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ORIGINAL ARTICLE

Protective Potential of Conjugated *P. aeruginosa* LPS –PLGA Nanoparticles in Mice as a Nanovaccine

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ABSTRACT

Background: *Pseudomonas aeruginosa* has an important role in nosocomial infections. **Objective:** To evaluate biological activity of the detoxified LPS (D-LPS) entrapped into Poly lactic-co-glycolic acid (PLGA) nanoparticles. **Materials:** LPS was extracted and detoxified from the *P. aeruginosa* strain PAO1. The D-LPS, conjugated to the PLGA nanoparticles with 1-ethyl-3-dimethyl aminopropyl carbodiimide (EDAC) and N-hydroxy-succinimide (NHS). The connection was evaluated by FTIR (Fourier transform infrared), Zetasizer, and Atomic Force Microscope (AFM). The BALB/c mice injected intramuscularly with the D-LPS-PLGA with two-week intervals and then challenged two weeks after the last immunization. The bioactivity of the induced specific antisera and cytokines responses against D-LPS-PLGA antigen was assessed by ELISA. **Results:** D-LPS-PLGA conjugation was confirmed by FTIR, Zetasizer, and AFM. The ELISA results showed that D-LPS was successful in the stimulation of the humoral immune response. The immune responses raised against the D-LPS-PLGA, significantly decreased bacterial titer in the spleen of the immunized mice after challenge with PAO1 strain in comparison with the control groups. **Conclusion:** The conjugation of the bacterial LPS to the PLGA nanoparticle increased their functional activity by decrease in bacterial dissemination and increase the killing of opsonized bacteria.

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Keywords: LPS, Nanovaccine, PLGA, *Pseudomonas Aeruginosa*

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INTRODUCTION

P. aeruginosa is the most common opportunistic pathogen isolated from patients who have been hospitalized longer than one week, and it is associated with acute respiratory infections in immunocompromised patients, chronic respiratory infections in cystic fibrosis (CF) patients, infections of the urinary tract, and burned skin and wound infections. *Pseudomonas* infections are often resistant to treatment (1,2). In recent years, we observed advances in the therapy of *Pseudomonas* infection; however, effective treatment remains a constant problem. *Pseudomonas* uses different mechanisms to acquire resistance against antimicrobial agents (3). Extensive drug-resistant isolates have been considered as prevalent causes in CF and hospitalized patients (4); therefore, the development of new therapeutic options against *P. aeruginosa* has been a challenge for several years (5,6). The vaccine candidates have long been desired as a preventing method to infection in susceptible patients (7). *P. aeruginosa* is a potentially pathogenic bacteria with several products such as toxins, adhesions, and invasion factors for local and systemic diseases (8). Some of the virulence factors which can contribute to one or several stages of pathogenesis, including ucoid exopolysaccharide (alginate), Lipopolysaccharide (LPS) and exotoxin A (ETA) (9). Alginate and LPS are the major surface components acting as an adhesion to anchor the bacterium to the respiratory epithelium and causing chronic pulmonary infections in CF patients. In this microorganism, LPS is a key factor in the pathogenesis and innate immune response through binding to Toll-like receptor 4 (TLR4) and acquiring immunity to infection (10). Polymeric nanoparticles are synthetic or natural polymers often used as suitable carriers. These nanoparticles are either derived from polylactic acid (PLA) or formed by the copolymer of lactic and glycolic acid (PLGA). These polymers are widely employed in antigen-controlled release owing to their biodegradability, biocompatibility, and ability to effectively cross biological barriers (11). As the first FDA approved products, PLGA polymers are utilized to entrap antigens and antibiotics in nanoparticle formulations. Peptides and proteins conjugated in PLGA display advantages including improved biological effects and delivery, low degradation and toxicity and site-specific (12). Recently, it has been reported that loaded of amikacin or ciprofloxacin in PLGA nanoparticles can reduce drug toxicity while increasing drug efficacy against *P. aeruginosa* (13,14). In the current study, for the first time, we synthesized and characterized conjugate vaccines based on detoxified lipopolysaccharide through the use of PLGA nanoparticles. The efficacy of the PLGA-conjugated antigens in stimulating the immune responses was assessed *in vitro* and *in vivo.*

MATERIALS AND METHODS

Bacterial Strains. The standard strain PAO1 of *P. aeruginosa* was used in this study (kindly provided by Biological Research Center, Zanjan Branch, Islamic Azad University, Zanjan, Iran).

