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Acellular pertussis vaccine based on outer membrane vesicles capable of conferring both long-lasting immunity and protection against different strain genotypes

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ABSTRACT

Despite high vaccination coverage rates, pertussis continues to be a global concern, with increased incidence widely noted. The current pertussis epidemiologic situation has been mainly attributed to waning immunity and pathogen adaptation. To improve the disease control, a new generation of vaccines capable to overcome those weaknesses associated to the current vaccines need to be developed. Previously we have demonstrated that the outer membrane vesicles obtained from the recombinant *Bordetella pertussis* strain expressing PagL enzyme ($OMVs_{BpPagL}$) are good vaccine candidates to protect against pertussis. In this work the $OMVs_{BpPagL}$ formulated with diphtheria and tetanus toxoids ($Tdap_{OMVsBpPagL}$) was used to evaluate its capacity to offer protection against Argentinean clinical isolates and to induce long-term immunity. To these aims BALB/c mice were immunized with $Tdap_{OMVsBpPagL}$ and challenged with sublethal doses of the clinical isolate *Bp*106 selected as a representative circulating isolate. Comparisons with a current commercial Tdap vaccine used at a dose in which pertussis toxin level was equivalent to that of $Tdap_{OMVsBpPagL}$ were performed. With the normalized doses of both vaccines we observed that $Tdap_{OMVsBpPagL}$ protected against the clinical isolate infection, whereas current commercial Tdap vaccine showed little protection against such pathogen. Regarding long-term immunity we observed that the $Tdap_{OMVsBpPagL}$ protective capacity against the recommended WHO reference strain persisted at least 9 months. In agreement with these results $Tdap_{OMVsBpPagL}$ induced Th1 and Th2 immune response. In contrast, commercial Tdap induced Th2 but weak Th1 responses. All results presented here showed that $Tdap_{OMVsBpPagL}$ is an interesting formulation to be considered for the development of novel acellular multi-antigen vaccine.

1. Introduction

Before specific vaccines were introduced in the 1940s, the respiratory disease called pertussis was a major cause of infant death worldwide. Widespread vaccination with the first pertussis whole-cell vaccine (wP) significantly reduced morbidity and mortality. However, in the 1970s, some concerns about the wP's adverse effects led, in some countries, to rising rates of vaccine refusal and consequently to increase pertussis incidence. Since the 1980s, many countries replaced wP by less reactogenic acellular vaccines (aP). In 1981, Japan started using aP containing pertussis toxin (PTx), identified as a major protective antigen [1]. Current aP contain the PTx and, in addition, other surface proteins. In general, five-component vaccines are considered more effective than two- or three-component vaccines [2,3].

Despite relatively high vaccination rates, in recent years the pathogen has come roaring back to infect people in numbers not seen since the pre-vaccine days. Indeed, pertussis is now recognized as a frequent infection not only in newborn and infants, but also in adults [4–9]. The increase in pertussis cases has been mostly

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attributed to waning vaccine-induced immunity and pathogen adaptation [4]. The exact duration of immunity is difficult to estimate universally since it depends on the burden of the disease, surveillance systems, and case definitions used. However, Wendelboe et al. [5] estimated that infection-acquired immunity wanes after 4–20 years and protective immunity after vaccination wanes after 4–12 years. It was recently reported in US that children vaccinated with aP had a remarkable increased risk to get the disease than children vaccinated with the discontinued wP [6].

Pathogen adaptation also seems to play a role in pertussis persistence and resurgence [7]. Mooi et al. [8] proposed that antigenic divergence between circulating bacteria and vaccine strains would affect both memory recall and antibodies efficacy, while higher PTx levels may increase suppression of the innate and acquired immune systems. Moreover the authors proposed that these bacterial adaptations have decreased the period in which pertussis vaccines are effective and thus enhanced the waning of immunity.

Altogether, the epidemiological situation has led to intensified research on the design of new vaccines capable of conferring both long-lasting immunity and protection against different strain genotypes. Recently, we have demonstrated that outer membrane vesicles derived from *Bordetella pertussis* expressing the lipid A deacylase PagL (OMVs*Bp*PagL) can protect against intranasal pertussis challenge when administered in a mouse infection model [9]. OMVs seems to have important advantages in vaccine development over the currently used aP since the native OMVs carry a wide spectrum of endogenous antigens, in addition to the natural self-adjuvanticity exerted by several innate immune response activating components, such as outer membrane proteins and lipopolysaccharide (LPS).

Here, an established murine immunization model was used to examine the induction and longevity of immunological memory and protection against different *B. pertussis* bacterial background.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. pertussis Tohama phase I strain, the WHO reference strain *Bp*18323 [10], the Argentinean clinical isolate *Bp*106 [11], a PTx defective *B. pertussis* mutant strain [12] and *B. pertussis* Tohama strain carrying the broad-host-range vector pMMB67EH containing *pagL* gene of *Bordetella bronchiseptica* [13] (hereafter referred as *Bp*PagL), were used throughout this study. The *B. pertussis* strains were grown on Bordet Gengou (BG) agar medium (Difco, Houston, US) supplemented with 10% (v/v) defibrinated sheep's blood (BGA plates) [11].

2.2. Isolation of outer membrane vesicles (OMVs)

To obtain OMVs from bacterial cells we used the method previously described [14,15]. OMVs were negatively stained and examined with an electron microscope [14,15]. Protein content was estimated by the Lowry method [16] using bovine serum albumin as standard.

2.3. Cloning and expression of the recombinant subunit A of pertussis toxin (PTxA)

PCR product of subunit A of PTx obtained using Pfx Platinum polymerase (0.5 units, Invitrogen) was cloned into pET-TOPO 200 vector according to the manufacturer's instructions (Invitrogen). The recombinant clones were confirmed by PCR and sequenced. The pET-TOPO 200::*ptxA* vectors were transformed first into TOP10 *Escherichia coli* competent cells and then into expression host cells, BL21Star. For PTxA expression, selected clones were grown in LB medium containing Km (50 μg/ml) and IPTG 1 mM.

