INFECTION AND IMMUNITY, Aug. 2002, p. 4215–4225 Vol. 70, No. 8
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.8.4215–4225.2002

Immunization with a Polyprotein Vaccine Consisting of the T-Cell Antigens Thiol-Specific Antioxidant, *Leishmania major* Stress-Inducible Protein 1, and *Leishmania* Elongation Initiation Factor Protects against Leishmaniasis

Rhea N. Coler,[1]* Yasir A. W. Skeiky,[2] Karen Bernards,[1] Kay Greeson,[1] Darrick Carter,[2] Charisa D. Cornellison,[2] Farrokh Modabber,[1] Antonio Campos-Neto,[1] and Steven G. Reed[1,2]

Infectious Disease Research Institute[1] and Corixa Corporation,[2] Seattle, Washington 98104

Received 13 February 2002/Returned for modification 2 May 2002/Accepted 13 May 2002

Development of an effective vaccine against *Leishmania* infection is a priority of tropical disease research. We have recently demonstrated protection against *Leishmania major* in the murine and nonhuman primate models with individual or combinations of purified leishmanial recombinant antigens delivered as plasmid DNA constructs or formulated with recombinant interleukin-12 (IL-12) as adjuvant. In the present study, we immunized BALB/c mice with a recombinant polyprotein comprising a tandem fusion of the leishmanial antigens thiol-specific antioxidant, *L. major* stress-inducible protein 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF) delivered with adjuvants suitable for human use. Aspects of the safety, immunogenicity, and vaccine efficacy of formulations with each individual component, as well as the polyprotein referred to as Leish-111f, were assessed by using the *L. major* challenge model with BALB/c mice. No adverse reactions were observed when three subcutaneous injections of the Leish-111f polyprotein formulated with either MPL-squalene (SE) or Ribi 529-SE were given to BALB/c mice. A predominant Th1 immune response characterized by in vitro lymphocyte proliferation, gamma interferon production, and immunoglobulin G2A antibodies was observed with little, if any, IL-4. Moreover, Leish-111f formulated with MPL-SE conferred immunity to leishmaniasis for at least 3 months. These data demonstrate success at designing and developing a prophylactic leishmaniasis vaccine that proved effective in a preclinical model using multiple leishmanial antigens produced as a single protein delivered with a powerful Th1 adjuvant suitable for human use.

Protozoan parasites of the genus *Leishmania* cause a wide spectrum of clinical manifestations, ranging from subclinical or self-healing cutaneous infections to progressive fatal visceral disease, depending on the parasite species and the immune system of the patient (3). With an estimated 350 million people at risk for acquiring infection with *Leishmania* parasites and 12 million cases worldwide, the World Health Organization considers leishmaniasis to be one of the most serious, epidemic-prone parasitic infectious diseases afflicting the poor and disadvantaged. For this reason, a reinforced focus on leishmaniasis control, particularly the development of an effective vaccine, is needed.

No defined vaccine against leishmaniasis has been found, and killed or crude antigenic preparations of *Leishmania* promastigotes, so-called "first-generation vaccines," have been found to induce variable levels of protection in humans (26). Although chemotherapies against leishmaniasis do exist, they are few, are not affordable by those who need them, require daily injections for weeks, and are associated with side effects (29). In addition, drug resistance is becoming an increasing problem. Control of the vector, *Phlebotomus* sand flies, is not feasible in certain foci (forests, open desert areas), and where it is possible, the infrastructure and sustainability cost is prohibitively high. Vaccination through controlled infection with viable parasites or "leishmanization," which has been practiced in the Middle East (28), is being discontinued as a result of undesirable side effects and clinical complications (13, 14). Thus, systematic vaccine development may be the best control measure against leishmaniasis (11, 38).

Presumably, successful prophylactic immunization against at least one of the four main forms of leishmaniasis, cutaneous leishmaniasis (CL), should be amenable to vaccine-induced immunity given that this disease can be self-limiting and may be followed by resistance to reinfection. In recent years, several leishmanial antigens have been identified and evaluated as vaccine candidates in murine models of CL or visceral leishmaniasis (VL), with various degrees of protection (1, 5, 8, 9, 16, 17, 21, 24, 27, 30, 32, 33, 39, 42, 43, 48, 51). Thus, there is ample evidence that leishmanial antigens protect against disease in the murine model of the human disease.

Over the last decade, there has been considerable progress in understanding the immune responses involved in conferring protective immunity against leishmaniasis. In the murine model of leishmaniasis, interleukin-12 (IL-12)-driven Th1-type immune responses with gamma interferon (IFN-γ), IL-2, and tumor necrosis factor beta production are associated with protection and self-cure while the Th2 phenotype with IL-4, IL-5, IL-10, IL-13, and transforming growth factor beta production is associated with susceptibility to disease (2, 12, 18, 19, 22, 23, 34, 35, 36). In the human disease, there is evidence that mixed

* Corresponding author. Mailing address: Infectious Disease Research Institute, 1124 Columbia St., Suite 600, Seattle, WA 98104. Phone: (206) 381-0883. Fax: (206) 381-3678. E-mail: coler@idri.org.

Th cytokine profiles are present, while healing and protection against reinfection are associated with dominant Th1 and/or $CD8^+$ T cells. These findings suggest that it is the balance between cytokines that activate or suppress activation of macrophages harboring *Leishmania* parasites that determines the outcome of the infection. Thus, treatments or antigen-adjuvant formulations that can alter the type of T-helper response may change the course of disease progression.

In previous studies, we identified and characterized three T-cell antigens, *Leishmania* elongation initiation factor (LeIF), *Leishmania major* stress-inducible protein 1 (LmSTI1), and thiol-specific antioxidant (TSA), that were found to be conserved among various *Leishmania* species and that elicit primarily a Th1-type immune response in murine or human cells (31, 40, 41, 49, 50). We have shown that two of these antigens, recombinant LmSTI1 (rLmSTI1) and recombinant TSA (rTSA), individually or when combined and delivered in the presence of recombinant IL-12 (rIL-12) or as a DNA-based vaccine, elicit protection against CL in the murine and nonhuman primate models (4, 25). The third antigen, recombinant LeIF (rLeIF), has been found to provide partial therapeutic protection against leishmaniasis and has adjuvant properties (41), including elicitation of IL-12. IL-12 is a potent inducer of IFN-γ (37, 47), maintains memory/effector Th1 cells (44), and is required for primary immunity to leishmaniasis (20, 45).

A vaccine suitable for use in humans and consisting of a single protein would be less costly to manufacture and has the advantage of a practical production process and more straightforward quality control testing than a vaccine consisting of several recombinant proteins. For this reason, a polyprotein composed of these three priority candidate antigens fused in tandem was made that is referred to as Leish-111f (Y. A. Skeiky, R. Coler, J. R. Webb, D. Carter, A. Campos-Neto, and S. G. Reed, 2nd Int. Congr. Leishmania Leishmaniasis [WorldLeishII], abstr. 34, 2001). Recombinant Leish-111f (rLeish-111f) adjuvanted with rIL-12 has been evaluated in the mouse model of *Leishmania major* and shown to be protective (Skeiky et al., 2nd Int. Congr. Leishmania Leishmaniasis). Because IL-12 has limitations as an adjuvant for human use, we have tested our polyprotein antigen in the presence of various adjuvants that may be more suitable for human use.

In the present report, we show that Leish-111f has relatively low inherent immunogenicity and requires a strong adjuvant to prime a specific immune response. We evaluated and compared the immunogenic and protective efficacies of formulating Leish-111f with either the naturally derived disaccharide adjuvant of *Salmonella minnesota* monophosphoryl lipid A (MPL) with squalene (MPL-SE) or a synthetic monosaccharide analog of MPL, termed Ribi 529, with SE (Ribi 529-SE). MPL has been used as an adjuvant in several human clinical trials of safety and immunogenicity, including vaccines for malaria (10), hepatitis B (46), genital herpes (G. Leroux-Roels, E. Moreau, B. Verhasselt, S. Biernaux, V. Brulein, M. Francotte, P. Pala, M. Slaopui, and P. Vandepapeliere, Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1209, 1993), and allergy desensitization. These studies all found that MPL was well tolerated and there was no evidence of systemic toxicity. In combination with MPL-SE, Leish-111f induces a very potent immune response that protects mice against CL when they are challenged 3 or 12 weeks postimmunization. Our data therefore emphasize the importance of adjuvant for the screening of candidate antigens for *Leishmania* vaccines and demonstrate that Leish-111f shows great promise for inclusion in a future leishmaniasis vaccine.