LPS Isolation and Purification. LPS was extracted from PAO1 strain *P. aeruginosa* by the hot phenol-water method as described previously (15). Briefly, bacterial suspensions (10^8 CFUs/ml) were centrifuged (8000 × g for 10 min) and the cells were washed twice in PBS (phosphate-buffered saline) containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. Afterwards, the pellets were suspended in PBS and sonicated for 10 min on

ice. Contaminant proteins and nucleic acids were eliminated by enzymatic treatment with proteinase K (100 µg/ml), DNase (40 µg/ml), and RNase (40 µg/ml) prior to the extraction step. The mixtures were suspended in an equal volume of hot phenol 90% followed by vigorous shaking at 65-70°C for 15 min. Next, the suspensions were cooled on ice and centrifuged at 2600 × g for 45 min at 4°C. The aqueous phase was mixed with trichloroacetic acid (TCA) (10% final concentration) and 10 volumes of 95% cold ethanol and stored at -20°C overnight to precipitate LPS. The samples were then centrifuged (2000 × g 4°C for 10 min) and the precipitate was dissolved in 0.2 N NaOH and heated at 100°C for 2 hours. Free fatty acids were removed via dialyzing extensively against dH_2O at 4°C for 2 days with six changes of water. After that, the detoxified LPS (D-LPS) was harvested by absolute ethanol precipitation.

Conjugation of D-LPS and PLGA. The conjugation of D-LPS with nanoparticle was performed according to the previous reports (16,17). The solution of PLGA (Sigma) nanoparticle was prepared in Dimethyl sulfoxide (or DMSO, Sigma) (15 mg/ 5 ml) and kept at lab temperature for 1 hour. Then, 150 mg of N-(3-Dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDAC, Sigma) and 50 mg of N-Hydroxysuccinimide (NHS)(Sigma) was added and maintained at lab temperature for one hour. D-LPS antigen (25 mg) was then added and incubated (37°C, for seven days). Thereafter, LPS-PLGA was dialyzed against dH_2O (4°C, 72 hours, with 3-time change of water), and LPS-PLG nanoparticles were purified through gel filtration chromatography (Sephacryl S-200 HR).

Fourier Transform Infrared. The characterization of the chemical groups present in nanoparticles obtained by Fourier transform infrared (FTIR). The spectra absorption was collected from 4000 - 400 cm^{-1} for PLGA and conjugated D-LPS-PLGA by Bruker FTIR spectrophotometer (please add the name of the instrument here, Germany).

Atomic-force microscopy (AFM). Atomic Force Microscopy (AFM) for analyzing surface topography of nanoparticles was taken using NanoWizard, Germany. AFM images were analyzed using JPK lab analysis software. Particle size and surface electric charge analysis by Zetasizer (Nano ZS, England). The electric charge on the particle surface was calculated as zeta potential which revealed a colloidal dispersion stability of approximately83%. PLGA nanoparticles were analyzed before and after conjugating with D-LPS.

Immunization of Mice. The immunization of animals was performed according to previously published reports (17). Four groups for the immunogenicity assay defined, including D-LPS-PLGA, D-LPS, PLGA, and control group (NMS) which in each group ten mice were located. The mice were immunized three times with fourteen days' interval. The route of administration vaccine was IM injection of 100 µg antigens to anesthetized mice (injected intraperitoneally (IP), mixture of Ketamine (80 mg/kg) and Xylazine (10 mg/kg)). All animal experiments in this research were according to the Animal Care and Use Protocol of Razi Vaccine and Serum Research Institute. In addition, this research has the ethics code for work on animals: IR. IAU. ZANJAN. REC. 1396.2.

