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ORIGINAL ARTICLE

# Evaluation of immunological responses against outer membrane vesicles (OMV) of nontypeable *Haemophilus influenzae* using MPLA-CpG adjuvant as a vaccine candidate

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**ABSTRACT**

**Background and Objectives:** Nontypeable *Haemophilus influenzae* (NTHi) are major causes of non-invasive infections, including otitis media and sinusitis and it can also contribute to respiratory infections of all ages. Currently, there is no licensed vaccine against NTHi commercially available. Many studies have been conducted on the use of OMV as a vaccine against NTHi. The purpose of this study is to achieve an immunogenic vaccine against NTHi.

**Materials and Methods:** In this study, standard OMV *Haemophilus* (ATCC49766) with adjuncts CpG and MPLA was used and after infusion into BALB/c mice, the levels of antibodies and cytokines were measured on serum of immunized mice.

**Results:** The results showed that total IgG antibody and IgG1 and IgG2a isotypes in OMV immunized mice with mixture of CpG-MPLA adjuvant had a significant increase. Also, the results of cytokines (IL-10, IL-4 and IFN-γ) showed that IL-4 had the highest rate.

**Conclusion:** These findings indicate that OMVs derived from NTHi strains have a high potential to act as a vaccine against NTHi infections.

**Keywords:** Non typeable *Haemophilus influenza*; Outer membrane vesicle; Vaccine

## INTRODUCTION

Nontypeable *Haemophilus influenzae* (NTHi) is one of the most important causes in the development of acute otitis media in children and respiratory diseases in adults (1). From the introduction of conjugated capsular vaccine against the *H. influenzae* type B virus (Hib) in the late 1980s, Hib invasive diseases have been significantly reduced in developing countries, and invasive diseases caused by NTHi strains became prevalent (2). Lack of protective capsule, high antigenic heterogeneity and high levels of changes in many surface antigens have been described as a limitation to produce vaccines against NTHi (3). Therefore, studies regarding the vaccine development have focused on the protection of outer membrane proteins, lipooligosaccharide and pili (4). Bacteria can produce outer membrane vesicles (OMV) during the growth stages. OMVs is a spherical bilayer structure which contains various components such as lipopolysaccharide, outer membrane proteins, periplasmic proteins, cytoplasmic proteins, DNA and RNA. In this regard, outer membrane vesicles (OMV) can be considered as a new vaccine candidate. Due to the various compounds in these structures, acting as carriers of several native bacterial antigenic compounds, these structures have been considered in the development of the vaccine

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(5, 6). Due to the necessity of designing an effective vaccine against non-capsular strains and lack of suitable animal model and considering the importance of OMV in virulence and its role in internalizing, survival of bacteria in intracellular conditions and bacterial binding, and ultimately with the knowledge of the importance of bacterial binding in the pathogenicity (7), in the present study, we attempted to purify the OMVs in bacteria and due to the high lipopolysaccharide (LPS) content in these structures, LPS removal methods have been used.

## MATERIALS AND METHODS

**Bacterial culture.** In order to extract the OMVs, standard strain of *H. influenzae* ATCC49766 was purchased from the microbial bank of Pasteur Institute of Tehran, Iran and grown in the specific blood agar media, containing NAD and Hemin, as well as the BHI medium, in order to achieve the proper cell mass, large-scale culture was grown in the fermenter.

**OMV extraction.** OMV of standard strain of *H. influenzae* (ATCC49766) was extracted as follows. The inactivated Haemophilus cells were centrifuged for 1 hour at 6000 rpm and 4°C. The sediment was suspended in sodium chloride buffer and homogenized for 30 minutes and the wet weight was determined. This suspension was recentrifuged at 6500 rpm for 1 h at 4°C and the pellet was stabilized in a volume, 7.5 times of its wet weight, with a 1.0 M Tris buffer, containing 10 mM EDTA (w/v). The suspension was supplemented with a volume of 1:20 of 0.1 M Tris buffer solution, containing EDTA and 100 g/L sodium deoxycholate. After 10 minutes, the pellet suspended in deoxycholate, then it was separated by ultracentrifuge (Backman L8, 80M) for 1 h at 16500 rpm, at 4°C. Then, cell-free supernatant was centrifuged for 2 h at 42,000 rpm and 4°C. The OMV pellet was dissolved in 3% sucrose, and sterilized, after passing through the Millipore filter of 0.22 microns. After extraction, in order to confirm the OMVs, gel electrophoresis was performed on SDS-PAGE gel. In order to calculate the protein concentration, the Nanodrop and also Bradford assay were used. Then, the LAL kit was used to measure the amount of LPS in the sample and to ensure the removal of the LPS in OMV-LPS free structures. Ultimately, for the final confirmation, using the electron microscope, the uniformity of OMV was evaluated (8).