MATERIALS AND METHODS

Animals and parasites. Six- to eight-week-old female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Portage, Mich.). The animals were maintained under pathogen-free conditions at the Fred Hutchinson Cancer Research Center and used for experiments beginning at 8 to 10 weeks of age. Mice were transferred and maintained in a level 2 physical containment facility before infection with *L. major* or *L. amazonensis. L. major* and *L. amazonensis* were maintained by continuous passage in BALB/c mice. For preparation of *L. major* promastigotes, the parasites were grown at 25°C in medium 199 supplemented with 20% heat-inactivated fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 0.1 mM adenine, 40 mM HEPES, 0.25 mg of hemin per ml, and 0.31 mg of 6-biotin per ml. For the preparation of *L. amazonensis* promastigotes, the parasites were grown in Schneider's drosophila medium supplemented with 10% heat-inactivated fetal bovine serum, minimal essential medium (MEM) amino acid solution, MEM nonessential amino acid solution, MEM sodium pyruvate, gentamicin, and 2 mM L-glutamine. In all experiments, promastigotes were used after one to three passages in vitro.

Leishmanial antigens. rTSA, LmSTI1, LeIF, and Leish-111f were produced as described previously (40, 49, 50; Skeiky et al., 2nd Int. Congr. Leishmania Leishmaniasis). Briefly, Leish-111f protein was produced in recombinant *Escherichia coli* BL21(DE3)/pLysS (Novagen, Madison, Wis.). Stationary cultures of bacteria expressing Leish-111f were grown overnight with kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml) and then diluted in fresh medium and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The cells were harvested and lysed in the presence of Complete Protease Inhibitor Tablets (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 10 mM Tris (pH 8.0) with a French pressure cell at 16,000 lb/in^2. Inclusion bodies were harvested by centrifugation, washed once with 10 mM Tris (pH 8.0)–0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and then solubilized in 8 M urea–30 mM ethanolamine (pH 10.0) (buffer A). Solubilization was allowed to continue overnight with agitation, and the solubilized Leish-111f protein was loaded onto a column containing High Q Strong Anion Exchange resin (Bio-Rad, Hercules, Calif.). Fractionation occurred by elution with a gradient from buffer A to buffer A containing 1 M NaCl (50% in 70 min and then 100% in 10 min). The fractions containing the protein as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were bound to nickel-nitrilotriacetic acid resin (Qiagen Inc., Valencia, Calif.) overnight at 4°C. The recombinant-bound-resin was equilibrated at room temperature, washed with 10 mM Tris (pH 8.0)–8 M urea–0.5% deoxycholic acid–10 mM imidazole–250 mM NaH_2PO_4, and eluted with 8 M urea–10 mM Tris (pH 8.0)–300 mM imidazole. The eluted protein was dialyzed against excess 10 mM Tris (pH 8.0) containing 1 mM reduced glutathione and stored at −80°C for further analysis. The lipopolysaccharide content of each antigen preparation was measured by a *Limulus* amoebocyte lysate test (BioWhittaker, Walkersville, Md.) and shown to be below 10 endotoxin units (EU)/mg of protein.

Immunization and determination of parasite burden after challenge. Groups of five mice were immunized with 10 μg of the Leish-111f polyprotein containing TSA, LmSTI1, and LeIF alone or with 5, 10, or 20 μg of either MPL-SE or Ribi 529-SE (Corixa Corporation, Seattle, Wash.) in a volume of 0.1 ml. Control groups received either adjuvant alone or saline. Three subcutaneous (s.c.) injections were given in the right footpad and at the base of the tail at 3-week intervals. Mice were infected 3 or 12 weeks after completion of the immunization protocol. As a challenge, 2×10^5 or 4×10^5 *L. major* WHOM/IL/80/Friedlin metacyclic promastigotes or 1×10^6 *L. amazonensis* promastigotes were suspended in 25 μl of saline and injected s.c. into the left hind footpad. The progress of infection was followed by measuring footpad swelling or thickness with a metric caliper (Mitutoyo Measuring Instruments, Aurora, Ill.). Grading of lesion size was done by subtracting the thickness of the uninfected contralateral footpad from that of the infected one. Animals were euthanized when they were unable to walk or when the lesions became necrotic.

ELISA for anti-Leish-111f IgG. Serum samples were taken from all animals, and antigen-specific enzyme-linked immunosorbent assays (ELISAs) were performed for the identification of specific anti-rLeish-111f, anti-rTSA, anti-rLmSTI1, and anti-rLeIF IgG1 and IgG2a. Briefly, 96-well microtiter plates (Costar) were coated (100 μl/well) with each recombinant antigen at 2 μg/ml in phos-

phate-buffered saline (PBS) and incubated for 4 h at 37°C. Plates were washed and blocked overnight at 4°C with 200 μl of 10% fetal calf serum (FCS) in PBS-Tween per well. Serum samples were diluted to 1:100 with PBS–Tween–10% FCS and applied to plates in twofold serial dilutions. Plates were incubated at 37°C for 4 h. Plates were washed, horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates Ltd., Birmingham, Ala.) was added at a 1:2,000 dilution, and the mixture was incubated for 2 h at 37°C. Detection was done with the substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, Md.). Optical density at 450 nm was determined by using 570 nm as the reference wavelength.

Culture of spleen cells. Proliferative and cytokine responses were measured by preparing single-cell spleen suspensions by Lympholyte-M density gradient centrifugation (CedarLane Labs, Hornby, Ontario, Canada). Splenic mononuclear cells were resuspended in complete medium (RPMI 1640 medium supplemented with 10% FCS, 50 μg of gentamicin per ml, 2 mM L-glutamine, and 5×10^{-5} M β-mercaptoethanol) and plated at 2×10^6/ml in 96-well flat-bottom plates (Costar). The spleen mononuclear cells and lymph node cells were cultured in the presence of anti-IL-4R antibody (Immunex Corporation, Seattle, Wash.) at 1 μg/ml to increase the sensitivity of the IL-4 measurement. The spleen cells were stimulated in vitro at 37°C in 5% CO_2 with Leish-111f, rTSA, rLmSTI1, or rLeIF at 10 and 2 μg/ml; soluble leishmania antigen (SLA; 10 μg/ml); or medium alone. Concanavalin A, at a concentration of 1 μg/ml, was used in all experiments as a positive control for cell viability. Supernatants were collected after 48 and 72 h of culture and tested by ELISA for IL-4 and IFN-γ elicitation as previously described (7). Alternatively, after 5 days, the cultures were pulsed with 1 μCi of [^{3}H]thymidine for 18 h, harvested, and counted in a gas scintillation counter.

Infection of antigen-presenting cells (APCs) with *L. major*. Macrophages were obtained from bone marrow precursor cells eluted from the femurs of BALB/c mice. Bone marrow cells were washed once in Hanks balanced salt solution and plated at 2×10^5/ml in six-well plates (Costar) in Dulbecco MEM containing 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 ng of recombinant granulocyte-macrophage colony-stimulating factor (Immunex Corporation) per ml. The medium was changed on days 2 and 4 of culture. On day 5 of culture, adherent cells (macrophages) were washed twice with cold PBS, harvested on ice, and infected with metacyclic *L. major* promastigotes.

To infect APCs, parasites and 10^5 macrophages/well in a 48-well plate were centrifuged at $1,200 \times g$ for 1 h at room temperature to give a multiplicity of infection of two to four parasites per cell. At the end of infection, noninternalized protozoa were separated from macrophages by washing with warm Hanks balanced salt solution. An aliquot of infected APCs was fixed for 30 min in 1% glutaraldehyde and stained with Giemsa. The level of infection was measured by counting 1,000 cells in two separate cultures. Infected APCs were then cultured in fresh medium at 37°C in 5% CO_2 for 60 h before being used in T-cell stimulation assays.

Statistical methods. The efficacies of different vaccination formulations were compared by one-way analysis of variance of footpad swelling in millimeters. A P value of <0.05 was considered significant.

RESULTS

Comparative evaluation of experimental vaccines containing Leish-111f formulated with adjuvants suitable for use in humans. Protective immunity against leishmaniasis is believed to be associated with induction of Th1 cellular immunity. To monitor antigen-specific immune responses primed by immunization with Leish-111f, humoral and cellular responses were examined in immunized mice. Anti-Leish-111f immune responses were investigated in parallel with responses to the individual components LmSTI1, TSA, and LeIF.

Recombinant Leish-111f was formulated with MPL and Ribi 529 adjuvants in SE. These adjuvants have been proven to boost the potency of modern vaccines or antigens, including those used for various infectious diseases and allergy desensitization. MPL has demonstrated safety and efficacy in human clinical trials involving more than 30,000 doses. RC-529 is a synthetic monosaccharide product based on structure-function studies of the MPL adjuvant. Since it can be synthesized organically in large-scale, high-purity lots, Ribi 529 has the advantage of a facile production process and quality control testing. Ribi 529 also has been shown to have an excellent safety profile and enhances cellular and humoral immune responses, including cytotoxic T lymphocytes, IgG2a, and IgA. Extensive preclinical studies have shown that Ribi 529 compares favorably to MPL, and phase I clinical trails are ongoing.

