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Recombinant outer membrane protein Q and putative lipoprotein from *Bordetella pertussis* inducing strong humoral response were not protective alone in the murine lung colonization model

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Abstract: Despite high vaccination coverage after introduction of whole cell (wP) and acellular pertussis (aP) vaccines, pertussis resurgence has been reported in many countries. aP vaccines are commonly preferred due to side effects of wP vaccines and formulated with aluminum hydroxide (Alum), which is not an effective adjuvant to eliminate *Bordetella pertussis*. Low efficiency of current aP vaccines is thought to be the main reason for the resurgence for which newer generation aP vaccines are needed. In the present study, immunogenicity and protective efficacy of outer membrane protein Q (OmpQ) and a putative lipoprotein (Lpp) from *B. pertussis* were investigated in mice by using two different adjuvants, monophosphoryl lipid A (MPLA) or Alum. OmpQ and putative Lpp were cloned, expressed, and purified from *Escherichia coli.* The proteins were formulated to immunize mice. Both recombinant OmpQ and putative Lpp induced a significant increase in immunoglobulin G1 (IgG1) and immunoglobulin G2a (IgG2a) responses compared to the control group. Moreover, MPLA-adjuvanted formulations resulted in higher IgG2a levels than Alum-adjuvanted ones. However, there were no significant differences between test and control groups regarding interferon-gamma (IFN-γ) levels, and the mice lung colonization experiments indicated that neither rOmpQ nor rLpp could confer protection alone against *B. pertussis* challenge.

Key words: Adjuvants, *Bordetella pertussis*, pertussis vaccines, recombinant proteins

1. Introduction

Pertussis (whooping cough) is a highly contagious respiratory tract infection, the causative agent of which is a gram-negative, nonspore-forming, and encapsulated coccobacillus called *Bordetella pertussis* (Finger and von Koenig, 1996). The disease is common among infants and children, but increased incidence rate in older ages has also been reported, suggesting that adolescents and adults can serve as a reservoir to spread the pathogen to infants who are too young for vaccination but suffer from severe complications including death in rare cases (Mooi et al., 2007; Hewlett et al., 2014; Sealey et al., 2016). Despite high vaccination coverage (86% in 2015), 142,512 global pertussis cases were reported in 2015 and it is still an important public health problem due to pertussis outbreaks (Burns et al., 2014; WHO, 2017).

Whole cell pertussis (wP) vaccines combined with tetanus and diphtheria toxoids (DTwP) confer protection mainly through CD4+ T helper (Th) 1 cells, Th17 cells, interferon gamma (IFN-γ), and tumor necrosis factor (TNF-α), all of which provide effective clearance of *B. pertussis* (Edwards, 2014; Edwards and Berbers, 2014). Although the protective capacity of DTwP is quite efficient, it has already been replaced with acellular pertussis (DTaP) vaccines due to safety concerns (Warfel and Edwards, 2015). Several DTaP vaccines have been produced with 2 to 5 virulence factors of *B. pertussis* including pertussis toxins (PT), pertactin (PRN), fimbriae (Fim 2 and 3), and filamentous hemagglutinin (FHA) (Edwards et al., 1995). Variations due to polymorphisms have been reported in some virulence factors found in aP vaccines, possibly resulting in differences in antibody efficiencies and immunological memory against the disease (Mooi et al., 1998, 2009; King et al., 2001; Lam et al., 2012). Comparative whole-genome sequence analyses of dozens of *B. pertussis* strains from Finland, China, and the Netherlands indicated that evolution in this pathogen has been a major driving force (Xu et al., 2015). Thus, as already emphasized (Tefon et al., 2013), discovery of new protective antigens has been a widely accepted strategy in recent years.

It is well known that the Th1-type response, which mediates cellular immunity, is required for bacterial

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clearance in pertussis infection in addition to the Th2 response, which mainly induces antibody production from B cells. In particular, IFN-γ produced by Th1 cells is an essential element having a role in the prevention of bacterial spread (Fedel et al., 2015). Besides the antigen itself, adjuvants also play an important role in stimulation of the immune response against antigens. Current DTaP vaccines are adjuvanted with aluminum hydroxide (Alum), which predominantly helps to induce Th2-type immunity that is not as effective as Th1 response in clearance of *B. pertussis* (Edwards and Berbers, 2014). Therefore, substituting Alum with new adjuvants such as a TLR agonist is required to enhance protective immunity (Ross et al., 2013).

