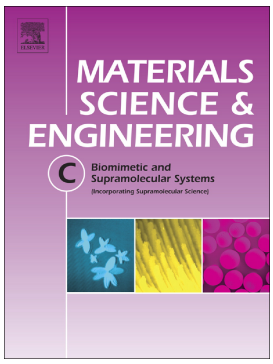
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Nano-Co-delivery of Lipophosphoglycan with Soluble and Autoclaved Leishmania Antigens into PLGA nanoparticles: Evaluation of in Vitro and in Vivo Immunostimulatory Effects against Visceral Leishmaniasis

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Nano-Co-delivery of Lipophosphoglycan with Soluble and Autoclaved Leishmania Antigens into PLGA nanoparticles: Evaluation of in Vitro and in Vivo Immunostimulatory Effects against Visceral Leishmaniasis

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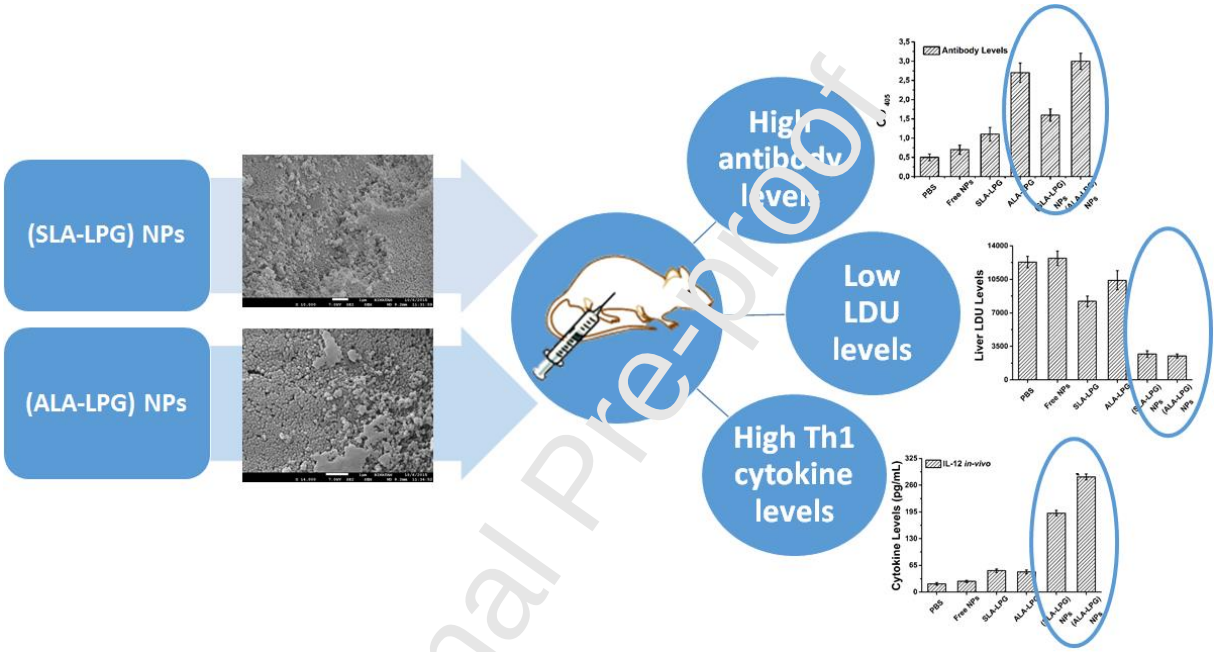
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Abstract

The aim of the present study was to encapsulate lipophosphoglycan molecule (LPG) which is one of the most immunogenic antigens of Leishmania parasites into PLGA nanoparticles with autoclaved or soluble leishmanial antigens, characterize synthetized nanoparticles with different methods and evaluate their in vitro/in vivo immunostimulatory activities to develop new vaccine candidates. PLGA nanoparticles including LPG and autoclaved leishmania antigen (ALA) or soluble leishmania antigen (ALA) were synthetized by double emulsion solvent evaporation method. The synthetized nanoparticles were characterized by SEM and Zeta-sizer instruments for determination of size, zeta potentials and polydispersity index (PDI) values. The antigen release profiles and encapsulation efficiencies were determined by UV-Vis spectroscopy. Griess reaction and ELISA tests were used for measurements of produced nitric oxide (NO) and cytokine levels of macrophages and splenocytes treated with nanoparticles. For determination of protective effects of nanoparticles, parasite reduction in livers and spleens of immunized mice were calculated by LDU values post-infection. According to results, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs possessed the sizes of 253 and 307 nm respectively. Antigen-loaded nanoparticles elevated the released NO amounts from macrophages for 14 and 18-folds in contrast to control. Furthermore, synthetized nanoparticles significantly triggered macrophages to produce excessive levels of IFN-γ and IL-12 cytokines. Besides it was detected that vaccination of mice with (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs elicited approximately 80% protection from Visceral Leishmaniasis. Furthermore, (SLA-LPG) PLGA NPs and (ALA-LPG)

PLGA NPs lead to 10 to 14-folds increase in secreted Th1 cytokine levels from splenocytes than control demonstrating abundantly stimulation of T cell response following to vaccination with nano-vaccine formulations. These results reveal that both (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs have excellent immunostimulatory activities and they are promising nanovaccine formulations for the prevention of leishmaniasis in near future.

Keywords: Leishmania, antigen, PLGA, nanoparticles, vaccine, delivery



Gaphical Abstract of "Nano-Co delivery of Lipophosphoglycan with Soluble and Autoclaved Leishmania Antigens into PLGA nanoparticles: Evaluation of in Vitro and in Vivo Immunostimulatory Effects against Visceral Leishmaniasis"

1. Introduction

Leishmania, the causative agent of leishmaniases, is an intracellular parasite transmitted by the bites of female *Phlebotomus* sandflies [1]. Leishmaniasis is a neglected public health problem that is endemic in more than 102 countries in Asia, Africa, Southern Europe, South America and Central America [2]. Leishmaniasis is represented by a broad spectrum of clinical manifestations ranging from ulcerative skin lesions (cutaneous leishmaniasis, CL) to life-threatening visceral disease (visceral leishmaniasis, VL). VL, also known as Kala-azar is the most severe form of the disease and it is estimated that 500.000

people in the world are being caught to VL in every year. Annual global mortality rate of VL is being reported as 50.000 people [3]. VL is a chronic infection seriously affecting the internal organs such as liver, spleen and bone marrow. The possible symptoms of the disease are known as prolonged fever, anemia, weight loss and hepatosplenomegaly. VL is frequently fatal if left untreated [4]. Poor housing, malnutrition and domestic sanitary conditions (such as living in humid houses or in vicinity of accumulated rubbish, sewerage and farms of livestock) can increase the risk of encountring leishmaniasis especially in non-developed countries. Furthermore, climate changes, wars and migrations are also important factors contributing parasite reproduction and eventually affecting the distribution of leishmaniasis [5].

Compared with other diseases, treatment of leishmaniasis is very problematic. Different conventional antileishmanial drugs such as pentavalent antimonials, liposomal amphotericin B, pentamidine and miltefosine are currently used for the remedy of human leishmaniasis in many endemic countries. However, there are some critical drawbacks of these medications. Undesired toxicity, recently developed resistance in parasites and high costlines limits the use of these drugs in the treatment of leishmaniasis. Existence of these limitations in treatment reveal the significance of applying alternative medications and also put forward huge necessity for development of effective, reliable and preventive vaccine strategies [1, 6, 7].

For more than 80 years various type of vaccine candidates (e.g., killed or attenuated whole parasites, synthetic or recombinant peptides, or recombinant live vaccine vectors), with or without cytokines or other adjuvants, are being investigated against leishmaniasis in three main categories [8]. First generation vaccines include killed or live attenuated parasites (leishmanization). Autoclaved Leishmania Antigens (ALA) and Soluble Leishmania Antigens (SLA) were repeatedly tested as vaccines against CL and VL. SLA and ALA contain many

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antigenic epitopes and target proteins in their structure so they can easily interact with numerous major compatibility complex (MHC) components to produce high immune responses [9, 10],. Besides, acquisition of these antigens is laborless and also cost-effective. However, to date these antigenic cocktails alone or with the use of various adjuvants showed low degrees of protection. In order to increase the immune responses against first generation vaccines, it is very important to discover more appropriate and effective adjuvants. On the other hand, leishmanial surface antigens, intracellular molecules, stress sensitive proteins and many other biomolecules that are important tools of parasite metabolic pathways are used as sub-unit antigens that are classified in second generation vaccines against Leishmaniasis [3]. Lipophosphoglycan (LPG) which is a glycoconjugate predominantly found at the surface of the parasite including flagellate is the most commonly studied antigen according to its high immunogenic feature among sub-unit antigens [11]. However, second generation vaccines alone cannot produce an adequate immune response and require for suitable adjuvant support. Third generation vaccines generally include DNA sequences encoding leishmanial proteins. Besides their extensive benefits, DNA vaccines have some serious limitations such as specificity to only protein-mediated immunity, ability to insert into the host genome, leading to autoimmune diseases and affecting host cell growth. As seen, although substantial efforts have been spent for development of a reliable and effective vaccine so far, there is no available vaccine formulation against human leishmaniasis in market. On the other side, three licenced vaccines that are called as Leish-Tec, CaniLeish and Letifend were developed against canine visceral leishmanisis and are now marketed for veterinary use [12-14]. Among these vaccines, Leish-Tec and Canileish include combinations of leishmanial antigens and adjuvants indicating that recruitment of vaccine candidates with suitable immunomodulators can implement their protective efficacies against leishmaniasis. Today, new approaches are being investigated in the experimental models in order to discover antileishmanial vaccines

for human care. Combinations of several leishmanial antigens and enrichment with appropriate adjuvants are believed to induce both humoral and cellular immunity.

