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Kinetoplastid Membrane Protein-11 DNA Vaccination Induces Complete Protection against Both Pentavalent Antimonial-Sensitive and -Resistant Strains of *Leishmania donovani* That Correlates with Inducible Nitric Oxide Synthase Activity and IL-4 Generation: Evidence for Mixed Th1- and Th2-Like Responses in Visceral Leishmaniasis[1]

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The emergence of an increasing number of *Leishmania donovani* strains resistant to pentavalent antimonials (SbV), the first line of treatment for visceral leishmaniasis worldwide, accounts for decreasing efficacy of chemotherapeutic interventions. A kinetoplastid membrane protein-11 (KMP-11)-encoding construct protected extremely susceptible golden hamsters from both pentavalent antimony responsive (AG83) and antimony resistant (GE1F8R) virulent *L. donovani* challenge. All the KMP-11 DNA vaccinated hamsters continued to survive beyond 8 mo postinfection, with the majority showing sterile protection. Vaccinated hamsters showed reversal of T cell anergy with functional IL-2 generation along with vigorous specific anti-KMP-11 CTL-like response. Cytokines known to influence Th1- and Th2-like immune responses hinted toward a complex immune modulation in the presence of a mixed Th1/Th2 response in conferring protection against visceral leishmaniasis. KMP-11 DNA vaccinated hamsters were protected by a surge in IFN-γ, TNF-α, and IL-12 levels along with extreme down-regulation of IL-10. Surprisingly the prototype candidature of IL-4, known as a disease exacerbating cytokine, was found to have a positive correlation to protection. Contrary to some previous reports, inducible NO synthase was actively synthesized by macrophages of the protected hamsters with concomitant high levels of NO production. This is the first report of a vaccine conferring protection to both antimony responsive and resistant *Leishmania* strains reflecting several aspects of clinical visceral leishmaniasis. *The Journal of Immunology,* 2005, 174: 7160–7171.

Leishmaniasis caused by the most genetically diverse intracellular protozoan parasite is exemplified by its diversity and complexity. Visceral leishmaniasis (VL),[4] also known as kala-azar, is caused by members of *Leishmania donovani* complex resulting in clinical symptoms like fever, cachexia, hepatosplenomegaly, and blood cytopenia. Active VL is associated with the absence of parasite-specific cell-mediated immune response (1, 2). VL has also been increasingly recognized as an opportunistic infection in individuals with HIV infection (3). World Health Organization has identified leishmaniasis as a major and increasing public health problem (4). The visceral form of leishmaniasis is fatal if left untreated, with recent epidemics in Sudan and India resulting in over 100,000 deaths (5). Failure of the pentavalent antimonials, the present main form of chemotherapeutic treatment worldwide, is attributed to the emergence of antimonial-resistant *Leishmania* strains resulting in frequent relapses after treatment (6, 7). In India, antimony is no longer useful as a drug because 65% of VL patients fail to respond or promptly relapse (8). Alternative chemotherapeutic treatments with amphotericin B and its lipid formulation have severe limitations due to their toxic effect and prohibitive high cost of treatment (7). An in vitro study has shown that *Leishmania* also developed resistance against miltefosine, a recently approved effective oral drug for treatment of VL (9). Growing limitations in available chemotherapeutic strategies due to emerging resistant strains and lack of an effective vaccine strategy against VL deepens the crisis.

Immune response to *Leishmania*, influenced by forms of disease that can range from relatively mild localized to severe visceralizing life threatening, depends on several host- and parasite-related factors (10, 11). A realistic assessment of efficacy of vaccine against leishmaniasis therefore depends upon three important variables that we considered in our study: 1) genetic make up of the host, 2) nature of the Ag tested, and 3) the nature of vaccine. Considering the host, our goal of formulating a vaccine strategy was based on its implementation in a golden hamster model as these animals largely reflect the clinicopathological features of progressive human VL, including a relentless increase in visceral

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[4] Abbreviations used in this paper: VL, visceral leishmaniasis; KMP-11, kinetoplastid membrane protein-11; CL, cutaneous leishmaniasis; SLA, soluble leishmanial Ag; iNOS, inducible NO synthase; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates.

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burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia, and ultimately death (12).

Addressing the Ag variable was the basis of the selection of kinetoplastid membrane protein-11 (KMP-11) as a vaccine candidate Ag. KMP-11, a highly conserved surface membrane protein present in all members of the family Kinetoplastidae, is differentially expressed both in amastigote and promastigote forms of *Leishmania* (13–15). In a previous report, we have implicated an association of KMP-11 expression with the vaccine potential of an attenuated, avirulent *Leishmania* strain UR6 (16). Moreover, the ability of KMP-11 to induce IFN-γ from PBMC derived from Kenyan patients cured of VL has been reported (17). KMP-11 is the only Ag that was uniquely recognized by the sera of all asymptomatic *L. infantum*-challenged golden hamsters (18). Several findings indicate a disparate host response to different parasite Ags in cutaneous leishmaniasis (CL) and VL. *Leishmania* glycoprotein 63 failed to induce significant IFN-γ from lymphocytes of patients cured of VL (17). In contrast the same Ag, glycoprotein 63, induced high levels of IFN-γ from lymphocytes of patients cured of CL (19). KMP-11, unlike glycoprotein 63, induced significant production of IFN-γ from lymphocytes of patients cured of VL (17). Thus the nature of Ag influencing the host immune response to different *Leishmania* species comes under scrutiny.

The third variable regarding the nature of vaccination was addressed in the selection of a genetic form of immunization instead of protein vaccination, as DNA vaccination elicits a comparable Th response but a stronger CTL response due to its ability to present endogenously processed Ag through a MHC class I-restricted pathway. $CD8^+$ CTLs causing direct lysis of infected target cells resulting in healing of lesion was reported in a murine CL model (20). Recently $CD8^+$ T cells have been shown to be essential for development of naturally acquired immunity to primary infection with *L. major* (21). Moreover depletion of $CD8^+$ T cells at the time of immunization abrogated the protective efficacy of a *Leishmania* homologue of receptor for activated C kinase (LACK)-DNA vaccine in *L. major* model making it worthwhile to assess the role of Ag-specific CTL response in generating protective immunity in experimental VL model (22).

It has been suggested that, unlike in CL, disease susceptibility in VL is determined to be due to the lack of Th1 response rather than the presence of Th2 response (23). In clinical model *Leishmania* Ag-specific expansion of both Th1 and Th2 subsets capable of producing IFN-γ as well as IL-4 and IL-10 are found in cured VL patients (24, 25). In this report we formulated an effective DNA vaccine encoding KMP-11 and dissected its protective effector mechanisms against both antimonial-sensitive and -resistant virulent *L. donovani* strains. Therefore studies were performed to assess whether the resultant protective ability of genetically immunized hamsters was due to a polarized Th1-like response, as mostly observed in protected experimental VL models, or to a mixed Th1/Th2-like response, representative of a clinically cured VL scenario. Our studies gave rise to some hitherto unknown aspects of complex cytokine interplay and effector mechanisms in experimental VL. Our results indicate that copresence of Th1- and Th2-like cytokines, without the necessity of an absolute polarized Th bias, can tilt the immune system toward either a progressive or protective response in VL depending on T cell functionality, Ag-specific CTL-like response, and induction of leishmanicidal effector molecules.

Materials and Methods

Reagents

HEPES, penicillin-streptomycin, sodium bicarbonate, 2-ME, BSA, ABTS, sulfanilamide, *N*-(1-naphthylethylene diamine hydrochloride) were purchased from Sigma-Aldrich. Tritiated thymidine [^{3}H]thymidine was obtained from New England Nuclear. Biotin-conjugated mouse anti-Armenian and anti-Syrian hamster IgG1 mAb, mouse anti-Syrian hamster IgG2 mAb, FITC-conjugated anti-mouse IgG mAb, and avidin-HRP conjugate were obtained from BD Biosciences, and H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) was obtained from Molecular Probes.

Cell culture

IL-2-dependent murine cell line HT-2 bearing high affinity IL-2R (26) was obtained from American Type Culture Collection. Cells were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM $NaHCO_3$, 20 mM HEPES, 5×10^{-5} M 2-ME along with 10% FCS (Invitrogen Life Technologies) at 37°C in the presence of 5% CO_2 supply.

Animals, parasite and infection, immunization and dose, and tissue response

Golden hamsters at 4- to 6-wk-old, reared in institute facilities, were used for experimental purposes with prior approval of the animal ethics committee of the Indian Institute of Chemical Biology (Kolkata, India).

Two strains of *L. donovani*, pentavalent antimonial-responsive AG83 (MHOM/IN/83/AG83) and pentavalent antimonial-resistant GE1F8R, were used for experimental purposes (27). AG83 was originally obtained from Indian kala-azar patients and GE1F8R, an in vitro developed isolate, was originally obtained from patient *L. donovani* isolates GE1 (MHOM/IN/89/GE1). GE1F8R, derived from a Stibanate-responsive patient, was made Stibanate-resistant by repeated passage in hamsters followed by suboptimal doses of Stibanate. EC_{50} values showed that GE1F8R is 10 times more resistant to Stibanate-sensitive clone GE1C6S and 1.3 times more resistant than RS1 clone derived from Stibanate-unresponsive patient. GE1F8R retained their phenotypes in vivo as amastigotes (28). Both strains were maintained in golden hamsters as previously described (27). Promastigotes obtained after transforming amastigotes from infected spleen were maintained in M199 (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS at 22°C. For infection, 4- to 6-wk-old golden hamsters were injected with 1×10^6 second-passage promastigotes suspended in saline through intracardiac route using a 28-gauge needle.