In immunoproteomic studies on *B. pertussis* conducted in our laboratory (Altındiş et al., 2009; Tefon et al., 2011), outer membrane protein Q (OmpQ) and a putative lipoprotein (Lpp), BP2919, were among immunogenic surface proteins of the pathogen. Subsequent metareverse vaccinology analysis of our data (Emrah Altındiş, personal communication) in pangenomic databases suggested the potential utility of these proteins as vaccine component candidates, prioritizing them for experimental testing. Protective immunogenicity of outer membrane proteins and lipoproteins has been demonstrated in several microorganisms including *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Pasteurella multocida*, and *Escherichia coli* (Mansouri et al., 1999; Fletcher et al., 2004; Wu et al., 2007; Okay et al., 2012; Guan et al., 2015). The present study aims to investigate immune responses to recombinant OmpQ and putative Lpp from *B. pertussis* and their protectivity in a mouse model by using Alum- and monophosphoryl lipid A (MPLA)-adjuvanted formulations of these proteins.

2. Materials and methods

2.1. Bacterial strains and vectors

B. pertussis Tohama I, a vaccine strain, and Saadet, a local strain in Turkey, were used in the study. Cloning and expression studies were carried out in *E. coli* DH5α (ATCC) and *E. coli* BL21 (DE3) (Novagen, Germany), respectively. While pGEMT Easy (Promega, USA) was used for PCR cloning, expression of recombinant proteins was performed with the pET28a (+) vector (Novagen, Germany).

2.2. Cloning of ompQ and BP2919

The *ompQ* and *BP2919* genes were amplified from *B. pertussis* Tohama I genomic DNA through PCR with specifically designed primers including *BamH*I and *Bgl*II restriction sites. The primers of *ompQ* were as follows: forward primer 5' - ggatccatgcgtcgtcttctcgtc - 3' and reverse primer 5' - agatcttcagaagcgctgggtcattcc - 3'. The primers of *BP2919* were as follows: forward primer 5' - ggatccgtgccccgaatcgcg - 3' and reverse primer 5' - agatcttcagcggcggggcaag - 3'. After PCR, the products were cloned into pGEM®-T Easy in *E. coli* DH5α. The expression vector pET-28a (+) was digested with *BamH*I and the genes were then cloned into pET-28a (+) in *E. coli* BL21 (DE3) (pET28-OmpQ and pET28-Lpp). Ligation reactions contained 500 ng of DNA samples, 2 μL of vector, 1 μL of 10X ligation buffer, 1 μL of T4 ligase, and sterile dH_2O to complete the volume. The mixtures were incubated at 4 °C for 16 h. Verification of the cloned DNA sequences was carried out at RefGen Inc. using the chain termination method (Ankara, Turkey) and the BLAST search of the NCBI website was used to compare the deduced nucleotide sequences.

2.3. Expression of recombinant OmpQ and putative Lpp

Ligation product (10 μL) was mixed with 100 μL of competent *E. coli* BL21 cells. After incubation on ice for 20 min, a heat shock was applied at 42 °C for ~70 s. Then the mixture was incubated on ice for 5 min. After addition of 900 μL of Luria broth (LB; Merck, Germany) and incubation for 80 min at 37 °C, the pellets were obtained through centrifugation. The pellets were dissolved in LB and the cells were inoculated onto LB agar plates supplemented with 30 μg/m: kanamycin (Sigma, Germany).

Stocks of *E. coli* BL21 (DE3) cells at –80 °C transformed with pET28-OmpQ or pET28-Lpp were inoculated on LB agar plates containing 30 μg/mL kanamycin. After overnight incubation, a single colony was selected to be inoculated on 10 mL of LB supplemented with kanamycin. After incubation with shaking for 16–18 h, 3 mL of seed culture was added into two volumes of 150 mL of fresh LB containing kanamycin. The cultures were incubated at 37 °C at 200 rpm until OD_{600} was around 0.6. One culture remained as an uninduced control and the other was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, Germany) at a final concentration of 1 mM for production of the recombinant proteins. After 5 h of induction, the cells were harvested and resuspended in denaturing solubilization buffer (DSB; 1 M NaCl, 50 mM NaH_2PO_4, and 8 M urea, pH 8.0). After 2 cycles of freeze-thaw, the samples were lysed through sonication using a CP70T Ultrasonic Processor (Cole-Parmer, USA) on ice. They were then centrifuged and the supernatants were collected to purify the recombinant proteins.