Besides, recent advances in nanotechnology have brought a new dimension to vaccine researches. Nanoparticular antigen-carrying systems allow controlled and effective release of antigens without degredation in the biological system. The most important feature of these platforms is that two or more antigens encapsulated into nanoparticles can be efficiently uptaken by the same antigen-presenting cell. Therefore, these nanoparticular tools can mediate the generation of stronger immune response and protection from the infections. Poly(lactic-co-glycolic) Acid (PLGA) which is a FDA-approved polymer is commonly used as nanoparticular carrier systems because of their special features such as biocompatiblity, biodegradablity, low-toxicity and also adjuvanticity [15, 16]. There are several studies in the literature investigating in vitro and in vivo immunogenicities of PLGA based vaccine formulations loaded with single leishmanial antigen. On the other hand, researches in association with examining vaccine efficacies of PLGA nanoparticles carrying dual Leishmania antigens are lacking.

Considering the possible strong immunostimulatory effects of using dual Leishmania antigens and also favorable adjuvant properties of PLGA polymers, we anticipate that PLGA nanoparticles loaded with combination of first-generation vaccine (SLA or ALA) and second-generation vaccine (LPG) components could stimulate the genaration of significant humoral and cellular immune response. We also thought thatlong-term and sustainable release of antigens from PLGA nanoparticles would provide strong protection against VL. Hence, the aim of this study was to prepare vaccine formulations by encapsulation of SLA-LPG and ALA-LPG dual antigens into PLGA nanoparticles, characterize synthetized nanoparticles with different methods and evaluate their in vitro and in vivo immunostimulatory activities against visceral leishmaniasis.

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2. Materials and Methods

2.1. Materials

Roswell Park Memorial Institute medium (RPMI 1640) and heatinactivated Fetal bovine sera (FBS) were purchased from GIBCO (Life Technologies, USA). Penicillin, streptomycin, Hemin, Adenosine, PLGA (molar ratio of D, L-lactic to glycolic acid, 50:50, Mw 38,000-54,000 Da), polyvinyl alcohol (PVA) (87-90% hydrolyzed, average MW 30.000–70.000 Da), Methylthiazolyltetrazolium (MTT) and methanol were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM, hypergrade ≥ 99.9 %) and Giemsa stain were obtained from Merck (Darmstadt, Germany). All cytokine kits were purchased from eBioscience. Ultra-pure water was obtained from Millipore MilliQ gradient system.

2.2. Parasite Culture

Leishmania infantum strain (MCAN/TR/2005/LV126) was used in all in vitro and in vivo experiments. L.infantum promastigotes were cultured in RPMI medium supplemented with 10% FBS and gentamicin (80 mg/mL) at 27°C until late log phase. Large amounts of parasite biomass are required in order to obtain soluble Leishmania antigen (SLA), autoclaved Leishmania antigen (ALA) and lipophosphoglycan (LPG) antigens for the preparation of vaccine formulations. A large scale culture procedure was employed for that process. Briefly *L. infantum* parasite culture was primarily transferred to a 75 cm^2 flask (with number of parasites at 10^7/ml) and then transferred into the one liter erlenmeyer with Brain Heart Infusion Medium, supplemented with %5 FBS, Hemin and Adenosine and the cultivation was performed on a shaker at 27°C. The process yielded 2 L of *L. infantum* culture with density of 25–30 million parasites per 1 mililiter. Continuous *L. infantum* culture was passaged once in a two week period.

2.2.1. Extraction and purification of LPG

The isolation, extraction and purification steps of LPG was described as previously [17].

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Briefly, metacyclic forms of parasites, collected from large scale culture, were rinsed twice with PBS. Washed parasite biomass divided into seperate tubes and a chloroform methanol (1:2) mixture was added tubes with a 3.75 fold volume. After that, the mixture was sonicated in an ice bath in order to make lysis of the cells. Afterwards, parasite suspensions were incubated for one hour at the room temperature. Then the suspensions were centrifuged at 4000 rpm at 4 °C for 30 min and all the supernatants were collected into volumetric flasks. The remaining pellet was extracted with 4 ml 1-Propanol (9%) and sonicated again. At the end of the sonication process suspensions were centrifuged at 14000 rpm for 20 min. All supernatants were collected in a balloon and evaporated until 1-Propanol was completely removed. Following, the remaining extact was frozen at -80°C and then lyophilized overnight, resuspended in 5% 1-propanol/0.1 M ammonium acetate. Afterwards, resuspended LPG sample was transferred onto a column with 2 mL of octyl-Sepharose (12 × 3 cm), equilibrated within the same buffer and incubated at room temperature for overnight. Following to incubation, the column was washed with 50 mL of 5% 1-propanol/0.1M ammonium acetate mixture and then eluted with 1-propanol/0.1M ammonium acetate mixture with a gradient ranging between 5% and 60% 1-propanol. The elution fractions were collected as 120 drops for each tube. Finally, tubes were tested for carbohydrate existence via thin layer chromatography (TLC). Purple spots were regarded as positive and these positive fractions were brought together to determine the amount of LPG using phenol sulphuric acid method.

2.2.2. Preparation of Soluble Leishmania Antigen (SLA) and Autoclaved Leishmania Antigen (ALA)

SLA and ALA preparation was performed using the protocol developed by Tafagodi et al., with minor modifications [18]. At the begining metacyclic Leishmania promastigotes were washed three times with PBS buffer and centrifugated 4000 rpm for 20 min. The final pellet was reconstituted with HEPES buffer (pH 7.5) solution. The suspension was divided into two

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tubes for preparation of SLA and ALA separately. For acquisition of SLA, parasite suspension was lysed by freeze-thaw method for five cycles, and then the lysate was centrifuged at 12000 rpm for 15 min. Afterwards, the supernatant was collected and stored at -40 °C until its use. The protein concentration of the SLA was measured by UV-Vis spectrophotometer. For the prepation of ALA, parasite suspension was autoclaved at 121°C under 1.1 atm pressure for 15 minutes. The amount of antigenic protein in one mililiter was measured by UV-Vis spectrophotometer and then antigens were stored at -40 °C until their use.

2.3. Synthesis and Characterization of Nanovaccine Formulations

In this study nanovaccine formulations were synthetized by dual encapsulation of SLA-LPG and ALA-LPG antigen cocktails into PLGA nanoparticles with minor modification of our previous study [19]. (LPG) PLGA, (SLA-LPG) PLGA and (ALA-LPG) PLGA nanoformulations were prepared by double emulsion solvent evaporation technique (w/o/w). Briefly, 100 mg of PLGA was dissolved in 2.5 ml dichloromethane (DCM). LPG alone, SLA-LPG and ALA-LPG antigen combinations were all dissolved in distilled water. The dissolved antigens were added to polymer solutions in DCM and the mixtures for each two formulations were then emulsified with probe sonicator (Bandelin Sonopuls, Germany) in ice bath for 90 seconds (80 W amputul). Afterwards the emulsion was poured into 10 ml (3%, w/v) of an aqueous phase containing polyvinilyalcohol (PVA) as a stabilizer. The organic and aqueous phases were again emulsified for 5 min with sonicator at the same conditions. The obtained double emulsion (w/o/w) was then poured into 35 ml of an PVA (0.1 %, w/v) and stirred overnight at 350 rpm for the completely evaporation of DCM from the emulsion. Following that (LPG) PLGA, (SLA-LPG) PLGA and (ALA-LPG) PLGA NPs were both centrifuged at 14.000 rpm for 30 min. Obtained pellet was washed twice with deionized distilled water and the collected pellets was lyophilized for 48 hr.

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The encapsulation efficiencies of nanoformulations was calculated for SLA, ALA and LPG using following equation:

$$\textbf{Encapsulation efficiency}\ \% = \frac{\text{Amount of encapsulated antigens}}{\text{Initial amount of antigens used in formula}} \times 100 \qquad (1)$$

For this purpose, the concentration of non-encapsulated SLA and ALA was measured by bicinchoninic acid (BCA) protein assay using UV spectroscopy [19], whereas LPG amount was measured via gel permation chromatography (GPC) [17].