**Immunization of mice with outer membrane vesicles (OMV).** Fifty micrograms of purified OMV was injected subcutaneously, in three doses (at days 0, 14 and 28), at intervals of every 2 weeks, in a final volume of 100 µl. The mice were divided into four groups of ten, the first group receiving OMV-LPS, the second group receiving OMV-LPS with a mixture of adjuvant CpG and MPLA and the third and fourth groups, as the control group received PBS and adjuvant CpG- MPLA, respectively. Blood samples were taken on days 14, 28 and 42. Then the sera were kept at -70°C for further analysis.

**Evaluation of IgG, IgG1 and IgG2a isotypes in mouse serum.** Total IgG and IgG2a and IgG1 isotypes were measured in mouse serum, by indirect ELISA, using 96- well plates. In brief, 100 µl of PBS at concentration of 10 g/ml at pH = 7.4, was added to each well (1 g/well) and incubated overnight at 4°C. Then, it was washed 3 times with Phosphate Buffered Saline with Tween (PBS-T). Mouse sera were diluted 1:100 and 100 µl was added to the wells and incubated for 2 h at 37°C. After washing, 100 µl of peroxidase-conjugated antibody with 1:100000 dilutions were added to each well and incubated for 90 minutes at 37°C. Following few washing steps, 100 µl of chromogenic substrate (TMB /$H_2O_2$, Razi-institute, Iran) was added to each well, the enzyme activity was stopped, using 1N sulfuric acid (Merck-Germany), and the absorbance was read at 450 nm.

**Evaluation of proliferation in stimulating cells.** The spleens of healthy and infected mice were dissected, stored in suspension in sterile PBS buffer, in cone-shaped polyethylene tubes (Falcon), collected separately for each mouse and stored on ice. The cells were collected by centrifugation at 350 g for 14 minutes at 4°C and washed twice with PBS again. After the last wash, the supernatant was discarded completely and the cells were kept in a small amount of residual buffer in the tube. The hypotonic red blood cell lysis buffer was added to the cellular contents of each tube. After 12 minutes exposure at room temperature, it was neutralized by adding PBS, containing 5% FBS, and then centrifuged at 350 g for 15 minutes at 4°C. The white blood cells in the tubes were washed twice with PBS, containing 5% FBS, and finally suspended in complete RPMI me-

dium, supplemented with 10% FBS, unnecessary amino acids, penicillin-streptomycin and glutamine, and stored at 4-8°C. The number of lymphocytes was counted and uniform suspensions of $4 \times 10^6$ cells/mL with a final volume of 1 ml was cultured in each well, of a 24-well plate in triplicate. The final concentration of 5 µg / mL of the antigen were added to each well. The cultures were kept in an incubator, containing 5% $CO_2$ for 72 h. Then, the supernatant of the cell culture was centrifuged, collected, and stored at -70°C. The IL-10, IFN-γ and IL-4 levels were measured in cell culture, using the mouse cytokine kit (R & D). ConA was also used as a positive control (9).

**Statistical analysis.** Data collected from each series of measurements were collected and archived. The data analysis and comparison were performed, using SPSS software (v.20) and the standard student t-test. The p value <0.05 was considered statistically significant.

## RESULTS

**OMV extraction and purification.** As shown in Fig. 1, the electrophoretic pattern of OMVs was evaluated on 12% gel by comparing with standard molecular weight protein markers. The protein content was measured using Bradford assay as 1.08 mg / mL, and using the LAL kit, the endotoxin level was determined as 0.8 mg/dL. The morphological characteristics of OMV, after passage through different stages of extraction, and purification with a negative contrast staining with potassium phosphotungstate were studied by electron microscopy. As shown, the vesicles have a size of 50-150 nm, and more than 80% of them maintained their natural and spatial shape during the purification stages, and no impurity was observed between the vesicles (Fig. 2).

