Mucosal Immunization with Shigella flexneri Outer Membrane Vesicles induced protection in mice

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1. INTRODUCTION

According to World Health Organization (WHO), approximately 2.5 billion cases of diarrhea occurred worldwide which results in 1.5 million deaths among children under

the age of five. It is a common cause of death in developing countries and the second most common cause of infant deaths. Among the main causes, Shigellosis is responsible of more than 165 million cases annually, leading to 1.2 million deaths [1]. Furthermore, many cases progress into serious damages in their intestinal epithelium that will limit the correct nutrient absorption with the subsequent sequel for life. *Shigella* spread massively within the community and from person to person, and hence, prevention relies on basic sanitary measures, which unfortunately may be not possible applied for many countries. In addition, the increasing problem of antibiotic resistance alerts on the urgent need of protective vaccines. In fact, the World Health Organization has made the development of a safe and effective vaccine against *Shigella* a high priority [1].

The efforts have been mainly focussed on live oral vaccines with several vaccine candidates on clinical trials [2]. However, development of such safe *Shigella* vaccine is being problematical, and no vaccine is still available[3].

Currently, most vaccines in development are acellular vaccines which [2;4] in comparison to live-attenuated or whole inactivated organism, are safer. However, these prototypes require adjuvants to achieve a more effective immune response. The challenge is the designing of formulations able to enhance the immunogenicity of associated antigens, through the right activation of the immune system, and susceptible to be administered by mucosal routes. Previous studies of our group have evaluated the adjuvant capability of nanoparticles made from the copolymer of methyl vinyl ether and maleic anhydride (Gantrez AN®). These nanoparticles demonstrated their ability to initiate a strong and balanced mucosal immune response and then, to efficiently induce Th-1 subset [5]. In addition, these nanoparticles loaded with different antigens have showed to be effective against experimental challenges with *Salmonella* or *Brucella* [6-9].

In this work we propose the use of outer membrane vesicles (OMVs) from *Shigella* as the source of relevant antigens to be included in the acellular vaccine. OMVs are secreted from the outer membrane of a large variety of gram negative bacteria, during *in vitro* culture and during infection [10]. Currently, there have been described many functions for this blebbing process. Functions proposed vary from facilitating the intracellular bacterial growth within phagocytes [11], to the delivery of effectors molecules critical for pathogen dissemination such as pathogen-associated molecular patterns (PAMPs) and other virulence factors to host cells [12-14].

We therefore describe here the preparation, characterization and evaluation of *Shigella flexneri* outer membrane vesicles in order to be used in vaccination. We obtained the OMVs from *S. flexneri* 2a, being this the most common cause of shigellosis. In fact, it's responsible for 25 to 50 percent of all cases in the developing world [2]. The protective efficacy of OMVs either in their free form or adjuvanted in NP were tested in the murine pneumonia model [15] after immunization with one single dose by intradermal or mucosal routes.

The OMVs formulations obtained and characterized here were found to induce protection in mice after one single dose against a lethal dose of *S. flexneri* 2a.

2. MATERIAL AND METHODS

Preparation and characterization of outer membrane vesicles

OMVs were obtained from *S. flexneri 2a* (clinical isolate from Hospital de Navarra, Pamplona, Spain). Vesicles were purified from a method adapted from Horstman and

Kuehn [16]. Bacteria were grown in LB broth overnight to early stationary phase. Then, bacteria were inactivated with a solution of binary ethylenimine and formaldehyde (6 mM BEI- 0, 06% FA, 6 h, 37 ºC). BEI was prepared as a 0.1 M solution by cyclization of 0.1 M 2-bromoethylamine hydrobromide (Sigma) in 0.175 M NaOH solution for one hour following the method of Bahnemann [17]. Cells were removed by pelleting (10,000 × g, 10 min). Supernatant was filtered through a 0.45 μm Durapore PVDF filter (Millipore) and purified by ultradiafiltration via a 300-kDa tangential filtration concentration unit (Millipore). The retentate was freezed in order to induce larger blebs formed through reassociation of the smaller ones into multimicelles, as had been proposed previously [18]. Final product was recovered by centrifugation at 40,000 × g, 2 h. Total protein content was determined by the method of Lowry, with bovine serum albumin as standard. Lypopolysaccharide (LPS) content was determined by Purpald assay[19;20]. Briefly, to 50 μL of LPS samples or standards [21] in each of the duplicate wells in a 96-well tissue culture plate, 50 μL of 32 mM NaIO4 was added, and the plate was incubated for 25 min followed by addition of 50 μL of 136 mM purpald reagent in 2 N NaOH. After further incubation for 20 min, 50 μL of 64mM NaIO4 was added, and the plate was incubated for another 20 min. The foam in each well can be eliminated by addition of 20 μL 2-propanol. The absorbance of each well was measured by a plate reader at 550 nm. Finally, extract was resuspended in sample buffer 1× and analyzed by SDS-PAGE and immunoblotting, using polyclonal pool sera from patient infected with *S. flexneri* (Clínica Universidad de Navarra) or anti IpaC mAb (kindly provided by A. *Phalipon*, Institut Pasteur). The morphology of the vesicles was examined by Field Emission Scanning Electron Microscope.

