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Outer membrane vesicles obtained from *Bordetella pertussis* Tohama expressing the lipid A deacylase PagL as a novel acellular vaccine candidate

Cristian J.A. Asensio[a,1], María Emilia Gaillard[a,1], Griselda Moreno[c], Daniela Bottero[a], Eugenia Zurita[a], Martin Rumbo[c], Peter van der Ley[b], Arno van der Ark[b], Daniela Hozbor[a,*]

[a] *Instituto de Biotecnología y Biología Molecular (IBBM), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Centro Científico Tecnológico CONICET La Plata, Calles 47 y 115, 1900 La Plata, Buenos Aires, Argentina*
[b] *Netherlands Vaccine Institute, 3720 AL Bilthoven, The Netherlands*
[c] *Laboratorio de Investigaciones del Sistema Inmune (LISIN), Facultad de Ciencias Exactas, UNLP 47 y 115 (1900) La Plata, Argentina*

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ABSTRACT

In an effort to devise a safer and effective pertussis acelullar vaccine, outer membrane vesicles (OMVs) were engineered to decrease their endotoxicity. The *pag*L gene from *Bordetella bronchiseptica*, which encodes a lipid A 3-deacylase, was expressed in *Bordetella pertussis* strain Tohama I. The resulting OMVs, designated OMVs$_{Bp\text{PagL}}$, contain tetra- instead of penta-acylated LOS, in addition to pertussis surface immunogens such as pertactin and pertussis toxin, as the wild type OMVs. The characterized pertussis OMVs$_{Bp\text{PagL}}$ were used in murine *B. pertussis* intranasal (i.n.) challenge model to examine their protective capacity when delivered by i.n. routes. Immunized BALB/c mice were challenged with sublethal doses of *B. pertussis*. Significant differences between immunized animals and the PBS treated group were observed ($p < 0.001$). Adequate elimination rates ($p < 0.005$) were observed in mice immunized either with OMVs$_{Bp\text{PagL}}$ and wild type OMVs. All OMV preparations tested were non toxic according to WHO criteria; however, OMVs$_{Bp\text{PagL}}$ displayed almost no weight loss at 3 days post administration, indicating less toxicity when compared with wild type OMVs. Induction of IL6- and IL1-expression in lung after i.n. delivery as well as neutrophil recruitment to airways showed coincident results, with a lower induction of the proinflammatory cytokines and lower recruitment in the case of OMVs$_{Bp\text{PagL}}$ compared to wild type OMVs.

Given their lower endotoxic activity and retained protective capacity in the mouse model, OMVs$_{Bp\text{PagL}}$ obtained from *B. pertussis* seem as interesting candidates to be considered for the development of novel multi-antigen vaccine.

1. Introduction

Protein subunit vaccines (non replicating system or acellular vaccines) are particularly attractive among traditional vaccine designs, since they induce a protective immune response avoiding the safety limitations of either attenuated or killed whole microorganism vaccines. Despite their advantages in safety, a major limitation of protein subunit antigens is their inability to stimulate strong immune responses *in vivo* when administered alone. Subunit antigens are combined with adjuvant, conjugated to polysaccharide or protein carriers, or formulated in controlled-release systems in order to improve their immunogenicity [1–7]. Controlled-release technologies have emerged as promising strategies for antigen delivery, because they are similar in geometry to naturally occurring pathogens and are readily internalized by antigen-presenting cells. These delivery systems primarily comprise polymer particles [4], immune-stimulating complexes [5], liposomes, proteosomes, and related vesicles [6,8,9]. Although these formulations seem to be technically appealing solutions, the complex manufacturing steps required to purify and encapsulate antigens in particulate delivery systems can render these approaches economically expensive affecting its potential use, especially in developing countries [10].

An interesting alternative that can replace those strategies consists in the use of outer membrane vesicles (OMVs) which contain naturally incorporated bacterial surface antigens. In fact, there are currently two vaccines for serogroup B meningococcal disease that are OMVs that naturally bleb from the outer membrane of the Gram-negative bacteria, and they contain components derived from the *Neisseria meningitidis* outer membrane and periplasm [11]. The proven safety and efficacy records of these OMVs vaccines [12,13] together with the knowledge that vesicles are produced by nearly all species of Gram-negative bacteria during both planktonic growth and in surface attached biofilm communities [14],

* Corresponding author. Tel.: +54 221 425 0497x31.
E-mail address: hozbor@biol.unlp.edu.ar (D. Hozbor).
[1] These authors equally have contributed to the work.

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present the possibility of employing the OMV vaccination strategy for prevention of other diseases. In this context, we have recently demonstrated that OMVs derived from *Bordetella pertussis* can protect against intranasal pertussis challenge when administered by either intraperitoneal or intranasal route in a mouse model of infection [15]. The composition of these OMVs was determined by SDS-PAGE, immunoblot techniques and gel electrophoresis associated to tandem mass spectrometry. These techniques revealed the presence of the main well known *B. pertussis* surface immunogens in the OMVs such as pertactin, adenylate cyclase–haemolysin, pertussis toxin, as well as the lipo-oligosaccharide (LOS) and other proteins predicted to have outer membrane or periplasmic location.

