokine mRNAs or secreted cytokines. For cytokine mRNA analysis, total RNA was isolated from PBMC and cDNA synthesized using poly(dT) (Pharmacia, Piscataway, NJ) and AMV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in a final volume of 20 μl. cDNA samples were brought to 200 μl with water. After normalization to β-actin, 12–20 μl of diluted cDNA was amplified by PCR using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) with 0.2 μM of the respective 5' and 3' external primers in a reaction volume of 50 μl. The conditions used were: denaturation at 94°C (1 min for β-actin, IL-2, and IL-4; 45 s for IFN-γ; and 30 s for IL-10), annealing at 55°C (1 min for β-actin, IL-2, and IL-4; 30 s for IL-10) or 60°C for 45 s for IFN-γ and elongation at 72°C. We verified that our PCR conditions were within the semi-quantitative range by initially performing serial dilutions of cDNA and varying the number of cycles used for PCR. In all subsequent experiments, 30 cycles were used in the amplification reactions for β-actin, IL-2, IL-4, and IFN-γ. In the case of IL-10 PCR, 25 cycles were used. The primer pairs used were derived from published information: β-actin, IL-2, IL-4, and IFN-γ (15) and IL-10 (16). The nucleotide sequences for the 5' and 3' oligonucleotide primers, respectively, are as follows: β-actin, TGACGGGGTCACC-CACACTGTGCCCATCTA and CTAGAAGCATTGCGGTGG-ACGATGGAGGG; IL-2, ATGTACAGGATGCAACTCCTGTCTT and GTCAGTGTTGAGATGATGCTTTGAC; IL-4, ATGGGTCT-CACCTCCCAACTGCT and CGAACACTTTGAATATTTCTC-TCTCAT; IFN-γ, ATGAAATATACAAGTTATATCTTGGCTTT and GATGCTCTTCGACCTCGAAACAGCAT; IL-10, TCTCAA-GGGGCTGGGTCAGCTATCCCA and ATGCCCCAAGCTGAG-AACCAAGACCCA.

Inserts were isolated from plasmids containing the human sequences for IL-2, IFN-γ, IL-4, IL-10, and β-actin and radiolabeled $^{32}$P-probes generated as described (17). PCR products were analyzed by electrophoresis on 1.5% agarose gels, transferred to nylon membranes, and probed with the appropriate $^{32}$P-labeled DNA insert. Hybridizations were at 55°C overnight. Posthybridization washes were at 55°C for 20 min twice each with 2× and 1× SSC containing 0.2% SSC.

*Nucleotide Sequence Accession Number.* The nucleotide sequence of pLeIF has been submitted to the EMBL/GenBank/DDBJ/Data Bank under accession number U19888.

## Results

*Molecular Cloning and Sequence Characterization of L. braziliensis Ribosomal eIF4A.* As part of a strategy for identifying leishmanial Ag, we screened a L. braziliensis genomic expression library with sera from a ML patient, purified immunoreactive rAg, and analyzed them in patient T cell assays for their ability to stimulate proliferative responses and preferential Th1 cytokine production. This led to the identification of several cDNAs. One of these was identified by GenBank sequence comparison as the L. braziliensis homologue of the eukaryotic initiation factor 4A (LeIF).

The open reading frame of the full-length LeIF encodes a protein of 403 amino acids and a predicted molecular mass of 45.3 kD (not shown). The lengths (403, 413, 407, and 395

amino acids), molecular mass (45.3, 46.8, 46.4, and 44.7 kD), and isoelectric points (5.9, 5.4, 5.5, and 4.9) of LeIF, and the homologous proteins from tobacco, mouse, and yeast respectively (18–20) are similar. LeIF contains sequence elements characteristic of several demonstrated or putative ATP-dependent RNA helicases represented by the eukaryotic initiation factor 4A (eIF4A). The eIF4A is one of the best characterized members of the "DEAD box" family of RNA helicases. These proteins share a series of conserved motifs, with two of them representing specialized versions of the A and B motifs previously described in other ATP-binding proteins (21). The four amino acid sequence Asp-Glu-Ala-Asp (DEAD) is part of the specialized version of the B motif.

Southern analysis of L. braziliensis genomic DNA digested with enzymes that cut both within (PstI and NotI) and outside (BamHI, EcoRI, EcoRV, HindIII, PvuII, and SstI) of LeIF and probed with an ∼0.94-kb fragment (nucleotides 143 to 1083) of the coding region of LeIF revealed at least two copies of LeIF (Fig. 1). The same figure also illustrates the cross-species conservation between LeIF of L. braziliensis and other Leishmania species. Northern analysis revealed that LeIF is transcribed as ∼2.5 kb mRNA species (not shown).