All vaccines were given three times at 3-week intervals, and the antigen-specific immune responses primed by immunization with vaccine formulations or controls were investigated. The titers of antibodies against Leish-111f and its individual components were determined in sera harvested from immunized mice. The isotype profile of antibody responses depends on the cytokines produced by antigen-specific T cells. Production of IgG2a is dependent on IFN-γ, whereas IL-4 is associated with high levels of IgG1 (6). The relative production of immunoglobulin isotypes is thus a marker for the induction of Th1-like versus Th2-like immune responses. Anti-Leish111f IgG1 and IgG2a titers were measured in the sera taken from all mice receiving Leish-111f vaccines, regardless of the adjuvant or the dose of the adjuvant administered. The highest levels of IgG1 were observed when Leish-111f was formulated with 20 μg of Ribi 529-SE (IgG1 titer, 2.174 ± 0.087) versus 20 μg of MPL-SE (IgG1 titer, 1.587 ± 0.1164) (Fig. 1). In contrast, the highest levels of IgG2a were measured in mice receiving Leish-111f plus various concentrations of MPL-SE (anti-MPL-SE titer, 2.24 ± 0.158; anti-Ribi 529-SE titer, 1.409 ± 0.133) (Fig. 1A to C). These mice also all had both IgG1 and IgG2a responses to the individual components of Leish-111f, suggesting that an immune response to each component is mounted when the polyprotein fusion molecule is formulated with the MPL-SE and Ribi 529-SE adjuvants and used for vaccination. These results also suggest that no strong antigenic competition occurs among TSA, LmSTI1, and LeIF, at least when immune responses are measured after administration of three immunizing doses of the vaccine. Sera from animals receiving saline or different amounts (5, 10, or 20 μg) of adjuvant alone were essentially negative for Leish-111f-specific IgG1 and IgG2a.

Mice immunized with Leish-111f alone (in the absence of adjuvant) developed lower titers of specific anti-Leish-111f and anti-LmSTI1 IgG1 antibodies than did mice receiving Leish-111f formulated with adjuvant. The observed IgG2a antibody responses to these two antigens were particularly affected by the absence of the MPL-SE and Ribi 529-SE adjuvants. Few, if any, antigen-specific humoral responses were elicited against rTSA or rLeIF in mice immunized with Leish-111f in the absence of adjuvant (data not shown). These results suggest that, in combination with Leish-111f, the MPL-SE and Ribi 529-SE adjuvants facilitate polarization to a greater Th1 response.

To further investigate the phenotype (Th1 versus Th2) of the immune response elicited by immunization of Leish-111f, suspensions of spleen cells from immunized mice were restimulated in vitro with Leish-111f, its components, or controls and then proliferative and cytokine levels were measured. Splenocytes harvested from mice immunized with Leish-111f formulated with the MPL-SE or Ribi 529-SE adjuvant proliferated and produced IFN-γ with little IL-4 when stimulated in vitro with SLA (10 μg/ml) or rLeish-111f, TSA, LmSTI1, or LeIF (10 and 2 μg/ml) (Fig. 2A and B). These results confirm that

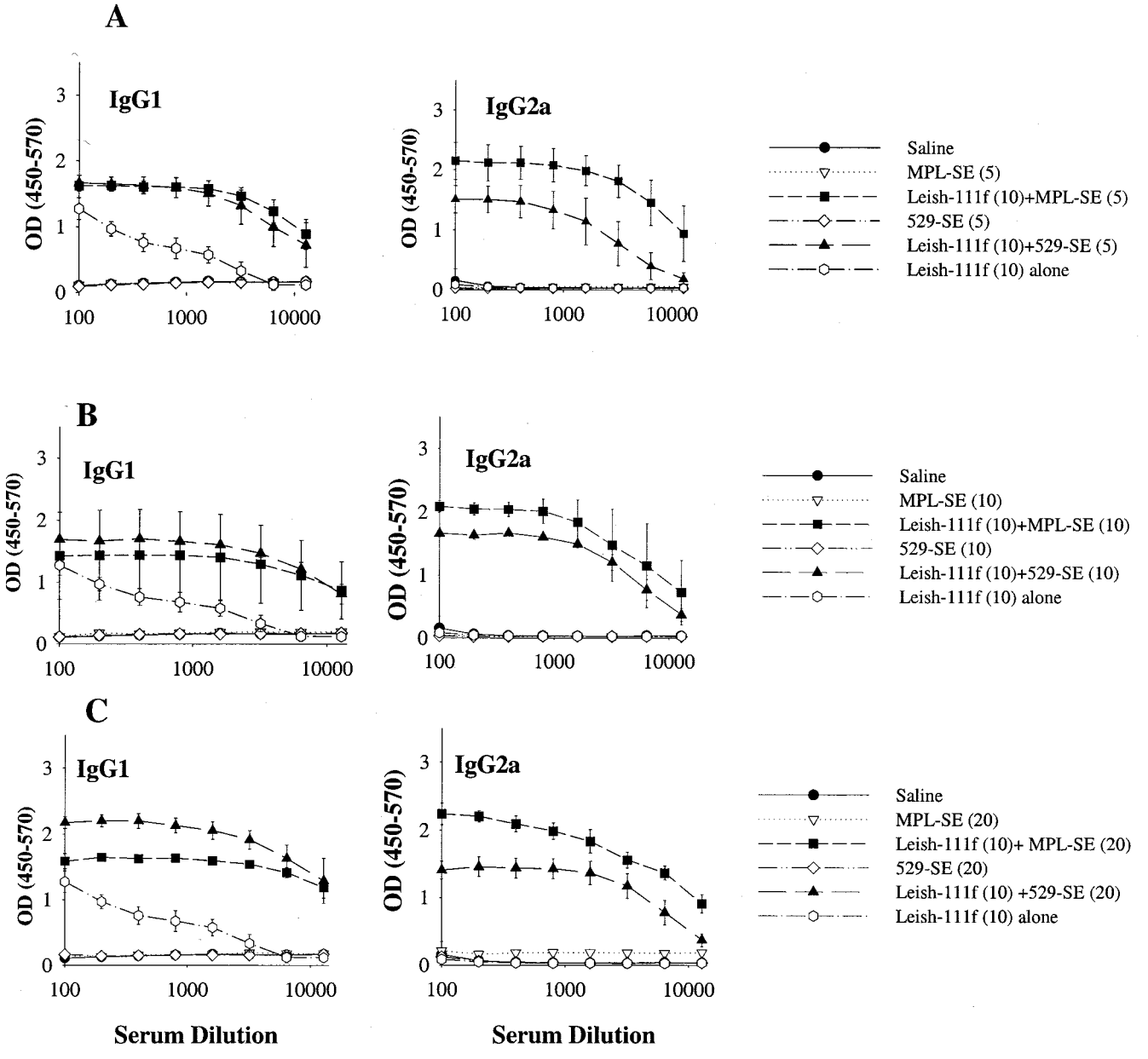

FIG. 1. Serological responses to Leish-111f in vaccinated mice. Groups of eight BALB/c mice were immunized three times at 3-week intervals by s.c. injection of 10 μg of Leish-111f with or without 5 (A), 10 (B), or 20 (C) μg of either MPL-SE or Ribi 529-SE adjuvant, with adjuvant alone, or with saline. Serum samples were collected 2 weeks after the last injection and analyzed by ELISA for the presence of anti-Leish-111f IgG1 and IgG2a. Each point represents the mean and standard error of the mean of data from eight individual mice. This experiment was repeated with similar results. OD (450-570), optical density at 450 to 570 nm.

immunization with rLeish-111f plus the MPL-SE or Ribi 529-SE adjuvant induces an immunity that is polarized toward a Th1 phenotype.

Recognition of Leish-111f after *L. major* infection in vivo and in vitro. To investigate whether Leish-111f and its component antigens are targets for the immune response during leishmanial infection, splenocytes from mice at day 48 after *L. major* infection were stimulated in vitro with these recombinant antigens and controls. Peripheral blood lymphocytes purified from mice infected with *L. major* and restimulated in vitro secreted high levels of IFN-γ when cultured with SLA (16,200 pg of IFN-γ/ml) or with the purified antigen rLmSTI1 (6,600 pg of IFN-γ/ml), rTSA (12,500 pg of IFN-γ/ml), rLeIF (22,000 pg of IFN-γ/ml), or Leish-111f (6,100 pg of IFN-γ/ml) (Fig. 3A). Furthermore, in the presence of APCs infected at a multiplicity of infection of 2 to 4 and T-cell lines generated against Leish-111f or SLA, high levels of proliferation and IFN-γ were measured (Fig. 3B and C). Uninfected APCs cultured with Leish-111f-specific T-cell lines did not proliferate or produce IFN-γ (Fig. 3B and C).

