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Characterization of the immune response induced by pertussis OMVs-based vaccine

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ABSTRACT

For the development of a third generation of pertussis vaccine that could improve the control of the disease, it was proposed that the immune responses induced by the classic whole cell vaccine (wP) or after infection should be used as a reference point. We have recently identified a vaccine candidate based on outer membrane vesicles (OMVs) derived from the disease etiologic agent that have been shown to be safe and protective in mice model of infection. Here we characterized OMVs-mediated immunity and the safety of our new candidate. We also deepen the knowledge of the induced humoral response contribution in pertussis protection. Regarding the safety of the OMVs based vaccine ($Tdap_{OMVsBp}$,) the *in vitro* whole blood human assay here performed, showed that the low toxicity of OMVs-based vaccine previously detected in mice could be extended to human samples.

Stimulation of splenocytes from immunized mice evidenced the presence of IFN-γ and IL-17-producing cells, indicated that OMVs induces both Th1 and Th17 response. Interestingly $Tdap_{OMVsBp}$-raised antibodies such as those induced by wP and commercial acellular vaccines (aP) which contribute to induce protection against *Bordetella pertussis* infection. As occurs with wP-induced antibodies, the TdapOMVsBp-induced serum antibodies efficiently opsonized *B. pertussis*. All the data here obtained shows that OMVs based vaccine is able to induce Th1/Th17 and Th2 mixed profile with robust humoral response involved in protection, positioning this candidate among the different possibilities to constitute the third generation of anti-pertussis vaccines.

1. Introduction

Pertussis is a vaccine preventable respiratory disease that currently needs attention. This disease mainly provoked by *Bordetella pertussis* affects all age groups being infants the most vulnerable population group [1]. Despite the massive use of vaccines for more than 60 years the pathogen represents a major global health problem and one of the top 10 causes of childhood mortality [2]. The first-generation of vaccines still in use in many countries consists of detoxified killed whole bacteria (wP). With its massive use from 1950s the incidence and mortality associated with pertussis fell to very low levels [3]. However, reports on safety concerns in the 1970s shed doubt on the value of wP and contributed to reduce vaccine acceptance in different countries [4–6]. This drawback on wP has prompted the development of a second generation of vaccines, the acellular vaccines (aP) that contain purified proteic antigenic components of *B. pertussis* [6,7]. Although these aP vaccines seemed to keep pertussis under control, during the last two decades the epidemiologic situation of the disease has changed and several outbreaks were reported [8]. Many of these outbreaks were detected in developed countries where wP vaccines composed of a wide range of antigens and used in the primary series were replaced by aP vaccines composed of three to five *B. pertussis* antigens. To explain this outcome, it was speculated that the loss of the vaccine antigen pertactin (PRN) in *B. pertussis* circulating isolates, provides a selective advantage for bacterial survival in aP vaccinated populations [9,10]. Furthermore, the faster waning immunity detected in aP vaccinated population probably due to the Th2 response mainly induced by

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this type of vaccine might also contributes to the increase of pertussis cases. In contrast the wP vaccines and also the natural infection that induce a more long lasting immune response characterized by a mixed Th1/Th17-type CD4+ T-cell response seem to diminish the risk of pertussis [11–16]. The consequences of these differences between the immunity induced by both vaccines have become evident in baboons infection model [17] and humans [18]. This whole scenario points out the need for a third-generation of aP vaccines capable of overcoming the weaknesses associated with the current formulations to improve the disease control. In particular the next-generation aP vaccines should not only induce CD4+ T cells with a mixed Th1/Th17-cell profile, like those induced by infection or immunization with wP vaccine but also must contain more than few purified immunogenic proteins. Following this rationale, our group has recently identified the outer membrane vesicles (OMVs) as a new aP vaccine [19–21]. This vaccine, containing a greater number of immunogens than the current aPs and conformations close to those found in bacteria, behaves safe and highly protective against *B. pertussis* challenge in mouse models. In this study, we investigated with more detail the safety and the immune response induced by OMVs-based vaccine. Comparisons with the commercial aP and wP vaccines currently used are also presented.

2. Materials and methods

2.1. Ethics statement

The studies have been approved by Ethical Committee for Animal Experiments of the Faculty of Science at La Plata National University (approval number 004-06-15 and 003-06-15).

