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(54) **Title:** A DNA VACCINE FORMULATION IN CATIONIC LIPOSOME VEHICLE USEFUL FOR MAXIMIZING VACCINE POTENCY FOR *LEISHMANIASIS*

(57) **Abstract:** The present invention discloses DNA vaccination with plasmid encoding the cysteine protease C (cpc) gene of *Leishmania donovani* to induce effective protection against visceral leishmaniasis (VL). The DNA vaccine entrapped in the cationic liposomes comprises of a plasmid vector pVAX1 encoding *L. donovani* cpc gene operably linked to transcriptional regulatory sequences. The invention relates to efficacy of liposomal formulation containing the bilayer-associated adjuvant MPLA, encapsulating the DNA construct pVAX1-cpc, against lethal *L. donovani* infection model in BALB/c mice. This formulation of liposomal DNA vaccine is highly effective in inducing robust cell-mediated immune response against leishmanial parasites upon intramuscular administration.



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A DNA VACCINE FORMULATION IN CATIONIC LIPOSOME VEHICLE USEFUL FOR MAXIMIZING VACCINE POTENCY FOR *LEISHMANIASIS*

TECHNICAL FIELD OF THE INVENTION

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The present invention relates to a DNA vaccine formulation in cationic liposome vehicle useful for maximizing vaccine potency for *Leishmaniasis*. More particularly, it relates to cationic liposomes containing intrabilayer monophosphoryl lipid A (MPLA) and cysteine protease C (cpc) gene useful for vaccination for *Leishmaniasis*. The present invention further relates to method of preparation, characterization and use of DSPC (1, 2-distearoyl-sn-glycero-3-phosphocholine) bearing cationic liposomes containing intrabilayer MPLA and cpc gene for vaccination against *Leishmania sp.*

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The proposed adjuvant formulation of cationic liposomes containing MPLA is highly promising immunostimulatory agent and efficient delivery vehicle maximizing vaccine potency against various pathogens including *Leishmania*. This vaccine formulation combines safety, efficacy alongwith cost-effectiveness to provide new possibilities for prophylactic and therapeutic vaccines.

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20 BACKGROUND AND PRIOR ART OF THE INVENTION

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Protozoan parasites of the genus *Leishmania* cause a wide spectrum of diseases collectively termed leishmaniasis that varies in their clinical manifestations in the form of cutaneous, mucocutaneous and visceral *Leishmaniasis*. Visceral leishmaniasis (VL) or kala-azar, caused by *Leishmania donovani*, is a fatal parasitic disease causing 25,000–40,000 cases and 200–300 deaths annually in Indian subcontinent alone [Joshi et al., 2008]. VL is endemic in tropical and subtropical areas of Latin America, Europe, Africa and Asia and has emerged as an important opportunistic HIV coinfection. Control of leishmaniasis thus remains a source of grave concern worldwide. There are few drugs available for chemotherapy and treatments are still long-drawn, highly toxic and expensive. Combined therapies advocated to combat the drug resistant parasites remain costly for the majority of patients [Croft and Olliaro, 2011].

Leishmania vaccines continue to hold promise due to the fact that cured patients from VL are subsequently protected against reinfection. Impressive clinical responses to cutaneous leishmaniasis have been observed in the few recent human clinical trials, although the overall track record for *Leishmania* vaccines, particularly VL is less impressive [Evans and Kedzierski, 2012]. VL is of higher priority than CL since anthroponotic VL foci are the origin of frequent and deadly epidemics. Although a series of vaccine candidates have been tested against *Leishmania* in the past 10 years, none has successfully emerged for human use. Improvements in vaccine delivery systems are thus an important component of current research efforts against *Leishmania* to realize their full potential. Previous VL vaccine strategies have included immunization with whole parasite or cell lysate vaccines, peptide vaccines, dendritic cell (DC) vaccines, viral vectored and plasmid DNA vaccines [Modabber 10 2010; Das and Ali, 2012]. Relative ease of production, stability, safety, low cost and ability to induce cellular immunity has made the plasmid DNA vaccines more attractive over conventional protein vaccines. Although DNA vaccines are able to elicit both humoral and cellular immune responses, genetic immunization against *Leishmania* has often been hampered by the failure of the vaccine to elicit a cytotoxic T-cell response strong enough for protection against the disease [Melby et al., 2001; Saldarriaga et al., 2006]. This can probably be overcome by coupling the DNA vaccine with an efficient vaccine delivery system and immunomodulator capable of greater activation of innate immunity during vaccination. A better protection for the plasmid DNA against degradation by cellular nucleases is also achieved by cationic liposomal entrapment due to DNA condensation and covering of DNA strands with lipid bilayers [Radler et al., 1997]. Cationic liposomes have been previously shown to markedly potentiate the ability of plasmid DNA to activate innate immune responses by endosomal targeting, thereby increasing the TLR9 activation and efficient MHC class I-restricted antigen presentation [Zuhorn et al., 2002; Yasuda et al., 2005]. Despite variations in lipids, cationic surface charge enhance antigen uptake when delivered via such liposomes [Korsholm et al., 2007]. However, protection with cationic liposomal protein vaccine was limited mostly to intraperitoneal route of administration [Afrin et al., 2002; Ravindran et al., 2010; Bhowmick et al., 2010]. Therefore, additional adjuvant or immunomodulator was required along with the liposomes for successful protection in human 30 administrable route of vaccination such as subcutaneous, intramuscular and intravenous. Monophosphoryl lipid A (MPLA), which targets intracellular TLR4, have previously exhibited considerable potency and safety in human trials with a variety of candidate vaccines, including vaccines to malaria, hepatitis B, HIV-1 and several different types of

cancer [Agnandji et al., 2012; Cluff 2010; Di Paolo et al., 2010; Fries et al., 1992; Garcon, 2011]. Moreover, liposomal antigen presentation and macrophage recruitment have been shown to be augmented by intraperitoneal administration of liposomes containing MPLA compared to those lacking MPLA [Verma et al., 1992]. To optimize the route of immunization in addition to improved immune stimulation, MPL-TDM (monophosphoryl lipid – trehalose dicorynomycolate) has been used for liposomal gp63 antigen which resulted in robust Th1 immune responses for protection against VL [Majumder et al., 2011]. The present invention shows further advancement of this strategy by incorporating MPLA within lipid bilayer of cationic liposomes for delivery of a DNA vaccine, thereby making the delivery system more compact and combining all the advantages of DNA vaccination over protein vaccines.

Cysteine proteases (CPs) are enzymes known to play critical roles in the pathogenesis of *Leishmania* and other parasitic infections [Vermelho et al., 2010; Silva-Almeida et al., 2012]. Extensive studies have shown that leishmanial CPs are involved in parasitic survival, replication, autophagy, metacyclogenesis and transformation to amastigotes essential for onset of disease. Thus CPs have been identified as putative vaccine candidates and as potential drug targets against leishmaniasis. Although cysteine protease A, B and C have shown to be protective singly or in combination against *L. major* and *L. infantum* in different studies, their efficacy has not been studied against *L. donovani*.

OBJECTIVES OF THE INVENTION

The main object of the present invention is to provide a DNA vaccine formulation in cationic liposome vehicle useful for maximizing vaccine potency for *leishmaniasis*.

Another object of the present invention is to provide a process for the preparation of an adjuvant cum vaccine delivery system containing liposomal vesicles with intralamellar MPLA thus combining antigen presentation along with TLR 4 signaling.

Yet another object is to provide a process wherein the cationic liposomes composed of DSPC, stearylamine, cholesterol and MPLA, are designed to encapsulate desired antigen/ vaccine candidate.

Yet another object is to provide a process wherein the vaccine formulation will augment T cell response thereby enhancing immunogenicity of a subunit protein/ DNA vaccine.

Yet another object is to provide a stable, safe and biodegradable submicron adjuvant system for entrapment of both lipophilic and hydrophilic biomolecules (particularly vaccines) for their delivery and sustained release.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a DNA vaccine formulation in cationic liposome vehicle useful for maximizing vaccine potency for leishmaniasis

In accordance with a first of its aspects, the invention provides a cationic liposomal formulation containing intralamellar MPLA, encapsulating Ldcpc (*Leishmania donovani* cpc) DNA (GenBank Accession no. JX968801.1, Seq ID 3) for targeting leishmanial parasites prophylactically and which may also be therapeutically active. Cationic liposomes thus provided, incorporating TLR-4 agonist in membrane, possess immunostimulatory activity which is further improved by entrapping DNA vaccine construct into them. Further, the present disclosure provides a pharmaceutical composition comprising MPLA coupled cationic liposomes, as physiologically acceptable carrier as well as adjuvant for DNA and protein vaccine formulations.

In a preferred embodiment, the present invention provides a novel MPLA containing DSPC bearing cationic liposomes as vaccine carriers as well as promising adjuvants. Site-specific delivery of charged molecules like plasmid DNA can be effectively mediated by the cationic liposomes. This invention primarily focuses on a DNA vaccine using plasmid encoding cpc gene of *L. donovani* whose site specific delivery and efficacy is due to its liposomal encapsulation.

The liposome containing MPLA that has been used in the present invention can also be effectively used as efficient delivery vehicle for various other biomolecules, particularly drugs and vaccines against other diseases requiring predominantly cell mediated response. Additional immunomodulatory activities of such liposomes can further be exploited to

generate strong humoral and cellular immunity required to combat various pathogens and cancer.

In another embodiment of the present invention, it provides a process of cloning a pVAX1-cpc plasmid comprising amplification of *cpc* gene with primers; ligating *cpc* gene into pVAX1 vector; transforming the said pVAX1 vector into competent *E. coli* bacteria; transcription and translation of pVAX1-cpc clones.

Yet another embodiment of invention provides the liposomal DNA vaccine formulation described herein as a cost-effective and health-promoting intervention for control, prevention and/or treatment of Leishmaniasis. The vaccine can substantially ameliorate morbidity, mortality and difficulties in providing medical care globally wherever there is prevalence of VL. The vaccine can also be useful for decreasing the severity of the disease when administered after initial infection with *L. donovani*.

Yet another embodiment of invention provides a candidate cross-protective gene useful as a DNA vaccine, or for production of a recombinant protein in various expression systems against *Leishmania*. Yet another embodiment of invention provides a DNA vaccine formulation in cationic liposome vehicle useful for maximizing vaccine potency for leishmaniasis characterized in containing intralamellar monophosphoryl lipid A (MPLA) and cysteine protease C (*cpc*) gene.

Yet another embodiment of invention provides a DNA vaccine formulation wherein a plasmid DNA containing Seq ID 3 encoding cysteine protease C (*cpc*) gene of *L. donovani* is encapsulated in the cationic liposome vehicle.

Yet another embodiment of invention provides a DNA vaccine formulation wherein the cationic liposome is a multilamellar vesicle comprising of:

- a) a neutral lipid preferably distearylphosphatidylcholine (DSPC);
- b) a cationic lipid preferably stearylamine (SA);
- c) a second neutral lipid preferably cholesterol; and
- d) a TLR agonist preferably monophosphoryl lipid A (MPLA);

wherein the neutral lipid, the cationic lipid, the second neutral lipid and the TLR agonist are formulated in the range of 7:1.5:1.5:0.0016 to 7:2.5:2.5:0.003 molar ratio..

5 Yet another embodiment of invention provides a DNA vaccine formulation wherein the plasmid DNA is present in the range of 700 to 1000 µg/ml.

Yet another embodiment of invention provides a DNA vaccine formulation is administered to a subject via intra-venous, intra-muscular or intranasal route wherein said
10 subject is a mammal including human.

