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Complete Cure of Experimental Visceral Leishmaniasis with Amphotericin B in Stearylamine-Bearing Cationic Liposomes Involves Down-Regulation of IL-10 and Favorable T Cell Responses[1]

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Visceral leishmaniasis caused by *Leishmania donovani* is a life-threatening disease involving uncontrolled parasitization of liver, spleen, and bone marrow. Most available drugs are toxic. Moreover, relapse after seemingly successful therapy remains a chronic problem. In this study, we evaluated a new therapeutic approach based on combination of a low dose of amphotericin B (AmB) in association with suboptimum dose of stearylamine (SA)-bearing cationic liposomes, itself having leishmanicidal activity. We demonstrate that a single-shot therapy with this formulation caused clearance of parasites from liver and spleen below the level of detection in the selected piece of the organs of BALB/c mice. The combination was superior to free AmB and AmBisome for therapy, as well as for prevention of relapse and reinfection. Besides having better killing activity, AmB in SA liposomes, in contrast to AmBisome, maintained the immunomodulatory effect of free AmB on $CD4^+$ and $CD8^+$ T cells for IFN-γ production, at the same time reducing the toxic effects of the drug, reflected through decline in TNF-α. In addition, IL-10 was down-regulated to almost negligible levels, most efficiently through therapy with SA-bearing cationic liposomes-AmB. This IL-10-deficient environment of IFN-γ-secreting T cells probably up-regulated the enhanced IL-12 and NO production observed in splenic culture supernatants of these mice, correlating with prolonged disease suppression better than free AmB and AmBisome. The ability of the formulation to elicit protective immunity was reconfirmed in a prophylactic model. Our results emphasize the requirement of effective immune stimulation, additionally, by antileishmanials for persistent disease protection, demonstrated by this liposomal AmB formulation. *The Journal of Immunology,* **2008, 181: 1386–1398.**

Leishmaniasis is currently endemic in 88 countries, and is a threat to 350 million people with a worldwide prevalence of 12 million cases (1). Clinical manifestation of leishmaniasis depends upon the causative species of *Leishmania* and the immune status of the host, and ranges from a self-healing skin lesion to a lethal systemic form of disease (2). Among all the disease forms, visceral leishmaniasis (VL)[3] or kala-azar, caused by the parasite *Leishmania donovani*, is fatal if left untreated. The currently available drugs for treatment of VL, including pentavalent antimonials, amphotericin B (AmB), miltefosine, paromomycin, etc., have limitations of unresponsiveness, relapse, specific toxicities, multiple injections, or parenteral administrations (3). Hence, there is an urgent need for nontoxic, single-shot treatment strategy having long-lasting protective effect to prevent disease resurgence and reinfection. Disease outcome of VL is associated with immunological dysfunctions of T cells, NK cells, and macrophages (4–6). Both experimental (7, 8) and clinical (9–13) data support a pathogenetic role of IL-10 in VL. Endogenous IL-10, known to suppress IFN-γ synthesis, is capable of derailing Th1-type response and deactivating macrophages (8). Effective defense toward *L. donovani* infection depends strictly upon T (Th1) cells, and acquired resistance is governed by T cell- and macrophage-activating cytokines (14). Among the latter, IL-12 initiates and drives the expansion of the Th1 subset with production of cytokines such as IFN-γ that activate macrophages for parasite killing through release of NO (15). Previous studies demonstrated that immunotherapy with anti-IL-10R Ab induced IL-12- and IFN-γ-dependent parasite killing in experimental murine VL (16). Moreover, treatment with anti-IL-10R Ab in combination with a low dose of AmB produced remarkable synergistic effect toward the clearance of liver parasites (17). Unexpectedly, however, combination therapy with exogenous IFN-γ and pentavalent antimonials in India kala-azar patients showed discouraging results (18). Thus, rather than exogenous administration of Th1 cytokine, stimulation of endogenous production of Th1-cell type immune response or a kind of therapy that frees up curative Th1 cell-type mechanisms from the suppressive effect of IL-10 will probably be the effective treatment strategy.

Expression of the in vivo leishmanicidal effect of pentavalent antimonials in parasitized tissues is not direct. Instead, it requires the preceding mechanism with interdigitation of the following: 1) T (Th1) cells and T cell costimulation via CD40L and CD40; 2) IFN-γ and allied activating, inflammatory cytokines (IL-12 and TNF); and 3) influxing blood monocytes as primary effector cells within epithelioid granulomas (17). Although AmB is known to be

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[3] Abbreviations used in this paper: VL, visceral leishmaniasis; AmB, amphotericin B; DTH, delayed-type hypersensitivity; LAg, leishmanial Ag; LDU, Leishman-Donovan unit; PC-SA, stearylamine-bearing cationic liposome; PKC, protein kinase C; SA, stearylamine.

directly leishmanicidal via a specific binding to ergosterol (19), and the effect is independent of the host immune response, it has been shown that similar targeting of Th1 cell mechanism can enhance its efficacy and reduce its doses to achieve comparable activities (17). In addition, AmB appears to have some important immunomodulatory effects. AmB has been shown to increase production of cytokines, including IL-1β (20–24), TNF-α (20, 24–26), IL-6 (27, 28), and M-CSF (29) from monocytes and macrophages. It has been postulated that the higher infusion-related toxicity of AmB is partially related to the exacerbated production of these cytokines through activation of the innate immune system (30). Liposomal formulations of AmB reduce the toxicities observed with free AmB. However, it is also possible that different lipid-associated formulations of AmB may modify the immunomodulatory activity of the drug (31, 32), thus masking the beneficial immunomodulatory properties of AmB. Despite its high cost, liposomal AmB (AmBisome) remains the best antileishmanial formulation for the treatment of VL (33). However, necessity of a very high dose (7.5–20 mg/kg) to cure VL (33), possibility of relapse with its very fast early response (34), loss of efficacy in chronic murine VL even with the dose of 8 mg/kg (35), and relapse of disease in chronic murine VL despite therapy with a total dose of 72 mg/kg AmBisome (36) leave open the opportunity to explore new lipid-associated formulations of AmB that, in addition to reducing its toxicity, maintains its beneficial leishmanicidal immune responses.

Earlier we had shown that stearylamine (SA)-bearing cationic liposomes (PC-SA) alone had a leishmanicidal effect both in vitro and in vivo (37, 38). We, therefore, envisaged that entrapment of AmB within these vesicles would, besides reducing its toxicity, enhance the activity of AmB synergistically toward direct killing of the parasites in the liver and spleen. Moreover, based on our prior observations that positively charged liposome formulated with SA could greatly augment the immunogenicity of the Ag associated with it (39), we reasoned that AmB complexed in these cationic vesicles would also be effective in maintaining and enhancing the inherent immunostimulatory activity of AmB. We, therefore, conducted studies to investigate the therapeutic effect of AmB associated with SA-bearing cationic liposomes against both established and chronic murine *L. donovani* infection. Our studies have also included an examination of the probable mechanism of this AmB liposomal formulation to redirect the disease-associated immune response to establish and maintain a curative response against VL. We found that a single-shot cationic liposome-associated AmB could almost completely eliminate the parasites from the infected liver and spleen of BALB/c mice. Cationic liposomes permitted outstanding leishmanicidal activity with a remarkably low dose of AmB through enhancement of a Th1-biased curative immune response and almost complete blockade of IL-10 secretion.

Materials and Methods

Reagents and chemicals

HEPES, penicillin-streptomycin, sodium bicarbonate, BSA, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, brefeldin A, and phosphatidylcholine were purchased from Sigma-Aldrich. SA was purchased from Fluka. AmB deoxycholate and AmB (Sarabhai Piramal Pharmaceuticals) were gifts from R. T. Goswami and B. Saha of School of Tropical Medicine, Kolkata and V. Vobalaboina, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India, respectively. Conventional liposomal formulation of AmB (AmBisome) was purchased from Gilead Science. [^{3}H]Thymidine was obtained from Amersham Biosciences. The following mAbs obtained from BD Pharmingen were used: HRP-conjugated anti-mouse IgG, IgG1, IgG2a, anti-mouse CD4 (FITC, L3T4, H129.19), anti-mouse CD8a (FITC, Ly-2, 53-6.7), anti-mouse CD19 (FITC), anti-mouse CD11b (FITC), anti-mouse IFN-γ (PE), and anti-mouse IL-10 (allophycocyanin).

Parasite culture and maintenance in animal

L. donovani (MHOM/IN/1983/AG83) parasites were cultured as promastigotes in medium 199 (Sigma-Aldrich)-supplemented 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Sigma-Aldrich) at 22°C. Golden hamsters, 4 wk old, and BALB/c mice reared in institute facilities were used for the purpose of parasite maintenance with prior approval of the Animal Ethics Committee of Indian Institute of Chemical Biology.

Preparation of AmB-encapsulated SA-bearing liposome

Liposomal AmB was prepared by incorporation of AmB into the lipid bilayer of SA-bearing choline liposome (lipid ratio, 7:0.9), as previously described (40). In brief, liposomes were prepared by adding methanolic solution of the drug to the lipids in chloroform, followed by evaporating the organic solvents to form a thin film in a round-bottom flask. The film was dried overnight in a vacuum desiccator. The film was rehydrated in 20 mM PBS (pH 7.4), and the suspension was sonicated for 30 s in an ultrasonicator, followed by incubation for 2 h at 4°C. The unentrapped drug was removed by centrifugation. The amount of AmB intercalated into liposome was determined spectrophotometrically at 405 nm after dissolving an aliquot of liposomal preparation in methanol (41). Intercalation efficacy of AmB was estimated to be 70% ($\pm$5) for the liposome prepared with PC-SA in the molar ratio of 7:0.9. We observed that PC-SA-associated AmB formulation remained stable up to 2 wk when stored at 4°C, and its leishmanicidal activity remained intact.