2.2. Bacterial strains and growth conditions

B. pertussis Tohama phase I strain (CIP 8132) was used throughout this study. *B. pertussis* was grown in Bordet–Gengou agar incubated at 36 °C or Stainer–Scholte liquid medium (SS) as previously indicated [22].

2.3. Isolation of outer membrane vesicles (OMVs)

OMVs were produced as previously described [21,23,24]. Briefly, culture samples from the decelerating growth phase were centrifuged and the bacterial pellet obtained was resuspended in 20 mM Tris–HCl, 2 mM EDTA pH 8.5. The suspension was sonicated in cool water for 20 min. After two centrifugations at 10,000 × *g* for 20 min at 4 °C, the supernatant was pelleted at 100,000 × *g* for 2 h at 4 °C. This pellet was resuspended Tris 20 mM pH 7.6. The samples obtained were negatively stained for electron microscope examination [21]. Protein content was estimated by the Bradford method using bovine serum albumin as standard [25].

2.4. Active/pasive immunization and intranasal challenge

Groups of 5 female BALB/c mice (4 weeks of age) mice were immunized systemically with OMVs-based vaccine formulated as previously described [20], commercial wP (Vaksin DTwP) or aP (Adacel™) (1/10 human dose (HD)) using a two-dose schedule over a period of 2 weeks. Two weeks after the second immunization, mice were intranasally challenged with a sublethal dose (10^6–10^8 CFU 40 μl^{-1}) of *B. pertussis* Tohama phase I.

To study protection after by passive transfer experiments, serum (100 μl) from non-immunized mice or mice immunized with two doses of $Tdap_{OMVsBp}$, commercial wP or aP were injected intraperitoneally to BALB/c mice. Twenty-four hours later, the mice were infected with *B. pertussis* Tohama phase I strain (10^6–10^8 CFU 40 μl^{-1}). In all cases, bacterial counts were performed 7 days after the challenge as described previously [19,20]. At least three independent assays were performed in experimental settings.

2.5. Mouse weight gain test (MWG)

The MWG-test was carried out using groups of 8 BALB/c outbred mice (15–20 g) as described [19]. Vaccines were considered non-toxic when passing the WHO and EP requirements [26].

2.6. Stimulation of human whole blood

The whole blood IL-6 cytokine release assay was performed as described by Stoddard et al. [27]. Briefly, a 245 μl of blood sample was dispensed into each well of a 96-well tissue culture plate. Serial dilutions of each vaccine were prepared in RPMI 1640 cell culture medium (GIBCO) in a range of 5–300 ng/ml, incubated with the whole blood and then briefly centrifuged. A sample of 55 μl of plasma from each well was removed and frozen pending quantification of the cytokines. IL-6 levels were measured by ELISA using BD OptiEIA (BD Biosciences) following manufacturer instructions.

2.7. ELISA

Plates (Nunc A/S, Roskilde, Denmark) were coated with sonicated *B. pertussis* Tohama phase I (whole cell lysates) or purified recombinant PTxA both at 3 μg/ml in 0.5 M carbonate buffer, pH 9.5 by overnight incubation at 4 °C. The plates were then blocked with 3% skimmed milk (2 h 37 °C) and incubated with serially diluted mice serum samples (1 h 37 °C). Bound IgG was detected after 2 h incubation with anti mouse IgG -horseradish peroxidase (HRP) produced in goat (Invitrogen, USA). For measuring IgG isotypes, detection of bound antibody was determined using HRP labeled subclass-specific anti-mouse IgG1 (1:8000) or IgG2a (1:1000) (Sigma, Aldrich). Optical densities (OD) were measured with Titertek Multiskan 340 microplate reader (ICN, USA) at 492 nm, and a curve describing the relationship of the OD *vs* the log of the reciprocal of serum dilution was plotted. The inflection point of the curve was determined by GraphPad Prism software. Titers were determined as the reciprocal of serum dilution giving a OD corresponding to the inflection point of the curve.

2.8. Cell line growth

Macrophage-like RAW 264.7 (ATCC TIB-71) were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic solution (penicillin 100 units/ml, streptomycin 100 μg/ml) at 37 °C in 5% CO_2.

