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A new approach for development of vaccine against visceral leishmaniasis: Lipophosphoglycan and polyacrylic acid conjugates

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ABSTRACT

Objective: To determine the antileishmanial vaccine effectiveness of lipophosphoglycan (LPG) and polyacrylic acids (PAA) conjugates on *in vivo* mice models.
Methods: LPG molecule was isolated and purified from large-scale *Leishmania donovani* parasite culture. Protection efficacies of LPG alone, in combination with Freund's adjuvant, in a physical mixture and in conjugate (consisting of various LPG concentrations) with PAA, were comparatively determined by various techniques, such as cultivation with the micro-culture method, assessment of *in vitro* infection rates of peritoneal macrophages, determination of parasite load in liver with Leishman-Donovan Units, and detection of cytokine responses.
Results: Obtained results demonstrated that the highest vaccine-mediated immune protection was provided by LPG-PAA conjugate due to all parameters investigated. According to the Leishman-Donovan Units results, the sharpest decline in parasite load was seen with a ratio of 81.17% when 35 μg LPG containing conjugate was applied. This value was 44.93% for the control group immunized only with LPG. Moreover, decreases in parasite load were 53.37%, 55.2% and 65.8% for the groups immunized with 10 μg LPG containing LPG-PAA conjugate, a physical mixture of the LPG–PAA, and a mixture of LPG + Freund's adjuvant, respectively. Furthermore, cytokine results supported that Th1 mediated protection occurred when mice were immunized with LPG-PAA conjugate.
Conclusions: It has been demonstrated in this study that conjugate of LPG and PAA has an antileishmanial vaccine effect against visceral leishmaniasis. In this respect, the present study may lead to new vaccine approaches based on high immunogenic LPG molecule and adjuvant polymers in fighting against *Leishmania* infection.

1. Introduction

Leishmaniasis, which is caused by the *Leishmania* species, are intracellular parasites of mammals, and is one of the largest public health problems in 98 countries and territories around the world, including Turkey. It is known that nearly 350 million people are at risk for this infection. Every year, approximately 1–1.5 million cases of cutaneous leishmaniasis and 500 000 cases of visceral leishmaniasis occur worldwide [1,2]. Visceral leishmaniasis (VL), or kala-azar, is known as the most serious form of leishmaniasis and is the second most deadly parasitic infection, following malaria. Treatment with chemotherapy is prolonged, costly, and toxic and requires frequent monitoring and infrastructure that may be beyond the capacity of health systems where VL is endemic [3–7]. There is currently no vaccine available for any form of leishmaniasis [8]. Therefore, development of a vaccine against leishmaniasis has been

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insistently advocated by the World Health Organization. One of the basic reasons for this suggestion is the recognition of vaccination as the only prevention strategy because of the unquestionable role of the immune system in controlling *Leishmania* infection. Hence, various vaccine approaches have been pursued for many years [9].

To date, developed vaccines were investigated in three groups: first-, second- and third-generation vaccines. Vaccinations with live virulent parasites (termed leishmanization) or with killed parasites are considered first-generation vaccines; vaccinations with subunits, purified fractions, recombinant vaccines in heterologous microbial vectors, and genetically or otherwise attenuated live parasites are considered second-generation vaccines; and DNA-based vaccines are considered third-generation vaccines [10]. However, all of these vaccine trials had serious disadvantages despite their advantages. Among first-generation vaccines, the use of killed parasites is considered to be confident, but their efficacies were demonstrated to be low. The use of attenuated live *Leishmania* parasites (called leishmanization) was suspended by reason of the fact that parasites can achieve virulence again, even many years later [11,12]. In recent times, genetically modified *Leishmania* parasites have been used as live-attenuated vaccine candidates. In general, genetically modified live *Leishmania* parasites are obtained by the elimination of virulence genes such as dyhydrofolate reductase, biopterin reductase, cystein proteases or heat shock proteins and vaccination with these antigens achieved promising results in animal studies. However, safety concerns are still an obstacle that prevents their use in clinical trials [13–15]. Second-generation vaccines using antigen fragments of parasites provide efficient protection against leishmaniasis compared to other groups of vaccines. These vaccine candidates are obtained in two different ways: one is isolation and purification of native antigens from a parasite culture, the other one is producing antigens by using recombinant DNA technology [11]. The success of subunit vaccines based on recombinant proteins or peptides which are found in second-generation vaccines, has been demonstrated, but was also variable to poor [16]. Despite the fact that the immunogenicities of several recombinant antigens were investigated on animal models, only a few of them achieved progression to clinical trials that were performed on primates, dogs and humans (preclinical studies) [17,18]. In one of these efforts, recombinant A2 protein of *Leishmania chagasi*, which was used in combination with saponin adjuvant, provided partial (40%) protection against canine leishmaniasis and this protection rate was found to be sufficient to develop a licensed canine vaccine that is named Leish-tec® [19]. Clinical trials of other recombinant proteins such as Leish-111F/MPL-SE have been done on humans, however, there are currently no licensed human vaccines based on recombinant antigens [20,21].