*Expression, Purification, and Immunoblot Analysis of LeIF Ag.* Fig. 2 shows the expression and purification of the ∼45 kD LeIF gene product. Immunoblots of lysates from L. braziliensis as well as other Leishmania species (promastigote and amastigote stages) were performed with the polyclonal rabbit anti-LeIF serum which detected one dominant protein species of ∼45 kD. The immune rabbit sera did not react
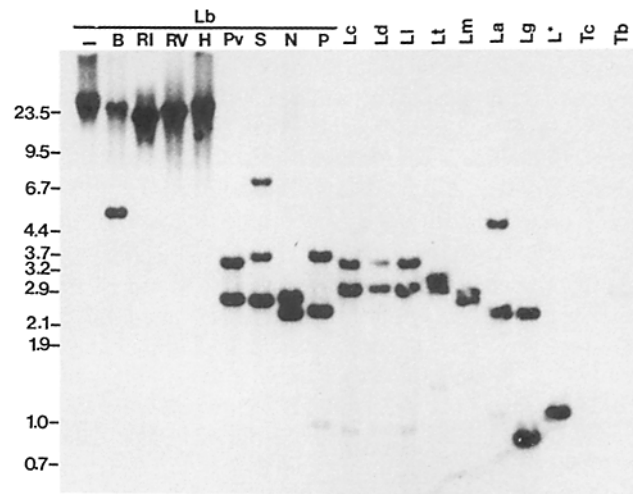


**Figure 1.** Genomic organization of Leishmania eIF4A. L. braziliensis DNA (lanes Lb, 2.5 μg per lane) was digested with BamHI (lane B), EcoRI (lane RI), EcoRV (lane RV), HindIII (lane H), PvuII (lane Pv), SstI (lane S), NotI (lane N), and PstI (lane P). DNA from L. chagasi (lane Lc), L. donovani (lane Ld), L. infantum (lane Li), L. tropica (lane Lt), L. major (lane Lm), L. amazonensis (lane La), L. guyanensis (lane Lg), an uncharacterized protozoan species (lane L*), T. cruzi (lane Tc), and T. brucei (lane Tb) were digested with PstI. The blot was probed with a ∼0.94-kb restriction fragment of LeIF comprising only coding sequences. Numberings indicate the sizes in kb pairs of HindII/HincII–digested λ DNA.
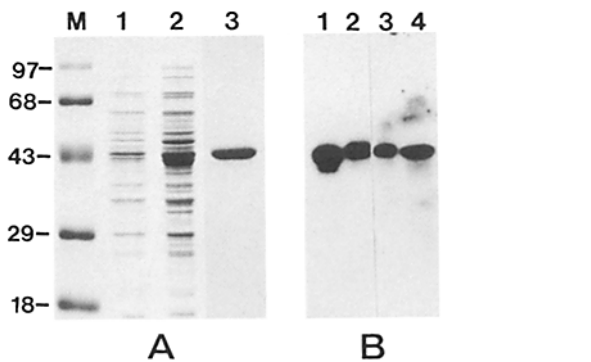
**Figure 2.** Expression, purification, and immunoblot analysis of LeIF Ag. (*A*) Coomassie blue-stained 12% SDS-PAGE of mol wt markers (lanes *M*), *E. coli* lysates from uninduced (lanes *1*) and induced (lane *2*) cultures and the purified rAg (lanes *3*). (*B*) Reactivities of a rabbit anti-rLeIF serum on *L. braziliensis* and *L. chagasi* promastigote lysates (lanes *1* and *2*, respectively), or *L. amazonensis* promastigote and amastigote stages (lanes *3* and *4*, respectively).

with *E. coli* lysate (not shown), demonstrating the lack of *E. coli* proteins in the purified rLeIF.

*rLeIF Stimulates PBMC from Leishmaniasis Patients to Proliferate and to Express a Predominant Th1 Cytokine Profile.* Purified rLeIF was tested for its effectiveness in stimulating T cells from *L. braziliensis*–infected individuals. Proliferation and cytokine responses of PBMC from patients with active or healed CL, ML, or individuals with self-healing CL were compared using rLeIF and *L. braziliensis* lysates. PBMC from most (>70%) ML and CL patients proliferated to rLeIF with stimulation indices ranging from 12 to 233 and 2 to 64, respectively (Table 1). PBMC from all six individuals with self-healing CL proliferated in response to rLeIF with stimulation indices (16–198) comparable to those of ML patients. PBMC from two of the self-healing individuals (MS and DJ), proliferated to LeIF to a greater degree than to lysate. Cells from normal individuals were only marginally stimulated by LeIF. The results demonstrate that rLeIF is a potent T cell Ag recognized by a majority of *L. braziliensis*–infected individuals in different stages or manifestations of infection.

A more detailed analysis of cytokine patterns of PBMC from patients with confirmed cases of *L. braziliensis* infection was performed using PCR. Fig. 3 *A* shows the PCR results of cytokine mRNA for three of the six ML patients' PBMC analyzed (JV, SZ, and TE). Cytokine mRNA analyses were performed with freshly isolated PBMC (lanes *0*), or PBMC cultured in the absence (lanes −) or presence of rLeIF. In three of six patients (TE, Fig. 3 *A*; NO and EO, not shown), PBMC not cultured in vitro had detectable levels of mRNA for IFN-γ and IL-4, and two were positive for IL-2 (patients TE and EO). IL-10 mRNA was not detected in uncultured ("resting") PBMC from any of the ML patients. However, after in vitro culture without Ag, IL-10 mRNA was upregulated in most of the ML patient PBMC analyzed. In addition, the levels of other cytokine mRNAs detected in the resting PBMC of patients TE, NO, and EO, decreased to background levels. In three of six patients, leish-

manial lysate stimulated the expression of mRNA for the Th1 cytokines (IFN-γ and IL-2) as well as for the Th2 cytokine IL-4. Increased IL-10 mRNA was detected in one of the patients' PBMC (SZ, Fig. 3 *A*) after culture with leishmanial lysate. In contrast, rLeIF Ag elicited increased mRNA for IFN-γ and IL-2 from all ML patient PBMC, an exclusive Th1 cytokine profile. In fact, the addition of LeIF downregulated the production of IL-10 mRNA in the cultured PBMC of most ML patients (Fig. 3 *A*, lanes *eIF*).

