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Liposomal-lipopolysaccharide vaccine extracted from *Proteus mirabilis* induces moderate TLR4 and CD14 production

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ABSTRACT

Proteus mirabilis is a common cause of recurrent urinary tract infections in individuals with functional or structural abnormalities. It also forms bladder and kidney stones. Lipopolysaccharide (LPS) is a potential *Proteus* virulence factor that plays a key role in pathogenesis, as well as in stimulating innate immune response. Therefore, this study aimed to extract LPS from a highly resistant isolate and incorporate it in a delivery system (liposome) to stimulate an immune response against virulent pathogens. In the work, 50 isolates of *P. mirabilis* were taken from 200 urine specimens obtained from recurrent-urinary tract infections (UTI) of patients of AL-Sadar Hospital. Specimens were cultured on specific media, and then bacterial isolates were identified via morphological, biochemical and Vitek-2 systems. The results showed that *P. mirabilis* was expressed in 11 (22%), 30 (60%) and 9 (18%) recurrent UTI, kidney stone and catheter samples, respectively. All isolates were assessed through antibiogram testing, with the results revealing that most isolates were multidrug resistant to more than 3 classes of antibiotics. Herein, *P. mirabilis* NO 50 revealed particularly high resistance, so it was chosen for LPS extraction. Lethal dose 50 (LD50) observations indicated that a live suspension of *P. mirabilis* was at 4.5×10^7 CFU/ml, while LPS was at 270 µg/ml. LPS was used as an immunogenic to stimulate the immune system through injecting Rats intraperitoneally (I.P.) with 1 ml of LD50%. Subsequently, the efficiency of immunogenes in stimulating the immune response was evaluated by determining the Toll-like receptor and CD14 levels. The results indicate that LPS incorporated in the Liposome released moderate levels of Toll-like receptors-4 (TLR4) that enabled the immune system to clear pathogens. The LPS+ complete Freund's adjuvant (CFA) and LPS vaccinated groups recorded hyper production for TLR4 (52.2 and 40.9 pg/ml, respectively), this was followed by liposome (LIP) and bacterial suspension (11 and 20.5 pg/ml, respectively) in ranking effectiveness. This study reveals a mean of CD14 that was higher in both LPS and LPS+CFA and moderate in LPS+LIP, in comparison with control and liposome groups. In conclusion, LPS-Liposomes are a promising nanomedicine for modulating the hyper response of LPS. This may lead to tissue inflammation but appeared beneficial in stimulating the immune response at moderate levels so as to eradicate infection without tissue damage.

INTRODUCTION

Proteus mirabilis is a gram negative, facultative anaerobic, polymorphic and nosocomial opportunistic rod bacteria belonging to the *Enterobacteriaceae* family. *P. mirabilis* are widely disseminated in the environment with reservoirs in soil, tainted water and sewage [1]. It is one of the essential

reasons for UTIs in hospital patients with indwelling urinary Catheters [2], in neonatal sepsis, wound infection, meningitis and pneumonia [3,4]. Infections with *Proteus* spp. have seen increasing importance as a cause of serious nosocomial outbreaks that are difficult to treat using most common antibiotics [5]. Indeed, the number of multi-drug resistant (MDR) bacterial isolates has exploded in recent years. This affects the prognosis and survival of hospitalized patients [6,7].

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Nagano *et al.* [8] have observed that the increasing nosocomial outbreak caused by CTX-M-2-producing *P. mirabilis* is particularly difficult to treat through current antibiotic therapy. Thus, this isolate has become a serious pan-global clinical problem. This highlights the need to increase the preventability of *Proteus* infections and to avoid failure to mitigate recurrent infections and allergic reactions to certain antibiotics. This situation leads to the high priority of drug development research seeking new therapeutics as alternative therapy to control infections caused by *P. mirabilis* [9].

One of the most promising approaches against bacterial infection is vaccination by antigenic materials that stimulate immunity that have been extracted from pathogenic bacteria [10]. That pure lipopolysaccharides (LPS) from *P. mirabilis* can induce both systemic and humeral immunity, has been confirmed by [11]. Darweesh [12] too, has observed that exo-polysaccharides extracted from G-ve bacteria modulate the production of pro-inflammatory biomarkers to prevent the over-production of cytokine that can affect the host.