Recently, second generation-vaccine development studies have also focused on the native surface glycoconjugates of parasites. One of the important surface antigens of *Leishmania*, which is called the Fucose-Mannose Ligand (FML) possess high immunogenic features. By considering the antigenicity of FML, researchers prepared a vaccine formulation including FML and a saponin adjuvant that was isolated from Quillaja saponaria. This vaccine candidate underwent Phase I-III clinical trials and has been licensed as Leishmune® [22]. In different endemic regions of the world, this vaccine is being used in humans with success indicating that antigenic molecules isolated from Leishmanial parasite surfaces have great potential to be formulated as vaccine candidates and provide strong protection.

Like FML, lipophosphoglycan (LPG) is another important surface glycoprotein of *Leishmania* parasites. LPG covers all surfaces of parasites including flagella and plays an important role in the survival of parasites, both in humans and in vector organisms. The basic LPG structure in all *Leishmania* species consists of a 1-O-alkyl-2-lyso-phosphatidyl inositol lipid anchor, a heptasaccharide glycan core, a long phosphoglycan (PG) polymer composed of (Galb1-4Mana1-PO4) n repeat units (n = 10–40), and a small oligosaccharide cap [23].

In one study, it was shown that intranasal vaccination with *Leishmania amazonensis* LPG was an important immunomodulatory molecule [24]. Other experiments have shown that LPG provided protection to *Leishmania major* (*L. major*) infections in BALB/c mice [25–27]. However, protection was demonstrated to be dependent upon the use of adjuvants such as liposomes or killed *Corynebacterium parvum* and the integrity of the molecule. Therefore, LPG may be a good vaccine candidate only when it is used with appropriate adjuvants.

As compared with whole-cell or virus-based vaccines, subunit vaccines are poorly immunogenic and require the presence of adjuvants to stimulate protective immunity [28,29]. However, the most effective adjuvants generally cause significant inflammation. This may be essential for adjuvanticity, but their use in humans may be precluded because of unacceptable side effects. For approximately the past two decades, vaccine research has been focused on the alternation of the alum type of an adjuvant in order to increase immunogenicity. Biodegradable polymers are being used as adjuvants and drug carriers, because of their biocompatible, nontoxic nature and their biodegradable properties. Polymers that are chosen as excipients (adjuvants) for parenterally administered vaccines should meet some requirements, including being biodegradable, safe, antigen compatible and permeable, stable *in vitro*, easy to process and, ideally, inexpensive [30].

Polyacrylic acids (PAA) that are strongly negatively charged compounds with high molecular weight demonstrate adjuvant effects for both humoral and cell-mediated immunity [31,32]. Previously, synthetic polymers of PAA and more hydrophobic derivatives containing alkyl-esters significantly enhanced the antibody response against numerous inactivated model protein antigens [33,34]. These fully synthetic constructs are potentially safe in that they have not induced adverse effects in animal models and display potentially low cytotoxicity [35]. There are only a limited number of vaccine studies in the extant literature demonstrating the adjuvant properties of PAA against infectious diseases. In one of these studies, PAA conjugate was reported as a sufficient adjuvant for protection against haemorrhagic nephritis enteritis, a disease caused by a polyomavirus, since it increased the antibody response significantly in gooses [36]. However, to date, its efficacy as an adjuvant has not been investigated against leishmaniasis.

Considering the high immunogenic properties of the LPG molecule, adjuvant features of PAA and the convenience in chemical structures of each molecule to compose a conjugation, we suggest that a formulation that includes LPG-PAA conjugates could be an important vaccine candidate against Visceral Leishmaniasis. However, to the best of our knowledge, there are no antileishmanial vaccine studies based on LPG-PAA conjugates in the literature. Therefore, for the first time, this study aimed to implement conjugation between PAA and the highly immunogenic LPG molecule found on the surface of *Leishmania* parasites, to investigate the effectiveness of LPG-PAA conjugates as a vaccine candidate on animal models and to reveal their role in the development of new vaccines against leishmaniasis.

2. Material and methods

2.1. Parasite culture

Leishmania donovani (*L. donovani*) (HOM/IN/83/AG83) promastigotes were cultured in RPMI 1640 medium with L-glutamine (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Sigma) and gentamicin (80 mg/mL) at 27 °C. In order to obtain large scale cultivation, *L. donovani* parasites grown in RPMI 1640 media were transferred into 75 cm^2 culture flasks with serum-free Brain Heart Infusion Medium, including Hemin (Sigma, H9039) and Adenosine (Sigma, A9251). After two weeks, parasites (25–30 million parasites/mL) were transferred into 250 mL of glass bottles on a shaker. The process yielded 3–5 L of *L. donovani* culture with a volume of 25–30 million parasites/mL. Continuous *L. donovani* culture was passaged once in a two week period.

2.2. Isolation and purification of LPG

In order to isolate the LPG molecule, chloroform-methanol extraction and sonication processes were performed on a stationary phase Leishmania pellet. Initially, a chloroform-methanol (1:2) mixture was added to a pellet containing tubes with a 3.75 fold volume. A sonication process was then maintained in order to suspend the pellet. Following 1 h incubation at room temperature, the tubes were centrifuged at 4 000 rpm at +4 °C for 30 min and the supernatants were transferred into volumetric flasks. Then the remaining pellet was re-suspended with 4 mL 9% 1-Propanol and the tubes were sonicated again. After a sonication process, homogenized solutions were centrifuged at 14 000 rpm for 20 min. All collected supernatants were then evaporated, and 9% 1-Propanol was removed. Later, 1 mL of the remaining isolate was frozen at −40 °C and then lyophilized overnight. The obtained sample was stored at −40 °C until the column chromatography process.