2.9. Opsonophagocytosis assay

GFP-expressing *B. pertussis* (*B. pertussis* Tohama phase I strain carrying pCW504 kindly provided by Dr. Weiss) were opsonized by incubation at 37 °C with 20% wP-induced/TdapOMVs or aP/naive-induced serum samples for 30 min in a final volume of 40 μl. Serum opsonized GFP-expressing bacteria were incubated with RAW cells at multiplicity of infection (MOI) of 70 for 30 min at 37 °C to allow binding and internalization. After extensive washing to remove non-attached bacteria, samples were analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and phagocytosis was estimated by the mean fluorescence intensity. Results were expressed as mean fluorescence intensity. Statistics analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPadPrims®). Differences were considered to be significant when $p < 0.001$.

2.10. Gel electrophoresis and immunoblots

SDS-PAGE separated proteins of OMVs, bacterial lysate of *B. pertussis* Tohama phase I and purified recombinant PTxA, Fim2 and PRN were transferred onto PVDF (Immobilon P, Millipore) at constant voltage (100 V). 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.

2.11. Immunoproteomics assays

OMVs and bacterial lysate samples were subjected to 1D gel electrophoresis in triplicate as described [21]. The unstained 1-D gels were electro blotted onto PVDF membranes, which were then incubated with sera from each treatment (1/500 dilution) and the immune reactivity was finally detected as above described. Membranes were then scanned, and the profile of immune-reactive proteins was matched to 1D gel images of the same sample and selected protein bands were excised from the stained 1D gels, digested *in situ* with trypsin and subjected to mass spectrometry fingerprinting (MALDI TOF). Immune reactive proteins present in the vesicles were identified as previously reported [28,29].

2.12. Analysis of cellular response elicited by vaccination

Cellular response was analyzed as described [20]. Briefly, spleen cells from mice immunized with $Tdap_{OMVsBp}$ or commercial wP or aP were harvested 8 weeks after the last immunization. 10^6 splenocytes were seeded in 48-well culture plates in a final volume of 500 μl/well RPMI 1640 with 10% fetal bovine serum (Invitrogen) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. All cell samples were stimulated with heat killed bacteria suspension (10^6 UFC/ml), OMVs*Bp* (5 μg/ml), PTx (2.5 μg/ml) or medium alone. Supernatants were removed after 72 h of incubation at 37 °C and 5% CO_2, and IFN-γ, IL-17 and IL-5 production was determined by ELISA (BD Biosciences, San Diego, USA), using conditions recommended by the manufacturer.

2.13. Statistical analysis

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

3. Results

Following our standardized protocols to evaluate the quality of *B. pertussis* OMVs as vaccines, shapes and OMVs-sizes were examined through electron microscopy. In addition, the presence of the known protective immunogens pertussis toxin (PTx) and pertactin (PRN) were assessed by specific immunoblot (not shown). After formulating the OMVs-based vaccine ($Tdap_{OMVsBp}$), the safety of the vaccine was evaluated by mouse gain weight test (MGW) and the human whole blood (WB) IL-6 release assays previously described for *Neisseria* vaccines [27]. The $Tdap_{OMVsBp}$ fulfilled the WHO criteria of safety for the MGW test [20]. For mice immunized with $Tdap_{OMVsBp}$, only small weight loss (<1.6% of the original weight) was observed at 16 h. This decrease was higher in wP immunized mice (3.2% of the original weight). Regardless of the treatment used, at day 3 after vaccination, all mice regain their weight registered before the first immunization dose. Furthermore, at day 7 all immunized mice exceeded their weight in more than 16.0 ± 5.3% of the weight registered at day 3. Results from WB assays also showed that the wP exhibits a higher endotoxic activity compared with the other two vaccines here used (Fig. 1). The averaged IL-6 cytokine release values are plotted against the concentration of stimulating antigen in Fig. 1. The $Tdap_{OMVsBp}$ vaccine was found to be significantly less active than the wP vaccine ($Tdap_{OMVsBp}$ 100 ng/ml: 5.3 ± 0.9 ng/ml *vs* wP 100 ng/ml: 9.5 ± 0.6 ng/ml). No cytokine stimulating activity was detected for the commercial aP vaccine which in principle does contain only traces of LPS (Fig. 1).

