

## gp63 in Stable Cationic Liposomes Confers Sustained Vaccine Immunity to Susceptible BALB/c Mice Infected with *Leishmania donovani*<sup>∇</sup>

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Visceral leishmaniasis is deadly if not treated, and development of a vaccine with long-term immunity remains a challenge. In this study, we showed that cationic distearoyl phosphatidylcholine (DSPC) liposomes, when used as vaccine adjuvant with the immunodominant 63-kDa glycoprotein (gp63) of *Leishmania donovani* promastigotes, induced significant protection against progressive visceral leishmaniasis in susceptible BALB/c mice. gp63 used without adjuvant elicited partial protection but in association with liposomes exhibited marked resistance in both the livers and spleens of the mice challenged 10 days after the last vaccination. The protective efficacy of liposomal gp63 vaccination was dose dependent, with 2.5 μg of protein showing optimal protection. The immunity conferred by this vaccine formulation was durable, as mice challenged 12 weeks after immunization were still protected, and the infection was controlled for at least 3 months postchallenge. Production of gamma interferon (IFN-γ) and interleukin-4 (IL-4) by splenic T cells, and of serum immunoglobulin G1 (IgG1) and IgG2a following immunization, suggested that a mixed Th1/Th2 response had been induced following immunization. However, control of disease progression and parasitic burden in mice vaccinated with gp63 in cationic DSPC liposomes was associated with enhancement of antigen-specific IFN-γ and downregulation of IL-4, demonstrating a Th1 bias. Long-term immunity elicited by this vaccine corresponded to, in addition to the presence of antigen-specific Th1, CD8<sup>+</sup> T-cell responses. Our results demonstrated that stable cationic liposomes containing gp63 acted as a potent adjuvant for protein antigen to induce long-term protection against *L. donovani* that represents an alternative to DNA vaccination.

Leishmaniasis is an infectious disease complex caused by several species that are members of the protozoan parasite genus *Leishmania*. In humans, disease manifestation ranges from self-healing cutaneous lesions to life-threatening visceral leishmaniasis (VL). This disease complex affects 12 million people, and there are 1.5 million new cases annually (42). VL, caused by *Leishmania donovani* and *Leishmania chagasi*, remains the main agent of morbidity and mortality in leishmaniasis, with hundreds and thousands of people dying in recent epidemics in Sudan and India.

The parasite has a simple life cycle, and abundant clinical and experimental evidence indicates that of all the parasitic diseases, leishmaniasis in particular should be an appropriate target for effective control through vaccination. There are, however, no vaccines in routine use against any form of the disease (29, 42). The fundamental basis of vaccination is the generation and maintenance of an antigen-specific immune response sufficient to mediate protection upon infectious challenge. Currently available vaccines against a variety of infectious diseases mediate protection by a long-lived humoral response through the production of antibodies. For diseases such as tuberculosis, malaria, human immunodeficiency virus infection, and leishmaniasis, however, the cellular immune response comprising primarily Th1 and CD8<sup>+</sup> effector T cells has been

shown to be critical for mediating protection against infection (60). In experimental models of cutaneous leishmaniasis (CL) in which CD4-positive (CD4<sup>+</sup>) Th responses are driven toward a polarized Th1, protection can be achieved by vaccination. But such vaccines do not generate potent and durable responses, and protection wanes after a few weeks (14, 18). Induction of long-term protection against leishmaniasis requires the generation of memory T cells, probably of both CD4<sup>+</sup> and CD8<sup>+</sup> lineages (16, 27, 77). These are maintained by the continued presence of antigens through the activity of live persistent parasites or DNA vaccines (2, 15, 69). Thus, deliberate inoculation of virulent organisms by a process known as leishmanization is still used in some countries and remains the gold standard for vaccination and life-long protection (30). However, logistic problems of safety and manufacturing considerations restrict the use of this procedure, and DNA vaccines are far less potent for inducing immune responses in humans than in rodents (15). One recent approach for generating sustained cellular immunity in vivo against leishmanial proteins is the use of Toll-like receptor agonists as nonreplicating vaccine adjuvants. CpG oligonucleotides, which represent one of the potent inducers of innate and adaptive immunity, are effective when high doses of antigens are administered (39, 53). Therefore, there remains a need for new nonreplicating vaccine adjuvants capable of eliciting strong cellular immune responses.

Cationic liposomes have been shown to markedly potentiate activation of immune response to plasmid DNA and oligonucleotides (24, 75). More recently, the induction of cell-mediated immune response to poorly immunogenic protein and peptide antigens has been made possible through the use of positively charged liposome carriers (5, 44). Although these

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vesicles alone are relatively nonimmunogenic, their adjuvant potency toward the associated antigen is due in part to the efficient delivery of the antigen to professional antigen-presenting cells (APCs), including macrophages and dendritic cells (11, 45). It is well established that liposomes channel protein and peptide antigens into the major histocompatibility complex class II pathways of APCs, resulting in enhanced antibody and antigen-specific T-cell proliferative response (50). In addition, there have been reports of the use of cationic liposomes for the generation of CD8<sup>+</sup> T-cell response, which requires antigen presentation in the context of the major histocompatibility complex class I pathway (5, 17, 44). Stable liposomes formulated with distearoyl phosphatidylcholine (DSPC), a saturated phospholipid with a high transition temperature and with cholesterol, reduced clearance from blood and enhanced cationic lipid-mediated endosomal membrane destabilization (17, 73). Thus, we believe that cationic DSPC liposomes with cholesterol would not only deliver antigens more efficiently to the cytosol for eliciting a CD8<sup>+</sup> T-cell response but would also have the added advantage of persistent antigen presentation through prolonged circulation, enabling the possibility of durable immunity induced by protein-based vaccination.

Although genome-sequencing projects now provide a plethora of potential vaccine candidates for treatment of leishmaniasis, few criteria exist for selecting among them, and choosing the antigen remains a largely empirical procedure (42). Extensive studies in the last decade have, nonetheless, led to the identification of a number of defined protein antigens having significant vaccine potential. These studies, however, have focused almost entirely on treatment of CL, and VL has remained a neglected disease. However, the limited experimental and clinical evidence available indicates that the same vaccine/antigen might not work for all cases of leishmaniasis (29, 37). For example, differences have been observed with the 63-kDa (gp63) glycoprotein and with the *Leishmania* homologue of the receptor for activated C kinase (LACK), antigens that have received maximum attention in studies of CL treatment (36). Although vaccination with gp63, in its protein and DNA forms, has been largely unsuccessful or only partially protective, immunization with LACK induced strong protective and durable immunity against *L. major* (36). Interestingly, *L. donovani* LACK failed to induce protection against VL (37) and induced no response in the peripheral blood mononuclear cells of individuals cured of *L. infantum* or *L. donovani* infection (33). Whereas LACK is an essential molecule for *L. major* survival in mammalian hosts (28), gp63 expression corresponds to avirulence in these parasites (25, 26, 43). Conversely, gp63 corresponds to the infective stage and virulence of diffused cutaneous and visceralizing species of *Leishmania* (67, 72). Moreover, T cells from patients who had recovered from leishmaniasis responded in vitro to gp63 (57, 58). Further, kala-azar patients responded largely through a humoral response directed toward gp63 (32, 51, 52). gp63 could thus be the key antigen for vaccination against VL, but apart from our preliminary report (1), no vaccine study of any form of *L. donovani* gp63 has been carried out for this disease.

In this study, we investigated whether gp63 purified from *L. donovani* formulated in cationic DSPC liposomes could induce a protective response to a visceral disease in BALB/c mice. We

report that *L. donovani* gp63 is highly immunogenic in association with these vesicles and can prevent infection in virulent challenges using *L. donovani* parasites not only after a short vaccination protocol but also 12 weeks after immunization. Interestingly, we find that the protective immune response induced by gp63 contained within liposomes is dependent on the dose of the antigen, with the optimum dose eliciting maximum immunogenicity and protection. Our report further demonstrates sustained immunity through the use of gp63-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells that control chronic infection.