Particle size measurements and evaluation of polydispersity indexes for each formulations were performed by photon correlation spectroscopy (PCS) using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) [20]. Besides, zeta potentials of the samples were assessed with the same instrument. For morphological analysis of synthetized nanoparticles scanned images were obtained by using a Evo LS10 (Zeiss, Welwyn Garden City, UK) scanning electron microscope (SEM) at an accelerating voltage of 7 kV [20]. To obtain release profiles of LPG, SLA-LPG and ALA-LPG antigens, 10 mg particles for each sample were dispersed in 5 ml PBS at pH 7.4 and then incubated in a shaker at 37°C. At separate periods, sample solutions were filtered by amicon filter (10 kDa) and centrifuged for 15 min at 7000 rpm. The released amounts of SLA and ALA were determined by BCA assay, while released LPG was measured by GPC.

2.4. Cell Viability Assay

The murine macrophage cell line (J774) were grown in a 25T culture flasks (Corning) with RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FBS, and 1 M HEPES. Cell cultivation was maintained at 37°C in a humidified atmosphere with 5% CO_2. Following to deattachment procedure, 1 x 10^4 J774 macrophage cells (100 µl per well) were seeded in 96 well plates and incubated overnight to adhere at 37℃. Then non-adherent cells were removed and 100 µl of fresh culture medium containing free PLGA NPs, (LPG) PLGA NPs, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs were supplemented in various

concentrations ranging between 50 and 1000 µg/ml. Cells that were not exposed to nanoparticular formulations were used as the control. The cells in the control group was only exposed to PBS. Following to 144 hours of incubation, viabilities of macrophage cells were determined by using MTT assay. Briefly, 10 µl MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added into each wells of the microplates and cells were incubated for four hours at 37 °C. Afterwards, formazan crystals generated in viable cells were dissolved by adding 100 µl DMSO and the plates were incubated at room temperature for 30 minutes. Then optical density (OD) values in each well were determined by reading the absorbance at 570 nm wavelength in ELISA reader equipment. Finally, cell viabilities in each group were calculated with the following equation:

$$\textbf{Cell viability}\ \% = \frac{\text{OD values in experimental group}}{\text{OD values in control group}} \times 100 \quad (2)$$

2.5. Nitric Oxide Measurements

Griess reaction was employed to determine secreted nitric oxide amounts in macrophages treated with different formulations including LPG, SLA-LPG, ALA-LPG, free PLGA NPs, (LPG) PLGA NPs, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs. J774 macrophages (1×10^4/mL) were seeded into 96-well plates and cultured for 24 h at 37°C. Then medium was refreshed and cells were co-incubated with suspensions containing LPG, SLA-LPG, ALA-LPG, free PLGA NPs, (LPG) PLGA NPs, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs in 100 µl for 48 h. LPS was used as positive control. After incubation, suspensions in each well were collected and restored at - 40 °C until use in order to evaluate the levels of NO production.

The nitrite levels were evaluated by the Griess method. Briefly, standard nitrate solution ($NaNO_2$) was gradually diluted with PBS in a 96-well plate. Subsequently, 50 µl of sample supernatants were also included into separate wells. Afterwards, equal volumes of Griess reagents (1% sulfanilamide, 0.1% naphthylethylenedamine, and 2.5% H_3PO_4) were added into

wells including standard solutions and experimental samples and then the microplate was incubated for 10 minutes at room temperature. In pursuit of incubation the absorbance values in each well were measured at 540 nm in an ELISA reader.

2.6. Measurements of In Vitro Cytokine Production

In this experiment, in vitro immunostimulatory effects of synthetized nanoparticles were evaluated in J774 macrophage culture and their efficacies were compared with used free antigens. Same procedure like evaluation of produced nitric oxide amounts was conducted on macrophae cell in order to trigger them to release cytokines such as IL-4, IL-10, IL-12 and IFN-gamma. The concentration of mentioned cytokines in the culture supernatants of macrophage cells that were exposed to free, antigens, free nanoparticles and also antigen loaded nanoparticles were independently determined by enzyme-linked immunosorbent assay (ELISA) kit by following the instructions from the manufacturer. Optical density (OD) for each cytokine kit was measured at 450 nm. The cytokine levels for distinct groups were reported as pg/ml according to the plotted standard curve.

2.7. Immunization groups of BALB/c Mice

In vivo studies were carried out in Experimental Animal Research Center of Bezmialem University. Thirty female six weeks-old BALB/c mice were used in the experiments. Mice were fed with tap water and laboratory pellet chow in 12 light /12 dark cycle condition. All procedures associated with the animals were approved by the Institutional Animal Care and Use Committee of the Bezmialem University. The animals were separated into 6 groups including 5 mice per each. Afterwards, animals were intraperitoneally immunized five times at two weeks intervals with the following vaccine formulations; LPG, SLA-LPG, ALA-LPG, (LPG) PLGA NPs, (SLA-LPG) PLGA NPs, (ALA-LPG) PLGA NPs, free PLGA NPs and PBS. In each immunization, 200 µl formulations prepared in PBS were administerred to animals. Two weeks after the booster dose inoculation blood samples were obtained from

every mice in order to measure serum antibody levels of animals immunized with vaccine formulations.

2.8. Infection and Disease Development by Giemsa method and Micro Culture Method (MCM) in Peripheral Blood

Two weeks after the booster dose administration animals in each group were challenged with infective L.infantum promastigotes in order to visualize and compare vaccine efficacies of all formulations. For this purpose, 1 x 10^6 metacyclic *L. infantum* promastigotes were suspended in 200 µl PBS and subcutaneously injected to mice in separate groups. The pysical observation were performed in every week within a month to observe development of the infection and also the peripheral blood were obtained from submandibular region of mice for parasite detection with Giemsa and Micro Culture Method (MCM) that was described by Allahverdiyev et al [21].

2.9. Parasite Burden in Liver and Spleen

Following to observation of large amounts of parasites in peripheral blood particularly in control group, sacrification process were held to investigate the parasite existence in visceral organs of mice in each group. Therefore, all mice were sacrified at the 30th day of infection challenge and visceral organs of each animal such as liver and spleen were removed. Both liver and spleen cell smears were prepared by the method prescribed by Bradley and Kirkley [22]. Briefly smears were fixed in methanol (100%) for 5 minutes, then stained with 10% Giemsa stain prepared in distilled water and finally the number of amastigotes per 200 host cell nuclei were counted under light microscope at 100 X dimension. Leishman Donovan units (LDU) were calculated using the formula below:

$$\mathbf{LDU} = \frac{\text{number of amastigotes}}{\text{number of tissue nuclei}}\ x\ \text{Weight of organ (mg)} \quad (3)$$

Also the percentage of the reduction in liver and spleen parasite burden was calculated as

follows:

$$\textbf{Percentage of Reduction} = \mathbf{100} - \left(\frac{\text{LDU values of immunized groups}}{\text{LDU values of control groups}}\ x\ 100\right) \qquad (4)$$

2.10. In Vivo Cytokine Assay

This experiment was performed in order to investigate the alterations in cytokine levels within spleens of mice immunized with different vaccine formulations. For that purpose, splenocytes were isolated from murine spleens by using cell strainers and plungers. Isolated splenocytes were counted with trypan blue staining and 1 x 10^6 cells per ml were cultivated within 24 well plates including phenol red free RPMI-1640 medium for 24 h at 37°C. After the incubation, cells were stimulated with 30 µg/mL of the LPG, SLA-LPG and ALA-LPG antigen cocktails and splenocytes were incubated for 72 h. Subsequently, culture supernatants were collected in order to measure produced cytokine levels in stimulated splenocytes. The evaluation of amounts of cytokines such as IFN-γ, IL-12, IL-4 and IL-10 were carried out in triplicate by using sandwich ELISA kits according to the manufacturer's instructions.

2.11. Statistical Analysis

The data were analyzed for statistical significance using analysis of variance (ANOVA), and the difference among means was compared by the high-range statistical domain using Tukey's test. A level of probability was accepted significantly when the P value was below 0.05. Additionally, results associated with nitric oxide levels are also presented as mean±SD of the triplicate measurement.

3. Results

3.1. PLGA NPs characterization

The reaction yield, encapsulation efficiency and loading yield results of synthesized nanoparticles are presented in the table below.

After the nanoparticles were obtained by double emulsion method, each nanoformulation was dried and weighed. In addition, phenol-sulfuric acid method was applied to calculate the

loading efficiency of the LPG molecule in nanofaromulations [(LPG) PLGA NPs, (SLA-LPG) PLGA NPs, (ALA-LPG) PLGA NPs] from the supernatants formed during the washing and purification of nanoformulations. However, the results could not be obtained in these analyzes. Therefore, the amount of LPG in the supernatants was calculated via GPC. SLA and ALA antigens were determined by bicinchoninic acid assay (BCA). Obtained encapsulation efficiency and loading yield were given in Table 1.

Table 1: The physicochemical properties of synthesized nanoparticles.