Yet another embodiment of invention provides a cationic liposome containing MPLA is useful as efficient delivery vehicle for biomolecules and vaccines

15 Yet another embodiment of invention provides a process for the preparation of the DNA vaccine formulation wherein said process comprising the steps of

- a. cloning cysteine protease C (cpc) gene having Seq Id. no. 3 into pET28a vector;
- b. sub-cloning cpc clone obtained in step a into pVAX1 vector to make pVAX1-cpc
20 plasmid construct;
- c. dissolving a neutral lipid, a cationic lipid, a second neutral lipid and a TLR agonist in the range of 7:1.5:1.5:0.0016 to 7:2.5:2.5:0.003 molar ratio in an organic solvent preferably chloroform to obtain a solution followed by removing solvent using evaporator to obtain a lipid film;
- 25 d. desiccating the lipid film as obtained in step (c) at a temperature in the range of 25 to 28°C for a period in the range of 12 to 16 hours to obtain a dried lipid film;
- e. dissolving the plasmid DNA in the range of 1 to 2 mg/ml obtained in step b in a physiologically acceptable aqueous medium;
- 30 f. hydrating the dried lipid film as obtained in step (d) with the solution obtained in step (e);

- g. vortexing the solution obtained in step (f) for a period ranging between 10 to 15 min and sonicating for a period in the range of 30 to 45 sec at 4 to 5 Hz at a temperature in the range of 3 to 4°C to obtain a liposomal formulation;
- h. removing the excess free plasmid DNA from the liposomal formulation obtained in step (g) by ultracentrifugation to obtain a DNA vaccine formulation.

Yet another embodiment of invention provides a process for the preparation of the DNA vaccine formulation wherein physiologically acceptable aqueous medium is Phosphate Buffer Saline solution in a molar ratio in the range of 0.01 to 0.02.

Yet another embodiment of invention provides use of the DNA vaccine formulation as a vaccine against leishmaniasis for maximizing vaccine potency in mammals against *Leishmania* parasite

Yet another embodiment of invention provides use of the cationic liposome to generate strong humoral and cellular immunity required to combat various pathogens and cancer.

Yet another embodiment of invention provides use of the cationic liposome for drug screening against leishmaniasis.

Yet another embodiment of invention provides a pharmaceutical composition comprising a therapeutically effective amount of the DNA vaccine formulation in a pharmaceutically acceptable carrier.

The following ABBREVIATIONS have been used:

DSPC- 1, 2-distearoyl-sn-glycero-3-phosphocholine

SA- stearylamine

PBS- Phosphate buffer saline

VL- Visceral leishmaniasis

MPLA- monophosphoryl lipid A

TLR- Toll-like receptor

MLV- multilamellar vesicles

DNA- deoxyribonucleic acid

pDNA- plasmid DNA

5 rCPC- recombinant cysteine protease C protein

cpc - cysteine protease C gene

MPL-LIP – MPLA containing liposome

LIP – Liposome

LDU – Leishmania Donovanii Units

10 DTH – Delayed Type Hypersensitivity

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts cloning, overexpression and purification of recombinant cysteine protease C protein (rCPC). **a.** Schematic representation of pET28a-cpc; **b.** Clone confirmation of cpc in pET28a vector. Lane 1 & 7, GeneRuler™ 1 kb DNA ladder (Fermentas, Canada) as marker, lane 2, vector pET28a, lane 3, NdeI/BamHI digested pET28a-cpc construct, lane 4, PCR of cpc from genomic DNA (insert), lanes 5 & 6, PCR of cloned construct; **c.** Coomassie Blue staining of 10% SDS-PAGE, Lane 1, molecular mass marker, lane 2, overexpressed recombinant cpc in *E. coli* BL21 (DE3), lane 3, purified recombinant cpc.

FIG. 2 shows the cloning and expression of *L. donovani* cpc in mammalian expression vector: **(a).** Schematic representation of pVAX1-cpc; **(b)** Clone confirmation of cpc in pVAX1 vector. Lane 1, λ DNA digested with Hind III marker, lane 2 & 3, HindIII/BamHI digested pVAX1-cpc construct, lane 4, PCR of cloned construct, lanes 5, GeneRuler™ 1 kb DNA ladder (Fermentas, Canada) as marker; **(c)** RT-PCR analysis for cpc in the pVAX1-cpc transfected HEK293T cells. Lane 1, GeneRuler™ 1 kb DNA Ladder, lane 2, HEK293T cells without transfection, lane 3, HEK293T cells transfected with vector pVAX1, lane 4 & 5, HEK293T cells transfected with pVAX1-cpc; **(d)** Western blot analysis for expression of cpc in transfected HEK293T cell line, using rabbit anti-CPC antibody. Lane 1, western blot of

pVAX1-cpc construct transfected in HEK293T cell line, lane 2, western blot of pVAX1 vector transfected in HEK293T cell line.

FIG. 3 is the representative of Acoustic Alternative current (AAC) mode atomic forced microscopy (AFM) images of empty DSPC liposomes with intralamellar MPLA and same liposomes entrapping plasmid pVAX1-cpc.

FIG. 4 shows the resistance of cationic liposomes to DNase. Lane 1: naked plasmid DNA (pVAX1-cpc). Lanes 2 & 3: naked plasmid DNA (pVAX1-cpc) incubated with DNase I for 5min and 1 hour respectively. Lane 4: liposomal pVAX1-cpc incubated with DNase I for 1 hour and subsequently extracted with phenol: chloroform (1:1). Lane 5: Liposomal pVAX1-cpc incubated with DNase I for 1 hour and run in gel without phenol: chloroform extraction.

FIG. 5 represents in vitro cell viability assay. Empty cationic liposomes with intrabilayer MPLA (3.2-200 $\mu\text{g/ml}$ with respect to DSPC) were incubated with murine peritoneal macrophages and 1h, 4 h; 12h later MTT assay was performed. Values presented are the mean $\pm$ S.E. of four replicates.

FIG. 6 is the toxicity analysis after intravenous administration of liposomal DNA. Serum samples were collected from mice ($n=5$), fifteen days after intravenous administration of liposomal DNA vaccine formulation and assayed for the enzymes aspartate transferase (AST), alanine transferase (ALT) and alkaline phosphatase (ALP) as indicators of hepatotoxicity.

FIG. 7 shows the DTH responses in differently vaccinated mice. Mice were immunized twice at 2-week interval. Ten days after immunization with recombinant CPC protein -specific DTH responses were measured. The response is expressed as the difference (in mm) between the thickness of the test (recombinant CPC protein -injected) and control (PBS-injected) footpads at 24 h. Each bar represents the mean $\pm$ S.E. for five individual mice per group at designated time point. The results are those from one experiment representative of two performed. Asterisks over each bar indicate significant differences in comparison to control groups. Asterisks over line indicate significant differences between groups. * - $P < 0.05$; *** - $P < 0.001$.

FIG. 8 shows the antibody production by liposomal DNA vaccine. Mice were immunized with PBS, empty liposomes containing intrabilayer MPL, vector pVAX1, naked or liposomal pVAX1-cpc DNA. Individual mouse sera from each group (n=5) were collected before infectious challenge. Recombinant CPC protein -specific production of IgG, IgG1 and IgG2a was assayed by ELISA and an average of duplicate absorbance values of each serum sample recorded. Values are given as mean of four measurements. *, $P < 0.05$; significant differences from each other (Tukey post test).

FIG. 9 shows the cytokine responses in vaccinated mice. Mice were immunized twice at 2-week intervals. Ten days after last immunization spleens were collected from mice and restimulated in vitro with recombinant CPC protein (5 $\mu\text{g/ml}$). After 72 h supernatants were collected and concentrations of released IFN- γ , IL-4, IL-2, IL-10 and IL-12 levels were determined by ELISA. Each sample was examined in duplicate. Each bar represents the mean $\pm$ S.E. for five individual mice per group. The results are those from one experiment representative of two performed. Asterisks over each bar indicate significant differences in comparison to PBS control group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG. 10 is evaluation of protection against *L. donovani* in vaccinated mice. Kinetics of liver and spleen parasite burden of mice immunized intramuscularly twice at 2-week interval with naked pDNA (pVAX1-cpc), only vector (pVAX1) and pDNA entrapped in cationic liposomes (pVAX1-cpc/MPL-LIP). Control animals received PBS or adjuvant (MPL-LIP) only. At 10 days after the last immunization, mice were challenged intravenously with 2.5×10^7 promastigotes of *L. donovani*. At the designated time mice were sacrificed and LDU were calculated from the weight and microscopic examination of impression smears of liver and spleen tissues. Each bar represents the mean $\pm$ S.E. for five individual mice per group. The results are those from one experiment representative of two performed. Asterisks over each bar indicate significant differences in comparison to control groups. ***, $P < 0.001$.

DETAILED DESCRIPTION OF THE INVENTION

Despite the past 15 years of research, human DNA vaccines have yet to fulfill their clinical success in the areas of infectious disease and cancer. Use of traditional adjuvants like alum, lipopolysaccharides (LPS), CpG motifs etc. to increase the immunogenicity of DNA vaccines have been modestly effective showing mixed results. Vaccine formulation

comprising of plasmid DNA entrapped in cationic liposomal vesicles with intralamellar immunomodulator MPLA, as disclosed in the present invention, gets closer to the level of immunogenicity required for human use. In general, any vaccine or drug molecule must reach its intended site of action, mostly inside the cell for its action avoiding non-specific interactions. Therefore, the problem of intracellular gene delivery involves not only the cellular uptake of large charged molecules like DNA but also their intracellular availability at the target sites, traversing the biological barrier of plasma membrane, lysosomal degradation and nuclear envelop. Multilamellar positively charged liposomes have emerged as ideal delivery system carrying DNA in its core, interacting with the negatively charged cell surface for successful gene delivery after endocytosis-mediated uptake [Almofiti et al., 2003)]. In addition, cationic liposomes provide a protective role against extra- and intracellular nucleases due to the compaction and covering of DNA by the lipid bilayers [Ibanez et al., 1996, Eastman et al., 1997]. In brief, manipulating DNA into liposomal delivery system makes it more efficient to stimulate both humoral and cell-mediated responses. Since liposomal efficacy largely depends on suitable immunostimulatory adjuvants to generate the appropriate adaptive immune response, the adjuvant monophosphoryl lipid A (MPLA) has been incorporated in the lipid-bilayer of delivery vesicles. MPLA is a detoxified form of the endotoxin lipopolysaccharide from *Salmonella minnesota*, and is among the first of a new generation of Toll-like receptor (TLR) agonists to be used in several human clinical trials [Garcon and Van Mechelen, 2011].

Vaccine formulation comprising of plasmid DNA entrapped in cationic liposomal vesicles with intralamellar immunomodulator MPLA, as disclosed in the present invention, gets closer to the level of immunogenicity required for human use. Liposomes and MPLA were chosen as adjuvants in this study as they are human-compatible inducers of cell-mediated immunity. Although liposomes containing MPL have shown considerable safety and potency in human trials in several diseases including malaria, HIV-1 and cancer [Alving et al., 2012], their adjuvant potency has never been tried against VL.

The present invention relates to the development of vaccination strategies with cationic liposomal vesicles containing intrabilayer MPLA through intramuscular route of administration for DNA vaccination against experimental VL. Cysteine proteinase has been known to be protective against *L. infantum* infection in BALB/c mice [Khoshgoo et al., 2008} and thus, was chosen as the vaccine candidate. The potentiating effects of

distearoylphosphatidyl choline (DSPC)-bearing cationic liposomes containing MPLA have been analyzed for formulating a DNA vaccine. It has been observed that current adjuvant formulation of cationic liposomes with intrabilayer MPLA enhanced the Th1 based cellular immunity mediated by DNA vaccine alone without compromising the safety. This is consistent with some early studies [Gustafson et al., 1992; Rickman et al., 1991; Majumder et al., 2011] which relates the adjuvant activity of MPL to activation of macrophages and its ability to induce IFN- γ , IL-2, and IL-12 known to be essential for the induction of Th1-derived cell mediated immunity [Nijkamp and Parnham, 2011]. Predominant activation of the Th1-derived immune system elicits a strong DTH response and IgG2a production in liposomal pVAX1-cpc vaccinated mice as shown in the results. Findings from the current invention suggest the advantages of using liposomal carriers with immunomodulatory activity over naked pDNA for inducing strong systemic immunity against expressed antigen. Liposomal encapsulation protects DNA against nuclease present in serum thereby increasing their retention time, transfection efficacy and enhanced antigen presentation. Intramuscular immunization with naked pDNA (pVAX1-cpc) alone could not elicit optimum protection. The liposome-mediated pDNA delivery on the other hand resulted in robust protection, 34.18 and 54.25 times higher than naked pVAX1-cpc in liver and spleen respectively. Hence, genetic vaccination using pDNA entrapped in immunomodulatory liposomes containing MPLA represents an important advantage in inducing protection against leishmaniasis.