Lipid nanomedicine-based approaches have also attracted increasing attention. The intent of such drug therapy is to improve or modulate immune responses, chiefly, cell mediated immunity (CMI), to assorted antigens [13]. Liposomes are small, spherical vesicles made of phospholipid bilayers and have the ability to entrap hydrophilic therapeutic agents in their aqueous central compartment and hydrophobic therapeutic agents within their phospholipid bilayers or liposome surfaces [14]. In [15], the authors explain that LPS-liposome conjugates induce high levels of IgG against *Shigella* infections. CD14 and Toll-like receptor-4 (TLR-4) are crucial for recognizing LPS and, hence, they also contribute to the host defense against them by eliciting innate and then adaptive immune responses [16].

This study aimed to evaluate the immunomodulator activity of LPS-Liposome and LPS extracted from *P. mirabilis* for preventing infection and for protecting the infected host from hyper-immune responses that lead to destruction of infected tissue.

MATERIALS AND METHODS

Bacterial Characterization

A total of 200 urine specimens were collected under aseptic conditions from patients at Al-Sadar Medical City in AL-Najaf province who were suffering from recurrent UTI, kidney stone and long-used catheter. These specimens were then inoculated on MacConkey agar and Blood agar and incubated at 37°C for 24 h. The morphological characteristics of the colonies including size, shape and color were recorded. The various *Proteus* strains were subsequently identified and assessed through biochemical testing [17] and confirmed by using Vitek-2 Compact (Bio Mérieux, France). An antibiotic susceptibility test was then done for all isolates by using the Vitek-2 system with the AST-XN05 card.

Extraction of lipopolysaccharide (LPS)

A. Cell Preparation

Proteus mirabilis isolates were cultured all night in a flask containing 25 ml of Luria Broth (LB) (for bacterial

activation) at 37°C for 18 hours. The fresh cultures were then inoculated into 3.5 L of LB broth suspended in 500 ml conical flasks containing 200 ml broth. Incubation followed at 37°C for 24 hours, with shaking at 150 rpm. Following centrifugation at 3000 rpm for 15 minutes, the pellet was washed twice with phosphate buffer. Cell pellets were then suspended in phosphate buffer containing 0.5% formalin (pH= 7.2) and were kept at 4°C for 18 hours. The precipitate were centrifuged at 3000 rpm for 15 minutes and washed with phosphate buffer. Finally, the cells were dried using cold acetone by ten times the sample volume [18].

B. Lipopolysaccharide Extraction

The *Proteus mirabilis* LPS was extracted from the MDR isolate by the hot EDTA method given by [19].

Partial Purification of LPS by Gel Filtration (Sephacryl S-300):

Sephacryl S-300 gel was primed according to the information of the manufacturer company (Sigma, Germany). The LPS extraction was subsequently washed and suspended in 0.025 M of phosphate buffer (PB) (pH 7.2), degassed by using a vacuum pump, then poured with care (to avoid bubbles) into a column with dimension of 75×2 cm. The final volume of the column was 235.5 cm³. The column was equilibrated with 0.025 M of phosphate buffer saline (PBS) (pH 7.2) and the flow rate was 75 ml/hour. The extracted sample was chemically analyzed to define the content of carbohydrate according to [20] and total protein according to [21]. Sterility was determined by culturing each vaccine on blood and nutrient agar. In order to assess safety, 3 rats were injected with 1 ml of each vaccine for 1 week and monitored. Finally, the toxicity of LPS and LPS-Liposome was detected according to [22].

Determination of LD₅₀ of Bacterial Suspension

Four groups of male rats with five replicates for each group were injected I.P. with 1 ml of bacterial suspension in one of (10⁶, 10⁷, 10⁸, 10⁹, 10¹⁰) CFU/ml dosages, respectively. The control groups were injected with PBS. Live and dead rats were estimated proportionally to total number of rats for each dose, after 5 days. Lethal dose was estimated according to [23].