For immunization, hamsters were divided into two groups: one immunized with KMP-11 containing pCMV-LIC mammalian expression vector (pCMV-LIC/KMP-11) and the other with blank vector construct not harboring KMP-11 gene (pCMV-LIC). Endotoxin-free plasmid DNA was isolated using EndoFree Plasmid Mega kit (Qiagen) according to the manufacturer's instruction. Seven and 15 days before lethal parasite challenge with AG83 or GE1F8R, 100 μg of endotoxin-free plasmid DNA construct (pCMV-LIC, pCMV-LIC KMP-11) dissolved in saline and injected i.m. in the hind leg thigh muscle was used for immunization of golden hamsters using 28-gauge needle.

For studying early (2 and 4 wk postinfection) hepatic tissue response in hamsters infected with AG83 and GE1F8R, liver sections from KMP-11 DNA immunized and infected hamsters were assessed microscopically after staining with H&E as previously described (29). Briefly, portions of liver tissue were first fixed in 10% formalin, and after standard processing like paraffin embedding, histologic sectioning and mounting on glass slides, tissue sections were stained. Sections from each liver ($n = 5$ hamsters per group) were examined by counting 25 consecutive ×40 microscopic fields per section. Besides early histologic response study, all the assays were undertaken between 90 and 120 days postinfection because the disease is progressively acute and reaches near its peak in terms of organ parasite burden by this time.

Determination of splenic and hepatic parasite burden by serial dilution method

The parasite burden was quantified in spleen and liver tissue by serial dilution assay as previously described (30). A weighed piece of spleen or liver from experimental hamsters was first homogenized between two sterile frosted glass slides in complete M199 medium and diluted with the same medium to a final concentration of 1 mg/ml. Ten-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 a 3-day interval, 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.

Cloning

pCMV-LIC vector used for cloning was purchased from BD Pharmingen. *L. donovani* genomic DNA was isolated from 10^8 promastigotes, which were washed with PBS, pH 7.4, (1.37 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and STE (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA), were suspended in STE buffer and incubated with proteinase K (1 mg/ml; Invitrogen Life Technologies) and 0.5% SDS at 50°C for 4 h. Nucleic acids were extracted by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Genomic DNA was spooled and subjected to RNase (100 μg/ml) treatment. KMP-11 gene was amplified using High Fidelity *Taq* Polymerase (Invitrogen Life Technologies) having a 3′-5′ exonuclease activity. PCR was performed using the following reagents: 50 mM Tris-Cl, 1.5 mM $MgCl_2$, and 10 μM each of dNTPs (Invitrogen Life Technologies) and KMP-11-specific primers with an additional 13 bp sequence homology to the vector plasmid forward, 5′-CT GGTTCCGGCGAATGGCCACCACGTACGAGGAG-3′; reverse, 5′-CTCGCTCCGGCGATTACTTGGACGGGTACTGCGC-3′ for 35 cycles in a Thermocycler (Gene Amp PCR System 9700; Applied Biosystems) under conditions at 94°C for 2 min, 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. Amplified PCR products were electrophoresed in agarose gel and eluted from the gel by GenElute Minus EtBr Spin Columns (Sigma-Aldrich). For the generation of vector compatible 13 bp single stranded overhangs, 100 μg of purified DNA was treated with 5 U of T4 DNA polymerase (Invitrogen Life Technologies) in a 10-μl reaction volume (165 mM Tris acetate, 330 mM sodium acetate, 50 mM magnesium acetate, 2.5 mM DTT, and 100 μg/ml BSA) in the presence of 0.5 mM dTTP at 37°C for 20 min. The linear pCMV-LIC (7.5 ng) with 13 bp single stranded homologous sequence and T4 DNA polymerase-treated PCR products (50 ng) were mixed in 10 μl and prepared for annealing at 22°C for 30 min. After heat inactivation of T4 DNA polymerase, 5 μl of annealing mix was used to transform competent DH5α cells. The transformants were screened for the presence of recombinant plasmids with KMP-11 insert by PCR under similar conditions as previously mentioned. Isolated positive clones were sequenced by DNA sequencer (ABI PRISM, model 377; Applied Biosystems).

Control vector without insert was generated by double restriction digest of pCMV-LIC KMP-11 at *Eco*R1 and *Apa*I sites flanking the insert. The 2 μg of construct was initially digested with 10 U of *Eco*R1 at 37°C in a total volume of 40 μl (500 mM Tris, pH 8.0, 100 mM $MgCl_2$, 500 mM NaCl) followed by 5′ end filling with Klenow fragment in the presence of dNTPs in a total volume of 20 μl (0.5 mM dNTP mix, 2 U Klenow fragment) and incubated for 10 min at 37°C. After purification, the DNA was digested with *Apa*I at 30°C for 1 h followed by removal of 3′ overhangs generated by *Apa*I. A reaction mixture containing purified DNA, 1 × T4 DNA polymerase reaction buffer and 20 μM each dNTPs were added along with 1 U of T4 DNA polymerase and incubated at 15°C for 30 min. After heat inactivation of T4 DNA polymerase at 75°C for 10 min, the DNA was purified as mentioned. Finally 50 ng of linear DNA was ligated by blunt-end ligation in a final volume of 50 μl (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% polyethylene glycol 8000 solution (w/v), 2 U of T4 DNA ligase) at 14°C for 24 h. T4 DNA ligase was inactivated by heating at 70°C for 10 min. The reaction mixture was used directly for transformation and the colonies negative for KMP-11 were checked by PCR and selected.

Parasite soluble Ag preparation

Soluble leishmanial Ag (SLA), used in ELISA, T cell proliferation assay, IL-2 assay, nitrite, and superoxide measurements, was prepared from stationary phase promastigotes of *L. donovani* strains AG83 and GE1F8R separately following published protocol (31). Leishmanial lysate from washed promastigotes (10^9/ml) was prepared by several cycles (minimum six) of freezing (−70°C) and thawing (37°C) followed by 5 min incubation on ice. Partially lysed promastigotes were then disrupted in a sonicator thrice for 30 s each (Soniprep 150; MSE) and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant containing soluble Ag was collected and the protein concentration was determined by Bradford Protein Assay method (Bio-Rad). Prepared Ag was stored at −20°C until further use.

T cell proliferation assay

T cell proliferation assay was performed as described (32). Single cell suspension of splenocytes from different experimental groups of hamsters 90 days postinfection with AG83 or GE1F8R were prepared after Ficoll density gradient centrifugation and then suspended in complete RPMI 1640. Cells were plated in triplicate at 10^5 cell/well concentrations in 96-well plates and allowed to proliferate for 3 days at 37°C in 5% CO_2 incubator in the presence of different SLA concentrations (0.5, 5, and 50 μg/ml) and also without SLA. At 18 h before they were harvested, cells were pulsed with 1 μCi (6.7 Ci/M) [^{3}H]thymidine/well. [^{3}H]Thymidine uptake, as an index of proliferation, was measured by liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

IL-2 assay

Splenocytes (5×10^5 cells/well) of hamsters derived from different groups of experimental hamsters 90 days postinfected with either AG83 or GE1F8R were stimulated for 24 h with SLA (0.5, 5, and 50 μg/ml) or without SLA in complete RPMI 1640 medium in 5% CO_2 incubator at 37°C. The culture supernatants were analyzed for the presence of IL-2 by proliferation of IL-2-dependent murine cell line (HT-2), and the extent of proliferation was measured by [^{3}H]thymidine uptake. A total of 10^4 HT-2 cells/well were incubated with 100 μl of culture supernatant for 48 h. The cells were then pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol) for 18 h (32). Incorporation of radioactive thymidine was assessed by liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

CTL assay

Nonadherent splenocytes from different groups of experimental hamsters 105 days postinfection were stimulated with SLA for 7 days followed by incubation with ^{51}Cr-labeled targets (splenic macrophages derived from normal hamster spleen and transfected with pCMV-LIC, pCMV-LIC KMP-11, and pEGFP-N1) in round-bottom 96-well plates (200 μl) at a different E:T ratio (6:1, 12.5:1, 25:1, and 50:1) for 4 h. Target cells (10^6) were labeled with 100 μCi Na_2CrO_4 (BARC) for 1 h at 37°C in 5% CO_2 incubator and washed several times until no gamma irradiation count were detected in the supernatant. After 4 h incubation, 100 μl of culture supernatant was collected and counted in triplicates in liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument). Specific lysis was calculated according to the formula: Percentage of specific lysis = ((sample − spontaneous release)/(maximum release − spontaneous release)) × 100.