Infection of mice, treatment schedules, doses, and toxicity

For experimental infections, BALB/c mice (4–6 wk) were injected via the tail vein with 2.5×10^7 hamster spleen-derived *L. donovani* amastigotes, and for reinfection, the same number of amastigotes was injected 12 wk after the first infection. Infected mice (5 mice/group) received i.v. injection of a single-shot treatment of either free AmB (2.5 mg/kg body weight), drug-free PC-SA liposome (20.3 mg/mouse), AmBisome (3.5 mg/kg), and PC-SA-AmB (3.5 mg/kg AmB associated with 20.3 mg of PC-SA liposome) after 8 (established) and 12 (chronic) wk of infection. For prophylactic studies, groups of 4- to 6-wk-old BALB/c (5 mice/group) mice were treated i.v. with a single dose of either free AmB (2.5 mg/kg), AmBisome (3.5 mg/kg), drug-free PC-SA liposome (20.3 mg/mouse), or PC-SA-AmB (3.5 mg/kg AmB associated with 20.3 mg of PC-SA liposome). Mice were treated once and infected 10 days after treatment with 2.5×10^7 *L. donovani* amastigotes.

Determination of splenic, hepatic, and bone marrow parasite burden

Spleen and liver were removed after 4 wk of treatment in both the infection models (established and chronic infection), and multiple impression smears were prepared and stained with Giemsa. Organ parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of parasites per 1000 nucleated cells $\times$ organ weight (in mg). Infection in the bone marrow was calculated as number of parasites/1000 host cell nuclei. Reinfected mice were sacrificed 20 wk of initial infection. To follow up the protective effect of PC-SA-AmB liposome on disease recurrence, batches of five mice of PC-SA-AmB-treated group were sacrificed further 8 and 12 wk after treatment and compared with the untreated, infected mice. To study the protective effect of the liposomal formulation of AmB, pretreated mice were sacrificed after 12 wk of infection. To further evaluate whether the spleen and liver contained live parasites, in selected experiments the parasite burden was quantified in these tissue by serial dilution assay, as previously described (42). Briefly, a weighed piece of spleen or liver from experimental mice was first homogenized in Schneider's medium supplemented with 10% FCS, and then diluted with the same medium to a final concentration of 1 mg/ml. Five-fold serial dilutions of the homogenized tissue suspensions were then plated in 96-well plates and incubated at 22°C for 2–3 wk. Wells were examined for viable and motile promastigotes at 7-day intervals, and the reciprocal of the highest dilution that was positive for parasites was considered to be the parasite concentration per milligram of tissue. The total organ parasite burden was calculated using the weight of the respective organs.

Parasite membrane Ag preparation

Stationary-phase promastigotes, harvested after the third or fourth passage in liquid culture, were washed four times in cold PBS and resuspended at a concentration of 1.0 g of cell pellet in 50 ml of cold 5 mM Tris-HCl buffer

(pH 7.6). The suspension was vortexed six times for 2 min each, with a 10-min interval of cooling on ice between each vortexing. The parasite suspension was then centrifuged at 2310 × *g* for 10 min. The crude ghost membrane pellet obtained was suspended in 5 ml of 5 mM Tris buffer (pH 7.6) and sonicated three times for 1 min each at 4°C in an ultrasonicator. The suspension was finally centrifuged for 30 min at 5190 × *g*. The supernatant containing leishmanial Ags (LAg) was harvested and stored at −70°C until used. The protein content in the supernatant was measured by the procedure described by Lowry's method.

Determination of delayed-type hypersensitivity (DTH)

DTH in posttreated 12-wk-infected mice was determined as an index of cell-mediated immunity. The response was evaluated by measuring the difference between the footpad swelling at 24 h following intradermal inoculation of the test footpad with 50 μl (800 μg/ml) of LAg and the swelling of the control (PBS-injected) footpad.

T cell proliferation assay

T cell proliferation assay was performed on splenocytes, from different experimental groups of posttreated and pretreated mice, and prepared by mechanical disruption, followed by NH_4Cl lysis of RBC. After several washings in RPMI 1640 medium, cells were resuspended in complete medium (RPMI 1640 with penicillin and streptomycin and 10% FCS). Cells were plated in triplicate at 2×10^5 cell/well concentrations in 96-well plates and allowed to proliferate for 48 h at 37°C in 5% CO_2 incubator in the presence (10 μg/ml) and absence of LAg. Cells pulsed with 1 μCi of [^{3}H]thymidine/well for an additional 18 h were harvested onto filter mats. [^{3}H]Thymidine uptake, an index of cell proliferation, was measured in a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

Analysis of cytokines by ELISA

Cytokine concentrations in the splenocyte culture supernatants of differently treated normal and infected mice, in response to Con A/LPS and LAg stimulation, respectively, were determined by sandwich ELISA (BD Pharmingen kit), as recommended by the manufacturer. IFN-γ and IL-10 were determined in normal mice, and TNF-α, IL-12, and IL-4 were measured additionally in infected mice. To investigate the direct effect of AmB, PC-SA, AmBisome, and PC-SA-AmB to modulate cytokine production, splenocytes (1×10^6/ml) from healthy control mice were incubated with various concentrations of AmB (0, 0.015, 0.031, 0.06, 0.12, and 0.25 μg/ml), PC-SA (0, 5, 10, 20, 40, and 80 μg/ml), AmBisome (0, 0.015, 0.03, 0.06, 0.12, and 0.25 μg/ml), or PC-SA-associated AmB (0/0, 5/0.015, 10/0.03, 20/0.06, 40/0.12, and 80/0.25 μg/ml, respectively) with or without LPS (2.5 μg/ml) or Con A (2.5 μg/ml) for 48 h at 37°C in 95% humidified air with 5% CO_2. LPS-specific IL-10 and Con A-specific IFN-γ were determined in cell-free culture supernatants, as described above.

Quantification of NO

Splenocytes (2×10^5/well) from different groups of experimental mice were incubated with (10 μg/ml) or without LAg for 48 h in 5% CO_2 incubator at 37°C. The culture supernatants were analyzed for their contents of nitrite (NO_2^-) according to the method of Ding et al. (43). Briefly, the mixture of Greiss reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) and culture supernatant at 1:1 ratio was incubated for 15 min at room temperature, and the OD was determined at 550 nm by ELISA reader (Labsystems Multiscan MS). Sodium nitrite ($NaNO_2$) diluted in culture medium served as standard.

Flow cytometry

Two-color flow cytometry was performed for intracellular analysis of IFN-γ produced by $CD4^+$ and $CD8^+$ T lymphocytes in normal untreated and treated, and infected and cured mice, and IL-10 produced by $CD4^+$, $CD8^+$, $CD19^+$, and $CD11b^+$ cells in normal untreated and treated mice at the single cell level. Splenocytes from different groups of experimental mice were stimulated for 20–24 h with LAg (10 μg/ml; infected and cured), and for 8 h either with Con A (2.5 μg/ml) or LPS (2.5 μg/ml) (normal untreated and treated mice). Brefeldin A (10 μg/ml) was added to the cultures 2 h before harvest. The cells were washed in PBS containing 0.1% NaN_3/1% FCS at 4°C and stained with either FITC-conjugated anti-CD4, anti-CD8, anti-CD11b, or anti-CD19 mAb. The cells were then permeabilized by treatment with FACS permeable solution (BD Pharmingen) and stained with PE-conjugated anti-mouse IFN-γ mAb, allophycocyanin-conjugated anti-mouse IL-10 mAb, or isotype-matched control mAb, and analyzed on a flow cytometer (FACSCalibur) using the CellQuest program on at least 10,000 events.

Determination of IgG isotype and IgE through ELISA

Mice were bled 4 wk after treatment, and sera were stored at −20°C until use. Ag-specific serum IgG isotype Ab response was measured by conventional ELISA. Wells of ELISA plates (Nunc) were coated with LAg at a concentration of 2.5 μg/well and incubated overnight at 4°C. Sera were added at 1000-fold dilutions, followed by washes and addition of peroxidase-conjugated isotype-specific secondary Abs (goat anti-mouse IgG, IgG1, or IgG2a; BD Pharmingen). Wells were then washed and incubated with substrate solution (*o*-phenylenediamine dihydrochloride, 0.8 mg/ml in phosphate-citrate buffer (pH 5.0), containing 0.04% H_2O_2) for 30 min, and absorbance was read on an ELISA plate reader at 450 nm. Total serum IgE was determined through ELISA kit (BD Pharmingen), as per manufacturer's instructions.

Statistical analyses

The in vitro experiments were performed at least in triplicates. A minimum of five mice per group was used for any in vivo experiment. The statistical significance of differences between groups was determined as described in figure legends using the program GraphPad Prism (version 3.03). A value of $p < 0.05$ was considered statistically significant. Error bars represent the SEM.