## MATERIALS AND METHODS

**Reagents and antibodies.** M199 medium, RPMI 1640 medium, HEPES, penicillin-streptomycin, sodium bicarbonate, glutamine, fetal bovine serum (FBS), leupeptin, EDTA, iodoacetamide, phenylmethylsulfonyl fluoride, octyl- $\beta$ -D-glucopyranoside,  $\alpha$ -methyl-D-mannoside, Zwittergent, DEAE-cellulose, complete Freund's adjuvant, incomplete Freund's adjuvant, DSPC, bovine serum albumin, 3,3'-diaminobenzidine tetrahydrochloride,  $\beta$ -mercaptoethanol, sulfanilamide, *N*-(1-naphthylethylene diamine hydrochloride), *o*-phenylene diamine dihydrochloride, Tween 20, brefeldin A, sodium azide, saponin, paraformaldehyde, and poly-L-lysine were purchased from Sigma-Aldrich, St. Louis, MO. Cholesterol and stearylamine were obtained from the Center for Biotechnology, New Delhi, India, and Fluka, Switzerland, respectively. Concanavalin A (ConA)-Sephadex resin was obtained from Amersham Biosciences, Piscataway, NJ. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G1 (IgG1) and IgG2a, fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAbs) to mouse CD4 and CD8, phycoerythrin-labeled anti-mouse gamma interferon (IFN- $\gamma$ ) and interleukin-4 (IL-4), and Perm-2 buffer were purchased from BD Biosciences, San Diego, CA.

**Animals and parasites.** BALB/c mice, reared in the animal care facility of the institute under pathogen-free conditions, were used at 4 to 6 weeks for experimental purposes with prior approval from the Animal Ethics Committee of the Indian Institute of Chemical Biology. An Indian strain of *L. donovani* (MHOM/IN/83/AG83) was maintained by passage in Syrian hamsters. Amastigotes were isolated from infected hamster spleens and allowed to transform to promastigotes by cultivation at 22°C in M199 medium (pH 7.4) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml of penicillin G-sodium and 100  $\mu$ g/ml of streptomycin sulfate. Parasites were checked and enumerated by counting in a hemocytometer on day 6 after first the transformation and every 72 h thereafter. Designated parasites from stationary-phase cultures were diluted in fresh medium with the same composition as that mentioned above to maintain an average density of  $2 \times 10^6$  cells/ml (34).

**Isolation of gp63.** Soluble leishmanial antigens (SLAg) were extracted from stationary-phase *L. donovani* promastigotes harvested after the second passage as detailed earlier (1). Briefly,  $1 \times 10^{10}$  cells were suspended in cold 5 mM Tris-HCl buffer (pH 7.6) containing 5  $\mu$ g/ml of leupeptin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide (lysis buffer). The suspension was vortexed six times for 2 min each time with a 10-min interval on ice. The parasite suspension was then centrifuged at  $2,310 \times g$  for 10 min, and the pellet was collected. The crude ghost-membrane pellet was resuspended in 10 ml of the same buffer and sonicated three times for 1 min each time on ice. The suspension thus obtained was solubilized with lysis buffer containing 1% (wt/vol) octyl- $\beta$ -D-glucopyranoside overnight at 4°C and was finally ultracentrifuged for 1 h at  $100,000 \times g$  (1). The supernatant containing SLAg was used for purification of gp63 as described previously (55) with slight modifications. Two milligrams of SLAg was applied at a flow rate of 3 ml/h to 1 ml ConA-Sephadex resin preequilibrated in the buffer used for SLAg preparation containing 1% octyl- $\beta$ -D-glucopyranoside and maintained at 4°C. Unbound protein was washed off the column with the same buffer, and bound material was eluted at 10 ml/h with linear 0 to 0.5 M  $\alpha$ -methyl-D-mannoside gradient in wash buffer. The amount of protein was estimated in the fractions (0.5 ml) collected during elution and analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (1). The gp63-containing peak fractions were pooled, concentrated, and dialyzed against 5 mM Tris-HCl (pH 8.5) with 0.2% Zwittergent 3.12. The sample (800  $\mu$ g of protein) was run on a 5-ml DEAE-cellulose column that was pre-equilibrated in the same buffer used for dialysis. Under these conditions the anion exchanger readily bound gp63. Following thorough washing, the bound protein was eluted with a gradient of 0 to 500 mM NaCl in the same buffer at a flow rate of 6 ml/h. Peak fractions obtained with NaCl elution were run on 10% SDS-PAGE, and the gel was stained with ammoniacal silver nitrate solution (74).

The purity of the protein was further confirmed by two-dimensional PAGE (46), which included staining with ammoniacal silver nitrate solution.

Pure gp63 was used to raise antiserum in a rabbit (immunization with 150  $\mu$ g of gp63 in complete Freund's adjuvant and boosting twice with 100  $\mu$ g of protein in incomplete Freund's adjuvant). Native gp63 was obtained in mass by incubating SLAg with anti-gp63 rabbit IgG coupled to CNBr-activated Sepharose. The protein was subjected to NH<sub>2</sub>-terminal sequencing using a Procise sequence analyzer (Applied Biosystems).

**Entrapment of gp63 in liposomes.** Liposomes containing gp63 were prepared by detergent dialysis method as described earlier (55, 56) with slight modification. DSPC, cholesterol, and stearylamine (at a molar ratio of 7:2:2) mixed with octyl- $\beta$ -D-glucopyranoside (15 mg/10 mg lipids) were dissolved in chloroform and dried to produce a thin film. gp63 (100 to 120  $\mu$ g) isolated from an anti-gp63 immunoaffinity column was added to 10 mg lipid film and sonicated in an ultrasonicator for 30 s. The lipid-protein mixture was dialyzed overnight to remove detergent, and excess free antigen was removed by centrifugation at  $100,000 \times g$  for 1 h at 4°C. The level of incorporation ranged between 80 and 85%.

**Injection of mice.** BALB/c mice were immunized by three intraperitoneal (i.p.) injections at 2-week intervals with graded doses (0.6 to 10  $\mu$ g) or 2.5  $\mu$ g of gp63 free in phosphate-buffered saline (PBS) or entrapped in liposomes (200  $\mu$ l). Animals receiving only PBS or empty liposomes served as controls. At 10 days or 12 weeks postimmunization, groups of mice were either sacrificed for immunological assays or challenged intravenously with  $2.5 \times 10^7$  freshly transformed *L. donovani* promastigotes (34).

The protective capacity of spleen cells from mice that had been vaccinated with PBS or with free gp63 or gp63 in association with liposomes was assessed by adoptive transfer experiments (69). Single-cell suspensions of 20 million pooled splenocytes from three mice from each experimental group were injected i.p. into syngeneic BALB/c mice, and infections were detected 7 days later and monitored for 3 months.

**Delayed-type hypersensitivity (DTH).** Levels of DTH were determined as an index of cell-mediated immunity as described earlier (1). The response was evaluated at 24 h by measuring the difference in swelling between two hind footpads, one injected with 50  $\mu$ l of PBS alone and the other with PBS containing *L. donovani* membrane antigens (LAg) (800  $\mu$ g/ml).

**Measurement of gp63-specific antibody responses.** Serum samples from individual mice of each group were obtained before and after infection and analyzed for the presence of gp63-specific antibodies. Ninety-six-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with gp63 (5  $\mu$ g/ml) diluted in 0.02 M phosphate buffer (pH 7.5) overnight at 4°C. Plates were blocked with 1% bovine serum albumin in PBS at room temperature for 3 h to prevent nonspecific binding. After being washed with PBS containing 0.05% Tween 20, the plates were incubated overnight with a 1:100 dilution of mice serum samples at 4°C. The next day, the plates were incubated for 3 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a diluted 1:1,000 in blocking buffer. Substrate solution (0.8 mg/ml *o*-phenylene diamine dihydrochloride–0.05 M phosphate-citrate buffer [pH 5.0] containing 0.04% H<sub>2</sub>O<sub>2</sub>) (100  $\mu$ l) was added for 30 min, and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystems Multiscan MS, Finland) at 450 nm (1).

**Immunoblot analysis.** Four micrograms of gp63 were subjected to 10% SDS-PAGE and electrophoretically transferred to nitrocellulose for immunoblotting. Immunoblot assays were performed as described previously (54) with slight modifications. The nitrocellulose strips were first saturated and then blocked overnight at room temperature in 100 mM Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 and washed once for 15 min with 0.05% Tween 20 in 100 mM Tris-buffered saline (pH 7.6) (washing buffer) with shaking. Incubation of the nitrocellulose strips with a 1:500 dilution of serum samples from mice immunized with gp63 in liposomes, free gp63, or PBS was carried out at room temperature followed by three washes performed for 20 min per wash. The blots were then washed and incubated with peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) at a 1:500 dilution in the washing buffer for 1 h at room temperature; this was followed by three washes performed as described above. The last wash was done without Tween 20. Enzymatic activity was revealed by use of 15 mg of 3,3'-diaminobenzidine tetrahydrochloride in 30 ml of Tris-buffered saline (pH 7.6) containing 15  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub>.