RNA isolation and semiquantitative RT-PCR of cytokine and inducible NO synthase (iNOS)

RNA from splenocytes of different groups of experimental hamsters at 105 days postinfection was isolated following RNeasy Minikit Isolation Procedure (Qiagen) as described. The following forward and reverse primers were used to amplify the following cytokines as described by Melby et al. (12): IL-12, forward 5′-GTACACCTGYCACAAAGGAG-3′, reverse 5′-GATGTCCCTGATGAAGAAGC-3′ (430 bp product); IL-10, forward 5′-ACAATAACTGCACCCACTTC-3′, reverse 5′-AGGCTTCTATGCAGT TGATG-3′ (432 bp product); IL-4, forward 5′-CATTGCATYGTTAGCR TCTC-3′, reverse 5′-TTCCAGGAAGTCTTTCAGTG-3′ (463 bp product); IFN-γ, forward 5′-GGATATCTGGAGGAACTGGC-3′, reverse 5′-CGACTCCTTTTCCGCTTCCT-3′ (309 bp product); TNF-α, forward 5′-GACCACAGAAAGCATGATCC-3′, reverse 5′-TGACTCCAAAG TAGACCTGC-3′ (695 bp product); TGF-β, forward 5′-CCCTGGAYAC CAACTATTGC-3′, reverse 5′-ATGTTGGACARCTGCTCCAC-3′ (310 bp product). To obtain specific amplifications for iNOS, specific primers were designed: forward 5′-GCAGAATGTGACCATCATGG-3′; reverse 5′-CTCGAYCTGGTAGTAGTAGAA-3′ (198 bp product). For HGPRT (hypoxanthine–guanine phosphoribosyltransferase) amplification, the following primer was designed: forward 5′-ATCACATTATGGCCCTCT GTG-3′, reverse 5′-CTGATAAAATCTACAGTYATGG-3′ (125 bp product). Degenerate bases are indicated by the appropriate International Union of Pure and Applied Chemistry designation (Y = C or T, R = A or G). Using platinum quantitative RT-PCR Thermoscript One Step system kit (Invitrogen Life Technologies), 0.5 μg of RNA was amplified by RT-PCR under the following condition: 55°C for 35 min, 5 min at 95°C for cDNA synthesis, and 95°C for 15 s, 55°C for 40 s, 72°C for 40 s for 40 cycles for amplification of the genes. The identities of the PCR amplified products were verified by agarose gel electrophoresis. Identical aliquots were processed in parallel without addition of reverse transcriptase to rule out the presence of residual genomic DNA contamination in PCR amplification. Densitometry analyses were done using Quantity One software (Bio-Rad) after agarose gel electrophoresis of the PCR products and subsequent ethidium bromide staining and visualization under UV transilluminator. For densitometry calculations, the same band area was taken for band intensity and was normalized to HGPRT.

Quantification of NO

Splenocytes (10^6/ml) from different groups of experimental hamsters 105 days postinfection were incubated with 5 μg/ml SLA or without SLA for 72 h in 5% CO_2 incubator at 37°C. The culture supernatant was analyzed

for their contents of nitrite (NO_2^-) by Griess Reagent containing 1% sulfanilamide in 5% H_3PO_4 and 0.1% aqueous solution of *N*-1-naphthylethylene diamine hydrochloride. The mixture of Griess Reagent and culture supernatant at 1:1 ratio was incubated for 15 min at room temperature in the dark, and the OD was determined at 550 nm by spectrophotometer (SmartSpec 3000; Bio-Rad).

Measurement of reactive oxygen species (ROS)

To monitor the level of ROS, the cell permeant probe H_2DCFDA was used as previously described (33). Splenocytes from different groups of hamsters 105 days postinfection were stimulated with SLA (5 μg/ml) for 72 h and without SLA stimulation, suspended in DMEM and incubated with H_2DCFDA (2 μg/ml) at room temperature for 20 min in the dark. Relative fluorescence was measured in a PerkinElmer LS50B Spectrofluorometer at an excitation wavelength of 510 nm and emission wavelength of 525 nm. For each experiment, fluorometric measurements were performed in triplicate and expressed as fluorescence intensity unit.

Measurement of anti-KMP-11 Ab responses

KMP-11 protein under native condition was purified from pQE-30 plasmid (a kind gift from Prof. H. Moll, University of Wurzburg, Wurzburg, Germany) containing KMP-11 ORF of *L. infantum* aligned in frame with His tag (34). Briefly pQE-30 containing the KMP-11 gene along with pREP4 were introduced into DH5α strain of *Escherichia coli* and induced by isopropyl β-D-thiogalactoside (1 mM) resulting in high-level expression of the recombinant His-tagged protein and allowing purification of the KMP-11 protein by passage of the whole bacterial cell extract through a Ni-NTA affinity column (Qiagen). The purity and the yield of KMP-11 were analyzed in SDS-PAGE and using the Bradford method, respectively. Serum samples from different groups of hamsters (n = 5/group) were obtained between 90 and 120 days of parasite challenge and were analyzed for the KMP-11-specific Ab titer. The 96-well ELISA plates (Nunc) were coated with KMP-11 (2 μg/ml) in PBS overnight at 4°C. Plates were blocked with 5% FCS in PBS at room temperature for 1 h to prevent nonspecific binding. Sera from different groups of hamsters was added at various dilutions (1/10, 1/100, 1/1000, and 1/10000) and incubated for 2 h at room temperature. Biotin-conjugated mouse anti-hamster IgG1 and mouse anti-Armenian and anti-Syrian hamster IgG2 was added for 1 h at room temperature followed by another 1 h incubation with detection reagent (avidin-conjugated HRP). ABTS as peroxidase substrate in citrate buffer (0.1 M, pH 4.3) with 0.1% H_2O_2 was added and absorbance was read on ELISA plate reader (Multiskan MS; Labsystems) at 405 nm.

Statistical analysis

Paired two-tailed t test was used for statistical analysis of the data. Values of $p < 0.05$ were considered. Results are represented as mean ± SD or SE as indicated in figures.

Results

KMP-11 DNA vaccination causes complete clearance of splenic and hepatic parasite burden following AG83 and GE1F8R strains of L. donovani *challenge with early hepatic histopathological responses*

KMP-11 gene was cloned from *L. donovani* genomic DNA under CMV promoter in a mammalian expression vector pCMV-LIC and expression status of the cloned gene was checked in vitro both at mRNA and protein level and in vivo at mRNA level from immunized hamster tissues (data not shown). To study the efficacy of KMP-11 DNA vaccination in VL, we immunized and challenged golden hamsters with either of two different virulent strains of *L. donovani*, AG83, a pentavalent antimonial-sensitive strain, or GE1F8R, a pentavalent antimonial-resistant strain. All the vaccinated hamsters immunized with KMP-11 DNA survived the lethal challenge of AG83 and GE1F8R and remained healthy until the termination of the experiment at 8 mo postinfection, which was the end of our study period, whereas all nonimmunized and blank vector-immunized hamsters succumbed to virulent *L. donovani* challenge within 6 mo (Fig. 1, *A* and *B*). Remarkably, in all hamsters of both the KMP-11 DNA vaccinated groups, there was complete absence of amastigotes in the impressions of stamp smears of transverse sections of spleens and livers when observed under light microscopy (data not shown). Serial dilution assay confirmed complete sterile protection in the hepatic parasite burden in all the 20 hamsters immunized with KMP-11 DNA and challenged either with AG83 or GE1F8R at 2 and 4 mo postinfection (Fig. 1, *E* and *F*). Consistently over 80% of hamsters in both AG83 and GE1F8R challenged KMP-11 DNA vaccinated groups showed sterile protection both at 2 and 4 mo postinfection with respect to their splenic parasite burden (Fig. 1, *C* and *D*). In the case of AG83-infected KMP-11 DNA vaccinated hamsters, 16 of 20 at 2 mo postinfection and 18 of 20 at 4 mo postinfection showed absence of promastigote in the serially diluted culture for 21 days of observation. In case of GE1F8R-infected KMP-11 DNA vaccinated hamsters, 17 of 20 both at 2 and 4 mo postinfection showed sterile protection, verified similarly by serial dilution assay (data not shown). Those KMP-11 DNA vaccinated hamsters that did not show sterile protection in serial dilution assay, showed >99% reduction of splenic parasite burden at 2 and 4 mo of study when compared with respective blank vector-immunized and infected controls.

Because successful resistance to *L. donovani* is reflected by early hepatic histologic reactions (35–39), we examined the liver sections of AG83- and GE1F8R-infected and KMP-11 DNA vaccinated hamsters. In 2 wk time parasitized Kupffer cells were seen with few surrounding mononuclear cells and infiltrating lymphocytes in both AG83-infected (Fig. 2*A*) and GE1F8R-infected (data not shown) hamsters. By 4 wk, heavily parasitized Kupffer cells were seen with congregation of cells surrounding parasitized core forming a granuloma-like structure but with few infiltrating lymphocytes (Fig. 2*B*). In KMP-11 DNA vaccinated hamsters, parasitized Kupffer cells were not observed and well-formed granuloma comprised of mononuclear cells and surrounding lymphocytes were observed at 2 wk postinfection (Fig. 2*C*). In 4 wk time, complete absence of parasitized Kupffer cells with a higher number of lymphocyte infiltrates was observed (Fig. 2*D*).