**ELISA data analysis.** Data presented in the Fig. 3 shows that the difference in the mean optical absorbance of IgG between the OMV-LPS receiving groups with a mixture of CpG-MPLA adjuvant, was significantly different, compared to the control group (P <0.0001). Optical absorbance in the groups receiving OMV-LPS with a mixture of CpG-MPLA adjuvant, shows a significant increase, compared to the other groups (P <0.0001). It was also found that IgG1 levels were higher in all groups, compared to IgG2a, and this was significantly higher in the OMV-LPS + MLPA-CpG group, compared to the other groups (P <0.0001) (Fig. 4). Meanwhile, according to the results obtained from the antibody isotypes, the ratio of IgG1 to IgG2a in the OMV-LPS + MLPA-CpG receiving group has a range of 1.3 to 2.54, which can

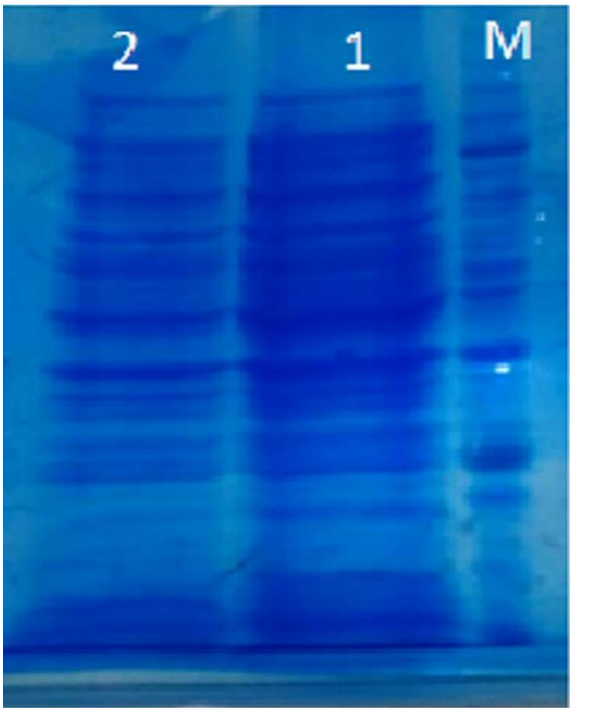


**Fig. 1.** The 12% SDS-PAGE, representing OMVs from *H. influenzae* (ATCC49766); column one and two have different concentrations of OMV. Columns 1 and 2 have different concentrations of OMV of *Haemophilus* bacteria.

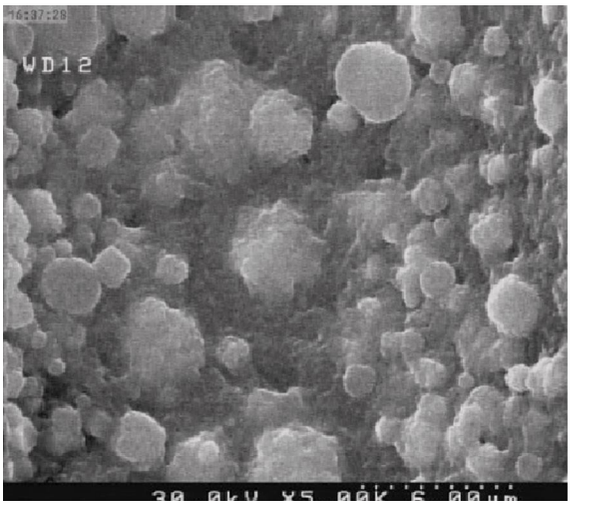


**Fig. 2.** Electron micrograph of vesicles prepared from an OMV of standard strain of *H. influenzae* (ATCC49766)

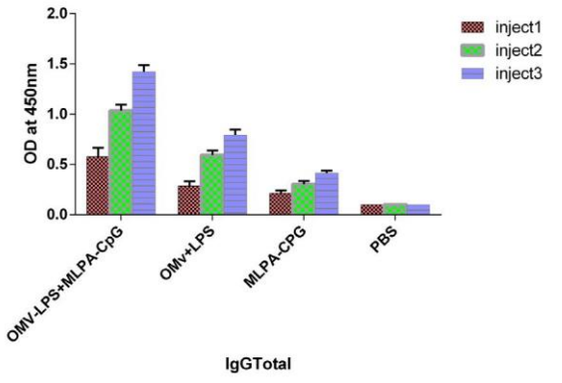


**Fig. 3.** After Immunization of BALB/c mice with different antigens which prepared in 1:1000 dilution, we evaluated immune responses by ELISA technique. The results are presented as the mean of optical absorbance at 450 nm.