The respiratory disease caused by *B. pertussis* is currently prevented by administration of different pertussis acellular vaccines (Pa) composed up to five antigens (filamentous hemagglutinin [FHA], pertactin [PRN], pertussis toxin [PT], and two fimbrial proteins [Fim]) [16]. Though these vaccines have proven to be successful in reducing morbidity and mortality of pertussis they need to be improved since their efficacy rounds 70–90%, depending on the formulation considered and the technical definition of a clinical case [17–19]. Moreover, Pa composed of only a few bacterial proteins may be less efficacious because of recently observed vaccine-induced antigenic shifts and adaptations. Such concerns are heightened by the recent discovery of circulating strains that are either deficient in two of the antigens included in the acellular vaccines, PT and PRN [20] or present alterations in the *ptx* promoter that result in increased production of PT [21]. In this respect, OMVs seems to have important advantages in vaccine development over those acellular vaccines since the native OMVs have a multi-immunogenic capacity to carry a wide spectrum of endogenous antigens, in addition to the natural self-adjuvanticity that is exerted by several innate immune response activating components inherent to OMVs, such as outer membrane proteins (OMPs) and lipopolysaccharide (LPS).

In order to achieve the use of OMVs as vaccine, an important aspect to be addressed and improved is the safety issue since native OMVs contain endotoxic LPS (LOS, for the case of *B. pertussis*), which could provoke excessive secretion of proinflammatory cytokines in the host [22]. The endotoxic activity and adjuvant activity of LOS are properties based both upon the recognition of the LOS by the host Toll-like receptor (TLR) complex TLR4/MD-2 and the subsequent activation of NFk-β [23]. The reactogenicity of the classic whole cell pertussis vaccines has been associated with proinflammatory cytokines [24,25]. Hence, a straightforward approach to reducing reactogenicity would be the generation of a pertussis vaccine with less toxic lipid A or a reduced quantity of LOS. In efforts to refine the *B. pertussis* OMV in this sense, we employed a recombinant *B. pertussis* strain carrying the lipid A-modifying enzyme, PagL. This enzyme hydrolyzes the ester bond at the 3 position of lipid A, thereby modulating the recognition of lipid A by the TLR4/MD-2 receptor complex and the resulting endotoxic activity [26]. Here, we studied the consequence of PagL expression for the endotoxic activity and the protective capacity of *B. pertussis* OMVs. The goal of this study was to investigate whether PagL might be a useful tool for decreasing the LOS-mediated reactogenicity, without altering the previously demonstrated action of OMVs as a good acelullar vaccine.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. pertussis Tohama strain (CIP 8132) and *B. pertussis* Tohama strain carrying the broad-host-range vector pMMB67EH containing *pagL* gene of *Bordetella bronchiseptica* (hereafter referred as *Bp*PagL), were used throughout this study. *B. pertussis* Tohama strain carrying the empty vector pMMB67EH (hereafter referred as *Bp*Plasmid) was used as a control in many of the experiments described below. *B. pertussis* strains were grown in Stainer–Scholte liquid medium (SS) as indicated previously [27], with ampicillin (100 μg/ml) and IPTG (1 mM) for the plasmid-carrying derivatives.

For expression in *B. pertussis* the *pag*L, Geurtsen et al. [31] subcloned the gene of *B. bronchiseptica* [pagL(Bb)] into the broad-host-range, low-copy vector pMMB67EH [28]. Plasmid with the correct insert were designated by those author pMMB67EH-PagL(Bb).

2.2. Lipo-oligosaccaride extraction from bacterial cells

The LOS either from wild type and recombinant PagL *B. pertussis* strains was isolated by the hot phenol–water method along with the modifications previously described [29]. The obtained samples of LOS were dialyzed and lyophilized. Dry weight measures were used to determine the amounts of the LOS obtained. The quality of each sample was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, not shown) [30].

LOS modification of *Bp*PagL was checked on whole bacteria suspended in isobutyric acid-ammonium hydroxide 1 M (5:3, v/v) [31]. Lipid A was extracted as described previously [32] with slight modifications. The lipid A structure was subsequently analyzed by nanoelectrospray tandem mass spectrometry (MS/MS) on a Finnigan LCQ in the negative (MS) or positive (MS/MS) ion mode.

2.3. Isolation of outer membrane vesicles (OMVs)

OMVs were isolated from bacterial cells as previously described [15,33]. Briefly, culture samples from the decelerating growth phase were centrifuged at 10,000 × *g* for 20 min at 4 °C and the bacterial pellet obtained was resuspended in 20 mM Tris–HCl, 2 mM EDTA pH 8.5 (TE buffer). Five milliliters of TE buffer were used to resuspend approximately 1 g (wet weight) of bacteria. 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 in 1.5% (w/v) deoxycholate (DOC) in TE buffer. Six milliliters of this suspension were added on 2 ml of sucrose 60% (w/v).

After centrifugation at 100,000 × *g* for 2 h at 4 °C, the OMV band was observed at TE/sucrose interphase. The OMVs were stored with glycerol 1% and sodium azide 0.001%° at 4 °C. The samples obtained for all the *B. pertussis* strains used were negatively stained and then examined with an electron microscope.

2.4. Electron microscope negative stains

Electron microscopy was performed by suspending OMVs in 0.1 M ammonium acetate (pH 7.0). A droplet of this suspension was placed on a grid coated with a carbon-reinforced fomvar film. After 30 s, the excess fluid was removed by absorbing with filter paper and the grids stained with 2% (w/v) phosphotungstic acid pH 5.2 (with KOH). Examination was done with a JEM 1200 EX *Jeol* microscope.