Similar PCR analyses were performed on PBMC derived from CL patients. The resting PBMC from three (VS, JP, and CA) of the four patients analyzed revealed readily detectable mRNA for both Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) associated cytokines (Fig. 3 *B*). Little or no mRNA for IFN-γ, IL-2, IL-10, or IL-4 was detected in the resting PBMC of the fourth (AS) CL patient. Therefore, in contrast to ML patients, most patients with CL had RNA for IL-10 as well as for IL-4, IL-2, and IFN-γ. For most patients, whereas the mRNA for IL-2 and IFN-γ were reduced to barely detectable levels after the in vitro culturing of PBMC in the absence of Ag, mRNA for IL-10 remained either unaffected or increased.

All CL patients tested responded to rLeIF or to leishmanial lysate by upregulating the expression of mRNA for IL-2 and IFN-γ. However, IL-4 mRNA was also enhanced in one of the CL patients (AS) after stimulation with LeIF. In three of four patients (JP, VS, and AS), the level of IL-4 mRNA also increased after stimulation with parasite lysate. In the three patients (VS, JP, and CA) with resting levels of IL-10 mRNA, rLeIF downregulated their expression of IL-10 mRNA. We also studied PBMC from a diffuse cutaneous leishmaniasis (DCL) patient (*L. amazonensis* infection). Interestingly, as with PBMC from patients with *L. braziliensis* infection, LeIF downregulated IL-10 mRNA while increasing IFN-γ and IL-2 mRNA in the DCL patient VA (Fig. 3 *B*). PBMC from patient VA also proliferated in the presence of LeIF but not in response to parasite lysate (Table 1). In general, the PCR products for IL-2 and IFN-γ of patient PBMC after stimulation with LeIF or parasite lysate were readily detected on ethidium bromide–stained gels, suggesting relative abundance, whereas IL-4 and IL-10 were detected only by radioactive probing of the resolved PCR products.

The cytokine mRNA profiles of PBMC from self-healing CL were similar to those of ML patients in that (*a*), except for one individual (HS) with detectable levels of IL-10 mRNA, resting PBMC from three of the four patients (GS, AH, DJ, and HS) analyzed had detectable levels of IL-2, IFN-γ, and IL-4 but little or no IL-10 mRNA; (*b*) IL-10 mRNA was upregulated after culture of PBMC without Ag whereas those of IL-2, IFN-γ, and IL-4 decreased to background levels and; (*c*), leishmanial lysate stimulated the expression of a mixed Th1/Th2 cytokine profile whereas LeIF elicited increased mRNA expression of only the Th1-type cytokines and downregulated the expression of IL-10 mRNA in the cultured PBMC of most self-healing CL individuals (not shown).

To complement the cytokine mRNA analysis, we assayed PBMC culture supernatants for IFN-γ, TNF-α, IL-4, and IL-10. Cells from all three patient groups as well as from

**Table 1.** *In Vitro Proliferation of PBMC from L. braziliensis–infected Individuals in Response to Parasite Lysate and rLbeIF4A Ag*

| | $[^3H]TdR$ incorporation (mean cpm [SD] × $10^{-3}$) | | | | |
|---|---|---|---|---|---|
| Patients | Media | Lysate | | LeIF | |
| | | | *SI* | | *SI* |
| ML | | | | | |
| JV | 0.15 (0.0) | 41.30 (1.3) | 294 | 11.90 (4.8) | 81 |
| SZ | 0.45 (0.1) | 140.60 (7.6) | 308 | 105.90 (5.6) | 233 |
| AB | 0.42 (0.3) | 44.20 (0.5) | 104 | 5.00 (1.3) | 12 |
| NO | 0.38 (0.1) | 52.70 (3.3) | 138 | 12.80 (1.6) | 33 |
| TE | 0.18 (0.0) | 27.40 (1.5) | 150 | 8.80 (0.3) | 48 |
| MB | 0.18 (0.0) | 300.10 (9.4) | 1634 | 41.50 (4.5) | 226 |
| OM | 0.28 (0.0) | 35.40 (3.2) | 124 | 6.90 (2.5) | 24 |
| CL | | | | | |
| AS | 0.22 (0.0) | 19.14 (1.3) | 87 | 14.30 (2.3) | 64 |
| JP | 0.25 (0.0) | 55.63 (8.6) | 218 | 4.40 (0.3) | 17 |
| VS | 0.17 (0.0) | 0.26 (0.0) | 1.5 | 0.30 (0.0) | 2 |
| RJ | 0.10 (0.0) | 0.32 (0.2) | 3.0 | 1.50 (0.6) | 15 |
| JA | 0.16 (0.0) | 0.77 (0.1) | 4.7 | 2.50 (0.2) | 16 |
| AD | 4.20 (1.0) | 4.01 (1.0) | 2.0 | 14.10 (2.2) | 3.5 |
| HN | 0.36 (0.0) | 4.73 (1.7) | 13 | 4.69 (1.7) | 13 |
| DCL | | | | | |
| VAL | 0.22 (0.0) | 0.51 (0.3) | 2.0 | 2.12 (0.2) | 9.0 |
| SH-CL | | | | | |
| GS | 0.21 (0.0) | 19.70 (4.4) | 94 | 41.50 (2.8) | 198 |
| MS | 0.09 (0.0) | 0.60 (0.1) | 6.5 | 5.10 (2.1) | 57 |
| AH | 0.11 (0.0) | 59.60 (7.1) | 519 | 9.60 (4.7) | 83 |
| DJ | 0.12 (0.0) | 0.20 (0.1) | 1.6 | 19.00 (6.7) | 151 |
| HS | 0.12 (0.0) | 27.10 (2.0) | 225 | 12.40 (2.7) | 103 |
| MCT | 0.38 (0.0) | 130.30 (14) | 340 | 6.20 (1.5) | 16 |
| Normal | | | | | |
| LV | 0.14 (0.0) | 0.19 (0.0) | 1.4 | 0.71 (0.1) | 4.0 |
| VV | 0.18 (0.0) | 0.31 (0.1) | 1.7 | 0.28 (0.1) | 1.5 |
| N3 | 0.14 (0.0) | 0.36 (0.1) | 2.6 | 0.27 (0.1) | 1.9 |
| N4 | 0.59 (0.1) | 2.00 (0.3) | 3.8 | 0.56 (0.0) | 1.0 |