2.4. Purification of recombinant His_6-PTxA

The PTxA recombinant protein was purified using Ni-NTA columns (Invitrogen) equilibrated in denaturing lysis buffer (Invitrogen) according with the manufacturer's protocols. His_6-PTxA purity was judged by SDS-PAGE [17]. The identity of the expressed protein was confirmed by MALDI-TOF mass spectrometry [17]. For immunoblots assays, polyclonal antiserum (Ab) against PTxA was obtained in mice after two immunizations with 5 μg of the purified recombinant His_6-PTxA protein each time.

2.5. Gel electrophoresis and immunoblots

SDS-PAGE separated proteins of OMVs and different quantities of purified recombinant PTxA were transferred onto PVDF (Immobilon P, Millipore) at constant voltage (100 V). After transfer, PVDF membranes were placed into Odyssey blocking buffer (OBB, Li-COR) at 4 °C. Primary and secondary antibodies conjugated to IRDye 800CW (Li-COR) were diluted 1/500 and 1:15,000 in OBB 0.1% Tween-20, respectively. Membranes were scanned and analyzed using Odyssey imaging software 3.0.

2.6. Formulation of acellular vaccines, $Tdap_{OMVsBpPagL}$

The vesicles preparations were detoxified with formalin (0.37% at 37 °C overnight). To use the OMVs as a combined acellular vaccines, vesicles (1.75 μg of total OMVs protein), were formulated with diphtheria (1 to 3 Lf/dose with an output of 0.1 UIA/ml serum) and tetanus toxoids (5 to 7 Lf/dose with a power greater than or equal to 2 UIA/ml serum) in the amounts described below, besides aluminum hydroxide did not exceed 1.25 mg/dose.

2.7. Mouse weight gain test (MWG)

The MWG-test was carried out using groups of 8 Balb/c mice outbred mice (15–20 g) which were i.p. vaccinated with 1.75 μg of detoxified OMVs obtained from *Bp*PagL strain, formulated with diphtheria and tetanus toxoids ($Tdap_{OMVsBpPagL}$) or 1/10 human dose (HD) of TdaP (Boostrix™). Control group received an equal volume of sterile PBS. Animals were observed for 7 days and body weight was recorded after 16 h, 3 and 7 days. Vaccines were considered non-toxic when passing the WHO and EP requirements [18].

2.8. Induction of inflammatory markers upon vaccination

Serum was collected 4 h after each immunization by submandibular bleeding and IL-6 levels were measured by ELISA using BD OptiEIA (BD Biosciences) following manufacturer instructions.

2.9. Active immunization and intranasal challenge

Animal protocol using female BALB/c mice (4 weeks of age) was performed as we previously described [11]. Groups of five mice were immunized systemically with $Tdap_{OMVsBpPagL}$ (1.75 μg of total OMVs protein), 1/10 human dose (HD) or 1/200 human dose (HD) of commercial TdaP (Boostrix™) using a two-dose schedule over a period of 2 weeks. Mice were challenged 2 weeks after the second immunization by nasal challenge with sublethal dose (10^6–10^8 CFU/ 40 μl) of Tohama phase I, *Bp*18323 or *Bp*106 clinical isolate. To analyze long lasting immunity, OMVs vaccinated 2, 5 and 9 months after the second immunization mice were challenged intranasally with sublethal dose (10^6–10^8 CFU/ 40 μl) of *Bp*18323.

In all cases, bacterial counts were performed 7 days after the challenge. Lungs were aseptically removed, homogenized in the sterile PBS, serially diluted and plated on BGA plates to determine bacterial recoveries. At least three independent experiments were performed in each case.

3. ELISAs

Plates were coated with 1 μg/200 μl of OMVsPagL in 0.5 M carbonate buffer, pH 9.5 by overnight incubation at 4 °C. The plates were then blocked with 5% skimmed milk (2 h 37 °C) and incubated with serially diluted serum samples (1 h 37 °C). Bound IgG was detected after 2 h incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (from Jackson ImmunoResearch, Baltimore Pike). For measuring IgG isotypes, detection of bound antibody was determined using HRP labeled subclass-specific anti-mouse IgG1 (1:2000) or IgG2a (1:3,000) (Santa Cruz Biotechnology, Santa Cruz, CA).

3.1. Analysis of cellular response elicited by vaccination

For cytokines analysis, spleen cells from mice immunized with TdapOMVs*Bp*PagL or commercial Tdap as described above were harvested 8 weeks after the last immunization. 10^6 splenocytes were seeded in U-shaped 48-well culture plates in a final volume of 500 μl/well RPMI 1640 (GIBCO) with 10% fetal bovine serum (Invitrogen, Carlsbad, California) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. As control, spleen cells from naïve mice were used. All cell samples were stimulated with OMVs*Bp* (5 μg/ml) or medium alone. After incubation for 72 h at 37 °C and 5%CO_2, the supernatants were collected and analyzed for IFN-γ and IL-5 production by ELISA (BD Biosciences, San Diego, USA), using conditions recommended by the manufacturer.

3.2. Statistical analysis

Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey test (GraphPadPrims®). Differences were considered to be significant when $p < 0.05$.