2.5. Protein assay

Protein content was estimated by the Bradford method [34] using bovine serum albumin as standard.

2.6. Immunoblots

Samples of OMV obtained from *B. pertussis* cells were treated with Laemmli sample buffer [30] and run on 15% SDS gels. The electrophoresis was performed at room temperature and constant voltage, with molecular weights being estimated by means of

the Pharmacia Calibration Kit. After electrophoresis, the proteins were transferred from the polyacrylamide to a polivinylidenphosphate membrane (Immobilon P, Millipore) and incubated with mouse polyclonal immune sera directed against PT of *B. pertussis*. As a control commercial pertussis toxin (Sigma) was used. The immunochemical detection was performed using alkaline phosphatase-labeled sheep anti-mouse immunoglobulins.

2.7. Lipo-oligosaccharide extraction from OMVs and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

LOS from OMVs were solubilized in the sample buffer described above and heated at 100 °C for 10 min. Twenty-five micrograms of proteinase K in 10 μl of buffer were added per 50 μl of LOS suspension. The mixtures were incubated in a water bath at 60 °C for 1 h, occasionally mixing. Proteinase K-treated samples were applied to gels. Electrophoresis was performed at room temperature and constant voltage. The LOS was visualized by the BioRad silver-staining technique.

2.8. 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 intranasal vaccinated with detoxified (detoxification with 0.37% formalin 20 h at 37 °C) OMVs obtained from *B. pertussis* parental and recombinant strains. Two quantities of OMVs expressed in μg of proteins, 3 and 20 μg 40 μl^{-1}, were used in these experiments. 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 following requirements (WHO and EP requirements): (a) the total weight of the mice from the vaccine group 3 days after treatment was the same or higher than the initial weight, (b) at the end of 7 days the average weight gain of the vaccine group was not less than 60% of the control group and (c) not more than 5% of the animals died during the test period. The data obtained in all cases were represented by looking at each individual mouse over time and assessing weight loss or gain. ANOVA test was used to compare the data obtained.

2.9. Expression of lung inflammatory markers upon intranasal delivery of OMVs

2.9.1. Tissue processing and RNA isolation

Fifty microliters of OMV preparation containing 3 μg of total protein was administered intranasally to BALB/c mice. At different times upon stimulation mice were sacrificed by cervical dislocation. Inferior lobe of right lung was immediately processed for total RNA isolation on using Nuclespin RNAII system (GE, USA) following manufacturer instructions.

2.9.2. Reverse transcription and quantitative real-time PCR

RT-qPCR was performed as described [15], using 100 ng of total RNA and MMLV-RT (Promega). Resulting cDNA was amplified in triplicates using SYBR® Green PCR assay (Bio Rad Laboratories, Hercules, CA, USA) and products were detected on an ICycler (Bio Rad). PCR samples were incubated for 2 min at 50 °C and for 10 min at 95 °C, followed by 40 amplification cycles with 1 min annealing/extension at 60 °C and 15 s denaturation at 95 °C. The β-actin expression was used as normalizer. Specificity of PCR was checked by melting curves. Relative mRNA levels were determined by comparing the normalized PCR cycle threshold (Ct) between cDNA samples of the gene of interest as previously described [35]. Specific primers for IL1b and IL6 used are: IL1 β fwd: 5′ AAT CTA TAC CTG TCC TGT CCT GTG TAA TGA AAG AC, IL1 β rev: TGG GTA TTG CTT GGG ATC CA 3′; IL6 fwd: 5′ GTT CTC TGG GAA ATC GTG GAA A, IL6 rev: AAG TGC ATC ATC GTT GTT CAT ACA 3′. Animal experiments were performed at least in triplicates.

2.10. Bronchoalveolar lavage (BAL) cells analysis

Mice treated with either purified wild type LOS or LOS PagL (1 μg) were euthanized and the thoracic cavity was dissected. After severing the descending aorta, the blood in the lungs was cleared by perfusion through the heart right lobe with PBS at room temperature until the lungs became pale. To perform the BAL, the trachea was partially cut and 1 ml of sterile PBS containing 0.1% BSA was flushed into the lungs and then withdrawn. This procedure was repeated 3 times. To remove red blood cells, BAL fluid was treated with Red Cell Lysis Buffer (Becton Dickinson, USA) for 3 min at room temperature. The resultant cells were then washed, resuspended and counted using a Neubauer chamber.

Cells from the BAL were stained with fluorescent antibodies for 1 h at 4 °C, and flow cytometry analysis was performed using a FACSCalibur from Becton Dickinson. FITC-, PE-, APC-, and PerCP-conjugated monoclonal specific antibodies for CD11c (clone N418, hamster IgG, eBioscience), GR1 (clone RB6-8C5, Rat IgG2b, eBioscience), CD11b (clone M1/70.15, Rat IgG2b, CALTAG LABORATORIES), Ly-6G (clone 1A8, Rat IgG2a, BD Pharmingen) and Ly6C (AL-21, Rat IgM, BD Pharmingen) were used to label the cells. Results were processed using FlowJo software (TriStar Inc., Oregon, USA).

