Intranasal and Intramuscular Immunization with Outer Membrane Vesicles from Serogroup C Meningococci Induced Functional Antibodies and Immunologic Memory

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ABSTRACT

Background: Immunization is the key to prevent invasive meningococcal disease (IMD), caused by Neisseria meningitidis. Outer membrane vesicles (OMVs) can be used as meningococcal antigens.

Methods: Isogenic mice A/Sn (H2a) were immunized with low antigenic doses of OMVs of an N. meningitidis C:2a:P1.5 strain, via intranasal/intramuscular route, adjuvanted by cholera toxin subunit B (CTB) or via intramuscular route only, adjuvanted by aluminium hydroxide (AH). Mice were followed until old age and humoral and cellular responses were assessed by ELISA, Immunoblotting, Dot-blot, Serum-bactericidal assay, Immunohistochemistry and ELISpot.

Results: OMV+CTB and OMV+AH groups presented statistically higher antibodies titers, which persisted until middle and old ages. IgG isotypes point to a Th2 type of response. Avidity indexes were considered high, regardless of adjuvant use, but only groups immunized with OMVs and adjuvants (OMV+CTB and OMV+AH) presented bactericidal activity. The antibodies recognized antigens of molecular weights attributed to porin and cross-reactivity proteins. Although the spleen of old mice did not present differences in immunohistochemistry marking of CD68+, CD4+, CD79+ and CD25+ cells, splenocytes of immune groups secreted IL-4 and IL-17 when stimulated with OMVs and meningococcal C polysaccharide.

Conclusion: We concluded that both adjuvants, CTB and AH, improved the immunogenicity of low doses of OMVs and contributed to a persistent immune response. Even though AH is well established in the vaccinology area, CTB seems to be a promising adjuvant candidate for meningococcal vaccines: it is suitable for mucosal delivery and supports a Th2 type of response. Therefore, OMVs are still a relevant vaccine platform.

KEYWORDS

Aluminium hydroxide; cholera toxin subunit B; Neisseria meningitidis; outer membrane vesicles; prime-booster immunization

Introduction

Neisseria meningitidis, or meningococcus, is a Gram-negative bacterium, which can colonize the nasopharynx of health carriers or cause invasive meningococcal disease (IMD). It is classified into 12 serogroups according to the nature of its polysaccharide capsule, from which 6 (A, B, C, W, X, and Y) cause IMD more often (Rouphael and Stephens 2012). The
A pathogen can cause outbreaks and the IMD develops fast, leading to high morbidity and mortality rates, therefore, prevention by immunization is the key to control it (Borrow et al. 2017). Currently, there are polysaccharide-conjugated vaccines against serogroups A, C, W, and Y, and recombinant protein vaccines against serogroup B (Pizza et al. 2020).

Meningococci outer membrane vesicles (OMVs) present several antigens that can trigger an immune response (Balhuizen et al. 2021).

Thus, OMVs are suitable for mucosal delivery, a promising route that elicits mucosal and systemic immunity and facilitates administration (Acevedo et al. 2014). The mucosa immunization is capable of eliciting both mucosal and systemic immunity, but, when mucosa and parenteral routes are combined, the response is even better (Reyes et al. 2020; Woodland 2004). Adjuvants improve the immunogenicity of antigens even at low concentrations and ideal adjuvants should be affordable, adequate to the immunization route and capable of modulating the desired type of immune response (Savelkoul et al. 2015). Increasing life expectancy is also relevant in this context, since adjuvants benefit a long-term response (Trzewikoski de Lima and De Gaspari 2019).

Currently, the meningococcal vaccines are administered intramuscularly and use aluminum hydroxide as an adjuvant – hence, such vaccines do not prevent N. meningitidis carriage. On the other hand, intranasal vaccines could improve that aspect eliciting mucosal immunity (Pizza et al. 2020). Also, the elderly were suggested as promising targets for meningococcal vaccine studies (Currie and Gray-Owen 2021; Trzewikoski de Lima and De Gaspari 2019).