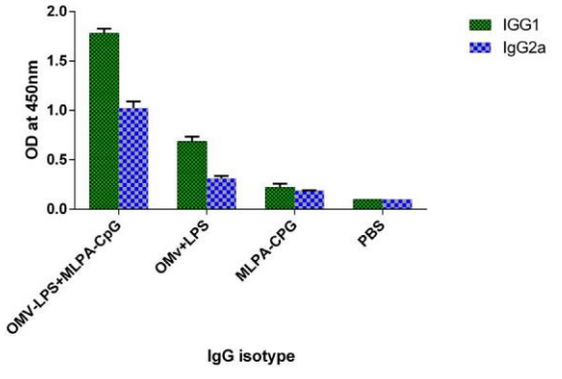


**Fig. 4.** Evaluation of subclass antibody responses in immunized BALB/c mice carried out after the booster injection by ELISA technique. The results are based on an average of optical absorbance at 450 nm.

be an indication of overcoming humoral immune response to a cellular response.

**Cytokine assay.** Two weeks after the third injection on day 42 and after re-stimulation with the antigens for 3 days, the level of cytokines, such as IL-10, IL-4 and IFN-γ was measured in the supernatant of the spleen cell culture, using ELISA. The evaluation of IFN-γ (as an indicator of the activity of Th1 lymphocytes), IL-4 (as an indicator of the activity of Th2 lymphocytes) and IL-10 (an anti-inflammatory and regulatory cytokine that inhibits the secretion of cytokines, such as IFN-γ showed that the level of the three cytokines tested in the OMV-LPS group with a mixture of CpG and MPLA adjuvant were increased, compared to the other groups, but in comparison with the positive control group *(conA)*, they show a decrease level of cytokine (Fig. 5).

Therefore, it can be concluded that OMV-LPS, with a mixture of adjuvant CpG and MPLA, has been able to properly stimulate immune responses and increase the amount of cytokines involved. Considering the significant increase in both cytokines, IFN-γ and IL-4, it can be suggested that a mixture of cellular immune responses (Th1) and humoral immunity (Th2) have been established, but because of high levels of IL-4, compared to interleukin 10 and IFN-γ, immune responses tend to be more toward humoral immunity (Th2) (Fig. 5d).

## DISCUSSION

Although, the non-typeable *Haemophilus* (capsule-free) acts as a normal flora of the respiratory tract, some serotypes are causing infections such as otitis media, pneumonia, as well as chronic obstructive pulmonary disease (COPD) (10). COPD as a major public health problem is mostly observed in newborn infants and one third of children will be expected to die per year, by 2020 from this devastating disease (11). Due to the loss of protective capsules in non-capsular strains, as well as the presence of variable phenotypes and genotypes, effective and potent vaccine against NTHi strains is not available yet, and according to reports of aggressive diseases and increased drug resistance against these strains, it seems necessary to make an effective vaccine against these strains (9, 12).

Initial vaccines against Hib that contained only PRP, due to their physical and chemical nature, could not stimulate cellular immunity and T lymphocytes and were classified as independent T-cell antigen (13); thus, they were not able to provide immunity against meningitis infection caused by *Haemophilus* in infants. *Haemophilus* vaccines, based on the capsular polysaccharide type b, *H. influenzae* conjugated to diphtheria toxoid, tetanus toxoid or protein in the outer membrane of *Neisseria meningitidis*, were effective in reducing meningitis caused by Hib, but they were not effective against other capsular serotypes and non-capsular strains of *H. influenzae*. In addition, infectious diseases caused by non-capsular strains of *H. influenzae* are also increasing worldwide, which are considered as one of the most im-