2.3. Column chromatography

For purification of the LPG molecule, an octyl sepharose column was used in the chromatography. First, a lyophilized sample was re-suspended with 2 mL of distilled water. Then 1-propanol as a 4 fold volume of LPG isolate, was added into tubes. After that, the sample was centrifuged at 18 000 rpm for 15 min and then the supernatant was re-suspended with 0.1 M ammonium acetate. Octyl sepharose was poured into the column and was packed with 100 mL of 5% 1-propanol/0.1 M ammonium acetate. Following that, the LPG sample was loaded into a column and then was incubated overnight at room temperature in order to increase the LPG binding capacity of octyl sepharose. After incubation, the column was washed with another 100 mL of 5% 1-propanol/0.1 M ammonium acetate mixture and the fractions were collected as 120 drops for each tube. Finally, the column was washed with a gradient starting from 5% 1-propanol/0.1 M ammonium acetate to 60% 1-propanol/0.1 M ammonium acetate.

2.4. Thin layer chromatography method

In order to detect carbohydrate existence in fractions, thin layer chromatography was performed. Paper pieces coated with silicate were numbered. On each fraction, 2 μL of the sample was deposited and the paper pieces were dried at room temperature. Afterward, Orcinol was sprayed on the silicate paper and allowed to dry. Following this, a 15% sulfuric acid solution was sprayed and allowed to dry at 100 °C. Purple spots were regarded as positive.

2.5. Phenol sulphuric acid method

This method was used to determine the amount of LPG. To obtain a standard curve, concentrations of 10, 20, 40, 60 and 80 μg/mL glucose solution were used. The amount of LPG purified by the octyl sepharose column was determined by reading absorbance values at 490 nm. According to the standard curve prepared depending on the glucose solution, the amount of sugar contained in LPG was calculated [37].

2.6. Conjugation of LPG and PAA

Hydroxyl groups of LPG were oxidized to aldehyde groups with 5 or 10 mM $NaIO_4$ in 0.02 M phosphate buffer for 6 h. After the reaction, the solution was dialyzed against water for 24 h at 4 °C. Carbonyl groups of oxidized LPG were reductively aminated by the reaction with ethylenediamine and $NaCNBH_3$ in 0.02 M phosphate buffer for 12 h. The reaction was followed by the dialysis against 0.02 M phosphate buffer for 24 h at 4 °C.

In the next step, LPG and PAA were conjugated by using water soluble EDC in the concentration ratio of $C_{PAA}/C_{LPG} = 0.5$. In this reaction, carboxylic acid groups of PAA (0.5 mg/mL) were activated with water soluble EDC [(EDC)/[−COOH) = 1] at pH 5.0 for 30 min. Under vigorous stirring at room temperature. Aminated LPG, dissolved in the same volume of PAA solution, was added to the activated PAA solution, and pH was adjusted to 7.0. The reaction solution was gently stirred overnight at 4 °C.

2.7. Gel permeation chromatography analysis

PAA and PAA-LPG conjugates were analyzed using gel permeation chromatography with a triple detection system. Triple detection consists of refractive index, right angle light scattering and viscosimetry detectors, which were calibrated with a PEO (22 kDa) standard solution. Gel permeation chromatography analysis was performed with a Shimadzu Shim-Pack Diol-300 [(50 × 0.79) cm] column at room temperature. PBS (pH 7.1) was used as a mobile phase, and the flow rate was 1.0 mL/min.

2.8. MALDI-TOF-MS analysis

Mass spectra of PAA, LPG and LPG-PAA conjugate were acquired on a Voyager-DE™ PRO MALDI-TOF mass spectrometer (Applied Biosystems, USA) equipped with a nitrogen UV-laser operating at 337 nm. Spectra were recorded in reflectron mode with an average of 50 shots. Dithranol was used as a MALDI matrix. Matrix and sample solutions were mixed to obtain a W_{sample}/W_{matrix} ratio of 1:10. One mL of the matrix/sample mixture was deposited on the sample plate, dried at room temperature and analyzed. Results of characterization analysis for LPG-PAA conjugates were demonstrated in our previous paper [38].

2.9. Vaccine administration

Various vaccine formulations were prepared in the present study: LPG-polymer conjugate, LPG-polymer physical mixture,

and LPG + Freund's complete adjuvant. The LPG alone was also used as a vaccine candidate for immunization. A vaccination dose of 10 μg and a higher dose (35 μg) were selected for animal experiments. The LPG-polymer physical mixture formulation was prepared by mixing 10 μg and 35 μg of the LPG antigen together with 20 μg and 70 μg of PAA, respectively. LPG-polymer conjugates were used at a concentration of 30 and 105 μg (containing 10 μg and 35 μg of LPG, respectively). Additionally, 10 μg and 35 μg of LPG were used together with 50 μL Freund incomplete adjuvant. For each group, five BalB/c mice were used and the animals were subcutaneously immunized with the mentioned formulations. Vaccine formulations were applied four times at 15 d intervals. After two months from first immunization, mice in each group were infected with stationary *L. donovani* promastigotes.

2.10. Infection of animals

The animals were intravenously infected with 1×10^7 stationary *L. donovani* promastigotes. The peripheral bloods were obtained in weeks 1, 2, 3 and 4 after infection by cutting the tail about 1 cm from the tip to permit a free flow of blood. Obtained blood samples were used for parasite detection in PB with Giemsa and Micro Culture Method (MCM).