Enzyme-linked Immunosorbent Assay (ELISA). The elicited antibody in response to the vaccine candidates was measured by indirect ELISA. After coating the plate (96-well microtiter plate, Nunc MaxiSorp) with antigen (1 µg D-LPS in 100 mM sodium carbonate/sodium bicarbonate buffer, pH 9.6), it was incubated (4ºC, overnight) and then three times rinsed with PBS-T (PBS buffer containing 0.05% Tween 20). Afterwards, 100 µl PBS-T containing 5% skimmed milk was added to each well and

incubated at 37ºC for two more hours. The well was also rinsed three times with PBS-T. In the next step, a serial dilution (from 1:2 to 1:16384) of immunized mice sera isolated in blocking buffer was prepared and 100 µl of each one was added to each well. The plates were then incubated for two hours and washed with PBS-T. After that, the HRP-labeled goat anti-mouse IgG (1:10000-diluted) was added to each well and incubated for one hour. Briefly, 96-well microtiter plates (Nunc MaxiSorp) were coated with 1 µg of D-LPS in a coating buffer (100 mM sodium carbonate/sodium bicarbonate buffer, pH 9.6) and incubated overnight at 4ºC. Next, the plates were rinsed three times with PBS-T (Phosphate-buffered saline containing 0.05% Tween 20) and incubated with 5% skimmed milk in PBS-T for 2 hours at 37ºC. After three times washing with PBS-T, a serial dilution (from 1:2 to 1:16384) of experimental mouse sera and normal mice sera in blocking buffer prepared and 100 µl of each one was added to each well. After 2 hours' incubation at 37ºC, the rinsed plates incubated with 1:10000-diluted HRP-labeled goat anti-mouse IgG at 37ºC for 1 h. After that, 100 µl TMB (3, 3, 5, 5'-tetramethylbenzidine) was added as an enzyme-substrate and incubated at room temperature for 30 min and, with adding 100 µl of 2N H_2SO_4 to each well for stopping the reaction. The optical density with an ELISA plate reader (Awareness Stat Fax 4200, USA) read at 450 nm.

Bacterial Inoculums and Challenge Study. Fourteen days after the third vaccination, the anesthetized mice of immunized and control group were subjected to the injection of the PAO1 strain of *P. aeruginosa* (1.5×10^8 CFUs) via the IP route. After 72 hours of challenge, the mice were sacrificed and the serial dilutions of homogenized spleens in of PBS (pH 7.4), plated on to Nutrient agar. After incubation at 37°C for 48 hours, the CFUs were calculated.

Opsonophagocytosis Assay. The opsonic killing activity of immunized mice polymorphonuclears (PMNs) was performed according to previous reports (5,17). Briefly, following the third immunization, the blood samples were taken, the sera were isolated, and the complement was inactivated by heat (56°C for 30 min). The 10 µl/well diluted sera by Hanks buffer containing 0.1% gelatin with ratio 1:4were added to a 96-well plate. After that, 2×10^3 CFUs of the PAO1 strain of *P. aeruginosa* added. As a complement source, baby rabbit serum 12.5% (final concentration) was added and the plate was kept at 37°C for 45 min under agitation. Thereafter, 80 µl/well of mouse PMNs (polymorphonuclears) (PMNs) isolated with Ficoll-Histopaque (Sigma, USA) was added and incubated (90 min, at 37°C, under agitation). Finally, NaCl 0.9% (80 µl/well) was added to each well and incubated (30 min, at 37°C). After this step, the samples were plated and incubated for 48 hours at 37°C. The following formula was used to calculate the opsonic activity:

[1 - (CFU immune serum at 90 min/CFU of pre-immune serum at 90 min)] × 100

The sample in each well was diluted with an equal volume of 0.9% NaCl (80 µl/well) and incubated for 30 min. Finally, the samples were plated (48 hours incubation at 37°C) for bacterial enumeration. The opsonic activity of the serum calculated as the following formula:

[1 - (CFU immune serum at 90 min/CFU of pre-immune serum at 90 min)] × 100

Cytokine Assay. Mice were intramuscularly immunized three times, with fourteen days intervals. Fourteen days after the last immunization, the blood samples were taken, centrifuged (10000g, 10 min) and frozen before performing the cytokine assay. The cytokines, i.e. TNF-a, IL-4 and INF-γ were quantified via the ELISA method. Cytokine assays performed using different kits (all from Mabtech Ebioscience R&D, USA)

according to the manufacturer's instructions. Briefly, the wells were coated with 100 µl of specific antibodies (1: 1000). After 24 hours, the well contents were removed, and the wells were washed. Next, 200 µl of the blocking buffer was added to each well and left at room temperature for 1 h. Thereafter, standards and samples (in triplicate) were added to the wells and keep at room temperature for two hours. The wells were then washed and 100 µl of (0.5 µg/ml) appropriate detection secondary-biotin antibodies was added and kept at room temperature for 1 h. Then, wells were washed and 100 µl of Streptavidin-HRP (1:1000) was added and left at room temperature for 1 h. After washing, 100 µl of the substrate (TMB) was dispensed and the plates were kept in dark for 20-30 min. Afterwards, 100 µl of H_2SO_4 was added and OD levels were read at 450 nm. The titer of cytokines was obtained in pg/ml according to their standard curve.