NP	Reaction Yield (%)	Encapsulation Efficacy (%)	Loading Yield (%)	Particle Size (nm)	Zeta Potential (mV)	PDI
Empty NP	44.61	-	-	154.1 ± 2.19	-27.6 ± 0.89	0.182 ± 0.035
(LPG) PLGA NPs	37.33	8.4	LPG: 13	159.3 ± 2.07	-34.2 ± 0.99	0.142 ± 0.01
(SLA-LPG) PLGA NPs	18.67	12.8 27.6	LPG: 5.6 SLA: 12	306.9 ± 26.25	-28.0 ± 1.36	0.403 ± 0.079
(ALA-LPG) PLGA NPs]	43.13	14.0 32.4	LPG: 12 ALA: 28	152.8 ± 2.65	-30.9 ± 1.67	0.187 ± 0.024

According to the DLS results (Figure 1 A and B) the obtained nanoparticles had a mean hydrodynamic diameter as 306.9 ± 26.25 nm and 152.8 ± 2.65 nm. Zeta potential is one of the important parameters affecting the stability of potential nanoparticles. The nanoparticles produced were negatively charged and their zetapotential values ranged from -27.6 ± 0.89 mV to -34.2 ± 0.99 mV. This expected and measured negative zetapotential value is derived from the carboxyl groups on the surface of the nanoparticles and polyvinylalcohol (PVA) used as the stabilizer. At the same time, the high negative zeta potential value increases the dispersion of the nanoparticle in water or physiological environment.

The morphological investigation of (SLA-LPG) PLGA NPs, (ALA-LPG) PLGA NPs was carried out using SEM, it was observed that nanoparticles were in a spherical and smooth morphology, and size distributions were generally in a narrow range (Figure 1 C and D).

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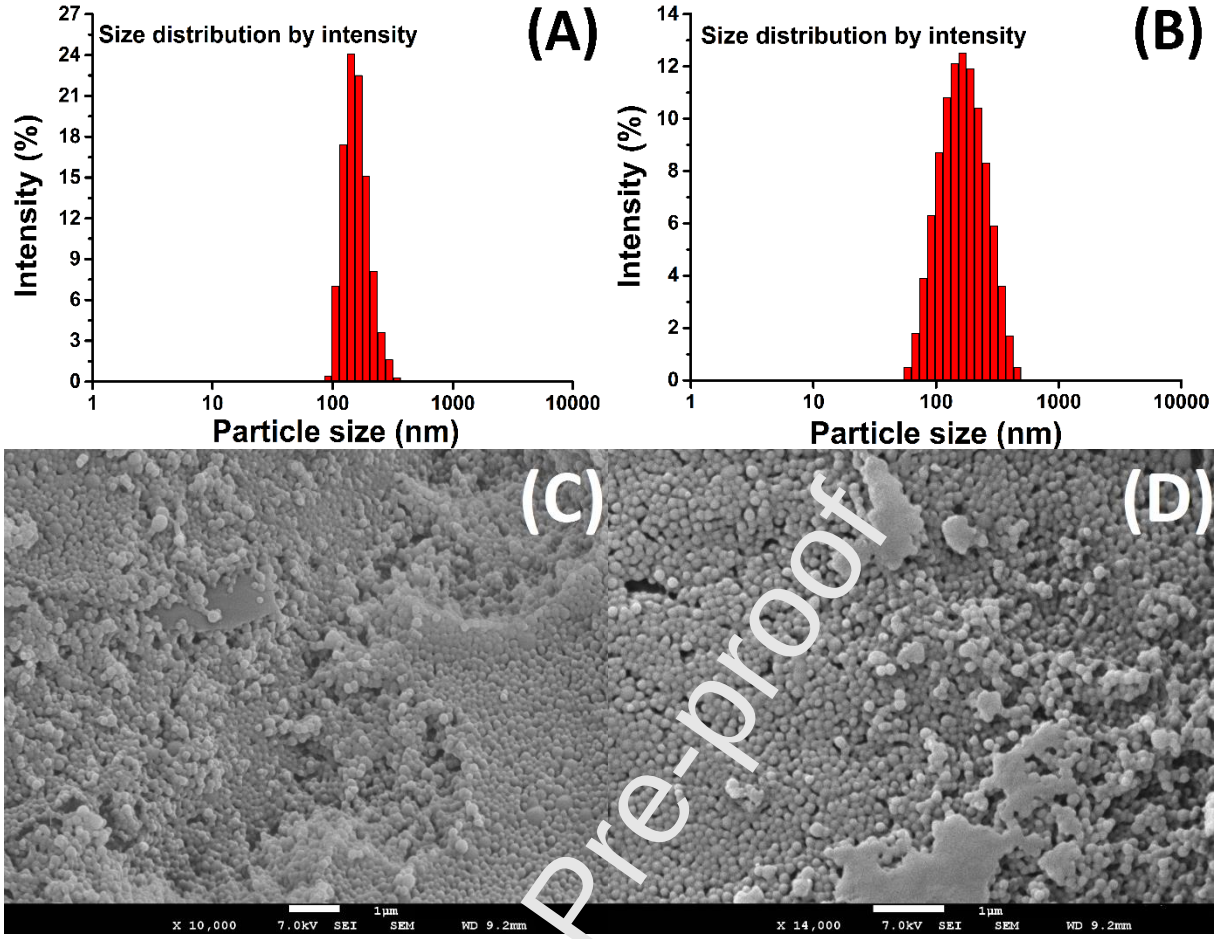


Figure 1. Particle size distributions of (SLA-LPG) (A) and (ALA-LPG) (B), SEM micrographs of (SLA-LPG) (C) and (ALA-LPG) (D) loaded PLGA nanoparticles.

In vitro release

The nanoformulations [(LPG) PLGA NPs, (SLA-LPG) PLGA NPs, (ALA-LPG) PLGA NPs] were incubated horizontally with shaker (150 rpm) at 37 °C in a physiological ambient (pH = 7.4). At certain selected times, the amount of antigen from the release medium was analyzed. As a result of each analysis the cumulative release plotted (Figure 2). As can be seen from the figure, it was determined that nanoformulations encapsulated with various antigens were released in vitro and the antigens were released in approximately 48.0%, 64.1% and 69.9% in the first 14 days.

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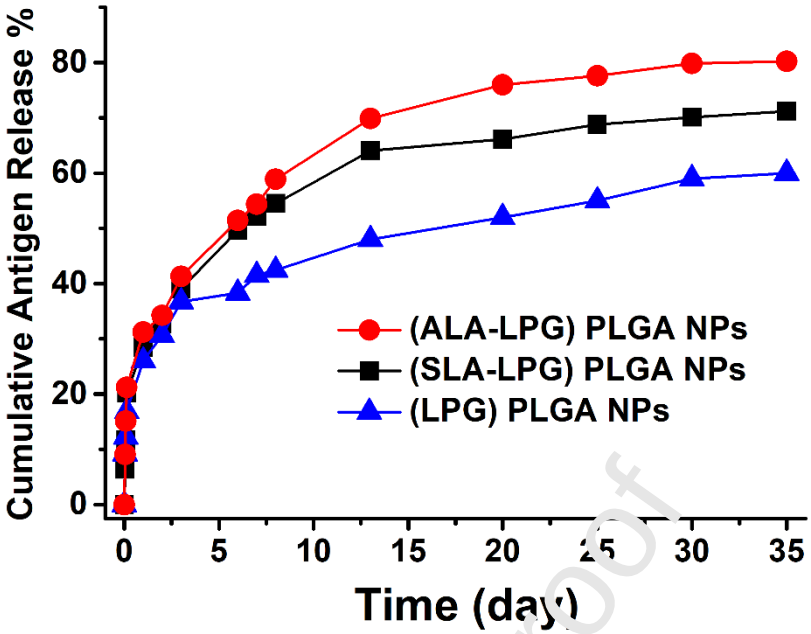


Figure 2. Controlled release pattern of (LPG) PLGA NPs, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs

3.2. Cytotoxicity Evaluation

The toxic effects of synthesized formulations on J774 murine macrophage cells were examined by MTT method. Different concentrations of PLGA NPs, (LPG) PLGA NPs, (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs were applied on macrophage cell culture and cellular viability rates of macrophages were evaluated following to exposure to synthetized nanoparticles. As it is seen from the Figure 3, antigen free and antigen-loaded PLGA nanoparticles did not lead to any serious toxic effects on macrophage cells. It was evaluated that IC_{50} values for each nanoparticles applied were more than 1000 µg/ml ($p<0.05$). There were slight alterations in cell viability rates of macrophages when they were exposed to nanoparticles at concentrations changing between 10 and 500 µg/ml and only 20% decrease was detected in viability amounts at these concentrations. However, cell viability ratios diminished to less than 70% when macrophages were exposed to antigen loaded PLGA nanoparticles at concentration of 1000 µg/ml. Therefore, nanoparticle concentration at 500

µg/ml was determined as non-toxic for each formulation and this dosage was used in following experiments.