Outer membrane proteins (OMPs) from *S. flexneri* were prepared by sequential detergent extraction of cell envelopes [18]. Briefly, after the disruption of cells by

sonication (4 pulses × 5 min, power 2, Branson Sonifier 450), whole bacteria were removed by centrifugation at 6000 × g, 15 min. Cell envelopes were recovered from supernatant by centrifugation (40 000 × g, 1h). Pellet was resuspended in 1% Sarkosyl (N-Lauryl sarcosine, Sigma Chemical Co., St. Louis, USA), incubated for 30 min and further centrifuged at 40 000g, 1h, twice. The enriched sediment in outer membrane proteins was suspended in 0.5 M Tris-HCl (pH 6.8) with 10% SDS (Lauryl sulfate, Sigma) and boiled for 15 min and finally, centrifuged (20 000 × g; 30 min). The OMPs of *S.* flexneri were present in the final supernatant.

Ipa (invasion plasmid antigens) proteins secretion assay. Secretion of Ipa proteins through the TTSS (Type three secretion system) was induced using a Congo Red secretion assay [22]. Exponential-phase bacteria were harvested, resuspended in 10 μM Congo Red/PBS, and incubated at 37 ºC for 30 min. Following incubation, bacteria were pelleted by centrifugation, and supernatants were collected and passed through a 0.22 μm-pore filter. Proteins in the supernatants, which represent proteins secreted through the TTSS, were then concentrated by tricholoroacetic acid precipitation. Finally, extract was resuspended in sample buffer 1× and analyzed by SDS-PAGE and immunoblotting using anti-IpaB or -IpaC mAb (kindly provided by A. *Phalipon*, Institut Pasteur).

Preparation and characterization of nanoparticles

Poly (anhydride) nanoparticles were prepared by a modification of the solvent displacement method [6;23]. Briefly, 100 mg of the copolymer of methyl vinyl ether and maleic anhydride (PVM/MA) (Gantrez®AN 119; M.W. 200 KDa) was dissolved in 5 ml acetone under magnetic stirring at room temperature. On the other hand, 5 mg OMVs were dispersed by ultrasonication with a probe Microson TM (Misonix Inc.,

New York, USA) in 10 ml water for 1 min. After dispersion, nanoparticles were formed by addition of this water phase containing OMVs. The agitation was maintained during 15 min in order to allow the stabilization of the system. Organic solvents were removed under reduced pressure (Büchi R-144, Switzerland). The obtained nanoparticles were collected by centrifugation (27.000 × g, 20 min, 4 ºC) and washed with water twice. Finally, particles were freeze-dried using sucrose 5% as crioprotector.

The preparation of empty nanoparticles was performed in the same way in the absence of OMVs.

Characterization of nanoparticles. The particle size and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1/10) and measured at 25ºC by dynamic light scattering angle of 90ºC. The zeta potential was determined as follows: 200 µL of the samples was diluted in 2 mL of a 0.1 mM KCl solution adjusted to pH 7.4. The morphology of the vesicles was examined by Field Emission Scanning Electron Microscope (Carl Zeiss, model Ultra Plus). For this purpose freeze-dried formulations were resuspended in ultrapure water and centrifuged at $27,000 \times g$ for 20 min at 4 °C. Then, supernatants were rejected and the obtained pellets were mounted on TEM grids. The yield of the nanoparticles preparation process was determined by gravimetry as described previously [23]. Briefly, poly (anhydride) nanoparticles, freshly prepared, were freeze-dried. Then, the yield was calculated as the difference between the initial amount of the polymer used to prepare nanoparticles and the weight of the freeze-dried carriers.