2.4. Purification of recombinant OmpQ and putative Lpp

Protino Ni-TED 2000 packed columns (Macherey-Nagel, Germany) were used for the purification process. After equilibration with 4 mL of DSB, the supernatants were passed through columns containing nickel ions that the polyhistidine parts of tagged proteins can bind. The columns were rinsed with DSB three times and then the recombinant proteins were eluted with 3 mL of denaturing elution buffer (DEB; 8 M urea, 50 mM NaH_2PO_4, 1 M

NaCl, and 250 mM imidazole, pH 8.0). Dialysis of the solutions containing eluted proteins was performed in dialysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, and 4 M urea, pH 8.0). After filter sterilization with 0.2 μm filters, concentrations of recombinant OmpQ (rOmpQ) and recombinant putative Lpp (rLpp) were evaluated by Bradford assay as described by Ramagli and Rodriguez (1985).

2.5. SDS-PAGE and western blotting

To visualize the purified proteins, 12% acrylamide/bis-acrylamide gels were prepared with a Bio-Rad cell system (Bio-Rad, USA). The gels were stained with colloidal Coomassie blue. Immunogenicity of the recombinant proteins was confirmed through western blotting as described by Altındiş et al. (2009). The antisera against *B. pertussis* Tohama I used in western blot analyses were obtained as previously described (Tefon et al., 2011).

2.6. Vaccine formulations and mice immunization

rOmpQ or rLpp at 40 μg/mL was mixed with Alum (InvivoGen, USA) or MPLA (InvivoGen, USA) for mice immunization experiments. Fifteen BALB/c female mice weighing between 16 and 18 g were used for each group and intraperitoneally immunized with 40 μg/mL rOmpQ-Alum, rOmpQ-MPLA, rLpp-Alum, rLpp-MPLA, or phosphate-buffered saline (PBS) solution as negative control. The injections were performed at day 0 and day 21. All animal experiments were performed under the approval of the Ethics Committee on Animal Experimentation, Middle East Technical University, Ankara, Turkey (METU Etik-2015/10).

2.7. Measurement of antibody levels

Sera were obtained from the tail veins of mice after the first and second immunizations (day 20 and day 30, respectively). Test sera were then used to measure specific immunoglobulin (Ig) G types: IgG1 and IgG2a. rOmpQ or rLpp at 4 μg/well in carbonate/bicarbonate (0.05 M, pH 9.6) buffer was used to coat each well of 96-well microplates. After incubation at 4 °C overnight, the microplates were washed three times with washing solution (1X PBS, 1% Tween 20). Blocking solution (4% BSA, 5% sucrose in PBS) was added to each well and incubated at 37 °C for 1 h. Murine sera diluted from 1:100 to 1:102,400 were added to the wells and the microplates were incubated at 37 °C for 1 h. After washing three times, alkaline phosphatase conjugated rat antimouse IgG1 or IgG2a (Southern Biotech, UK) diluted in blocking solution was added to each well and the microplates were incubated at 37 °C for 1 h. After washing, p-nitrophenyl phosphate disodium salt (Thermo Scientific, USA) was used to develop color detectable at 405 nm. Antibody titers were calculated as the reciprocal of the last dilution that gave a signal.

2.8. Determination of interferon-gamma (IFN-γ) levels

The spleens of three immunized mice from each group were excised at day 30 (before bacterial challenge) and resuspended in RPMI 1640 medium containing 10% FBS (Biochrom, UK) and 1% penicillin/streptomycin (Biochrom, UK). After homogenization, splenocytes were diluted at a concentration of 1×10^6 cells/well and placed into each well of a 96-well microplate. After incubation in a CO_2 incubator for 1 h at 37 °C, the cells were induced with PBS as a negative control, concanavalin A (ConA) (Sigma, USA) as a positive control, or 30 mg/mL rOmpQ or rLpp. After 3 days, the supernatants were collected and IFN-γ level was evaluated with a mouse IFN-γ ELISA development kit (Mabtech, USA) according to the manufacturer's protocol.

2.9. Bacterial challenge of mice

Immunized mice were intranasally challenged at day 31 with the live *B. pertussis* Saadet strain, which is more virulent than Tohama I. A suspension of 50 μL containing 2.5×10^9 CFU was administered to each nostril of the mice.