2.11. Parasite detection in PB with Giemsa method and MCM

After infection of the BALB/c mice with *L. donovani* promastigotes, blood samples were taken for four weeks. Smears were prepared with 10 μL of these blood samples. After fixation at room temperature for 5 min, the slides were stained with a 1:10 diluted Giemsa solution (Merck, 109204) for 25 min. Washed and dried slides were observed under light microscope with 100× magnification using immersion oil.

The parasite existence in test and control groups was also detected by MCM that was described by Allahverdiyev *et al.* [39]. Briefly, anti-coagulated 100 μL blood samples were transferred to capillary tubes and centrifuged at 3 000 rpm for 5 min. After the centrifuge, the buffy coat was extracted and mixed with RPMI 1640, which included 15% FCS. The mixed solution was then transferred to micro capillary tubes, closed by sterile wax and incubated at 27 °C. After a 24-h incubation period, the micro capillary tubes were observed under inverted microscope with 40× magnification.

2.12. Sacrifice of animals

Five mice from each immunized group and control group were sacrificed at the 30th day after infection with *L. donovani* parasites. First, peritoneal exudate cells were extracted using an injector and were then cultured on clean 24-well plates in RPMI + 10% FCS. Peritoneal macrophages were used for *in vitro* infection of peritoneal macrophages. The liver and spleen of each animal were removed and weighed. The liver and spleen were used for calculation of the Leishman-Donovan Unit (LDU).

2.13. Infection of peritoneal macrophages with L. donovani promastigotes

Peritoneal macrophages were isolated from the BALB/c mice in all experimental groups. The peritoneal macrophages were grown in plastic 25 cm^2 culture flasks in RPMI 1640 medium (Sigma) containing *L*-glutamine, buffered with 10 mM HEPES, and were supplemented with 10% heat inactivated FCS and gentamicin (80 mg/mL) in a humidified incubator 5% CO_2 at 37 °C and were subpassaged once a week.

The promastigotes of *L. donovani* in the stationary phase were washed twice by centrifugation (3 000 rpm for 5 min) in PBS, at pH 7.2, and their concentration was adjusted to 10×10^6 parasites/mL. The infection of macrophage cells was based on a ratio of 10 parasites per macrophage, and this procedure was used in triplicate for the strain. After 24 h of macrophage-*Leishmania* interaction in culture, the coverslips were washed in PBS, fixed in absolute methyl alcohol (5 min), and stained using Giemsa's method to determine the infection index. The percentage of reduction of the infection index was evaluated as follows:

$$100-\left(\frac{\textit{Infection index of immunised mouse}}{\textit{Infection index of control mouse}}\times 100\right)$$

2.14. Assessment of parasite load

Mice from each immunized group and control group were sacrificed on the 30th day after infection, and the liver and spleen of each animal was removed and weighed. A cut section of liver and spleen was rinsed in RPMI 1640 (pH 7.2), and impression smears were made on clean slides by touching homogenously the fragments of mentioned organs. The slides that were prepared for livers and spleens were dried and then fixed with methanol for 5 min. Following this, the slides were stained with Giemsa (1:10) for 10 min at 37 °C, washed with water and viewed by light microscopy under oil immersion (100×). The number of amastigotes was determined by analyzing 200 cells from each slide. Parasite loads were monitored in LDU (LDU = number of amastigotes/number of liver cells nuclei × mg organ weight). The results were assessed by comparing the parasite burden of immunized animals with that of the control animals. The percentage of the reduction in liver parasite burden was evaluated as follows:

$$100-\left[\left(\frac{\text{LDU values of immunized groups}}{\text{LDU values of Control groups}}\right)\times 100\right]$$

2.15. Evaluation of cytokine production in spleen

Spleen cells isolated from the vaccinated and control mice were cultivated in RPMI 1640 medium for evaluation of cytokine responses. Spleen cells that were isolated from immunized mice were stimulated with 10 μg LPG for 72 h. Supernatants of cultured spleen cells belong to all experimental and control groups mice were used for cytokine analysis. Briefly, the captured antibody was diluted with PBS, and 100 μL from this dilution was added into a 96-well microplate. The plate was incubated at room temperature overnight. The following day, the wells were rinsed three times with a cleaning solution. Non-specific binding was prevented due to the addition of a blocking solution, and the plate was incubated for 1 h at room temperature. This was followed by another washing process, after which 100 μL of samples diluted in reaction solution and standards were added, and the microplate was again incubated

for 2 h at room temperature. Following the binding into antibodies, another washing process was performed, and detection antibody was added. After a 2-h incubation period, 100 μL 97 Streptavidin-HRP was added into all of the wells, and the plate was again incubated for 20 min at room temperature. Following another washing process, 100 μL substrate solution was added to all of the wells. After a 20-min incubation period, 50 μL of stopping solution were added, and the plate was read at a 450 nm wavelength.

2.16. Ethics statement

Inbred, five to six-week-old BALB/c mice were used for this study. The ethical clearance and approval of the animal protocol for conducting experiments on animals was granted from Yeditepe University in Turkey by the Yeditepe University Experimental Animal Ethics Committee.