4. Results

We evaluated the protective capacity of the OMVs$_{Bp\text{PagL}}$ against current circulating bacteria. To this end the protective dose of OMVs (1.75 μg of protein) was formulated with tetanus and diphtheria toxoids (Tdap$_{\text{OMVs}Bp\text{PagL}}$) as the current commercial combined Tdap vaccine. For comparisons, we used one of the licensed current acellular Tdap vaccines (Boostrix™) in two different doses: one that equalized the PTx content to the protective dose of Tdap$_{\text{OMVs}Bp\text{PagL}}$ and the other, a dose that, although high (1/10 of the human dose, HD) for the weight of the mouse, is widely used. We selected PTx antigen to equalize the doses of vaccines because we observed that the OMVs derived from a *B. pertussis* mutant strain that does not expresses PTx, diminished their protective capacity in more than two logs, being an important determinant in OMVs protective capacity (Fig. 1).

The PTx level in the OMVs was determined by quantitative immunoblot (Fig. 2). Recombinant purified subunit A of PTx cloned in pET200 vector by us was used to perform the calibration curve (Fig. 2B). The fluorescent signal obtained was directly proportional to the amount of target protein present and correspond roughly to 10.5 ng ± 2.5 ng of PTxA per dose of Tdap$_{\text{OMVs}Bp\text{PagL}}$ (Fig. 2), which is equivalent to 47.1 ng ± 11.2 ng of the complete PTx protein (MW of PTx 117 kDa, MW of PTxA 26 kDa). This amount of PTx corresponds to 1/200 of a human dose of commercial Tdap. Therefore comparisons experiments between Tdap$_{\text{OMVs}Bp\text{PagL}}$ and commercial Tdap were made using 1.75 μg of OMVs and 1/200 human dose of Tdap.

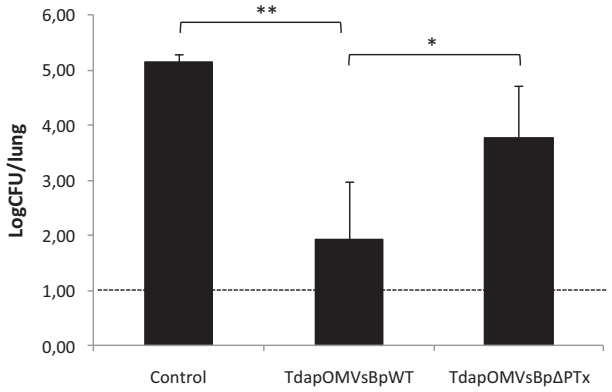

Fig. 1. Effect of immunization with OMVs formulated as Tdap vaccines in the mouse intranasal challenge model. OMVs were obtained from both the PTx defective *B. pertussis* strain and *B. pertussis* Tohama parental strain. Immunization protocols comprised a two-dose schedule over a period of 2 weeks. Mice were challenged 2 weeks after the second immunization by nasal challenge with sublethal dose (10^6–10^8 CFU 40 μl) of *Bp* Tohama phase I. Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. * $p < 0.001$ vs control, ** $p < 0.005$ vs TdapOMVs*Bp*WT.

4.1. Protective capacity of Tdap$_{OMVsBpPagL}$ against current circulating bacteria carrying the predominant genotype ptxP3, ptxA1, prn2 and fim3-2

To evaluate the protective capacity of Tdap$_{\text{OMVs}Bp\text{PagL}}$ and the selected two doses of commercial Tdap, mice were immunized twice with each formulation and challenged 2 weeks after the second immunization with sublethal dose of *B. pertussis* strain. For the challenge we first used bacteria expressing vaccine/reference type genes: *ptx*P1, *ptx*A2, *prn*1, *fim*3-1 (*B. pertussis* Tohama phase I) and *ptx*P4, *ptx*A4, *prn*6, *fim*3-1 (WHO reference *Bp*18323). As negative and positive controls we used mice treated with PBS and mice immunized with high dose of commercial Tdap vaccine (1/10 of the HD), respectively. For both bacterial challenge strains, significant differences in lung *B. pertussis* bacteria counts between immunized animals with Tdap$_{\text{OMVs}Bp\text{PagL}}$ and negative control group were observed ($p < 0.001$) (Fig. 3A and B). More than 3 log differences between vaccinated and non-immunized mice were detected. These differences were also observed between Tdap$_{\text{OMVs}Bp\text{PagL}}$ and commercial Tdap vaccine used in 1/200 HD. Commercial Tdap vaccine in high dose (1/10 HD) offered high protection level against both *B. pertussis* strains: the *B. pertussis* Tohama strain and the WHO recommended *Bp*18323 strain.

When Tdap$_{\text{OMVs}Bp\text{PagL}}$ immunized mice were challenged with bacteria carrying non-vaccine-type alleles, *ptx*P3, *ptx*A1, *prn*2, *fim*3-2 (Argentinean circulating strain *Bp*106) adequate elimination rate ($p < 0.001$) was observed (Fig. 3C). Again, in mice immunized with commercial Tdap vaccine (1/200 HD) for this bacterial challenge strain, the number of lung-recovered colonies at day 7-post challenge was similar to the counts in non-immunized mice (Fig. 3C). In non-immunized mice the number of recovered colonies was high (10^5 CFU/lung). Commercial Tdap vaccine used in high dose, offered good protection level against the Argentinean clinical isolate.

All results presented here showed that Tdap$_{\text{OMVs}Bp\text{PagL}}$ has a good protective capacity against strains both expressing vaccine/reference type, *ptx*P1, *ptx*A2, *prn*1, *fim*3-1 (*B. pertussis* Tohama)