2.10. Evaluation of bacterial colonization in mice lungs

The lungs of four immunized mice from each group were removed at day 5, 8, and 14, respectively, after bacterial challenge. They were homogenized and diluted in 0.85% saline solution supplemented with 1% casamino acid. Serially diluted samples were inoculated into Cohen–Wheeler agar plates containing cephalexin (40 mg/L) and incubated at 37 °C for 3 to 4 days. The $\log_{10}$ weighted mean numbers of CFU/lung were calculated for each day.

2.11. Statistical analysis

The results were represented as means and standard deviations and they were analyzed through ANOVA along with Tukey's test for comparison of datasets.

3. Results

3.1. Expression and purification of the recombinant proteins

After amplification of the genes and cloning into the pET-28a (+) expression vector, pET28-OmpQ and pET28-Lpp were transformed into *E. coli* BL21 (DE3) and His-tagged recombinant proteins were expressed upon IPTG induction. The recombinant proteins (OmpQ, ~39 kDa; Lpp, ~25.5 kDa) were purified and analyzed by SDS-PAGE after staining with Coomassie blue (Figures 1A and 1B).

3.2. Western blot assays of the recombinant proteins

Immunogenicity of purified rOmpQ and rLpp was analyzed through western blotting by using sera collected from mice subcutaneously immunized with heat-inactivated *B. pertussis* Tohama I. The blots verified the immunogenicity of the recombinant proteins (Figures 2A and 2B). The ca. 60 kDa band seen in uninduced cell lysate most probably corresponds to Hsp60 (GroEL) of *E. coli*. It is known that

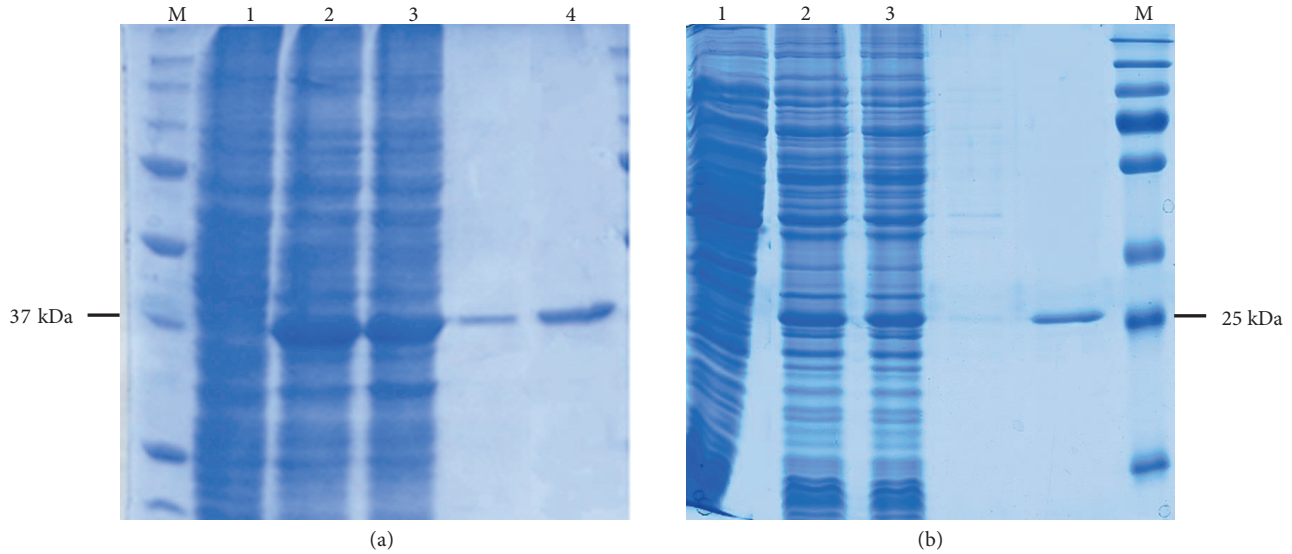

Figure 1. SDS-PAGE of recombinant OmpQ and Lpp. A) Lane 1: Uninduced cell lysate, lanes 2 and 3: isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced cell lysate, lane 4: purified recombinant OmpQ (~39 kDa), M: Precision Plus Protein Unstained Standards, #161-0363. B) Lane 1: Uninduced cell lysate, lanes 2 and 3: IPTG-induced cell lysate, lane 4: purified recombinant Lpp (~25.5 kDa), M: Pageruler Plus Prestained Protein Ladder, #SM1811.