For immunoblotting of macrophage membrane fractions, macrophages from the vaccinated mice were isolated in ice-cold PBS and suspended at a concentration of  $2 \times 10^6$  cells in 1 ml of 5 mM cold Tris-HCl buffer (pH 7.6). The macrophage membrane fractions were prepared as described previously (34) for LAg isolation from *L. donovani* promastigotes. Immunoblotting was carried out as described above using rabbit anti-gp63 IgG (1:500 dilution) as the first anti-

body and peroxidase-conjugated anti-rabbit IgG (1:1,500 dilution) as the second antibody (Sigma-Aldrich).

**Evaluation of infection.** At 3 months postchallenge, the course of infection was evaluated by microscopic examination of Giemsa-stained impression smears of liver and spleen samples. The parasite load was expressed in Leishman-Donovan units and was calculated by the following formula: number of amastigotes per 1,000 cell nuclei  $\times$  organ weight (in milligrams) (34).

**Macrophage infections.** Macrophages collected by peritoneal lavage from immunized BALB/c mice 10 days after the last booster injection were allowed to adhere to glass coverslips (18 mm<sup>2</sup>; 10<sup>6</sup> macrophages/coverslip) in 0.5 ml RPMI 1640 containing 10% FBS at 37°C in a humidified chamber containing 5% CO<sub>2</sub>. After 2 h, the nonadherent cells were removed by washing with warm PBS and adhered macrophages were infected with promastigotes at a ratio of approximately 10 cells per macrophage for another 3 h. The unphagocytosed parasites were removed by PBS washing, and the infected macrophages were further incubated in complete medium at 37°C for 72 h. The cells were then fixed in methanol and stained with Giemsa for determination of intracellular parasite numbers. Prior to fixation, culture supernatant fluids were removed at 72 h and frozen at –70°C for future analysis (34).

**gp63-specific cytokine production.** Spleens were removed aseptically from individual mice of each group at 10 days or 12 weeks after vaccination and at 3 months postinfection. Each spleen was filtered through a 20- $\mu$ m-pore-size sieve for preparation of a single-cell suspension in complete medium prepared with RPMI 1640 containing 10 mM NaHCO<sub>3</sub>, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 50  $\mu$ M  $\beta$ -mercaptoethanol, and 10% FBS. Erythrocytes were removed by lysis with 0.14 M Tris-buffered NH<sub>4</sub>Cl. The splenocytes were washed twice and resuspended in the culture medium, and viable mononuclear cell numbers were determined by trypan blue exclusion (1). Aliquots of viable cells were incubated with anti-CD4 and anti-CD8 monoclonal antibodies or the respective control IgGs at a concentration of 1  $\mu$ g/million cells for 1 h at 4°C and washed twice in complete medium. The efficiency of depletion was checked for each experiment by flow cytometry. Total and CD4- or CD8-depleted splenocytes (14) were stimulated in vitro with medium alone or with purified native gp63 (1.25  $\mu$ g/ml) for 72 h. The supernatants collected were stored at –70°C for cytokine analysis.

Measurement of IFN- $\gamma$ , IL-4, and IL-12 levels was carried out as detailed in the instructions supplied with a cytokine ELISA kit (BD Biosciences). The lower limits of detection for IFN- $\gamma$ , IL-4, and IL-12 were 15 pg/ml, 7.8 pg/ml, and 7.8 pg/ml, respectively.

**Measurement of NO production.** Nitric oxide (NO) levels, quantified by the accumulation of nitrite in the culture medium, were measured as described previously (34). Briefly, 100  $\mu$ l of culture supernatants was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylene diamine hydrochloride in 50% H<sub>3</sub>PO<sub>4</sub>) and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured. The amount of nitrite released was quantified by comparison with the NaNO<sub>2</sub> level as the standard.

**Flow cytometry.** All the monoclonal antibodies used were purchased from BD Biosciences. Appropriately labeled isotype controls and single- or double-color-stained cells were always used to define the specific gates. Single-cell splenocyte suspensions were stimulated overnight with 5  $\mu$ g/ml gp63 or left unstimulated. Following incubation with brefeldin A (10  $\mu$ g/ml) for 4 h, cells were washed with PBS–1% FCS–0.01% sodium azide, blocked with 10% normal mouse serum, and incubated with FITC-conjugated anti-CD4 or CD8 MAbs at 4°C for 45 min. For intracellular staining, cells were permeabilized with Perm-2 followed by washing with the above-described buffer containing 0.1% saponin and were then reacted with phycoerythrin-conjugated anti-IFN- $\gamma$  or anti-IL-4 MAbs. After proper washing, cells were fixed in 4% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. Typically, 0.5 million cells were analyzed per sample. Specific confidence intervals at a *P* value of  $\leq 0.05$  are noted in the text.

**Microscopy.** An aliquot (40 to 50  $\mu$ l) of *L. donovani* promastigotes or amastigotes ( $2$  to  $4 \times 10^6$ /ml) in PBS was immobilized on poly-L-lysine-coated coverglasses at 4°C for 30 min. Peritoneal macrophages from liposomal gp63-immunized or normal mice were collected and allowed to adhere on glass coverslips for 2 h at 37°C as described above for macrophage infections.

For confocal microscopy, the cells were fixed with 1% formaldehyde in PBS for 15 min at room temperature. Following PBS washing and blocking with 3% bovine serum albumin for 30 min, cells were reacted with FITC-anti-gp63 IgG or FITC-normal IgG (20  $\mu$ g/ml) for 30 min at room temperature. After the last washing, cells were mounted using 10% glycerol–PBS and immediately viewed and photographed using a TCS-SP Leica (Heidelberg, Germany) confocal microscope system.



**Statistical analysis.** Data comparisons were tested for significance by using an unpaired, two-tailed Student's *t* test and two-way or one-way analysis of variance (ANOVA) as required. *P* values of <0.05 were considered significant.

## RESULTS

***L. donovani* gp63 isolation and characterization.** We purified gp63 from *L. donovani* promastigotes through three steps (detergent solubilization, ConA-Sepharose affinity chromatography, and DEAE-cellulose ion-exchange chromatography) (Fig. 1A) and confirmed the purity of the protein by two-dimensional PAGE (Fig. 1B). Recombinant technology offers easy large-scale isolation of proteins, but it involves the loss of the native structure of the molecules. Expression of functional gp63 requires extensive posttranslational modifications, which are absent when gp63 expression is generated externally (35). The native structure of gp63 has been reported to be important for vaccination, as recombinant gp63 either failed to induce protection or induced only partial protection compared to the better protection observed with native protein (19, 22, 56). Hence, in our vaccination experiment we used native gp63, which was obtained in mass by incubating SLAg with anti-gp63 rabbit IgG coupled to CNBr-activated Sepharose. The sequence of gp63 from *L. donovani* strain AG83 differs in two amino acids from predicted gp63 sequences of *L. donovani*, *L. chagasi*, and *L. infantum* (the species causing VL), three from that of *L. amazonensis*, four from that of *L. major* native gp63, and more from those of other cutaneous and mucocutaneous *Leishmania* species with respect to their 16 NH<sub>2</sub>-terminal amino acid sequences (Fig. 1B) (76). These differences may be of significance for the immunogenicity of the protein, as a single amino acid substitution in this region can cause drastic change in the molecule or alter the nature of the immune response (35, 57). We also demonstrated the presence of gp63 on both promastigote and amastigote forms of the parasite (Fig. 1C) by immunofluorescence, further identifying it as a good candidate molecule for the study of vaccine efficacy.

***L. donovani* gp63 encapsulated in liposomes protects BALB/c mice in a dose-dependent manner.** The dose level of a foreign antigen determines the outcome of an immune response irrespective of its nature and route of immunization and might be a crucial factor in optimizing long-term T-cell immunity (3, 49, 60). To address the possible contribution of the level of a gp63 dose, we immunized mice with graded doses of gp63 (free or entrapped in liposomes) and infected them 10 days after the last immunization. Protection induced by liposomal gp63 in the liver was higher (*P* < 0.05, as determined by two-way ANOVA) at all doses compared to that obtained with free gp63 (Fig. 2A). Mice receiving 2.5 μg gp63 in liposomes acquired the highest resistance to hepatic infection with *L. donovani* (86%; *P* < 0.05) compared to control results. Immunizing mice with liposomal gp63 at lower (0.6 and 1.25 μg) and higher (5 and 10 μg) doses led to significantly higher (as determined by one-way ANOVA) parasitic burden than a dose of 2.5 μg. Similarly, free gp63 conferred maximum (55%) protection at the intermediate dose of 2.5 μg, and higher and lower doses resulted in increased parasitemia. In the spleen, the protection, although higher when liposomal gp63 was used, demonstrated statistical significance at doses of 1.25 and 2.5 μg compared to free gp63 results. A dose of 2.5 μg of gp63 in