Loading capacity of nanoparticles. The yield of nanoparticles was calculated from the difference between the initial amount of the polymer used to prepare the particles and the weight of the freeze-dried samples. The ability of PVM/MA nanoparticles to entrap the complex antigen was directly determined after degradation of loaded nanoparticles with NaOH. Briefly, OMVs-loaded Gantrez nanoparticles (15 mg) were dispersed in water vortexing 1 min. After centrifugation (27.000 × g, 15 min) pellet was resupended in NaOH 0,1 M sonicated *(Microson^{TM} Ultrasonic cell disruptor)* and incubated for 1 h to assess the total delivery of the associated antigen. After this time, the amount of antigen released from the nanoparticles was determined using microbicin choninic acid (microBCA) protein assay (Pierce, Rockford, CA, USA). In order to avoid interferences of the process, calibration curves were made with degraded blank nanoparticles, and all measurements were performed in triplicate.

Determination of the structural integrity and antigenity of OMVs. Western-blot analysis was used as a qualitative tool to examine the structure of the antigens, complementing the quantification performed by microBCA. To accomplish this analysis, the protocol for nanoparticle degradation was modified in order to avoid any interference of the enzyme. In this case, after nanoparticle isolation, 15 mg of loaded nanoparticles were dispersed in water vortexing 1 min. After centrifugation (27 000 × g, 15 min) pellet was resupended in 2 ml of a mixed of dimethilformamide: acetone (1:3) (-80 ºC, 1h). After centrifugation, pellet was resuspended in acetone (-80 ºC, 30 min). Finally, extract was resuspended in sample buffer 1× and analyzed by SDS-PAGE and immunoblotting using polyclonal sera from hyperimmunized rabbit with *S. flexneri* [24].

SDS-PAGE and Immunoblotting

SDS-PAGE was performed in 12% acrylamide slabs (Criterion XT, Bio Rad Laboratories, CA) with the discontinuous buffer system of Laemmli and gels stained with Coomassie blue or silver staining. After electrophoresis, gels were electroblotted to a PVDF (polyvinylidene fluoride) membrane at 0.8 mA/cm^2 for 30 min. Then, membranes were soaked overnight in a blocking solution containing 3% (w/v) of non-fat milk and then incubated in the presence of different sera, described above. After the incubation, the membranes were washed five times; the anti-rabbit or human Ig-alkaline phosphatase conjugate was added, followed by incubation for an additional hour. The membranes were exhaustively washed and the antibody–antigen complexes were visualized after addition of the substrate/chromogen solution (H_2O_2/cloronaftol).

Active immunization and challenge.

All mice were treated in accordance with institutional guidelines for treatment of animals (Protocol 087/06 of animal treatment, approved in 1 October 2007 by the Ethical Comity for the Animal Experimentation, CEEA, of the University of Navarra). Nine-week-old BALB/c mice (20±1 g) were separated in randomized groups of 6 animals and immunized with OMVs either free or encapsulated in PVM/MA NPs by intradermal, nasal, ocular (20 µg of extract) or oral route (100 µg of extract). The scheme of administration and doses are summarized in Table 1.

Challenge infection was performed on day 35 intranasally with a lethal dose of 1×10^7 UFC/Mouse of *Shigella flexneri* 2a (clinical isolate) grown to logarithmic phase and suspended in 20 µl of prewarmed PBS. The number of dead mice after challenge was recorded daily.

Measurement of immune response in the mouse.

Blood samples were collected from the reto-orbital plexures of anesthetized mice

ELISA. The antibody response was measured by an enzyme-linked immunosorbent assay (ELISA). In brief, 96-well microtiter plates (MaxiSorb; Nunc, Wiesbaden, Germany) were coated with 100 uL of 10 μg/ml OMVs in coating buffer (60 mM carbonate buffer, pH 9.6). Afterwards, unspecific binding sites were saturated with 3% bovine serum albumin (BSA) in PBS for 1 h at RT. Sera from mice were serially diluted in PBS with 1% BSA and incubated overnight at RT. After intense washing with PBS Tween 20 (PBS-T) buffer, the alkaline phosphatase (AP)-conjugated detection antibody, class-specific goat anti-mouse IgG/IgA (Sigma) for sera, was added for 1h at 37ºC. The detection reaction was performed by incubating the sample with ABTS substrate for 20 min at room temperature. Absorbance was measured with an ELISA reader (Sunrise remote; Tecan-Austria, Groeding, Austria) at a wavelength of 405 nm.

Quantification of cytokines from sera. Cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, IL17, IFN-γ, and tumor necrosis factor) were quantified from serum by luminex-based multiplex assay (Milliplex; Millipore, Billerica, MA) using a Bioplex analyzer (Bio-Rad, Hercules, CA).