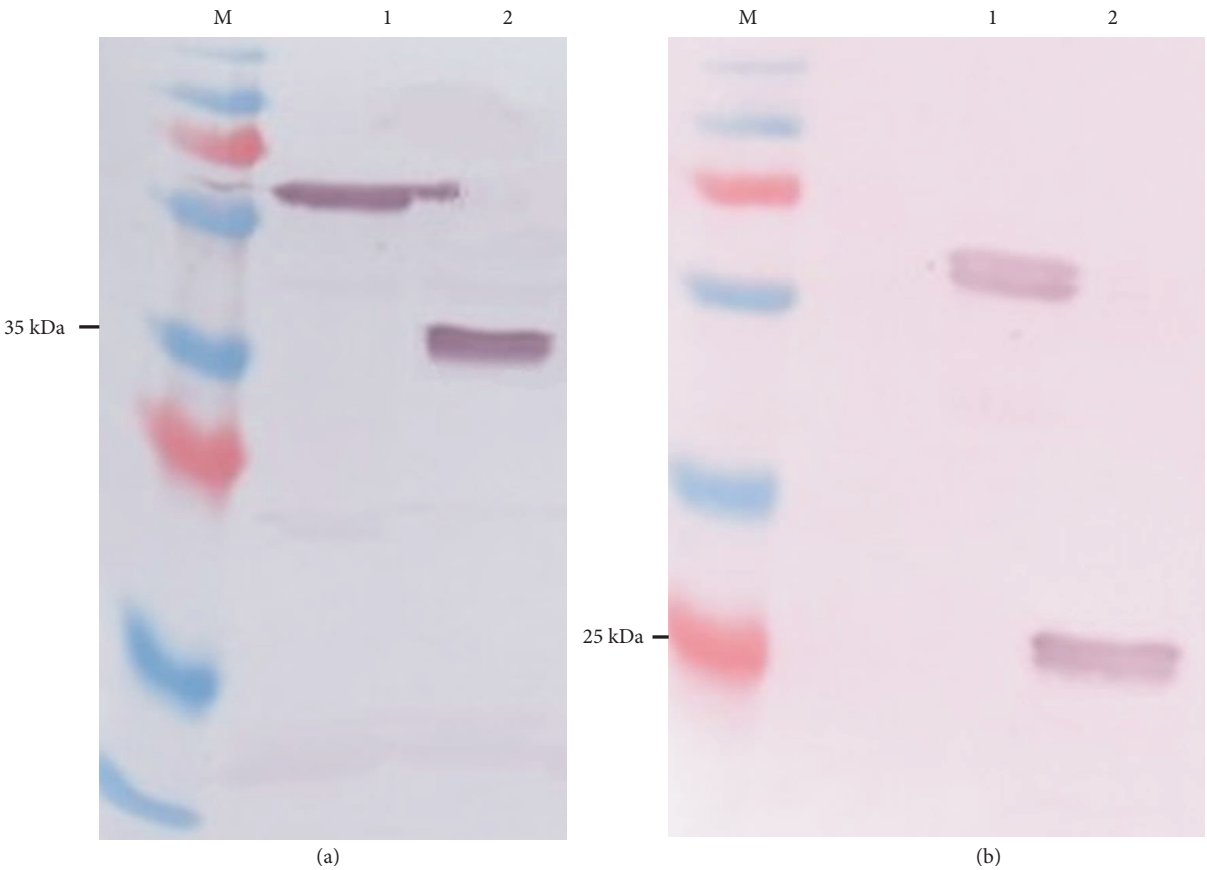

Figure 2. Western blot analyses with antisera against *Bordetella pertussis* Tohama I. A) Lane 1: Uninduced cell lysate, lane 2: purified recombinant OmpQ. B) Lane 1: Uninduced cell lysate, Lane 2: purified recombinant Lpp. M: Pageruler Plus Prestained Protein Ladder, #SM1811.

high sequence similarities are present in Hsp60 proteins among species (Maleki et al., 2016) and cross-reactivity can be observed (Hinode et al., 1998).