Results

Combination therapy with PC-SA and AmB causes complete clearance of splenic and hepatic parasite burden of established murine VL

We had earlier reported on the multiple-dose treatment of drug-free, positively charged liposomes comprising PC and SA for the elimination of parasites from the liver and spleen of BALB/c mice with established *L. donovani* infection (38). In this study, the efficacy of this leishmanicidal liposome as a delivery tool as well as combination therapy with AmB, to achieve sterile cure of parasites from spleen and liver with single-dose treatment, has been determined. The dose titration experiment with different doses of AmB (0.4, 0.8, 1.6, 2.5, 3, 3.5, 4 mg/kg) entrapped within fixed 7:0.9 molar ratio of PC-SA liposome (20.3 mg/mouse) showed the efficacy of the combination therapy against established (8-wk) infections without any obvious adverse effect on normal function of liver and kidney (our unpublished data). PC-SA liposome in combination with 3.5 mg/kg AmB (PC-SA-AmB; 7:0.9:0.037) completely cleared parasites from liver and spleen. Remarkably, in all mice (experiment repeated four times using fresh liposomal preparations for all experiments) of this treated group, there was complete absence of amastigotes in the impression of stamp smears of transverse sections of both spleen and liver when observed under light microscopy (Fig. 1, *A* and *B*). Parasite load was below the level of detection in the selected piece of liver and splenic tissue even with limiting dilution assay (data not shown) in all the mice treated with 3.5 mg/kg AmB in association with 20.3 mg/mouse of PC-SA liposome. The parasite burden in both the organs also decreased significantly following treatment with 2.5 mg/kg free AmB (72%, $p < 0.001$ and 83%, $p < 0.001$, liver and spleen, respectively) and equivalent dose (3.5 mg/kg) of AmBisome (88%, $p < 0.001$ and 92%, $p < 0.001$ in liver and spleen, respectively) compared with untreated, infected controls. Drug-free PC-SA liposome at the dose of 20.3 mg/mouse reduced only the splenic parasite burden marginally, and the differences were not statistically significant. Previously, we reported that empty liposomes composed of PC-SA in a 7:2 molar ratio reduced parasite burden up to 48% in liver and 60% in spleen 4 wk after treatment (44). In this study, we chose 7:0.9 molar ratio of PC-SA because this ratio is nontoxic with different doses of AmB (our unpublished data). In the present study, reduction in the killing activity of PC-SA liposome alone (7:0.9, 20.3 mg/mouse) is due to the reduction in the SA content of the leishmanicidal vesicle used earlier (7:2) (45, 46). Whereas free

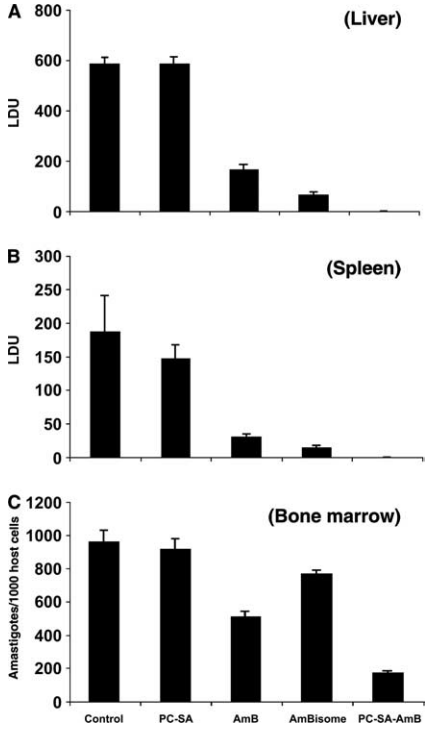

FIGURE 1. Complete cure is induced by PC-SA-associated AmB treatment in spleen and liver of established *L. donovani* infection in BALB/c mice. Effect of PC-SA (7:0.9, 20.3 mg/mouse)-associated AmB (3.5 mg/kg) was compared with identical dose (3.5 mg/kg) of AmBisome, drug-free PC-SA (20.3 mg/mouse) liposomes, and free AmB (2.5 mg/kg)-treated 8-wk-infected mice. Untreated, infected mice were considered as controls. *A–C*, Hepatic (*A*) and splenic (*B*) parasite burden were determined by stamp-smear method and expressed as LDU, and bone marrow parasite load (*C*) was determined in cell smear prepared from femur bone marrow and expressed as amastigotes/1000 bone marrow nuclei. Data represent the mean ± SE for five animals per group. Results are one representative of four experiments.

AmB and AmBisome could eliminate deep-sited bone marrow parasites, only 47 and 20%, respectively, PC-SA liposome associated with AmB suppressed parasite burden to 82% ± 5, from this site.

Combination therapy with PC-SA and AmB causes almost complete clearance of splenic, hepatic, and bone marrow parasite burden of BALB/c mice with chronic L. donovani *infection*

In chronically infected mice, spleen and bone marrow parasite burden is better expressed than in established infection (35), and the most obvious difference is the greater hepatosplenomegaly typical of VL. Hence, we examined the efficacy of combined treatment on 12-wk chronic infection after 4 wk of treatment. Almost complete clearance of parasites from spleen (99.6% ± 1.19) as well as liver (98.99% ± 6.89) and bone marrow (81%) was still achieved with the combination therapy in this model. In contrast, efficiency of free AmB and AmBisome to reduce parasite burden declined in all three sites, especially in spleen and bone marrow (69% ± 13.49 and 80% ± 10.63 in spleen, and 29% ± 356.4 and 24% ± 283.2 in bone marrow, respectively). Empty liposome treatment reduced parasite burden in spleen only marginally (35.35% ± 5).

Treatment with PC-SA liposome-associated AmB suppresses relapse of the disease and controls reinfection

No chemotherapy is thought to eradicate all tissue parasites in any form of leishmaniasis (47). Moreover, relapse after an apparent

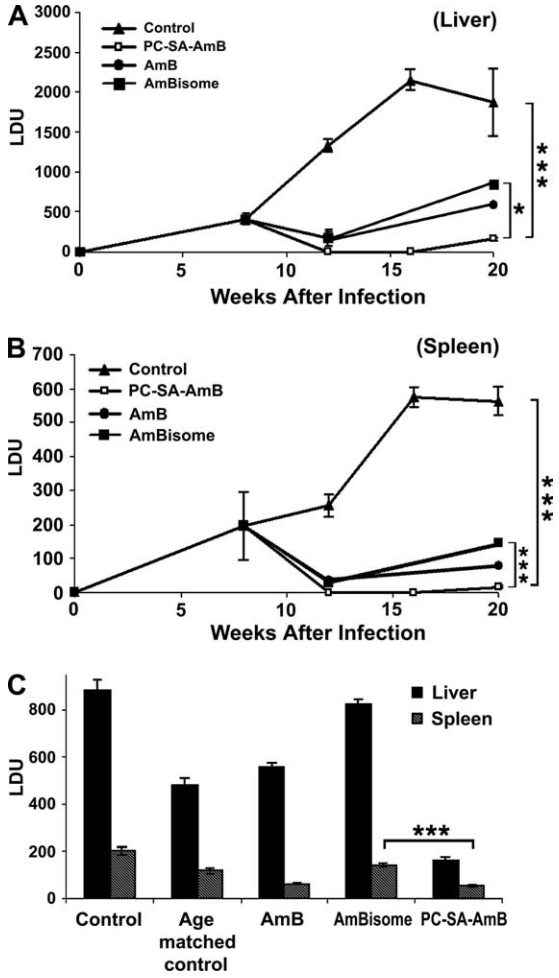

FIGURE 2. Protective effect of a single-dose treatment with PC-SA-associated AmB on disease relapse and reinfection with *L. donovani*. *A* and *B*, *L. donovani*-infected BALB/c mice received a single-shot indicated treatment (8 wk postinfection) and were sacrificed every 4 wk up to 20 wk of postinfection and compared with untreated, infected controls at corresponding time points. Hepatic (*A*) and splenic (*B*) parasite burden were expressed as LDU. *C*, Reinfection of BALB/c mice with *L. donovani*, previously treated with curative doses of PC-SA-associated AmB. Cured mice (PC-SA-associated AmB, free AmB, and AmBisome treated) and naive age-matched BALB/c mice (Control 2) were i.v. administered with 2×10^7 amastigotes of *L. donovani* through tail vein. Untreated, infected control mice were not reinfected (Control 1). The progress of infection was determined in liver and spleen after 8 wk of reinfection and expressed as LDU. Data represent the mean ± SE for five animals per group. Data were tested by ANOVA. Differences between mean were assessed for statistical significance by Tukey's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

response to any drug regimen is well recognized in kala-azar. Because cationic liposome-associated AmB showed outstanding leishmanicidal activity against both established and chronic infection, we tested the capability of PC-SA-AmB to prevent disease relapse. Our data demonstrate that a single-shot PC-SA-AmB injection reduced established *L. donovani* infection of BALB/c from liver and spleen by 100% at week 12 (Fig. 2, *A* and *B*). Sixteen weeks later, parasite burden in treated animals was almost totally absent. After 20 wk, some parasites were detected in liver and spleens (LDU, 158 ± 20 and 13 ± 4.5, respectively, $n = 5$), but the level of parasites was negligible in comparison with 20-wk untreated, infected mice (LDU, 1871 ± 426, and 561 ± 56.89, liver and spleen, respectively, $n = 5$) (Fig. 2, *A* and *B*). In contrast, higher levels of parasite burdens were detected in free AmB (LDU,

582 ± 144.91, and 78 ± 21.21, liver and spleen, respectively)- and AmBisome (LDU, 851.29 ± 177.03, and 144 ± 13.35, liver and spleen, respectively)-treated 20-wk-infected mice. To further ascertain that the combination regimen has conferred long-standing immunity, 8-wk-infected BALB/c mice treated with PC-SA-AmB, free AmB, and AmBisome were later reinfected i.v. 12 wk after primary infection. One group ($n = 5$) of age-matched normal mice was also infected simultaneously and was considered as age-matched, control-infected group. As observed up to 20 wk, only a slight and transient increase in organ parasite burden resulted in PC-SA-AmB-treated group. In comparison, free AmB reduced reinfection only to a marginal level. However, AmBisome failed to control reinfection (Fig. 2*C*).