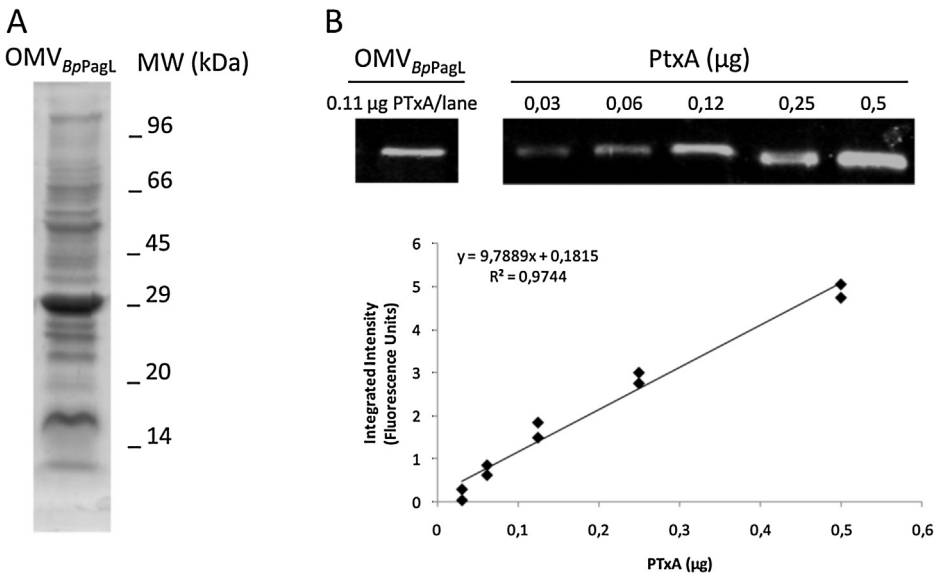

Fig. 2. (A) SDS-PAGE 12.5% of OMVs*Bp*PagL: Molecular weights are indicated at the left. (B) Upper Panel: Immunoblot using anti-pertussis toxin subunit A (PTxA) antibodies on OMVs*Bp*PagL and different quantities of purified recombinant PTxA. For this assay, three batches of OMVs independently obtained were used. To develop the blotting, secondary antibodies conjugated to IRDye 800CW was used.
Lower Panel: Quantification of PTxA level in the OMVs. The fluorescent signal obtained was directly proportional to the amount of target protein present content. Antibody signals were analyzed as integrated intensities of regions defined around the bands of interest.

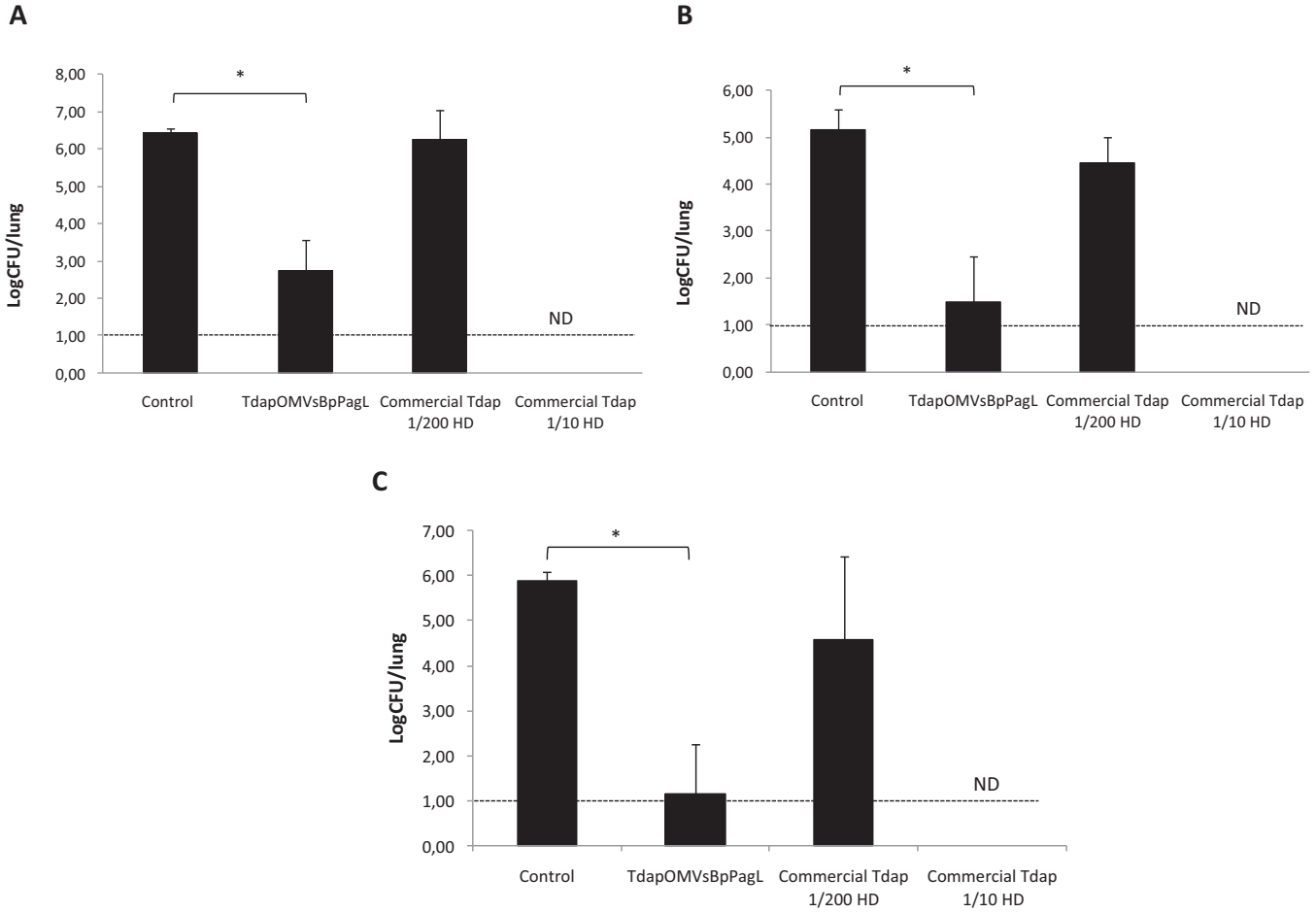

Fig. 3. Effect of immunization with $Tdap_{OMVBpPagL}$ and commercial Tdap vaccine in the mouse intranasal challenge model. Commercial Tdap vaccine was used in two different dose: 1/10 and 1/200 of the human dose. Immunization protocols comprised a two-dose schedule over a period of 2 weeks. Mice were challenged 2 weeks after the second immunization by nasal challenge with sublethal dose (10^6–10^8 CFU/ 40 µl) of *Bp* 18323 (Panel A), Tohama phase I (Panel B), or *Bp*106 clinical isolate (Panel C). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. * $p < 0.001$. ND: non detected.