2.17. Statistical analysis

The results were expressed as mean ± SD. Statistical Packages of Social Sciences (SPSS 16.0 version for Windows) software with parametric tests (paired samples *t*-test, analysis of variance, and Tukey's *post-hoc* test) was used for statistical analysis. A $P < 0.05$ value was considered statistically significant.

3. Results

3.1. Effect of different vaccine formulations on parasite load in blood

Parasite loads in BALB/c mice immunized with different vaccine formulations were detected by Giemsa and MCM. No parasite was detected by the Giemsa staining method in any of the immunized groups (Table 1). Only in the control group starting from the second week of the infection was the existence of parasites determined by the Giemsa method (Table 1). In comparison, MCM yielded positive results and detected the existence of parasites in all blood samples taken from each immunized group during all of the experimental periods, and was in contrast to the Giemsa results (Table 1). The parasite intensity in the control group was much greater than in each vaccinated group. Moreover, a very low amount of parasites was detected in the blood of animals vaccinated with LPG-PAA conjugates. It was also demonstrated that parasite numbers were again low in samples of other immunized groups, contrary to the control group, when they were analyzed with MCM.

3.2. Infection of peritoneal macrophages with L. donovani promastigotes

According to the infection index results, there was a massive reduction in infection rates of peritoneal macrophages isolated from immunized animals when compared to non-immunized animals. The maximum reduction in infection rate was seen in mice immunized with LPG-PAA conjugates (87.4% with 10 μg LPG; 89.3% with 35 μg LPG). This was respectively followed by the LPG-PAA physical mixture (65.1% with 10 μg LPG; 67.2% with 35 μg LPG), LPG + Freund's adjuvant group (46.2% for 10 μg LPG; 43.1% for 35 μg LPG) (Table 2). As seen, the LPG-PAA conjugation demonstrated meaningful efficacy in reducing the number of parasites in peritoneal macrophages in contrast to other immunized mice ($P < 0.01$) (Table 2). Furthermore, there was no significant difference between the LPG and LPG + Freund's adjuvant group in terms of infection indexes, indicating that protection against the infection is due to conjugation of LPG with PAA ($P < 0.01$).

Reduction of parasites was also clearly seen in microscopic examination following Giemsa staining of peritoneal macro-

Table 1

Evaluation of parasite loads in mice peripheral blood by Giemsa and MCM.

Group	Giemsa				MCM			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Control	0	2+	2+	3+	6+	6+	6+	6+
LPG	0	0	0	0	2+	2+	2+	3+
LPG + PAA (conjugate)	0	0	0	0	2+	2+	1+	1+
LPG + Freund's adjuvant	0	0	0	0	2+	2+	3+	3+
LPG + PAA (physical mixture)	0	0	0	0	2+	2+	2+	2+

Table 2

Infection index values decrease (%) of peritoneal macrophages immunized with different vaccine formulations including 10 and 35 μg LPG, and the reduction (%) in liver and spleen parasite burdens of the mice vaccinated with different formulations following infection with *L. donovani* promastigotes.

Group	LPG dose (μg)	Decrease in peritoneal macrophages infection index	Reduction in liver parasite burdens	Reduction in spleen parasite burdens
LPG	10	40.7 ± 1.2	33.5 ± 1.4	34.3 ± 0.8
	35	43.4 ± 0.9	44.9 ± 1.6	48.7 ± 1.1
LPG + Freund's adjuvant	10	46.2 ± 1.8	24.8 ± 1.0	41.3 ± 1.5
	35	43.1 ± 1.4	65.8 ± 1.5	53.3 ± 1.3
LPG + PAA (physical mixture)	10	65.1 ± 2.0	36.2 ± 1.9	38.8 ± 1.0
	35	67.2 ± 0.8	55.2 ± 1.1	51.3 ± 1.5
LPG + PAA (conjugate)	10	87.4 ± 2.3	53.4 ± 1.5	60.1 ± 2.3
	35	89.3 ± 1.9	81.2 ± 2.4	73.4 ± 1.7

phages (Figure 1). In Figure 1A, it was observed that macrophages isolated from immunized mice resisted infection with *L. donovani* promastigotes since the number of parasites in the vacuoles of macrophages was very low. In comparison, *L. donovani* promastigotes successfully infected macrophages isolated from non-immunized mice (Figure 1B). This result indicated that parasites could not survive within macrophages activated by LPG-PAA conjugates and this is a sign of strong protection.

3.3. Parasitic load

The reduction of parasitic load in the liver following each LPG vaccine formulation is shown in Table 2. Immunization with the LPG + PAA conjugate that contains 35 μg LPG antigen significantly ($P < 0.05$) reduced the parasite load at the rate of 81.2% in all immunized animals as compared to control animals. In comparison, the LPG + PAA conjugate that contained 10 μg LPG antigen reduced the parasite load at the rate of 53.4%.

Additionally, the effects of vaccine formulations including 35 μg LPG alone, its combination with Freund's adjuvant, and its physical mixture with PAA on the reduction of parasite load were also determined. Reduction rates for LPG + Freund's adjuvant vaccine, a physical mixture of LPG + PAA vaccine, and an LPG containing vaccine alone were 65.8%, 55.2% and 44.93%, respectively (Table 2). Once again, protection rates were low in groups immunized with different vaccine formulations, including 10 μg LPG.