Immunomodulatory effects of PC-SA-AmB on splenocytes of normal mice in vitro and in vivo

The experiments above demonstrated that PC-SA-AmB not only induced remarkable curative effect on established and chronic *L. donovani* infection, but also showed protection against relapse and reinfection. These findings, along with the fact that effective resolution of VL is generally associated with a protective immune response of the host, made us curious to investigate whether PC-SA-AmB, besides having a leishmanicidal role, could induce any protective immunomodulation in splenocytes. For an in-depth study of this efficacy, and to compare this with PC-SA, free AmB, and AmBisome, in vitro and in vivo investigations on healthy normal BALB/c mice were conducted. Our strategy was to study whether these formulations can induce IFN-γ from splenocytes suboptimally treated with Con A or down-regulate the production of LPS-stimulated IL-10 from splenocytes.

In the in vitro experiments, splenocytes isolated from healthy mice were cultured for 48 h in presence or absence of 2.5 μg/ml LPS or 2.5 μg/ml Con A (suboptimal dose), and were simultaneously treated with various concentrations of drugs (Fig. 3, *A–D*). It was found that PC-SA-AmB suppressed LPS-stimulated IL-10 maximally in a dose-dependent manner (Fig. 3*D*), followed by AmB (Fig. 3*C*). Notably, at the dose of 0.06 μg/ml free AmB reduced IL-10 from 150 pg/ml ± 9.5 to 80 pg/ml ± 9.8. The same dose of AmB when associated with 20 μg/ml PC-SA could reduce IL-10 up to 65 pg/ml ± 10.2. Even the basal levels of IL-10 in culture supernatant without LPS stimulation (32 ± 5.5) were reduced to levels that could not be detected with 0.06 μg/ml AmB associated with 20 μg/ml PC-SA, whereas free AmB reduced it to 15 pg/ml ± 2.07. PC-SA and AmBisome were unable to alter LPS-stimulated IL-10 levels significantly (Fig. 3, *A* and *B*). Our results, therefore, indicate that association of PC-SA with AmB enhanced the IL-10-suppressive efficacy of free AmB, which can well correlate with the better parasite removal efficacy of PC-SA-AmB than free AmB. The IL-10-reducing ability of free AmB from human PBMC was shown in a recent report from our laboratory (45). Conversely, lipid association with AmB in AmBisome almost completely masked the IL-10-reducing ability of AmB. Presumably, this inability of AmBisome to reduce IL-10 explains its failure to protect against relapse and reinfection in our experiments. Notably, none of these drugs was directly effective in vitro in significant augmentation of IFN-γ from the splenocytes of healthy mice stimulated with suboptimal dose of Con A (data not shown).

From previous reports, it is known that the conventional preparation of AmB enhances macrophage-dependent activation of NK cells and T lymphocytes (48). In the in vivo experiments, we therefore studied the immunomodulatory ability of PC-SA-AmB, and further investigated its involvement on the different cell populations in the splenocytes. Normal uninfected mice were treated with PC-SA-AmB in a single-shot dose, parallelly with free PC-SA, free AmB, and AmBisome. Ten days after the treatment, mice were sacrificed, and the ability of the splenocytes to induce Con A (suboptimal)-specific IFN-γ and to reduce LPS-specific IL-10 was evaluated in vitro. Our results showed that PC-SA-AmB induced significant elevation of IFN-γ ($p < 0.001$) in comparison with control (Fig. 3*E*). Although lower, significant ($p < 0.05$) induction of IFN-γ was also observed in PC-SA- and AmBisome-treated mice. Notably, an exacerbated production of IFN-γ (16,200 pg/ml ± 668.9) was observed in free AmB, indicating possible toxic effects. Thus, these results demonstrate that PC-SA in association with AmB induced a positive immunomodulation, suppressing the toxic effect of the free drug. Moreover, splenocytes of PC-SA as well as AmB-treated normal mice secreted reduced levels of IL-10 ($p < 0.05$ and $p < 0.001$, respectively) than the normal controls under the influence of LPS stimulation in vitro, with PC-SA-AmB showing maximum reduction ($p < 0.001$). Thus, PC-SA and AmB showed a synergistic effect when injected as PC-SA-AmB in reducing IL-10 secretion. Notably, AmBisome was unable to reduce IL-10 (Fig. 3*F*) to a significant level in comparison with untreated controls.

To evaluate the possible alterations in the different spleen cell populations, our flow cytometric study revealed that 10 days after the single-shot treatment in normal mice, PC-SA-AmB could not significantly alter the total number of T cells ($CD4^+$ and $CD8^+$), B cells ($CD19^+$), and macrophages ($CD11b^+$) per spleen, in comparison with untreated controls (data not shown). We were, therefore, interested to know the possible functional alterations of these cell populations that might be responsible for the protective immunomodulation induced by PC-SA-AmB. Ten days after treatment, intracellular assessment of IFN-γ and IL-10 production under in vitro stimulation of Con A (suboptimal) or LPS (as applicable, see *Materials and Methods*) from these cell types was performed by flow cytometry. Notably, there were nearly 12- and 8.3-fold enhancement in the frequencies of IFN-γ^+CD4^+ cells per total $CD4^+$ cells and IFN-γ^+CD8^+ cells per total $CD8^+$ cells, respectively (Fig. 3, *G* and *H*), in the group that received PC-SA-AmB treatment in comparison with untreated controls. Moreover, this group showed 11-, 5.7-, 11-, and 6.3-fold reduction in the frequencies of IL-10^+CD4^+, IL-10^+CD8^+, IL-10^+CD19^+, and IL-10^+CD11b^+ cells per total respective cell types relative to controls (Fig. 3, *I*, *J*, *K*, and *L*). In other treatment groups (PC-SA and AmB), IFN-γ-producing $CD4^+$ and $CD8^+$ cell populations were also increased (Fig. 3, *G* and *H*), and IL-10-producing $CD4^+$, $CD8^+$, $CD19^+$, and $CD11b^+$ cells (Fig. 3, *I*, *J*, *K*, and *L*) were decreased with respect to untreated controls, but to an extent lesser than PC-SA-AmB group.

PC-SA-AmB treatment on L. donovani*-infected BALB/c mice induces LAg-specific DTH, and lymphoproliferation in the splenocytes after successful cure*

Aforesaid experiments in normal mice clearly indicated that AmB associated with PC-SA has positive immunomodulatory capacity. Hence, we were interested to investigate the possible immunological alterations induced by the treatment of PC-SA-AmB in *L. donovani*-infected mice upon almost complete clearance of the parasites at cure, and in prevention of relapse and reinfection. Chemotherapeutic intervention and cure from VL are generally associated with the acquisition of a DTH response and, consequently, classical cell-mediated immunity (49). Therefore, we investigated the LAg-induced DTH responses in BALB/c mice with established infection after cure with PC-SA