a DCL patient (VA) secreted IFN-γ and TNF-α after stimulation with rLeIF (Fig. 4). The levels of both IFN-γ and TNF-α in the supernatants of patient PBMC (0.3–26 ng/ml and 0.1–3 ng/ml, respectively) were significantly higher compared to those from uninfected controls. In the absence of rLeIF stimulation, only PBMC from ML patients (five of six) produced detectable levels of supernatant TNF-α (60–190 pg/ml). Little or no IL-4 or IL-10 were detected in any of the supernatants analyzed (not shown), indicating levels below the detection limit of the ELISA assay employed. By comparison, leishmanial lysate also stimulated patient PBMC to secrete IFN-γ and TNF-α and, in some patients, IL-10 was also detected (not shown). Therefore, and in agreement with the PCR cytokine analysis, whereas the cytokine profile of patient PBMC stimulated with LeIF reflects an exclusive Th1 pattern, parasite lysate stimulates a mixed Th1/Th2 cytokine profile.

*LeIF Downregulates the LPS-induced IL-10 Production by Patient PBMC.* Taken together, the above results demonstrate that rLeIF elicits a predominant Th1 cytokine profile from the PBMC of ML, CL, or DCL patient while downregulating the expression of IL-10 mRNA. To further demonstrate
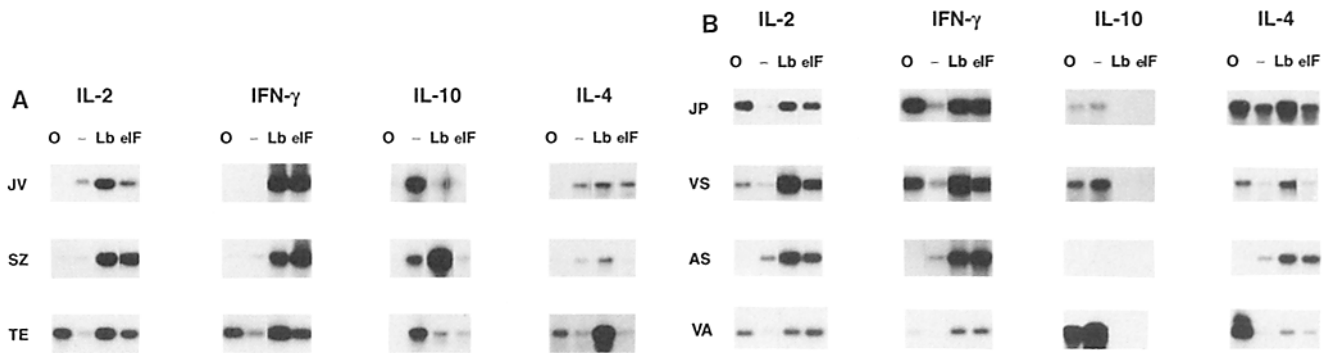
**Figure 3.** PCR amplification of cytokine mRNAs (*IL-2*, *IFN-γ*, *IL-10*, and *IL-4*) isolated from ML (*A*) and CL (*B*) patients' PBMC before and after stimulation with the indicated Ag. The amount of cDNA synthesis reactions used in the cytokine PCR was normalized to the β-actin PCR product (not shown). (Lanes O and −) PCR products at the initiation of culture or after 72 h of culture in the absence of Ag. PCR products after culturing of PBMC with *L. braziliensis* lysate (lanes *Lb*), and rLeIF (lanes *eIF*) Ag. After hybridization with the respective cytokine probes, the autoradiographs were exposed either at room temperature (*IL-2* and *IFN-γ*) for 30 min to 1 h or at −70°C for 6 h to overnight (*IL-10* and *IL-4*).
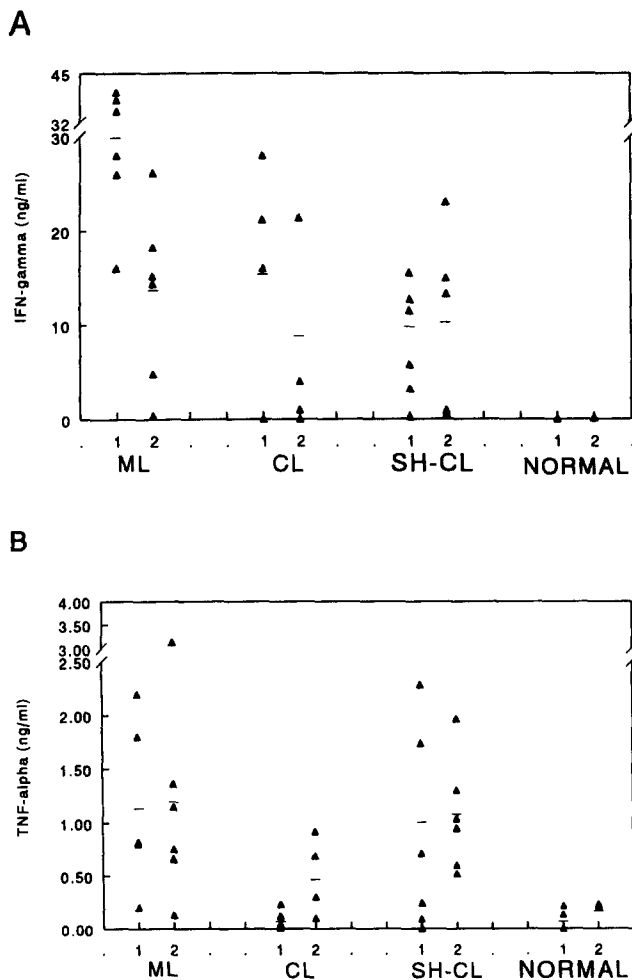