Vaccination with Leish-111f protects mice against *L. major* and *L. amazonensis* infection. BALB/c mice were immunized with three injections of Leish-111f and either MPL-SE or Ribi 529-SE or with the adjuvants alone. Immunized mice were

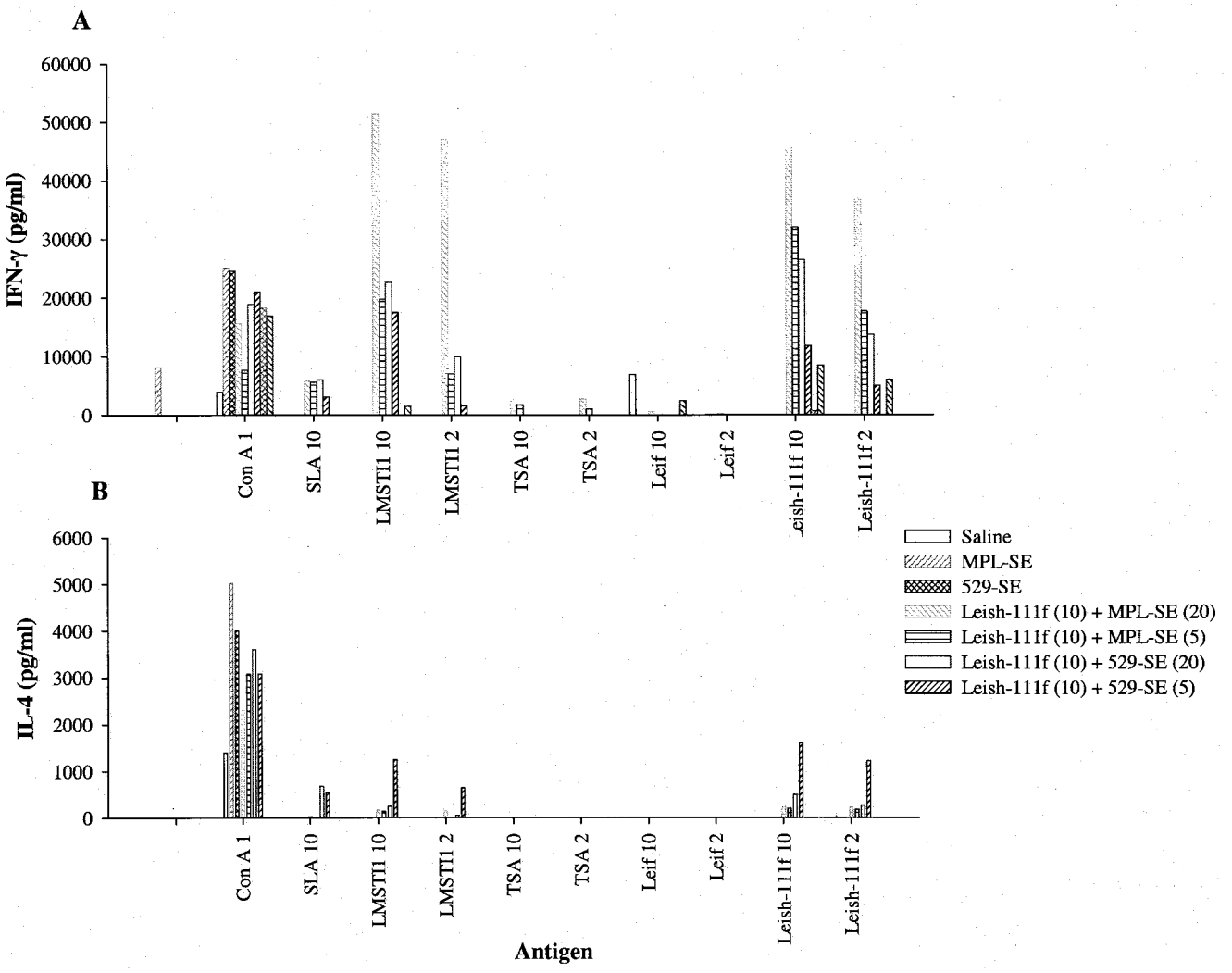

FIG. 2. T-cell responses of BALB/c mice immunized with Leish-111f formulated with adjuvants suitable for human use. BALB/c mice were immunized s.c. in the right rear footpad and at the base of the tail with 10 μg of Leish-111f formulated with 5, 10, or 20 μg of either MPL-SE or Ribi 529-SE (Corixa Corporation). Mice were boosted twice with a 3-week interval, and 10 days later, their spleens were removed and the splenocytes were stimulated in vitro with Leish-111f, TSA, LmSTI1, LeIF, and SLA (all at 10 μg/ml), concanavalin A (Con A; 1 μg/ml), or medium alone. Elicitation of IFN-γ (A) and IL-4 (B) was assessed by sandwich ELISA with supernatants removed after 72 h of in vitro incubation. Cells from three mice per group were pooled. Each bar represents the mean and standard error of triplicate wells. The data shown are the means of duplicate wells. This experiment was repeated with similar results.

subsequently challenged with *L. major* metacyclic promastigotes after the last boost, and lesion development was scored weekly (Fig. 4). Infections progressed similarly in vaccinated and control groups for the first 2 weeks. Thereafter, lesion size progressed at a more rapid rate in mice injected with saline or adjuvant alone than in mice immunized with Leish-111f in combination with MPL-SE or Ribi 529-SE (Fig. 4). These findings correlate with the results shown in Fig. 1 and 2, which show that mice that developed an effective protective immune response (Leish-111f plus MPL-SE or Leish-111f plus Ribi 529-SE) had substantially higher levels of IgG2a antibody titers and IFN-γ production than did nonprotected or nonhealing mice. Mice immunized with Leish-111f in combination with other adjuvants, such as alum or MPL adsorbed to alum, developed strong Th2-polarized antigen-specific immune responses and did not develop a protective immune response upon a challenge with *L. major* (data not shown). The most effective combination was Leish-111f formulated with 20 μg of MPL-SE, which afforded protection for at least 14 weeks (Fig. 4A).

On the basis of these results, the combination of rLeish-111f and MPL-SE was selected for further studies of the efficacy of this vaccine in protecting against an *L. amazonensis* challenge (Fig. 5), studies of the protective dose response of rLeish-111f (2 versus 10 μg) with 20 μg of MPL-SE, delayed-challenge studies, and studies of the efficacy of protection against a larger challenge dose of parasites.

C57BL/6 mice were immunized with Leish-111f in combination with MPL-SE or with the adjuvant alone. Immunized mice were subsequently challenged with 10^6 *L. amazonensis* promastigotes, and lesion development was scored weekly (Fig. 5). Infections progressed similarly in vaccinated and control