Statistics

Statistical analyses were performed using GraphPad Prism 5 for Mac OS X. All experiments were performed with n=6. Statistical comparisons between antibody serum levels were performed using Kruskal-Wallis test, followed by Dunn´s post-hoc test. The statistical significance was set at $P < 0.05$. For cytokine levels, it was performed using single-factor analysis of variance, followed by Turkey´s post hoc test. The statistical significance was set at $P<0.001$. The Kaplan-Meyer curves were used for analysis of the protection experiment.

3. RESULTS

Isolation and characterization of *Shigella flexneri* OMVs.

The scanning electron microscopy showed that the OMVs secreted *in vitro* by *S. flexneri* were spherical, with an average diameter of 50 nm (Fig. 1A). The yield obtained was 18 ± 0, 04 μg/ mg determined after lyophilisation and referred to the original cell culture dry weight. Quantitative analysis showed that protein content was 54.52 ± 3.2 %, whereas the LPS content was 37.6 ± 4.8 %. A comparative SDS-PAGE analysis of the OMVs revealed that contained proteins corresponded to the OmpA, 34 KDa; OmpC/OmpF, 38/42 kDa; VirG, 120 KDa (Fig. 2) already described by other authors as the main inmunodominant antigenic proteins [25;26]. As expected, the outer membrane protein enriched fraction and the purified OMVs showed a similar profile. Furthermore, OMVs contained bands at 62 KDa, 42 Kda and 38kDa that correspond with IpaB, IpaC and IpaD respectively (Fig. 2) [27]. Immunoblot assay using a monoclonal antibody specific to IpaB or IpaC demonstrated that these proteins were located on vesicles (Fig. 2), confirming the observation of Kadurugamuwa and Beveridge [28] .

Characterization of OMVs-containig nanoparticles

The yield of the OMV antigen-loaded NPs manufactured in relation to the initial amount of polymer employed was consistent (89%). Vaccine formulations were homogeneous and spherically shaped (Fig 1A). The average size of NP-OMV was 197 nm with a polydispersity index of 0.06.

The Z potential of NP was tested before and after OMV encapsulation. Results suggest that OMV is at least partially bound on the NP surface, indicated by the change in Z of NP. Zeta potential of free OMVs was -14.1 +/- 3 mV. The encapsulation of the extract in nanoparticles resulted in a change of Z potential from -44 +/- 4 mV to -27 +/- 4 mV when OMVs were loaded into PVM/MA nanoparticles.

To further confirm OMV encapsulation into NPs, BCA protein determination and SDS-PAGE/immunoblotting were also performed. The procedure involved the use of a purification step in order to discard unbound OMV. *S. flexneri* OMVs were efficiently associated with PVM/MA nanoparticles, as they showed a loading encapsulation of 20 μg OMVs/ mg of polymer. Besides, an immunoblotting was carried out using sera from rabbit hyper-immunized with *S. flexneri*. Results indicate that entrapment in nanoparticles did not alter its antigenic properties (Fig. 1B).

Evaluation of the immunogenicity and protection conferred by OMVs vaccine.

Groups of 6 mice were immunized once by intradermal or mucosal routes with OMVs (20 μg/mouse), either free or encapsulated in NPs. A control group of non-immunized mice was also included. All animals immunized by nasal or ocular routes remained in good health, exhibiting no respiratory difficulties, changes in body temperature, or abnormal behaviour. Oral immunized mice showed a transient abdominal swelling a few hours after immunization. By contrast, mice immunized intradermally experimented sweating and lethargy during 2 days post-immunization, which disappeared thereafter.

Specific IgG2a and IgG1 against OMVs antigens were determined by indirect-ELISA at days 0, 15 and 35 post-immunization (Fig. 3). Results expressed that the OMV

immunization by either route elicited significant levels of serum IgG1 and IgG2a with respect control mice (Fig. 3). Higher levels of IgG were found in groups immunized intradermally. Overall the levels of IgG2a (Th1 response) were higher than that those of IgG1 (Th2). An adjuvant effect after encapsulation was observed on the immunogenicity (global specific antibody response) especially after oral immunization. There were not found significant differences in the mucosal levels of the IgA elicited after intradermal or mucosal deliveries.

Levels of serum cytokines were determined at day 15 post-immunization (Fig. 4). The encapsulation of OMVs in NPs induced an increase in the level of IL-12 (p40) and a decrease of IL-10 with respect to the free form, by intradermal or oral delivery. In contrast, after ocular or nasal immunization, the inverse switching phenomenon was observed.

At day 35 after immunization, mice were challenged with *S. flexneri* via intranasal route and monitored for survival over 30 days (*n* = 6 mice/group) (Fig. 5). Nasal or ocular immunizations with free OMVs provided complete protection. Non-significant differences were found between OMV free or nano-encapsulated in groups immunized by nasal, ocular or oral route. In contrast, the intradermally delivery of free OMVs was not protective, while the encapsulated extract conferred full protection.