Statistical analysis

One-way ANOVA analysis (Multiple comparisons Tukey's post hoc test) was performed using the GraphPad Prism 5.0 software for Windows (GraphPad Software, San Diego CA). Results were considered significant when probability values were < 0.05 .

EXAMPLES

The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention.

Example 1

Cloning and purification of recombinant CPC protein from *L. donovani*

Genomic DNA isolated from *L. donovani* promastigotes (MHOM/IN/1983/AG83 originally isolated from an Indian kala-azar patient and maintained in hamsters and BALB/c mice at the Indian Institute of chemical Biology, 4, Raja S.C. Mullick Road, Kolkata, West Bengal, India, Pin:-700 032) was subjected to polymerase chain reaction (PCR) with sets of gene specific primers corresponding to *cpc* (~1 kb) gene. The primers used were Seq ID 1 (**forward**): 5' GGA ATT CCA TAT GCCA GCG ACG TCA AGC GCC GCT 3' and Seq ID 2 (**reverse**): 5'CGG GAT CC CTA CTC CTG CGC GGG TAT GCC AGC3' PCR conditions were one cycle of 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min 20 s at 58°C, and 1 min 10 s at 72°C, followed by a final cycle of 7 min at 72°C. The PCR amplified fragment was cloned in HindIII/BamHI site of bacterial expression vector pET28a (Novagen). Screening for recombinant clones was performed by growing randomly selected colonies overnight at 37°C in 5.0ml of LB broth (with 50µg/µl kanamycin). Cells were pelleted and plasmid DNA extracted using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. For clone confirmation, approximately 1µg plasmid DNA from individual miniprep was double digested with the appropriate restriction enzymes and the digest loaded onto a 1% agarose gel, in parallel with the molecular weight marker: GeneRuler™ 1 kb DNA ladder (Fermentas, Canada). Positive clones were selected on the basis of the size of insert and confirmed by DNA sequencing (ABI Prism, Model 377; Applied Biosystems). *Escherichia coli* BL21 (DE3) (parental strain for BL21 deficient in the lon and ompT proteases, from Novagen, Cat No 69450) transformed with pET28a-cpc construct was grown in 500ml culture medium at 37 °C until OD at 600nm reached 0.6. The host includes BL21 (DE3) and/or any of its precursors. Protein production was induced by adding isopropyl β-d-thiogalactoside (IPTG) to a final concentration of 0.5 mM, and incubating for an additional 4 h at 30 °C. The culture was then harvested by centrifugation at 6,000g, for 6 min, at 4 °C, and the cell pellet was resuspended in 6 ml of resuspension buffer (25 mM Tris-HCl, 500 mM NaCl, and 1 mg/ ml of Lysozyme, pH 8.0). The cell lysate was sonicated on ice for 5 min with 1 min pulse and 1 min interval between pulses using an ultrasonicator (Misonix). The pellet containing inclusion bodies was solubilised with solubilization buffer [50 mM CAPS {3-(Cyclohexylamino)-1-propanesulfonic acid} buffer (pH 11.0), 300 mM NaCl, and 0.5 % sarkosyl], kept at room temperature for 30 min and finally centrifuged at 12,000 g for 30 min at 4°C. The supernatant containing solubilised proteins were loaded separately onto Ni²⁺-nitrilotriacetic acid-agarose (Ni-NTA) column

(Qiagen, Valencia, CA) and purified under denaturing condition. The Ni-NTA column was pre equilibrated with equilibration buffer [50 mM CAPS buffer (pH 11.0), 150 mM NaCl, 0.5 % sarkosyl and 10 mM imidazole]. The column was washed with wash buffer [50 mM CAPS buffer (pH 11.0), 150 mM NaCl, 0.5 % sarkosyl and 20 mM imidazole] and eluted with elution buffer [50 mM CAPS buffer (pH 11.0), 150 mM NaCl, 0.5 % sarkosyl and 300 mM imidazole]. To refold, the purified materials were diluted 2 fold in dilution buffer containing 50 mM CAPS buffer (pH 11.0), 150 mM NaCl and 300 mM imidazole, and then dialyzed against 25 mM Tris-HCl, 250 mM NaCl, pH 8.0 and finally in 0.02M PBS for 6 hrs at 4 °C [Tao et al., 2010]. Protein concentrations were determined using Lowry's method [Lowry et al., 1951]. Purity and homogeneity of purified proteins were checked by using SDS-PAGE, and the gel was subsequently stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA).

The over expressed protein from *E. coli* BL21 (DE3) cells containing plasmid pET28a-cpc was purified through Ni²⁺-NTA agarose column under denaturing conditions finally yielding refolded protein in native state. The recombinant protein was refolded, dialyzed and finally concentrated by freeze-drying. The yield of purified protein was approximately 2.5 mg per liter of culture. Analysis of the purified recombinant CPC protein showed that the protein was essentially homogeneous (Figure 1c).

Example 2

Cloning and expression of *L. donovani* cpc for DNA vaccine

The gene encoding full-length of *L. donovani* cpc (Seq ID 3; GenBank accession number JX968801.1) was subcloned from pET28a in frame into pVAX1 (Invitrogen, San Diego, CA) at the HindIII/BamHI restriction sites. The full length cpc was amplified with cpc-specific primers. The primers used were Seq ID 4 (forward): 5' CCC AAG CTT GGA ATG GGA GCC CTC CGC GCC AAG TCT 3', and Seq ID 2 (reverse) 5' CGG GAT CC CTA CTC CTG CGC GGG TAT GCC AGC 3' in a Thermocycler (Gene Amp PCR System 9700; Applied Biosystems) using pfx Taq DNA polymerase (Invitrogen, San Diego, CA). PCR conditions were one cycle of 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min 20 s at 58°C, and 1 min 10 s at 72°C, followed by a final cycle of 7 min at 72°C. Amplified PCR

product was electrophoresed in agarose gel and eluted from the gel (QIA quick gel extraction kit, Qiagen, Valencia, CA). The eluted product was subsequently cloned into mammalian expression vector pVAX1 and transformed into competent *E. coli* TOP10 cells. The transformants were screened for the presence of recombinant plasmids. Growth was initiated by adding 20 ml of an overnight culture from a single colony to 1 L LB broth and incubating overnight in presence of 50 µg/mL kanamycin (Himedia, Mumbai, India) at 37°C with constant shaking at 225 rpm. Isolated positive clones were sequenced by DNA sequencer (ABI Prism, Model 377; Applied Biosystems). Recombinant plasmids were then maintained and propagated in TOP10 *E. coli*. Endotoxin-free plasmid DNA was isolated using Endo-free plasmid isolation kit (Qiagen, Valencia, CA) according to manufacturer's protocol and used for in vitro transfection and vaccination studies in BALB/c mice (BALB/C An.N (I.B) from National Institute of Nutrition, Hyderabad and reared in IICB animal care facility).

The plasmid construct referred to as pVAX1-cpc (Fig. 2a) places cDNA for *L. donovani* cpc under the transcriptional control of human CMV (cytomegalovirus) immediate-early enhancer/promoter element. The pVAX1-cpc plasmid expresses cysteine protease c from *L. donovani* strain (Fig. 2b &c), and can be obtained by using standard techniques based on the complete sequence disclosed herein and in GenBank accession number JX968801. The positive clones were selected using PCR and restriction enzyme digestion analysis (Figure 2 b), and the PCR product was further sent for sequencing. The results showed that the recombinants were correctly constructed and could be expressed in mammalian cell line.

Example 3

Transfection of plasmid constructs and Western blot

Human Embryonic Kidney 293 cells (HEK293T from Thermo Scientific, Cat NO: HCL4517) were maintained in DMEM medium (Invitrogen, San Diego, CA) supplemented with 10% FBS. The expression of cpc was detected in mammalian cell by transfecting pVAX1-cpc construct in HEK293T cells using lipofectamine 2000 (Invitrogen, San Diego, CA) according to the manufacturer's instructions with slight modifications. Briefly, HEK293T cells were cultured at 2×10^5 per well in 12-well plates to produce 85-90% confluence on the day of transfection. Lipofectamine 2000 and both pVAX1 vector and pVAX1-cpc construct were diluted in serum-free Opti-MEM media (Invitrogen, San Diego,

CA) at 1µg/200 µl and 2µg/200 µl, respectively. The diluted lipofectamine 2000 and plasmid DNA were mixed together and incubated for 30 min at room temperature. The mixture was then added drop wise onto the cell under gentle rocking condition, and incubated for 45 min at room temperature. The transfected cells were incubated 4-6 h at 37 °C with 5% CO₂ and medium was replaced by 1ml of DMEM (Gibco) supplemented with 10% FCS (Gibco). The cells were incubated at 37°C in a CO₂ incubator for 18-24 hours post-transfection before assaying for transgene expression. The media was replaced 24 h later with fresh media and transfected cells were maintained in presence of 250 µg/ml of G418.

The transcription level of the cpc gene was detected by reverse transcription-PCR (RT-PCR) using the cpc –specific primers mentioned above (Seq ID 4 and Seq ID 2) and the transient expression was analyzed by Western blotting. Briefly, total cellular RNA from pVAX1-cpc-transformed HEK293T cells was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The extracted RNA was treated with DNase I (Roche, Mannheim, Germany) to completely remove any residual DNA. cDNA was synthesized from 100ng of total RNA using SuperScript® III First-Strand Synthesis kit (Invitrogen, San Diego, CA). The cDNA was amplified by PCR with *L. donovani* cpc specific primers mentioned above (Seq ID no 4 and 2). After amplification, 10 µl of each PCR product was applied to 1% agarose gel and visualized by ethidium bromide staining. For Western blotting, the protein extracts from the transfected 293T cells were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. To detect the expressed protein, a primary polyclonal antibody against recombinant cpc was used at 1:1000 dilution followed by 1: 1000 dilution of HRP-conjugated goat anti-rabbit IgG secondary antibody (Bangalore Genei, Bangalore, India).

Example 4

Preparation of cationic liposomes with MPLA and entrapment of plasmid DNA within it:

Lipids used herein were obtained from Sigma and Fluka. MPLA was bought from Invivogen, San Diego, California. All other chemicals were of analytical reagent grade. Initially the solution of lipid was prepared by dissolving 20 mg DSPC, 3mg cholesterol, 2mg

SA and 10 μ g MPLA in approximately 1ml chloroform. The molar ratio of DSPC: Cholesterol: SA is 7:2:2 with 10 μ g/ml MPLA dissolved in chloroform followed by evaporating the organic solvents to form a thin film. The uniform lipid film is made in round bottomed flask with rotary evaporator. The lipid film is then desiccated in vacuum desiccator for almost 16 hours at a temperature of 25°C. Empty and plasmid DNA entrapped liposomes were prepared by dispersion of lipid film in either 1 ml of 0.02 M PBS (pH 7.4) alone or containing 1mg/ml of pVAX1-cpc. The mixture was then vortexed for 15 mins and sonicated in an ultrasonicator (Misonix, New York, USA) for 30 s at 5 Hz, followed by incubation at 4°C for 2 h. The excess free plasmid DNA was removed by ultracentrifugation at 105,000 $\times$ g for 1 h at 4°C twice. Estimation of percent liposomal DNA entrapment was based on its quantification in the unentrapped fraction by absorbance at 260 nm. The level of DNA incorporation ranged between 75–80%. The liposomes thus prepared, with or without DNA, were stored at 4°C until characterized and/or used.