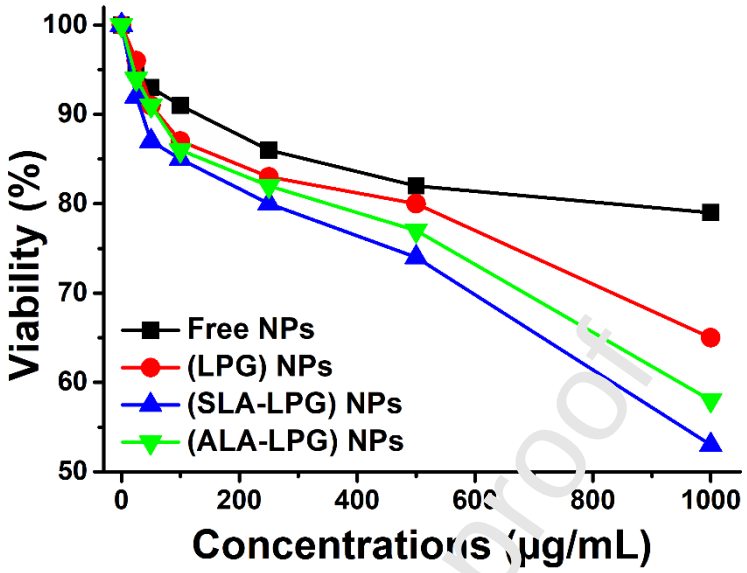


Figure 3. The effect of different concentrations of different nanoformulations on the viability of J774 cells

3.3. Measurements of macrophage nitric oxide production

In this experiment, we evaluated the secreted nitric oxide amounts from macrophages that was triggered by free and antigen loaded PLGA nanoparticles. No vaccine formulation was applied in control group. It was ascertained that the amounts of nitric oxide released by macrophages in negative control group was 4 ng/ml and positive control group was 43 ng/ml. On the other side, the amounts of nitric oxide secreted by macrophages were measured as 6, 23, 56 and 71 ng/ml for free PLGA NPs, (LPG) PLGA NPs, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs, respectively (Table 2). As it is seen, exposure to (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs resulted in 14- and 18-folds increase in secreted nitric oxide quantities from macrophages, respectively in contrast to control group ($p<0.01$). Strikingly, produced nitric oxide amounts from macrophages were relatively after exposure to (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs in contrast to use of LPS as positive control.

Thses data show us the superior immunomodulatory performances of nanoparticular vaccine delivery system including leishmanial antigen combinations. Finally, no significant increase was detected in nitric oxide levels after macrophages were treated with free NPs as compared to control.

Table 2: The amounts of secreted NO from macrophages treated with different vaccine formulations

Groups	**NO amounts (pg/mL)**
PBS (Negative Control)	4±0.21
LPS (Positive Control)	43±1.57
Free NPs	6±0.48
SLA-LPG	44±2.89
ALA-LPG	50±3.25
(LPG) PLGA NPs	23±1.47
(SLA-LPG) PLGA NPs	56±2.95
(ALA-LPG) PLGA NPs	71±3.76

3.4. In vitro Cytokine Production Assay

The amounts of IL-4, IL-10, IL-12 and IFN-γ cytokines that were produced by macrophages treated with free antigens, free nanoparticles and antigen-loaded nanoparticles were assessed by using ELISA kits. In Figure 4A and Figure 4B the produced levels of IL-12 and IFN-γ cytokines were demonstrated. As it was clearly seen from the figures, there was significant enhancements in the levels of IL-12 and IFN-γ in the experimental groups that macrophages were exposed to antigen encapsulated PLGA nanoparticles as compared with the control group. On the other hand, there was no meaningful change in the levels of IL-4 and IL-10 cytokines when macrophages were exposed to free antigens or nanoparticular formulations in contrast to control. Figure 4A exhibits the amounts of IL-12 levels of macrophages treated with PBS, free antigens and antigen loaded nanoparticles. As it is obviously seen, the levels of IL-12 produced by macrophages exposed to both non-encapsulated and encapsulated SLA-LPG and ALA-LPG antigens were significantly higher than those of exposed to PBS in

control group. Furhermore, an advanced increase was observed at IL-12 levels when macrophages were treated with free or encapsulated antigens than empty PLGA nanoparticles. The highest IL-12 levels were detected in the groups exposed free SLA-LPG and ALA-LPG antigens. It was exhibited that SLA-LPG and ALA-LPG antigens elevated the produced IL-12 amounts about 8-folds in contrast to control ($p<0.01$). Moreover, exposure to (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs also lead to significant advancements in IL-12 levels compared to empty nanoparticles and the control. It was shown that antigen loaded PLGA nanoparticles triggered macrophages to produce 2 to 6-folds higher IL-12 cytokine in contrast to free nanoparticles and the control ($p<0.01$). It was also demonstrated that produced IL-12 cytokine levels in macrophages exposed to (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs were nearly same when compared with macrophages that were treated with LPS as positive control. On the other hand, when the levels of IFN-γ produced by macrophages were examined, similar results were obtained (Figure 4B). Thus, no meaningful change was recorded in released IFN-γ levels from macrophages that were exposed to empty PLGA nanoparticles and PBS. In contrast, SLA-LPG and ALA-LPG antigens stimulated the highest IFN-γ production among all applied formulations except positive control. Exposure to SLA-LPG and ALA-LPG antigen combinations gave rise to approximately 3-folds increment in produced IFN-γ amounts compared to control ($p<0.01$). On the other hand, it was determined that antigen loaded PLGA nanoparticles enhanced secreted IFN-γ levels by 2-times than free nanoparticles and PBS application ($p<0.05$). The results obtained from in vitro cytokine analysis exhibited that encapsulation of antigen cocktails into PLGA nanoparticles did not stimulate the production of IL-4 and IL-10 cytokfaines by macrophages (Figure 4C and Figure 4D) while substantially lifting IL-12 and IFN-γ amounts. On the contrary, free antigen formulations demonstrated superior immunostimulatory activities than (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs since they stimulated much more IL-12 and IFN-γ

cytokines.

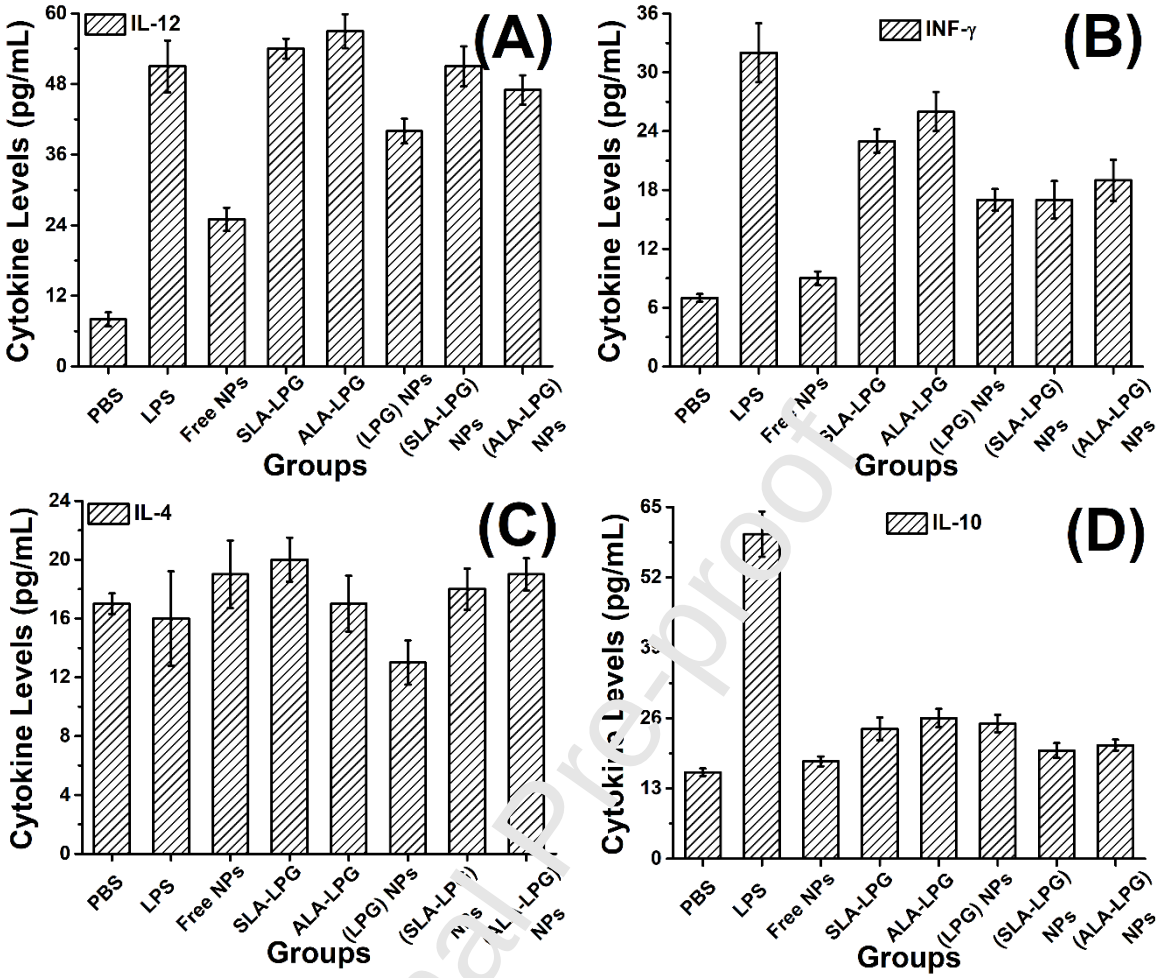


Figure 4. Secreted IL-12 (A), IFN-γ (B), IL-4 (C), and IL-10 (D) levels from macrophages that were treated wih different formulations