2.11. Active immunization and intranasal challenge

Female BALB/c mice were obtained at 4 weeks of age from Biol. SAIC, Argentina. Animal protocol was performed as we previously described [36]. OMVs from either *B. pertussis* Tohama phase I parental strain (CIP8132) or the recombinants derivatives strains carrying the plasmid pMMB67EH or pMMB67EH::*pagL*were detoxified with formalin (0.37% at 37 °C overnight).

Groups of 15 BALB/c mice were immunized intranasally with detoxified OMVs (3 or 20 μg of protein in 40 μl) without adjuvant. 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^{-1}) of Tohama strain. Lungs of challenged mice were collected for bacterial counts 2 h, 5 days and 8 days after the challenge. The lungs were aseptically removed, homogenized in the sterile PBS, were serially diluted, and then plated on Bordet–Gengou plates supplemented with defibrinated sheep blood (10%, v/v) to determine bacterial recoveries at different time points during the course of infection. At least three independent experiments were performed.

2.12. Statistical analysis

Means and standard deviations were calculated from Log_{10}-transformed CFU numbers. Differences among means were assessed by one-way and two-way ANOVA with significance accepted at the $p < 0.05$ level. Differences between groups were analyzed using Bonferroni Test (GraphPad Prims®).

3. Results and discussion

The isolation and characterization of *B. pertussis* OMVs has been described by us a few years ago [15,33]. OMVs contain many of the most important surface antigens used for immunization. Hence, we have already investigated the use of OMVs derived from *B. pertussis* as a potential vaccine candidate [15]. However, the fact that the OMVs contain in their composition LOS is a hurdle aspect that gives some shade to this vaccine formulation as the LOS exhibits endotoxic activity. Therefore, efforts should be made in finding ways of

rendering the LOS molecules non-endotoxic. This problem associated to the LOS was also considered for cellular pertussis vaccine by other researchers and for instance, the LOS has been removed by the whole cell pertussis vaccine [37]. This is a different approach aiming the same result, a safer pertussis vaccine targeting the same LOS molecule.

Increasing the activity of the outer membrane-located PagL enzyme was the strategy that we chose to render the LOS molecules non-endotoxic since this enzyme causes a deacylation of lipid A with a marked reduction of LOS endotoxicity of many bacteria. PagL, was first discovered in *Salmonella enterica serovar Typhimurium* and then in a wide range of other Gram-negative bacteria, including *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* [38]. However, in *B. pertussis*, the *pag*L open reading frame is disrupted by a frameshift, whereas in *B. bronchiseptica* and *B. parapertussis* the open reading frame was intact. Therefore in order to produce PagL in *B. pertussis* strain Tohama, Geurtsen et al. [31] introduced the *pag*L gene of *B. bronchiseptica* [*pag*L(Bb)] into *B. pertussis* on the plasmid vector pMMB67. The LOS modifications of this recombinant strain were corroborated by ESI-MS in the negative-ion mode, which showed a highly effective removal of the 3-linked C10-3OH acyl chain [26]. After PagL expression, only tetra-acylated forms lacking the C10-3OH acyl chain were observed, with two major peaks at 1310 and 1390 representing the mono- and di-phosphorylated forms, respectively. Here we used this strategy to produce and test efficacy and toxicity of OMVs derived from a PagL over-expressing *B. pertussis* strain Tohama phase I (CIP 8132).

3.1. OMVs isolation and characterization

As described in our previous work, vesicle formation from cell pellets could be induced by sonication [33]. Following this methodology, OMVs from *B. pertussis* Tohama phase I strain (CIP 8132) and *B. pertussis* Tohama phase I strain carrying either the broad-host-range vector pMMB67EH or this vector containing *pagL* gene of *B. bronchiseptica* were obtained. In electron microscopic observations (Fig. 1A), OMVs from the three *B. pertussis* strains were visible as nanosphere vesicles with variable sizes ranging from 40 to 200 nm in diameter. The protein profiles of these OMVs analyzed by SDS PAGE were very similar (Fig. 1B) and the presence of well known surface immunogens such as PRN (Fig. 1C) and PT (Fig. 1D) and also of LOS (Fig. 1E) could be detected in the three OMVs preparations (Fig. 1B). As shown in Fig. 1C the amount of PRN normalized to the amount of serum resistance protein Bp3494 is maintained in the three preparations of OMVs showing that ectopic expression of PagL not induce a significant change in the amount of PRN.

At least 6 independent replicates of the OMVs isolation and characterization procedure were carried out. In all cases similar