Example 5

Atomic forced microscopy for structural analysis of liposomes

For AFM imaging of liposomal samples, 10 μ l of the sample was deposited onto freshly cleaved muscovite Ruby mica sheet (ASTM V1 Grade Ruby Mica from MICAFAFAB, Chennai) for 15-30 minutes. Mica sheets are basically negatively charged so samples binds strongly on the mica surface. After 15 min, the sample was dried by using vacuum dryer. The sample was gently washed with 0.5ml Milli-Q water to remove the molecules that were not firmly attached to the mica and the sample was dried as mentioned above.

AAC mode AFM was performed using a Pico plus 5500 ILM AFM (Agilent Technologies, USA) with a piezoscanner maximum range of 9 μ m. Micro fabricated silicon cantilevers of 225 μ m in length with a nominal spring force constant of 21-98 N/m were used from Nano sensors, USA. Cantilever oscillation frequency was tuned into resonance frequency. The cantilever resonance frequency was 150-300 kHz. The images (512 by 512 pixels) were captured with a scan size of between 0.5 and 2 μ m at the scan speed rate of 0.5lines/S. Images were processed by flatten using Pico view1.4 version software (Agilent

Technologies, USA). Image processing and analyzation has been done through Pico Image Advanced version software (Agilent Technologies, USA).

5 The AFM images of liposomes on mica are shown in Figure 3 AFM in the acoustic alternative current (AAC) mode approaches allows the observation of the liposomal morphology avoiding any sample manipulation such as staining, labeling, or fixation. Flattening of vesicles on the mica support just few minutes after deposition indicates a moderate stability of the liposomes on a mica substrate. AFM images clearly depict the spherical, well-defined shape of liposomes with visible multilamellar structures. The
10 liposomal DNA complex was seen to be compacted with very few aggregations.

Example 6

Zeta potential Measurement

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The zeta potential, which is an indirect measurement of the vesicle surface charge, was measured at room temperature by half-diluting the liposomes in 20 mM PBS using Nano ZS Zetasizer (Malvern Instruments, Worcestershire, UK). A polydispersity index value of 0.0 represents a homogeneous particle population, while a value of 1.0 indicates the
20 heterogeneity of the liposome preparations.

Values of the zeta-potential of liposomes indirectly reflect vesicle surface net charge and therefore are used to evaluate the extent of interaction between the surface charges of cationic liposomes and the anionic charges of DNA. On this basis, the question of DNA
25 entrapped versus empty DSPC liposomes with MPLA was investigated before and after encapsulating the desired plasmid DNA. The positively charged liposomes make a strong complex with negatively charged phosphate moiety on the sugar backbone of DNA. Hence, entrapment of plasmid DNA into cationic liposomes probably led to neutralization of cationic charges and compaction leading to clear reduction of the zeta-potential values and size for the
30 formulation studied (Table 01). The liposomal formulations were heterogeneous, size of empty LIP (MPL) = 134 ± 23.86 nm; size of pVAX1-cpc/LIP (MPL) = 96.33 ± 14.31 nm and the zeta potential (z-p) of about 24.3 ± 3.1 mV and 22.6 ± 1.2 respectively.

Table 1. Incorporation of Plasmid DNA into Liposomes: Vesicle Size, zeta Potential and entrapment. pVAX1-cpc was entrapped in cationic liposomes as shown. Results represent mean $\pm$ S.E., n = 3.

Formulation	Vesicle size (nm $\pm$ S.E.)	Zeta potential (mV $\pm$ S.E.)	DNA encapsulation (% total $\pm$ S.E.)
Empty liposome	134 $\pm$ 23.86	24.3 $\pm$ 3.1	-
Liposomes with entrapped pVAX1-cpc	96.33 $\pm$ 14.31	22.6 $\pm$ 1.2	77.7 $\pm$ 6.5

Example 7

DNase Protection Assay

The plasmid DNA gets easily degraded by endonucleases such as DNase I, which is one of the obstacles for the delivery of plasmid DNA in vitro or in vivo. Therefore, the stability in the presence of DNase I is one of the essential parameters of systemic gene delivery. To confirm that liposomes described herein protects plasmid from nuclease, DNase I protection assay was carried out using a method modified slightly from that reported previously (Wheeler et al., 1999). The following samples were tested: naked plasmid DNA and plasmid DNA entrapped in liposomal vesicles. A 1 μ g DNA aliquot of each sample was treated with 1 μ l DNase I (1U/ μ l) (Invitrogen, San Diego, CA), 1 μ l of 10x DNase I reaction buffer, and water to 10 μ l total volume and incubated at 37°C for 15-60 min. The complexes were subsequently extracted with phenol-chloroform and analyzed on 1% agarose gel (Tris-acetate buffer system, pH 8.2) and stained with ethidium bromide post electrophoresis.

Fig 4. reveals that most of the plasmid DNA incorporated in the cationic liposomes [pVAX1-cpc/LIP (MPL)] could not be degraded by DNase I. In contrast, naked plasmid DNA (pVAX1-cpc) was degraded with few minutes upon exposure to the enzyme. This may be attributed to condensed DNA state of the plasmid within the cationic liposomes which is resistant to DNase action ((Legendre and Szoka, 1995). Results of liposomal DNA vulnerability to degradation by DNase were largely confirmed by agarose gel electrophoresis of samples of naked or liposomal DNA exposed to DNase I (Fig 4.). Based on intensity of

staining and the appearance of smearing, it can be seen that, whereas naked plasmid DNA was completely digested (Fig 4. lanes 2 and 3), DNA entrapped in cationic liposomes under study was fully protected (Fig 4. lanes 4 and 5). Most of the DNA was entrapped in the liposomes and retarded in the gel as no free DNA is seen in lane 5.

5

Example 8

In vitro cytotoxicity assay

10 To test the effect of liposomes on cell viability, MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma-Aldrich) assay was performed in normal murine peritoneal macrophages. Freshly harvested intraperitoneal macrophages from healthy BALB/c mice were plated at a density of 1×10^6 cells/well in a 96 well microtiter plates at 37°C in 5% CO₂ in RPMI as growth medium and incubated at 37°C overnight in 5% CO₂ atmosphere. After overnight incubation, empty cationic liposomes (3.2-200 µg/ml with respect to DSPC) were added to the medium and cells were incubated for another 1, 4 or 24 h. Cells incubated with only medium served as controls (100% viability). 100 µl MTT solution (2mg/ml) per 1 ml medium was added to each well after removing the media for 4 h at 37°C. The reduced, insoluble formazan crystals were solubilized in DMSO and absorption was measured at 550 nm on Thermo MULTISCAN EX plate reader. Viability was expressed in percent in comparison to untreated cells (100% survival). Experiments were performed in triplicates and repeated at least twice. Relative cell viability was calculated by dividing [Abs₅₅₀] (mean absorbance) of treated cells to [Abs₅₅₀] of the control cells. Results of the cell viability assays after liposome incubation are expressed in Fig. 5. Very high liposome concentrations between 100–200 µg/ml reduced the viability by 10–15% after 4 h of incubation, but did not affect the cell viability further even after 12h indicating that the invented formulation is totally safe for human use (Fig. 5).

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Example 9

Toxicity studies in vivo

The toxicity profiling of the developed formulation (liposomal pVAX1-cpc) was carried out using different toxicity markers. 5-week-old male BALB/c mice (n=5) were

treated with liposomes containing 50 µg or 100 µg plasmid DNA (with 1-2 mg of lipid w.r.t DSPC/animal/dose) in a 100µL injection volume in PBS, administered by lateral tail vein injection, in a single, double or triple dose. Animals were sacrificed 15 days after final treatment. Plasma alanine aminotransferase (ALT), aspartate amiotransferase (AST) and alkaline phosphatase (ALP) levels were determined for evaluating hepatotoxicity in plasma samples collected from different animal groups (using diagnostic kits from Randox, Ardmore, UK) [Heyes et al., 2006].

The relative toxicity of the new cationic liposomal formulation is of utmost interest for intended systemic administration. The toxicity, if any, after intravenous delivery of liposomal pVAX1-cpc vaccine was examined by detecting the levels of serum AST, ALT and ALP in mice. AST is an enzyme involved in the transfer of an amino group from aspartate to alpha ketoglutarate to produce oxaloacetic acid and glutamate, present in highest concentration in liver followed by heart, skeletal muscle, kidney etc. Elevation in AST level in serum generally indicates systemic tissue damage. ALP is an enzyme that comes mainly from the cells lining bile ducts and is normally eliminated in bile. Increase in serum ALP level than normal is suggestive of liver or bile-duct disease due to the body's inability to excrete it through bile. ALT is primarily found in the liver, making it a more specific test for detecting hepatocellular damage. The enzyme levels were elevated compared to normal controls only after triple dose of the vaccine which was not statistically significant as shown in Fig 6. AST, ALT and ALP levels actually remained within the normal limits with 50µg liposomal pVAX1-cpc injected twice within 48 hrs (administered dose) indicating that the invented formulation is totally safe for human use.

Example 10

Immunization of mice and challenge infection

For immunization, ten 4-6 weeks-old, healthy BALB/c mice were injected intramuscularly in the hind leg thigh muscle with 50 µg (in 50 µl of PBS) of pVAX1-cpc, empty liposome or PBS. For all immunization study, all groups were boosted once at 2-week interval. Ten days after the booster the mice were challenged intravenous with 2.5×10^7 freshly transformed stationary phase promastigotes in 200 µl PBS injected intravenously as described earlier [Mazumdar et al., 2004].

Example 11**Delayed type Hypersensitivity**

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One week after the last vaccination, delayed-type hypersensitivity (DTH) was determined as an index of cell-mediated immunity. The response was evaluated by measuring the difference in the footpad swelling at 24 h following intradermal inoculation of the test footpad with 50 µl of recombinant CPC protein (5 µg/animal) from that of control (PBS-injected) footpad with a constant pressure caliper (Starret, Anthol, USA) [Afrin et al., 1997].

10

DTH response was measured in vaccinated mice 10 days after the last immunization. Vaccinated mice with naked pVAX1-cpc pDNA and its encapsulation in cationic liposomes displayed significant DTH responses in comparison to control groups (Figure 7; $P < 0.05$ and $P < 0.01$ respectively). However, the response by pVAX1-cpc was significantly lower ($P < 0.05$) than the response induced by liposomal pVAX1-cpc immunization.

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Example 12**rCPC specific humoral response**

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Serum samples of individual mice were obtained before infection, ten days post vaccination. Sera of individual mice were assayed for the presence of cpc-specific total IgG, IgG1 and IgG2a antibodies using enzyme-linked immunosorbent assay (ELISA) as described earlier [Afrin et al., 1997]. In brief, 96-well microtiter plates (Nunc, Naperville, IL) were coated with recombinant CPC protein (50 µg/ml) and blocked to prevent nonspecific binding. The plates were then incubated with sera at a 1:200 dilution, followed by horseradish peroxidase (HRP)-conjugated goat IgG1, and IgG2a (1:1000) (BD Pharmingen, San Diego, CA). The color reaction was developed, and the absorbance was read in an ELISA plate reader (Thermo, Waltham, MA) at 450 nm [Afrin et al., 1997]

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In VL, increased IgG1 would characterize the Th2 response and disease progression in *L. donovani* -infected animals, while increase in IgG2 is indicative of Th1 response and resistance to infection [Deplazes et al., 1995]. Moreover, antibody isotype profile correlates

with type of cytokines produced from antigen-specific T cells. Switching of the IgG isotype to IgG2a is brought about by IFN- γ while IL-4 is associated with IgG1 production [Coffman et al., 1993]. IgG levels did not differ significantly between controls and liposomal DNA vaccinated animals. The combined IgG1 and IgG2a production with significantly high ratio of IgG2a:IgG1 as a measure of Th1:Th2 balance, discloses a Th1-biased immune protection with liposomal pVAX1-cpc DNA vaccine. In contrast, mice immunized with empty liposomes and naked pVAX1-cpc had similar IgG1 and IgG2a ratio lower than pVAX1-cpc entrapped in liposomes revealing a lack of Th1 dominance (Figure 8).