SLA induces proliferation of splenocytes and IL-2 generation from KMP-11 DNA vaccinated hamsters

Impairment of cell-mediated immune response in active VL patients is reflected by marked T cell anergy specific to *Leishmania* Ags as found in Indian kala-azar and South American VL as well as in experimental models (40–42). As it is generally noted that in vitro T cell proliferation is impaired in VL, we performed a T cell proliferation assay. Splenocytes from AG83-challenged KMP-11 DNA immunized hamsters at 90 days postinfection showed ~17-fold enhanced T cell proliferation than infected and blank vector-immunized hamsters at 5 μg/ml SLA concentration ($p < 0.0005$). At a similar Ag concentration, GE1F8R-challenged KMP-11 DNA immunized animals showed ~13 times greater proliferation ($p < 0.0005$) compared with infected and blank vector-immunized hamsters (Fig. 3*A*).

It has been shown that impairment of IL-2 generation and depressed splenic T cell response are associated in experimental as well as clinical VL (2, 41, 43). Thus we addressed the functional activity of IL-2 in KMP-11 DNA immunized and infected hamsters. When spleen cells from all groups hamster 90 days postinfection were stimulated with and without SLA for 24 h and the supernatants were tested for IL-2 activity (in terms of [^{3}H]thymidine uptake in HT-2 cell line), it was seen that the culture supernatants from splenocytes of KMP-11 DNA immunized hamsters contained significant level of IL-2 compared with normal as well as infected hamsters (Fig. 3*B*). In case of AG83 infection, KMP-11 DNA vaccinated hamsters showed 3.66-fold ($p < 0.001$), 5.16-fold ($p < 0.0001$), and 2.82-fold ($p < 0.0005$) more IL-2 production at 0.5, 5, and 50 μg/ml SLA concentration, respectively,

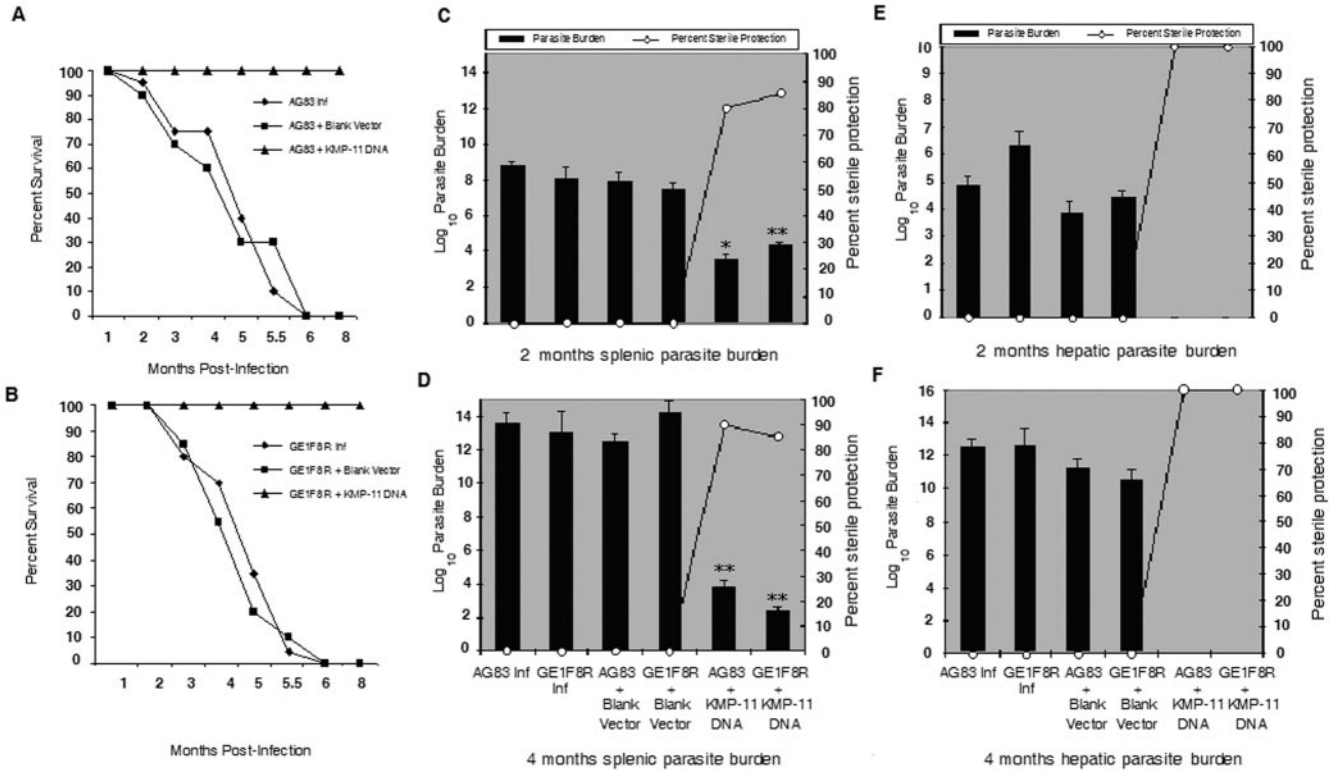

FIGURE 1. Sterile protection is induced by KMP-11 DNA immunization against lethal challenge of pentavalent antimonial-sensitive and -resistant virulent strains of *L. donovani*. *A* and *B*, Survival kinetics of KMP-11 DNA vaccinated hamsters following challenge with either AG83 (*A*) or GE1F8R (*B*) compared with respective blank vector-immunized and infected hamsters. *C–F*, Splenic and hepatic parasite burden of KMP-11 DNA immunized hamsters challenged either with AG83 or GE1F8R compared with respective blank vector-immunized and infected control groups of hamsters at 2 mo postinfection (*C* and *E*) and 4 mo postinfection (*D* and *F*). For vaccination, hamsters were prophylactically injected twice 7 days apart with 100 μg of pCMV-LIC KMP-11 construct or empty plasmid DNA through i.m. route. All the infected and DNA immunized hamsters were challenged with either AG83 or GE1F8R (1×10^6) live promastigotes through intracardiac route. Organ parasite burden was determined by serial dilution assay. The reciprocal of the highest dilution that was positive for parasite growth was considered to be the concentration of parasites per milligram of tissue. Total organ parasite burden was calculated from spleen or liver weight. Results are expressed as log of total organ parasite burden. Data represent the mean ± SD for 20 animals per group. ∗, Level of significant variance $p < 0.001$ compared with AG83-infected (Inf) control group of hamsters at 2 mo postinfection; ∗∗, level of significant variance $p < 0.0001$ in comparison with respective infected control groups.

than corresponding infected group, whereas, KMP-11 DNA immunized GE1F8R-infected hamsters showed ~3.57-fold ($p < 0.001$), 7.69-fold ($p < 0.0001$), and 2.52-fold ($p < 0.005$) more IL-2 production at 0.5, 5, and 50 μg/ml SLA concentration, respectively, than corresponding infected hamsters. KMP-11 DNA vaccinated hamster splenocytes showed insignificant level of IL-2 production without SLA stimulation ($p < 0.5$).

Immunization with KMP-11 DNA generates anti-KMP-11 CTL-like response in vaccinated hamsters

The i.m. administration of expression plasmid construct carrying Ag cDNA resulted in activation of Ag-specific CTLs due to their MHC class I-mediated processing and CTL priming as shown against several viral proteins (44) and also against malarial circumsporozoite protein tested in human volunteers (45). We thus became interested to see whether the effector mechanism involves specific CTL-like response against KMP-11 in addition to generation of Th response. Earlier observation that $CD8^+$ T cell depletion at time of infection resulted in abrogated protective efficacy of LACK-DNA vaccination in murine CL model suggested a DNA vaccination induced a dominant role of $CD8^+$ T cell-mediated protective immune response (22). In our case, SLA stimulated splenocytes for 7 days from KMP-11 DNA immunized hamsters (105 days postinfection) challenged with either AG83 or GE1F8R showed a 52.5 and 63.7% lysis of ^{51}Cr-labeled KMP-11 transfected autologous splenic macrophages, respectively, at 50:1 E:T ratio ($p < 0.0001$ compared with respective infected controls) (Fig. 4). The infected and blank vector-immunized control hamsters showed <17% lysis of labeled targets at 50:1 E:T ratio. This basal level of lysis in the infected and blank vector-immunized infected hamsters might indicate the presence of a low marginal threshold of anti-KMP-11-specific CTL-like response even at an acute stage of infection that might not be sufficient to bring about a protective response. Although in hamsters, CTLs have not been characterized, we consider the phenomenon akin to the prototype CTL generation known in other experimental models. This result was further corroborated by the failure of the nonadherent splenocytes from KMP-11 DNA vaccinated hamsters to lyse autologous macrophages transfected with enhanced GFP expressing pEGFP-N1 construct validating KMP-11 specificity of the cytotoxic cells generated in vaccinated hamster splenocytes (data not shown).