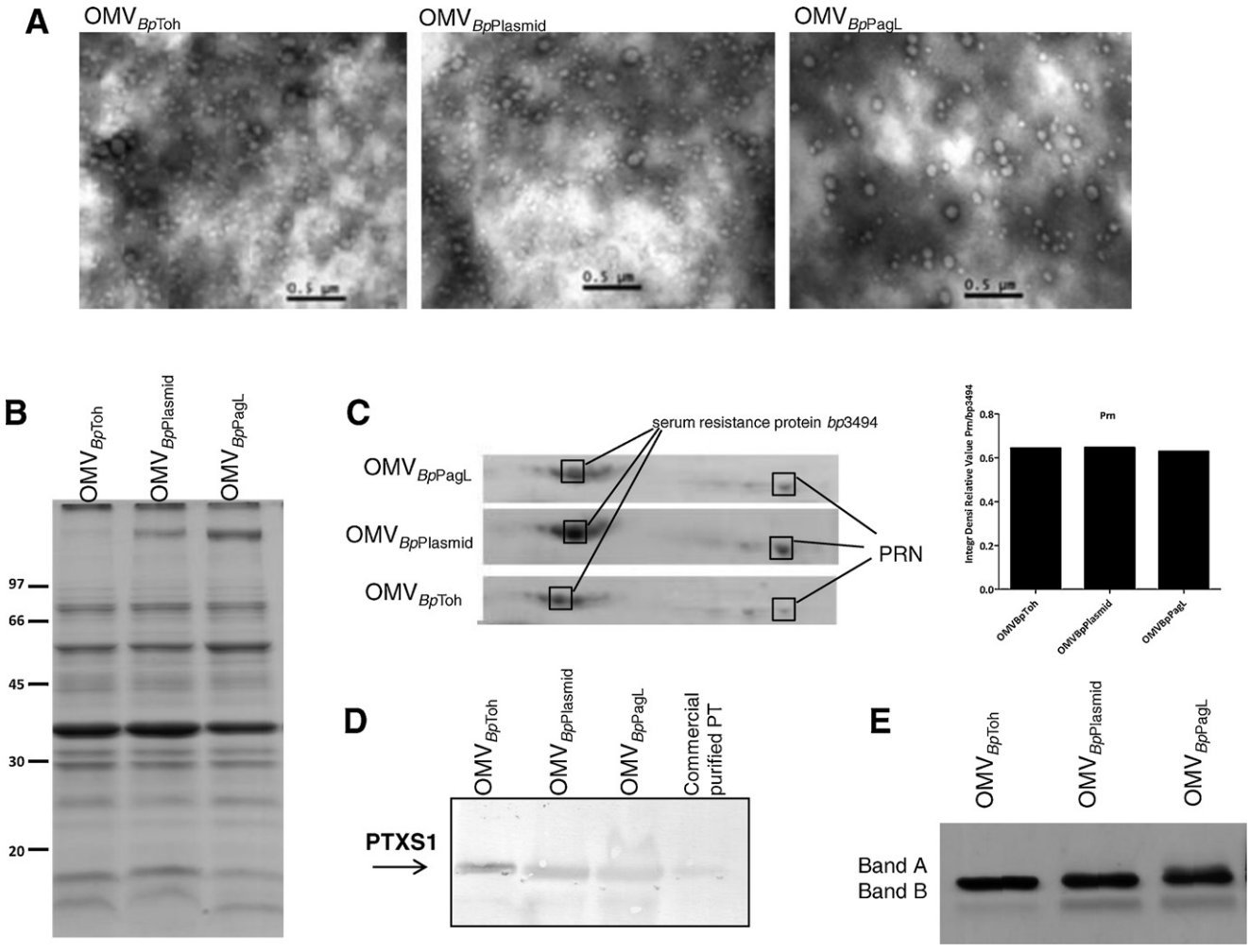

Fig. 1. (A) Electron micrograph of negative stained OMVs obtained from *Bordetella pertussis* Tohama I (OMV_{BpToh}), *B. pertussis* Tohama I containing the empty plasmid pMMB67EH ($OMV_{BpPlasmid}$) and *B. pertussis* Tohama containing the recombinant plasmid pMMB67EH::pagL (OMV_{BpPagL}) (scale bar: 150 nm). (B) SDS-PAGE 12.5% of OMV_{BpToh}, $OMV_{BpPlasmid}$ and OMV_{BpPagL}. Molecular weights are indicated at the left. (C) Left panel: section of 2D Electrophoresis gel containing pertactin spots of OMV_{BpToh}, $OMV_{BpPlasmid}$ and OMV_{BpPagL}. Right panel: relative densitometric values of PRN/bp3494 from the three OMVs. (D) Western blot with anti-pertussis toxin subunit 1 antibodies of OMV suspensions from cultures of *Bp*Toh, *Bp*Plasmid and *Bp*PagL. (E) LOS content in OMV_{BpToh}, $OMV_{BpPlasmid}$ and OMV_{BpPagL} suspensions normalized by total protein content.

morphology, size distribution and presence of surface immunogens were observed. This result means that changes introduced in LOS by expression of the PagL enzyme are not affecting the tested parameters on OMVs obtained from *B. pertussis*. The verify the effective deacylation of LOS by the introduced *pag*L gene, lipid A was isolated and analyzed by MS. After PagL expression, only tetraacylated forms lacking the C10-3OH acyl chain were observed, with two major peaks at 1310 and 1390 representing the mono- and di-phosphorylated forms, respectively.