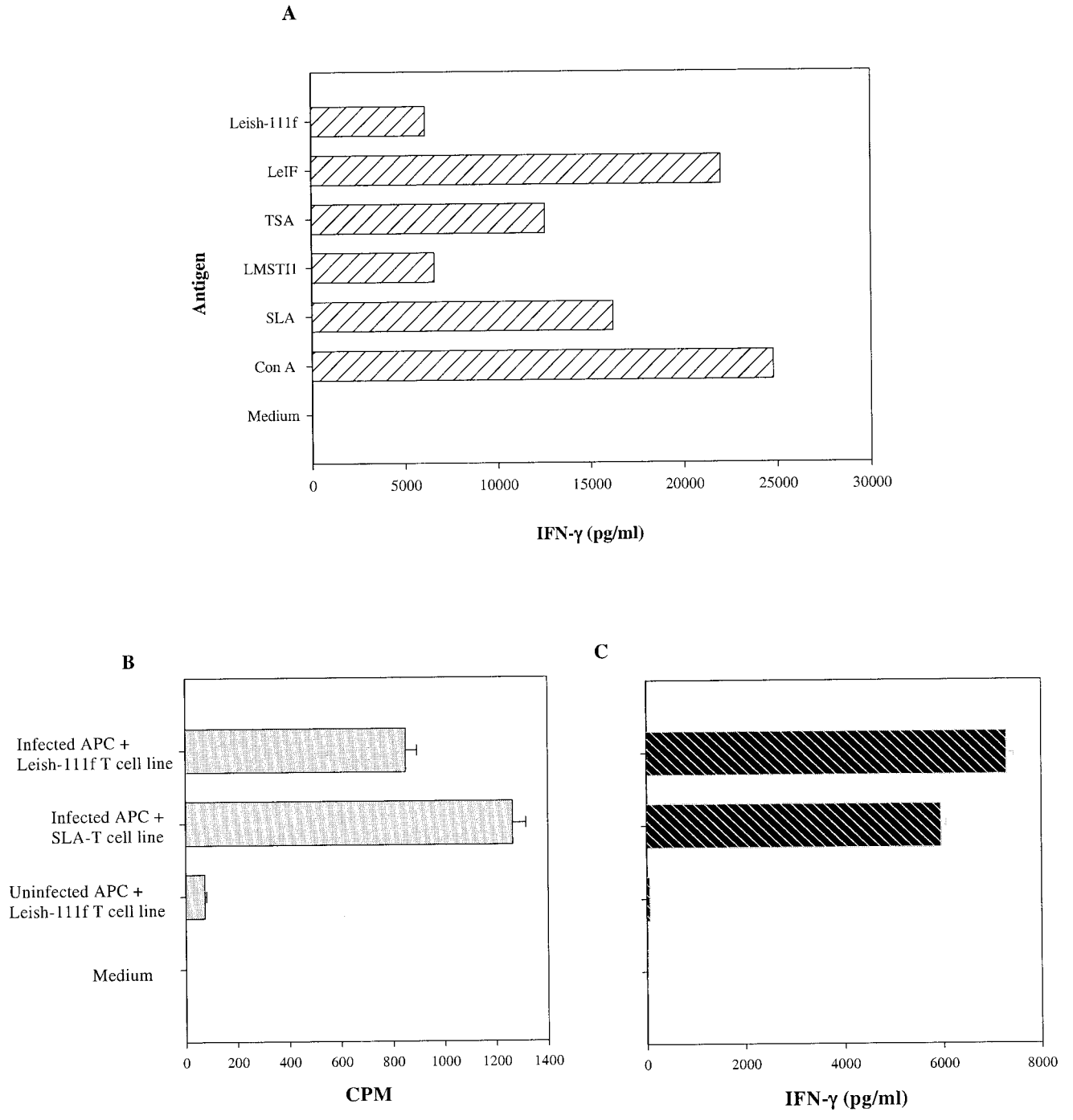

FIG. 3. APCs infected with metacyclic *L. major* promastigotes recognize Leish-111f. Leish-111f-specific T-cell lines or SLA-specific T-cell lines generated from mice immunized with Leish-111f formulated with MPL-SE or SLA formulated with murine rIL-12, respectively, were stimulated for 96 h with bone marrow-derived macrophages that were uninfected or had been previously infected at a multiplicity of infection of two to four parasites per cell. Proliferation (A) was measured by incorporation of [^{3}H]thymidine. After 72 h of culture, supernatants were taken and assessed by ELISA for production of IFN-γ (B). The data shown are the means of triplicate wells. These experiments were repeated with similar results. Con A, concanavalin A.

groups for the first 9 weeks. Thereafter, lesion size progressed at a more rapid rate in mice injected with saline or MPL-SE alone, whereas the majority of the animals (three of five) receiving the Leish-111f vaccine started healing over the next several weeks.

Mice immunized with both 2 and 10 μg of Leish-111f plus MPL-SE were protected against an *L. major* challenge for at least 10 weeks, at which point all of the mice in the saline and adjuvant control groups had to be euthanized. At 10 weeks postchallenge, the difference in the mean level of protection

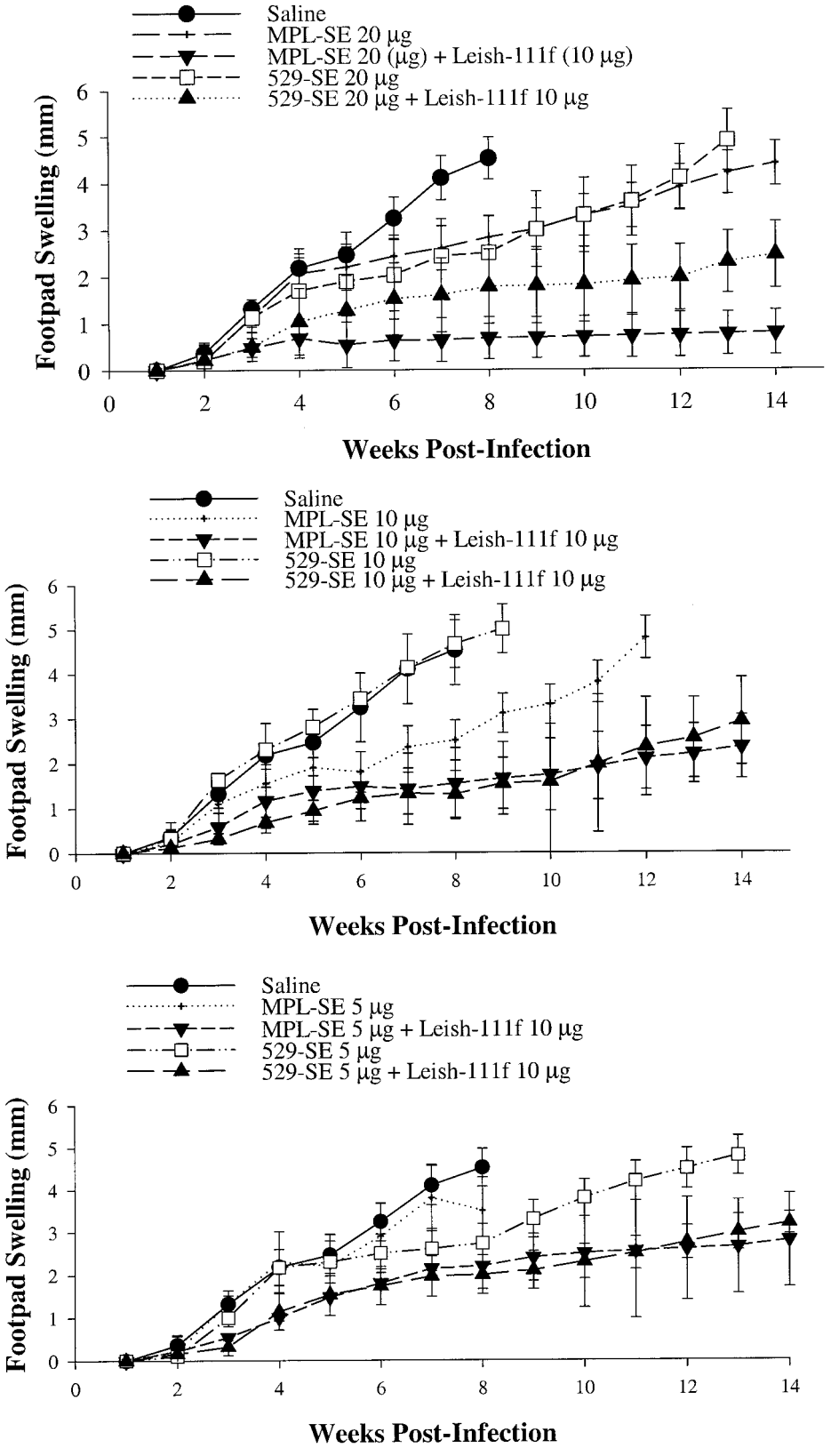

FIG. 4. Selection of adjuvant and adjuvant dose for the Leish-111f vaccine formulation. Groups of five mice were immunized three times at 3-week intervals by s.c. injection in the right hind footpad and at the base of the tail with 10 μg of Leish-111f plus adjuvant (either 5, 10, or 20 μg of MPL-SE or Ribi 529-SE). Control animals received either adjuvant alone or saline. Three weeks after the third immunization, mice were challenged s.c. in the left hind footpad with 2×10^5 metacyclic *L. major* promastigotes and footpad enlargement was measured weekly thereafter.