4. DISCUSSION

Currently, live vaccines provide better protection as compared to the inactivated vaccines, including the acellular ones[2;29]. However, it is always difficult to properly calibrate attenuation to achieve the minimum of toxicity with the optimal immunogenicity. Besides, the use of live *Shigella* vaccines is questionable since this

pathogen is able to strongly interfere with the immune response, by inducing an immunosuppressive condition that favors infective process. In our present experimental study, we support the use of mucosal immunization with acellular vaccines. Results demonstrated a significant efficacy and no reactogenicity in the mice pulmonary model.

The best prophylactic measure probably would be to prevent bacterial invasion by neutralizing key surface virulence factors. The outer membrane (OM) of *Shigella* contains several main virulence factors, including outer membrane proteins (OMP), protein adhesins, the highly conserved virulence-plasmid-encoded Ipa proteins [28] as well as LPS. These are essential components in the invasion process, and can alter the course of infection and the host responses, and therefore their neutralization for the host will succeed in protective immunity. [25;30-33].

Outer membrane vesicles (OMVs) consist of OM and soluble periplasmic components shed from gram-negative bacteria. This blebbing process is considered as a peculiar bacterial extracellular secretion system than enable bacterial colonization and impairs host immune response [34]. Therefore, it is plausible to think on *Shigella* OMVs as ideal candidates for an acellular vaccine. The capacity of OMV-based vaccines to stimulate a protective immune response has already been exploited against several bacterial pathogens, such as *Brucella ovis* [18], *S. typhimurium* [35], *Flavobacterium [36] Porfiromonas* [37] or *Neisseria meningitides* B, with over 55 million doses administered to date of the former [38].

As many gram-negative bacteria, *Shigella* bleb off membrane vesicles during normal growth. Kadurugamuwa, *et al.* already obtained and characterized membrane vesicles from *S. flexneri [39].* In order to obtain this material massively, we developed an extraction protocol that also maximize OMV purity. Vesicles were isolated from

concentrated, cell-free culture supernatant leading to an appropriate antigenic profile as well as high purity grade. Besides, final product was ultradiafiltered in order to avoid interferences in the encapsulation process.

OMV used here contain key alarm signals such as LPS, OMPs and Ipa recognized by the innate immune system, including epthelial cells, MALT and antigen presenting cells[40;41], and therefore have the capacity to either enhance bacterial clearance or cause host tissue damage by activating an inflammatory response. It is interesting to note that these components provide a prolonged stimulation of the inflammatory response that, at first instance, facilitates bacterial survival in the tissues. However, this fact will lead to the bacteria elimination by the host immune system[42].

In fact, our results indicate that a single dose of non-adjuvated OMVs delivered by mucosal routes is able to protect against a lethal challenge with *S. flexneri*. Vaccines that stimulate protective mucosal immune responses often need an adjuvant for proper delivery and presentation to the mucosal immune tissues. The mechanisms underlying the effectiveness of free OMV without external adjuvant may be explained by the nature of some individual components contained within this "proteoliposome" or/and by the biophysical properties of these vesicles [43]. Besides, Ipa containing OMVs may contribute to its adjuvanticity by their ability to interact with host cell receptors which facilitate OMVs transcytosis across mucosal epithelial barriers [27]. On the other hand, the amphipatic properties of OMVs may facilitate its own movement through mucosal tissues, enhancing antigen presentation to drive a protective response.

In this study, we measured the levels of cytokines in OMVs vaccinated mice two weeks after the immunization. Then, we analyzed their association with the challenge outcome. A strong association between the ratio of IL-12p40/ IL-10 and protection was found.

Moreover, low levels of IFN-γ correlated with protection. However, conclusions from these particular data must be taken with caution since cytokine levels were measured directly from serum. At this point, further studies are being carried out to really establish a correlation of these parameters and protection.

After oral administration, under steady-state conditions, some factors released by enterocytes, such as retinoic acid, thymic stromal lymphopoietin and TGF-β, will "condition" non-activated resident DCs to elicit a Th2 or regulatory responses [44]. However, following an inflammatory stimulus, a recruitment of DC expressing CX3CR1 to the mucosal tissues is observed, increasing the number of DC extending dendrites into intestinal lumen. Under this state of high activation, DC-expressing massively co-stimulatory molecules, present the antigenic determinant to the specific T naïve cells in the T area MALT. The substantial distinctive release of IL-12 from those DCs will also contribute to the further differentiation of naïve cells to Th1/Th2/Th17, linked to an inflammatory response. Actually, our results would support it since OMVs adjuvanted into NPs induced increasing levels of IL-12 (p40) and decreasing IL-10 with respect to the free form, either by intradermal or oral delivery. NPs can enhance the delivery of the loaded antigen to the gut lymphoid cells due to their ability to be captured and internalized by cells of the gut-associated lymphoid tissue (GALT), and to induced maturation of DCs with a significant upregulation of CD40, CD80, and CD86 and a Th1 response in animal models. The mechanisms responsible for DC maturation may be related to TLR-NP specific interaction [5].