We tested an immunization approach using meningococci OMVs via intranasal (IN) prime and intramuscular (IM) booster, both adjuvanted by Cholera-toxin subunit B (CTB), an effective and safe adjuvant, suitable for mucosa and parental routes. As a control, this scheme was compared to two IM doses, adjuvanted by aluminum hydroxide, which is representative of the current meningococcal vaccines. Mice were followed until old age to verify the contributions of each immunization approach in the long term.

**Material and methods**

**Antigenic preparations**

We used OMVs obtained from the meningococci strain C:2a:P1.5 because it presents the main serogroup isolated in Brazil (serogroup C) and a phenotypic characterization common to hyperinvasive strains (serotype 2a, subtype P1.5) (Mustapha et al. 2015; Presa et al. 2019). The N. meningitidis C:2a:P1.5 strain was isolated from a sample of a Brazilian patient, at the Bacteriology Center of Adolfo Lutz Institute, São Paulo, SP, Brazil. Antigens were obtained by centrifugation, as described in De Gaspari and Zollinger (De Gaspari and Zollinger 2001).

Briefly, the strain was cultivated in Tryptic Soy Broth (TSB) (Difco Laboratories) supplemented with 10% of heat-inactivated horse serum (Sigma-Aldrich), for 24 hours, at 37°C and 5% CO2 atmosphere. Then, it was incubated with a solution of 0.1 M sodium acetate and 0.2 M lithium chloride (pH 5.8), and then taken to a shaker at 45°C, for 2 hours at 300 rpm. Afterward, the outer membrane complexes were isolated by centrifugation at 12,000 rpm for 15 minutes at 4°C (Sorvall Instruments). The supernatant, where the OMVs remain, was dialyzed overnight in 0.15 M NaCl and
detoxified from Lipopolysaccharide (LPS) using a Polymyxin B Sepharose 4B column (Pierce); then, it was dialyzed again. Finally, OMVs were tested for LPS content using the Limulus Amebocyte Lysate (LAL) Gel-Clot Endosafe Kit (Charles River Laboratories), and the result was negative for LPS contamination. As adjuvants, we used Cholera toxin subunit B (CTB) (Sigma-Aldrich) and Aluminum hydroxide (AH) (Rehydrogel).

**Animals and samples collection**

Adult females (4 months old) of A/Sn mice (H2a) were obtained from the breeding facilities of Adolfo Lutz Institute (São Paulo, SP, Brazil). Animal experimentation was conducted following current legislation. The study was approved by the Ethics Committee on Animal Use of Adolfo Lutz Institute (CEUA/IAL number 04B–2021). Serum samples were collected by puncture of the retro-orbital plexus. When mice were old, they were anesthetized with Ketamine-Xylazine 10 mg/kg and euthanized by cervical dislocation. Spleens were collected under sterile conditions.

**Immunization**

We separated 5 groups (OMV+CTB, CTB, OMV+AH, AH, and OMV, n = 5 for each group) to test different immunization routes and suitable adjuvants. The prime-booster group received 4 intranasal (IN) doses containing 0.2 µg OMVs +0.1 µg CTB, during 4 consecutive days (one dose per day), and one intramuscular (IM) booster after 15 days containing 0.2 µg OMVs +0.2 µg CTB. The constant stimuli of the 4 IN doses favor the mucosal activation and the maturation of the immune response, while the heterologous booster delivered by a different route of immunization improves the systemic immune response (De Gaspari 2012; Woodland 2004). Nevertheless, our group has published interesting results combining multiple IN and parenteral doses for immunization strategies (Carmo et al. 2009; de Oliveira Santos et al. 2018; Gaspar et al. 2021; Ito et al. 2009). Parenteral groups received 2 IM doses, 15 days apart, containing 0.2 µg OMVs +0.1 mM AH. This schedule was chosen to consider the current meningococcal vaccines delivery (Pizza et al. 2020).