Example 13

Cytokine assay

For determination of recombinant CPC protein specific total cytokine production, spleens were removed aseptically from experimental mice of each group at 10 days after the last immunization. Each spleen was teased between 20 μ m pore size sieve to prepare single cell suspension in complete medium prepared with RPMI 1640 (Sigma) supplemented with 10 mM NaHCO₃, 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin sulphate, 50 μ M 2-ME (Sigma) and 10% heat inactivated fetal bovine serum. Erythrocytes were removed by lysis with 0.14 M Tris buffered NH₄Cl. The splenocytes were washed twice, resuspended in the culture medium and viable mononuclear cell number was determined by Trypan blue exclusion. Splenocytes were then incubated in a 96-well ELISA plate (Nunc) at a density of 2×10^5 cells/well in a final volume of 200 μ l with or without recombinant CPC protein (2.5 μ g/well). The cells were incubated for 96 h at 37 °C in a humidified chamber containing 5% CO₂. After 72 h incubation supernatants were collected and cytokine concentrations of IFN- γ , interleukin (IL)-4, IL-2, IL-10, and IL-12 were quantitated using Opt EIA kits (BD Pharmingen) in accordance with manufacturer's instructions.

The quality and magnitude of the immune response is probably regulated by the synergistic effect of the cytokines. We evaluated the Th1 and Th2 cytokine responses in groups of vaccinated mice. Splenocytes from immunized mice were isolated 10 days after immunization and IFN- γ , IL-2, IL-10, IL-12 and IL-4 levels were measured in vitro following restimulation with recombinant CPC protein. While IL-12, IFN- γ and IL-2, are regarded as crucial for parasite control and resolution of disease, growing evidences suggests importance

of IL-10 in down modulating type 1 responses, resulting in parasite persistence in the host and progressive VL. CD4⁺ and CD8⁺ Th1 cells, via IFN- γ and IL-2, direct the response towards cell-mediated immunity involving cytotoxicity and macrophage activation, whereas Th2 cells, via IL-4 and IL-10, direct the response towards antibody production. The two poles are counter-regulatory such that antibody formation is inhibited by IFN- γ and macrophage activation is largely inhibited by IL-4 and IL-10. Strong Th1 based immune protection was achieved with liposomal DNA formulation [pVAX1-cpc/LIP(MPL)] compared to PBS, adjuvant control and naked plasmid DNA (pVAX1-cpc) as reflected by increased IFN- γ , IL-2 and IL-12 along with low levels of Th2 promoting cytokines like IL-4 and IL-10 (Figure 9), after immunization. Even the mice immunized with empty liposomes containing MPLA shows increased IL-12 and IFN- γ as markers for Th1 response which enhanced with entrapping the plasmid DNA construct.

Example 14

Evaluation of parasite burden in liver and spleen

The efficacy of naked and liposome-encapsulated pVAX1-cpc plasmids to protect animals against lethal *L. donovani* challenge, by intramuscular immunization, was evaluated in murine model of progressive VL (Fig 10). After 3 months of challenge infection clearance of organ parasite burden was quantitated as LDU in liver and spleen biopsies. The course of infection was monitored by the microscopic examination of Giemsa-stained impression smears of liver and spleen. The parasite load was expressed as Leishman-Donovan units (LDU) and was calculated by the following formula: number of amastigotes per 1,000 cell nuclei $\times$ organ weight (in milligrams) (Stauber et al., 1958).

As shown in Figure 10, control mice receiving PBS or empty liposomes developed highest parasite load in the liver and spleen due to progressive disease. Both naked and liposomal pDNA (pVAX1-cpc) conferred protection against parasite challenge upon intramuscular immunization in spleen. However, protection was maximum and significantly increased in mice vaccinated with liposomal vaccine formulation over naked pDNA in liver ($P < 0.05$) and spleen ($P < 0.05$). In contrast, only vector and adjuvant control provided no protection in spleen. Interestingly, there was significant decrease in parasite load in mice

receiving only adjuvant (LIP-MPL) in liver ($P < 0.05$) after 3 months showing some protective capacity of cationic liposomes with MPLA alone. Liposomal p-DNA conferred 82.10 and 79.9 percent higher protection than empty liposomes, in liver and spleen respectively.

5

ADVANTAGES OF THE INVENTION

1. The adjuvant formulation comprising of MPLA incorporated in the lipid bilayer of cationic liposomes is a novel and safe strategy of combining TLR signaling with efficient delivery vehicle for antigen presentation and to promote CD4⁺ and CD8⁺ T cell-mediated protective immunity against *Leishmania*, through a route compatible with human administration.
2. In a further aspect, there is provided a composition comprising plasmid DNA with Th1 promoting adjuvant and/or carrier to prevent and/or treat any parasitic infection. The results obtained with encapsulating DNA can be extrapolated to other molecules like proteins/ peptides and may find application against intracellular pathogens like *Leishmania* and also against cancer.
3. The present invention is a breakthrough in the field of medical immunology and further to methods of preparation of vaccines and immunomodulators to combat parasitic diseases and cancer.
4. In the present invention incorporation of MPLA in the liposomal formulation provided better protection than too in very low dose of MPLA (~1µg/animal/dose) in comparison to the dose of MPL-TDM whose dose requirement is 25µg/dose/ animal [Majumder et al., 2011].
5. Compared to previous DNA vaccination with type I (cpa) and II (cpb) cysteine protease genes from *Leishmania major* within solid-lipid nanoparticles (J Pharm Pharmaceut Sci (www.cspsCanada.org) 13(3) 320 - 335, 2010), the present invention shows better immunopotentiality by incorporating MPLA in liposomes.

6. The liposome-mediated pDNA (pVAX1-cpc construct) delivery resulted in robust protection, 34.18 and 54.25 times higher than naked pVAX1-cpc in liver and spleen respectively.

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Seq ID 1 (forward): 5'GGA ATT CCA TAT G GCC CTC CGC GCC AAG TCT
GCC 3'

Seq ID 2 (reverse): 5'CGG GAT CC CTA CTC CTG CGC GGG TAT GCC AGC3'

5 Seq ID 3:

LOCUS JX968801 1038 bp DNA linear INV 28-OCT-2012

DEFINITION *Leishmania donovani* strain MHOM/TN/1983/AG83 cysteine protease C gene, complete cds.

ACCESSION JX968801

10 VERSION JX968801.1 GI: 409905639

SOURCE *Leishmania donovani*

ORGANISM *Leishmania donovani*

Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;

Leishmaniinae; *Leishmania*.

15 ORIGIN

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Seq ID 4 (forward): 5' CCC AAG CTT GGA ATG GGA GCC CTC CGC GCC AAG TCT

35 3'

CLAIMS

1. A DNA vaccine formulation in cationic liposome vehicle useful for maximizing vaccine potency for *leishmaniasis* characterized in containing intralamellar monophosphoryl lipid A (MPLA) and cysteine protease C (cpc) gene.
5
2. A DNA vaccine formulation as claimed in claim 1 wherein a plasmid DNA containing Seq ID 3 encoding cysteine protease C (cpc) gene of *L. donovani* is encapsulated in the cationic liposome vehicle.
10
3. A DNA vaccine formulation as claimed in claim 1 wherein the cationic liposome is a multilamellar vesicle comprising of:
 - a) a neutral lipid preferably distearylphosphatidylcholine (DSPC);
 - 15 b) a cationic lipid preferably stearylamine (SA);
 - c) a second neutral lipid preferably cholesterol; and
 - d) a TLR agonist preferably Monophosphoryl lipid A (MPLA).
4. A DNA vaccine formulation as claimed in claim 3 wherein the neutral lipid, the cationic lipid, the second neutral lipid and the TLR agonist are formulated in a range of 7:1.5:1.5:0.0016 to 7:2.5:2.5:0.003 molar ratio.
20
5. A DNA vaccine formulation as claimed in claim 2 wherein the plasmid DNA is present in the range of 700 to 1000 µg/ml.
25
6. A DNA vaccine formulation as claimed in claim 1 is administered to a subject via intra-venous, intra-muscular or intranasal route wherein said subject is a mammal including human.
- 30 7. The cationic liposome containing MPLA as claimed in claim 3 is useful as efficient delivery vehicle for biomolecules and vaccines.
8. A process for the preparation of the DNA vaccine formulation as claimed in claim 1 wherein said process comprising the steps of
35
 - a) cloning cysteine protease C (cpc) gene having Seq ID 3 into pET28a vector;

- b) sub-cloning cpc clone obtained in step (a) into pVAX1 vector to make pVAX1-cpc plasmid DNA construct;
- c) dissolving a neutral lipid, a cationic lipid, a second neutral lipid and a TLR agonist as claimed in claim 3 in a range of 7:1.5:1.5:0.0016 to 7:2.5:2.5:0.003 molar ratio in an organic solvent preferably chloroform to obtain a solution followed by removing solvent using evaporator to obtain a lipid film;
- d) desiccating the lipid film as obtained in step (c) at a temperature in the range of 25 to 28°C for a period in the range of 12 to 16 hours to obtain a dried lipid film;
- e) dissolving the plasmid DNA as obtained in step (b) in the range of 1 to 2 mg/ml in a physiologically acceptable aqueous medium to obtain a solution;
- f) hydrating the dried lipid film as obtained in step (d) with the solution as obtained in step (e) to obtain a solution;
- g) vortexing the solution obtained in step (f) for a period ranging between 10 to 15 min and sonicating for a period in the range of 30 to 45 sec at 4 to 5 Hz at a temperature in the range of 3 to 4°C to obtain a liposomal formulation;
- h) removing the excess free plasmid DNA from the liposomal formulation as obtained in step (g) by ultracentrifugation to obtain a DNA vaccine formulation.

9. A process for the preparation of the DNA vaccine formulation as claimed in step (e) of claim 8 wherein physiologically acceptable aqueous medium is Phosphate Buffer Saline solution in a molar ratio in the range of 0.01 to 0.02.

10. Use of the DNA vaccine formulation as claimed in claim 1 as a vaccine against leishmaniasis for maximizing vaccine potency in mammals against *Leishmania* parasite.

11. Use of the cationic liposome as claimed in claim 7 to generate strong humoral and cellular immunity required to combat various pathogens and cancer.

12. Use of the cationic liposome as claimed in claim 7 for drug screening against leishmaniasis.

13. A pharmaceutical composition comprising a therapeutically effective amount of the
5 DNA vaccine formulation of claim 1 in a pharmaceutically acceptable carrier.