DNA immunization elicits a mixed Th1- and Th2-like response in protected group of hamsters

As in *L. donovani*-infected murine models, a mixed Th1 and Th2 response has been noted in VL patients cured from the disease (23–25, 46). We made a detailed splenic cytokine analysis at 105 days postinfection period as VL is at an acutely progressive stage at this time point. Comparative cytokine profile showed that in

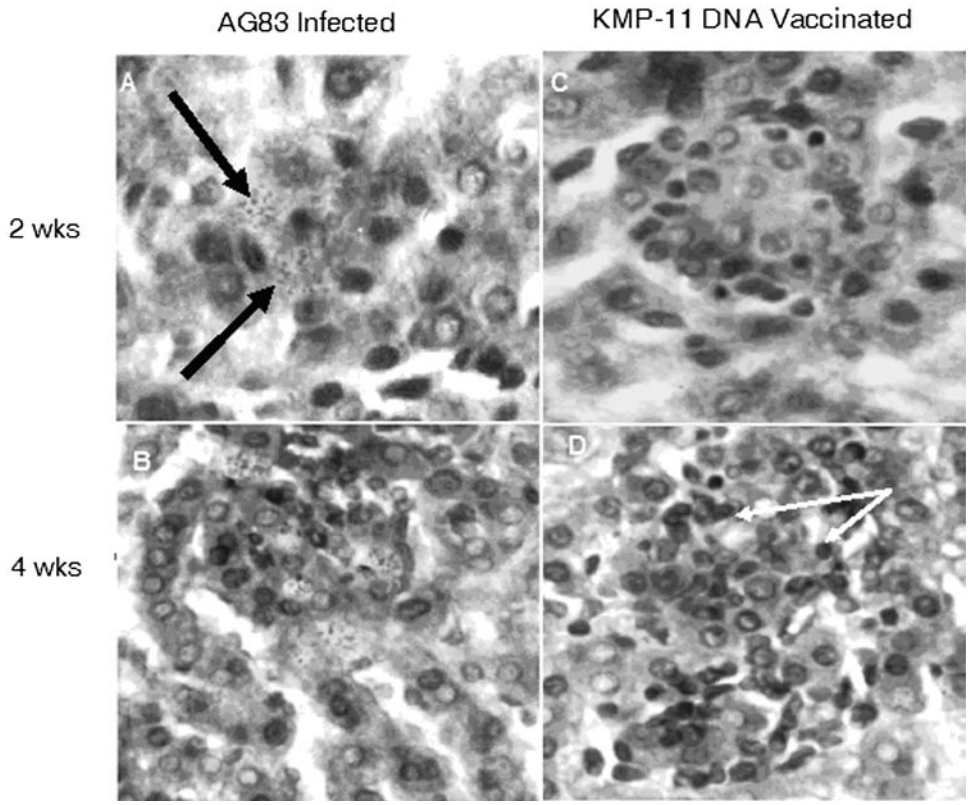

FIGURE 2. Early hepatic histologic response to *L. donovani*-infected and KMP-11-immunized hamsters. Magnification ×40. *A*, Two weeks after infection parasitized Kupffer cells (thick black arrows) with few epitheloid cells resemble ill-formed granuloma. *B*, 4 wk after infection, granuloma is more organized with heavily parasitized Kupffer cells and few infiltrating lymphocytes. *C*, In contrast, 2 wk after infection DNA immunized hamsters show well-organized granuloma free of parasites with surrounding epitheloid cells and lymphocyte. *D*, Four weeks after infection more infiltrating lymphocytes (thin white arrows) are seen in an enlarged structure resembling involuting granuloma.

both groups of vaccinated hamsters expression of IFN-γ transcripts were ~7- and 10-fold greater than in AG83- and GE1F8R-infected and blank vector-immunized control hamsters, respectively (Fig. 5, *A–C*). IL-12 transcripts in KMP-11 DNA vaccinated hamsters were 7.2- and 4.7-fold greater than in AG83- and GE1F8R-infected and blank vector-immunized control hamsters, respectively. Level of expression of Th1 suppressive cytokine, IL-10 was 11- and 10-fold more in AG83- and GE1F8R-infected groups, respectively, than corresponding vaccinated groups of hamsters reflecting its extreme down-regulation. TNF-α showed 9- and 8.71-fold increased expression in KMP-11 DNA vaccinated groups compared with AG83- and GE1F8R-infected and blank vector-immunized control groups of hamsters. Expression of TGF-β was moderate in both the infected groups whereas in case of KMP-11 DNA vaccinated hamsters its expression was significantly down-regulated. Intriguingly, expression of an established Th2 cytokine-like IL-4 showed ubiquitous association with the protection showing copious transcript generation from the spleen of both KMP-11 DNA immunized protected groups of hamsters challenged either with AG83 or GE1F8R. IL-4 transcript was mostly undetectable in both infected and blank vector-immunized hamsters. KMP-11 DNA immunized hamsters infected with AG83 or GE1F8R showed nearly 15- and 16.4-fold increased IL-4 transcript generation than respective infected and blank vector control groups. Apparently KMP-11 DNA vaccination conferred protection by the preferential induction of Th1- and Th2-like cytokine genes to generate antileishmanial immune response as absolute dominance of Th1- or Th2-like cytokine genes conferring protection or susceptibility could not be highlighted.

DNA immunization induces iNOS transcript in KMP-11 DNA vaccinated hamsters

Lack of detectable NO due to an impaired iNOS signaling pathway despite significant production of IFN-γ is attributed to increased susceptibility to *L. donovani* infection in both hamster and human macrophages (30, 47). In case of KMP-11 DNA immunized AG83-challenged hamsters, we found a 17-fold higher expression of iNOS transcript, whereas DNA immunized GE1F8R-challenged hamsters showed a 13.2-fold increase with respect to their infected controls at 105 days postinfection (Fig. 5, *D* and *E*). iNOS transcripts could not be readily detected from splenic macrophages of blank vector-immunized or infected hamsters. Previously iNOS activation and NO-mediated leishmanicidal activity was implicated in *L. donovani*-challenged golden hamsters treated with polyinosinic-polycytidylic acid and L-arginine that was inhibited by N-w-nitro-L-arginine, an inhibitor of iNOS (48). Our finding is commensurate with extremely elevated IFN-γ and TNF-α transcripts produced from the splenocytes of the KMP-11 DNA vaccinated hamsters. This experiment has revealed, for the first time, that iNOS expression, which is impaired in *L. donovani*-infected experimental hamster model, can be elicited by genetic immunization with KMP-11.

Measurement of reactive nitrogen intermediates (RNI) and ROS generation in KMP-11 DNA vaccinated hamsters

Nitrite and superoxides are two macrophage-derived oxidants that are critical in controlling *Leishmania* infection (30, 49–52). In the cytokine expression profile of both infected and vaccinated hamsters we got elevated levels of iNOS transcripts along with high TNF-α and IFN-γ transcripts in protected hamsters. Thus we became interested in whether these two cytokines can activate macrophages to an extent in which there is sufficient stimuli to generate NO in a down-regulated IL-10 environment. Moreover a recent report showed in an ex vivo study that superior efficacy of SLA in production of IFN-γ and TNF-α compared with LACK Ag alone was demonstrated (11). This prompted us to study the production of NO and ROS both with and without SLA stimulation. At three various SLA concentrations tested (0.5, 5, and 50 μg/ml) optimum

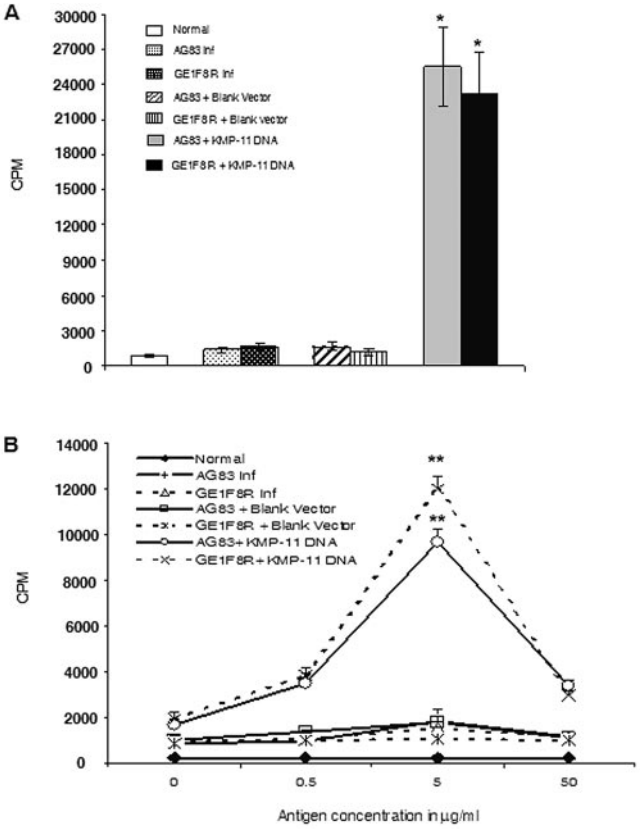

FIGURE 3. KMP-11 DNA vaccination overcomes impaired T cell proliferation with significant IL-2 production. *A*, Proliferative response to SLA (5 μg/ml) by splenocytes from KMP-11 DNA immunized hamsters challenged (Inf, infected) with AG83 or GE1F8R compared with respective blank vector-immunized and infected controls. Proliferation was measured by [^{3}H]thymidine incorporation. At 5 μg/ml SLA stimulation, optimal proliferation was obtained showing high T cell proliferation (*, $p < 0.0005$ compared with respective infected groups of hamsters). The results are representative of four individual experiments ($n = 5$/group) and data represent the mean of triplicate wells ± SE. *B*, Production of IL-2 by spleen cells from KMP-11 DNA immunized hamsters challenged with either AG83 or GE1F8R compared with respective blank vector-immunized and infected controls in terms of proliferation of an IL-2-dependent murine cell line HT-2 (i.e., proportional to [^{3}H]thymidine incorporation). The results are representative of four individual experiments ($n = 5$/group) and data represent the mean of triplicate wells ± SE. IL-2 production was found to be significant in KMP-11 DNA immunized hamsters challenged either with AG83 or GE1F8R compared with blank vector-immunized and respective infected control groups of hamsters (**, $p < 0.0001$ compared with respective infected controls).