**Figure 4.** Supernatant levels of IFN-γ (*A*) and TNF-α (*B*) determined by ELISA from 72 h cultures of PBMC from *Leishmania* infected (*ML*, *CL*, and self-healing CL [*SH-CL*]) and control (*NORMAL*) individuals in response to stimulation with parasite lysate (*1*) or rLeIF (*2*). Each value represents an individual patient. Mean values are indicated with a dash for each group.

directly the effect of LeIF in downregulating IL-10 production, PBMC from three ML patients were cultured in the presence of LPS with or without LeIF. LPS stimulated PBMC from all three individuals tested to secrete IL-10 with values ranging from 185 to 424 pg/ml (Fig. 5). However, in the presence of LeIF, the production of LPS-induced IL-10 secretion was reduced by 49–65% (83–166 pg/ml). Because IFN-γ can inhibit IL-10 production by activated macrophages (22) and LeIF stimulated patient PBMC to secrete IFN-γ, the secreted IFN-γ may be responsible for partially inhibiting the production of LPS-induced IL-10.

*LeIF Stimulates both Patient and Normal PBMC to Secrete IL-12.* IL-12 has been shown to play a pivotal immunoregulatory role in the development of cell-mediated immunity, including the generation of Th1 responses and IFN-γ production in intracellular bacterial or parasitic infections (23). IL-12 is a heterodimeric molecule comprised of p40 and p35 subunits which must be coexpressed for the production of biologically active IL-12 p70 (24). The p40 subunit is produced only by IL-12–producing cells and is induced in vitro and in vivo after bacterial and parasitic stimulation (13, 25), whereas the p35 subunit is both ubiquitous and constitutively expressed. Therefore, cells producing IL-12 p70 also have a large excess (10–100-fold) of biologically inactive free p40 chains (13). Because LeIF stimulated a dominant Th1 cytokine profile and downregulated Th2 responses, we explored a possible role for IL-12 in PBMC responses to rLeIF. Fig. 6 *A* shows that LeIF stimulated IL-12 p40 production from patient PBMC with values consistently higher than those observed with parasite lysate, and that IL-10 abrogated the production of IL-12 p40 by patient PBMC after stimulation with either LeIF or parasite lysate.

PBMC from uninfected individuals also produced IL-12 p40 when cultured with LeIF (Fig. 6 *B*), although no p40 was detected in response to parasite lysate. This may suggest a role for IFN-γ in the lysate-induced p40 observed in patient PBMC, which produced 5–100-fold more IFN-γ than normal PBMC after antigen stimulation (Fig. 4 *A*).
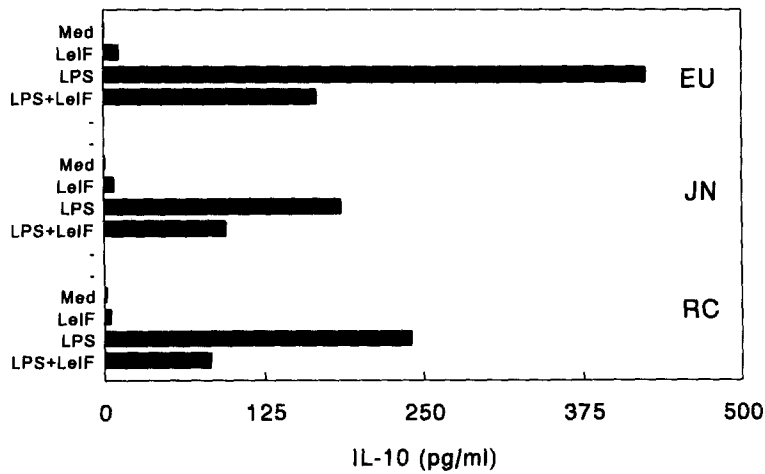
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**Figure 5.** LeIF downregulates the LPS-induced IL-10 production by PBMC. Cells from three ML patients (*EU*, *JN*, and *RC*) were cultured in the absence (*Med*) or presence of LeIF (10 µg/ml), LPS (1 µg/ml) or LPS and LeIF together and the supernatant levels of IL-10 determined after incubation for 3 d.