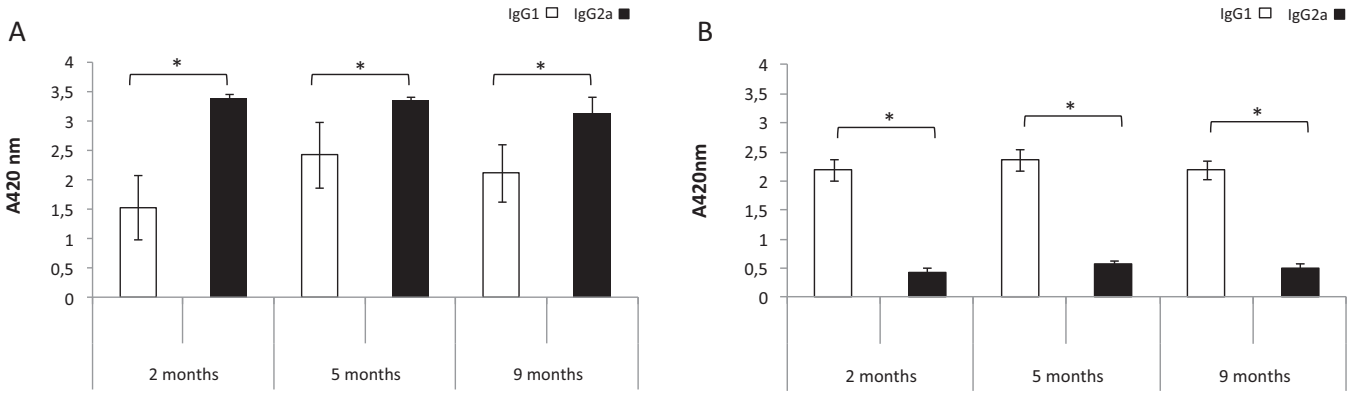

Fig. 4. Antibody response to Tdap$_{OMVsBpPagL}$ (Panel A) and commercial Tdap vaccines (Panel B). Anti-Tdap$_{OMVsBpPagL}$ and anti-commercial Tdap IgG1 and IgG2a were determined by ELISA. OMVsPagL (1 μg/200 μl) was used to coat the 96-well polystyrene microtiter plates. Detection of bound antibody was determined using HRP labeled subclass-specific anti-mouse IgG1 (1:2,000). Antibody amounts were measured for individual serum. The data show mean antibody amounts ± SD at 2, 5 and 9 months after the last immunization dose.

and *ptx*P4, *ptx*A4, *prn*6, *fim*3-1 (*B. pertussis* 18323) and non-vaccine-type alleles, *ptx*P3, *ptx*A1, *prn*2, *fim*3-2 (*Bp*106).

4.2. Tdap$_{OMVsBpPagL}$ safety

All the formulations here used for vaccination fulfilled the WHO criteria of safety for this test. In the case of mice immunized with Tdap$_{OMVsBpPagL}$, only small weight loss (less than 1.5% of the original weight) was observed at 16 h but at 3 days after vaccination, all treated mice regain their weight. Mice treated with Tdap$_{OMVsBpPagL}$ and commercial Tdap exceeded at day 3 their weight registered before the first dose of immunization in 5.5 ± 2.3% and 6.3 ± 2.2%, respectively. At day 7, all mice exceeded in more than 15.6 ± 5.3%, the weight registered at day 3 regardless of treatment used.

Furthermore, the levels of IL-6 used as indicator of proinflammatory response showed a rise to 127.16 ± 72.08 pg/ml at 4 h post immunization with Tdap$_{OMVsBpPagL}$. A similar behavior was observed for the commercial Tdap used at 1/10 of the human dose, 69.14 ± 9.42 pg/ml.

4.3. Long-term protection capacity against intranasal B. pertussis challenge after vaccination with Tdap$_{OMVsBpPagL}$

In order to evaluate the duration of protective capacity induced by the TdapOMVs*Bp*PagL, we characterized the nature and longevity of the immune response and analyzed the effect on subsequent colonization by the WHO recommended reference strain *Bp*18323 after 2, 5 and 9 months after two i.p. administrations of Tdap$_{OMVsBpPagL}$. Mice immunized with high dose of commercial Tdap vaccine was used as positive control.

To characterize the nature and longevity of the antibody response to Tdap$_{OMVsBpPagL}$ immunization, sera responses to OMVs were examined from long-term-immunized mice (Fig. 4A). The titers of antibody responses were quantitatively and qualitatively characterized. High serum levels of specific IgG1 were found at 2, 5 and 9 months after commercial Tdap priming, with no antibody detected in control animals (Fig. 4B). In contrast to commercial Tdap vaccine, Tdap$_{OMVsBpPagL}$ resulted in murine antibody responses dominated by IgG2a (Fig. 4A).

At 2 months after immunization, we also evaluated the IFN-γ and IL-5 levels produced by spleen cells. In agreement with the IgG1/IgG2a ratio responses, higher concentrations of IFN-γ were produced by spleen cells from Tdap$_{OMVsBpPagL}$ immunized mice in comparison with those of Tdap 1/10 HD immunized mice (Fig. 5A). In contrast IL-5 was produced at low concentrations by spleen cells from Tdap$_{OMVsBpPagL}$-immunized mice, but was induced at higher concentrations in Tdap 1/10 HD mice (Fig. 5B). All these findings indicate that TdapOMVsPagL induce a Th1 response.