3.2. Protection against intranasal B. pertussis challenge after vaccination with OMVs

In order to evaluate the protective capacity induced by the OMVs prepared from *B. pertussis*, Tohama strain carrying the recombinant vector pMMB67EH::*pag*L, animal assays using intranasal *B. pertussis* challenge were performed. In particular, the effect on subsequent colonization by Tohama strain (5×10^6 CFU 50 μl^{-1}) after two intranasal administrations of OMVs (either with 3 μg or 20 μg of protein in each immunization) was analyzed. The results obtained were compared with those obtained in mice intranasal immunized with OMVs obtained from *B. pertussis* Tohama parental strain and the recombinant strain carrying the empty vector used as a control. All OMVs vaccines were detoxified by formalin treatment. Animals vaccinated twice with PBS were used as negative control. Significant differences in lung bacterial counts between immunized animals either with 3 μg or 20 μg of each OMV and the control group were observed ($p < 0.001$) (Fig. 2). Adequate elimination rates ($p < 0.001$) were observed in mice immunized with all OMVs vaccines used. While in mice immunized with PBS the number of recovered colonies from lungs was high (10^5 CFU per lung), in mice immunized with OMVs (3 μg) either from parental or recombinant strains, the number of lung recovered colonies at day 5 post challenge dropped at least five orders of magnitude in relation to the counting in non immunized mice (Fig. 2). Similar results were obtained with 20 μg of each OMVs (data not shown). Efficacy of OMVs as protective immunogen is not affected by the changes introduced upon expression of PagL. The data presented here support the use of OMVs obtained from either parental or recombinant *B. pertussis* strains for cleared faster *B. pertussis* from the lungs of mice.

3.3. Mouse weight gain test

The mouse weight gain test is widely used to measure the toxicity of pertussis vaccine formulations. Results of mouse weight gain test obtained with OMVs formulation obtained from the *B. pertussis* PagL recombinant strain were compared with those of parental and recombinant *B. pertussis* strains containing only the empty vector. For these assays we used two different quantities of OMVs, 3 μg and 20 μg, which were given intranasally in different mice groups. Mice were weighed 16 h, 3 and 7 days after immunization. The lower dose of OMV used for vaccination resulted in no alteration in the weight gain curve, meaning that all formulations were non toxic. However, when we analyzed the toxicity of other protective OMVs quantity (20 μg) which is predicted to be more toxic due to the higher content of LOS, we observed that mice vaccinated with OMVs extracted from either the parental strain or recombinant strain carrying the empty plasmid, lose weight at 16 h after the immunization (Fig. 3). At 3 days post vaccination, the weight losses observed for those treatments were more evident.

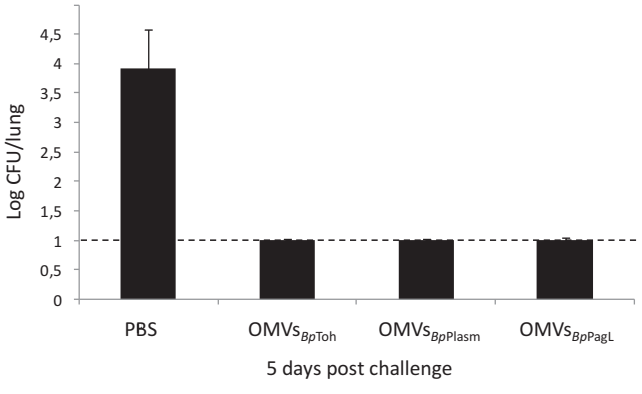

Fig. 2. Effect of intranasal immunization with OMV$_{BpToh}$, OMV$_{BpPlasmid}$ or OMV$_{BpPagL}$ in the mouse intranasal challenge model. The WHO reference strain *B. pertussis* Tohama was used as challenge bacteria (5×10^6 CFU 40 μl^{-1}). Three independent experiments were performed. Results from one representative experiment are shown. Results depicted are means of five mice per group at 5 days post challenge. The dashed line indicates the lower limit of detection. Bacterial numbers are expressed as the log 10 means ± standard errors (error bars).

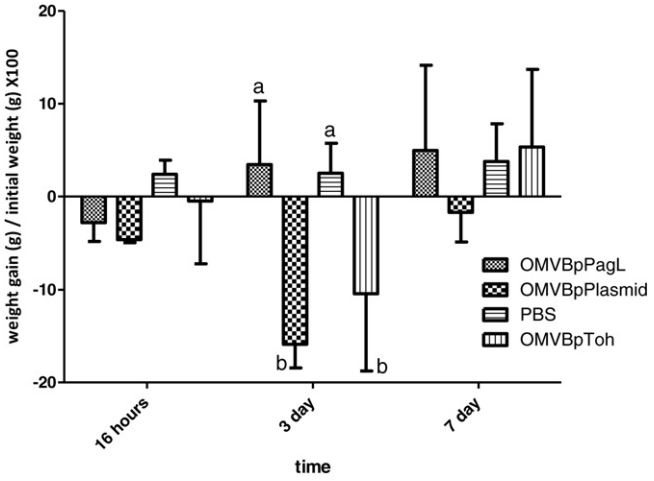

Fig. 3. Mouse weight gain test using groups of 8 Balb/c mice which were intranasal vaccinated with detoxified OMVs (20 μg/40 μl) obtained from *B. pertussis* parental and recombinant strains. Control group received an equal volume of sterile PBS. Animals were observed for 7 days and body weight was recorded after 16 h, 3 and 7 days. $p < 0.001$ comparison of PBS vs. OMV$_{BpPlasmid}$, $p < 0.05$ comparisons of PBS vs. OMV$_{BpToh}$ and OMV$_{BpToh}$ vs OMV$_{BpPagL}$, $p < 0.001$ comparison of OMV$_{BpPagL}$ vs. OMV$_{BpPlasmid}$.

In the case of mice immunized with the OMVs obtained from the strain that ectopically expresses the enzyme PagL, only small weight loss was observed at 16 h but at 3 days after vaccination, all treated mice regain their weight (Fig. 3).