Determination of LD₅₀ of LPS

The lethal dose 50% of LPS was determined by injecting six groups, with five rats in each group. Rats in each group were injected intraperitoneally with 1ml of (100, 150, 200, 250, 300, 400, 500 µg/rat) rates, respectively, while control groups were injected with PBS. The detection of LD50 was calculated using the Karber method [24].

Experimental Design

Laboratory Animals

Albino Swiss rats were supplied by Pharmaceutical control – Baghdad. Their age was 8-9 weeks, and their weight was 23-27 g. They were held in a quiet, air-conditioned room wherein the temperature was 30±5°C, and the light/dark periods was of 10/14 hours/day. The animals were housed inside plastic cages with hardwood chips

for bedding, and maintained on a special pellet diet, with free access to drink water during all experiments. They were given a week's time to get acclimatized to laboratory conditions before experimentation.

A. Immunization of Rats

The 36 rats were divided equally into six groups and immunized I.P. with either 1ml of 0.1 LD50% of LPS or LPS+ LIP or LPS + CFA vaccine or LIP bacterial suspension. Normal saline was used as control. Injection was given twice (1st and post dose) at 14 days intervals. At the end of each period, 3 rats were killed from each group and blood samples taken. Each sample was divided into two parts: one kept in ethylene diamine tetra acetic acid (EDTA) tubes in order to evaluate CD 14 by flow cytometry, and the second for Sera separation, in which case it was stored at -20°C until being quantified for TLR-4 level by ELISA as per manufacturer's instruction (Elabscience, USA).

B. Determination of Surface CD14 Expression by Flow Cytometry

Monocytes and macrophage surface CD14 were examined by flow cytometry (Mindary Medical International Limited, North America) by using Fluorochrome (Dako, U.K.) and conjugated monoclonal antibodies (Dako, U.K.). Here, 50 µ of anticoagulant whole blood was added to the bottom of a polystyrene tube, along with conjugated antibody, as per manufacturer's instruction, then vortexed and incubated in the dark at room temperature for the time specified. After this, 100 µ of reagent A was added to each sample and the tubes were re-vortexed and incubated for 10 min in the dark at room temperature. Subsequently, 1 ml of reagent B was added to each sample. This was then re-vortexed and incubated for 20 min in the dark at room temperature. Following this, the sample was centrifuged at 300 xg for 5 min, the supernatant poured off and the pellet re-suspended in 1 ml of PBS. The sample was then centrifuged once more at 300 xg for 5 min, the supernatant poured off and the pellet resuspended in 300 ml of PBS (this should contain paraformaldehyde for preservation if the samples are not analysed the same day). Finally, the samples were analyzed through flow cytometry, using WinMDI for analysis software.

Ethical Approved

This study was approved by the ethical and research committee of College of Medicine, University of Kufa, Ministry of High Education and Scientific Research.

Statistical analysis

The results are presented as means and statistical with standard error (S.E.) and were analyzed using one-way analysis of variance (ANOVA) test via Graphpad prism 5.04. $p < 0.05$ was considered significant.

RESULTS

1. Characterization of *Proteus mirabilis*

The results showed that 188 specimens (94%) out of 200 urine specimens gave positive results for bacterial growth on MacConky agar. Among the positive growths and depending

on the characteristics ascertained by means of microscopic, morphological, biochemical and Vitek-2 system examination, only 50 isolates were found to belong to *P. mirabilis*. Of these, 11 (22%), 30 (60%) and 9 (18%) were recovered from recurrent UTI – 67 (35.6%), kidney stone – 78 (41.4%) and catheter – 43 (22.8%) samples, respectively. The remaining isolates 138 (73.4%) showed the growth of *P. vul.* – 18 (13%), *Klebsiella* spp. – 60 (43.4%), *E. coli* – 52 (37.6%) and *Pseudomonas* spp. – 8 (5.7%).

2. Extraction of Lipopolysaccharide from the chosen isolate

The isolate that gave highest resistance to antibiotics was isolated from a recurrent UTI patient. The method used to extract LPS yielded a bacterial mass of 20 gm dry weight bacteria and obtained 130 mg of crude lipopolysaccharide.

3. Partial Purification

The results of chemical analysis of LPS in 1 ml of crude and partial purified LPS showed that the carbohydrate concentrations were 159 µg/ml and 230 µg/ml, respectively, while protein concentrations were recorded as 40 and 23 µg/ml, respectively.