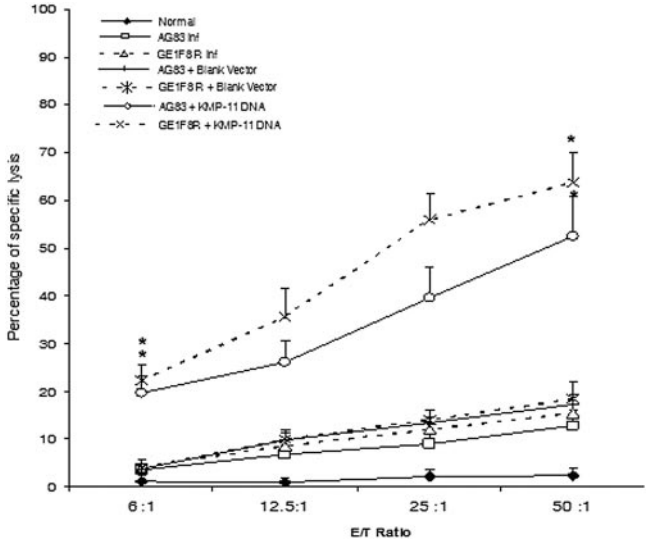

FIGURE 4. KMP-11 DNA immunization generates anti KMP-11 CTL-like response. Splenocytes (effector) from different groups of hamsters were pulsed with SLA for 7 days. Autologous macrophages derived from splenocytes of normal hamsters were cultured and labeled with ^{51}Cr after transfecting with pCMV-LIC/KMP-11 or pCMV-LIC plasmid DNA and used as target. Macrophages transfected with unrelated expression plasmid did not show >10% lysis (data not shown). Spontaneous release was <15%. Results shown are representative of five individual experiments ($n = 5$/group) and data represent the mean ± SE. At 50:1 and 6:1 E/T ratio both groups of DNA immunized hamsters showed high extent of specific lysis of the KMP-11 DNA transfected target cells. *, $p < 0.0001$ in comparison with respective infected control groups.

ROS and NO production were found at 5 μg/ml (data not shown). We found that at 105 days postinfection, AG83-infected hamsters in the presence of SLA stimulation showed 0.7 μM nitrite production and the GE1F8R-challenged hamsters showed 0.84 μM nitrite production (Fig. 6*A*). AG83-challenged hamsters immunized with KMP-11 DNA showed near 20-fold (14 μM) increase in nitrite production ($p < 0.0005$) and GE1F8R-challenged hamsters immunized with KMP-11 DNA showed 15 μM nitrite ($p < 0.0001$) when stimulated at 5 μg/ml SLA. Remarkably significant NO generation was detected from splenocytes of both of the vaccinated groups of hamsters (but not from infected and blank vector-immunized hamsters) even in the absence of SLA stimulation. RNI-like NO is itself sufficient to clear *L. donovani* infection although ROS contributes to the efficiency of parasite killing (53, 54). In the presence of SLA stimulation (5 μg/ml) both AG83- and GE1F8R-challenged KMP-11 DNA vaccinated hamsters showed nearly 6-fold ($p < 0.0005$) and 3-fold ($p < 0.005$) increase in production of superoxide, respectively, than corresponding infected controls reflecting the overall activated state of the splenic macrophages specific to *Leishmania* Ag (Fig. 6*B*).

KMP-11-specific production of IgG1 and IgG2 are increased in vaccinated hamsters challenged with AG83 and GE1F8R

In mice, IL-4 and IFN-γ are two cytokines that direct Ig class switching of IgG1 and IgG2a, respectively. Although in hamsters there are no distinct classifications of Ig, it is believed that hamster IgG2 corresponds to mouse IgG2a/IgG2b and hamster IgG1 corresponds to murine IgG1 (30). DNA vaccinated hamsters developed an effective immune response by showing substantially higher levels of KMP-11-specific IgG2 Ab titer, which is a measure of cell-mediated immune response (Fig. 7*B*). Although a highly significant difference was found in IgG2 titer between KMP-11 DNA vaccinated and infected control groups of hamsters ($p < 0.0001$), there was no significant difference in the KMP-11-specific IgG1 levels ($p < 0.5$) among the infected and the vaccinated hamsters (Fig. 7*A*). This insignificant difference of IgG1 titer between KMP-11 DNA vaccinated and infected control hamster sera might be due to enhanced IL-4 production associated with the vaccinated protected animals.

Discussion

Previous studies from this laboratory and others have hinted toward the immunodominant potential of KMP-11 in both experimental and clinical VL models (16–19, 55). In this study, we attempted to validate the candidature of KMP-11 as an immunodominant Ag by assessing its DNA vaccine potential

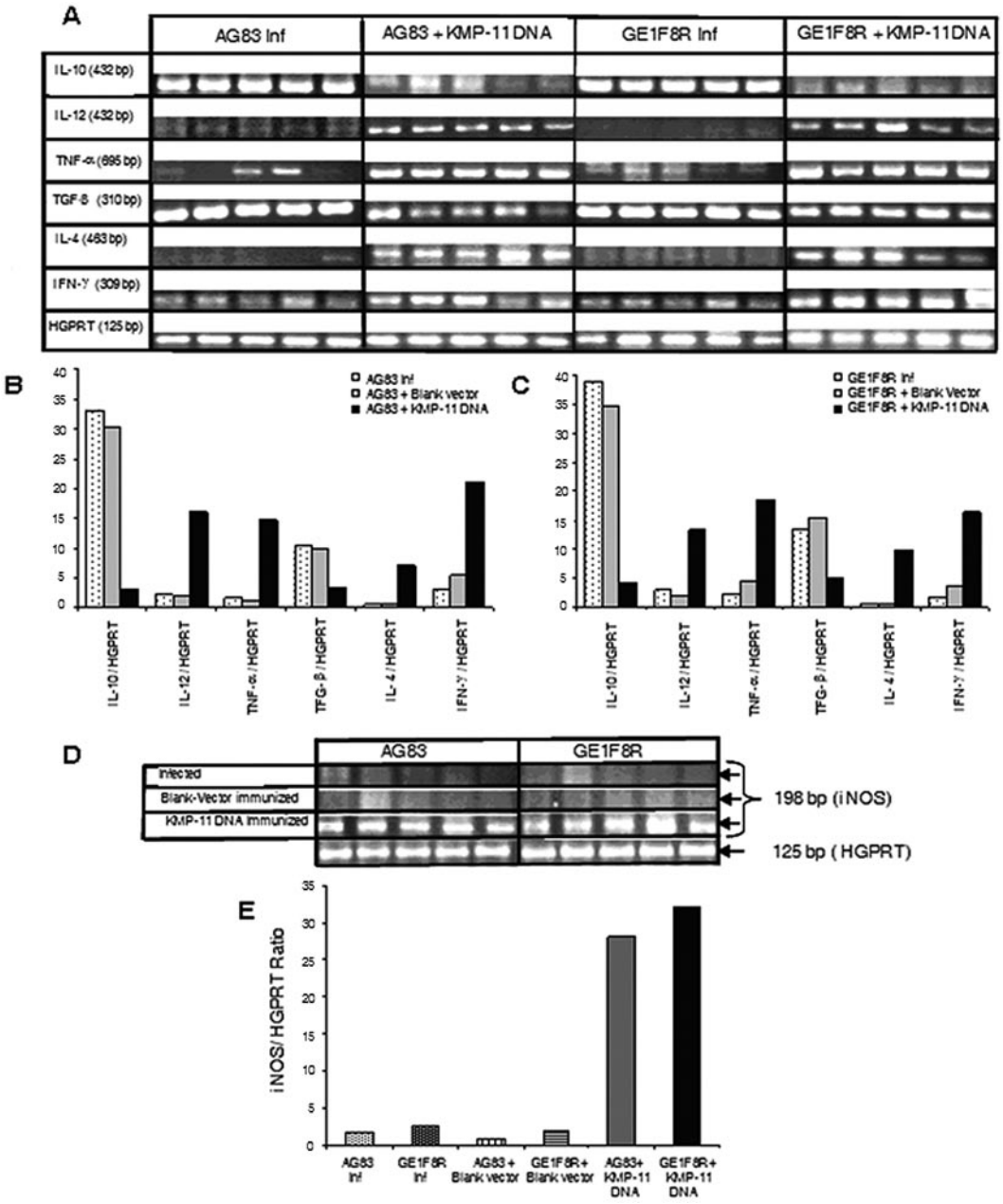

FIGURE 5. Protected KMP-11 DNA vaccinated hamsters produce both Th1 and Th2 cytokines with significant iNOS transcript generation. *A–C*, Cytokine profile analysis of KMP-11 DNA immunized hamsters challenged with either AG83 or GE1F8R compared with respective infected control groups by semiquantitative RT-PCR and densitometry. Five representative hamsters from each experimental group were randomly selected to analyze the splenic cytokine profile. Cytokine RT-PCR profile of normal, blank vector-immunized, and challenged AG83 or GE1F8R groups is not shown. Equivalent amount of RNA from splenic tissue of different groups of hamsters was used as input in RT-PCR analysis. In all the cases HGPRT was used as housekeeping gene control (*A*). *B* and *C*, Densitometry analysis of mRNA expression. Expression of each cytokine transcripts were expressed as a ratio of cytokine mRNA to HGPRT mRNA. *D* and *E*, Semiquantitative RT-PCR and densitometry analysis of iNOS transcripts produced from purified splenic macrophages of KMP-11 DNA immunized hamsters challenged with either AG83 or GE1F8R compared with respective blank vector-immunized and infected controls. Expression of iNOS transcripts were represented as a ratio of iNOS mRNA to HGPRT mRNA. Results are representative of five individual experiments ($n = 5$/group).

against both pentavalent antimonial-sensitive (AG83) and antimonial-resistant (GE1F8R) virulent *L. donovani* strains, taking into account the high incidence of emergence of pentavalent antimonial-resistant strains that result in conventional chemotherapeutic failure (7, 8).