3.5. Challenge results

Following to infection of immunized mice with metacyclic infective L. infantum promastigotes, disease progression was macroscopically examined in all groups, particularly at the control group. Subsequent to one month challenge with parasitic infection, we observed development of clinical symptoms associated with leishmaniasis such as hepatosplenomegaly, weight lose and wound occurrence in the head and neck of the mice within the control group. In order to confirm the progression of the disease, blood samples were obtained from all

experimental and control groups for the detection of the parasite existence in peripheral blood by MCM method. The density of parasites in peripheral blood obtained from mice vaccinated with antigen combinations and antigen containing nanoparticles were investigated in response to control group (data not shown). It was discovered that parasite density was at the highest level in the control group immunized with PBS alone. On the contrary, during the microscopic investigation, the number of parasites at the investigated zone decelerated in blood samples of mice immunized with antigen including vaccine formulations. Expectedly, parasite density in the blood was at the lowest degrees in groups vaccinated with (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs. Furthermore, it was determined that (ALA-LPG) PLGA NPs was the most successful vaccine formulation for the clearence of parasites from the peripheral blood due to the lowest parasite density following to infection challenge. Finally, it was also noteworthy that antigen encapsulated PLGA nanoparticles eliminated much more parasites in the peripheral blood than free antigen combinations indicating antigen delivery systems were more advantageous to stimulate immune system for parasite clearence.

3.5.1. Assessment of antibody response

Besides investigation of parasite density in blood of immunized mice, IgG antibody levels were also evaluated within obtained serum samples. It was recorded that there was a slight increase in the average antibody levels of mice in the groups vaccinated with either (ALA-LPG) PLGA NPs or (SLA-LPG) PLGA NPs in contrast to groups that were exposed to free antigen cocktails (Figure 5). Unexpectedly, antibody response against free ALA-LPG antigens was comparatively higher than those against (SLA-LPG) PLGA NPs. Among applied formulations, (ALA-LPG) PLGA NPs was the vaccine candidate that gave rise to the highest IgG response, followed by free ALA-LPG antigen combinations ($p<0.01$). These results indicate that free antigens are successful in stimulation of humoral immunity as much as antigen encapsulated PLGA nanoparticles are. Furthermore, both (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs lead to development of higher antibody titers in vaccinated mice when compared with (LPG) PLGA NPs. This result ascertained that nanoparticles

including antigen cocktails were more successful in elevating antibody levels in contrast to nanoparticles delivering single antigen.

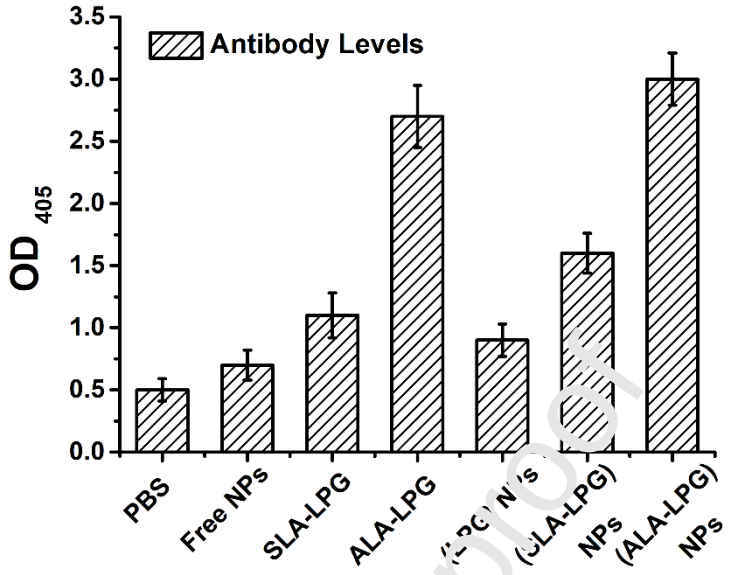


Figure 5. The antibody levels of mice treated with different vaccine formulations

3.5.2. Measurement of parasite burden in spleen and liver

The parasite burden in murine visceral organs such as spleen and liver were calculated by using LDU measurements following to sacrification of the animals in both experimental and control groups (Figure 6). Figure 6A indicates the average LDU values in livers of mice that were immunized with vaccine formulations. Unsurprisingly the highest liver LDU values were evaluated in the control group not immunized with antigen containing vaccine formulations. On the other side, application of non-encapsulated ALA-LPG and SLA-LPG antigens decreased LDU levels about 1.2 to 1.5-folds in contrast to control, respectively following to infection challenge. Figure 6 also demonstres that nanoparticular delivery platforms carrying LPG, LPG+SLA and LPG+ALA were more successful in parasite clearence from liver in contrast to free antigen applications. Besides, vaccination with (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs lead to the lowest parasite accumulation in livers of mice after infection process. It was determined that in the group immunized with

(SLA-LPG) PLGA NPs, mean LDU values in livers of mice diminished 4,5-folds compared to control ($p<0.01$). Similarly, vaccination with (ALA-LPG) PLGA NPs lead to 5-folds reduction in liver parasite burden in contrast to administration of PBS alone ($p<0.01$). Due to these data, it can be concluded that (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs provided approximately 80% protection against VL.

Similar results were obtained when spleen LDU values were calculated in the samples of immunized animals after infection challenge (Figure 6B). The highest reduction in parasite burden was measured in groups that were exposed to (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs formulations prior to parasite administration. It was recorded that vaccination with antigen encapsulated nanoparticles decreased the spleen LDU levels nearly 2-folds in contrast to control group ($p<0.05$). These results reveal that encapsulation of antigen combinations into nanoparticular systems increase their protection degrees when compared with freely administration of same antigens.

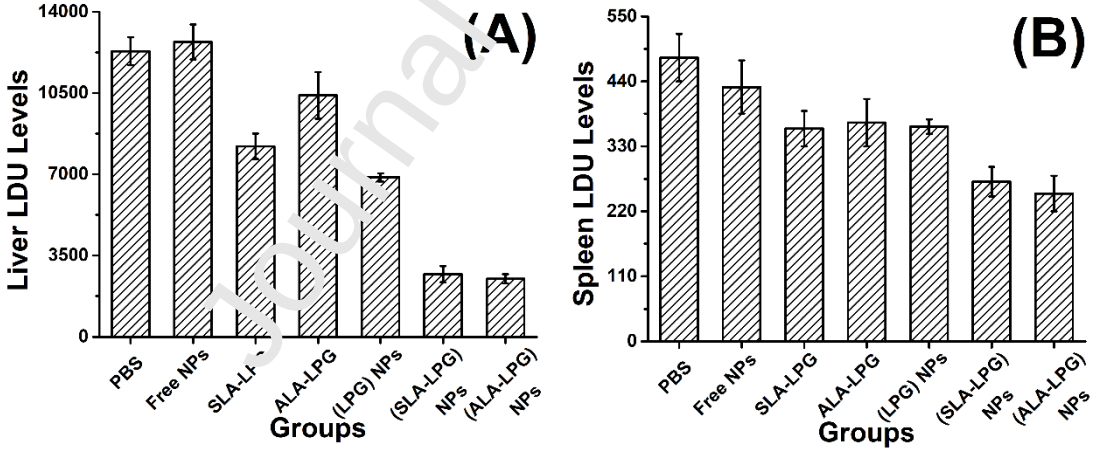


Figure 6. Liver (A) and spleen (B) LDU values of infected mice immunized in different vaccine formulations

3.5.3. Assessment of cytokine production by splenocytes

Subsequent to removal of spleens of immunized and non-immunized mice, isolated splenocytes were induced with antigens of SLA, ALA and LPG in order to measure released

IL-4, IL-10, IL-12 and IFN-γ levels for each group. According to the results, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs lead to spectacular increases at the levels of IL-12 and IFN-γ released by splenocytes (Figure 7A and B). On the contrary, no significant alteration was monitored in the amounts of IL-4 and IL-10 levels between vaccine administered groups and PBS administered group indicating that nanoparticle-based vaccine formulations did not stimulate splenocytes to produce IL-4 and IL-10 (Figure 7C and D). Figure 7A exhibits the produced IL-12 amounts from splenocytes isolated from immunized and non-immunized mice after sacrification. Accordingly, (SLA-LPG) PLGA NPs enhanced the produced IL-12 levels approximately 10-folds, while (ALA-LPG) PLGA NPs elevated same levels 14-folds in contrast to control ($p<0.01$). Furthermore, there was also a meaningful difference in IL-12 levels of splenocytes isolated from mice immunized with free antigens and antigen encapsulated nanoparticles. Thus, produced IL-12 amounts were 4-times higher in splenocytes of mice immunized with (SLA-LPG) PLGA NPs than those immunized with free SLA-LPG antigen combinations. Similarly, administration of (ALA-LPG) PLGA NPs raised produced IL-12 amounts by 5.5-folds in contrast to vaccination with ALA-LPG antigen cocktails.