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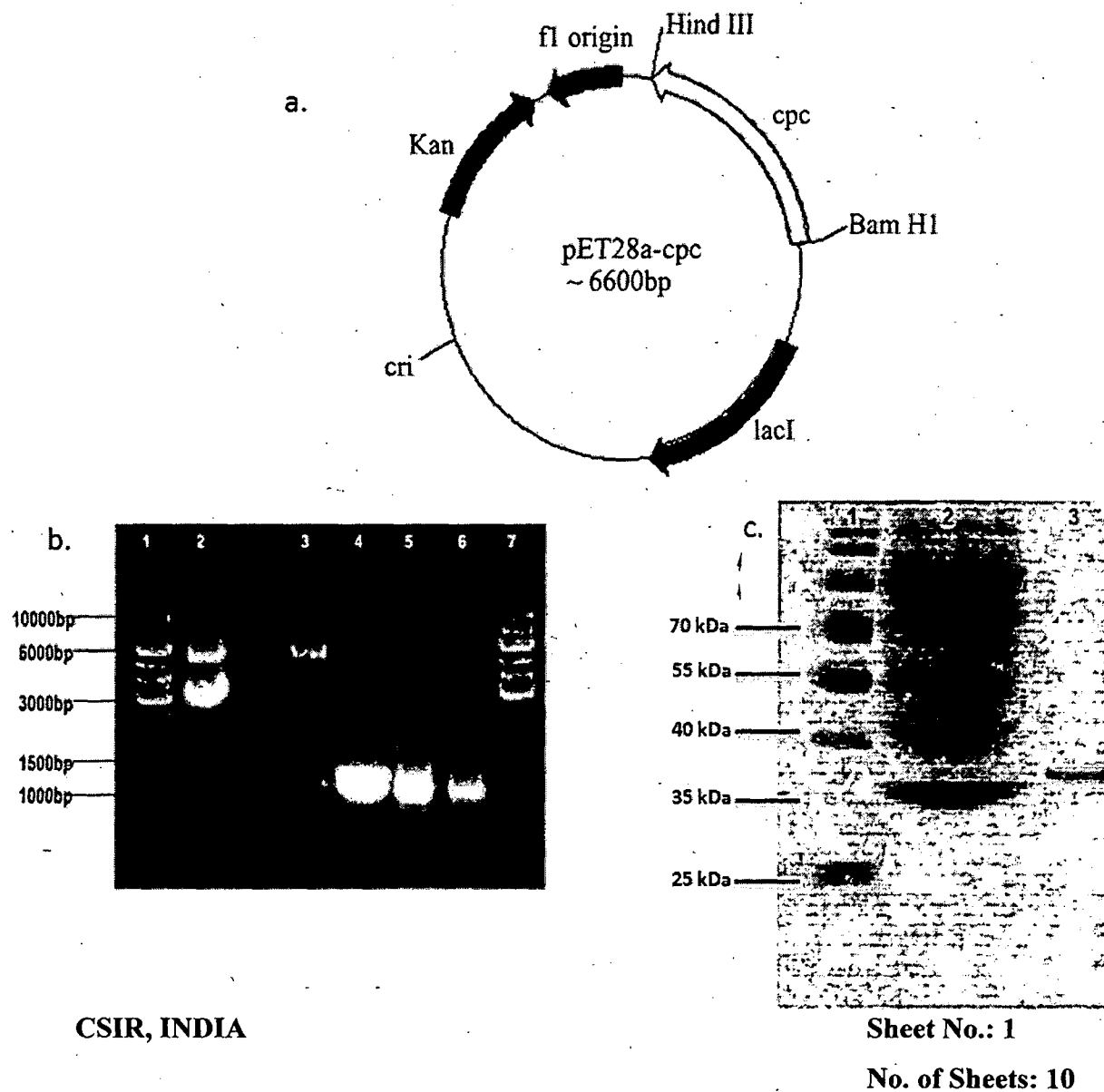


Figure 1

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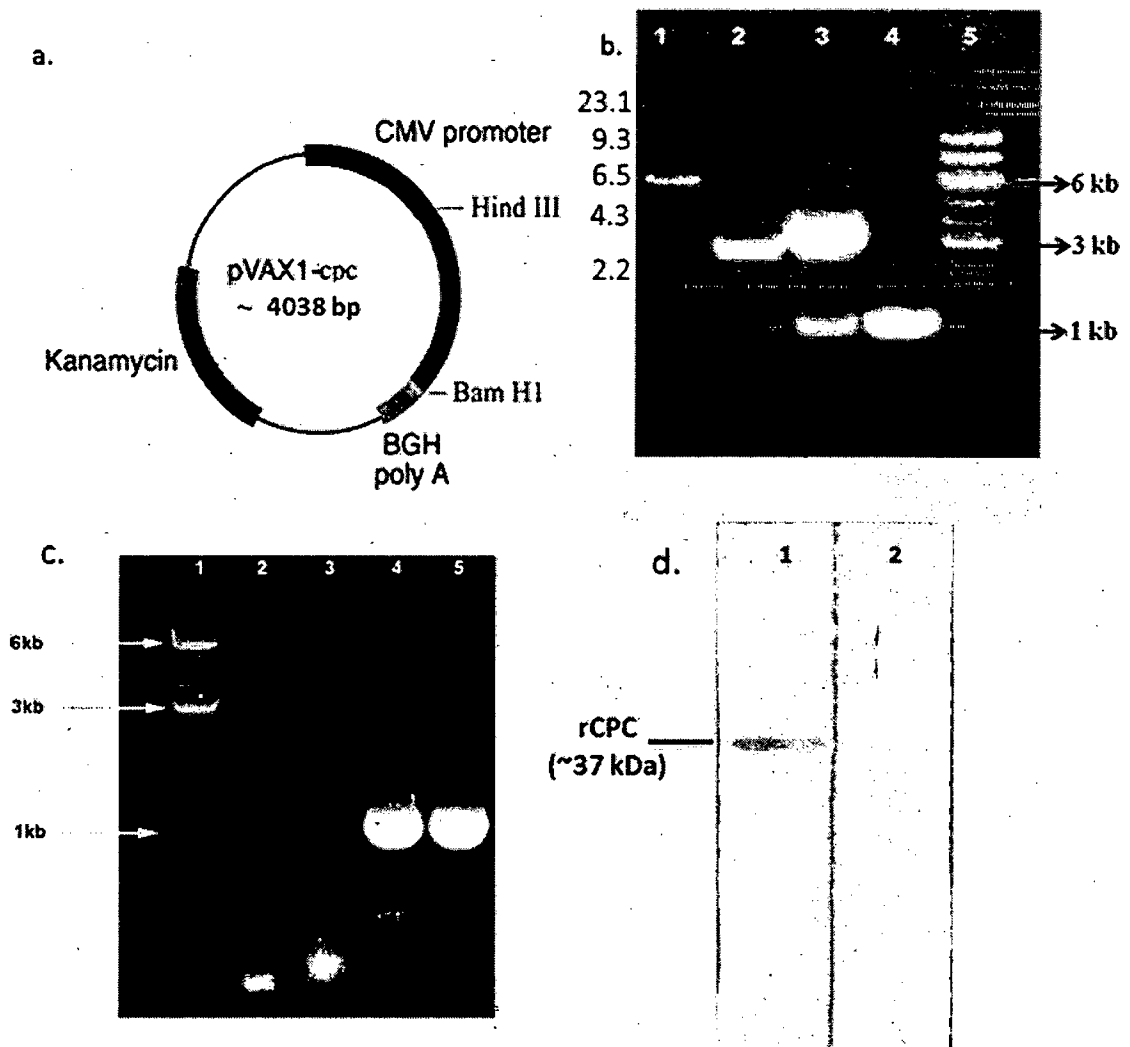


Figure 2

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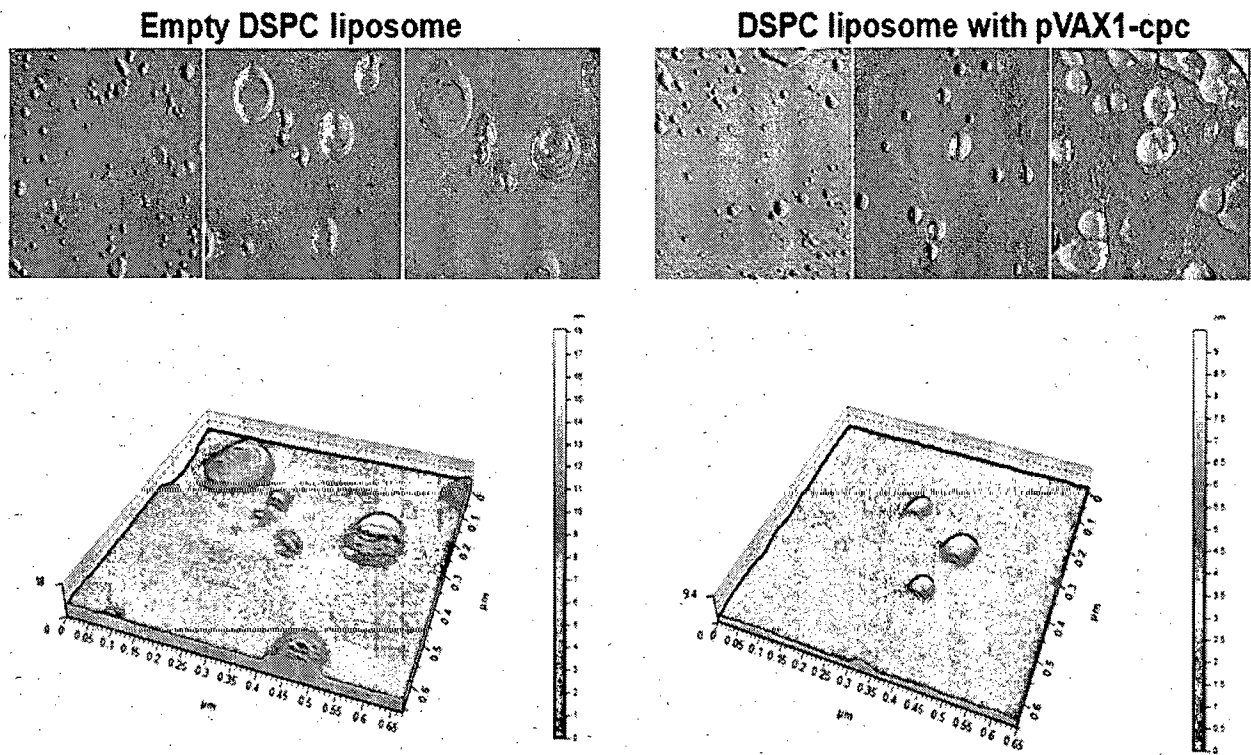


Figure 3

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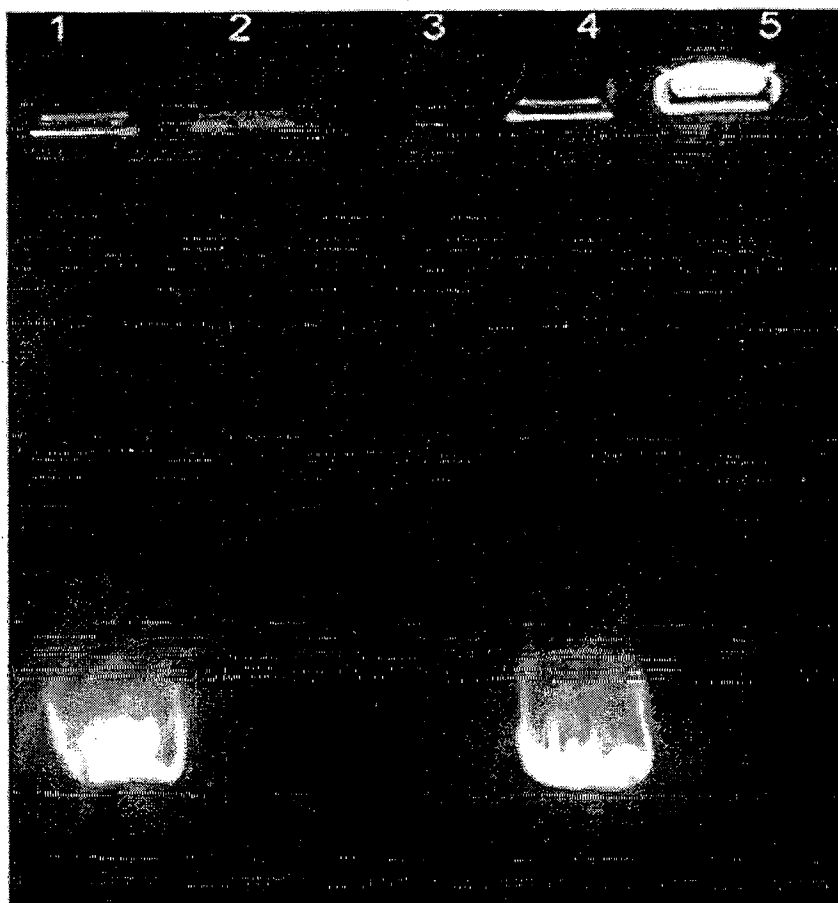