In contrast, the reduction rates in spleen parasite burdens of immunized and non-immunized mice are given in Table 2. As seen, the most significant reduction in parasite load was in groups that were vaccinated with LPG-PAA conjugates. According to LDU analysis, vaccination with LPG-PAA conjugates including 35 μg LPG lead to a 73.4% reduction in parasite load for spleens in contrast to control group ($P < 0.01$). In comparison, the reduction in parasite loads of spleens were evaluated as 60.1% when mice were immunized with LPG-PAA conjugates including 10 μg LPG. In addition, for formulations including 35 μg LPG, the parasite reduction rates in spleen of mice immunized with the LPG + PAA physical mixture, LPG + Freund's adjuvant combinations and LPG alone were determined as 51.3%, 55.3% and 48.7%, respectively ($P < 0.05$). In contrast, the impact of formulations including 10 μg LPG on spleen parasite load was very low when compared with the LPG-PAA conjugate. For these formulations, the parasite reduction rates in spleen of mice immunized with LPG + PAA physical mixture, LPG + Freund's adjuvant combinations and LPG alone were determined as 38.8%, 41.3% and 34.3%, respectively ($P < 0.05$). These results indicate that conjugates of LPG with PAA reduce parasite loads in livers and spleens much more than other formulations and the anti-leishmanial vaccine effect of the conjugate improves with an increase in the concentration of LPG.

3.4. Evaluation of cytokine production in spleen

Table 3 showed the IFN-γ levels of mice immunized with different vaccine formulations. The highest IFN-γ levels were detected in mice immunized with the conjugate. There was a remarkable difference between the control group and the conjugate group.

In comparison, Table 3 showed that IL-10 levels were significantly lower in mice immunized with the LPG-PAA conjugate in contrast to the control group. Furthermore, IL-10 levels were also at low values in mice immunized with LPG + Freund's adjuvant, but the difference between this group and the control group was not as significant as the difference between the LPG-PAA conjugate group and the control group.

Table 3

IFN-γ and IL-10 levels of Balb/c mice immunized with various vaccine formulations after 21 d following to parasite infection (pg/mL).

Group	IFN-γ	IL-10
Control	123.4 ± 17.2	89.3 ± 4.8
LPG	347.2 ± 31.3	58.7 ± 3.4
LPG + Freund's adjuvant	621.4 ± 44.6	71.3 ± 2.8
LPG + PAA (physical mixture)	490.3 ± 38.9	56.5 ± 3.0
LPG + PAA (conjugate)	743.8 ± 52.7	22.4 ± 2.4

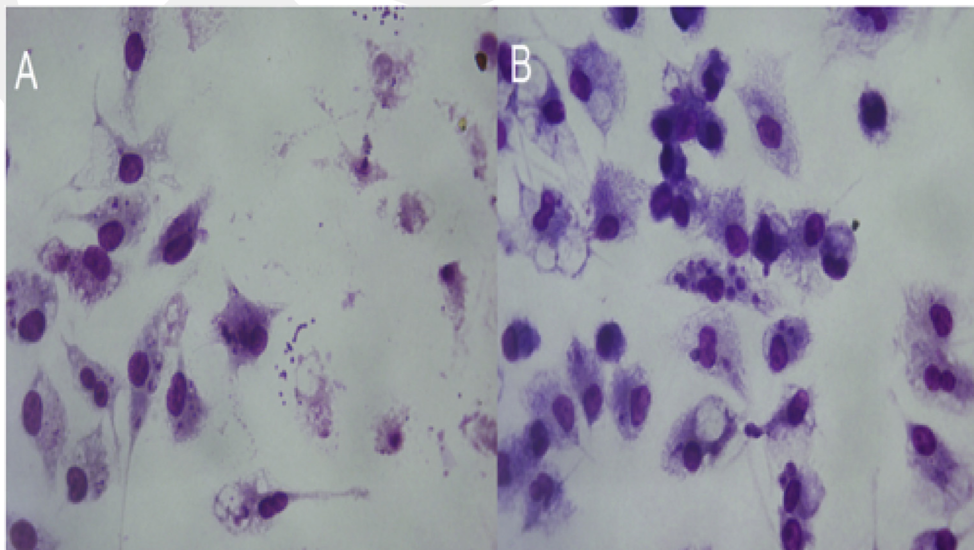

Figure 1. *In vitro* infection of peritoneal macrophages isolated from immunized or non-immunized mice with *L. donovani* promastigotes. (A) peritoneal macrophages of immunized mice with LPG-PAA conjugates (B) peritoneal macrophages of control group (any vaccination).

4. Discussion

In this study, the protective efficacy and immunogenicity of LPG-PAA conjugates were investigated in *L. donovani*-infected BALB/c mice by evaluation of parasite burden in peripheral blood, spleen and liver, assessment of cytokine levels in spleen and analysis of *in vitro* infection rates of macrophages isolated from immunized mice. The results showed that the parasite load in the sacrificed organs of immunized mice were meaningfully reduced in contrast to control. Especially in livers, the reduction of parasite load reached 80%. Additionally, following *in vitro* infection of peritoneal macrophages with *L. donovani* promastigotes, we determined that the infection index decreased at a rate of 83% in macrophages that were obtained from immunized mice with LPG-PAA conjugates. Furthermore, an enhanced Th1 immune response was detected in immunized mice against VL via evaluation of cytokine responses. IFN-γ levels significantly increased, while IL-10 cytokine levels sharply decreased. Therefore, all results reveal that vaccination with LPG-PAA conjugates resulted in significant protection against a progressive infection with *L. donovani*.