At day 7, all mice exceeded the weight registered at day 3 regardless of treatment used, only mice. Only mice treated with OMVs$_{BpPlasmid}$ did not regain the original weight.

For all the analyzed times, PBS-treated mice increased their weight. Altogether these results show that the OMVs obtained from the recombinant strain expressing PagL is less toxic than the OMVs obtained from either the parental strain or the recombinant strain containing the empty plasmid.

3.4. Activation of lung innate response and bronchoalveolar lavage (BAL) cells analysis

In order to compare the induction of proinflammatory markers in lung upon intranasal immunization, we evaluated the expression of IL6 and IL1b (Fig. 4). In all cases, administration of OMV preparation induces a consistent transcriptional response with rise in IL-6 and IL-1β specific mRNA. However, in the case of OMVs obtained from *B. pertussis* that expressed PagL, the levels of IL1b and IL6 were lower than the ones observed upon administration of wild type OMVs ($p \leq 0.05$), consistent with the lower inflammatory profile of this preparation.

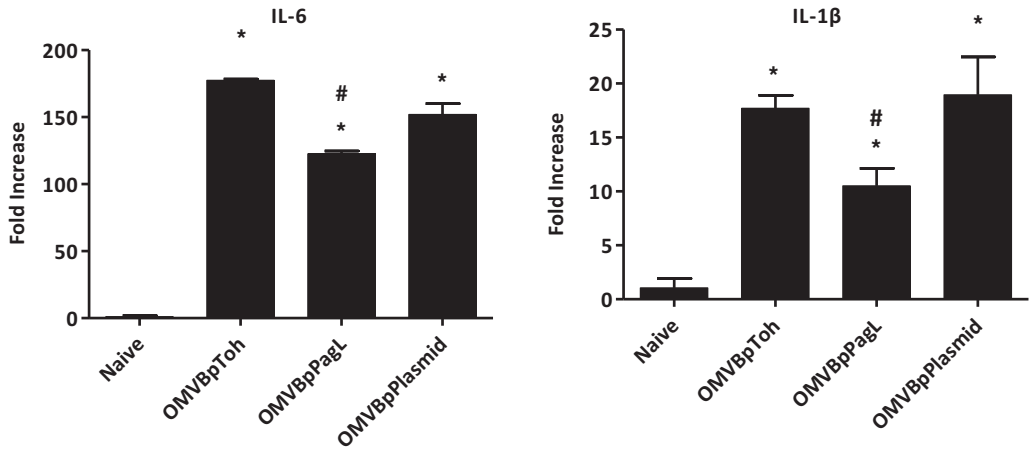

Fig. 4. Levels of inflammatory cytokines mRNA in lungs of mice intranasally treated with OMVs from wild type *B. pertussis* (Bp Toh), or from mock transfected *B. pertussis* (empty plasmid) or with plasmids containing functional PagL enzymes. Results are mean of five mice per group at each time. (A) mRNA levels of IL-6, (B) mRNA levels of IL-1β. $^{*}p \leq 0.05$ comparison of each condition vs. naïve control. $^{\#}p \leq 0.05$ OMVs of different conditions vs OMV_{BpToh}.

In order to confirm that changes in LOS structure are responsible for the different biological activity observed in the OMV preparations, we next analyzed the cell recruitment to the lung and airways after treated mice with purified LOS from either parental strain or recombinant *B. pertussis* strain expressing PagL enzyme. Cells from bronchoalveolar lavages (BAL) were sampled at different 2 and 24 h after infection, counted and analyzed by flow cytometry (Fig. 5). Prior to inoculation, alveolar macrophages defined as $CD11c^{+}$ $CD11b^{+}$ $Ly6G^{-}$ $Gr1^{-}$ cells were the predominant cell population. Following LOS treatment, different incoming cell populations could be identified on the basis of CD11c, CD11b, Gr1, Ly6C and/or Ly6G expression. Fast recruitment of neutrophils to the bronchoalveolar space is a hallmark of proinflammatory activation of airways [39]. Differences in neutrophils recruitment into the bronchoalveolar compartment of lungs between wild type LOS and PagL LOS were observed. Neutrophils, characterized as $CD11c^{-}$ $CD11b^{+}$ $Ly6G^{+}$ $Gr1^{+}$, were more massively recruited in mice treated with wild type LOS than those treated with PagL LOS. The magnitude of neutrophil recruitment was significantly increased at 6 h after inoculation and persisted 24 h after treatment. In both cases differences in neutrophil recruitment were observed among both LOS preparations. These results are in coincidence with the low toxicity observed in the mouse weight gain test and the diminished proinflammatory capacity induced by OMV_{BpPagL}.