On the other hand, the encapsulation of OMVs in NPs induced an increase in the level of IL-10 and a decrease of IL-12 (p40) with respect to the free form, by ocular or nasal routes, which is characteristic of mucosal adjuvants that usually stimulate a Th2

T-cell response [45-47], characterized by increased secretory IgA, high proportions of antigen-specific serum IgG1, and the stimulation and synthesis of IL-4, IL-5, and IL-10.

The specific immune mechanisms that mediate resistance to *Shigella* infection have not been clearly defined and are currently being debated. Thus, in humans, up regulation of both proinflammatory and anti-inflammatory are observed during the first stages of infection. Later, in relation with the convalescent stage of shigellosis, an increase in IFN-γ is observed. Summing up, although Th1 is effective to control infection, a Th2 response may be also as effective but shorter-lasting.

Concerning the antibody response elicited after OMV immunization, we can not establish a relation between antibody levels and protection. Serum and mucosal antibodies to LPS and the Ipa proteins have been demonstrated during human shigellosis [48;49]. However, it has not been established the role of these antibodies to limit the spread or severity of the infection. The apparent inconsistency between IgG subclass response and cytokine profile may be due to immune cells other than T helper cells.

The ultimate goal for vaccination is to stimulate long-lasting protective immunological memory. Toll-like receptors [50] generally promote adaptive immune responses indirectly by activating innate immune cells. It has been recently shown that the use of multiple TLR-agonists carried by nanoparticles influence in the induction of long-term memory cells [51].

Recent studies report that in a murine model of acute bacterial infection with *S. flexneri* the T cell response is dominated by the induction of long-term memory *Shigella*-specific Th17 cells that contribute to mediate protective immunity against reinfection [52]

Now, new research shows an unexpected direct role for TLR2 signalling in T cells themselves, promoting the differentiation and proliferation of T helper 17 (T$_H$17) cells [50]. Taking into account these data and together with previous results from our own group about the high ability of PVM/MA to stimulate TLR2 [5] suggest that these nanoparticles are good adjuvant candidate for further investigation. OMVs are safe and protective in mice, therefore, the use of OMVs adjuvanted into NP to trigger mucosal immunity and effectively neutralize *Shigella* infection open the door to safely deals with vaccination, especially critical when young children are the primary target.

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Figure legends

Figure 1. (A). Scanning electron micrograph images of outer membrane vesicles (OMVs) from *Shigella flexneri* 2a (up), or loaded in nanoparticles (NP-OMVs) (down). Scale bar indicates 200 nm. **(B) Integrity and antigenicity of the outer membrane vesicles components antigenic components after encapsulation into nanoparticles.** Panel shows the immunoblotting developed with a pool of sera from rabbit hyperimmunized with whole cells from S*higella flexneri*: lanes correspond with the following samples: (1) free OMVs, (2) OMVs released from OMV-loaded NPs.

Figure 2. Comparative analysis of *Shigella flexneri* outer membrane vesicles. SDS-PAGE with silver staining for proteins (A) or for LPS (B), and immunoblotting (C) of: (1) outer membrane vesicles (OMVs), (2) extract enrich in outer membrane proteins (OMPs), and (3) extract enrich in Ipa proteins. Immunoblots were developed with polyclonal antibodies from a patient infected with *S. flexneri* (lane a), anti-IpaC mAb (lanes b) and anti-IpaB mAb (lane c). Molecular weight markers and identity of some bands are indicated.

Figure 3. Antibody immune response induced after vaccination of BALB/c mice. Serum IgG1, IgG2a and IgA titers in vaccinated mice (n=6/group) with either free extract (OMVs) or loaded in nanoparticles (NP-OMVs) at weeks 0, 2 and 5 after immunization. Broken line indicates first dilution tested. Data are mean value (*, P < 0, 05 for immunized mice vs. control).