Statistical Analysis. The statistical data were analyzed with GraphPad Prism version 6.0 for Windows, (GraphPad Software, San Diego, CA, USA). The one-way ANOVA test (Tukey's test) was used for statistical analysis. The data were shown as mean ± SD, p-values <0.05 were considered as significant (Sig), and 95% confidence interval in supplementary table 1. It has been evaluated in relation to the vaccine group with the other groups in all immunogenicity tests. Survival data for the different groups were analyzed using Kaplan-Meier survival curves and the log-rank test. The statistical differences between mortality rates were specified using Fisher's exact test.

RESULTS

Nanoparticle characterization and analysis.

The analysis of the collected fraction of size exclusion chromatography (Sephacryl S-200 HR) showed that the fractions 20-21 containing the high value of D-LPS-PLGA nanoparticles (Figure 1). To confirm the formation of PLGA-conjugated nanoparticles, several methods used, including FTIR, AFM, and Zetasizer. The confirmed D-LPS-PLGA nanoparticles fractions used for immunization of mice.

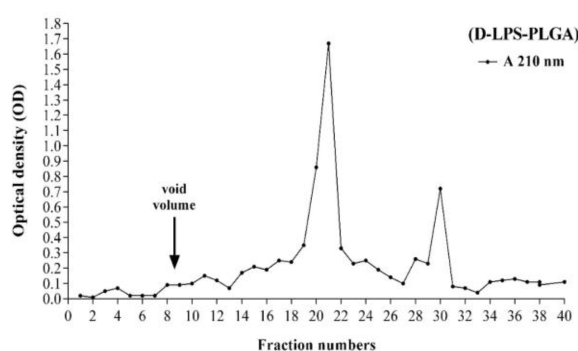

Figure 1. The evaluation of the gel filtration chromatography results for the purification of the D-LPS-PLGA.

FTIR spectroscopy results.

The structural analysis of the conjugated nanoparticles and conjugation bonds was conducted by FTIR spectroscopy. At results of FTIR peaks assigned between 1650 and

1800 cm^{-1} indicate the H-C=O group in PLGA, at 1660.41cm^{-1} and in D-LPS, at 1653.66 cm^{-}. After conjugation by PLGA nanoparticles, for D-LPS-PLGA, these peaks were 1099.20 cm^{-1} for D-LPS and 1273.75 cm^{-1} for PLGA. The changes in peaks indicated that the ester bonds formed in PLGA-D-LPS nanoparticles that confirmed the conjugation process (Figure 2).