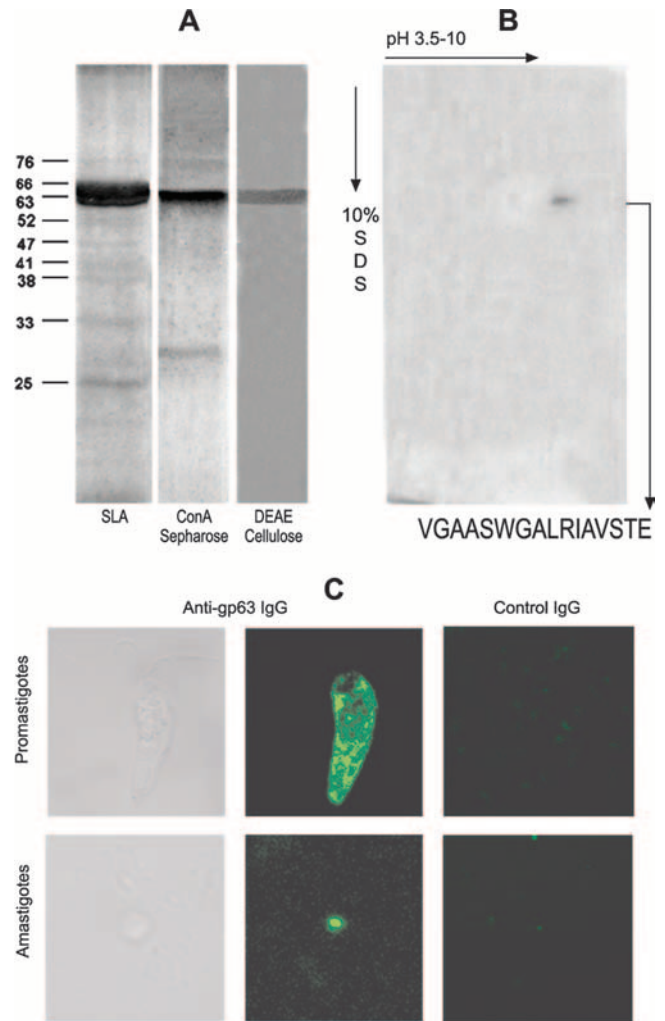


FIG. 1. Purification and characterization of *L. donovani* gp63. SLAg (SLA) from *L. donovani* promastigotes was subjected to affinity chromatography on ConA-Sepharose resin equilibrated with 5 mM Tris HCl (pH 7.6)–0.1% octyl-β-D-glucopyranoside, and the bound proteins were eluted in the same buffer with 0 to 500 mM linear methyl-α-D-mannopyranoside gradient. A pool of enriched ConA-Sepharose fractions was further loaded onto a DEAE-cellulose column equilibrated with 5 mM Tris-HCl (pH 8.5)–0.2% Zwittergent 3.12. The elution of gp63 was performed with a linear gradient of 0 to 500 mM NaCl. (A) Silver-stained 10% SDS-PAGE gels showing (from left to the right) SLAg (6 μg), proteins eluted from ConA Sepharose resin (6 μg), and purified gp63 (4 μg) through use of a DEAE-cellulose column. The numbers at the left represent apparent molecular masses in kilodaltons. (B) Two-dimensional PAGE of purified gp63 (4 μg) stained with ammoniacal silver nitrate solution. The arrow at the right side of the panel indicates the amino acid sequence of native gp63 at the NH<sub>2</sub> terminus. (C) *L. donovani* promastigotes and amastigotes were fixed in formaldehyde followed by staining with 20 μg/ml rabbit anti-gp63 FITC-IgG and studied by both phase-contrast and confocal microscopy. Cells treated with identical concentrations of normal rabbit-FITC IgG served as controls. The photographs are representative of the results of one of three similar studies.

liposomes induced the highest (81%) resistance to *L. donovani* compared to the results seen with lower and higher doses of antigen (*P* < 0.05). Dose-dependent protection in BALB/c mice correlated with cell-mediated immunity, as assessed by

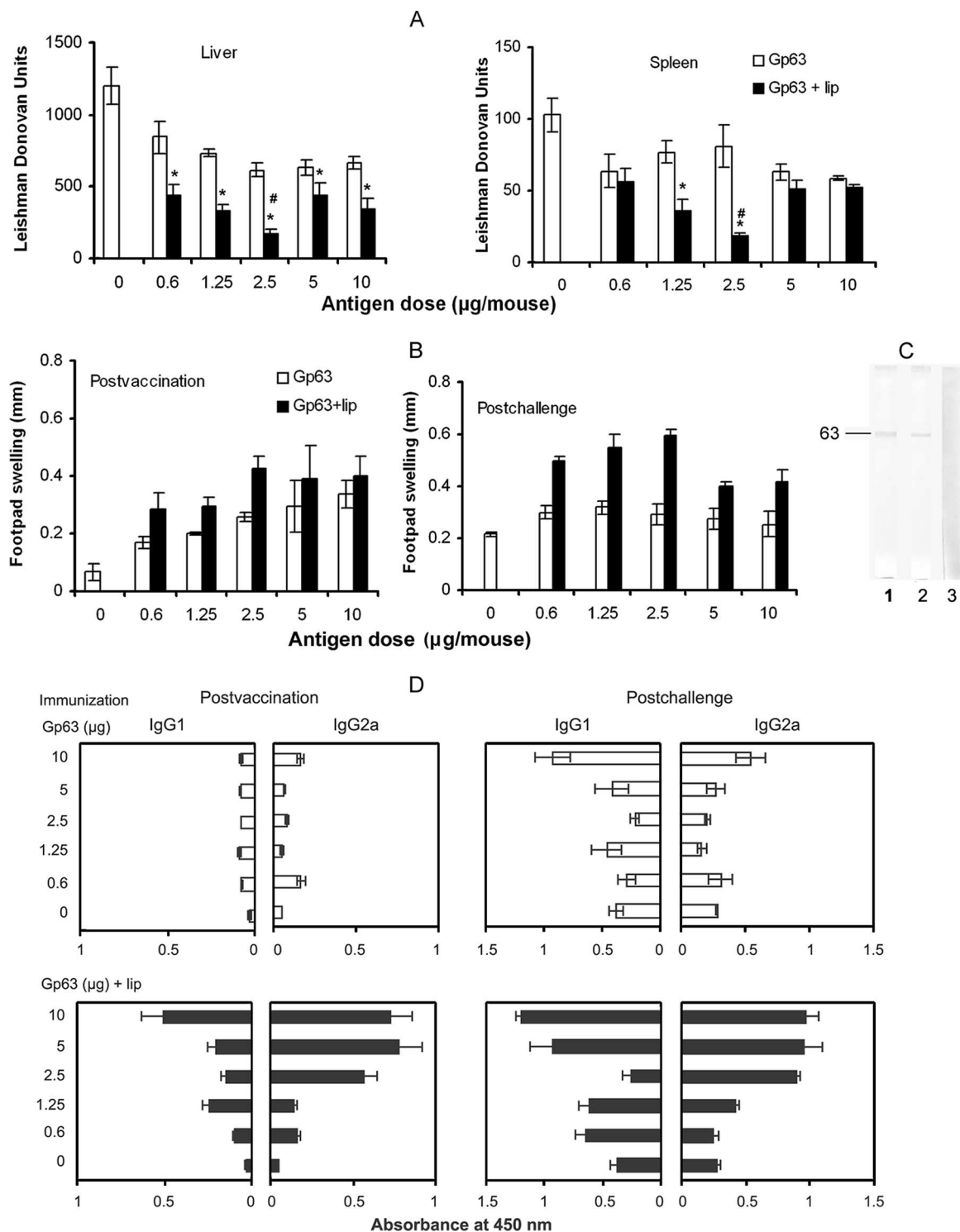


FIG. 2. Dose-dependent immune response to infection with *L. donovani* elicited by the use of gp63 in positively charged liposomes (lip). Mice were immunized i.p. with graded doses of gp63, free or in association with liposomes, and 10 days after the last immunization they were challenged intravenously with  $2 \times 10^7$  freshly transformed promastigotes. (A) Parasite burdens in the liver and spleen at 3 months postinfection. (B) LAg-specific DTH responses in immunized and challenged mice expressed as the differences (in millimeters) between the thicknesses of the test (LAg-injected) and control (PBS-injected) footpads. (C) Reactivity of gp63 with vaccinated mouse serum samples. Blots of gp63 (4 µg) were probed with 1:500 dilutions of serum samples from mice immunized with liposome-encapsulated gp63 (lane 1), free gp63 (lane 2), and PBS (lane 3). (D) Serum IgG1 and IgG2a antibody responses in vaccinated mice before and after *L. donovani* challenge were determined by ELISA using gp63-coated microtiter plates incubated overnight with a 1:100 dilution of sera as described in Materials and Methods. Data represent means  $\pm$  standard errors of the means of the results obtained with four individual mice. \*, ANOVA and Tukey's tests showed significant decreases in parasite load for mice immunized with different doses of liposome-encapsulated gp63 versus free gp63 ( $P < 0.05$ ). The results obtained in comparisons of the maximum decreases in parasite load in mice immunized with 2.5 µg gp63 in liposomes to the results obtained with other doses were statistically significant ( $P < 0.05$ ) (#, ANOVA and Tukey's tests).