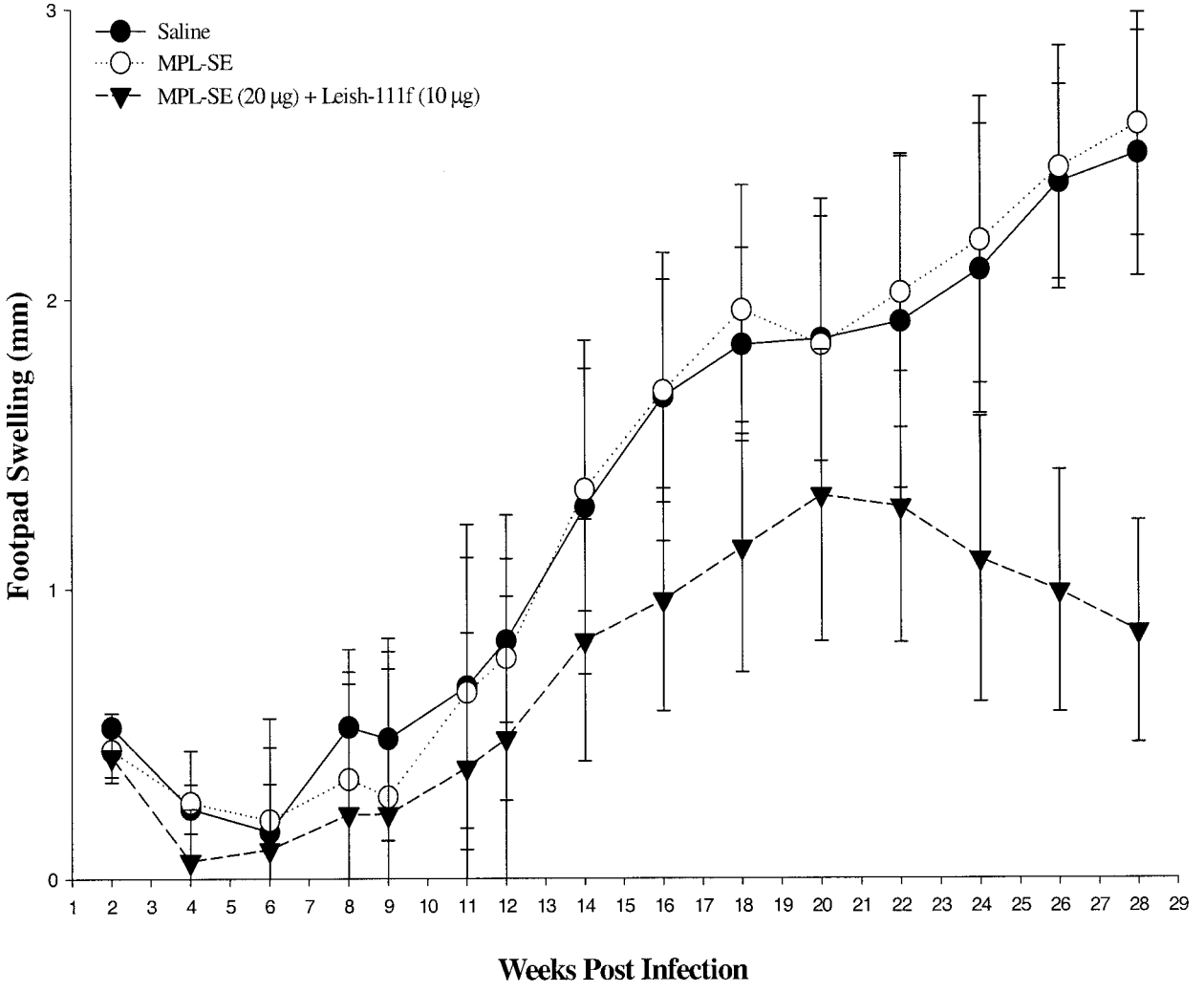

FIG. 5. Efficacy of Leish-111f plus MPL-SE against *L. amazonensis*. Groups of five C57BL/6 mice were immunized three times at 3-week intervals by s.c. injection in the right footpad and at the base of the tail with 10 μg of Leish-111f plus 20 μg of MPL-SE. Control animals received either adjuvant alone or saline. Three weeks after the third immunization, mice were challenged s.c. in the left hind footpad with 10^6 *L. amazonensis* promastigotes. Footpad enlargement was measured every second week thereafter.

observed in mice receiving 2 μg (0.38 ± 0.55) versus 10 μg (0.74 ± 0.57) of Leish-111f plus MPL-SE was not statistically significant (Fig. 6A). This experiment has been repeated twice with similar results. One experiment was extended for as long as 35 weeks postinfection, at which time the mean difference in footpad swelling measured in groups of mice immunized with 2 μg (1.7 ± 1.72 mm) versus 10 μg (1.74 ± 1.53 mm) of Leish-111f still did not reach statistical significance (data not shown). This suggests that in BALB/c mice, immunizing doses of 2 and 10 μg of Leish-111f with MPL-SE perform equally as well at eliciting protection against an *L. major* challenge.

Delaying the challenge by 3 months did not alter the protective capacity of the vaccine containing Leish-111f with MPL-SE. In fact, we monitored the footpad swelling of these BALB/c mice for at least 10 weeks postinfection (22 weeks after the third immunization) and the mice in the saline group developed more severe lesions (peak mean lesion size, 2.62 ± 0.44 mm) than the vaccinated group, in which only a small amount of swelling was observed (peak mean lesion size, 0.29 ± 0.19 mm) (Fig. 6B).

Changing the challenge dose from 2×10^5 to 4×10^5 *L. major* metacyclic promastigotes affected the kinetics of footpad swelling but did not change the protective effect of rLeish-111f with MPL-SE (data not shown). With the larger challenge dose, a significant difference in lesion size could be discerned between the control and vaccinated groups from week 2 to 3 onward, whereas with the smaller challenge dose, at least 4 weeks was required before a significant difference between these groups of mice became observable. Additional experiments showed that similar protection results were obtained when mice were given two instead of three s.c. immunizations of the Leish-111f vaccine followed by a challenge with 2×10^5 *L. major* promastigotes or 8×10^3 *L. major* amastigotes (data not shown).

DISCUSSION

Although CL caused by species such as *L. major* or *L. amazonensis* usually heal spontaneously within 6 to 14 months,

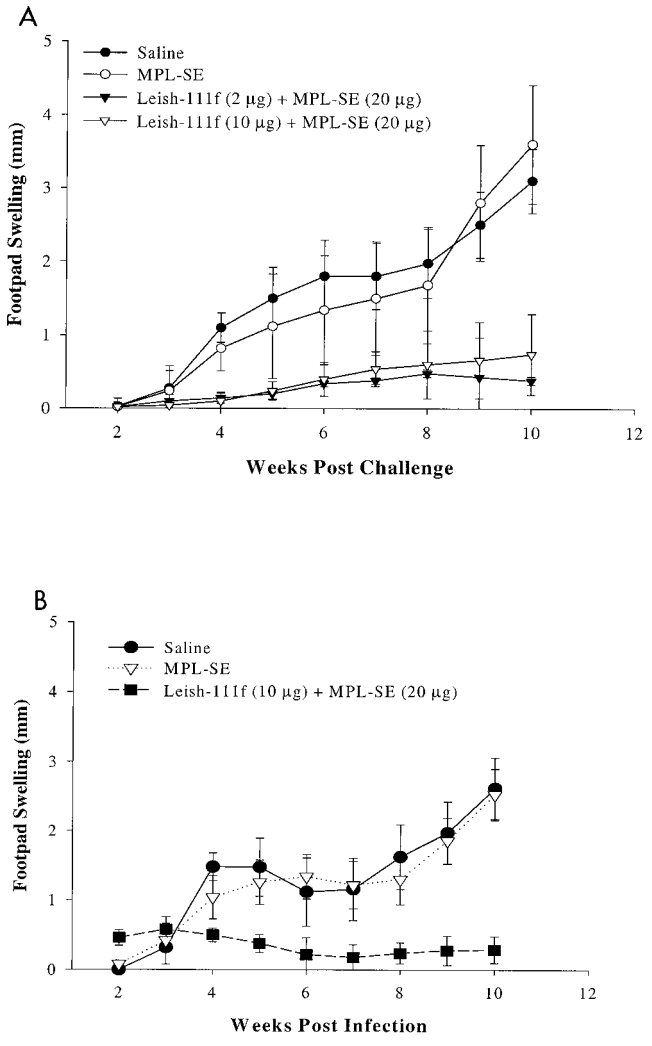

FIG. 6. Effective memory immunity against *L. major* challenge by immunization with Leish-111f vaccine. Groups of five BALB/c mice were immunized three times at 3-week intervals by s.c. injection in the right footpad and at the base of the tail with 10 μg of Leish-111f plus 20 μg of MPL-SE. Control animals received either adjuvant alone or saline. At 3 weeks (A) or 3 months (B) after the third immunization, mice were challenged s.c. in the left footpad with 4×10^5 *L. major* promastigotes and footpad enlargement was measured weekly thereafter and is expressed as mean swelling ± the standard error of the mean.

reinfections, secondary disfiguring lesions, and chronic nonhealing lesions are not unusual. Additionally, although human vaccination is currently ongoing with first-generation antileishmania vaccines that employ heat-killed parasites and BCG, their protective efficacy is very limited. Thus, there continues to be a need for identification and validation of candidate antigens for inclusion in a protein or DNA vaccine against leishmaniasis. More than likely, effective vaccination against a complex parasitic infection such as leishmaniasis would require a multivalent vaccine composed of several antigens to enhance the possibility of covering a good number of major histocompatibility complex types.