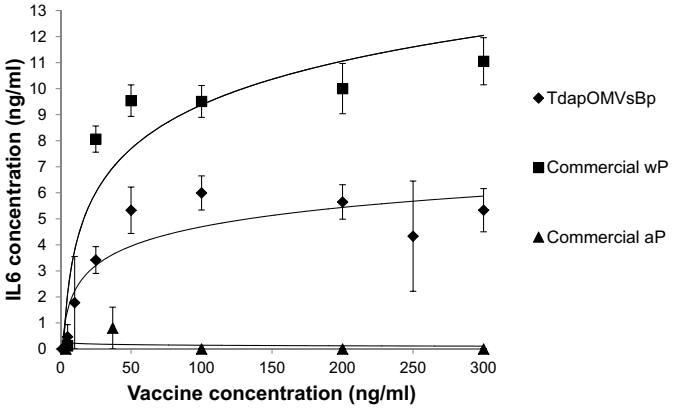

Fig. 1. IL-6 production in whole-blood stimulation assay. Average results of five assays comparing the responses to the commercial vaccines are shown. The bars represent the standard errors of the geometric means.

3.1. $Tdap_{OMVsBp}$ vaccination induces a mixed Th1, Th2 and also Th17 immune response

Since it was demonstrated the importance of Th17 cells in protection [16], in this work we investigated if $Tdap_{OMVsBp}$ vaccine exhibiting a mixed Th1/Th2 profile [20] could also induce an IL-17 response after active vaccination. At 2 months after mice immunization we found that in addition to the IFN-γ, IL-17 was also produced by spleen cells obtained from $Tdap_{OMVsBp}$ immunized mice (Fig. 2). The IL-17 concentrations detected in $Tdap_{OMVsBp}$ immunized mice were comparable to those detected for wP immunized mice when OMVs, or heat killed bacteria were used as stimulus (Fig. 2). Lower concentrations of both IFN-γ and IL-17 were detected in spleen cells obtained from commercial aP immunized mice (Fig. 2). In agreement with our previous report [20] IL-5 was produced at low concentrations by spleen cells from $Tdap_{OMVsBp}$-immunized mice, but it was induced at higher concentrations in aP immunized mice (Fig. 2). All these findings indicate that $Tdap_{OMVsBp}$ induce a mixed Th1, Th17 and Th2 response.

3.2. Serum from $Tdap_{OMVsBp}$-vaccinated mice protects against B. pertussis

In agreement with our previous results [20], this study evidences that $Tdap_{OMVsBp}$ vaccination induces a robust antibody response (Fig. 3). High IgG antibody titers against a *B. pertussis* whole cell lysate were detected in $Tdap_{OMVsBp}$ and wP vaccinated mice, whereas lower levels against this bacterial lysate were found in aP vaccinated mice (Fig. 3A). In contrast to commercial aP vaccine, $Tdap_{OMVsBp}$ triggered a murine antibody responses with a IgG2a/IgG1 > 1 (Fig. 3A). The levels of specific IgG against PTx (IgG-PTx) in aP immunized mice were higher than those from $Tdap_{OMVsBp}$ immunized mice (Fig. 3A). The lowest levels of IgG-PTx were detected in wP immunized mice (Fig. 3A). The contribution of these induced antibodies in the protection were analyzed by transfer experiments in mice. For comparison purposes equal volume of sera (100 μl) from mice immunized with two doses of the 3 vaccines here studied were transferred to groups of five