Estimation of lethal dose LD₅₀ of live bacterial

The results of LD₅₀ assessment for *P. mirabilis* live suspension were 4.5×10^7 CFU/ml. The estimation was done by calculating the number of dead and alive rats in each group after 5 days (Table. 1).

$$\text{Herein, } LD_{50} = 10^{7+0.66} = 4.5 \times 10^7 \text{ CFU/ml.}$$

Table 1. LD₅₀ of *P. mirabilis* live suspension

Con. of bacteria	Total no. of rats	Dead Rats	Living Rats	Accumulated number of living and dead Rats				
				D	S	D+S	D/D+S	%mortality
1010	5	5	0	14	0	14	14/14	100
109	5	4	1	9	1	10	9/10	90
108	5	3	2	5	3	8	5/8	62.5
107	5	2	3	2	6	8	2/8	25
106	5	0	5	0	11	11	0/11	0

The procedure was used for estimating the cytotoxicity of *P. mirabilis* lipopolysaccharide and the results revealed that the LD₅₀ was 270 µg/ml (Table 2).

$$\text{Herein, } LD_{50} = \text{Least lethal dose} - (\sum a*b/n); LD_{50} = 500 - (1150/5) = 270 \text{ µg/ml.}$$

Table 2. LD₅₀ for LPS extracted from *P. mirabilis*

Group	Dose (µg/ml)	Dose difference a	Dead	Mean b	Product a • b
1	500		5		
2	400	100	4	4.5	450
3	300	100	3	3.5	350
4	200	100	2	2.5	250
5	100	100	0	1	100
6	50	50	0	0	0
7	control	0	0	0	
Total					T=1150

4. Evaluation the TLR4 serum rate level in response to different types of immunogenic response

The results clearly indicated that the TLR-4 concentration was distributed differently in each of the investigated groups of rats as shown in Figure 1. In the first dose, the TLR4 concentration was higher in the vaccinated groups LPS+CFA and LPS, and was followed up by the LPS+ liposome and bacteria groups. The highest increase of TLR4 concentration was observed in LPS and LPS + ACF in post-dose immunization, while moderate elevation appeared in LPS + Liposome and no significant increased was seen in mean of bacteria and liposome, when compared with mean of control for the first and second dose.

5. Frequency of CD14 in the treated rats, as compared with control

CD14 showed diverse distributions in the investigated groups of rats (Table 3). Following the second dose, the highest CD14 was noted in LPS and LPS+CFA (0.78

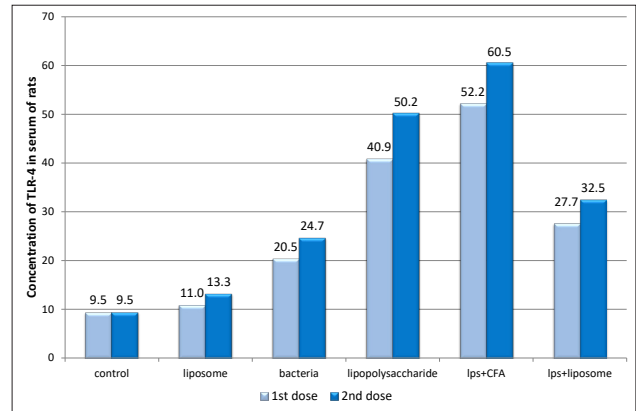


Figure 1. The effect of different types of vaccines in stimulating production of TLR-4 in treated rats

and 0.96 cells/cu. mm blood, correspondingly), followed by LPS+LIP and bacteria (0.49 and 0.30 cells/cu. mm blood), while the liposome group increased non-significantly compare with control (Fig. 2).