In this report, we have shown that a vaccine composed of MPL-SE and a recombinant polyprotein containing three different *Leishmania* antigens fused in tandem induced effective protection against *L. major* and *L. amazonensis* challenges in two different strains of mice. MPL-SE at 20 μg was the most potent of the six different adjuvant formulations tested at eliciting protection against leishmaniasis in BALB/c mice. Whether the vaccine contained 2 or 10 μg of rLeish-111f or whether the challenge dose was increased from 2×10^5 to 4×10^5 parasites was not relevant with regard to the protective effect. Furthermore, this vaccine formulation conferred durable protection against leishmaniasis for up to 14 weeks. Of particular importance is the finding that susceptible BALB/c mice immunized with the Leish-111f vaccine were protected whether they were challenged 3 or 12 weeks postvaccination. Achievement of such long-lasting protection against leishmaniasis with a protein-based vaccine has not been reported previously.

Effective vaccination against *Leishmania* infection depends on the generation of antigen-specific memory T cells that, upon exposure to infecting parasites, rapidly expand as effector Th1 T cells for production of IFN-γ. In turn, IFN-γ-mediated activation of infected macrophages should lead to killing of intracellular parasites if the recall response is rapid and of sufficient magnitude to overcome the adverse effects of the *Leishmania* infection on macrophage function. Maintenance of adequate vaccine-induced memory T cells for long-term protective immunity against leishmaniasis is another important consideration. It has been argued that the maintenance of memory T cells and lasting protective immunity against leishmaniasis depends on sustained IL-12 production and periodic antigen reexposure, either through natural exposure to *Leishmania* parasites, persistence of subclinical parasites, or repeated vaccine delivery. Thus, an important consideration in leishmaniasis vaccine design is delivery in a form that is likely to persist, such as DNA or formulation with bacterium-derived or slow-release adjuvants such as *Corynebacterium parvum* or alum, respectively. Remarkably, we found that the immune protection conferred by the Leish-111f–plus–MPL-SE vaccine was systemic and maintained for more than 3 months after infection. These data stand in contrast to previous reports that durable protection against leishmaniasis was not achieved with protein-based vaccines when the challenge was delayed for 12 weeks after primary immunization (25) unless IL-12 DNA was administered concurrently (15) or unless the mice were given supplemental (every 2 weeks) treatment with the recombinant protein and/or rIL-12 (44).

Several lines of evidence indicate that the protection induced by our multicomponent vaccine was due not to a nonspecific immunostimulatory effect of the adjuvants used but rather to immune responses induced by both the sequences specific to our recombinant polyprotein antigen and the MPL-SE adjuvant. The levels of spontaneous release of IFN-γ by spleen cells from vaccinated and control mice were similar. Control mice showed no evidence of antigen-specific cellular or humoral immune responses, reduction in lesion size, or reduction in parasite burden (data not shown). The finding that Leish-111f is not highly immunogenic or protective in the absence of adjuvant suggests that this molecule does not have sufficient inherent adjuvant activity in vivo. Furthermore, the antigen-specific antibody isotype profile shifts significantly to higher levels of IgG2a on addition of MPL-SE to rLeish-111f.

Thus, the protective ability of Leish-111f is adjuvant dependent.

MPL is thought to activate monocytes-macrophages and stimulate the release of several cytokines, including IL-12, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor. Through the action of these cytokines, it is thought that T cells and APCs are recruited to local lymphoid organs, where cellular interactions occur that result in induction of strong Th1-type cellular responses characterized by increased production of IFN-γ and IL-2, as well as production of IgG2a. We are currently investigating the various cell types and associated cytokines that are recruited by the Leish-111f vaccine and that are involved in conferring durable protection against leishmaniasis.

BALB/c mice are remarkably susceptible to *L. major* and develop infections relating to the induction of an intense Th2 response. Thus, it is significant that the Leish-111f vaccine is capable of eliciting robust protection against leishmaniasis in this animal model of the disease. It has been reported that IL-12 is the key endogenous initiator of the cell-mediated immune response that underlies defense against *L. major* (47). Correlating with long-term protection, we have found that mice immunized with Leish-111f plus MPL-SE have persistent memory cells that are capable of antigen-specific restimulation for at least 6 months.

In theory, linking these three antigens in tandem increases the number of epitopes available for inducing Th1-type immune responses and protective immunity in a heterogeneous human population with diverse major histocompatibility complex types. For subunit vaccine development, induction of protective immunity will likely require responses against different epitopes, probably from several antigens. Durable immunity is likely to require the delivery of the vaccine in such a way that there is sustained antigen release or antigen persistence, to mimic, as it were, the persistent antigenic stimulation of subclinical or posthealing infection. Our Leish-111f formulation satisfies these requirements for vaccination against leishmaniasis. For these reasons, we are currently testing the Leish-111f vaccine in a nonhuman primate model and plan to initiate phase I human safety and immunogenicity trials in the near future.

ACKNOWLEDGMENTS

This work was supported by grants AI25038 and AI36810 from the National Institutes of Health and the Bill and Melinda Gates Foundation. Rhea N. Coler was supported in part by National Institutes of Health Biotechnology Fellowship grant GM08347.