All of the KMP-11 DNA immunized hamsters challenged with either AG83 or GE1F8R survived the lethal challenge and remained healthy until the termination of the experiment at 8 mo postinfection, whereas all nonimmunized and blank vector-immunized hamsters succumbed to the lethal *L. donovani* challenge within 6 mo postinfection. In terms of hepatic parasite burden, KMP-11 DNA vaccination conferred sterile protection to all AG83- and GE1F8R-challenged hamsters studied at 2 and 4 mo postinfection. In terms of splenic parasite burden >80% hamsters showed absolute clearance of amastigotes at both time points of study. This extent of formidable protection is hitherto not achieved with any other DNA vaccination trial like LACK, A2 virulence gene, or ORFF Ags against *L. donovani* infection suggestive of the

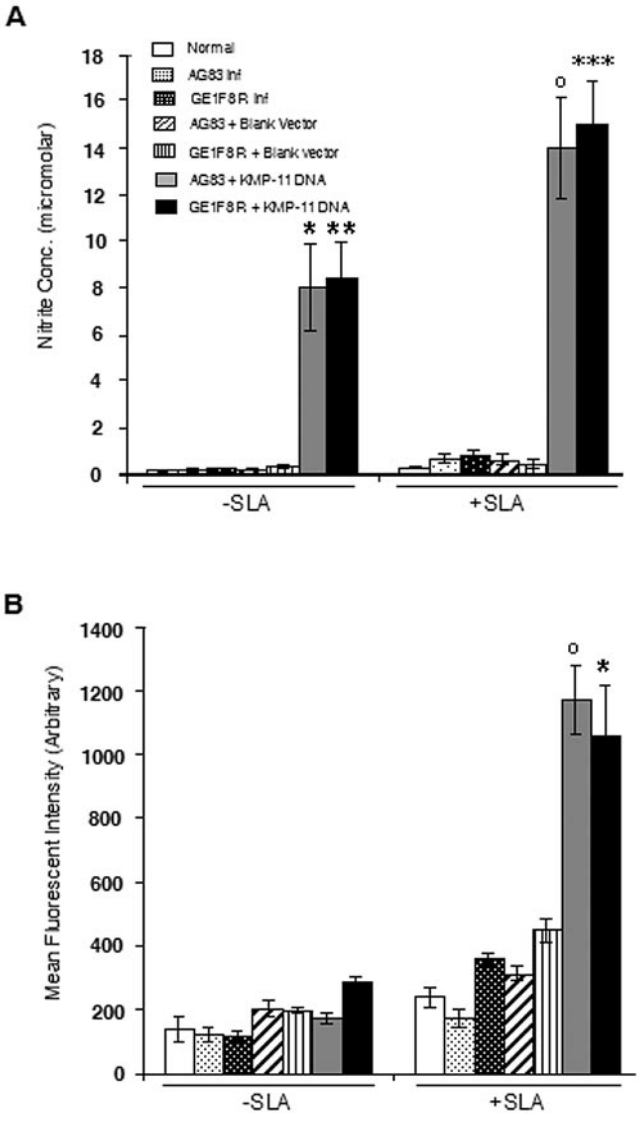

FIGURE 6. Production of leishmanicidal effector molecules in KMP-11 DNA immunized hamsters. *A*, Nitrite generation by supernatants of splenocytes (devoid of RBC) derived from different experimental groups of hamsters were stimulated with SLA (5 μg/ml) or without SLA for 72 h and assayed as described in *Materials and Methods*. KMP-11 DNA vaccinated hamsters challenged with either AG83 or GE1F8R showed a significant level of nitrite generation even without SLA stimulation compared with respective infected control groups (*, $p < 0.005$ and **, $p < 0.002$). In the presence of SLA, nitrite generation in each of the vaccinated groups was further enhanced (°, $p < 0.0005$ and ***, $p < 0.0001$). Results are representative of five experiments ($n = 5$/group) and data represent the mean ± SE. *B*, ROS generation measured by H_2DCFDA staining of the splenocytes (devoid of RBC) from different experimental groups of hamsters without and with SLA stimulation (5 μg/ml) for 72 h. In the presence of SLA stimulation only, ROS production varied significantly between the KMP-11 DNA immunized and the infected control groups of hamsters (°, $p < 0.0005$ and *, $p < 0.005$). Data represent the mean ± SE ($n = 5$/group) and results are representative of three experiments.

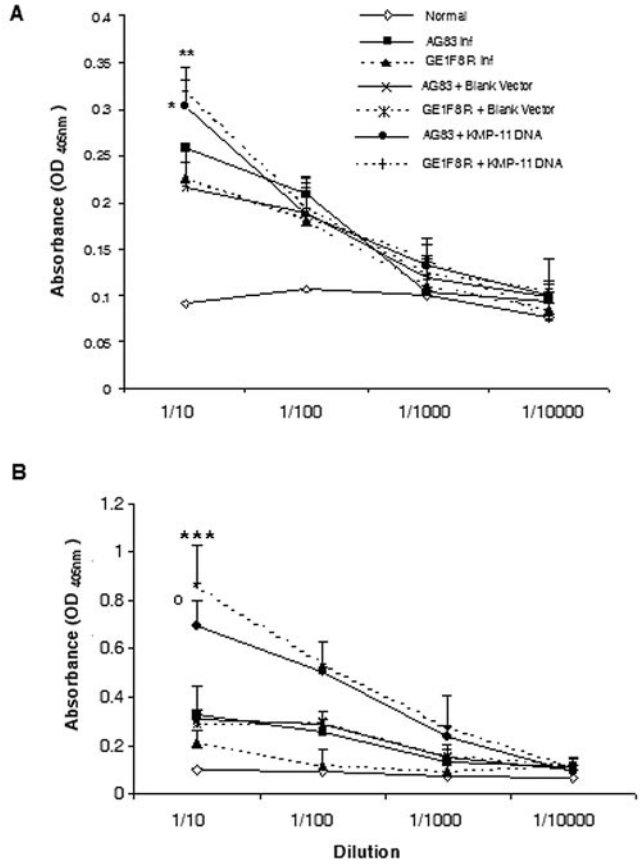

FIGURE 7. Anti-KMP-11 IgG1 and IgG2 Ab titers in infected and KMP-11 DNA vaccinated hamsters. Sera from KMP-11 DNA and blank vector-immunized hamsters challenged with either AG83 or GE1F8R and respective infected controls ($n = 5$/group) were analyzed individually for KMP-11-specific anti-IgG1 and anti-IgG2 Ab titers by ELISA. The results are representative of three experiments and data represent the mean ± SE. IgG1 titer (*A*) did not vary significantly among the vaccinated groups of hamsters compared with respective infected controls (*, $p < 0.5$ and **, $p < 0.1$). IgG2 titers (*B*) varied significantly between the DNA immunized and infected hamsters (°, $p < 0.05$ and ***, $p < 0.005$).

supreme importance of judging the proper Ag candidature as a means to achieve such robust immune response (56–58). Previously it has been reported that DNA immunization with KMP-11-HSP fusion construct conferred significant protection against *Trypanosoma cruzi* with elicitation of both anti-KMP-11-specific humoral and cytotoxic response (59).

Seminal studies by Murray et al. (35–39) have established early hepatic granuloma formation, with associated immune cell recruitment at parasitized focus, as an index of the ongoing nature of immune response in experimental VL. We have previously reported that ill-defined splenic granuloma was associated with slowing down of parasite replication at late stages of *L. donovani* infection in BALB/c mice (60). Early hepatic pathological changes in AG83- and GE1F8R-infected hamsters showed the presence of heavily parasitized Kupffer cells and ill-formed granuloma-like structures with very few lymphocyte infiltrates indicative of absence of protective cellular response. Complete absence of parasitized focus and well-formed granulomas observed at 2 wk postinfection and a high incidence of lymphocyte infiltration in a structure resembling involuting granuloma at 4 wk postinfection was suggestive of complete eradication of parasites by vigorous protective immune response in the vaccinated hamsters.