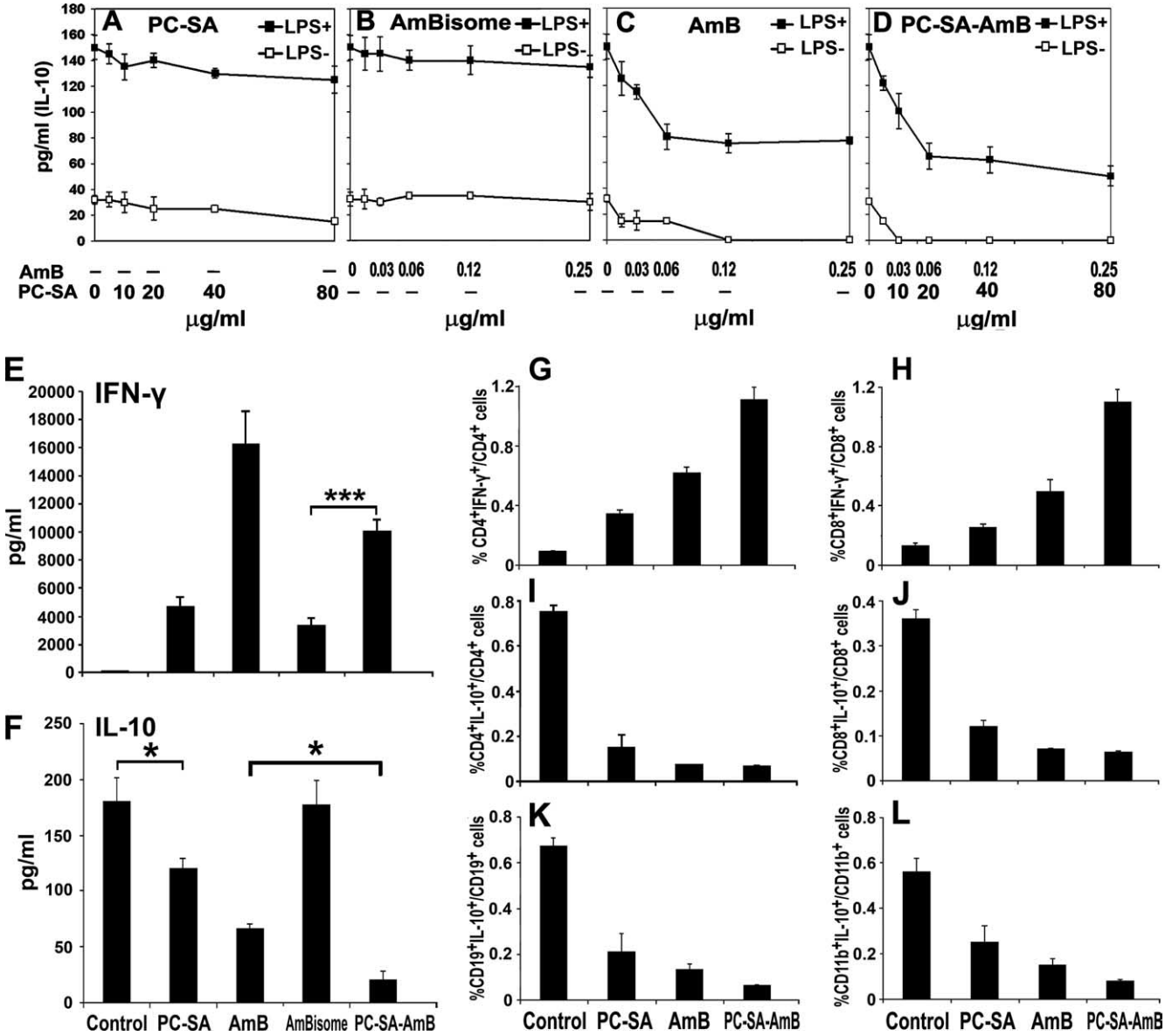

FIGURE 3. Immunomodulatory effect of PC-SA-AmB on splenocytes of normal mice in vitro and in vivo. *A–D*, Splenocytes (2.5×10^5 cells/well) of normal healthy mice were incubated with various concentrations of PC-SA (*A*), AmBisome (*B*), free AmB (*C*), and PC-SA-AmB (*D*) with or without LPS (2.5 μg/ml) for 48 h at 37°C in 95% humidified air with 5% CO_2. IL-10 was measured by ELISA from supernatants. Each symbol represents mean cytokine level (±SEM) at each dose of the drugs ($n = 3$). *E* and *F*, Normal mice were treated once with PC-SA (20.3 mg/mouse), AmB (2.5 mg/kg body weight), AmBisome (3.5 mg/kg body weight), and PC-SA-associated AmB (20.3 mg/mouse and 3.5 mg/kg body weight, respectively). Spleen cells of differently treated animals were isolated 10 days after treatment, plated aseptically (2.5×10^5 cells/well), and stimulated with Con A (2.5 μg/ml) or LPS (2.5 μg/ml) for 48 h. Levels of Con A-specific IFN-γ and LPS-specific IL-10 were determined by ELISA. Data represent the mean ± SE for five animals per group. Data were tested by ANOVA. Differences between mean were assessed for statistical significance by Tukey's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *G–L*, Splenocytes of PC-SA-, AmB-, and PC-SA-AmB-treated (at the doses mentioned above) and control mice were stimulated with Con A (2.5 μg/ml) or LPS (2.5 μg/ml). Surface phenotyping and intracellular staining were performed, as described in *Materials and Methods*, and cells were examined by flow cytometry. Mean frequencies of IFN-γ^+CD4$^+$ cells per total CD4$^+$ cells and IFN-γ^+CD8$^+$ cells per total CD8$^+$ cells (Con A specific) and IL-10$^+$CD4$^+$, IL-10$^+$CD8$^+$, IL-10$^+$CD19$^+$, and IL-10$^+$CD11b$^+$ cells per total respective cell types (LPS specific) in each group of untreated and differently treated ($n = 3$) BALB/c mice have been presented. Data represent the mean ± SE for three animals per group.

liposome-associated AmB treatment, as an index of cell-mediated immunity. Empty liposome- and AmBisome-treated animals showed marginal levels of DTH response. BALB/c mice treated with free AmB exhibited pronounced DTH ($p < 0.001$, vs untreated control). However, the PC-SA-AmB-treated mice showed the strongest DTH response (Fig. 4*A*). Because impairment of cell-mediated immune response in active VL patients is reflected by marked T cell anergy specific to *Leishmania* Ags (50–52), we therefore performed a LAg-specific T cell proliferation assay. Splenocytes from *L. donovani*-challenged PC-SA-AmB-treated mice at 4 wk posttreatment showed ~10-fold enhanced T cell proliferation than infected mice at 2.5 μg/well LAg concentration ($p < 0.001$). Remarkably, splenocytes from free AmB-treated animals also showed enhanced T cell proliferation ($p < 0.001$), but empty liposome and AmBisome treatment had little or no effect on splenocyte proliferation (Fig. 4*B*). Our results indicate that although cationic liposomes alone are relatively inert in terms of activating cell-mediated immunity,

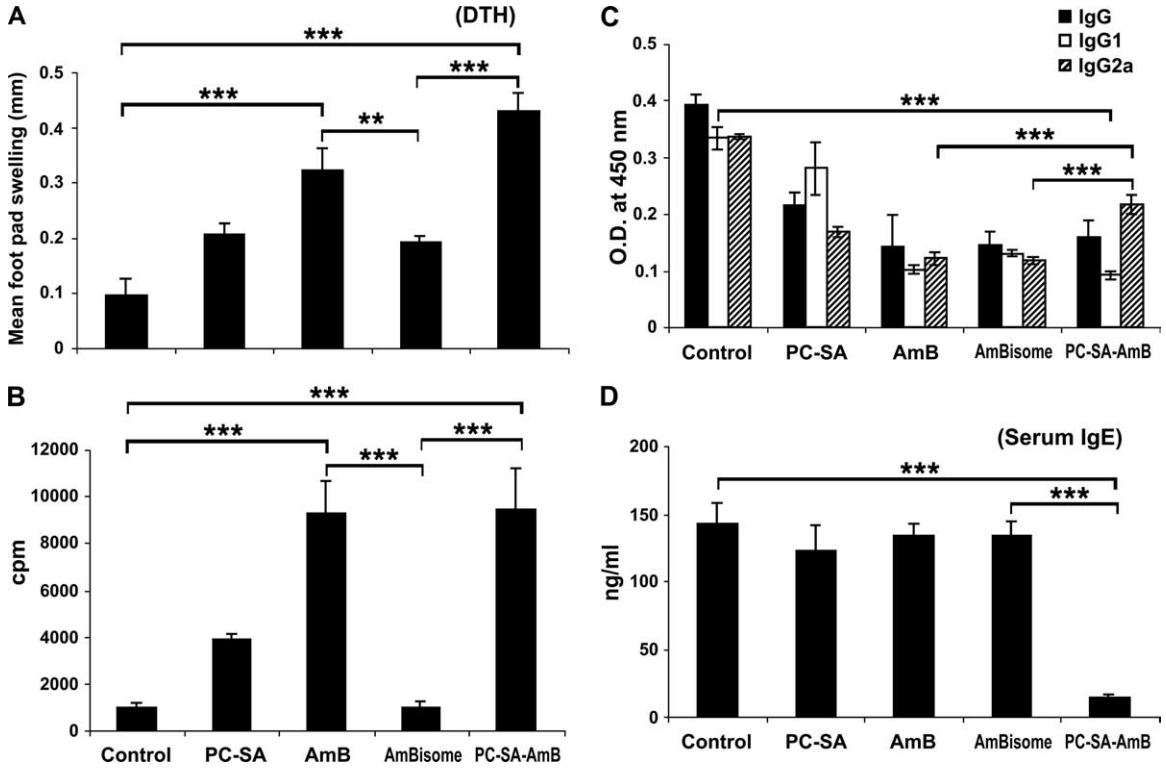

FIGURE 4. Cell-mediated and Ab responses in differently treated *L. donovani*-infected BALB/c mice. *A* and *B*, LAg-specific in vivo DTH, and in vitro proliferation of spleen cells of differently treated animals were determined after 4 wk of drug treatment. *C*, Sera from treated animals were analyzed for LAg-specific anti-IgG, anti-IgG1, and anti-IgG2a levels by ELISA. *D*, Total serum IgE levels in treated animals were determined by BD Optia ELISA kit. Data represent the mean ± SE for five mice per group. Data were tested by ANOVA. Differences between mean were assessed for statistical significance by Tukey's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

they can markedly potentiate activation of cell-mediated immunity in combination with AmB.

LAg-specific enhanced production of IgG2a and decreased IgG1 in PC-SA liposome-associated AmB-treated mice as a measure of combination therapy induced immunity

Previous studies demonstrate that IgG2a levels are dependent on IFN-γ, whereas IgG1 levels correlate with IL-4. IgG2a and IgG1 are therefore used as surrogate markers for Th1 and Th2 responses (46). Ag-specific IgG response was high in infected control mice. With treatment, the IgG response declined in all the groups. Notably, in free AmB-treated mice, IgG2a level was slightly higher than IgG1. However, in AmBisome-treated group, the level of IgG1 was almost equal to IgG2a. Strongest elicitation of IgG2a was found in PC-SA-AmB-treated animals (Fig. 4*C*). In this group, IgG2a level was significantly ($p < 0.001$) higher than IgG1. These results correlate with the high IFN-γ, low IL-4, and almost total absence of IL-10 in PC-SA-AmB-treated mice.

PC-SA-AmB treatment reduces total serum IgE production

The total serum IgE in PC-SA-AmB-treated BALB/c mice was significantly reduced in comparison with untreated control mice (Fig. 4*D*). However, the reduction in the total IgE levels in all the other treated groups was not significant in comparison with the untreated, infected mice. These results suggest the suppression of Th2 immune responses by the treatment of PC-SA-AmB.