Regarding colonization at 5 and 9 months after the last immunization with Tdap$_{OMVsBpPagL}$, the results obtained showed significant differences in lung bacterial counts between immunized animals with Tdap$_{OMVsBpPagL}$ and the control group ($p < 0.001$) (Fig. 6). While in mice treated with PBS the number of recovered colonies from lungs was high (10^5 CFU per lung), in mice immunized with Tdap$_{OMVsBpPagL}$ (1.75 μg of OMVs), the colony forming units recovered from lungs at 5 and 9 months post last immunization dropped, respectively, at least five and 3 orders of magnitude in relation to the counting in non immunized mice (Fig. 6). Significant decrease in the bacterial counts at 5 and 9 months post last immunization were obtained with the positive control consisting in a high dose of commercial Tdap vaccine.

Altogether the data presented here demonstrate the longevity of the Tdap$_{OMVsBpPagL}$ vaccine-induced immunity to *B. pertussis*.

5. Discussion

The new generation of aP should solve the weaknesses of existing vaccines, i.e. they should confer protection against circulating *B. pertussis* strains and must confer long-term immunity. Observations that genetic variability of circulating bacteria is higher during periods of low vaccine coverage [19] suggest that vaccination may induce clonal expansion of strains that have a selective advantage in vaccinated human populations. Mooi and colleagues reported polymorphisms in vaccine antigen genes, *ptx*P, *ptx*A, *prn* and *fim* [4,20,21]. Recently, the frequencies of *ptx*P3, *ptx*A1, *prn*2 and *fim*3-2, the non-vaccine-type alleles, have been increasing, and these alleles have become dominant in many countries [11,22–25].

This study explores the capacity of our previously designed aP, the Tdap$_{OMVsBpPagL}$ vaccine, to induce protection against different *B. pertussis* genotypes. Although mouse it is not a natural host of *B. pertussis*, we have used mice intranasal challenge model because highly significant correlation between bacterial clearance in immunized mice and vaccine efficacy in children was observed [26]. With this model we observed that Tdap$_{OMVsBpPagL}$ vaccine presented a good safety profile and was effective against strains expressing vaccine/reference type *ptx*P1, *ptx*A2, *prn*1, *fim*3-1 (*Bp* Tohama phase I) and *ptx*P4, *ptx*A4, *prn*6, *fim*3-1 (*Bp*18323), and non-vaccine-type alleles, *ptx*P3, *ptx*A1, *prn*2, *fim*3-2 (circulating strain *Bp*106) [11,27]. In contrast, commercial Tdap used in a dose in which PTx content was equivalent to Tdap$_{OMVsBpPagL}$, showed little protection effect against all genotypes tested. Geurtsen et al. [28] also observed a reduction in the protective capacity of commercial Tdap when it

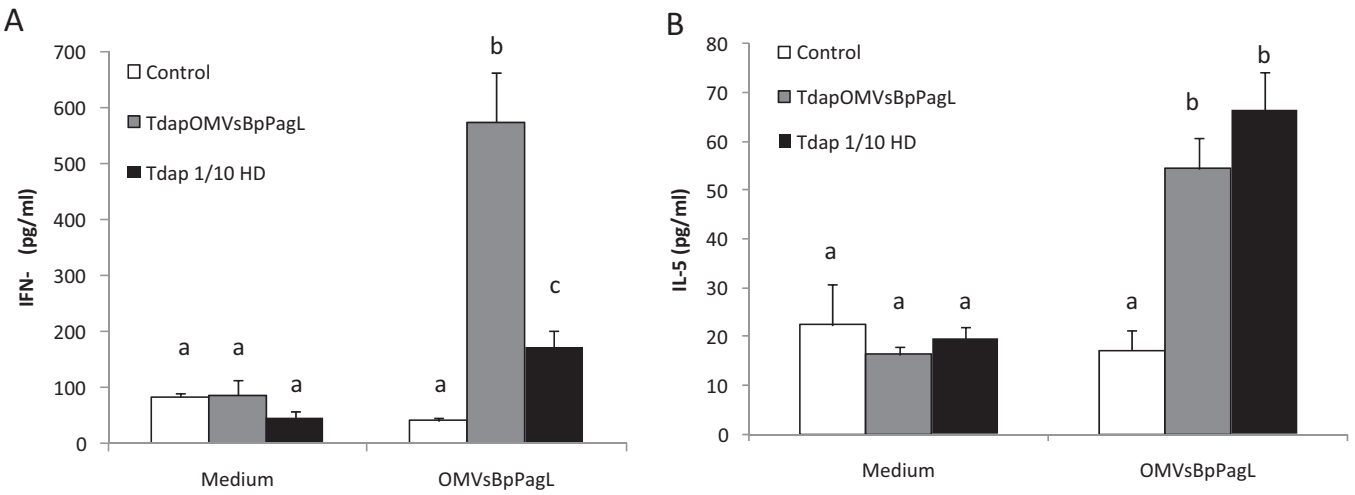

Fig. 5. Cytokines production by spleen cells of immunized mice. Spleen cells from $Tdap_{OMVBpPagL}$ or Tdap immunized mice obtained 2 months after the last immunization were stimulated *in vitro* with OMVs*Bp*PagL or medium alone. After incubation for 72 h at 37 °C and 5%CO_2, the supernatants were collected and analyzed for IFN-γ (Panel A) and IL-5 (Panel B) production by ELISA. Different letters were used to indicate statistical differences with $p < 0.05$. No differences were observed between conditions marked with the same letter. Each panel is representative of two independent experiments.