Most of the assays in this study were done between 90 and 120 days postinfection as the disease progression reaches its peak by this time. It appeared that all the KMP-11 DNA immunized hamsters challenged either with AG83 or GE1F8R strain have a specific active T cell response that was severely impeded in infected nonimmunized and blank vector-treated hamsters. This finding was supported by substantial IL-2 generation that was completely

abrogated in the infective state of the disease. The presence of class I-restricted cytotoxic $CD8^+$ T cells was demonstrated in murine CL models that healed their primary lesion (21). High percentage of specific lysis of the KMP-11 transfected macrophages was suggestive of a generation of KMP-11-specific CTLs in the DNA immunized hamsters. It should also be acknowledged that the absence of a distinct cellular marker of hamster immune cells makes it difficult to predict the exact phenotype of the cells involved in the target specific lysis.

Although murine macrophages and cell lines were shown to produce significant amounts of NO generation in response to IFN-γ and TNF-α, previous reports indicated that impairment of iNOS signaling pathway, leading to failure in NO generation in human and hamsters, contributed to their susceptibility to VL (30, 47, 49–51). Besides leishmanicidal activity, NO is also known to regulate immunologic pathways including endogenous IL-12 secretion (37, 61). We found that significant iNOS transcript production in both groups of KMP-11 DNA vaccinated hamsters correlated proportionally with NO generation that was considerably higher with SLA stimulation. iNOS transcripts could not be detected in infected or blank vector-immunized hamsters. As reported by Wei et al. (52) who found significant NO generation from $iNOS^{-/-}$ homozygous mutant mice without IFN-γ/LPS stimulation, we too found considerable NO generation from vaccinated hamster splenocytes (but not from infected control) even without SLA stimulation. This finding could be attributed to accumulation of NO produced by constitutive NO synthase or induction of NO that is severely impaired in heavily parasitized spleen. It is reported that cytokine-activated macrophages kill ingested *L. donovani* amastigotes by secreting reactive oxygen intermediates and/or RNI, which might act in concert against the parasite (53, 54) as inhibition of either RNI or reactive oxygen intermediates pathway prevented macrophage-mediated killing of *L. donovani* (62–64). We also found a high ROS generation from the SLA-stimulated splenocytes in both AG83- and GE1F8R-challenged KMP-11 DNA vaccinated hamsters that showed >3-fold enhancement in vaccinated groups of hamsters. It is possible that high SLA-specific ROS generation might have additionally contributed to the efficiency of parasite killing. The manifold enhancement in NO and ROS production in response to SLA stimulation from vaccinated hamster splenocytes is in agreement with Bottrel et al. (11) who in an ex vivo study showed an increase in IFN-γ and TNF-α production in response to SLA but not to LACK Ag.

The presence of a comparable existence of Th1 and Th2 clones producing both IFN-γ and IL-4 obtained from patients cured of VL prompted us to assess whether the protective response elicited by KMP-11 DNA vaccination in hamsters can reflect this attribute of clinical findings (24, 25, 46). IFN-γ, a signature cytokine of Th1-type response that has a dominant effect on macrophage microbicidal responses and other effector killing mechanisms, was found to be moderately expressed (30) along with low TNF-α production in infected hamsters. Transcripts of IFN-γ and TNF-α, often reported to act in concert to activate iNOS for the production of NO (51), showed manifold increase in the KMP-11 DNA immunized groups of hamsters. It is also suggested that TNF-α is one of the primary agents to stimulate macrophage to produce NO (50). High TNF-α levels present in the sera of active VL patients with IL-10-mediated suppressed T cell response may not be effective and beneficial for the patient (65–67). Thus it might be assumed that in a progressive disease state, dominant immunosuppressive environment is preventing NO induction by the combination of moderate IFN-γ and low TNF-α production. Likewise, a higher concentration of TNF-α might be required to mount a NO generating signal as observed during a protective response in vaccinated hamsters. IL-10, a key macrophage deactivating Th1 suppressive cytokine, is reported to have a definitive association with an acute phase of VL during which a progressive increase of IL-10 transcripts in tissues was generated but IL-10 mRNA was not detectable after successful chemotherapy (12, 68). Commensurate with these results we found extreme down-regulation of IL-10 mRNA levels in KMP-11 DNA vaccinated hamsters. The moderate IFN-γ transcript production from infected and blank vector-immunized hamsters correlated with the clinical findings in which presence of IL-10 as well as IFN-γ were reported in patients with acute VL whereby only IL-10 levels decreased remarkably with disease cure (69). All of these findings together with our study indicate a stoichiometric proportion of IFN-γ requirement along with TNF-α and immunosuppressive cytokine like IL-10, which can dictate the outcome of the disease. There are reports that primary Th1 cell-mediated antileishmanial events induced in $IL\text{-}10^{-/-}$ mice require IFN-γ that is largely induced by IL-12 (70). In our study IL-12 was completely down-regulated in the AG83- and GE1F8R-infected and blank vector-immunized hamster, whereas high levels of IL-12 mRNA transcripts were found in KMP-11 vaccinated hamsters. The synergism of IL-12 with IFN-γ might have an additional paramount effect on leishmanicidal activities of *L. donovani* in this tissue. Furthermore it may be inferred that high expression of IL-10 in infected hamsters might have a profound suppressive effect on these Th1-like cytokine-induced responses and thus infection continues unabated. TGF-β, a pleiotropic cytokine with diverse functions, is known to be expressed at a moderate level even in normal hamsters (12, 30). TGF-β is also known to inhibit the activities of immune cells and was found to be down-regulated in vaccinated hamsters compared with the infected and blank vector-immunized controls. Our surprise candidate was IL-4, a Th2 signature cytokine of *Leishmania* susceptible BALB/c mice. IL-4 transcripts could not be detected at all in splenocytes of >90% of the infected hamster. Previous findings have shown that visceral growth of *L. donovani* is not prompted by IL-4 and have implied a beneficial role of IL-4 in effective chemotherapy (71). IL-4 is not consistently expressed in human kala-azar (68, 72). Moreover IL-4 is needed to drive Th1 differentiation (73), to elicit Th1-associated CTL-mediated tumor immunity generation (74), to maintain IFN-γ production (75), and to prime IL-12 production (76). Our finding showed that all the KMP-11 DNA immunized hamsters produced significantly elevated IL-4 mRNA. This might indicate either a direct or indirect role of IL-4 in protective response. Nevertheless, IL-4 production as a consequence of restoration of T cell anergy during protective immune response remains a possibility. Our results might validate the implications of the finding in which $IL\text{-}4^{-/-}$ knockout mice were significantly more susceptible to *L. donovani*, suggesting a protective ability of this cytokine in VL (77). IL-4-mediated induction of nitrite production in human monocytes, induced by IFN-γ and inhibited by NMMA (N^G-monomethyl-L-arginine), indicated an existence of IL-4-dependent iNOS signaling pathway (78). Furthermore, synergism of IL-4 along with IFN-γ in microbicidal activity of macrophages has been reported by Bogdan et al. (79). In this context the iNOS-induced NO generation process in humans might be compared with hamsters that can be enhanced due to IL-4 up-regulation acting in synergy with IFN-γ. These combinatorial effects of Th1- and Th2-like cytokines have sufficient stimuli to produce iNOS mRNA that is expressed only in the vaccinated hamsters that further downplays the role of an overt dominance of Th1-like cytokine response or absence of Th1 cytokine response being responsible for protective and disease exacerbating mechanism, respectively, in *L. donovani* infection. Finally significantly elevated levels of KMP-11-specific IgG2 Ab titer of the protected hamsters along with an equivalent level of IgG1 Ab

levels compared with infected and blank vector-immunized hamsters also supported these implications.

Our study demonstrated an outstanding protective efficacy of a DNA vaccine encoding the immunodominant Ag KMP-11 against VL by generation of functionally active IL-2-producing T cells along with specific anti-KMP-11 CTL-like response and other leishmanicidal effector mechanisms. Significant up-regulation of IFN-γ along with manifold enhancement of TNF-α and IL-12 along with extreme down-regulation of IL-10 might be considered to revert the impaired iNOS signaling pathway resulting in detectable NO generation and concomitant protection that might additionally be directly or indirectly influenced by IL-4. Our finding suggests that a vaccine potential against VL can be truly assessed in the highly susceptible hamster model that showed protection being associated with mixed Th1/Th2 cytokine response, a finding similar to that of clinical VL in which a mixed Th1/Th2 cytokine response is found in patients cured of VL (24, 25, 46). We cannot discount the possibility that the apparent homogeneity in the mechanisms of protection conferred by KMP-11 DNA vaccination in AG83- and GE1F8R-infected hamsters might be attributed to limited extent of MHC polymorphism (80). This is the first ever report of a successful vaccine strategy that is effective against both antimonial-sensitive and -resistant *L. donovani* strains. Because recovered VL patients show Ab and T cell response against KMP-11 (17, 55), we tend to believe that KMP-11 DNA vaccine has a strong potential to be used in humans. This result is particularly significant in context of the decreasing efficacy of chemotherapeutic intervention along the face of increased emergence of drug resistant *L. donovani* strains and might lead to homogeneity in vaccine design against any forms of drug-resistant and drug-susceptible *Leishmania* strains.

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Disclosures

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