REFERENCES

1. **Aebischer, T., M. Wolfram, S. I. Patzer, T. Ilg, M. Wiese, and P. Overath.** 2000. Subunit vaccination of mice against new world cutaneous leishmaniasis: comparison of three proteins expressed in amastigotes and six adjuvants. Infect. Immun. **68:**1328–1336.
2. **Barral-Netto, M., A. Barral, C. E. Brownell, Y. A. Skeiky, L. R. Ellingsworth, D. R. Twardzik, and S. G. Reed.** 1992. Transforming growth factor-beta in leishmanial infection: a parasite escape mechanism. Science **257:**545–548.
3. **Berman, J. D.** 1997. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin. Infect. Dis. **24:**684–703.
4. **Campos-Neto, A., R. Porrozzi, K. Greeson, R. N. Coler, J. R. Webb, Y. A. W. Seiky, S. G. Reed, and G. Grimaldi, Jr.** 2001. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect. Immun. **69:**4103–4108.
5. **Champsi, J., and D. McMahon-Pratt.** 1988. Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. Infect. Immun. **56:**3272–3279.
6. **Coffman, R. L., D. A. Lebman, and P. Rothman.** 1993. Mechanism and regulation of immunoglobulin isotype switching. Adv. Immunol. **54:**229–270.
7. **Coler, R. N., Y. A. Skeiky, T. Vedvick, T. Bement, P. Ovendale, A. Campos-Neto, M. R. Alderson, and S. G. Reed.** 1998. Molecular cloning and immunologic reactivity of a novel low molecular mass antigen of Mycobacterium tuberculosis. J. Immunol. **161:**2356–2364.
8. **Connell, N. D., E. Medina-Acosta, W. R. McMaster, B. R. Bloom, and D. G. Russell.** 1993. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the Leishmania surface proteinase gp63. Proc. Natl. Acad. Sci. USA **90:**11473–11477.
9. **Dole, V. S., V. S. Raj, A. Ghosh, R. Madhubala, P. J. Myler, and K. D. Stuart.** 2000. Immunization with recombinant LD1 antigens protects against experimental leishmaniasis. Vaccine **19:**423–430.
10. **Fries, L. F., D. M. Gordon, R. L. Richards, J. E. Egan, M. R. Hollingdale, M. Gross, C. Silverman, and C. R. Alving.** 1992. Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy. Proc. Natl. Acad. Sci. USA **89:**358–362.
11. **Genaro, O., V. P. de Toledo, C. A. da Costa, M. V. Hermeto, L. C. Afonso, and W. Mayrink.** 1996. Vaccine for prophylaxis and immunotherapy, Brazil. Clin. Dermatol. **14:**503–512.
12. **Ghalib, H. W., M. R. Piuvezam, Y. A. Skeiky, M. Siddig, F. A. Hashim, A. M. El Hassan, D. M. Russo, and S. G. Reed.** 1993. Interleukin 10 production correlates with pathology in human Leishmania donovani infections. J. Clin. Investig. **92:**324–329.
13. **Greenblatt, C. L.** 1980. The present and future of vaccination for cutaneous leishmaniasis. Prog. Clin. Biol. Res. **47:**259–285.
14. **Gunders, A. E.** 1987. Vaccination: past and future role in control, p. 929–941. *In* W. Peters and R. Killick-Kendrick (ed.), The leishmaniases in biology and medicine. Academic Press, Inc., New York, N.Y.
15. **Gurunathan, S., C. Prussin, D. L. Sacks, and R. A. Seder.** 1998. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nat. Med. **4:**1409–1415.
16. **Gurunathan, S., D. L. Sacks, D. R. Brown, S. L. Reiner, H. Charest, N. Glaichenhaus, and R. A. Seder.** 1997. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with Leishmania major. J. Exp. Med. **186:**1137–1147.
17. **Handman, E., F. M. Symons, T. M. Baldwin, J. M. Curtis, and J.-P. Scheerlinck.** 1995. Protective vaccination with promastigote surface antigen 2 from *Leishmania major* is mediated by a TH1 type of immune response. Infect. Immun. **63:**4261–4267.
18. **Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley.** 1989. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper T cell subsets. J. Exp. Med. **169:**59–72.
19. **Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley.** 1991. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by $CD4^+$ lymphocytes in vivo during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci. USA **88:**7011–7015.
20. **Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately.** 1993. Recombinant interleukin 12 cures mice infected with Leishmania major. J. Exp. Med. **177:**1505–1509.
21. **Jardim, A., J. Alexander, H. S. Teh, D. Ou, and R. W. Olafson.** 1990. Immunoprotective Leishmania major synthetic T cell epitopes. J. Exp. Med. **172:**645–648.
22. **Locksley, R. M., and P. Scott.** 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunol. Today **12:**A58–A61.
23. **Louis, J., H. Himmelrich, C. Parra-Lopez, F. Tacchini-Cottier, and P. Launois.** 1998. Regulation of protective immunity against Leishmania major in mice. Curr. Opin. Immunol. **10:**459–464.
24. **McMahon-Pratt, D., D. Rodriguez, J. R. Rodriguez, Y. Zhang, K. Manson, C. Bergman, L. Rivas, J. F. Rodriguez, K. L. Lohman, and N. H. Ruddle.** 1993. Recombinant vaccinia viruses expressing GP46/M-2 protect against Leishmania infection. Infect. Immun. **61:**3351–3359.
25. **Mendez, S., S. Gurunathan, S. Kamhawi, Y. Belkaid, M. A. Moga, Y. A. Skeiky, A. Campos-Neto, S. Reed, R. A. Seder, and D. Sacks.** 2001. The potency and durability of DNA- and protein-based vaccines against Leishmania major evaluated using low-dose, intradermal challenge. J. Immunol. **166:**5122–5128.
26. **Modabber, F.** 2000. First generation leishmaniasis vaccines in clinical development: moving, but what next? Curr. Opin. Anti-infect. Investig. Drugs **2:**35–39.
27. **Mougneau, E., F. Altare, A. E. Wakil, S. Zheng, T. Coppola, Z. E. Wang, R. Waldmann, R. M. Locksley, and N. Glaichenhaus.** 1995. Expression cloning of a protective leishmania antigen. Science **268:**563–566.
28. **Nadim, A., E. Javadian, G. Tahvildar-Bidruni, and M. Ghorbani.** 1983. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. Bull. Soc. Pathol. Exot. Fil. **76:**377–383.
29. **Olliaro, P. L., and A. D. M. Bryceson.** 1993. Practical progress and new drugs for changing patterns of leishmaniasis. Parasitol. Today **9:**323–328.
30. **Piedrafita, D., D. Xu, D. Hunter, R. A. Harrison, and F. Y. Liew.** 1999.

Protective immune responses induced by vaccination with an expression genomic library of Leishmania major. J. Immunol. **163:**1467–1472.
31. **Probst, P., Y. A. Skeiky, M. Steeves, A. Gervassi, K. H. Grabstein, and S. G. Reed.** 1997. A leishmania protein that modulates interleukin (IL)-12, IL-10 and tumor necrosis factor-alpha production and expression of B7–1 in human monocyte-derived antigen-presenting cells. Eur. J. Immunol. **27:**2634–2642.
32. **Rachamim, N., and C. L. Jaffe.** 1993. Pure protein from Leishmania donovani protects mice against both cutaneous and visceral leishmaniasis. J. Immunol. **150:**2322–2331.
33. **Rafati, S., A. A. Baba, M. Bakhshayesh, and M. Vafa.** 2000. Vaccination of BALB/c mice with Leishmania major amastigote-specific cysteine proteinase. Clin. Exp. Immunol. **120:**134–138.
34. **Reed, S. G., and P. Scott.** 1993. T-cell and cytokine responses in leishmaniasis. Curr. Opin. Immunol. **5:**524–531.
35. **Scharton-Kersten, T., L. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott.** 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. J. Immunol. **154:**5320–5330.
36. **Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher.** 1988. Immunoregulation of cutaneous leishmaniasis: T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J. Exp. Med. **168:**1675–1684.
37. **Scott, P., and G. Trinchieri.** 1997. IL-12 as an adjuvant for cell-mediated immunity. Semin. Immunol. **9:**285–291.
38. **Sharifi, I., A. R. FeKri, M. R. Aflatonian, A. Khamesipour, A. Nadim, M. R. Mousavi, A. Z. Momeni, Y. Dowlati, T. Godal, F. Zicker, P. G. Smith, and F. Modabber.** 1998. Randomised vaccine trial of single dose of killed Leishmania major plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. Lancet **351:**1540–1543.
39. **Sjolander, A., T. M. Baldwin, J. M. Curtis, and E. Handman.** 1998. Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response are required for generation of immunity to leishmaniasis. J. Immunol. **160:**3949–3957.
40. **Skeiky, Y. A., J. A. Guderian, D. R. Benson, O. Bacelar, E. M. Carvalho, M. Kubin, R. Badaro, G. Trinchieri, and S. G. Reed.** 1995. A recombinant leishmania antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12. J. Exp. Med. **181:**1527–1537.
41. **Skeiky, Y. A., M. Kennedy, D. Kaufman, M. M. Borges, J. A. Guderian, J. K. Scholler, P. J. Ovendale, K. S. Picha, P. J. Morrissey, K. H. Grabstein, A. Campos-Neto, and S. G. Reed.** 1998. LeIF: a recombinant leishmania protein that induces an IL-12-mediated Th1 cytokine profile. J. Immunol. **161:**6171–6179.
42. **Solioz, N., U. Blum-Tirouvanziam, R. Jacquet, S. Rafati, G. Corradin, J. Mauel, and N. Fasel.** 1999. The protective capacities of histone H1 against experimental murine cutaneous leishmaniasis. Vaccine **18:**850–859.
43. **Stager, S., D. F. Smith, and P. M. Kaye.** 2000. Immunization with a recombinant stage-regulated surface protein from Leishmania donovani induces protection against visceral leishmaniasis. J. Immunol. **165:**7064–7071.
44. **Stobie, L., S. Gurunathan, C. Prussin, D. L. Sacks, N. Glaichenhaus, C. Y. Wu, and R. A. Seder.** 2000. The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc. Natl. Acad. Sci. USA **97:**8427–8432.
45. **Sypek, J. P., C. L. Chung, S. E. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. F. Wolf, and R. G. Schaub.** 1993. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J. Exp. Med. **177:**1797–1802.
46. **Thoelen, S., P. Van Damme, C. Mathei, G. Leroux-Roels, I. Desombere, A. Safary, P. Vandepapeliere, M. Slaoui, and A. Meheus.** 1998. Safety and immunogenicity of a hepatitis B vaccine formulated with a novel adjuvant system. Vaccine **16:**708–714.
47. **Trinchieri, G., and P. Scott.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. Res. Immunol. **146:**423–431.
48. **Walker, P. S., T. Scharton-Kersten, E. D. Rowton, U. Hengge, A. Bouloc, M. C. Udey, and J. C. Vogel.** 1998. Genetic immunization with glycoprotein 63 cDNA results in a helper T cell type 1 immune response and protection in a murine model of leishmaniasis. Hum. Gene Ther. **9:**1899–1907.
49. **Webb, J. R., A. Campos-Neto, P. J. Ovendale, T. I. Martin, E. J. Stromberg, R. Badaro, and S. G. Reed.** 1998. Human and murine immune responses to a novel *Leishmania major* recombinant protein encoded by members of a multicopy gene family. Infect. Immun. **66:**3279–3289.
50. **Webb, J. R., D. Kaufmann, A. Campos-Neto, and S. G. Reed.** 1996. Molecular cloning of a novel protein antigen of Leishmania major that elicits a potent immune response in experimental murine leishmaniasis. J. Immunol. **157:**5034–5041.
51. **Xu, D., and F. Y. Liew.** 1995. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of L. major. Immunology **84:**173–176.

Editor: B. B. Finlay