was used in a dose of 1/125 HD. Only when commercial Tdap vaccine was used in high dose for mice (1/10 of HD), the protection levels achieved against different *B. pertussis* strains were similar to those conferred by $Tdap_{OMVs\mathit{Bp}PagL}$. These last results are in agreement with a previous report that showed that with a high dose of tri-component aP, the efficacy of lung clearance of the circulating isolates was high [29]. The OMVs are constituted by a wider variety of antigens than the current aP, some of them even exert adjuvant activity. Although we have not identified all components responsible of the protective capacity of OMVs, it is likely contributed by multiple components, some of them present in the commercial formulations (PRN, FHA, PT, Fim2, Fim3), and also other less characterized components such as several membrane proteins (i.e. chaperonin GroEL and OMP porins) that are present in the OMVs [15]. We are currently working on the identification of other protective antigens present in the OMVs. Moreover, since the OMVs are nanoparticles, it is expected that they exert an improved uptake of the antigen by antigen presenting cells than the bacterial whole cell [30]. These properties could contribute to the shown efficacy of the $Tdap_{OMVs\mathit{Bp}PagL}$ against different genotypes of *B. pertussis* and positioned it above the current acellular vaccines.

While neither vaccine- nor infection-induced immunity to *B. pertussis* can be said to be life long, immunity conferred by commercial Tdap may wane after only few years in humans, whereas the immunity conferred by infection has been estimated to last at least 20 years [5]. In fact it was recently reported in US that children vaccinated with aP had a remarkably increased attack rate of the disease compared to children vaccinated with

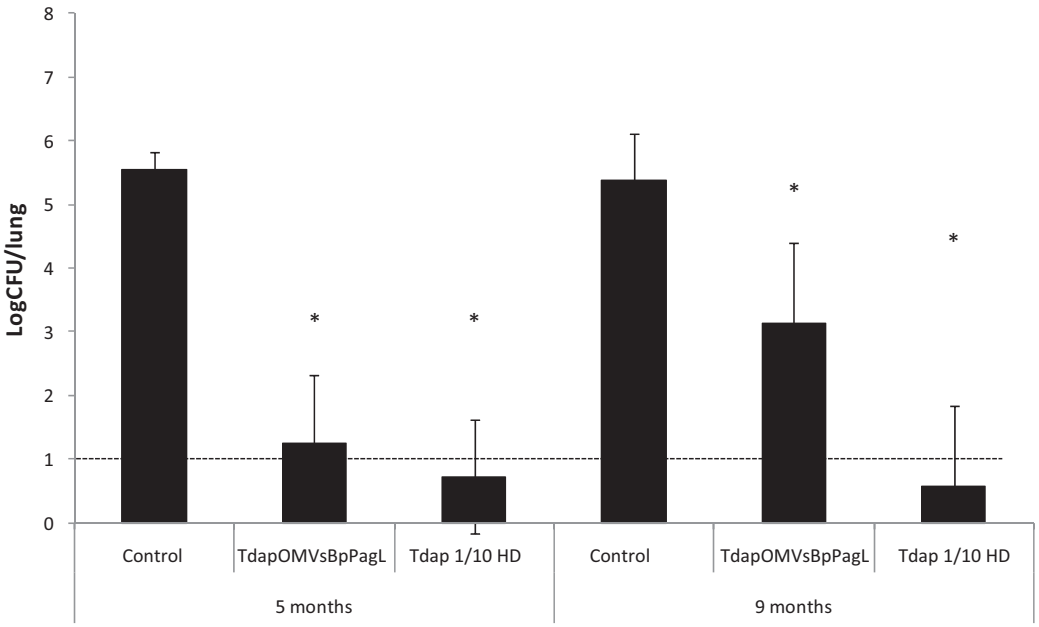

Fig. 6. Long lasting immunity induced in $Tdap_{OMVs\mathit{Bp}PagL}$ vaccinated mice. Immunization protocols comprised a two-dose schedule over a period of 2 weeks. Mice were challenged 5 and 9 months after the second immunization by nasal challenge with sublethal dose of *Bp* 18323. These experiments were designed in a way to make the challenge at the same time and with the same dose of *B. pertussis* (10^6–10^8 CFU in 40 μl). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of five mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log ± SD. * $p < 0.05$.

the discontinued wP [6]. In agreement with these observations, recently Ross et al. [31] examined the type of immune responses induced with aP compared with wP in an attempt to design a more effective vaccine. The authors observed that while wP induce Th17 cells and the required Th1 cells for optimum immunity [32,33], aP administered with alum as the adjuvant, induced Th2 and Th17 cells, but weak Th1 responses. The results here presented showed that $Tdap_{OMVs\textit{Bp}PagL}$ primed long-lasting *B. pertussis*-specific IgG responses. This antibody response was predominantly of the IgG2a subclass, which is consistent with a Th1 profile or immune response. Furthermore, at 2 months after immunization higher concentrations of IFN-γ were produced by spleen cells from $Tdap_{OMVs\textit{Bp}PagL}$ immunized mice in comparison with those of Tdap 1/10 HD immunized mice (Fig. 5A). In contrast, a current licensed Tdap administered with alum as the adjuvant induced Th2 but weak Th1 responses (higher IgG1/IgG2a ratio and lower INF-γ). This evidence also opens the possibility of envisaging the use of OMVs based vaccine as initial immunization strategy followed by boosters of other acellular formulations. Moreover, we observed that $Tdap_{OMVs\textit{Bp}PagL}$ could induce long-term protection in a murine model. $Tdap_{OMVs\textit{Bp}PagL}$ was shown to confer high level of protection against bacterial challenge at 5 and 9 months post immunization.

Considering the aforementioned epidemiological situation and the challenges to be solved by new developments, the data presented here strongly suggest that $Tdap_{OMVs\textit{Bp}PagL}$ is an attractive candidate for translation to human use as a novel acellular pertussis vaccine.