Figure 4

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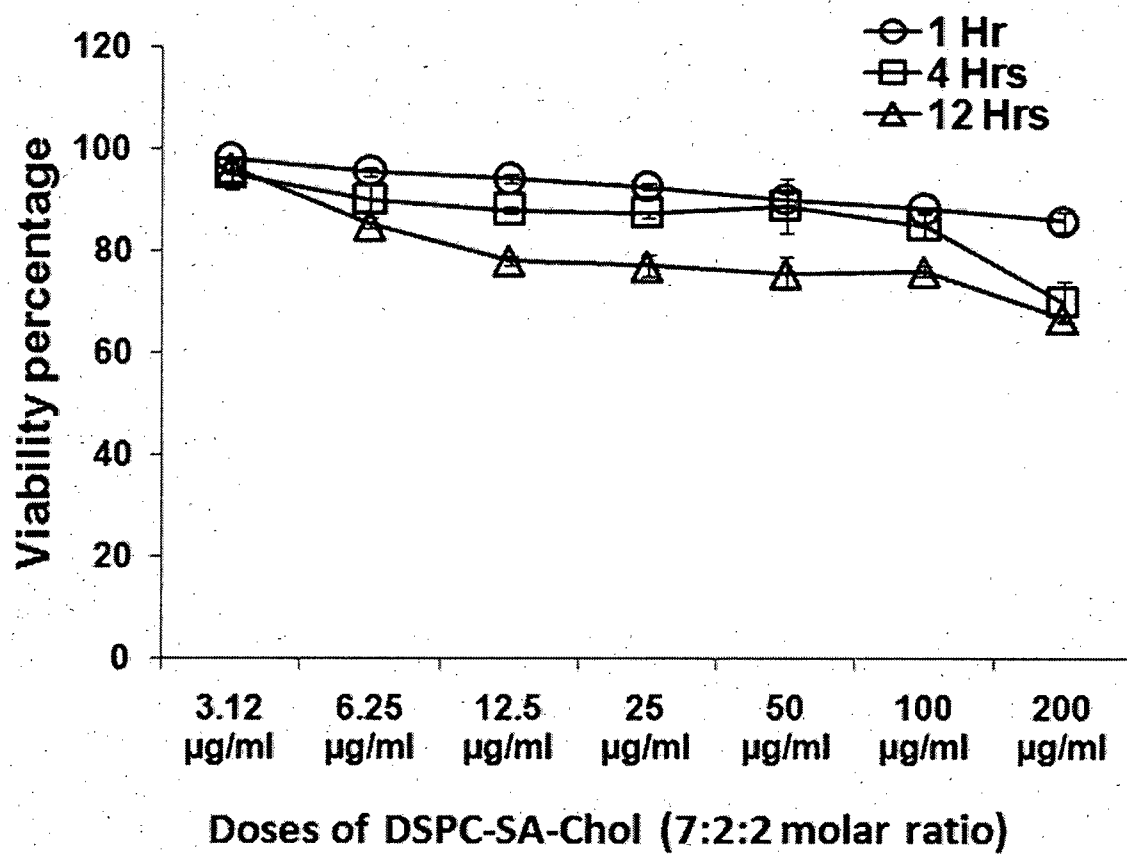


Figure 5

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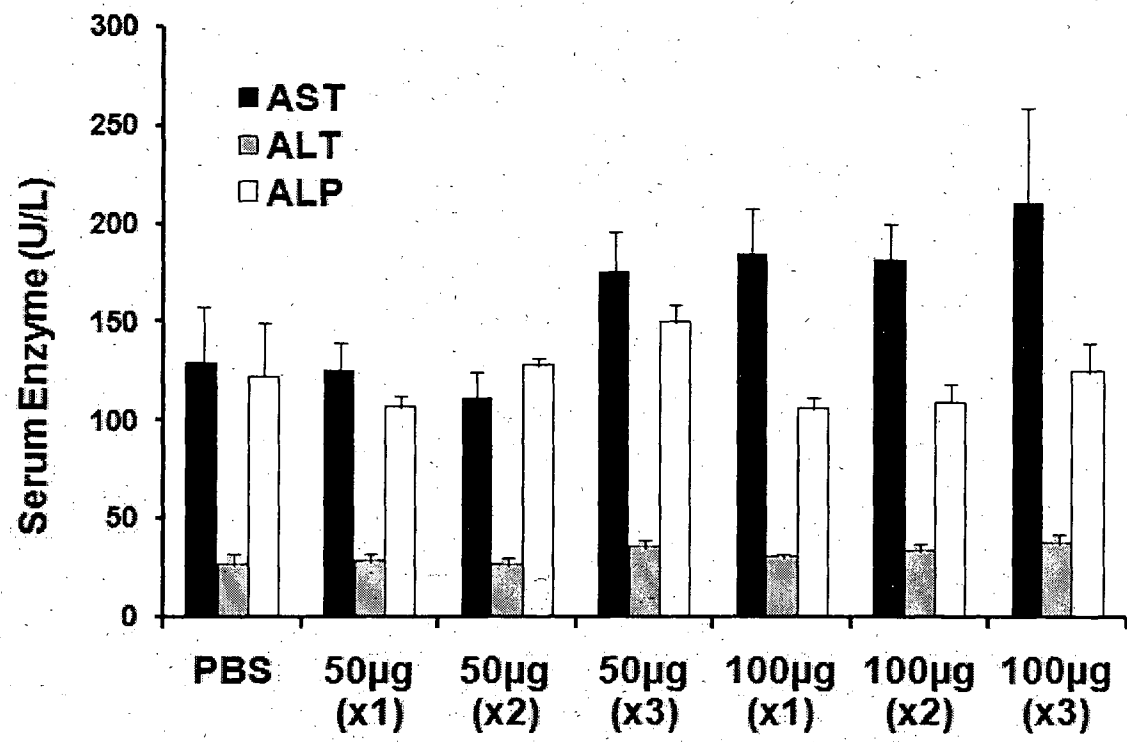
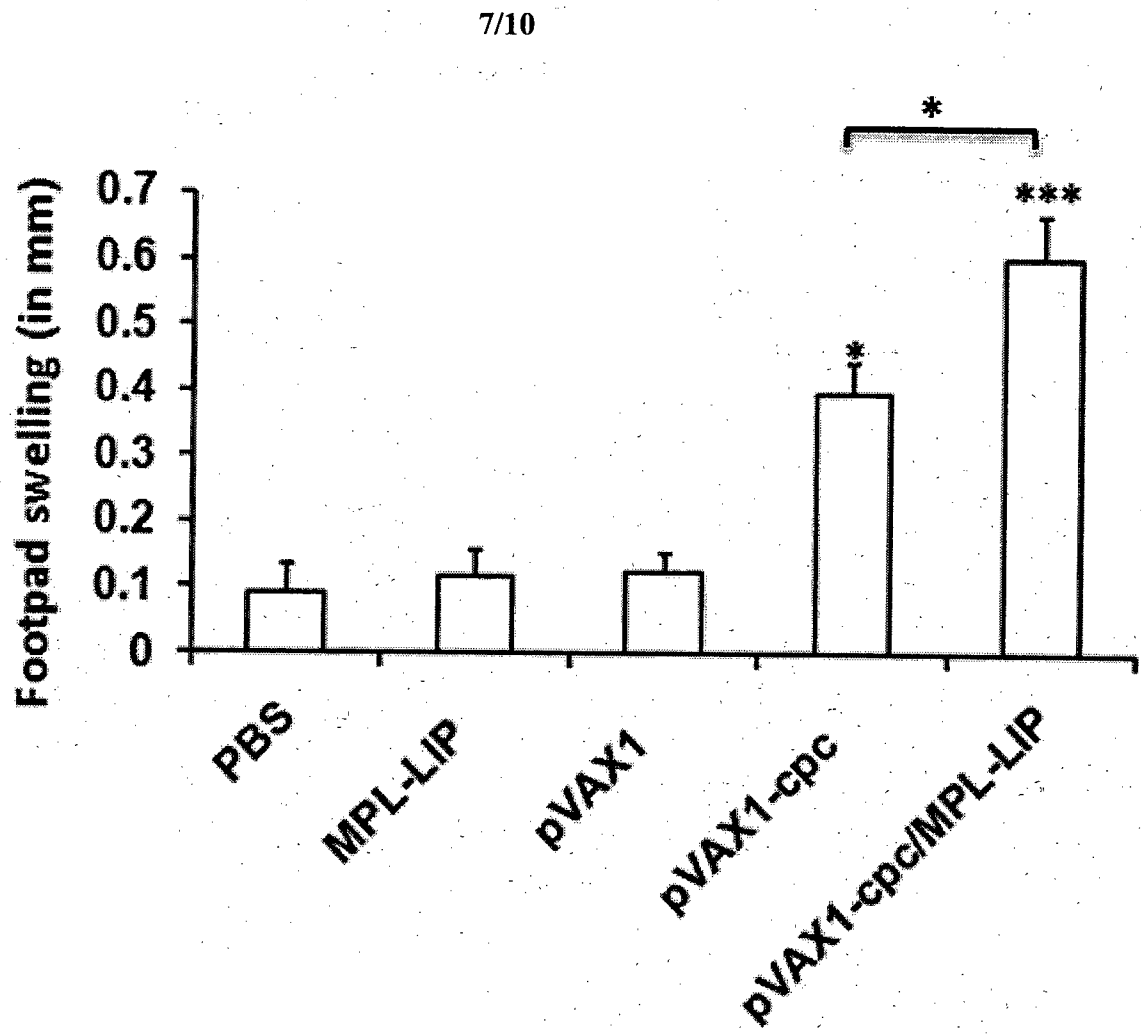


Figure 6

**Figure 7**

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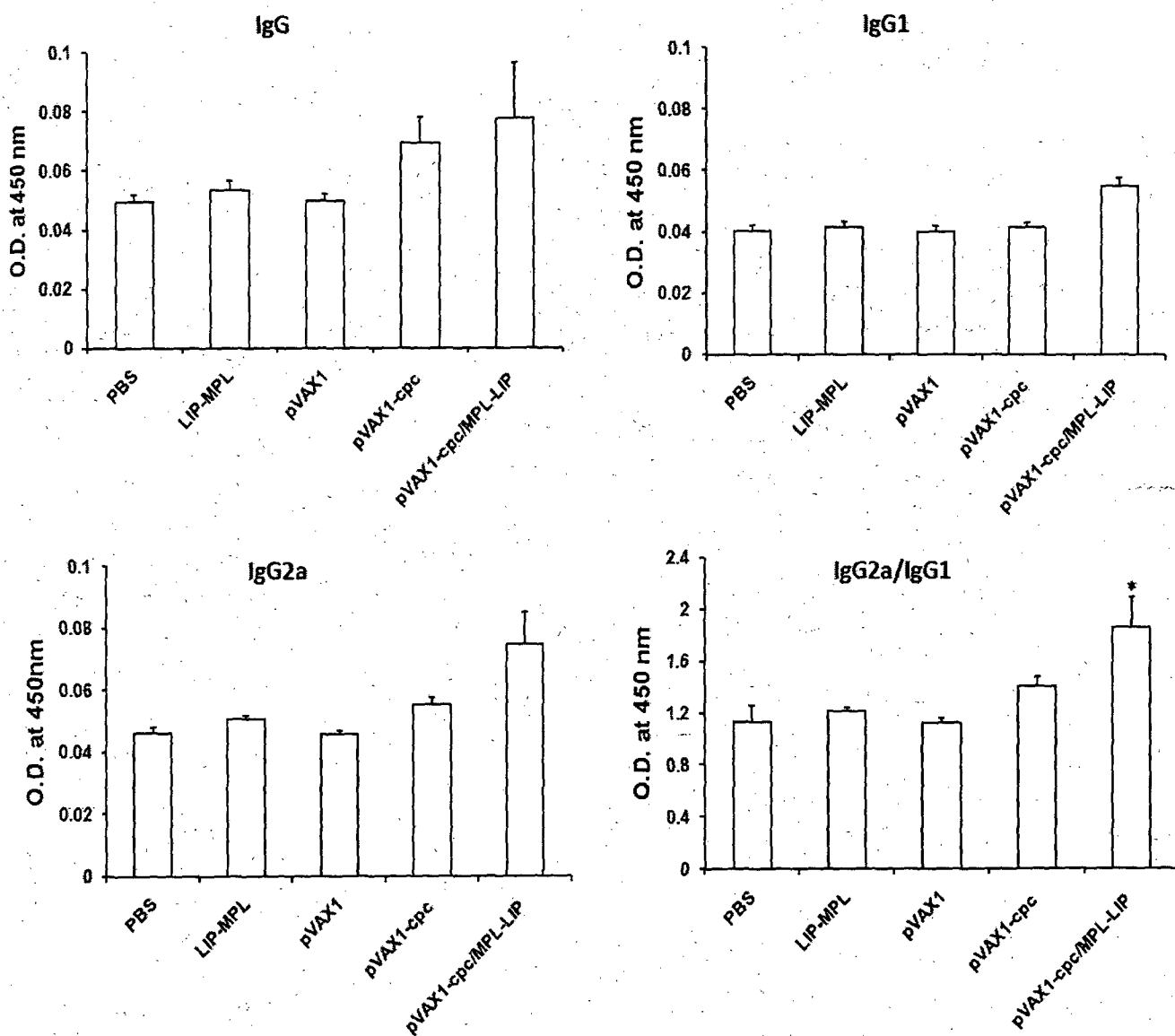