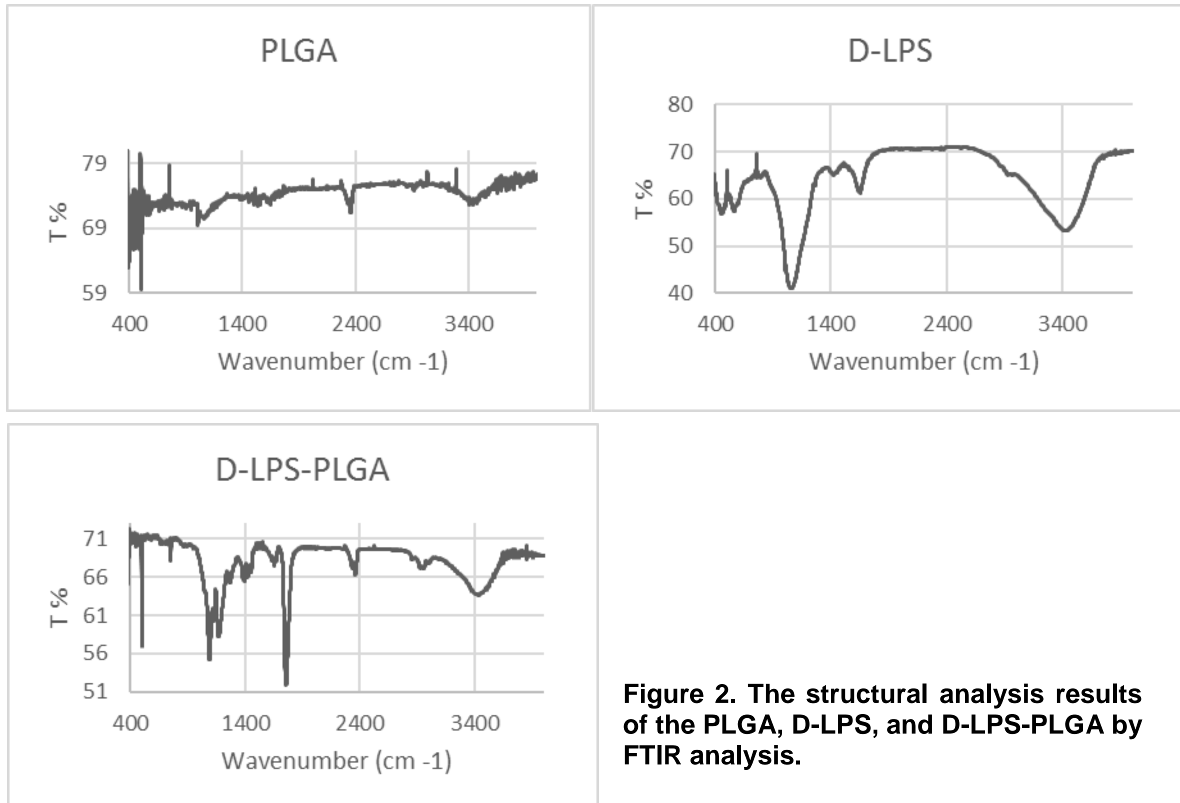

Figure 2. The structural analysis results of the PLGA, D-LPS, and D-LPS-PLGA by FTIR analysis.

Results of Zetasizer.
The average of the nanoparticles' hydrodynamic size, evaluated by Zetasizer. The results showed that the PLGA size and the surface charge was 105 nm and -4.21 mv, respectively, before conjugation. While the size and surface charge in the D-LPS-PLGA were 332 nm and -2.14 mv respectively, after conjugation. These changes indicated that the conjugation performed successfully.
AFM Results.
The surface topographic morphology of PLGA nanoparticles was studied by AFM and analyzed via JPK software. Results showed that the size of PLGA nanoparticles was 12-24 nm prior to the conjugation of antigens (Figure 3). Following the connection, the size of the nanoparticles increased to 247.4 nm in D-LPS-PLGA (Figure 3). The shape of connection sites on PLGA nanoparticles was sharpness before conjugation, whereas after conjugation, in D-LPS-PLGA nanoparticles the sites changed to inflate. All these results supported the successful connection formation.

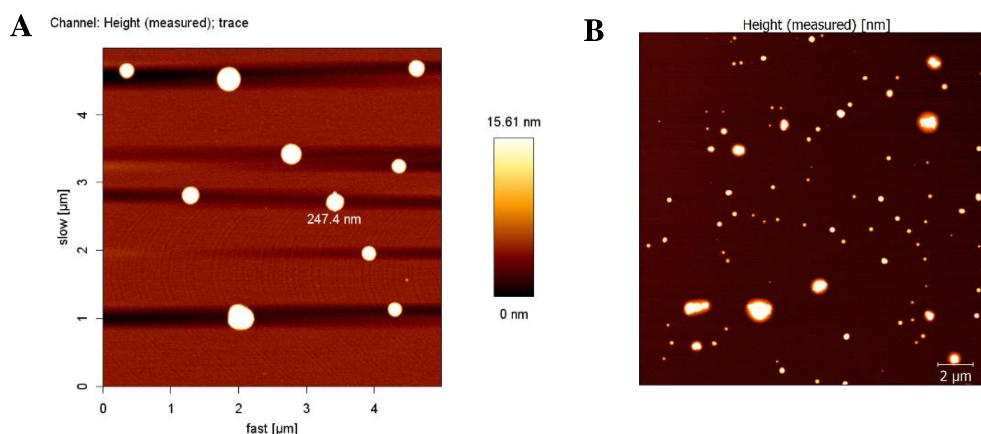

Figure 3. The images of D-LPS-PLGA after conjugation by Atomic force microscopy. (A) The two-dimension of the D-LPS-PLGA nanoparticles and B) three dimensions of the D-LPS-PLGA nanoparticles.