Similar results were obtained when released IFN-γ levels from splenocytes of immunized mice were evaluated (Figure 7B). The amounts of IFN-γ secreted from splenocytes of mice vaccinated with SLA-LPG antigens were 1,8-times higher than those in control group ($p<0.05$). It was also determined that ALA-LPG antigen combinations lead to production of approximately 2-folds more IFN-γ when compared to control ($p<0.05$). As expected, (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs were much more effective in regards to stimulate IFN-γ production in contrast to usage of free antigens and PBS. Hence, it was demonstrated that administration of (SLA-LPG) PLGA NPs cause 2 to 4-folds increase in IFN-γ levels in contrast to application of free antigens and control, respectively ($p<0.05$). The

highest IFN-γ levels were measured in the group including mice vaccinated with (ALA-LPG) PLGA NPs. It was detected that (ALA-LPG) PLGA NPs enhanced the released IFN-γ levels by 8-folds in contrast to control ($p<0.01$).

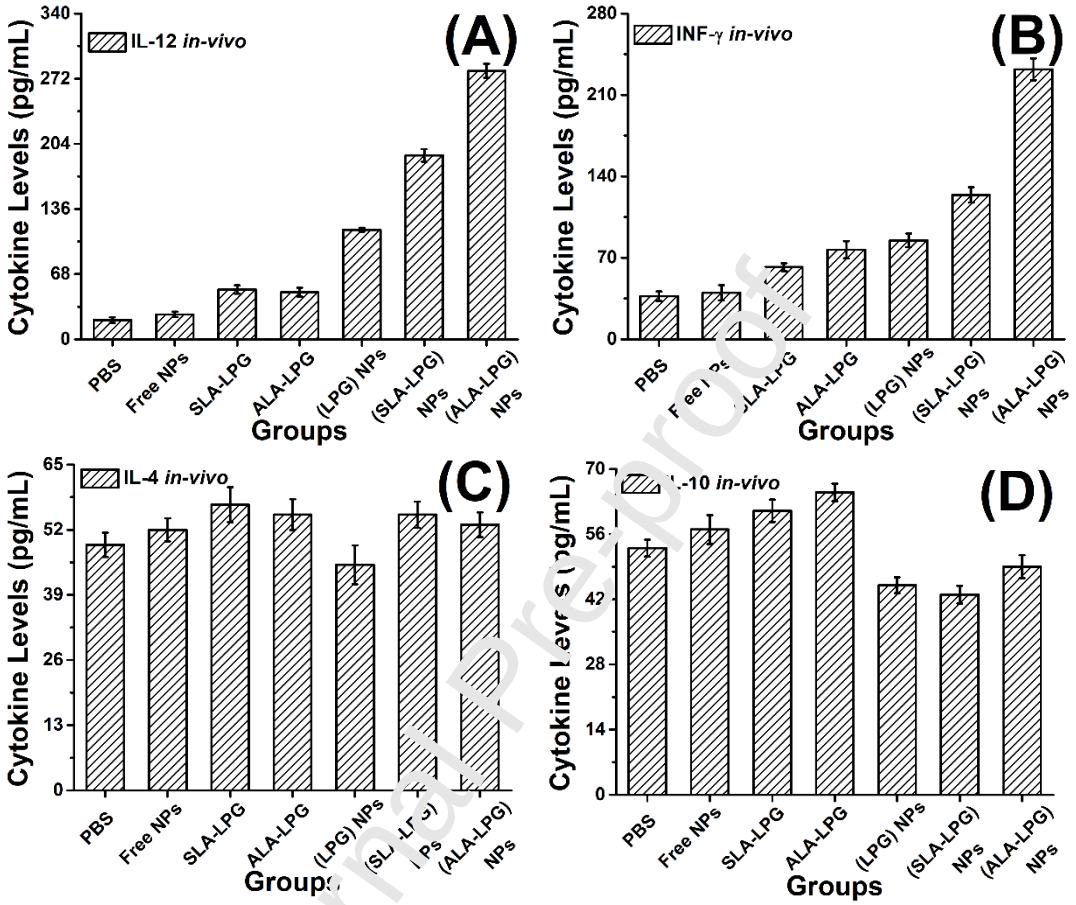


Figure 7. IL-12, IFN-γ, IL-10 and IL-4 cytokine responses of mice immunized with different vaccine formulations

4. Discussion

As the first time in this study, lipophosphoglycan molecules isolated from L.infantum promastigotes were co-encapsulated into PLGA nanoparticles together with either autoclaved Leishmania antigens (ALA) or soluble leishmania antigens (SLA) in order to evaluate immunostimulatory activities of synthetized formulations and investigate their protection facilities against visceral leishmaniasis, as well. It was revealed that both (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs excessively increased the nitric oxide amounts secreted from macrophages and

strongly stimulated them to produce IFN-gamma and IL-12 cytokines according to in vitro analysis results. Supportively each nanoparticular formulation exhibited excellent in vivo immunostimulatory efficacies by triggering cytokine production in splenocytes and providing largely clearence of parasites from visceral organs following to infection challenge. Furthermore, (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs elicited strong protection against murine visceral leishmaniasis model compared to administration of free antigens.

Nanovaccines are the promising new-generation tools to promote cellular and humoral immunity against several diseases at nano scales. Nanovaccines may be either delivery vehicles that implement passive or active cargo of various antigens into antigen presenting cells or helper adjuvant components for augmentation of immune responses against antigens administered together [23]. It was reported that sizes of nanoparticles are very important factor in regards to determination of immune response type. Hence, it is accepted that nanoparticles with the size ranging between 100 and 500 nm are preferantially uptaken by dendritic cells via endocytosis, while larger particles with size ranges between 500 nm and 5 micrometers are promptly engulfed by macrophages via phagocytosis. Targeting of nanoparticles into dendritic cells which are responsible for antigen presentation to T cells is believed to result in with strongly activation of cellular immunity. On the other hand, following to uptaken of nanoparticles by macrophages, humoral immunity is triggered to develop antibodies against the naoparticles and antigens that they carry [24, 25]. In our study, we used antigen loaded PLGA nanoparticles with sizes ranging between 253 and 308 nm. According to the results of measerument of antibody levels following to vaccination, it was determined that (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs did not lead to meaningful increase at antibody levels when compared with administration of free antigen combinations. We think that moderate elevation in humoral immunity might be associated with the sizes of nanoparticle based delivery systems. In a similar study, Gutierro et al prepared vacine formulations by encapsulating bovine serum albumin into PLGA nanoparticles at different size such as 200, 500 and 1000 nm. Researchers demonstrated that antigen loaded nanoparticles with the size of 1000 nm excessively stimulated humoral immunity by providing production of high levels of antibody. In contrast, IgG responses to nanoparticles with the size of 200 and 500 nm were relatively lower than larger nanoparticles [26]. Similarly, in another

study performed by Kanchan et al, hepatitis B surface antigens (HBsAg) were entrapped into PLA particles with different sizes ranging between 200 and 2000 nm. The authors detemined that microparticles with the size of 2000 nm strongly triggered antibody production against HBsAg, while nanoparticles at 200 nm did not lead to remarkable increase at the antibody levels as much as larger particles did [27]. In our present study, we also exhibited that administration of antigens within polymeric delivery systems significantly elevated the levels of cytokines associated with T cell response. Therefore, we assumed that (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs deliberately targeted antigen presenting cells and then lead to exacerbation of cellular immunity thanks to their small dimensions which contributed to their direct contact with dendritic cells as mentioned in previous studies.

There are several advantages of using various types of nanoparticles in immunotherapy and vaccination strategies that makes them preferable to applications of free sub-unit antigens. At first, nanovaccines can prevent enzymatic degradation of antigens in biological systems while raising their stabilities [28]. Moreover, both hydrophobic and hydrophilic molecules may be inserted in nanoparticular platforms as core-shell structures and this facilitates to resembling of different antigens by same antigen presenting cell for the augmented immunity as a response [29, 30]. Additionally, encapsulation of antigens into nano delivery systems may enhance their active or passive targeting into antigen presenting cells and by this way more efficiently processing of antigens might be achieved. Finally but perhaps more importantly nanovaccines enable cross-presentation of antigens into dendritic cells and this activates cytotoxic T cell responses by the help of CD8+ lymphocytes to extinguish infected cells with pathogenic microorganisms [31].

Among nanoparticular delivery vehicles, PLGA nanoparticles are the most favored tools thanks to their specific properties such as biodegradability, bioavailability, biocompatibility, and FDA approval. It was also reported in several studies that PLGA nanoparticles possess adjuvanticity which increase immunogenic features of antigens that are carried inside [32]. In our previous study, we encapsulated SLA and ALA antigens into PLGA nanoparticles and evaluate their in vitro/in vivo immunostimulatory performances [19]. In mentioned study, we determined that both (SLA) PLGA NPs and (ALA) PLGA NPs exhibited superior immunogenic features compared to free antigens and

we evaluated that (SLA) PLGA NPs as the most effective formulation achieved approximately 64 % protection rate against visceral leishmaniasis. In the current study, we hypothesized that co-encapsulation of whole leishmanial antigens and sub-unit molecules of parasites into PLGA nanoparticles could augment developed immune response and protection rates in contrast to single antigen containing nanoparticular systems. Lipophosphoglycan molecules are found at the surfaces of Leishmania parasites and promote the immunopathogenesis of this infection since they mediate the escape mechanisms of parasites from immune system cells via preventing the genaration of phagolysosomes [33]. Therefore LPG is accepted as one of the most powerful immunogenic molecules of Leishmania parasites and its potential in vaccine development against leishmaniasis was repeatedly proven [34]. On the other side, it was recorded that immunogenic features of LPG could be enhanced by using suitable adjuvants or combining with other antigenic molecules due to the limitations derived from carbohydrate characteristics of LPG instead of protein structure [35-38]. In the present study, combination of LPG with either ALA or SLA was encapsulated into PLGA nanoparticles expecting that dual antigen delivery would synergistically increase the immunogenic properties of whole and sub-unit antigens and also sustained release from PLGA nanoparticles would elicit long-term protection against VL.