In reviewing the literature, despite the fact that LPG is one of the most important surface glycoproteins of *Leishmania* parasites, there are only a limited number of studies investigating its use as a vaccine candidate. This limitation may be dependent on the complexity of the LPG isolation process from *Leishmania* parasites and the absence of its commercial sale. There are only a few studies in the extant literature examining its efficacy alone and with several adjuvants in protection against CL [40–42]. There are currently no studies about its use as a vaccine candidate against VL. In mentioned studies, it has been suggested that LPG had good immunogenicity and could be an important vaccine candidate when it is used together with an appropriate adjuvant.

In recent years, various types of polymers have been introduced for vaccine development research for different kinds of infection and other diseases [43,44]. For this purpose, the adjuvant features of PAA, which are found in the polyelectrolyte groups of polymers, have recently been indicated. When delivered to primed chickens, PAA enhanced secondary immune response to inactivated Newcastle disease virus, and immune-modulating activities of polyelectrolytes, including polyanions [45,46]. Early experiments carried out using polyacrylic acid in mice immunized with the red blood cells of sheep demonstrated higher specific antibody responses and general immune activation than with antigen alone.

Carbomers, a species of cross-linked polyacrylic acid, have been evaluated as adjuvants in veterinary vaccines against swine parvovirus [47] and circovirus type 2 [48], *Staphylococcus aureus* in sheep [49], and in an experimental contraceptive vaccine [50] and an equine influenza virus vaccine in horses [51]. These reports suggest that carbomers such as carbopol are not harmful in mammals and stimulate a more robust immune response than antigens alone [52]. However, PAA has not been applied alone or within a conjugate in vaccine development studies against any form of leishmaniasis, despite its potential ability to improve the immunogenicity of a molecule related to parasites and to provide concise protection. In addition to specific features of LPG and PAA, the most important factor that supports the basis of this study is the easy and strong interactions between the NH_2 group of LPG and COOH groups of PAA in order to generate a conjugate [38]. Taking all information into account, for the first time in this study, we investigated the efficacies of LPG-PAA conjugate formulations as vaccine candidates against VL.

In order to reach this goal, we applied methods that are generally used in vaccine studies, such as assessment of parasite load by the LDU [53], evaluation of infection rates of peritoneal macrophages [54] and detection of parasites in peripheral blood by Giemsa staining [55]. In addition to these methods, the MCM that was previously developed by our group was also utilized to detect Leishmania parasites in peripheral blood.

The basic advantage of MCM is its capacity to diagnose VL with high sensitivity, independent of the number of parasites [56]. This method is being used in several endemic regions of the world in order to diagnose VL and CL with high sensitivity and specificity. Furthermore, the success of this method in detecting parasite existence in donor blood and murine models with asymptomatic leishmaniasis has been demonstrated in previous studies [57–59].

In the present study, we investigated parasite existence in the peripheral blood of infected immunized and non-immunized mice by using the Giemsa method and MCM. Results demonstrated that the sensibility of the Giemsa method is not sufficient to assess parasite load, since it could not detect the presence of parasites, especially in the blood of immunized animals. In comparison, MCM demonstrated more clearly the differences in parasite intensity between the groups, including infected animals immunized with different vaccination processes and the non-immunized control group.

The MCM results showed that parasite intensity was least in the peripheral blood of infected mice vaccinated with the LPG-PAA conjugate, in contrast to mice vaccinated with LPG alone, LPG + Freund's adjuvant combination, the physical combination of LPG-PAA, and the control group. These finding revealed the first signals of protection of the LPG-PAA conjugate against leishmaniasis. These results also demonstrate that the MCM method can be used to monitor the effect of a vaccine candidate in a *in vivo* model without using invasive techniques and the sacrifice of animals.

The vaccine-induced protective immune response is associated with a reduction in the parasitic number of peritoneal macrophages and the parasite load in the liver and spleen of mice after a challenge infection. Macrophages play an important role in antigen-presenting cells during infection by *Leishmania*. Macrophages are also effector cells that kill Leishmania parasites when a protective Th1 type of immune response has been established [60]. Therefore, results obtained from *in vitro* infection of peritoneal macrophages has importance in determining whether a vaccine formulation activates peritoneal macrophages to inhibit multiplication and the survival of *L. donovani* parasites. In a previous study, Kavoosi *et al.* showed that *in vitro* stimulation of murine macrophage cells with purified LPG isolated from *L. major* significantly enhanced the production of nitric oxide, which is an antileishmanial agent that macrophages use to kill Leishmania parasites. Additionally, the authors also concluded that LPG can be a good vaccine candidate since it has potential to activate macrophages [61]. Similarly, in 2008, Bhomwick *et al.* investigated the parasite clearance rates of peritoneal macrophages that were immunized with free gp63 antigen and gp63 encapsulated cationic liposomes in comparison with free liposome following peritoneal macrophage isolation from mice and their *in vitro* infection with *L. donovani* promastigotes. It was determined that the number of surviving parasites within

peritoneal macrophages were significantly lower in groups that were immunized with free gp63 antigen and gp63 encapsulated cationic liposomes in contrast to a group immunized with free liposomes and the control group. The accumulation of gp63 molecules on surface receptors of peritoneal macrophages prevented the entry of promastigotes into host cells. Furthermore, it was suggested that activated macrophages with vaccine formulations produced high amounts of IL-12 and IFN-γ in order to inhibit parasites and therefore parasite clearance of peritoneal macrophages isolated from immunized mice following *in vitro* infection could be associated with intensive secretion of these cytokines [54]. In another study, Elcicek *et al.*, demonstrated that PAA exposure considerably increased nitric oxide production in macrophages, *in vitro* [62].