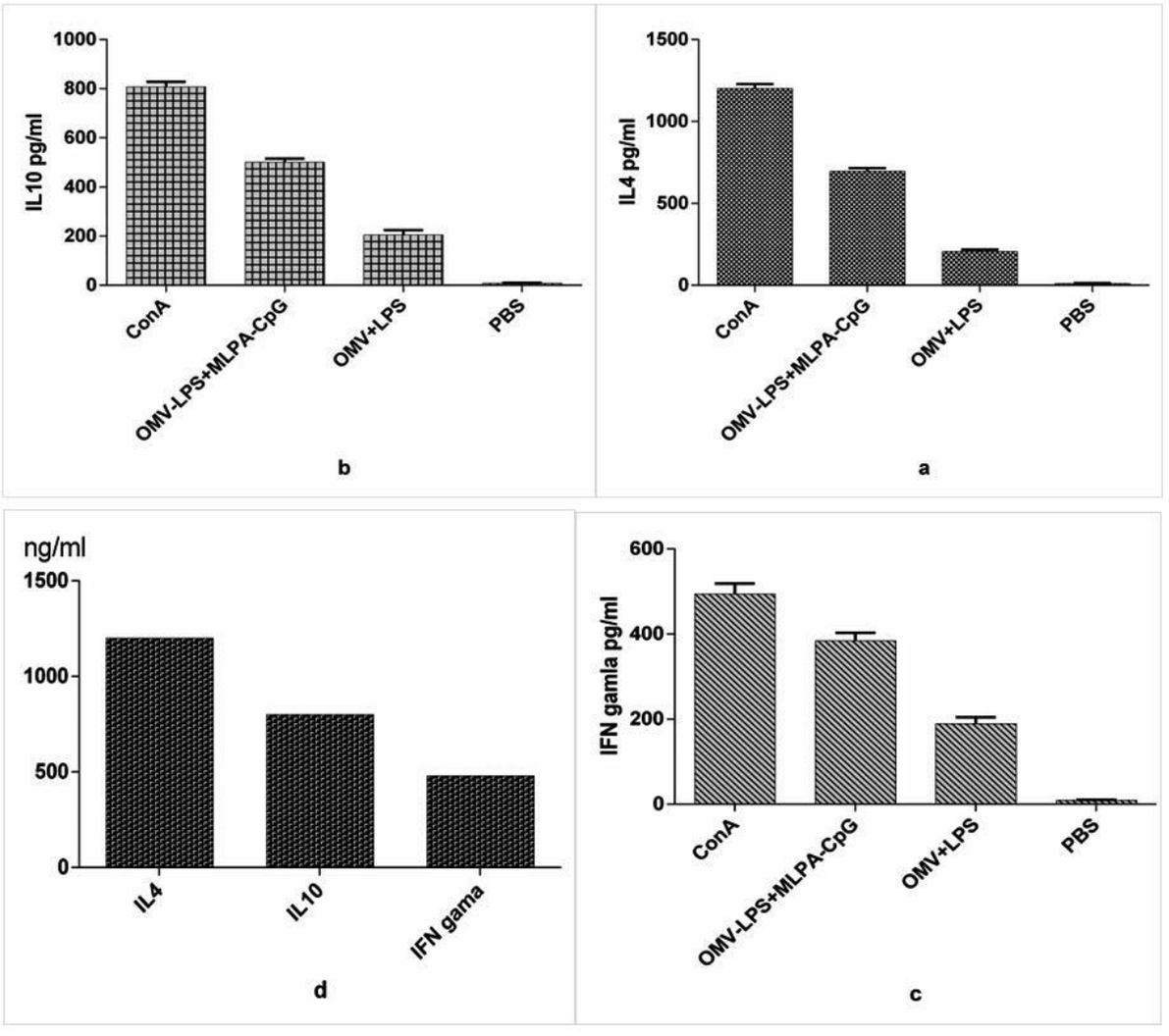


**Fig. 5.** Data regarding measurement of cytokine responses in mouse cells, stimulated with the antigens. Evaluating the response of IL-4 two weeks after the third injection in mice showed an increase, compared to the level of IL-10 and IFN-γ. In addition, OMV-LPS + MLPA-CPG receiving groups showed higher cytokine level, compared to the other groups.

portant pathogens in the human respiratory system (14).