PC-SA-associated AmB treatment elicits a Th1-like response in cured mice

Successful clearance of *Leishmania* parasites requires Th1 cells that secrete large amounts of IFN-γ (53) and enhance cellular immunity by activating macrophages and CTL. Resistance to the parasites is antagonized by type 2 cytokines, including IL-4 and IL-10. To evaluate the type of immunological response in *L. donovani*-infected mice after complete cure with PC-SA liposome-associated AmB treatment, detailed analysis of cytokine production was conducted in spleen cells of differently treated infected animals at the protein level by ELISA at 12 wk postinfection, because VL is at an acutely progressive stage at this time point. Comparative cytokine profiles (Fig. 5, *A–E*) clearly demonstrated a high level of IL-10 and some IL-4 secretion, and a low secretion level of IL-12, IFN-γ, and TNF-α cytokines in infected, untreated controls. In contrast, IL-12 and IFN-γ levels were ~10- and ~15-fold greater in PC-SA-AmB-treated mice and ~7- and ~11-fold greater in free AmB-treated mice compared with infected mice, respectively, and the difference between free AmB and PC-SA-AmB for IL-12 secretions was statistically significant ($p < 0.001$). However, in AmBisome-treated mice, IL-12 and IFN-γ level was only 5.6- and 3.3-fold higher than the untreated, infected mice. In empty liposome-treated mice, neither IL-12 nor IFN-γ secretion was enhanced at all. TNF-α level was high in only free AmB-treated mice. Other treatment groups, including the PC-SA-AmB-treated mice, showed marginal levels of TNF-α secretion. A lower level of IL-4 was detected in all the treatment groups in comparison with the untreated, infected group. However, the lowest level of IL-4 secretion was determined in the cultures of the cells from PC-SA-AmB-treated mice. Additionally, although treatment with free AmB and AmBisome reduced IL-10 production by ~6- and ~2-fold, respectively, in comparison with the untreated controls, treatment with PC-SA liposome-entrapped AmB led to an almost absolute decline of IL-10. Notably, difference between AmB and

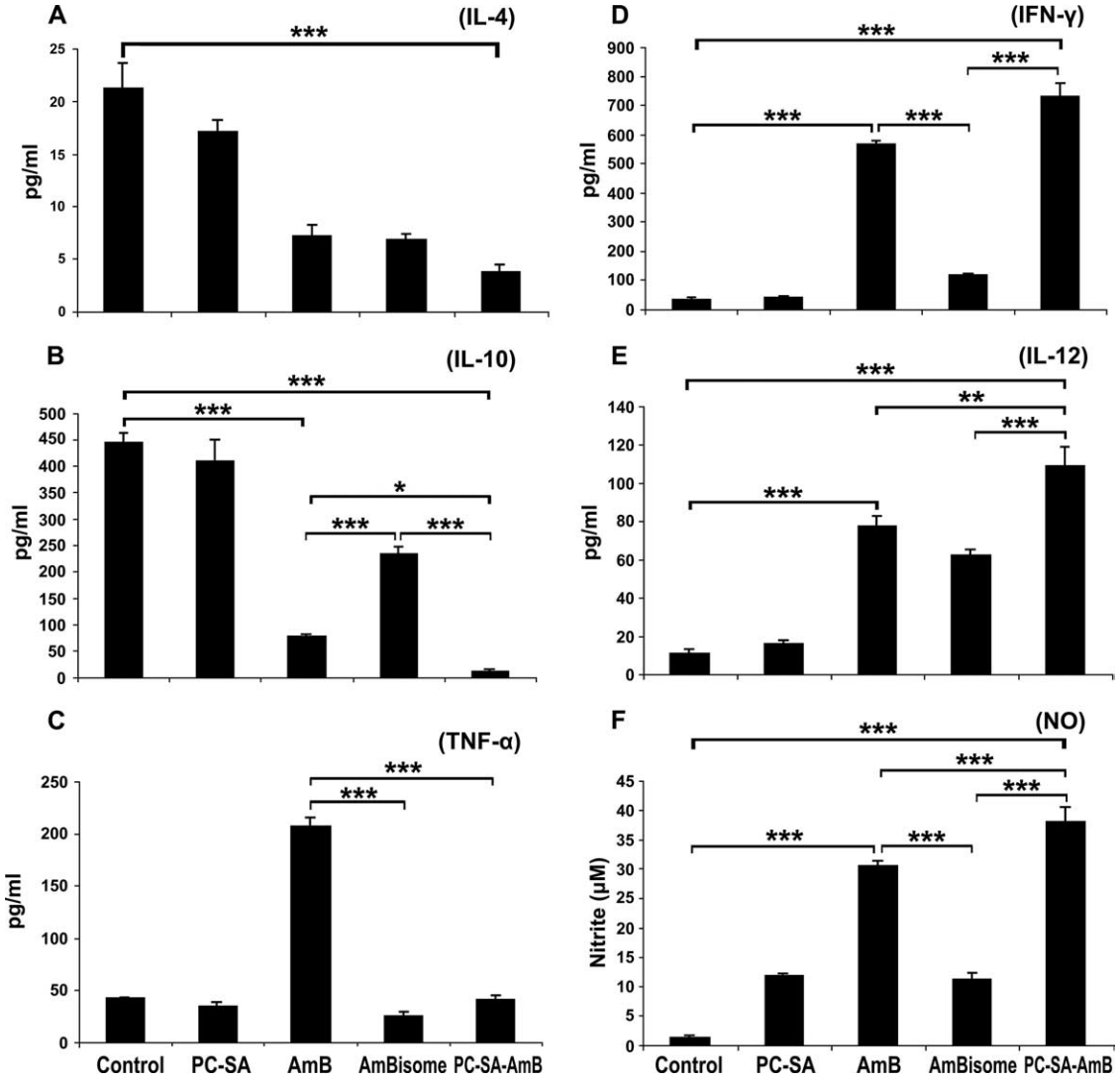

FIGURE 5. LAg-specific cytokine and NO levels in differently treated, infected mice. Spleen cells of indicated treated animals were isolated 4 wk of posttreatment, plated aseptically (2×10^5 cells/well), and stimulated with LAg at 10 μg/ml for 48 h. IL-4 (*A*), IL-10 (*B*), TNF-α (*C*), IFN-γ (*D*), IL-12 (*E*), and NO (*F*) in spleen cell culture supernatants of indicated treatment groups were determined by ELISA and Greiss assay method, respectively. Data represent the mean ± SE for five animals per group. Data were tested by ANOVA. Differences between mean were assessed for statistical significance by Tukey's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

PC-SA-AmB for IL-10 secretion was statistically significant ($p < 0.05$).

PC-SA-associated AmB treatment induces nitrite production in cured mice

NO is the crucial killing effector molecule against leishmaniasis produced by IFN-γ-stimulated and inducible NO synthase-induced, classical macrophages. To determine the influence of PC-SA liposome-associated AmB treatment on *L. donovani*-specific killing effector functions, NO was determined from splenic cell culture supernatants. In case of PC-SA liposome-associated AmB-treated *L. donovani*-challenged mice, we found ~20-fold higher level of nitrite production in comparison with infected controls. Free AmB-treated mice showed ~15-fold increase with respect to the infected control at 12 wk postinfection (Fig. 5*F*). Thus, PC-SA-AmB induced significantly higher NO production than free AmB ($p < 0.0001$). However, empty liposome and AmBisome treatment induced marginal levels of NO production. Our finding is commensurate with elevated IFN-γ produced from the splenocytes of the PC-SA-AmB, followed by free AmB-treated mice.

PC-SA liposome-associated AmB treatment elicits a high frequency of IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells

We used intracellular cytokine staining to examine the comparative frequency of effector T cells recruited into the spleen during established infection and cure in selected treatment groups. Mice with established *L. donovani* infection were treated i.v. with PC-SA liposome-associated AmB or free AmB, and spleens were removed after 4 wk posttreatment. Intracellular cytokine staining was performed for IFN-γ on T cells labeled for surface CD4$^+$ or CD8$^+$ (3 mice from each group). A low frequency of CD4$^+$ (1.84% ± 1.04) and CD8$^+$ (1.19% ± 1.12) T cells secreting IFN-γ (Fig. 6) was detected in spleen of mice bearing established *L. donovani* infection. The frequency of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells increased 4 wk after treatment with free AmB (5.47% ± 0.38 and 4.98% ± 0.58, respectively). The magnitude was highest with PC-SA-AmB treatment (8.21% ± 1.13 and 7.83% ± 1.35, respectively) (Fig. 6). These findings are consistent with enhanced IFN-γ production from splenocytes, as revealed through cytokine ELISA, as described previously, in PC-SA-AmB,

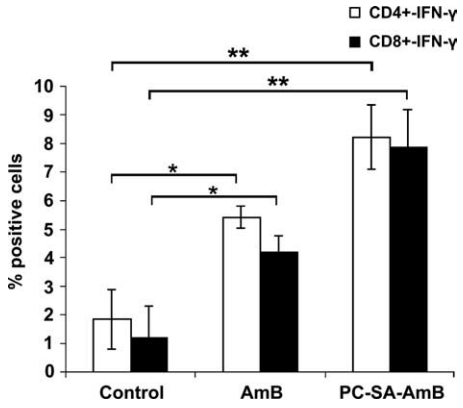

FIGURE 6. Percentage of LAg-stimulated $CD4^+$ and $CD8^+$ T cells of differently treated and untreated, infected BALB/c mice producing IFN-γ. Splenocytes were stimulated with LAg (10 μg/ml). Surface phenotyping and intracellular staining were performed, as described in *Materials and Methods*, and cells were examined by flow cytometry. Mean percentage of $CD4^+$ and $CD8^+$ cells producing IFN-γ in each group of untreated and cured ($n = 3$) BALB/c mice has been presented. The significance of difference between the means was determined by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

followed by free AmB-treated group. The findings also demonstrate a prominent incline toward Th1 effector function and involvement of both $CD4^+$ and $CD8^+$ T cells in producing IFN-γ in sterile cure condition.