3.3. Humoral responses against the recombinant proteins

IgG1 and IgG2a levels were detected via ELISA using the sera from mice immunized with rOmpQ-Alum, rOmpQ-MPLA, rLpp-Alum, rLpp-MPLA, and PBS. Both rOmpQ and rLpp adjuvanted with Alum or MPLA demonstrated strong IgG1 and IgG2a responses, especially after the second vaccinations, when compared to the control groups (Figures 3A and 3B). Moreover, IgG2a levels were clearly higher in MPLA-adjuvanted formulations than Alum-adjuvanted ones.

3.4. Antigen-specific interferon-gamma levels

Splenocyte culture was obtained from the spleens of immunized mice and antigen-specific IFN-γ level was evaluated. The measurement of IFN-γ levels demonstrated that there were no significant differences between negative controls and mice immunized with rOmpQ or rLpp, although a slight but not significant increase was observed in MPLA-adjuvanted formulations (Figure 4).

3.5. Bacterial colonization in the lungs of mice

The lungs of immunized and challenged mice were used to assess bacterial colonization. The results revealed that neither rOmpQ nor rLpp adjuvanted with Alum or MPLA alone induced a significant decrease in bacterial colonization, although immunization with MPLA-adjuvanted rOmpQ resulted in a slight but not significant decrease at day 14 (Figure 5).

4. Discussion

Pertussis is still a public health problem due to pertussis outbreaks in 3- or 4-year cycles although it is a vaccine-preventable disease. In 2015, 142,512 pertussis cases were globally reported even in the presence of a high vaccination rate (86%) (WHO, 2017). In addition to DTwP and DTaP, Tdap vaccines have been developed for adolescents and adults due to pertussis incidence among older ages (Tefon et al., 2013; Sealey et al., 2016). Despite the presence of vaccines and high vaccination coverage, pertussis outbreaks have been observed in many countries including the United Kingdom, the United States, Australia, and the Netherlands (Burns et al., 2014). The disease killed 10 babies in 2010 and a pertussis epidemic was declared with 9935 cases in 2014 in California, USA (CDC, 2014). In 2012, 10,000 pertussis cases were confirmed in the United Kingdom and 14 infants died. In the same year, Minnesota, USA, also experienced a large pertussis epidemic where 4144 pertussis cases were reported (Sealey et al., 2015). It is suggested that many factors contribute to the resurgence of pertussis including increased awareness and improved diagnosis, antigenic variations in circulating isolates, lower efficiency of current aP vaccines, and early waning immunity (Chiappini et al., 2015). Moreover, aP vaccines are adjuvanted with Alum, which is well known to mainly help to induce Th2-type immune response instead of Th1. Th2 response alone is not sufficient to completely eliminate *B. pertussis* that can survive intracellularly; Th1 response is also required in pertussis infection due to its ability to mediate activation of neutrophils and macrophages as well as formation of opsonizing antibodies such as IgG2a (McKee et al., 2007; Allen and Mills, 2014; Edwards and Berbers, 2014). While IgG1 has a role in the neutralization of toxins and prevention of bacterial adherence in the respiratory tract (Barnard et al., 1996), IFN-γ secreted by Th1 cells plays a key role in clearance of *B. pertussis* by enhancing phagocytosis and complement fixation (Higgs et al., 2012).