Figure 8

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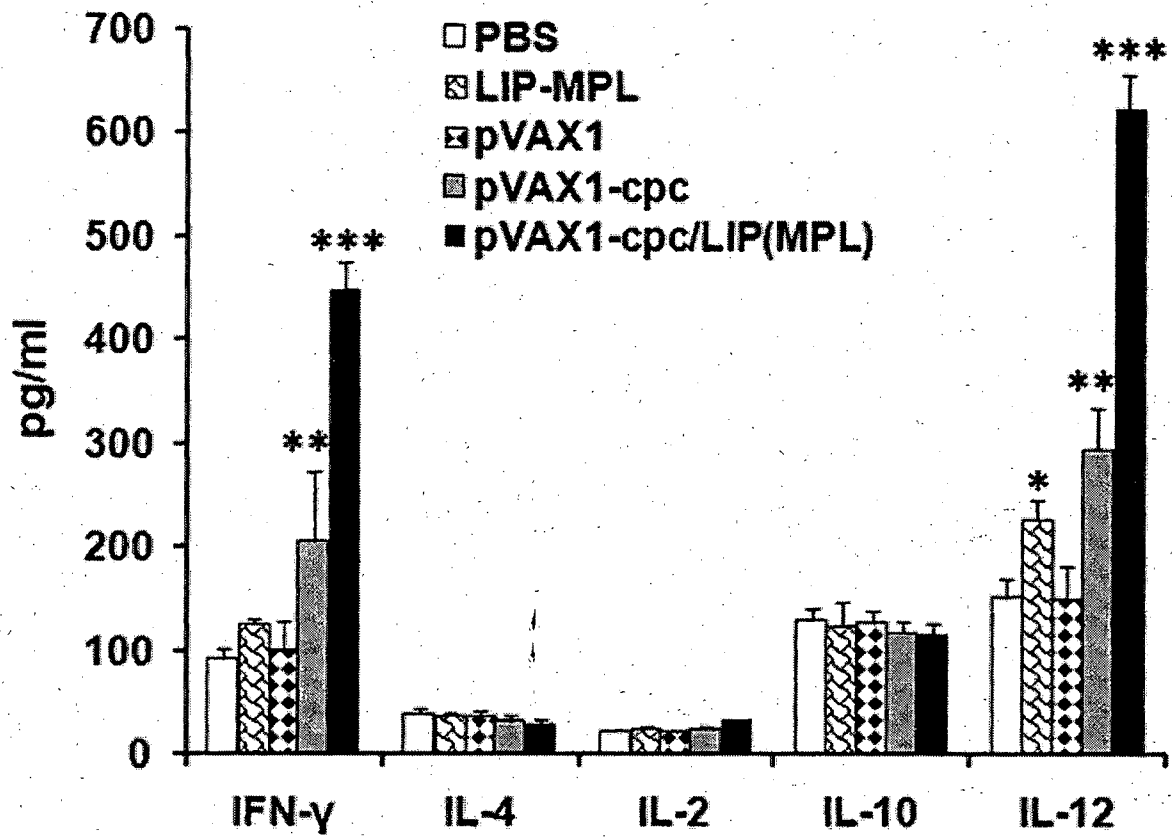


Figure 9

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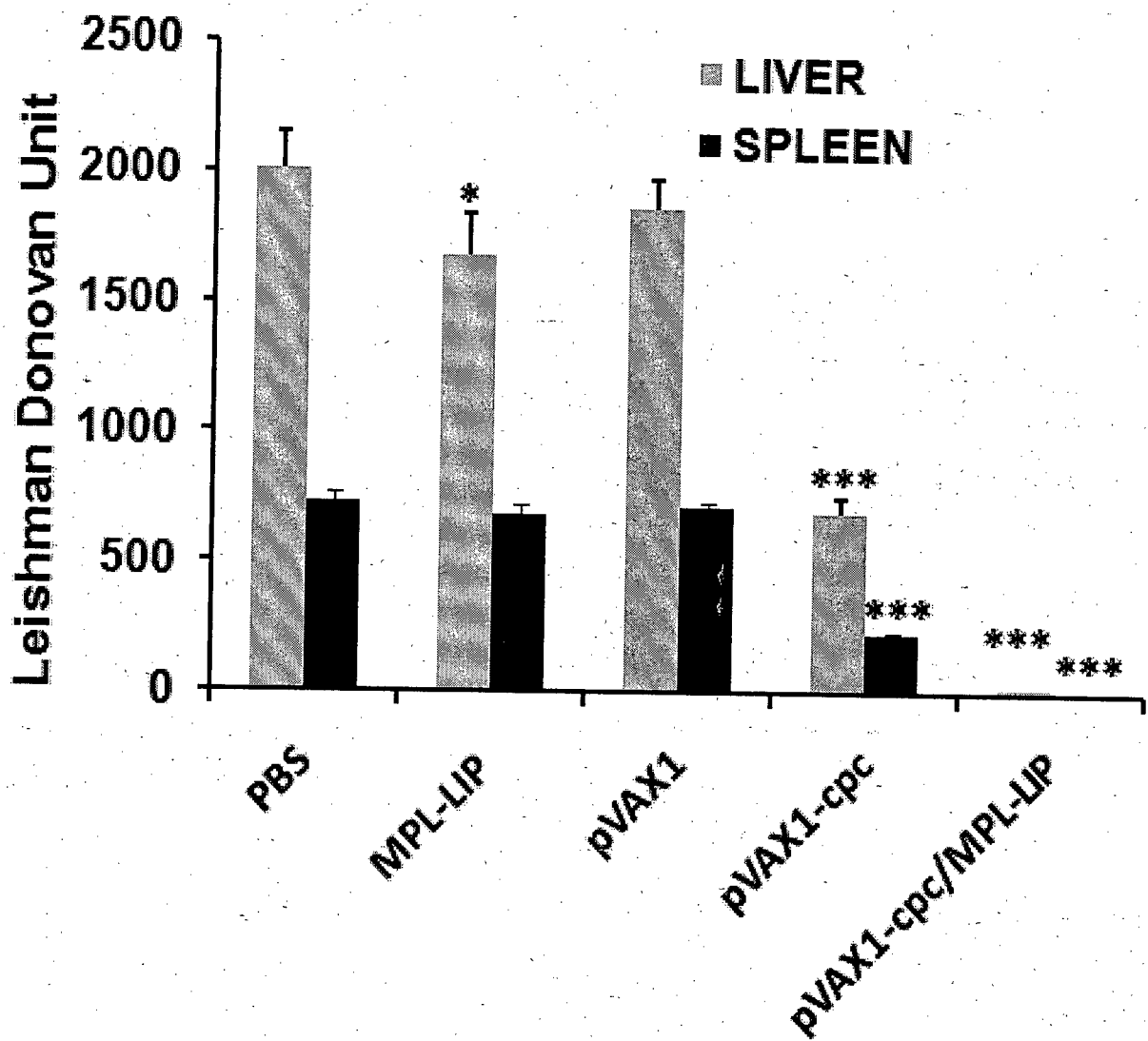


Figure 10

INTERNATIONAL SEARCH REPORT

International application No

PCT/IN2015/000186

A. CLASSIFICATION OF SUBJECT MATTER
 INV. A61K39/008 A61K39/39
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NAGHMEH KHOSHGOO ET AL: "Cysteine proteinase type III is protective against Leishmania infantum infection in BALB/c mice and highly antigenic in visceral leishmaniasis individuals", VACCINE, vol. 26, no. 46, 1 October 2008 (2008-10-01), pages 5822-5829, XP055206589, ISSN: 0264-410X, DOI: 10.1016/j.vaccine.2008.08.065 cited in the application the whole document ----- -/--	1-13



Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 August 2015

Date of mailing of the international search report

20/08/2015

Name and mailing address of the ISA/

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 Fax: (+31-70) 340-3016

Authorized officer

Renggli-Zulliger, N

INTERNATIONAL SEARCH REPORT

International application No

PCT/IN2015/000186

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Delaram Doroud ET AL: "Cationic solid lipid nanoparticles loaded by cysteine proteinase genes as a novel anti-leishmaniasis DNA vaccine delivery system: characterization and in vitro evaluations", Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Société canadienne des sciences pharmaceutiques, 5 September 2010 (2010-09-05), pages 320-335, XP055206628, Canada Retrieved from the Internet: URL: http://www.ncbi.nlm.nih.gov/pubmed/21092706 [retrieved on 2015-08-06] cited in the application abstract -----	1-13
Y	SAUMYABRATA MAZUMDER ET AL: "Potentiating Effects of MPL on DSPC Bearing Cationic Liposomes Promote Recombinant GP63 Vaccine Efficacy: High Immunogenicity and Protection", PLOS NEGLECTED TROPICAL DISEASES, vol. 5, no. 12, 20 December 2011 (2011-12-20), page e1429, XP055206574, ISSN: 1935-2727, DOI: 10.1371/journal.pntd.0001429 the whole document -----	1-13
A	BHOWMICK S ET AL: "Comparison of liposome based antigen delivery systems for protection against Leishmania donovani", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 141, no. 2, 25 January 2010 (2010-01-25), pages 199-207, XP026815093, ISSN: 0168-3659 [retrieved on 2009-10-07] 2.2 and 2.3; figures 2-5 -----	1-13
A	AMRITA DAS ET AL: "Vaccine Development Against Leishmania donovani", FRONTIERS IN IMMUNOLOGY, vol. 3, 1 January 2012 (2012-01-01), XP055206698, ISSN: 1664-3224, DOI: 10.3389/fimmu.2012.00099 abstract; tables 1,2 -----	1-13
	-/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2015/000186

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YVONNE PERRIE ET AL: "LIPOSOME (LIPODINE(TM))-MEDIATED DNA VACCINATION BY THE ORAL ROUTE", JOURNAL OF LIPOSOME RESEARCH, vol. 12, no. 1-2, 1 June 2002 (2002-06-01), pages 185-197, XP055206801, ISSN: 0898-2104, DOI: 10.1081/LPR-120004792 abstract -----	1-13
Y	WO 2011/115684 A2 (MASSACHUSETTS INST TECHNOLOGY [US]; IRVINE DARRELL J [US]; MOON JAEHYU) 22 September 2011 (2011-09-22) page 11 page 37 page 63 -----	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IN2015/000186

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