IgG total and the different IgG subtypes (IgG1, IgG3, IgG2a, and IgG2b) responses to PLGA-D-LPS conjugate.

The IgG total and subtypes of this antibody response to stimulation of D-LPS were increased in the D-LPS-PLGA (Figure 4). The differences of IgG total and subtypes response between the mice immunized with D-LPS-PLGA and D-LPS alone were significant and the significant increase in IgG total and subtypes (IgG1, IgG3, IgG2a, and IgG2b) were observed in D-LPS-PLGA immunized group. These results demonstrated that D-LPS-PLGA is a suitable immunogen to elicit the IgG.

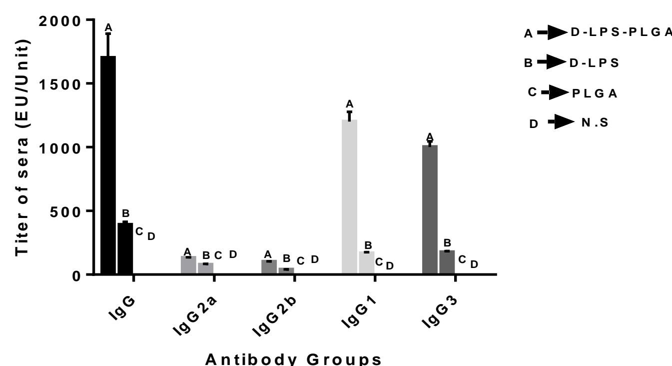

Figure 4. The determination of the total antibody and subtypes responses against D-LPS in the immunized mice. The D-LPS-specific IgG total and subtypes such as (IgG1, IgG2a, IgG2b, and IgG3) the results are the average of three mice in each group. The total antibody and all of the subtypes showed significant differences compared to the control groups (PLGA and N.S). There were significant at $p<0.05$ between D-LPS-PLGA group with the other groups. In addition, there were no significant differences at the $p<0.05$ between PLGA alone with the control group (N.S) ($p=1$).

Bacterial infection in the spleen.

To analysis the function of the specific antibodies and cytokines response raised against D-LPS-PLGA in the inhibition of the distribution of the bacteria to the internal organs,

we determined the spread of infection by evaluated the bacterial loads in the spleen. The bacterial load was distinguished in the spleen of immunized mice, after 72 hours of infection with the PAO1 strain of *P. aeruginosa*. Our results were shown that the antibodies and cytokines raised against D-LPS-PLGA in immunized mice infected with PAO1 strain, significantly reduced the bacterial load in the spleen compared to the control groups, normal saline, PLGA, and D-LPS alone groups (Figure 5).

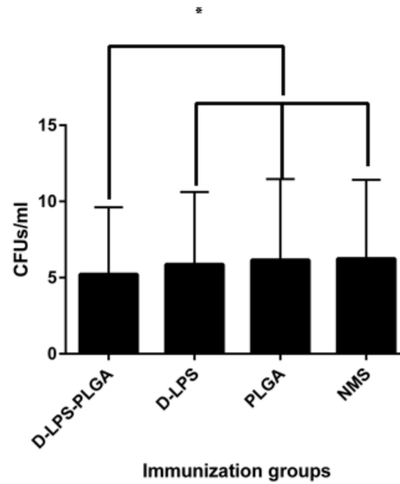

Figure 5. The bacterial load in the spleen. After 72 hours of infection with the PAO1 strain of *P. aeruginosa*, the spleen of mice was isolated and the serial diluted of the homogenates plated for CFUs counting. The * indicates the significant difference in the D-LPS-PLGA compared to other groups ($p<0.05$).

Opsonic killing activity.

The clearance of infection is closely related to the functional activity of the specific antibodies and cytokine responses that can mediate bacterial uptake by phagocytes. The antisera and cytokine production of immunized mice was evaluated for opsonic killing activity (*in vitro).*

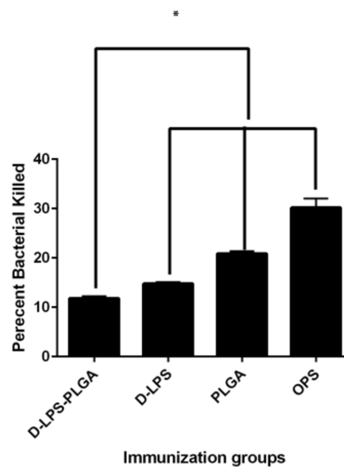

Figure 6. The evaluation of opsonic killing activity against *P. aeruginosa* strain PAO1. Fourteen days after the third immunization (Day 42), the strain incubated with specific antibodies (ratio 1:4) and PMNs in the presence of rabbit complement. The significant

differences in the opsonic killing activity observed in the D-LPS-PLGA immunized group compared to other groups ($p<0.05$).