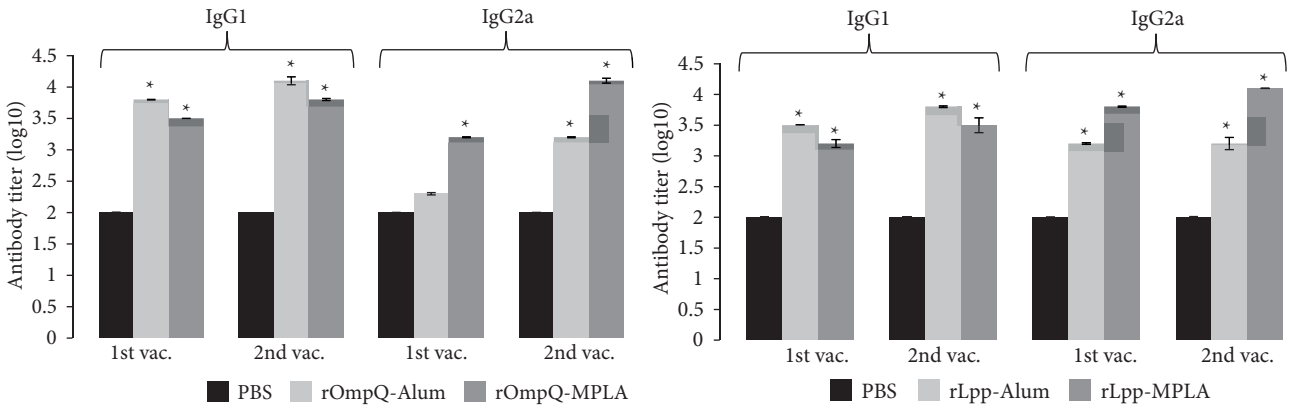

Figure 3. Antibody responses to recombinant OmpQ (rOmpQ) and Lpp (rLpp) formulated with Alum (aluminum hydroxide) or MPLA (monophosphoryl Lipid A). A) IgG1 and IgG2a responses to recombinant OmpQ-Alum and OmpQ-MPLA after first and second immunizations. B) IgG1 and IgG2a responses to recombinant Lpp-Alum and Lpp-MPLA after first and second immunizations. Control mice immunized with PBS were used as negative control. The results are represented as mean ± SD (** $P < 0.05$).

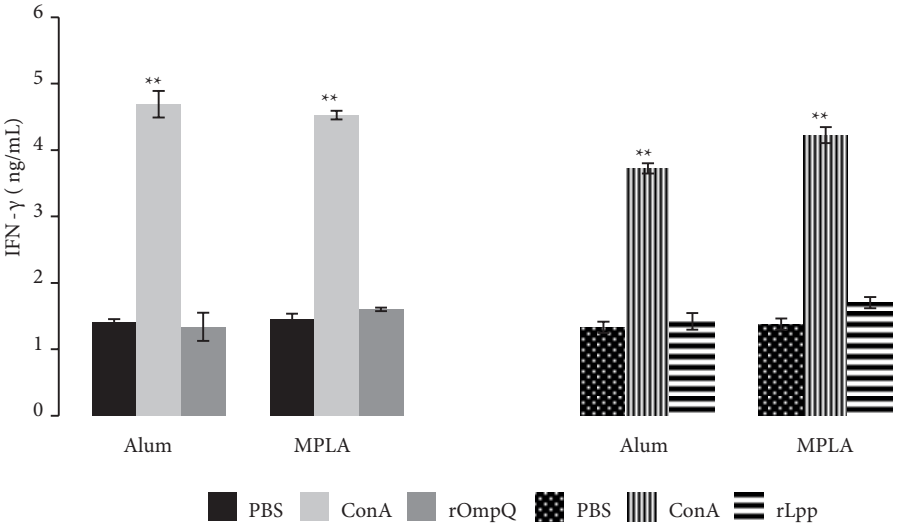

Figure 4. IFN-γ production by spleen cells of mice immunized with recombinant OmpQ (rOmpQ) or Lpp (rLpp) formulated with Alum (Aluminum hydroxide) or MPLA (monophosphoryl lipid A). Spleen cells induced with PBS and ConA were used as negative and positive controls, respectively. The results are represented as mean ± SD (** $P < 0.01$).

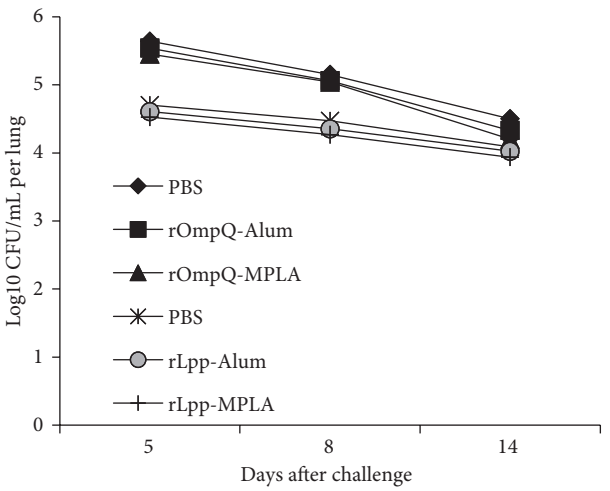

Figure 5. Mean numbers of CFU/mL per lung of groups of mice intraperitoneally immunized with recombinant OmpQ (rOmpQ) or Lpp (rLpp) formulated with Alum (aluminum hydroxide) or MPLA (monophosphoryl lipid A). Control mice immunized with PBS were used as negative control. The results are presented as mean CFU/mL per lung ± SD.