DTH response (Fig. 2B). We found a gradual increase in DTH response with increasing doses of free gp63 after 10 days of vaccination, but the responses became comparable at all doses after 3 months of infection. Immunization with 2.5  $\mu$ g liposomal gp63 induced the highest level of DTH before, as well as after, challenge infection, correlating with the highest resistance observed in this group. DTH levels dropped in mice with doses lower and higher than 2.5  $\mu$ g gp63 in liposomes. As DTH response correlates with cell-mediated immunity, its fall thus explains the reduced protection observed for groups of mice. Dose-dependent modulation of the humoral response was also observed in our vaccine study. Free gp63 induced a smaller amount of antibodies after immunization and an increased amount after infection (Fig. 2C). Following immunization with lower (0.6 and 1.25  $\mu$ g) doses of liposomal gp63, higher IgG1 and IgG2a levels were observed, more significantly in postchallenge serum samples. The intermediate dose (2.5  $\mu$ g) that corresponded to effective protection induced substantially higher levels of gp63-specific IgG2a than of IgG1. Further increases in antigen dose levels led to increases in IgG1 production. Consistent with previous observations (3), our results suggest that a low, 2.5- $\mu$ g dose of liposomal gp63 induces DTH, probably mediated by Th1 (as evidenced by IgG2a production), corresponding with protection. Higher doses initiate, in addition, induction of IgG1 antibodies, demonstrating a mixed Th1/Th2 response and a decline in resistance. Doses lower than 2.5  $\mu$ g are insufficient to promote cell-mediated responses and produce only low levels of IgG1 antibodies, probably by weak TCR ligation, which might result in differentiation of CD4 T cells with respect to Th2 (7). Our results therefore demonstrate that optimization of the antigen dose is crucial for designing a vaccine against such slowly replicating organisms.

**Vaccination with liposomal gp63 confers durable protection in BALB/c mice following challenge with *L. donovani*.** In contrast to the natural resistance to hepatic infection reported for other strains of *L. donovani* in infections of BALB/c mice (10), infection with *L. donovani* AG83 results in a progressive multiplication of parasites in the liver and spleen, causing pronounced hepatosplenomegaly (34). In this report we have shown that i.p. administration of leishmanial antigens (LAg) in liposomes was sufficient to confer protection in this susceptible BALB/c model (34). Immunization with the optimal dose of liposomal gp63 (2.5  $\mu$ g), an immunodominant component of LAg (1), further demonstrated that induction of protection in the liver and spleen was possible through use of this defined antigen when mice were challenged 10 days postvaccination (Fig. 3A). Protection induced by this vaccine also demonstrated a significant reduction ( $P < 0.05$ ) in the organ weight compared to the results seen with animals receiving only PBS or empty liposomes. In contrast, immunization with gp63 alone could arrest only hepatomegaly (Fig. 3A). Spleen cells of gp63-liposome-immunized animals were indeed immunogenic, as adoptive transfer of these cells to naive mice resulted in demonstrable reduction in parasite burden and organ weight at 3 months postinfection (Fig. 3B).

We next determined the durability of the immune response induced by vaccination with liposomal gp63. To this end, mice immunized for short-term protection were infected 12 weeks postvaccination and killed 3 months postinfection. Immunity

elicited by liposomal gp63, but not gp63 or liposome alone, was durable, with enhanced resistance even when mice were challenged 12 weeks after vaccination (Fig. 3C). Moreover, mice (10 per group) immunized with gp63 in liposomes showed 100% survival in response to *L. donovani* infection until 3 months postinfection, when 30 to 50% of gp63-, liposome-, and PBS-immunized mice died of heavy infection. These data, therefore, demonstrate that gp63, as a single antigen in liposomes, can induce effective protection not only against short-term but also against long-term infection that is sustained in the susceptible organs of BALB/c mice until at least 3 months of infection.

**Liposomal gp63 vaccination stimulates macrophages to produce IFN- $\gamma$ , IL-12, and nitric oxide and enhances IFN- $\gamma$  production from mice splenocytes in vitro.** To ascertain the vaccine-induced activation of macrophages to limit parasite multiplication, resident peritoneal macrophages were isolated from immunized BALB/c mice and incubated with *L. donovani* promastigotes for 3 h. The numbers of infected macrophages from mice immunized with free gp63 and liposomal gp63 were significantly lower (Fig. 4A) ( $P < 0.05$ ) compared to the results seen with PBS and empty liposomes, suggesting that immunization with gp63 affects the initial entry of the parasite into macrophages. gp63 remained localized on the surface of macrophages from gp63-liposome-immunized mice in a patched pattern, as revealed by FITC-labeled anti-gp63 antibody results and as additionally confirmed when macrophage ghost-membrane immunoblots were probed with anti-gp63 (Fig. 4B). In contrast, no binding of gp63 was detectable on macrophages from PBS-immunized mice. These data suggest that blocking of parasite uptake in macrophages from gp63-immunized mice might be due to loading of gp63 onto its surface receptors (4). In these macrophages, infection was arrested compared to control macrophage results, which showed parasites multiplying progressively (Fig. 4C). Since stimulated macrophages produce IL-12 and IFN- $\gamma$  in response to intracellular pathogens (68), we investigated the production of these cytokines in 72-h supernatants of the macrophages from immunized mice infected in vitro. While IFN- $\gamma$  was released by macrophages from both free gp63- and liposomal gp63-immunized mice, IL-12 was induced only by macrophages through gp63-liposome immunization (Fig. 4D). The simultaneous production of IFN- $\gamma$  and IL-12 in these macrophages correlated with enhanced production of NO (Fig. 4D) and almost complete elimination of the parasites. Together, these results imply in vivo stimulation of the macrophages by liposomal gp63 to produce Th1-promoting cytokines to facilitate *Leishmania* clearance by inducing NO production.

Although IL-12-dependent production of IFN- $\gamma$  is the chief mechanism of protection in vaccine-induced sustained immunity against *L. major* infection (59), a parasite-specific Th1 immune response is insufficient to confer protective immunity to *L. donovani* (37). Therefore, to assess the immune correlates of protection in this model, we evaluated antigen-specific IFN- $\gamma$ , IL-12, and IL-4 from splenocytes of mice after in vitro stimulation. IFN- $\gamma$  was detected postvaccination and postchallenge infection only in groups of mice able to control infection, that is, those given free gp63 and gp63 in liposomes and challenged 10 days later and those given gp63 in liposomes and challenged 12 weeks later (Fig. 4E and F). IL-12, however, was