The antisera and cytokines from mice immunized with D-LPS-PLGA after dilution with 1:4 ratio, mediated the significant killing activity at levels approaching 91% (Figure 6). These results suggest that D-LPS-PLGA, as a candidate vaccine, enhanced *P. aeruginosa* PAO1-specific antibody and cytokine response.

Cytokine responses to immunization.

Fourteen days after the last injection, the levels of TNF-a, INF-γ, and IL-4 were evaluated by ELISA assay. We observed the elevated cytokine profiles in immunized mice with D-LPS-PLGA. The cytokine includes TNF-α, INF-γ, and IL-4 levels were significantly ($p<0.05$) higher than those in the control groups (D-LPS, PLGA, and NMS) (Figure 7). According to the results, the conjugation of the PLGA to D-LPS enhanced the cytokine response in the immunized mice.

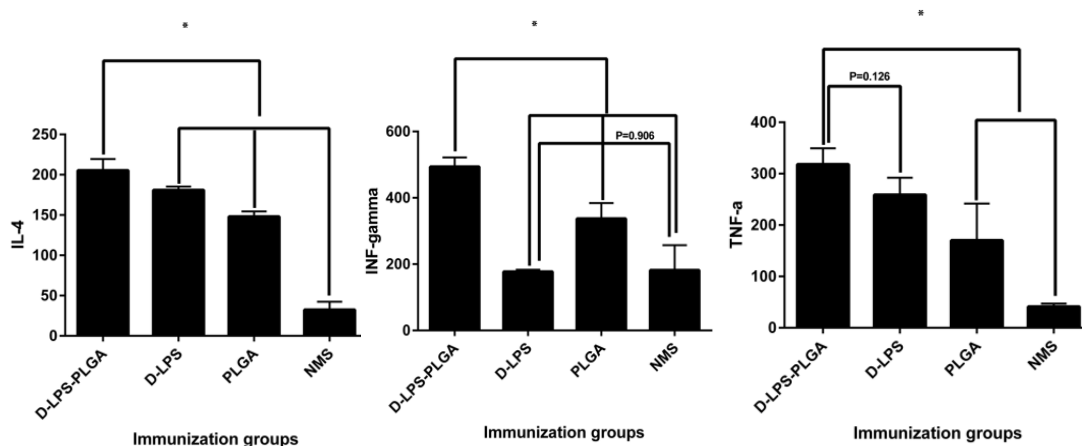

Figure 7. The determination of elicited cytokines in the immunized mice. (A) D-LPS-PLGA, D-LPS, and PLGA alone had higher IL-4, respectively and there were significant differences between the D-LPS- PLGA group with other groups. (B) The evaluation of the INF- γ responses in the immunized mice. The D-LPS-PLGA, PLGA alone and D-LPS had the highest results in the INF-γ cytokine response, respectively. There were no significant differences between the control groups and the D-LPS alone ($p=0.906$). (C) The TNF-α cytokine levels in the D-LPS- PLGA, D-LPS and the PLGA groups had significantly increased compared to the NMS group ($p<0.05$).

DISCUSSION

The high rates of resistance to commercially available antibiotics and frequent multidrug-resistance (MDR) isolates from *P.aeruginosa* infections have become a major problem worldwide. Therefore, the preventive measures for controlling the *P.aeruginosa* infections by the development of the vaccines seem necessary. In this research, we analyzed the novel D-LPS-PLAG vaccine against *P. aeruginosa.* The bacterial load in the internal organs was assessed in the mice immunized with the PAO1 strain of *P. aeruginosa* and the immunogenicity of the D-LPSPLAG nanoparticles was