Currently detoxified LOS is used as a conjugated molecules with different carrier proteins such as a tetanus toxoid and it can lead to increasing immune responses (14, 15). Recent studies have shown that P6 is one of the protected outer membrane proteins, but it is not protected in all NTHi strains and may not be exposed to the surface, so focusing on antigen alone may be a suitable approach to make an effective vaccine against strains of NTHi (16, 17). Instead, the presence of several heterologous antigens in the immune system can increase the effectiveness of the vaccine against NTHi strains (3, 18). In this context, OMVs can be considered as a new candidate for a vaccine. Due to the presence of various compounds, such as outer membrane proteins, lipopolysaccharide, peptidoglycan and the recent discovery of DNA and RNA in the OMV structure, it can be stated that OMV is a carrier of several native bacterial antigens and is therefore important for the development of the vaccine (9, 19). The significant properties of membrane antigens and their surface exposure in OMV have led to the confirmation of structural, physicochemical stability and the protective and genomic properties of OMV in many bacteria. OMV is involved in the formation of biofilms, binding to the host cell, intra and intercellular transmission, antibiotic resistance, competitive death responses among microorganisms, response to stress and the maturi-

ty of immune cells (9, 20). Considering the studies performed in these regards, and the role of OMV in binding and the increased pathogenicity of *H. influenzae*, and the possibility of stimulating the immune system; in the present study, we decided to use this structure as a new vaccine candidate, and to examine the protective immune responses and also, to evaluate the cytotoxicity effect in the presence of these structures.

The secretion of OMV by NTHI strains and the presence of 142 proteins have been confirmed in the 86-286NP strain. Because the pathogenesis of *H. influenzae* begins with the establishment in the nasopharynx, and then the organism spreads to other parts, including the middle ear, sinuses, and so on; thus by preventing the initial binding of bacteria to the host cell surface, the local infection, and ultimately, its dissemination to other parts of the body may also be prevented. Many studies emphasize on the role of OMV in connection caused by *H. influenzae*, which makes the hypothesis of being a vaccine candidate, much stronger (21, 22). Today, it is known that most adjuvants perform their own actions by mimicking pathogen-associated molecular patterns (PAMP) molecules, such as lipo-polysaccharide, compounds in the bacterial wall, dsrRNA, ssRNA, and DNA containing non-methylated CpG, in pathogens. As the immune system evolves to identify specific antigens, the mixture of an adjuvant with the vaccine enhances the acquired immune responses to the antigen, by enhancing the function of DCs, macrophages, lymphocytes, as a mimic of natural infection (23). Thus in this study, antibody and cytokine responses against OMV were evaluated in the presence of a mixture of CpG and MPLA adjuvants, and compared with other groups.

The results of this study indicate the ability to stimulate the humoral response and to induce cytokines such as IFN-γ and IL-10, IL-4 from lymphocyte against *Haemophilus* strain. Antibody levels indicate that the antigen used during the host mouse infection significantly interacts with the immune system and its level in the bacterial cell is so high that it can detect a significant amount of antibody. The induction of cytokines shows the power of this antigen in stimulating responses, and, on the other hand, the high level of IL-4 response indicates that this antigen is involved in inducing and enhancing humoral immune responses. Therefore, it seems that the use of this immunogenic antigen that can stimulate humoral and cellular immune responses may be useful in studies of *Haemophilus* vaccines.

In this study, in order to evaluate the type of responses, including humoral and cellular response IgG1 and IgG2a isotypes and their ratio in serum of immune mice were measured. The results show that the serum level of IgG1 and IgG2a antibodies, produced against the compounds used in immune groups significantly increased compared to the control groups. Meanwhile, the levels of these antibodies in the groups, receiving OMV plus MPLA + CpG adjuvant were higher, and the highest response rates for these antibodies belonged to the OMV-LPS + MLPA-CpG receiving group. Meanwhile, the IgG1 / IgG2a ratio of the studied compounds is greater than 1.3 and therefore, due to the increase of IgG1, it can be an indication of overcoming the humoral responses (Th2) to cellular responses (Th1).

Finally, with regard to the production of protective antibodies against infection and also the high level of IgG1 as a complement-activating antibody, it can be concluded that the combination of OMV-LPS + MLPA-CpG can be a suitable vaccine candidate for the *H. influenzae*.

## CONCLUSION

In this study, we used OMVs from *H. influenzae* with MPLA + CpG adjuvant and evaluated their ability to stimulate immunity and cytokine responses. We found that this combination could significantly increase IgG1 and IL-4 levels. Therefore, it could potentially be a suitable candidate for the development of a vaccine against *H. influenzae*.

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