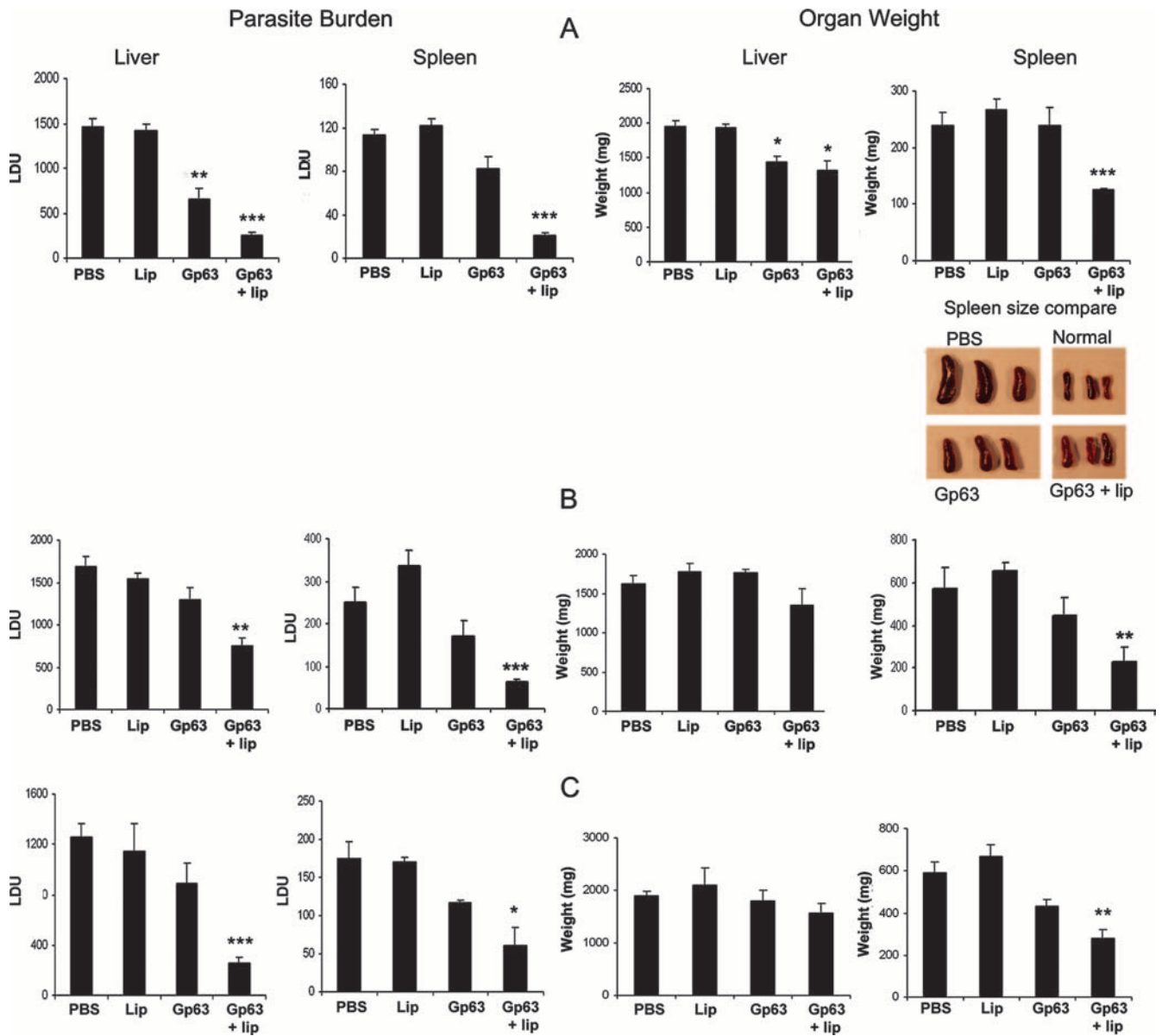


FIG. 3. Evaluation of protection against visceral leishmaniasis in BALB/c mice. Animals were immunized i.p. and were twice given booster injections at 2-week intervals with PBS, with empty liposomes (Lip), and with free gp63 or gp63 entrapped in cationic liposomes (lip). (A) Parasite burden and organ weights in the liver and spleen of mice challenged 10 days after the last immunization were determined at 3 months. Data are representative of the results of two independent experiments. (B) Splenocytes ( $2 \times 10^7$ ) of mice after 10 days of vaccination were transferred to naïve mice, and 7 days postvaccination the animals were challenged. The parasite load and organ weights were examined in the liver and spleen after 3 months. Photographs of the spleens of infected controls and vaccinated animals are shown at upper right. (C) In a separate experiment, mice were immunized with PBS, empty liposomes, or gp63 alone or in association with liposome and were challenged 12 weeks after vaccination. At the end of 3 months of infection, liver and spleen parasite loads and corresponding organ weights were determined. Data represent Leishman-Donovan units (LDU)  $\pm$  standard errors of the means and organ weights  $\pm$  standard errors of the means determined for individual mice ( $n = 3$  or  $4$ ). Significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ) differences in the results were obtained using vaccinated versus control mice, as determined by Student's *t* test.

produced only by cells from mice immunized with liposomal gp63 and vaccinated before infectious challenge and after infectious challenge at 10 days but not at 12 weeks (Fig. 4G), indicating that persistent IL-12 might not be required in this model. To investigate the cellular source of IFN- $\gamma$ , anti-CD4 and anti-CD8 monoclonal antibodies were added to the cultures. Addition of anti-CD4 substantially inhibited the

production of IFN- $\gamma$  from cells of all the groups able to control infection (Fig. 4E and F). Addition of anti-CD8 reduced IFN- $\gamma$  levels only for the cells of mice vaccinated with liposomal gp63, indicating a role for CD8<sup>+</sup> T cells in immunity induced in this group. CD4<sup>+</sup> T cells from mice vaccinated with free gp63 and with gp63 in liposomes also secreted IL-4, levels of which decreased substantially 3



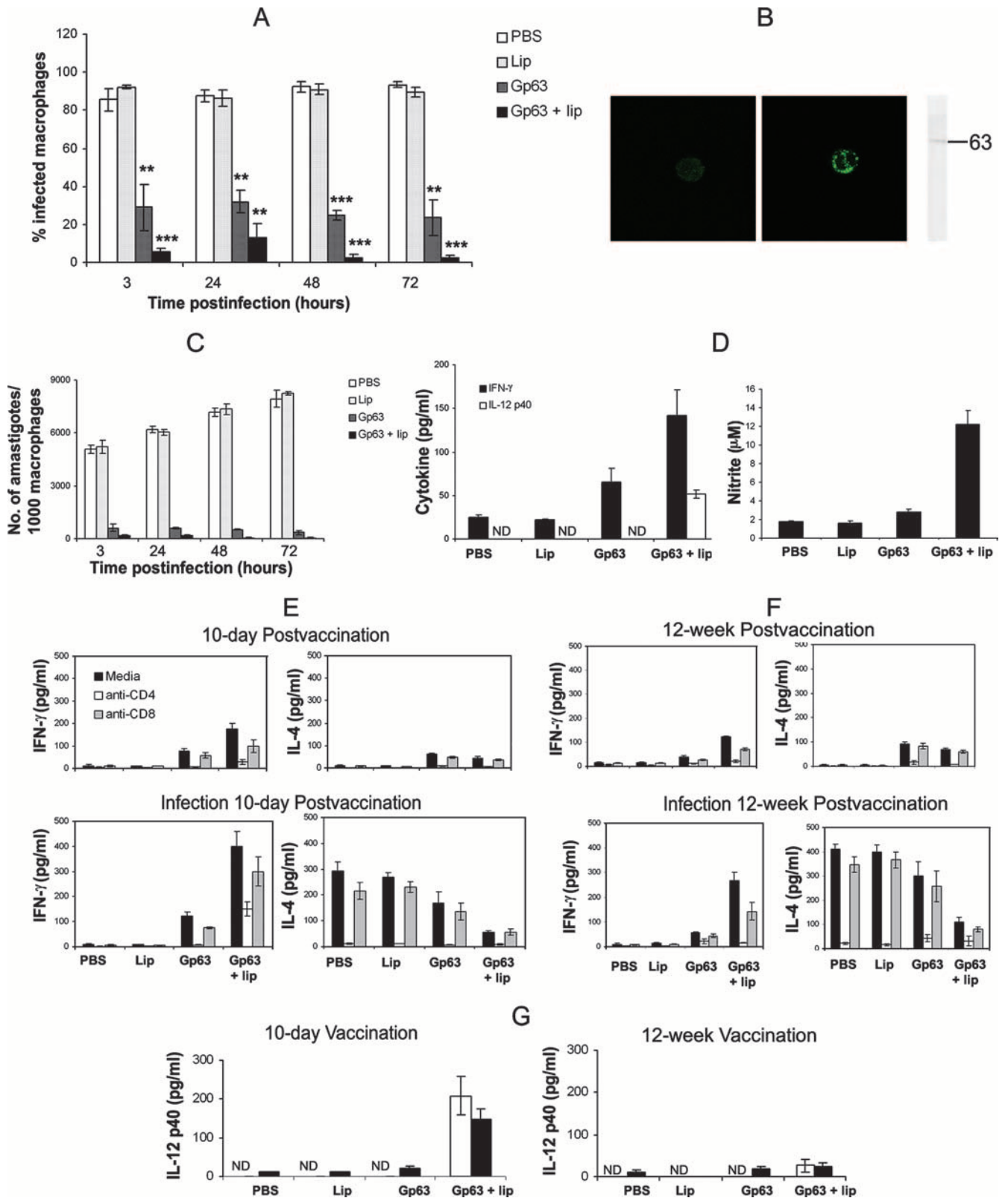


FIG. 4. Macrophages and T cells are involved in the control of *L. donovani* infection. BALB/c mice were immunized with free or liposome-encapsulated (lip) gp63 as described in Materials and Methods. Control mice received PBS and empty liposomes (Lip). (A) Peritoneal macrophages were collected from different groups of mice followed by in vitro infection with *L. donovani*, and percentages of infected macrophages were determined. \*, Student's *t* test revealed statistically significant (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ) decreases of infection in animals immunized with free- and liposome-encapsulated gp63 versus PBS control results. (B) Presence or absence of gp63 on macrophage surfaces of PBS-vaccinated mice (left panel) and liposomal gp63-vaccinated mice (right panel) as observed by confocal microscopy. A Western blot employing polyclonal anti-gp63



months after infection compared to the results seen with infected control mice (Fig. 4E and F).