To determine whether the IL-12 p40 production observed in Ag-stimulated PBMC cultures reflected biologically active cytokine, IL-12 p70 was also determined in these cultures (Fig. 6, *C* and *D*). In general, the p70 production pattern paralleled that of p40, demonstrating that biologically

active IL-12 was produced in response to LeIF in both patient and normal PBMC.

*Production of IFN-γ by LeIF-stimulated Patient PBMC is IL-12 Dependent and Is Downregulated by IL-10.* The above results indicated that the production of IFN-γ by patient PBMC
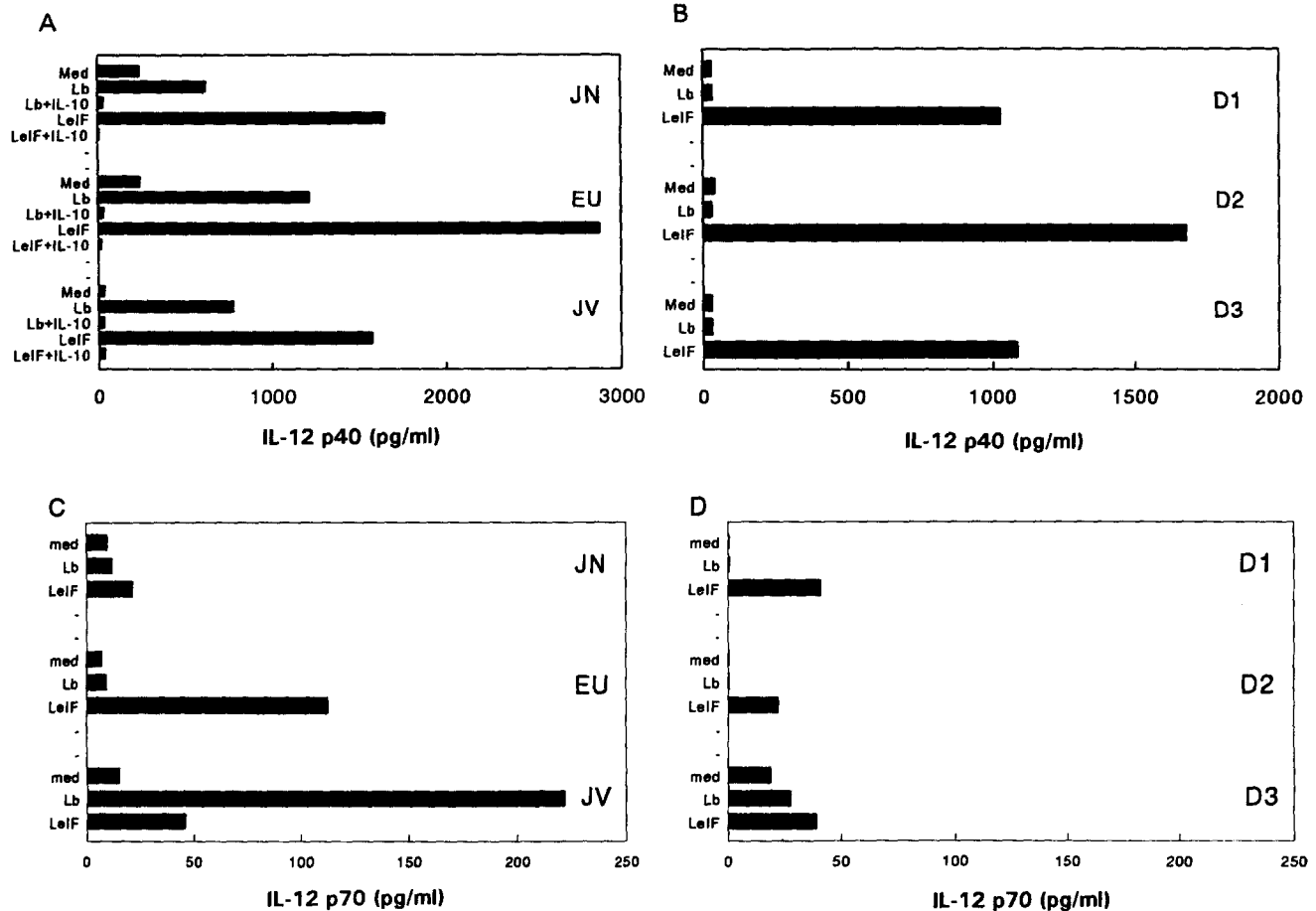