investigated by *in vitro* opsonophagocytosis test, antibodies titer, and cytokine responses. Previous works have reported that the conserved epitope of LPS in *P.aeruginosa* had efficacy in inducing cytokine responses such as TNF-α. Besides, LPS can bind to Toll-like receptor 4 (TLR4) on the surface of immune cells, thereby stimulating inflammatory immune responses. This endogenous pyrogen is capable of acting as a target for effective immunity (10). Nanoparticles are biodegradable and polymeric matrices. As a result, vaccines composed of nanoparticles are a new class of vaccines that are more effective and compatible with the tissues and more economical than conventional vaccines. Nanovaccine based on biodegradable polymers such as PLGA has some advantages including better antigen delivery, non-toxic and generating a strong immune response with a few antigens (18). The other applications of PLGA nanoparticles that can be pointed to increase absorption and drug penetration (18-22). The output of the connection of the antigens to PLGA nanoparticles is very valuable for containing the protein antigens such as LPS. The optimization of the protocols successfully upgrades the antigens conjugation efficiency. In this study, we showed the high yield antigens conjugation efficiency by measuring the average hydrodynamic size also, the third dimension topography of the PLGA nanoparticle prior to entrapment was significantly smaller than the PLGA-encapsulated nanoparticles after entrapment. Marasini *et al.* encapsulated a lipopeptide vaccine in PLGA nanoparticles and showed that they elicited high titers of specific systemic IgG and mucosal IgA immune responses compared with free lipopeptides or lipopeptide-coated PLGA, suggesting the importance of antigen encapsulation. Furthermore, the antibodies raised against lipopeptides encapsulated with PLGA were able to opsonize up to 95% of the clinical strains of Group A streptococci (20). Raphael Simon *et al*. prepared a new COPS-flagellin conjugate vaccine and investigated its biological activity. They reported that immunization with this vaccine in infants and young children in sub-Saharan Africa, successfully prevented *S.enteritidis* and *S.typhimurium* infections (23). Tan *et al.* observed that the oral delivery of acid-resistant HP55 entrapped into PLGA nanoparticles had some advantages; in the patients receiving HP55/PLGA nanoparticles, they reported the clearance of *H. pylori* mediated by the Th1/Th17 cells, and interference the infiltration of PMNs (21). Cruz *et al.* synthesized a new antimicrobial peptide (GIBIM-P5S9K) by double-emulsion solvent evaporation, successfully loading it in PLA and PLGA nanoparticles. They further showed that these nanoparticles were stable in human sera and had antibacterial activity against *P. aeruginosa*, *Escherichia coli O157: H7*, and Methicilin-resistance *Staphylococcus aureus* (MRSA)(22). Angela Ruyras *et al.* found that the immunostimulant-loaded nanoliposomes protected fish against bacterial (*P.aeruginosa* PAO1) or viral infections. The nanoliposomes accumulated in immune tissues and immunologically active cells such as macrophages (24). In this study, we found that the D-LPS was an appropriate immunogen to induce total IgG and subtypes antibodies (IgG1, IgG3, IgG2a, and IgG2b) and TNF-a cytokines mediated immune responses. In addition, we observed the *in vivo* evidence that specific antibodies and cytokines to the antigen-loaded in PLGA nanoparticles are versatile compared to PLGA alone in bacterial clearance and systemic spread of *P. aeruginosa* strain PAO1 infection in the immunized mice. The specific total and subtypes antibodies and cytokines responded against the vaccine, which among them, the total IgG, IgG1, and IgG3 subtypes antibodies showed more increase compared to the IgG2a and IgG2b and IL-4, TNF-a and INF-ɣ cytokines, respectively. The opsonic killing activity raised against D-LPS-PLGA showed the enhanced activity

compared to the D-LPS and PLGA. Furthermore, our results indicated that the TH2 and TH1 were produced IL-4 and INF-γ cytokine responses against D-LPS-PLGA. The *in vivo* results showed that D-LPS-PLGA nanovaccine was able to clear *P.aeruginosa* strain PAO1 by the recall of TH1/TH2 cells, which mediated the recruitment and infiltration of PMNs such as neutrophils. Taken all together, the conjugation of bacterial D-LPS to PLGA nanoparticle with NHS as spacer molecules increase the functional activities of the candidate vaccine by decrease the bacterial dissemination and increase in the killing of opsonized bacteria. This pilot study was a basis for the further development of a nano-vaccine for possible application in humans to protect infection by *P. aeruginosa.*

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