In the present study, the infection index of peritoneal macrophages isolated from mice immunized with LPG-PAA was remarkably lower than for murine macrophages immunized by other experimental groups and the control group. Since LPG is one of the most important surface molecules that are responsible for parasitic invasion into host cells, just like the gp63 molecule, parasite clearance in immunized macrophages following *in vitro* infection may be explained by the accumulation of LPG onto macrophage receptors, which prevents entry and promastigotes stimulation of macrophages. Additionally it can be assumed that application of combinations including LPG and PAA produced high amounts of nitric oxide and cytokines such as IL-12 and IFN-γ and this leads to strong inhibition of the parasites. Under any circumstance, the prevention of intracellular Leishmania survival by peritoneal macrophages represents another important signal indicating the success of LPG-PAA conjugates as a vaccine candidate.

The LDU is another significant parameter for detecting the efficacies of vaccines [63]. Therefore, we applied this method in our study in order to determine the parasite burden in immunized mice after infection. The LPG-PAA conjugate decreased parasite numbers at a rate of 81% in liver and 73% in spleen. In comparison, vaccination with LPG alone resulted in a 40–50% reduction in parasite burdens of the liver and spleen. In previous studies, it was shown that administration of LPG alone lead to partial protection in susceptible BalB/c mice and its combination with an appropriate adjuvant or polymers was recommended. Similarly, in a recent study, Abdian *et al.* investigated immunogenic features of recombinant LPG against *L. major* infection in BalB/c mice and determined that LPG vaccination provided partial protection, although a significant increase was detected in antibody and cytokine levels in mice after vaccination. In that study, the parasite burden in spleens of mice immunized with LPG was found to decrease at a rate of 50% at most in contrast to control, thus indicating partial protection [64]. Therefore, the data in the present study representing an approximately 50% reduction in parasite burden in visceral organs when mice were vaccinated with LPG alone can be considered consistent with the results of previous studies. Furthermore, a 70%–80% reduction in parasite loads of the liver and spleen of mice immunized with LPG-PAA conjugates elicited significant protection against a *L. donovani* infection challenge and the antileishmanial vaccine effectiveness of LPG-PAA conjugates. It may also be said that this effectiveness is dose-dependent, and higher doses of conjugate may provide complete protection.

Subsequently, we investigated cytokine response in spleens against application of formulations including LPG-PAA conjugates. As is known, immunity against leishmaniasis is T-cell mediated, but T cells were not thought to recognize or present non-protein antigens. Today, it is accepted that many novel and interesting microbial antigens, including mycobacterial glycolipids, can be recognized by T cells, and that these antigens are presented to T cells by a special subset of MHC class I proteins known as CD147. Therefore, it may be rewarding to reevaluate the potential of LPG as a vaccine candidate [65]. Alternatively, the Th1/Th2 paradigm is very critical for the progression of leishmaniasis. Cytokine such as IFN-γ induce a Th1 immune response indicating a cure of the infection, while IL-10 stimulates a Th2 response, which is a sign of parasite survival and progression of the infection [66,67]. Therefore, in vaccine studies, the determination of cytokine responses in spleens of immunized mice is very significant in regards to demonstrating the efficacies of an applied vaccine candidate.

In the present study, the results of cytokine analysis revealed that an accurate Th1 cell response arises in mice immunized with LPG-PAA conjugate. IFN-γ levels in this group were significantly diminished, while IL-10 levels considerably increased in contrast to the control. These results also support the idea that LPG would constitute a T-cell response.

Another significant result obtained from the current study was improvement of the immune response in mice when they were exposed to LPG-PAA conjugates in contrast to their exposure to a LPG + PAA physical mixture. This may be explained by more effective delivery of LPG antigens into antigen presenting cells in conjunction with a PAA polymer. Kabanov *et al.* [68] demonstrated that once polyelectrolyte-antigen conjugates interacted with B cells, polyelectrolyte chains provided clustering of membrane proteins while an antigen was presented to cell membrane receptors. Clustering of cell membrane proteins leads to alterations in the ion flux of cells and a signal is produced to trigger a cellular mechanism for elevating the immune response to the antigen that is found within the conjugate [68]. We propose that low immunogenicities of LPG + PAA physical mixtures as compared to their conjugates is associated with a lack of simultaneous and sufficient delivery of antigen and adjuvants to immune competent cells. Alternatively, conjugation with PAA is thought to provide sufficient transportation of LPG to desired immune cells and this results in a strong immune response against this antigen.

In conclusion, for the first time in this study, it was demonstrated that a formulation, including a conjugate of LPG obtained from *L. donovani* and PAA, has an antileishmanial vaccine effect against VL. This study also for the first time identifies the importance of polymer technology in vaccine strategies for leishmaniasis. In this respect, the present study may lead to new vaccine approaches based on high immunogenic LPG molecules and adjuvant polymers in the fight against Leishmania infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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