**Figure 6.** LeIF stimulates PBMC to secrete IL-12 p40 and p70. Cells from (*A* and *C*) ML patients (*JN*, *EU*, and *JV*) and (*B* and *D*) three uninfected individuals (*D1*, *D2*, and *D3*) were cultured in the absence (*Med*) or presence of leishmanial lysate (*Lb*), LeIF, or LeIF and IL-10 and the supernatants from 24-h cultures assayed for secreted IL-12 p40 and p70.
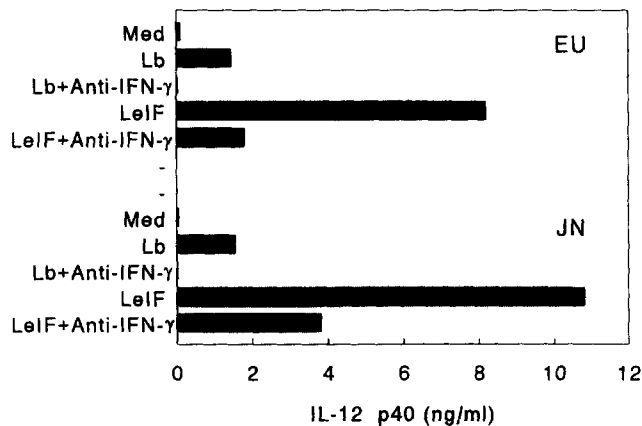
**Figure 7.** Dependence of IL-12 production on IFN-γ. Supernatants from ML PBMC were assayed for IL-12 p40 in the absence of Ag stimulation (*Med*), in the presence of Ag (*LeIF* and *Lb*) or Ag and anti-IFN-γ mAb.

cate that the production of IFN-γ by LeIF-stimulated patient PBMC is IL-12 dependent, whereas the production of IL-12 is regulated by both IFN-γ-dependent and -independent pathways.

## Discussion

We have cloned and characterized rLeIF, a *Leishmania* Ag, that elicits proliferation and a Th1-type cytokine profile from a leishmaniasis patient PBMC. Although resting and lysate stimulated PBMC from *L. braziliensis*-infected individuals have a mixture of Th1- and Th2-like cytokine profiles, rLeIF shifted this pattern towards an exclusive Th1 (IFN-γ and IL-2) cytokine profile. In addition, rLeIF stimulated both patient and normal PBMC to produce IL-12 whereas lysate did not stimulate the production of IL-12 in uninfected individuals. Finally, both rLeIF and parasite lysate stimulated PBMC from ML and self-healing CL patients to secrete TNF-α. The observation that LeIF stimulated T cells from all patients with self-healing CL to proliferate and produce IFN-γ suggests that PBMC responses to LeIF may be associated with protective immunity. In contrast, we have identified other *L. braziliensis* Ag that stimulate patient PBMC to produce a mixed Th1/Th2 or a predominant Th2 cytokine profile or that stimulate PBMC from ML patients but not from self-healing individuals (Skeiky, Y.A.W., et al., manuscript in preparation). Thus, LeIF appears to have unique immunological properties.

Cytokines appear to be essential mediators of immunity to *Leishmania* (27). IFN-γ and TNF-α may synergize to induce leishmanicidal activity in macrophages (28–30). In addition, TNF-α and IL-2 can also synergize with IL-12 to stimulate IFN-γ production by NK cells (31–33). Studies in mice have demonstrated that IL-12 (0.25–1 μg/d) can induce IL-10 production (34, 35). Another study using human monocytes has demonstrated that the addition of TNF-α (5–10 ng) can also induce IL-10 production (36). In our study, TNF-α and IL-12 but not IL-10 were produced by patient PBMC stimulated with LeIF. This may be due to the relatively lower level of either TNF-α or IL-12 produced by pa-
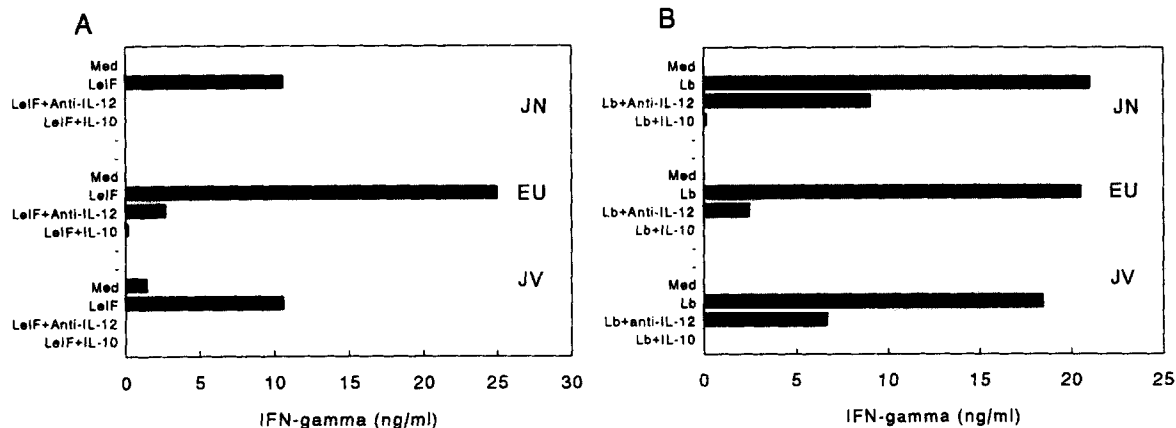
after stimulation with LeIF or parasite lysate may be mediated by IL-12. LeIF stimulated higher IL-12 p40 in patient PBMC than in normal PBMC indicating that the production of IL-12 may be upregulated by IFN-γ. To determine the interaction between IL-12, IFN-γ, and IL-10 in response to LeIF, PBMC from ML patients were stimulated in vitro with LeIF in the absence or presence of anti-IL-12, anti-IFN-γ mAb, or IL-10, and the cultured supernatants were assayed for IFN-γ and IL-12 p40 secretion. The addition of anti-IFN-γ mAb reduced the LeIF-induced production of IL-12 by 65 and 80% but abrogated the lysate-induced IL-12 (Fig. 7). Both anti-IL-12 mAb and IL-10 abrogated the production of LeIF-induced IFN-γ (Fig. 8 *A*) whereas anti-IL-12 mAb only partially decreased the production of IFN-γ after stimulation with leishmanial lysate (Fig. 8 *B*). Exogenous IL-10 abrogated IL-12 p40 production by patient PBMC in response to either LeIF or lysate (Fig. 8). IL-10 has also been shown to inhibit IL-12 production by human PBMC (26) and by human myeloid leukemia cell lines (14). These results indi-