Antigen loaded nanoparticles were synthetized with double emulsion solvent evaporation technique. Primarily physicochemical characteristics of nanoparticles were examined. According to dynamic light scattering analysis, antigen encapsulated PLGA nanoparticles were in size ranges between 254 to 307 nm, while average dimensions of empty nanoparticles were measured as 154 nm. The dimensional differences between free and antigen loaded PLGA nanoparticles may be accepted as the indicator of successfully encapsulation of whole and sub-unit leishmanial antigens into nanoparticular systems. These results are consistent with the results of previous studies on that subject [39]. Moreover, assessed PDI values of synthetized nanoparticles demonstrate that homogenously distribution of nanoparticles was achieved. Furthermore, measurements of negative zeta potentials for free and antigen containing nanoparticles were associated with general characteristics of the PLGA polymer and these results are compatible with the results of previous studies related to PLGA-based delivery platforms [40].

In subsequent to synthesis and characterization of nanoparticle based vaccine candidates, we examined their in vitro immunostimulatory efficacies on J774 macrophage cells by calculating the released NO and cytokine levels. It was determined that (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs increased the produced NO amounts by macrophages for 14 and 18-folds, respectively. Nitric oxide is one of the most important mediators of the innate immunity against intracellular pathogens. NO is a native microbiocidal molecule, which is produced by abundantly expression of iNOS genes following to infection of host cells with microbial agents such as bacteria, virus or protozoa. Its major role in innate immunity is controlling the replication of intracellular microorganisms and killing them within host cells [41]. On contrary, pathogenic microorganisms especially Leishmania parasites have developed several escape mechanisms from remarkable cytotoxic activities of nitric oxide. It was reported that Leishmania parasites can eliminate JAK/STAT pathway in the host cells and inhibit the expression of iNOS genes within macrophages to inhibit NO production [42-45]. By the way, they can easily invade host phagocytic cells and maintain thier proliferation. Therefore, external intervention involving parasite specific antigen administration as vaccine candidate is very substantial in order to stimulate macrophages to generate high amounts of NO. In the previous studies, it was demonstrated that iNOS genes were highly expressed to produce nitric oxide when naive macrophages were treated with purified LPG antigen and IFN-gamma cytokine as a result of synergistic activation [46, 47]. Similarly in our study, dual encapsulation of LPG and ALA/SLA antigens into polymeric nanoparticles with adjuvant feature exhibited significant increase in produced NO amounts from macrophages. We think that these results are the indicator of immunotherapeutic effectiveness of (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs since stimulation of NO-mediated killing of parasites is one of the most effective action mechanisms of applied therapeutic vaccine formulations.

Detection of the parasite reductions in visceral organs such as spleen and liver following to vaccine administration is one of the basic indicators for protective features of vaccine candidates against VL [48]. In the present study, we calculated that spleen and liver LDU values diminished approximately 2 and 5 folds in the groups that were vaccinated with (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs in contrast to control following to infection challenge. The reduction in liver

parasite burden of immunized mice with (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs corresponds to 80% protection ratios against murine VL. In our previous study, we separately loaded ALA and SLA into PLGA nanoparticles and determined that these formulations gave rise to 52% and 64% protection from VL, respectively [19]. As it is obviously seen, vaccine formulations prepared by encapsulation of LPG-ALA and LPG-SLA combinations into PLGA nanoparticles in the present study achieved to higher protection rates when compared to formulations including single whole leishmanial antigen-encapsulated PLGA nanoparticles. These results elicited that dual antigen encapsulated nanoparticles were more successful in stimulation of immune reponse against leishmanial infection and eliminating parasites than use of single antigen containing polymeric delivery systems.

Measurement of cytokine levels released by immune system cells is another significant factor indicating immunostimulatory activities of used vaccine formulations against leishmaniasis. A paradigm which is associated with Th1/Th2 balance is very important for immunopathogenesis of parasitic infection. When the synthesis of cytokines related to Th1 immune response such as IFN-γ and IL-12 is highly upregulated, this reveals that immune system is defeating the infection and strong parasite clearence is performed by T lymphocytes. On the other hand, enhancement at the levels of IL-4 and IL-10 from macrophages and B cells is the signature of the infection exacarbation. It is known that $CD4^+$ T cells are responsible for the control of Leishmania infection. These cells produce IFN-γ cytokines that will activate macrophages in order to produce high levels of NO and other reactive oxygen species for killing intracellular Leishmania parasites [49-52]. On the other sides, IL-12 cytokines that are released from dendritic cells are very important mediators of differentiation of CD 4^+ T cells to Th1 cells to produce IFN-γ for activation of macrophages. On the contrary, IL-4 and IL-10 cytokines are the mediators of Th2 immune reponse. They are responsible for deactivation of macrophages and stimulation of parasite growth in phagocytic blood cells [53-55]. Therefore, abundantly production of Th1 cytokines following to immunization is directly related to clearence of Leishmania infection [56]. We investigated the alterations in Th1 and Th2 cytokines both in in vitro and in vivo conditions. According to anlalysis results of in vtro cytokine profiles produced by macrophages, it was shown that free antigen cocktails induced higher amounts of IFN-γ and IL-12 cytokines than antigen encapsulated nanoparticles, while produced IL-4 and IL-10 levels remained

stable in each group including control. The slight increase in the produced IFN-γ and IL-12 levels due to exposure of macrophage to free antigens compared to nanoparticular formulations may be explained by sustainable release of antigens from PLGA nanoparticles. The immunostimulatory effects of released IFN-γ and IL-12 cytokines within 96 hours might not be as effective as direct contact of macrophages with free administered antigens.

In subsequent to infection challenge performed on immunized mice with synthetized vaccine formulations, spleens of animals were removed and produced cytokines within splenocytes were evaluated with ELISA test in order to analyze in vivo immunostimulatory activities of vaccine candidates. It was determined that (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs outstandingly increased the released amounts of IFN-γ and IL-12 cytokines, while there was no significant change at the levels of IL-4 and IL-10. The levels of produced IFN-γ were 10- and 20-times higher in the group vaccinated with (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs, respectively than control group. Besides, (SLA-LPG) PLGA NPs lifted produced IL-12 levels for about 10-folds, while (ALA-LPG) PLGA NPs lead to an increase by 14-folds in contrast to control. Moreover, it was also demonstrated that (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs were superior in stimulation of IFN-γ and IL-12 cytokines than free antigen combinations. Hence, 2- to 5.5-times lower IFN-γ and IL-12 cytokines were secreted from splenocytes in the group immunized with free antigen cocktails as compared to those vaccinated with encapsulated antigens. The differences in produced Th1 cytokine levels betwen the groups exposed to free and encapsulated antigens may be related to long-term and sustainable release of antigenic molecules from nanoparticular vehicles in biological systems. Nanoparticles might have actively targeted dendritic cells with higher yield, more successfulluly antigen presentation could have occurred and more significant cellular immunity have been achieved as a result.

Consequently, as a first time in this study, excellent immunostimulatory performances of (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs were shown resulting with considerably elevated Th1 cytokines and strongly elimination of parasites within visceral organs. Considering all obtained results, we suggests that both (SLA-LPG) PLGA NPs and (ALA-LPG) PLGA NPs are very promising nanoparticular delivery platforms and may be furher used successively in prevention of VL.

Acknowledgments

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Declaration of competing interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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HIGHLIGHTS

- Lack of targeting antigen presenting cells, degradation in biological system, low immunogenicity and severe side effects are the general limitations of the current vaccine candidates against leishmaniasis.
- PLGA nanoparticles are employed as antigen carrying systems in many vaccine studies providing safely delivery into antigen presenting cells, sustainable release and adjuvanicity to improve humoral and cellular immune response without leading to any toxic side effects.
- Encapsulation of LPG into PLGA nanoparticles with ALA and SLA demonstrated no cytotoxicity against macrophage cells while stimulating them to produce large amounts of nitric oxide and cytokines
- (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs excessively decreased the parasite burdens in murine visceral organs such as spleen and liver following to infection challenge.
- (ALA-LPG) PLGA NPs and (SLA-LPG) PLGA NPs elicited high levels of protection against leishmaniasis since they provide the killing of intracellular Leishmania parasites in the host cells and stimulation of Th1 cytokine secretions such as IL-12 and IFN-γ which are responsible for the resolution of leishmaniasis

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