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References

[1] Sato Y, Kimura M, Fukumi H. Development of a pertussis component vaccine in Japan. Lancet 1984;1:122–6.
[2] Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Engl J Med 1996;334:349–55.
[3] Olin P, Rasmussen F, Gustafsson L, Hallander HO, Heijbel H. Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine ad hoc group for the study of pertussis vaccines. Lancet 1997;350:77–1569.
[4] Bart MJ, van Gent M, van der Heide HG, Boekhorst J, Hermans P, Parkhill J, et al. Comparative genomics of prevaccination and modern *Bordetella pertussis* strains. BMC Genomics 2010;11:627.
[5] Wendelboe AM, Van Rie A, Salmaso S, Englund JA. Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J 2005;24:S58–61.
[6] Witt MA, Arias L, Katz PH, Truong ET, Witt DJ. Reduced risk of pertussis among persons ever vaccinated with whole cell pertussis vaccine compared to recipients of acellular pertussis vaccines in a large US cohort. Clin Infect Dis 2013;56:1248–54.
[7] Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, Heuvelman KJ, et al. Bordetella pertussis strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 2009;15:1206–13.
[8] Mooi FR, VDM NA, De Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation—two sides of the same coin. Epidemiol Infect 2013:1–10.
[9] Asensio CJ, Gaillard ME, Moreno G, Bottero D, Zurita E, Rumbo M, et al. Outer membrane vesicles obtained from *Bordetella pertussis* Tohama expressing the lipid A deacylase PagL as a novel acellular vaccine candidate. Vaccine 2011;29:1649–56.
[10] World Health Organization (WHO). Expert committee on biological standardization. In: WHO/BS/2011.2158 (English only). World Health Organization (WHO); 2011. p. 1–64.
[11] Bottero D, Gaillard ME, Fingermann M, Weltman G, Fernandez J, Sisti F, et al. Pulsed-field gel electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. Clin Vaccine Immunol 2007;14:1490–8.
[12] Antoine R, Locht C. Roles of the disulfide bond and the carboxy-terminal region of the S1 subunit in the assembly and biosynthesis of pertussis toxin. Infect Immun 1990;58:1518–26.
[13] Geurtsen J, Steeghs L, Hamstra HJ, Ten Hove J, de Haan A, Kuipers B, et al. Expression of the lipopolysaccharide-modifying enzymes PagP and PagL modulates the endotoxic activity of *Bordetella pertussis*. Infect Immun 2006;74:5574–85.
[14] Hozbor D, Rodriguez ME, Fernandez J, Lagares A, Guiso N, Yantorno O. Release of outer membrane vesicles from *Bordetella pertussis*. Curr Microbiol 1999;38:273–8.
[15] Roberts R, Moreno G, Bottero D, Gaillard ME, Fingermann M, Graieb A, et al. Outer membrane vesicles as acellular vaccine against pertussis. Vaccine 2008;26:4639–46.
[16] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[18] World Health Organization (WHO). Recommendations for whole-cell pertussis vaccine. In: Annex 6. World Health Organization (WHO); 2007.
[19] van Loo IH, van der Heide HG, Nagelkerke NJ, Verhoef J, Mooi FR. Temporal trends in the population structure of *Bordetella pertussis* during 1949–1996 in a highly vaccinated population. J Infect Dis 1999;179:915–23.
[20] Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, Willems RJ. Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun 1998;66:670–5.
[21] van Loo IH, Heuvelman KJ, King AJ, Mooi FR. Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. J Clin Microbiol 2002;40:1994–2001.
[22] Octavia S, Sintchenko V, Gilbert GL, Lawrence A, Keil AD, Hogg G, et al. Newly emerging clones of *Bordetella pertussis* carrying prn2 and ptxP3 alleles implicated in Australian pertussis epidemic in 2008–2010. J Infect Dis 2012;205:1220–4.
[23] Lam C, Octavia S, Bahrame Z, Sintchenko V, Gilbert GL, Lan R. Selection and emergence of pertussis toxin promoter ptxP3 allele in the evolution of *Bordetella pertussis*. Infect Genet Evol 2012;12:492–5.
[24] Advani A, Gustafsson L, Ahren C, Mooi FR, Hallander HO. Appearance of Fim3 and ptxP3-*Bordetella pertussis* strains, in two regions of Sweden with different vaccination programs. Vaccine 2011;29:3438–42.
[25] Hallander HO, Advani A, Donnelly D, Gustafsson L, Carlsson RM. Shifts of *Bordetella pertussis* variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J Clin Microbiol 2005;43:2856–65.
[26] Mills KH, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun 1998;66:594–602.
[27] Bottero D, Gaillard ME, Basile LA, Fritz M, Hozbor DF. Genotypic and phenotypic characterization of *Bordetella pertussis* strains used in different vaccine formulations in Latin America. J Appl Microbiol 2012;112:1266–76.
[28] Geurtsen J, Banus HA, Gremmer ER, Ferguson H, de la Fonteyne-Blankestijn LJ, Vermeulen JP, et al. Lipopolysaccharide analogs improve efficacy of acellular pertussis vaccine and reduce type I hypersensitivity in mice. Clin Vaccine Immunol 2007;14:821–9.
[29] Denoel P, Godfroid F, Guiso N, Hallander H, Poolman J. Comparison of acellular pertussis vaccines-induced immunity against infection due to *Bordetella pertussis* variant isolates in a mouse model. Vaccine 2005;23:5333–41.
[30] O'Hagan DT, MacKichan ML, Singh M. Recent developments in adjuvants for vaccines against infectious diseases. Biomol Eng 2001;18:69–85.
[31] Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog 2013;9:e1003264.
[32] Mills KH, Barnard A, Watkins J, Redhead K. Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun 1993;61:399–410.
[33] Ryan M, Murphy G, Ryan E, Nilsson L, Shackley F, Gothefors L, et al. Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. Immunology 1998;93:1–10.