Role of cytokines in inhibiting posttreatment recurrence of L. donovani *infection*

Multiple cytokines participate in initial as well as memory response in *L. donovani* infection (47). Earlier, Murray (47) demonstrated a role of IFN-γ to suppress relapse of liver parasite burden after AmB therapy without the requirement of IL-12. But he did not show the role of IL-10 in disease recurrence. Notably, we observed a high level of IFN-γ and IL-12 and almost total decline of IL-10 in mice almost completely cured 1 mo after treatment with PC-SA-AmB (Fig. 5). To further investigate the possible impact of enhanced IFN-γ and almost absolute absence of IL-10 after complete cure to inhibit the replication of residual parasites of bone marrow for disease resurge in liver and spleen, we measured the IFN-γ and IL-10 levels from 5-mo-infected splenocyte culture

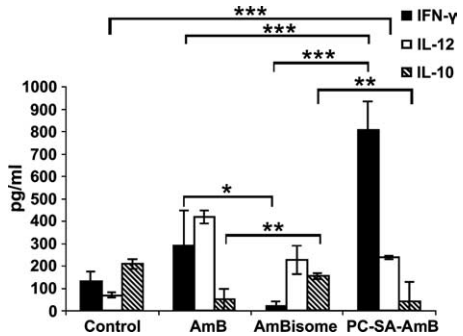

FIGURE 7. Levels of IFN-γ, IL-12, and IL-10 in spleen cell culture supernatants after prolonged parasite suppression (12 wk of posttreatment). Eight-week-infected animals were treated with free AmB, Ambisome, and PC-SA-AmB, and sacrificed 12 wk after treatment. IFN-γ, IL-12, and IL-10 were determined through ELISA. Data represent the mean ± SE for five animals per group. Data were tested by ANOVA. Differences between mean were assessed for statistical significance by Tukey's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

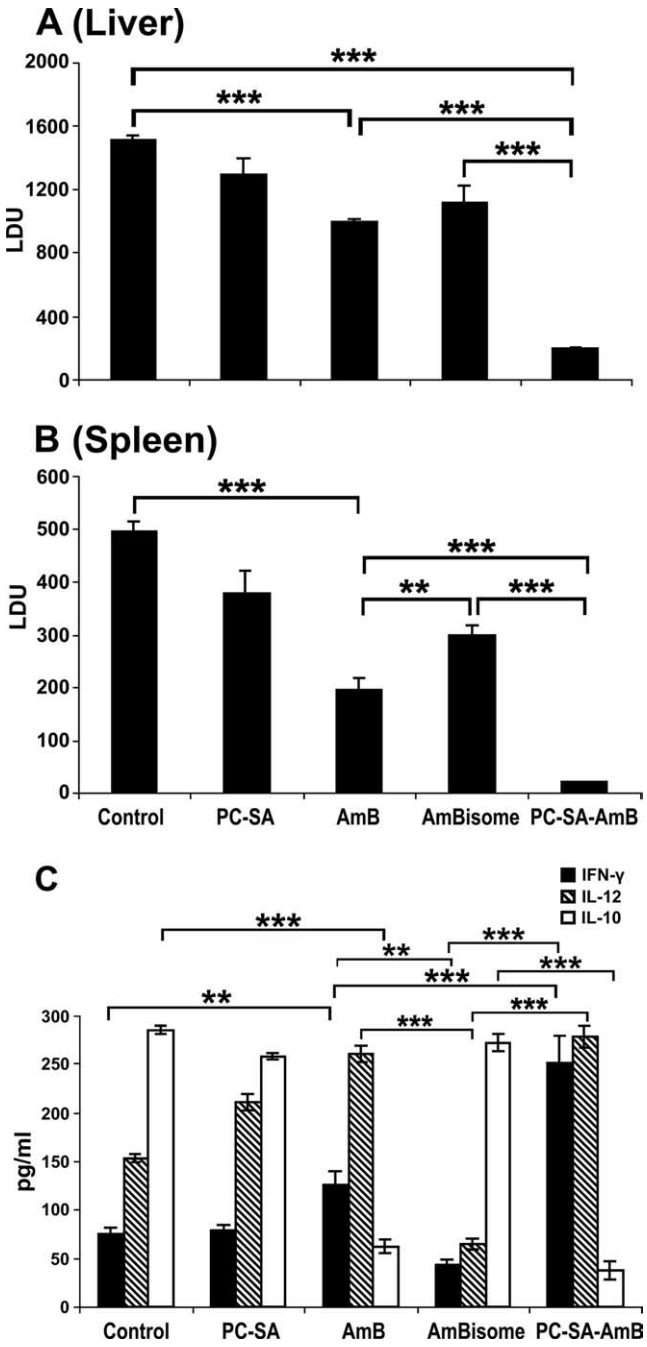

FIGURE 8. Evaluation of prophylactic effect of PC-SA-associated AmB. Normal mice were treated once with PC-SA, AmB, AmBisome, and PC-SA-AmB at doses as mentioned in Fig. 3. Animals were infected with parasites 10 days after treatment and sacrificed after 12 wk of infection. Liver (*A*) and spleen (*B*) parasite burden. *C*, IFN-γ and IL-12 were induced, and IL-10 was inhibited in mice prophylactically treated with PC-SA-AmB. Spleen cell culture supernatants of 12-wk-infected BALB/c mice after indicative treatments were analyzed for cytokine secretion through ELISA. Data represent the mean ± SE for five animals per group. Data were tested by ANOVA. Differences between mean were assessed for statistical significance by Tukey's test (**, $p < 0.01$; ***, $p < 0.001$).

supernatants of BALB/c mice in both untreated and PC-SA-AmB-treated groups. Interestingly, PC-SA-AmB treatment maintained remarkably low level of IL-10 and high level of IFN-γ, and thus inhibited disease relapse even after 3 mo posttreatment (Fig. 7). Moreover, significantly higher level of IFN-γ ($p < 0.0001$) in PC-SA-AmB in comparison with free AmB-treated mice after 3

mo posttreatment correlates with the better efficacy of PC-SA-AmB than free AmB to inhibit disease relapse, and inability of AmBisome to maintain high IFN-γ and low IL-10 correlates with its failure to inhibit disease relapse.

PC-SA-AmB treatment induces a prophylactic effect

So far in this study, our results provided evidence that the immunostimulatory effect of PC-SA-AmB plays an important role in maintaining long-standing parasite-suppressive effects, and therefore, indicated that treatment with PC-SA-AmB creates an immune environment unfavorable for parasite survival and growth. Hence, we were curious to investigate whether PC-SA-AmB treatment is also able to induce any prophylactic effect. Treatment of mice with PC-SA-associated AmB in vivo before *L. donovani* infection considerably reduced the degree of parasitemia in their spleen (96%) and liver (87%) compared with those of the untreated control group (Fig. 8, *A* and *B*). Interestingly, free AmB pretreatment also reduced the parasite burden significantly in comparison with untreated control by 35 and 60% in liver and spleen, respectively ($p < 0.001$). However, AmBisome induced only 29 and 40% protection.

Cytokine correlates of protective immunity

Our data showed evidence of induction of protective effect by cationic liposome-associated AmB. Hence, we were interested to evaluate the cellular immunity responsible for the induction of prophylaxis. We therefore analyzed the Ag-specific cytokine secretion in spleen cell culture supernatants in pretreated 3-mo-infected mice to determine the type of immunomodulation. Highest enhancement of splenocyte IFN-γ secretion (250 pg/ml ± 28.87) was seen in PC-SA-AmB-treated animals (Fig. 8*C*). In contrast, empty liposome- and AmBisome-treated mice secreted a basal level of IFN-γ almost equivalent to the infected group, although free AmB showed significantly higher level of IFN-γ ($p < 0.001$). Notably, PC-SA-AmB induced significantly higher ($p < 0.001$) levels of IFN-γ than free AmB. An elevated IL-12 response was found in spleen-cell culture supernatant of drug-free PC-SA (210.7 pg/ml ± 8.37)-, free AmB (260 pg/ml ± 8.66)-, and PC-SA-AmB (278 pg/ml ± 11.55)-treated mice in comparison with infected mice (152.7 pg/ml ± 4.33). However, in AmBisome-treated group, IL-12 level was low. IL-10 level remained unaltered in drug-free PC-SA liposome and AmBisome-pretreated mice infected with *L. donovani*, correlating with their failure to induce prophylaxis. Free AmB treatment group showed lower ($p < 0.001$, vs untreated control) level of IL-10 secretion in the splenocyte culture supernatant. The lowest level of IL-10 was detected in PC-SA-AmB-pretreated *L. donovani*-challenged mice. The cytokine data therefore correlate well with the prophylactic effect of the combination treatment.

Discussion

The major findings emerging from these studies are that our formulation of a low amount of AmB in SA-bearing cationic liposomes through single-shot administration for the first time demonstrates almost complete clearance of *L. donovani* from the liver and spleen of established and chronically infected BALB/c mice. Only a marginal level of parasites persists in the bone marrow. This mode of treatment generated a protective immunity through induction of IFN-γ and almost absolute decline in IL-10, which suggestively are responsible to prevent relapse and reinfection. Experimental infection with *L. donovani* is characterized by distinct organ-specific immune responses, and development of immune mechanisms in mouse liver has been extensively studied (54). Our present study may throw light on protective splenic cellular immune mechanisms in response to drug treatment.

Previous studies by Belkaid et al. (7) showed that sterile cure was achievable of IL-10-deficient and IL-10/IL-4-double-deficient C57BL/6 mice infected intradermally with low dose of *L. major* parasites at the site of infection. However, complete clearance of parasites did not occur even from liver when BALB/c mice infected with high dose of *L. donovani* parasites were treated with anti-IL-10 Ab in combination with pentavalent antimonials or AmB (8, 16, 17). Hence, it was assumed that neither chemotherapy nor satisfactorily expressed T cell-dependent host immune responses could eradicate all tissue parasites in any form of leishmaniasis (47). *L. donovani* infection in the liver of BALB/c mice is self limiting. However, this same strain of mice develops lifelong splenomegaly associated with parasite persistence (55). So, parasite clearance from spleen is a challenging aspect of chemotherapy. One reason for the almost complete cure achieved in liver and spleen by PC-SA liposome-associated AmB is that PC-SA liposomes can kill parasites by themselves (37, 38). Our recent report established that PC-SA liposomes possess direct leishmanicidal activity (56), which might act synergistically with AmB. On the other hand, profound impairment of the immune system of the infected host in VL is a major cause for partial success of the antileishmanial chemotherapy, and success of cure depends on combined effect of drug and immune status of the host (57). So, we questioned whether the remarkably effective elimination of parasites from both liver and spleen, found in this study, is the combined effect of direct killing and immunomodulatory activity of PC-SA-associated AmB.