Figure 4. Immune response induced after vaccination of BALB/c mice. Cytokines serum level (IL-10, IL-12 (p40), IL-12 (p70), IL-5, and IFN-γ) detected at day 15 after immunization with either free outer membrane vesicles (OMVs) (gray bars) or loaded in nanoparticles (NP-OMVs) (black bars). Broken line indicates serum level before immunization. Data are mean value (*, P < 0,001).

Figure 5. Protection study against *Shigella flexneri*. BALB/c mice (20 ±1 g) were immunized with 20 μg of outer membrane vesicles either free (OMVs) or loaded into nanoparticles of PVM/MA (NP-OMVs) by intradermal (■), nasal (▲), ocular ($\triangledown$) or oral (♦), routes. An extra group was included as non-immunized control (×). At day 35 after immunization, all groups received an intranasal lethal challenge of 10^7 UFC/mouse of *Shigella flexneri 2a* (clinical isolate). Graphs indicate the percentage of mice that survived the infective challenge at the indicated days after immunization (*, P<0, 01, Logrank test)
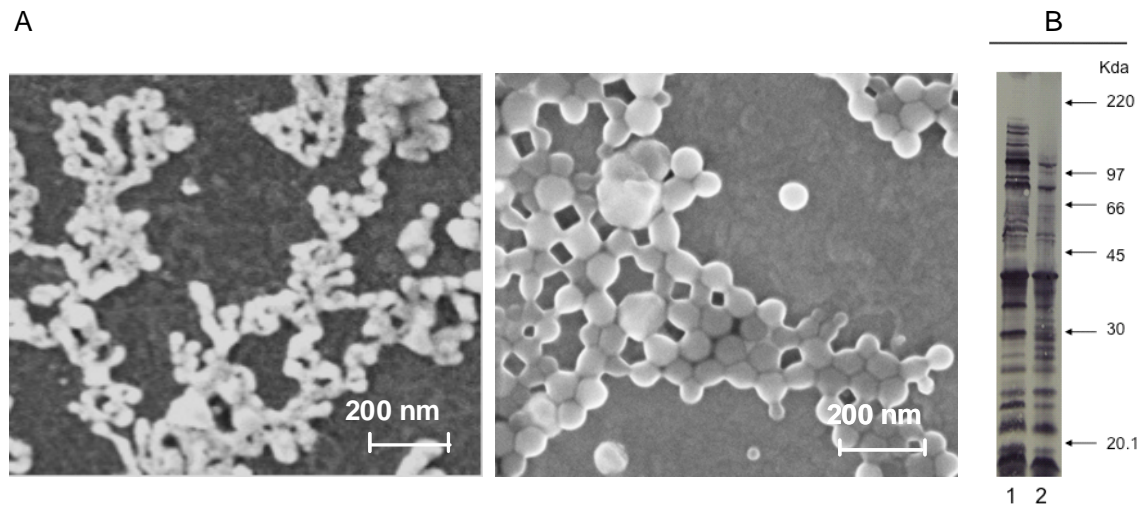
Figure 1