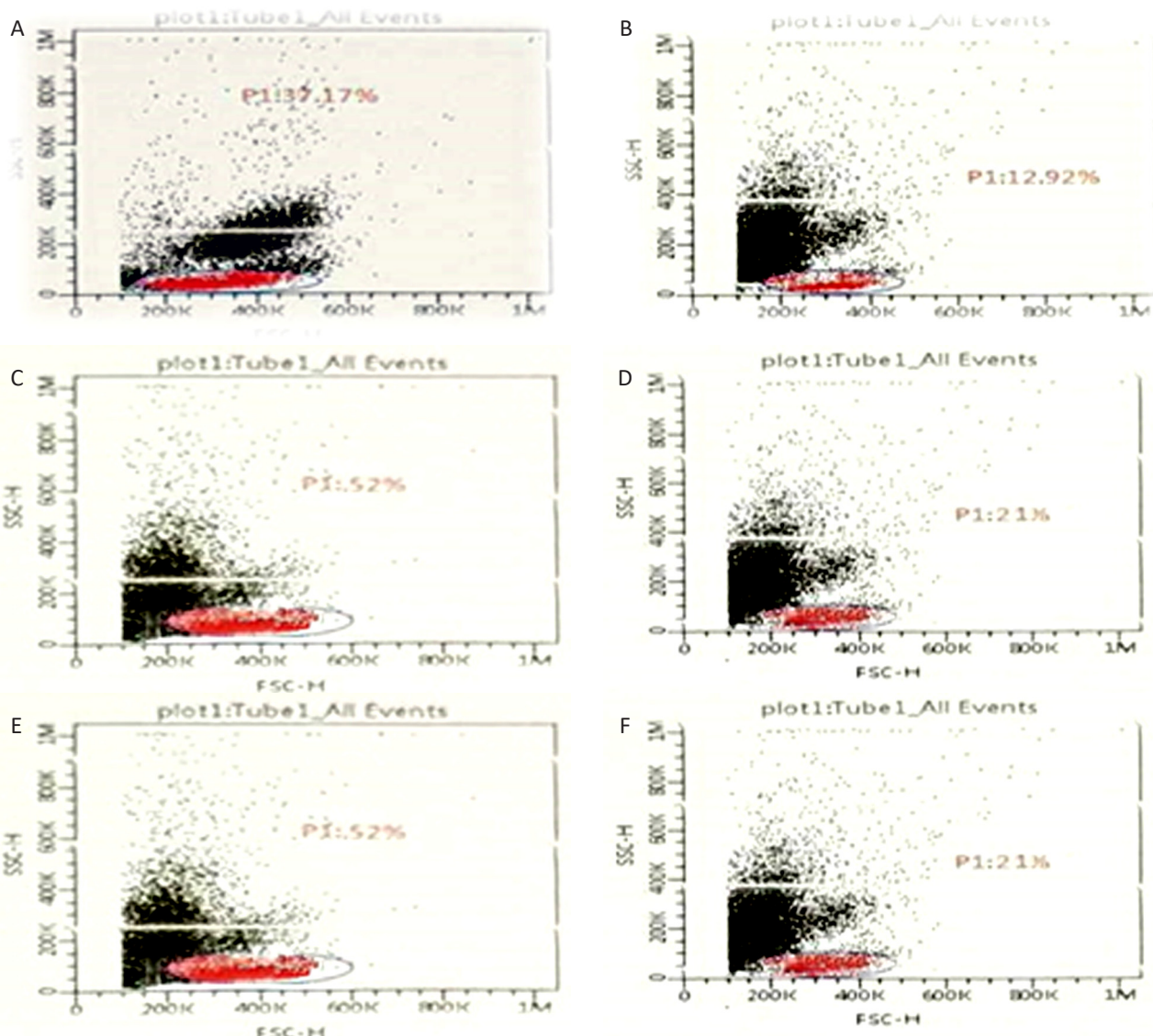


Figure 2. Flow-cytometric Analysis for CD14 Detection on monocytes in rats treated with: A – bacteria, B – Control, C – LPS, D – liposome, E – LPS+ liposome, F – LPS+CFA

Table 3. Frequency of CD14 by Flow Cytometry for vaccinated groups in comparison with control

Flow-cytometry	Group	N	mean	LSD
CD14	control	3	0.10 ± 0.04	0.18
	liposome	3	0.16 ± 0.07	
	Bacteria	3	0.30 ± 0.13	
	LPS	3	0.78 ± 0.28	
	LPS+ CFA	3	0.96 ± 0.30	
	LPS+ liposome	3	0.49 ± 0.21	