Even though leishmanicidal effect of AmB is direct and does not require host immune responses for activity, previous reports showed that AmB stimulated production of proinflammatory cytokines such as TNF-α, IL-6, IL-1Ra, IL-1β, chemokines (IL-8, MCP-1, MIP-1β, NO, PG, and ICAM-1) from murine and human immune cells (20). Moreover, targeting of Th1-cell mechanism increased the efficacy of AmB and permitted lower doses to be used with comparable activities (17). In addition to modifying macrophage function, AmB also alters T cell activity augmenting graft-vs-host reactions (58) and increases in vitro proliferative responses (59). In this study, 2.5 mg/kg free AmB treatment induced exacerbated IFN-γ production in normal mice splenocytes in response to suboptimal dose of Con A and reduced LPS-stimulated IL-10 production. Moreover, induction of DTH, lymphoproliferation, and $CD4^+$ and $CD8^+$ T cells producing IFN-γ, and inhibition of IL-10 were observed after treatment with free AmB in *L. donovani*-infected BALB/c mice. Cationic liposomes alone are relatively inert in terms of activating innate immune responses (60–62). Our previous study revealed that besides being an antileishmanial agent, SA-bearing cationic liposomes greatly augment the immunogenicity of the associated Ags (39). Present study showed that PC-SA liposomes used in 7:0.9 molar ratio of lipids, although inducing some level of IFN-γ and reduced LPS-specific IL-10 in normal mice, did not show any immunomodulatory effects in *L. donovani*-infected BALB/c mice. Treatment with PC-SA-associated AmB (3.5 mg/kg) enhanced moderate levels of IFN-γ and reduced maximal levels of LPS-specific IL-10 in normal mice. Notably, the combination therapy increased IFN-γ, IL-12, and NO production approximately by 1.3-fold, suppressed IL-4 by 3.4-fold, and almost completely prevented IL-10 production in comparison with free AmB (2.5 mg/kg) after cure. These observations therefore suggest that PC-SA liposomes in combination with AmB maintained and augmented inherent immunomodulatory efficacy of AmB and freed up Th1 immune responses by suppressing IL-4 and, most importantly, IL-10.

To evaluate the extent of therapeutic and immunomodulatory efficacy of the positive charge and lipid composition of our liposomal AmB formulation in *L. donovani*-infected mice, PC-SA-AmB was compared with an identical dose (3.5 mg/kg) of AmBisome. Therapeutic efficacy of AmBisome demonstrated a high degree of success against established infection model of VL in the liver and spleen, but not in bone marrow. Again, therapeutic efficacy observed with AmBisome in established infection model declined when evaluated against chronically infected mice. Interestingly, although some level of IL-12, IFN-γ, and NO was induced in AmBisome-treated mice in comparison with infected controls, the levels were much lower than both free and PC-SA-associated AmB-treated groups. The restoration of the IL-12 and IFN-γ response and down-regulation of IL-4 and IL-10 in free AmB and PC-SA-associated AmB-treated mice might not be a mere consequence of reduced tissue parasite burden. If it was so, then AmBisome-treated mice would elicit higher levels of IFN-γ and IL-12 and lower levels of IL-4 and IL-10 than the free AmB-treated mice, because lowering of parasite burden after AmBisome treatment was greater than free AmB. Moreover, there was no induction of TNF-α in AmBisome- and PC-SA-AmB-treated mice in contrast to high TNF-α in free AmB-treated mice. This suggests that both AmBisome and PC-SA were able to reduce TNF-α-induced toxicity associated with free AmB treatment. Besides reducing toxicity through inhibition of TNF-α secretion, AmBisome suggestively inhibited IL-12 as well as IFN-γ and NO production, thus masking the beneficial microbicidal immune responses of the encapsulated drug. In contrast, PC-SA-AmB helped to maintain the immune modulation induced by AmB. A previous report showed that AmB deoxycholate preferentially signals through TLR-2, thus favoring the production of TNF-α over that of IL-10, whereas negatively charged liposomes of AmBisome appears to work preferentially via TLR-4, thus skewing the balance toward production of IL-10 over that of TNF-α (63). The inability of AmBisome to down-regulate IL-10, further confirmed through our results (Fig. 3, *B* and *F*), is probably responsible for its lack of response against persistent parasites in bone marrow (Fig. 1*C*), and reduced efficacy against chronic infection, prevention of disease relapse, and failure to prevent reinfection and induce prophylaxis. In contrast, maximal cure of bone marrow parasites, highest cure of chronic infection, maximum protection against reinfection, and relapse and highest prophylactic efficacy induced by a single-dose treatment with PC-SA-associated AmB support that protective immunity is generated by this treatment.

It is well established that induction of long-lasting protective immunity against intracellular infections requires the activation and maintenance of cellular immune responses (64). A crucial role of IFN-γ in the activation of parasiticidal effector mechanisms in macrophages is well known (65–67). Reconstitution experiments using nude BALB/c mice and cell depletion experiments in euthymic mice using anti-CD4 or anti-CD8 mAbs showed the necessity for both $CD4^+$ and $CD8^+$ T cells in the protection against *L. donovani* infection (68). In *L. donovani*-infected BALB/c mice, $CD4^+$ cells are important in the initial 2 wk of infection, but later this cell population is replaced by $CD8^+$ cells when the progression of infection is controlled in liver (69). Our studies on normal mice showed that PC-SA-AmB is able to induce protective cell-mediated immune response mainly through induction of IFN-γ from $CD4^+$ and $CD8^+$ T cells and down-regulation of IL-10 from different splenic cells (see Fig. 3, *G–L*). Furthermore, therapeutic studies showed highest Ag-specific induction of lymphoproliferation; secretion of IL-12, IFN-γ, and NO; and, most importantly, total decline in IL-10 production in spleen of the PC-SA-associated AmB-treated mice. Consequently, highest induction of DTH in this group indicated that PC-SA-AmB was able to induce highest protective cellular immune response. Moreover, flow cytometric analysis revealed that both $CD4^+$ and $CD8^+$ T cells were simultaneously involved in the production of IFN-γ in spleen even 3 mo postinfection. Notably, free AmB also triggered both $CD4^+$ and $CD8^+$ to produce IFN-γ, although at a lesser frequency. These data, further supported by an in vitro depletion study of $CD4^+$ and $CD8^+$ cells that led to reduced IFN-γ production (data not shown), favor a role of these cell types in promoting a durable T cell response. Again, a high level of NO generation at cure suggests the induction of NO-mediated macrophage effector mechanism toward the control of parasite replication in response to PC-SA-associated AmB treatment. Evidence from previous studies showed the requirement of continuous presence of IL-12 for sustaining Th1 immunity, and thereby provides protective cell-mediated immune response against murine *L. major* infection, even in case of reactivation and rechallenge (70, 71). Our studies showing the negligible parasite burden in spleen and liver and high IL-12 and IFN-γ, even at 3 mo of posttreatment, substantiate these observations. In addition, almost total absence of IL-10, maintained even up to 3 mo posttreatment, was probably responsible for the ability of PC-SA-AmB to prevent disease relapse. Notably, prophylactic effect induced by this formulation through enhanced production of IFN-γ and IL-12 and down-regulation of IL-10 again confirms the mode of immune regulation by PC-SA-associated AmB in spleen.

In view of our in vitro and in vivo experiments on normal BALB/c mice, it is clear that AmB exerted an inhibitory effect on IL-10 production that was augmented by PC-SA association, thus freeing up the T cell immune mechanism with a biological outcome of significant IFN-γ production. How AmB down-regulates IL-10 and how positively charged PC-SA liposomes augmented IL-10-reducing capacity of AmB, however, remain to be investigated. A recent report indicates that AmB induces cell signaling via protein kinase C (PKC), Bruton tyrosine kinase, phospholipase C, c-Src, in addition to TLR2 (72). Among these components, PKC inhibition causes impairment in p38MAPK in *Leishmania*-infected macrophages (73) and favors the ERK-1/2-dependent IL-10 expression. PKC induced triggering of p38MAPK and IL-12 from macrophages and dendritic cells (74) probably initiated by AmB, favor IL-12-induced production of IFN-γ from T cells, and impair expression of ERK-1/2 from macrophages, resulting in down-regulation of IL-10.

Taken together, findings in this report support the view that although the leishmanicidal activity of AmB is direct and T cell independent, it has the capability to induce immunomodulation, thus imparting additional effects toward leishmanicidal activity. Our formulation of PC-SA-AmB liposomes not only reduces toxicity and induces delivery of AmB to the phagocytic cells of spleen, liver, and bone marrow, but also has antileishmanicidal activity of themselves. They can maintain and augment the beneficial immune modulatory activities of AmB toward T cells in such a way that the combination therapy eradicated parasites almost totally from liver, spleen, and bone marrow of infected BALB/c mice. Thus, not only direct killing by the drug, but also simultaneous generation of immunity against the disease is the better strategy for optimum efficacy of future drugs for nonhealing leishmaniasis as well as similar chronic infectious diseases.

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