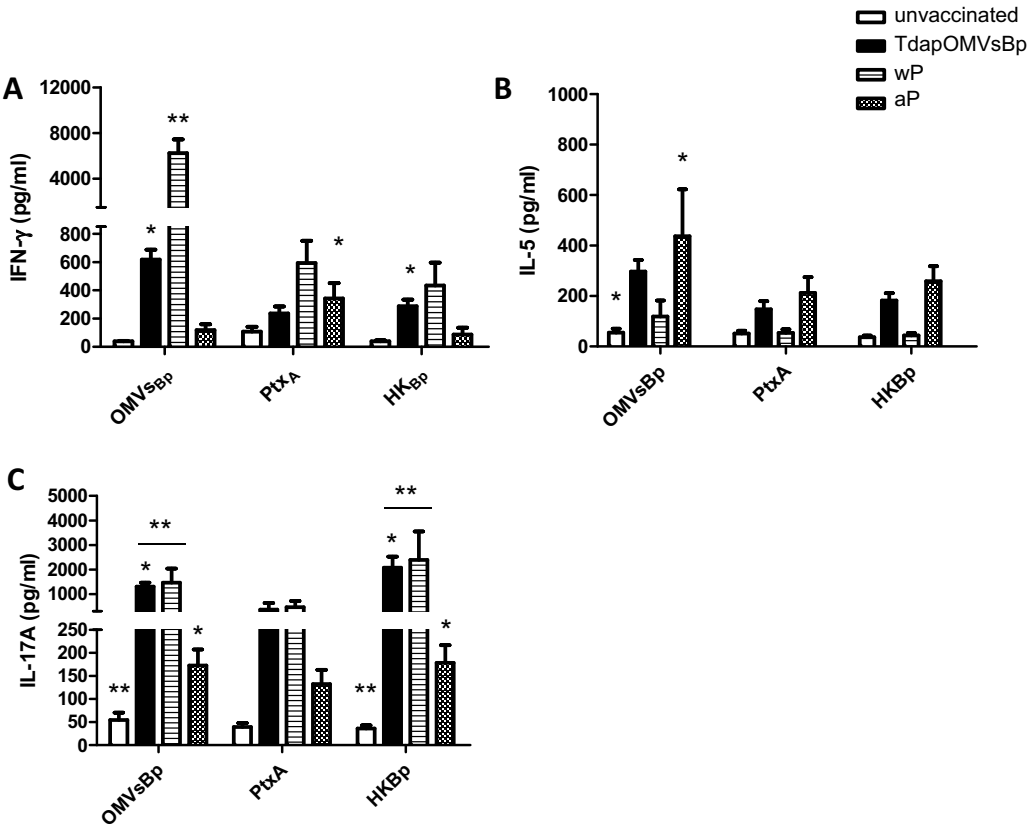

Fig. 2. Cytokine production by splenocytes from immunized mice. BALB/c mice were none immunized (unvaccinated) or immunized with two doses of $Tdap_{OMVsBp}$, wP or aP. Eight weeks after the last immunization, mice were sacrificed, and spleen cells were stimulated with OMVsBp, PTxA or HKBp. After 72 h of culture, the concentrations of IFN-γ (A), IL-5 (B) and IL-17A (C) were determined in the culture supernatant by ELISA. The results are expressed as mean values (±standard error) of three experiments with 4 mice per group. Significant differences were analyzed for each cytokine between different vaccines for each stimulus ($p \leq 0.05$).

female BALB/c mice. Twenty-four hours after transfer, mice were infected with *B. pertussis* and sacrificed 7 days later to determine the numbers of colony-forming units (CFU) in the lungs. Sera from non-immunized mice were used as a negative control of protection. As shown in Fig. 3B, transfer of 100 μl serum from $Tdap_{OMVsBp}$-immunized mice or from wP treated mice conferred adequate protection, as evidenced by a reduction in approximately 2.5 logs in CFU detected in the lungs compared to mice that had received sera from non-vaccinated donors (Fig. 3B). Higher level of protection was observed in mice that received sera from animals immunized with a high dose of commercial aP. No protection was achieved with serum transfer from non-immunized mice. As positive control of protection, active mice immunization was performed either with $Tdap_{OMVsBp}$, wP or aP vaccines. As shown in Fig. 3C all vaccines conferred strong protection, as approximately 2 logs of CFU were recovered from the lungs of immunized mice after challenge.