DISCUSSION

In this study, the isolate of *P. mirabilis* that gave high resistance to antibiotics according to the VITEK-2 Compact system results, was isolated from a recurrent UTI patient. The observed bacterial resistance was found to correlate to changes within the lipopolysaccharide. This is thought to act as a permeability barrier, making the outer membrane relatively impermeable to antibiotics or detergents, and any mutations in the LPS biosynthetic apparatus increase bacterial resistance to such agents [25]. Our work showed that extraction of LPS from antibiotic-resistant disease-causing organisms can be used to eradicate hazardous pathogens.

The results of extracted LPS demonstrated that the present engaged method yielded a bacterial mass of 20 gm dry weight bacteria, from which 130 mg of crude lipopolysaccharide was obtained. This is in agreement with Chandan and Fraser [19], who found that the EDTA method is a simpler approach with higher yield than the phenol method. The chemical analysis of LPS of 1 ml of crude and partial purified LPS showed that the carbohydrate concentrations were 159 µg/ml and 230 µg/ml, respectively, while protein concentrations were 40 and 23 µg/ml, respectively. These results nearly agree with [26], who ascertained that LPS extracted from *P. mirabilis* was at 153 µg/ml and 221 µg/ml in crude extract and in partial purified form.

Our investigation clearly indicates that the concentration of TLR-4 was distributed differently in each investigated group of rats. These results are consistent with the findings of Lan *et al.* [27], who revealed that serum TLR1 and TLR4 levels were robustly increased in pneumonia patients. Similarly, Ghosh and Bishayi [28] observed a significant increase in the level of TLR-4 protein expression in situations of endotoxaemia. Of note, LPS is known to activate immune cells to generate a diversity of host responses to abolish invading gram-negative infections. However, in higher concentrations, it generates significant morbidity by its ability to stimulate shock, fever, and severe inflammatory reactions [29].

Watanabe [30] discovered that LPS-liposomes carry LPS into the cell more efficiently and serve as safe and effective immune adjuvants. Moreover, he noted that the LPS-liposome can encapsulate antigen and stimulate adaptive responses more effectively than LPS plus antigen. In contrast, LPS-liposome did not stimulate high-minded inflammatory cytokines since LPS is highly concerted in the liposomes and the more complete integration of LPS into liposomes decreases its toxicity.

Moreover, a local study by Abid and Alwan [37] of an endocarditis patient that was infected with G-ve, indicated that the infection increased the mean of T regular (CD4, CD25 and Foxp3) cells in comparison to established norms, Furthermore [38], found that the median CD64 neutrophil and CD69 lymphocytes expression was significantly higher among sepsis patients infected with G-ve bacteria groups when compared to healthy controls.

CFA also induces the activation of different TLRs, leading to diverse cellular and cytokine responses that highly alter both the humoral and cellular responses [31,32]. In our study, CD14 showed varied distributions in the investigated groups of rats, with regard to this, [33] showed that the expression of CD14 increased in the bronchial epithelium and in some macrophages after inhalation of LPS. Herein, [34] noted the enhanced effect of LPS in heightening the secretions of CD14 (membranous and soluble CD14). This effect came earlier after a second LPS dose. What is more, [35] revealed that encapsulation of the core LPS extracted from *E. coli*, *Bacteroides fragilis* and *Pseudomonas aeruginosa* into liposome greatly reduces the capability of a given amount of LPS to stimulate tumor necrosis factor-alpha (TNF-alpha) production in vitro from human monocytes. He then suggested that the signaling of liposomal LPS can be partially restored by soluble CD14 or, to a minor extent, by lipopolysaccharide binding protein.

Hence, liposomal LPS demonstrates characteristics appropriate for utilization as a vaccine to be used in humans, and the incorporation of LPS into the liposome enhances the adjuvanticity of the liposome and increases vaccine efficacy in inducing appropriate immune response at moderate levels rather than hyper-stimulation. With regard to this, [36] confirmed that liposome offers higher protection than a vaccine containing the same components in an emulsion formulation. These results attest that bacterial infection increases the expression of multiple CD, which in turn enhances the immune response against infection.

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