A

B

		Kda
		← 220
		← 97
		← 66
		← 45
		← 30
		← 20.1

1   2

Figure 2

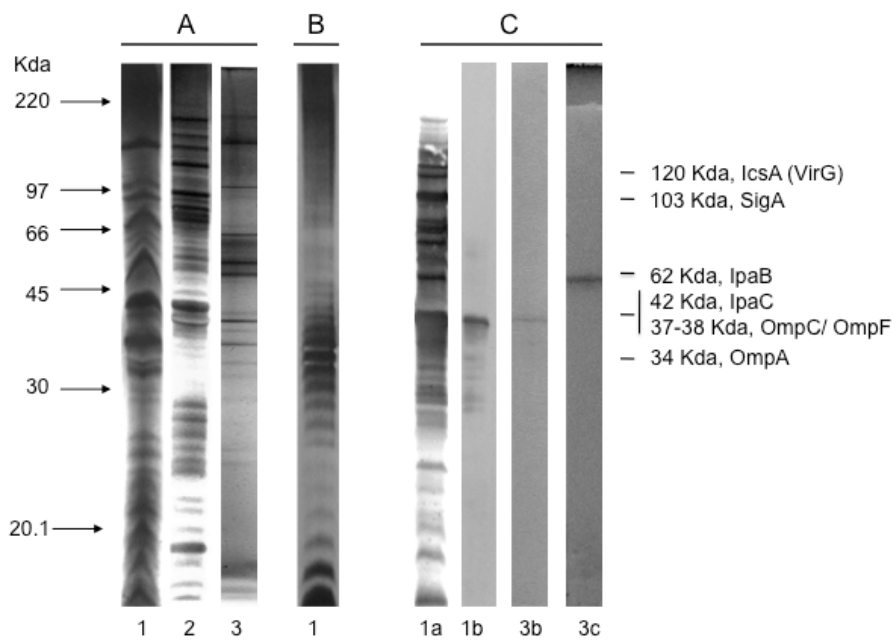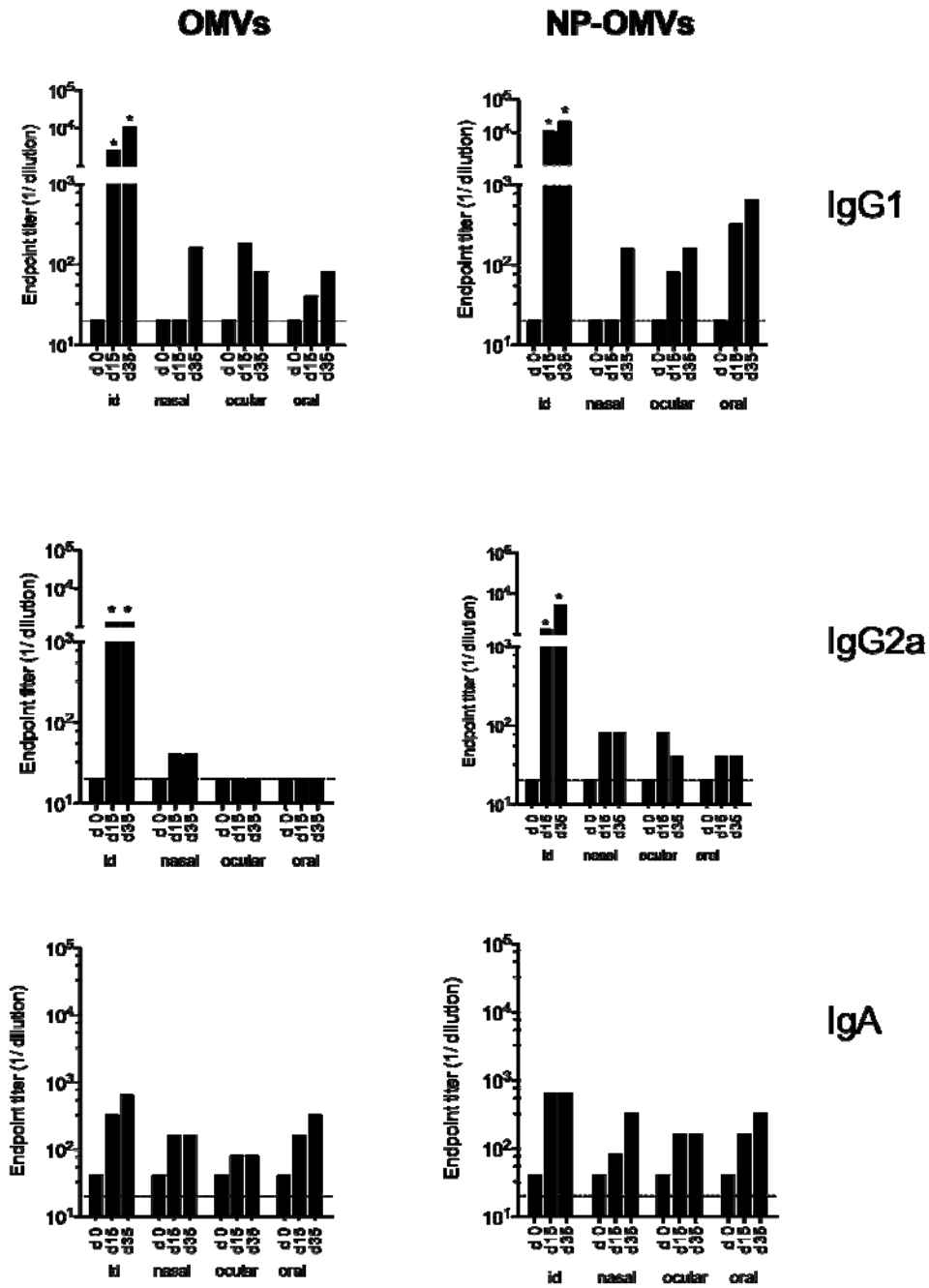|	
Kda	
220 →	
97 →	— 120 Kda, IcsA (VirG)
66 →	— 103 Kda, SigA
45 →	— 62 Kda, IpaB
30 →	— 42 Kda, IpaC
	37-38 Kda, OmpC/ OmpF
20.1 →	— 34 Kda, OmpA

A: 1 2 3   B: 1   C: 1a 1b 3b 3c

Figure 3

Figure 4

Figure 5