3.3. Characterization of the immune sera induced by the vaccines here used

Consistent with the ELISA-total IgG results obtained for $Tdap_{OMVsBp}$ and wP vaccines, *B. pertussis* was efficiently opsonized by both $Tdap_{OMVsBp}$ and wP-induced serum antibodies (Fig. 4). No significant internalization was detected either for bacteria opsonized with naïve serum or with aP-induced antibodies (Fig. 4). In agreement with these results, the recognition patterns obtained in blots from SDS-PAGE gels probed with $Tdap_{OMVsBp}$ and wP-induced sera were more complex than that of commercial aP (Fig. 5). The anti-$Tdap_{OMVsBp}$ sera reacted with the polypeptides present in both OMVs and bacterial lysate proteins. The recognition pattern was similar to that previously reported by us [30]. To identify the main recognized proteins, selected immune-reactive bands were excised from the 1D electrophoretic gels and then analyzed by mass spectrometry. Bands 1, 2 and 3 in Panel B of Fig. 5 were identified as GroEL-like protein (band 1, Panel B) and OMPc proteins (bands 2 and 3, Panel B), both of them very abundant in the outer membrane. Using purified recombinant proteins in the blots we detected that $Tdap_{OMVsBp}$ induced antibodies against PTxA, Fim2 and PRN (Fig. 5). Naïve sera were used as negative controls (not shown).

Sera obtained from commercial aP immunized mice recognized both OMVs and bacterial lysate proteins, a 69 kDa recognized protein corresponding to pertactin. In our experimental condition the sera obtained from aP immunized mice were able to recognize purified PTxA, PRN but not Fim2.

4. Discussion

In this work we examine in detail the immune response triggered by our recently developed OMV-based vaccine in comparison with wP and aP commercial formulations. As we previously reported the $Tdap_{OMVsBp}$ vaccine exhibits a mixed Th1/Th2 profile [20]. Additionally in this paper we demonstrate that $Tdap_{OMVsBp}$ vaccine also induces an IL-17 response after active vaccination. There is a consensus view that this mixed pattern of immune responses confers the best protective capacity against pertussis [31]. In fact there are several studies performed in the mouse model showing that wP vaccines and previous infection confer better protective immunity than aP vaccines because they induce Th1 cells and associated opsonizing antibodies, with a minor contribution by

A

Vaccine	Total IgG titer	IgG2a/IgG1	IgG-PTxA
Commercial wP	4710.00 ± 723.91	6.5 ± 0.90	15.12 ± 4.35
Commercial aP	58.56 ± 18.71	0.10 ± 0.10	67.21 ± 3.30
TdapOMVsBp	1387.41 ± 221.67	1.25 ± 0.43	58.15 ± 0.39

B

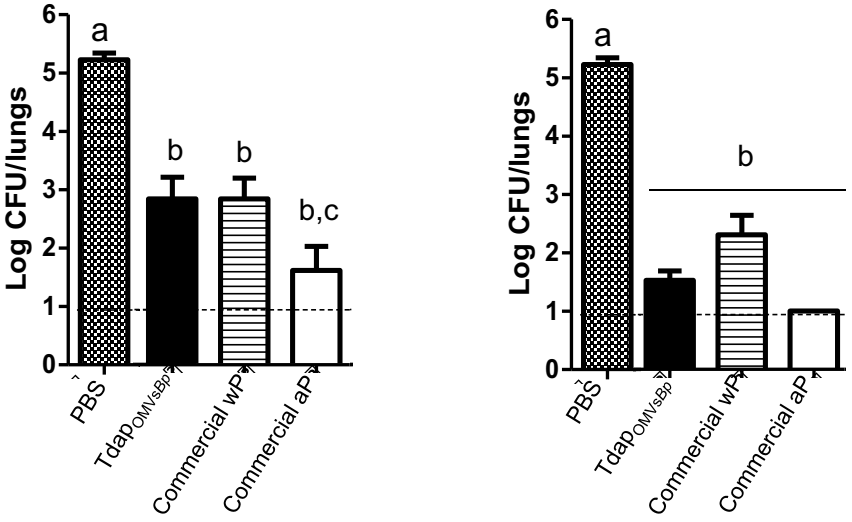

Fig. 3. Antibody titers and passive and active immunization induced by commercial wP and aP and TdapOMVsBp. Panel A shows both the total IgG titers and the IgG2a/IgG1 isotype ratio for the different vaccines used measured by indirect ELISA using either a *B. pertussis* whole cell lysate or purified PtxA subunit. Titers are expressed as geometric mean of each group ($n = 8$). Panel B and Panel C show the passive and active immunization, respectively, induced by the different treatments. *B. pertussis* Tohama was used as challenge bacteria (5×10^6 CFU 40 μl^{-1}). Three independent experiments were performed. Results depicted are means of four mice per group at 7 days post challenge. The dashed line indicates the lower limit of detection. The number of bacteria recovered from mouse lungs are expressed as the log 10 mean ± standard errors (error bars) of colony forming units per ml. Statistical analysis was performed by Bonferroni test. In Panel B, significant differences were detected for a *vs* b $p < 0.001$ and b *vs* c $p < 0.05$. In Panel C, different letters indicate significant differences with $p < 0.001$.