Studies have mainly been focused on improving current aP vaccines by taking two common approaches into consideration: discovery of novel vaccine antigens from *B. pertussis* and development of new vaccine formulations with more effective adjuvants. In an immunoproteomic analysis, Hayes et al. (2011) identified differentially expressed proteins under iron starvation. Some of the identified proteins were immunogenic and one protein called IRP1-3 strongly reacted with IgG purified from infected individuals. Moreover, it induced strong IgG1 and IgG2a production along with high IFN-γ and IL-10 levels in mice. The authors also demonstrated the protective capacity of another novel antigen from *B. pertussis* called AfuA, which is an iron-binding protein (Hayes et al., 2013). A proteomic analysis of *B. pertussis* biofilm content revealed 11 abundant proteins (de Gouw et al., 2014). When the protective activity of the most abundant protein, BipA, was investigated in mice, opsonization of the pathogen along with a decrease in bacterial colonization in the lungs was recorded. In another study, a novel antigen, iron superoxide dismutase (FeSOD), previously identified by our immunoproteomic studies, was evaluated in terms of immune response and protectivity in mouse models (Yılmaz et al., 2016). It was demonstrated that rFeSOD, especially when adjuvanted with MPLA, induced strong IgG1 and IgG2a production and stimulated production of high levels of IFN-γ along with a significant reduction in bacterial colonization in the lungs of immunized mice. As to the use of new adjuvant systems in aP vaccines rather than Alum, TLR4 ligands such as MPLA can be promising adjuvants because of their capacity to induce activation of Th1 cells, resulting in IFN-γ production and *B. pertussis* clearance (Ross et al., 2013; Fedel et al., 2015).

In the present study, immunizations with rOmpQ or rLpp adjuvanted with Alum or MPLA resulted in a significant increase in IgG1 and IgG2a levels as compared to the control groups. Furthermore, the levels of IgG2a were higher in MPLA-adjuvanted formulations than Alum

ones, suggesting the potential of MPLA as an effective adjuvant for aP vaccines. Although immunization with MPLA-adjuvanted formulations resulted only in a slight and insignificant increase in IFN-γ level, it is known that even a low level of IFN-γ is sufficient to induce class switching to IgG2a (Finkelman et al., 1988), which may explain high IgG2a titers obtained with these formulations. In protection studies related to *B. pertussis* infection, determination of bacterial colonization in the lungs of mice is an effective way to elucidate protective immunity against the pathogen because of the presence of a direct correlation between them (Hayes et al., 2011, 2013). In our study, no significant decrease in bacterial colonization was observed in the lungs of mice immunized with rOmpQ or rLpp adjuvanted with Alum or MPLA, although a slight decrease was present at day 14 in rOmpQ-MPLA immunization. The lack of decrease in bacterial colonization might be related to the low level of IFN-γ as it is crucial for elimination of *B. pertussis* from the lungs. There are examples in the literature of proteins that were found to be highly immunogenic but could not confer protection against the relevant infection, such as P55 of *Borrelia burgdorferi*, gpA of *Pneumocystis carinii*, and human metapneumovirus G protein (Feng et al., 1996; Gigliotti et al., 1998; Ryder et al., 2010). Moreover, in one study, while the vaccination of mice with recombinant autotransporter protein BrkA of *B. pertussis* as the only *B. pertussis* antigen did not protect against colonization by *B. pertussis*, it significantly increased the efficacy of the two-pertussis-component DTaP vaccine (PT, FHA) against *B. pertussis* in a sublethal intranasal murine respiratory challenge model (Marr et al., 2008). Its inclusion led to an antigen combination that was as efficacious in protecting mice as the commercial Infranrix vaccine that also contains PT and FHA but PRN instead of rBrkA. The authors suggested that BrkA is a promising candidate antigen to improve existing aP vaccines for use in humans. Although we did not test the protective efficacy of rOmpQ and/or rLpp when combined in two- or more-pertussis-component DTaP vaccines, our findings do not exclude the protective effectiveness of these proteins since the high amounts of IgG2a antibodies (high IgG2a/IgG1 ratio) induced by rOmpQ-MPLA and rLpp-MPLA are likely indicative of the existence of the Th1 type of immune response. Regarding the dominant effect of PT in aP vaccines (Xing et al., 2014), future experimental studies on new candidate components in mice models should investigate if they cause a significant contribution to the protectivity of the PT component rather than any substitutional effect of them on the other immunogenic proteins contained in commercial vaccines.

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