Although the toxicity of LOS from PagL-expressing *B. pertussis* has been previously shown to be reduced compared to wild type LOS by Geurtsen et al. [31], these authors did not observe the same behavior when they analyzed the endotoxic activity of the whole cells containing PagL-modified *B. pertussis* LOSs. They proposed that this paradoxical result may be explained, in part, by an increased

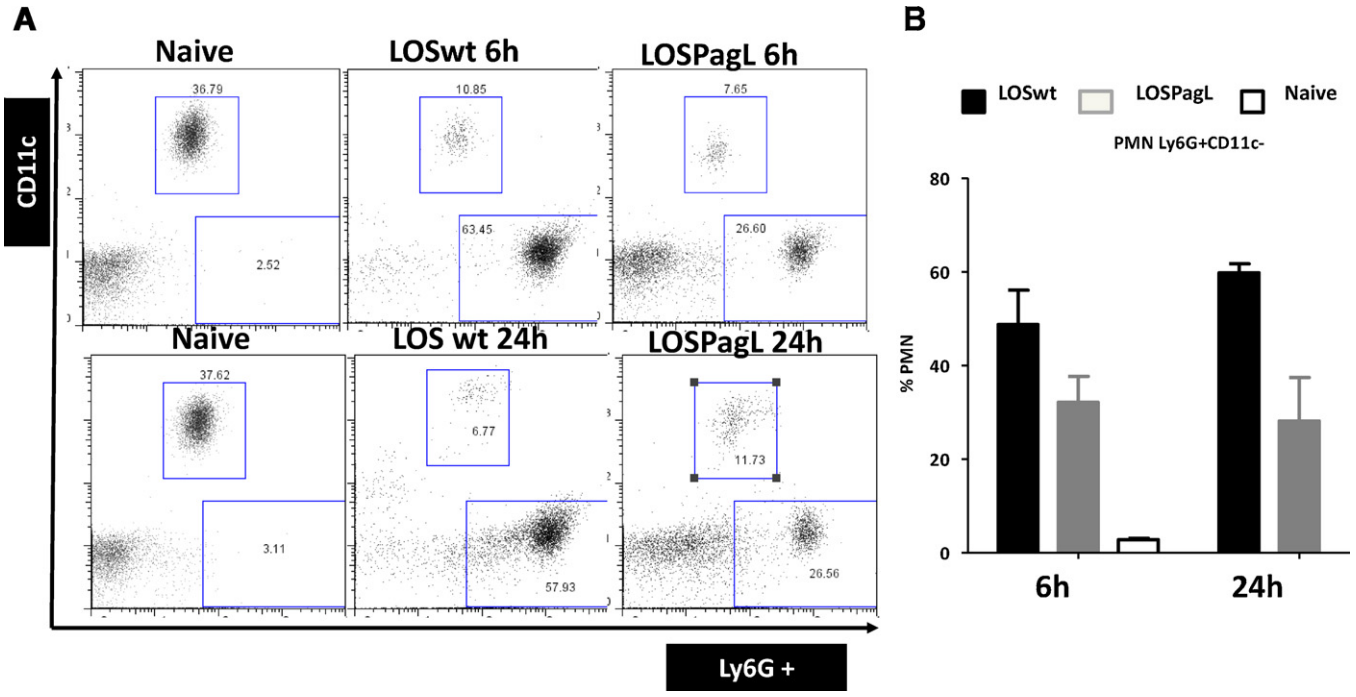

Fig. 5. Flow cytometry analysis of bronchoalveolar lavage (BAL) from mice after intranasal administration of 1 μg of LOS purified from *Bp*PagL compared with LOS from *Bp*Toh. Upon recovery, cells were stained with CD11c, CD11b, Gr1, Ly6C and/or Ly6G marked with different fluorochromes. Main populations of alveolar macrophages and neutrophils were defined as CD11c+, CD11b+, Gr1−, Ly6G− and CD11c−, CD11b+, Gr1+, Ly6G+, respectively. (A) Kinetic results from the dot plot analysis of cells gated for CD11b+, where alveolar macrophage and neutrophil gate are identified. LOS used for stimulation and time is indicated in the figure. (B) Percentage of neutrophils in BAL at different times after inoculation. Results shown are mean and standard deviation of three animals processed independently from a typical experiment performed twice.

release of LOS, which they observed with the PagL expressing cells. In the case of OMVs, the LOS present in the preparation would only derive from what is left in the outer membrane, as the centrifugation and extraction steps can be expected to have removed any free LOS. Consequently, toxicity of these preparations would only depend on the direct effect of modifications to LOS structure by the PagL enzyme, which indeed seems to be the case, according to results shown here.

Owing largely to their plasticity and also safety profile in humans, OMVs are attractive as vaccines and also as vehicles for vaccine delivery. After the first reports of OMV vaccines for serogroup B meningococcal disease, vesicles from *Salmonella thyphimurium* [40], *Pseudomonas aeruginosa* [41] and *B. pertussis* [15], containing surface antigens native to the pathogens have also been shown to exhibit immunogenic properties. In the case of *B. pertussis* we previously described the development and characterization of outer membrane vesicles (OMVs) as a good pertussis candidate to eliminate faster *B. pertussis* either for systemic and nasal administration. This last route has been considered as an alternative that may possibly improve pertussis vaccination [42,43]. Furthermore, the production of these OMVs has an important advantage over purified proteins since vesicle purification lies in simple ultracentrifugation steps, which effectively eliminates the need for costly infrastructure to support a priori purification of each of the antigens now included in the acellular pertussis vaccines. As the present study demonstrates, genetic engineering of the bacterial strains used for OMV production can be used to further improve their efficacy and/or safety.

Altogether, data presented here supports the use of OMVs obtained from recombinant *B. pertussis* strain expressing PagL as a good and safe formulation to improve *B. pertussis* elimination. This work indicates the potential of engineered OMV technology to overcome the significant safety and economic limitations that often arise in the course of acellular vaccine development.

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