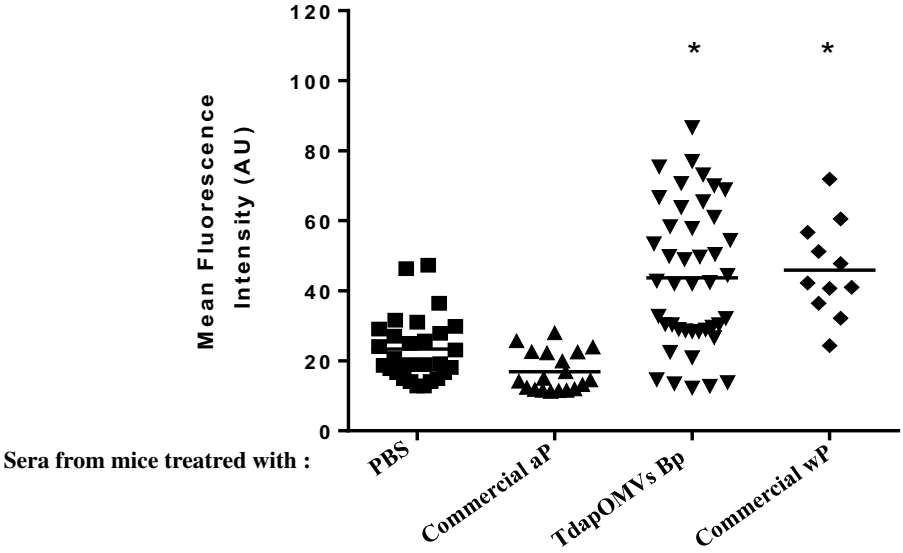

Fig. 4. Mean fluorescence intensity associated with macrophages incubated with GFP-expressing *B. pertussis* opsonized with serum from animals immunized with TdapOMVsBp, both commercial aP and wP or PBS. Each dot represents a different serum. AU indicates arbitrary units. * indicates $p \leq 0.01$.

Th17 cells [16,32]. In contrast, the less effective aP vaccines induce a robust Th2 response [16]. Recent evidence using the model of experimental pertussis in baboons [33] immunized with wP from different manufacturers showed that these vaccines were efficient to reduce pertussis burden, whereas aP, although able to prevent disease, was ineffective to reduce bacterial colonization [34]. This effect could be attributed not only to the wP capacity to elicit a Th17 response but to the broad response to different bacterial antigens as well.

Under this scenario, the Th1/Th17 and Th2 mixed immune response induced by $Tdap_{OMVsBp}$, which is close to the long lasting infection-acquired immunity [31], positions the OMV-based

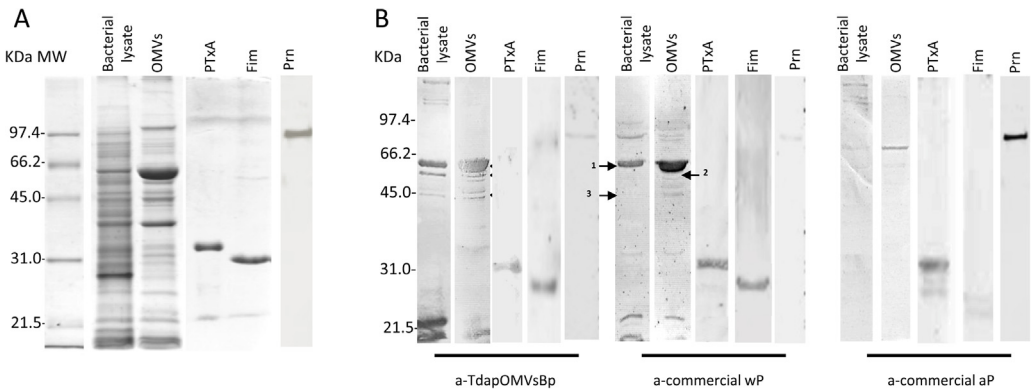

Fig. 5. (A) SDS-PAGE 12.5% (w/v) of bacterial lysates, OMVBp, purified recombinant PTxA, purified recombinant Fim2 and Prn. Molecular weights are indicated at the left. (B) Immunoblotting of the transferred SDS-PAGE proteins with anti-TdapOMVsBp induced antibodies (Panel at the left), anti-wP induced antibodies (Panel at the middle) and anti-aP induced antibodies (Panel at the right).