**Figure 8.** Interdependence of IFN-γ and IL-12 production and their abrogation by IL-10 after stimulation of patient PBMC with LeIF. (*A*) Production of IFN-γ by PBMC from three ML patients (*JN, EU,* and *JV*) cultured in the absence (*Med*) or presence of LeIF, LeIF, and anti-IL-12 mAb (*Anti-IL-12*) or LeIF and IL-10. (*B*) Same as in *A* but with parasite lysate.

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tient PBMC, as well as the ability of IFN-γ, produced in high amounts by patient PBMC, to inhibit the production of IL-10 (22). The use of patient PBMC, rather than purified cell populations, may be more relevant to the in vivo situation since the dominant cytokine pattern is dependent on the interplay of modulatory cytokines.

The presence of a mixture of Th1 and Th2 cytokine mRNAs in the resting PBMC of ML patients is similar to the cytokine profile reported in lesions of ML patients (37, 38), although in our study, IL-10 mRNA was not detected in the resting PBMC from any of the ML patients characterized. However, IL-10 mRNA was detected after in vitro culture of ML patient PBMC in the absence of Ag. In contrast, resting PBMC from CL patients had, in addition to mRNA for IL-4 and IFN-γ, detectable levels of IL-10 mRNA which remained generally unaffected after culture in the absence of Ag. Therefore, the levels of cytokine mRNA in unstimulated controls may reflect an activated state from in vivo exposure to parasite Ag. In this regard, rLeIF downregulated the resting/constitutive levels of IL-10 in patient PBMC's as well as in LPS-stimulated PBMC. 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 (23, 39–43) and is a potent inducer of IFN-γ (33) which can inhibit the production of IL-10 (22), we reasoned that rLeIF may be mediating a dominant Th1 cytokine profile by an IL-12–dependent mechanism. Manetti et al. (41) showed that patients with allergies who have mixed Th1/Th2 cytokine patterns could be shifted towards an experimentally induced Th1 phenotype by the addition of IL-12. A significant finding of the present study was the demonstration that rLeIF stimulated patient PBMC to produce IL-12 and that the production of IFN-γ was, at least in part, IL-12 dependent. Other studies have demonstrated a role for IL-12 in enhancing IFN-γ production in both mouse and human cells (44, 45). Recent studies from several groups have led to the conclusion that the dominant factor of Th1 cell development in response to infection

may be determined by the ability of the pathogen to stimulate IL-12 production by macrophages. In this regard, several pathogens including L. monocytogenes, Toxoplasma gondii, Mycobacterium tuberculosis, Staphylococcus aureus, and L. major, that induce a Th1 response correlate with their capacity to stimulate the production of IL-12 (13, 14, 24, 32, 43, 44, 46, 47). However, the components or factors responsible for stimulating IL-12 production remain to be identified.

Our results have demonstrated that a single component of Leishmania is capable of stimulating the production of IL-12. This study represents the first reported identification of a recombinant parasite protein that stimulates the production of IL-12 in both patient and normal PBMC. Therefore, our finding that LeIF can stimulate IL-12 production as well as the elicitation of an exclusive Th1 cytokine profile in patient PBMC indicates that LeIF could confer protective immunity against Leishmania infections and/or act as an adjuvant. In human peripheral blood, monocyte/macrophages are the major source of IL-12 although B cells and other minor populations of HLA-DR⁺ accessory cells also produce IL-12 (13). Our preliminary results indicate the monocytes/macrophages population of patient PBMC as the dominant IL-12–producing cell types after stimulation with LeIF. In addition, LeIF stimulated purified monocytes from uninfected individuals to produce IL-12 but not IL-10, thus suggesting the direct ability of LeIF to function as an adjuvant (Skeiky Y. A. W., M. Kubin, G. Trinchieri, and S. G. Reed, manuscript in preparation). The mechanisms by which LeIF induce IL-12 production are currently being investigated.

In summary, we have identified a Leishmania Ag that stimulates IL-12 and elicits a dominant Th1-type cytokine profile in human PBMC. An ideal prophylactic and therapeutic vaccine for leishmanial infection would be one that induces the expansion of Ag-specific Th1 cells. These cells would produce IFN-γ and IL-12 which would promote parasite destruction within the macrophage and downregulate Th2 responses. Because Leishmania LeIF induced a powerful Th1-type response, including the two cytokines most clearly associated with protection in experimental leishmaniasis, IFN-γ and IL-12, LeIF is an antigen that merits further study.

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