**Immunization with gp63 in liposomes induces CD4 and CD8 T-cell production of IFN- $\gamma$  in mice sustaining long-term immunity.** Direct evidence for CD4<sup>+</sup> and CD8<sup>+</sup> T-cell production of IFN- $\gamma$  was obtained by measuring the frequencies of these cells in the spleen. There were demonstrable numbers of CD4<sup>+</sup> cells producing IFN- $\gamma$  in mice vaccinated with both free and liposomal gp63 10 days after vaccination (Fig. 5A). However, CD4<sup>+</sup> T cells producing IFN- $\gamma$  were detected 12 weeks after vaccination only in cells of mice vaccinated with gp63 in liposomes. After 3 months of infection, the frequencies of CD4<sup>+</sup> IFN- $\gamma$ -producing cells from mice initially vaccinated with gp63 and gp63 in liposomes and infected 10 days later were enhanced further (Fig. 5B), correlating with protection in these groups. Cells of mice vaccinated with liposomal gp63 and infected 12 weeks later also demonstrated an increase in frequencies of CD4<sup>+</sup> IFN- $\gamma$ -producing T cells after infection. In addition, enhanced frequencies of CD8<sup>+</sup>-producing IFN- $\gamma$  cells were detected only in animals infected 12 weeks postvaccination, correlating with long-term protective immunity in this group. Immunization with gp63 alone is insufficient to maintain CD4<sup>+</sup> and CD8<sup>+</sup> T-effector cells producing IFN- $\gamma$ , resulting in failure to control infection 12 weeks after vaccination.

As for IL-4 production, mice vaccinated with liposomal gp63 had low numbers of CD4 cells producing IL-4 (Fig. 5C). The frequencies of IL-4-producing cells were similar for mice able to control infection 10 days after vaccination with free gp63 and vaccination with gp63 in liposomes, but these levels were lower than those seen with mice vaccinated with PBS (Fig. 5D). However, mice infected with free gp63 at 12 weeks after vaccination had higher frequencies of IL-4-producing CD4 cells. In contrast, mice vaccinated with liposomal gp63 had extremely low frequencies of cells producing IL-4 in comparison to mice in the control group.

## DISCUSSION

Herein we report, for the first time, the induction of long-term protective immunity by a defined protein antigen vaccine entrapped in cationic DSPC liposomes. Immunization of susceptible BALB/c mice with gp63 entrapped in these vesicles protected virtually all of the mice against progressive nonhealing infections with *L. donovani*. Moreover, the potent and durable protection was elicited by a low-dose protein antigen formulated in liposome carriers without the use of immunomodulating adjuvants such as

IL-12 DNA or CpG oligonucleotides. These data strongly suggest that effective protein vaccines can be successfully used for inducing durable immunity against diseases requiring cellular immune responses when delivered in association with cationic DSPC liposomes.

Our data demonstrating that gp63 encapsulated in stable cationic liposomes provides long-term protection are in contrast to previous studies of protein-based vaccines (14, 38). In a study by Gurunathan et al. (14), vaccination of BALB/c mice with both SLAg and recombinant LACK plus recombinant IL-12 failed to control infection when mice were challenged with *L. major* 12 weeks after immunization. Similarly, long-term protection against CL could not be demonstrated in a natural self-curing model (using C57BL/6 mice) when mice were vaccinated with leishmanial antigen and recombinant IL-12 (38). The elicitation of potent durable protection required vaccination with either plasmid DNAs encoding *Leishmania* antigen or antigens plus IL-12 DNA (14, 38). Although DNA vaccines have proved effective in the murine system, they are far less immunogenic in humans (15). CpG oligonucleotides can generate strong cellular responses against protein antigens. CpG oligonucleotides are effective as vaccine adjuvants when high doses of antigen are administered (39, 53). Moreover, there are several safety concerns regarding the clinical use of CpG oligonucleotides (31). Cationic DSPC liposomes, representing the vaccine adjuvant used in our study, are relatively inert in terms of activating immune responses, are nontoxic, and require low doses of antigen (5, 9). The experiments producing the results presented here showing that these liposomes provide long-term protection have been repeated with consistent results and little variability. Moreover, this vaccine adjuvant elicited sustained and durable protection when used with either LAG (unpublished observation) or purified protein.

Induction of significant short-term as well as long-term resistance in both livers and spleens of susceptible mice, in a model using purified antigen which closely mimics clinical pathology associated with human VL, has not been observed in other laboratory-based trials of VL vaccine. Only a few defined antigens (dp72, Lcr1, recombinant hydrophilic acid surface protein B1 [rHASP B1], A2, recombinant open-reading frame [rORFF]) have been evaluated as vaccine candidates for protection against VL (12, 23, 62, 65, 66). Of these, rHASP B1 and rORFF have been described as inducing long-term protection (62, 66). However, in these studies mice were challenged after only 3 weeks had passed after the last vaccination. Moreover, rORFF antigen conferred protection when coadministered with IL-12 DNA, an approach not desirable for

antibody for gp63 liposome-immunized macrophage membrane fractions was used to produce the photographs. (C) Immunization induced activation of macrophages to suppress intracellular amastigote proliferation 72 h after infection in vitro with *L. donovani*. (D) IFN- $\gamma$ , IL-12, and NO production by macrophages after 72 h of infection. ND, not detected. (E) Single-cell suspensions were prepared from harvested spleens of vaccinated animals before and after challenge infection, and portions were treated with media alone and anti-CD4 and anti-CD8 monoclonal antibodies as described in Materials and Methods. Total cells and T cells depleted of CD4<sup>+</sup> and CD8<sup>+</sup> were plated in 24-well tissue culture plates and stimulated in vitro with gp63 (1.25  $\mu$ g/ml). At 72 h later, supernatants were harvested and IFN- $\gamma$  and IL-4 levels were determined by ELISA. (F) Spleen cells of mice vaccinated at 12 weeks were harvested before and after infection with *L. donovani* and treated with anti-CD4 or anti-CD8 monoclonal antibodies as described above, and levels of in vitro production of IFN- $\gamma$  and IL-4 from 72-h culture supernatants were estimated. (G) Production of IL-12 by splenocytes before infection (open bar) or after infection (closed bars). The data represent means  $\pm$  standard errors of the means of the results obtained with three individual mice. ND, not detected.