vaccine as a promising candidate to replace current acellular vaccines. Besides, this rationale is also validated by the low toxicity detected in both the *in vivo* mouse weight gain test and the *in vitro* whole blood human assay (Fig. 1).

We here confirmed that $Tdap_{OMVsBp}$ vaccination induces, as wP, a robust antibody response with a high IgG2a/IgG1 rate (Fig. 3A). These two vaccines induced high levels of IgG measured against a broad mixture of pertussis antigens, whereas specific levels of IgG anti-PTx were higher in $Tdap_{OMVsBp}$ and aP vaccinated animals. Interestingly, we observed that *B. pertussis* was efficiently opsonized by both $Tdap_{OMVsBp}$ and wP-induced serum antibodies (Fig. 4). In spite of the difference in the quality of the antibody response elicited by the different vaccines here used, in all cases serum transference from a vaccinated to a naïve animal did partially protect against *B. pertussis* infection (Fig. 3B). There are several mechanisms by which antibodies may confer protection: complement mediated bacterial killing, enhancement of opsonophagocytosis direct bacterial killing, inhibition of bacterial adherence/infection and blocking of bacterial toxins, among others [35,36]. In the case of wP and $Tdap_{OMVsBp}$ the high IgG titers against a broad array of pertussis antigens, including opsonizing antibodies should be the main effectors in the passive transference of protection, as it has been suggested [37,38]. Regarding commercial aP vaccine, the induced antibodies failed to opsonize *B. pertussis* bacteria in the tested condition (Fig. 4), in coincidence with data that indicates that aP vaccination does not elicit serum bactericidal activity [39]. However, serum from aP vaccinated mice transferred protection against pertussis infection (Fig. 3), indicating that other antibody activities contribute to passive transference of protection in this case [40]. In this sense, the documented capacity to confer anti-pertussis protection through antibody transfer through placenta is mainly mediated by IgG1 isotype [41]. Moreover, it has been shown that this isotype correlates with protection after aP in mouse challenge model [42]. In fact, it was speculated that this isotype could contribute to passive protection by other effector functions in addition to complement fixation or opsonophagocytosis [43].

The main antigen targets of OMVs elicited response compared to aP and wP was also examined. In agreement with previous reports [30], the antibody responses induced by OMVs were directed against GroEL, OMPc but also PTx Fim and PRN. Thus wP multiantigenic composition elicits a broad humoral response [44], dominated by some particular proteins, among which the chaperonin GroEL triggers the highest response in different species [45]. Recently Raeven et al. have described the immunoproteomics profiling of a preparation of OMVs from *B. pertussis* compared with aP and wP [46]. Interestingly, they have shown that OMVs vaccination elicited a broad response characterized by production of different IgG isotypes against multiple antigens. While the protein composition of their OMV preparation differs from the one used here, probably due to the *B. pertussis* strains used for production, growing conditions or downstream processing, we could also observe broad antibody response elicited by our formulation. Interestingly, our preparation can elicit antibodies against the major antigens present in aP formulation, which are known to be key targets of anti-pertussis immunity. Taken together all the data on the comparative characterization of the humoral response elicited by OMVs show that this vaccine candidate can elicit a broad antibody response, with good levels against the key virulence factors and higher IgG2a/IgG1 ratio than aP and opsonophagocytic activity that contribute to protective capacity.

Considering the serious epidemiologic situation of pertussis worldwide, several strategies are followed to produce a third generation of anti-pertussis vaccines that should be safe and induce protective immunity [47]. Collectively, the data presented here show that the administration of OMVs-based vaccine triggers a wide spectrum of protective effector mechanisms associated with control of *B. pertussis* infection. These features of OMV-induced protective immunity position this candidate among the different possibilities to constitute the third generation of anti-pertussis vaccines.

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