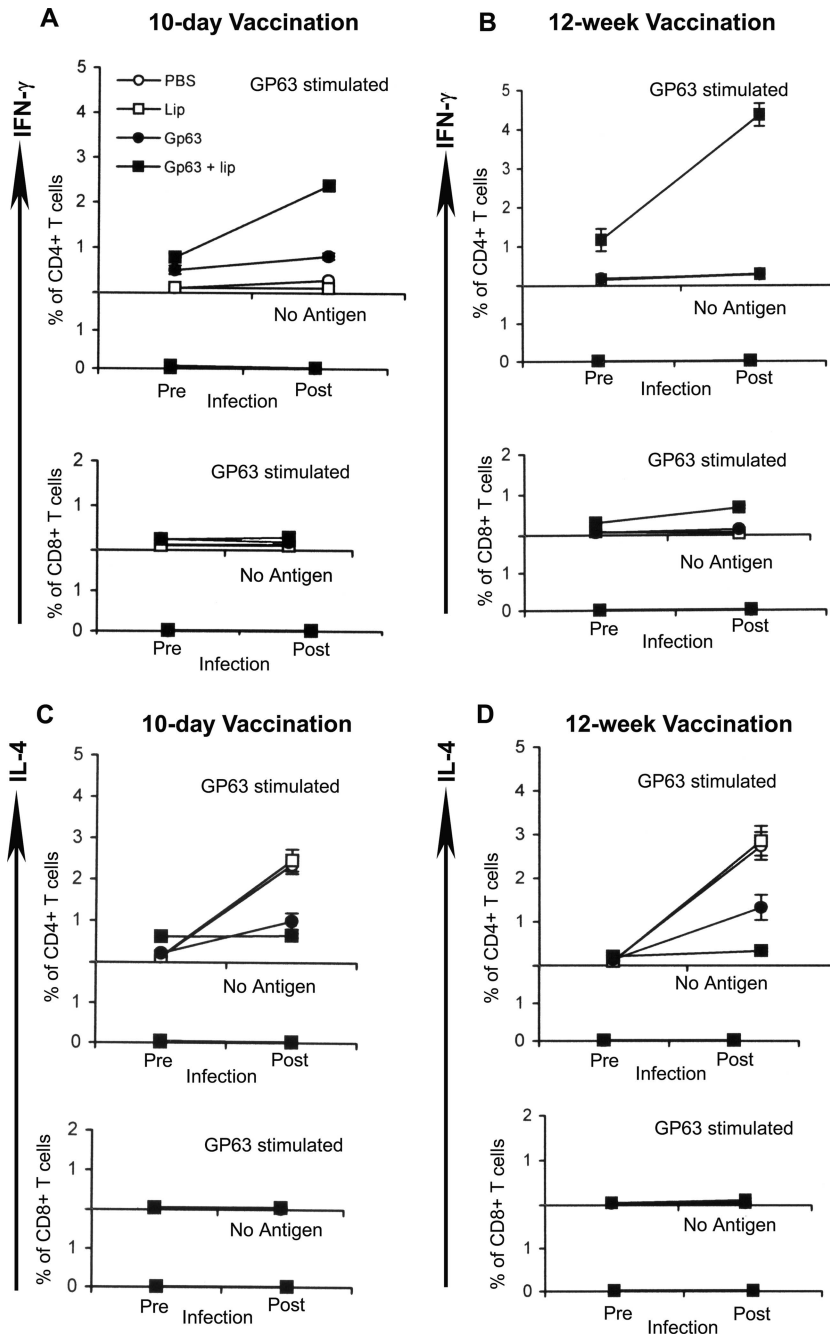


FIG. 5. Th1 responses that are dominant over Th2 responses control *L. donovani* infection. The frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  and IL-4 in the indicated groups were assessed using single-cell splenocyte suspensions left unstimulated or restimulated overnight with gp63 (5  $\mu$ g/ml). (A) The frequencies of cells producing IFN- $\gamma$  were assessed 10 days after vaccination both before and after 3 months of infection. lip, liposome encapsulation; Lip, empty liposomes. (B) The frequencies of cells producing IFN- $\gamma$  were assessed 12 weeks after vaccination both before and after 3 months of infection. (C) Mice were challenged with *L. donovani* at 10 days, and the frequencies of cells producing IL-4 were determined at 3 months both before and after infectious challenge. (D) Frequencies of cells producing intracellular IL-4 were also determined in animals at 12 weeks postimmunization both before and after 3 months of infectious challenge. Data represent means  $\pm$  standard errors of the means of the results obtained with three individual mice.

human administration. Protection induced by rHASP1 did not require an adjuvant. However, the level of protection did not exceed 50% at the peak liver burden. Immunization with gp63 purified from *L. donovani* promastigotes also resulted in partial protection in the absence of adjuvant in

BALB/c mice. The protective efficacy was markedly enhanced in association with liposomes inducing short-term as well as long-term resistance to challenge with virulent *L. donovani* parasites.

In terms of immune correlates of protection, the roles of

CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been well documented for long-term, vaccine-induced resistance to *Leishmania* spp. (16, 27, 77). Induction of potent Th1 responses by cationic liposomes (5, 24) is consistent with our findings that mice vaccinated with gp63 in liposomes exhibited striking enhancements of the frequency and production of IFN- $\gamma$  from CD4<sup>+</sup> T cells both before infectious challenge and at 10 days or 12 weeks after infectious challenge. Significant frequencies of antigen-specific CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells were demonstrable only in the protected mice challenged 12 weeks after vaccination, supporting the idea of the importance of CD8<sup>+</sup> T cells in mediating long-term immunity (16, 53). Our data demonstrating an ability to elicit CD8<sup>+</sup> T-cell responses by use of cationic DSPC liposomes with a defined protein antigen are comparable to those obtained with CpG ODN and DNA vaccination. Control of VL requires IFN- $\gamma$  (72), which was sufficiently produced and maintained for at least 12 weeks after vaccination with cationic liposome-encapsulated gp63, and the amount was enhanced at 3 months following *L. donovani* infection. IL-12, which is produced by APCs such as macrophages and dendritic cells at the initiation of immune response, is the inducer cytokine for IFN- $\gamma$  and activates macrophages and potentiates their microbicidal activities (60). However, in many experimental models of intracellular infections, including *L. major* infections, maintenance of CD4<sup>+</sup> IFN- $\gamma$  production has been reported to depend on the continuing presence of IL-12, although CD8<sup>+</sup> T-cell production of IFN- $\gamma$  may be IL-12 independent (14, 64). In contrast, studies of murine models of *L. donovani* infection demonstrating induction of comparable levels of IFN- $\gamma$  by rHASPBI immunization with and without IL-12 and resistance to infection in IL-12 p35 knockout mice have suggested that IL-12 is not required to maintain immunity against this parasite (41). Our data showing IL-12 production after short-term immunization indicate a role for IL-12 in at least the initiation of a Th1 response. The lack of IL-12 following long-term immunization suggests that once the protective responses are established, immunity in this model is sustained without IL-12. Disease progression in the present model corresponded to the presence of IL-4 and a lack of IFN- $\gamma$  production by splenic T cells. IL-4 was also produced in spleen cells of vaccinated mice after immunization. However, the level of IL-4 decreased significantly at 3 months postinfection compared to infected control results. Notably, our results, though not providing direct evidence, are in line with observations revealing that IL-4 is crucial for the priming of long-term CD8<sup>+</sup> T-cell memory responses (20, 40, 63). As IL-4 and IFN- $\gamma$  influence antibody class switching, assessment of antigen-specific production of antibody subtypes provides an indirect but physiologic correlate of the pattern of cytokine production in vivo. Protected mice had an increase in IgG2a levels (consistent with increased IFN- $\gamma$  levels). They also produced IgG1, although the level was significantly lower than that seen with unprotected mice, suggesting the presence of IL-4 as well. Vaccination with gp63 in association with cationic DSPC liposomes thus elicited both cell-mediated and humoral immune responses. After immunization, subsets of CD4 cells and of Th1 and Th2 cells were activated. Protection in vaccinated mice corresponded to a dominant Th1 response, a downregulation of Th2 response, and a CD8<sup>+</sup> T-cell response associated with long-term immunity.

The efficacy of our vaccine may be due to several factors. First, we used native gp63 versus recombinant gp63. Moreover, this antigen is immunodominant and associated with virulence of *L. donovani* (1, 71). Second, we determined that there is an antigen dose level that provides optimum protection, which confirms and extends previous observations that immunogen doses influence the generation of Th1/Th2 cells (3, 49, 60). Third, cationic liposomes demonstrate efficient interaction with APCs for a combination of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses (5, 11, 44, 45, 50). These vesicles have been observed to increase the immunogenicity of the antigens and prevent their degradation in vivo (5). Formulation of the antigen-containing cationic liposomes with DSPC, as shown in our studies, may serve to protect the antigen further and thereby improve antigen-delivering capacity (8, 13, 70). The presence of cholesterol in the vesicles facilitates cytoplasmic release of the antigens, avoiding lysosomal degradation (17). Increased in vivo stability of these vesicles may further promote stimulation of CD8<sup>+</sup> T-cell responses (21). Membrane antigens in DSPC liposomes have been reported to be transferred to plasma membrane of APCs without being processed (13, 70). Since we also obtained signals for the presence of intact gp63 on the surface of macrophages from immunized mice, we propose in addition that it is the direct transfer of gp63 onto macrophage surfaces, facilitated by the presence of its receptors (4), that may be responsible for prevention of the parasites from establishing infection.

Most licensed vaccines, particularly those incorporating attenuated live products or killed whole-cell products, contain all the components necessary for an integrated immune response. Thus, leishmanization remains the gold standard for vaccination and life-long protection against this disease. *Leishmania* parasites are probably never completely eliminated by therapy, and several studies of murine models have indicated the importance of persistent parasites in establishing an effective protective memory response (2, 69). Due to unacceptable adverse effects associated with whole parasites, attention has now turned toward safer and better-defined subunit vaccines (6). However, isolated antigens from such chronic infections often lack sufficient immunogenicity, thus requiring the addition of potent adjuvants which, in addition to enhancing antibody production, are specifically geared to generation of cell-mediated immunity (47, 48). Cationic liposomes are safe adjuvants, as demonstrated by the fact that they are exploited worldwide in human gene therapy (61). The observations demonstrating that stable cationic liposomes, when used as an adjuvant with purified protein antigen, can induce long-term protection against an intracellular infection have shown that these vesicles are potent vaccine adjuvants for diseases requiring cellular immunity.

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