Control groups received the same concentration of adjuvants alone. Given that OMVs are immunogenic per se, one group was immunized with two IM doses of 0.2 µg of OMVs, as antigen control.

The antigenic preparations were diluted in 10 µL (IN) or 100 µL (IM) sterile saline solution (0.1 M, pH 7.4). The OMVs were extracted in our laboratory as described above. CTB was purchased from Sigma-Aldrich and AH from Rehydrogel. The formulations were prepared on the day of use. The antigen and the adjuvants were allowed to interact during 1 hour before administration.

Sera were collected at different time points after immunization: 15 and 45 days, when mice were adult, 280 days, when mice were middle-aged, and 475 days, when mice were elderly. Figure 1a summarizes the experimental calendar.
We characterized 10 µg of OMVs in 10% polyacrylamide gel as described in Laemmli (1970), with a molecular weight (MW) marker (TrueColor High Range Protein Marker, Synapse) ranging from 11 to 245 kDa. The gel was stained with Coomassie Blue (PhastGel Blue R, Pharmacia) (Figure 1b).

**SDS-PAGE**

High binding ELISA-microtiter plates (Costar) were coated with 2 µg/ml of C:2a:P1.5 OMVs in Carbonate-Bicarbonate buffer (pH 9), overnight, at 4°C. The plates were blocked with PBS-Skimmed Milk 5% (La Serenissima) for 2 hours at 37°C. Individual mice sera were incubated for 2 hours at 37°C. HRP labeled-Anti-mouse IgG-γ chain (Kirkegaard & Perry Laboratories) diluted at 1:20,000; or HRP labeled-Anti-mouse IgG2a (Aviva Systems Biology) diluted at 1:10,000 were incubated for 2 hours at 37°C. To assess the IgG1 isotype, biotin labeled-Anti-mouse IgG1 (Aviva Systems Biology) diluted at 1:5,000 was incubated for 2 hours at 37°C, and HRP-streptavidin (Zymed Laboratories), diluted at 1:2,000, was incubated for 1 hour at 37°C. The reactions were developed with Tetramethylbenzidine (TMB) (Sigma-Aldrich) at 37°C for 20 minutes and stopped with sulfuric acid (H₂SO₄) 1N.

**Figure 1.** (a) Experimental calendar of mice’s immunization and sample collection. OMV+CTB and CTB groups were immunized with 4 in doses in consecutive days, receiving a booster dose 15 days after the beginning of immunization. OMV+AH, OMV and AH groups were immunized with 2 IM doses in a 15 days interval. Sera were collected before the immunization and 15, 45, 280 and 475 days after the beginning of the immunization, to represent different ages. After the last bled, mice were euthanized to collect the spleens. (b) electrophoretic profile of C:2a:P1.5 OMVs, used in immunization. AH: aluminium hydroxide; CTB: cholera toxin subunit B; OMV: outer membrane vesicles.

**Enzyme-linked immunosorbent assay (ELISA)**

We characterized 10 µg of OMVs in 10% polyacrylamide gel as described in Laemmli (1970), with a molecular weight (MW) marker (TrueColor High Range Protein Marker, Synapse) ranging from 11 to 245 kDa. The gel was stained with Coomassie Blue (PhastGel Blue R, Pharmacia) (Figure 1b).
OD was read at 450 nm using a microplate reader (Multiskan Labsystems). The titer of the sample was considered as the reciprocal dilution that provided an OD ≥ 0.160 (background of the plate +3 standards deviations) and converted to Log₁₀ for analysis.

**Avidity-ELISA**

ELISA was performed as described above, using samples collected 45, 280, and 475 days after the immunization, diluted at 1:100, so the linear point of the titration curve would be used (Dimitrov et al. 2011) (the titration curve is at supplementary material #1). The avidity index (AI) was assessed by adding the chaotropic agent potassium thiocyanate (KSCN) 1.5 M, at room temperature, for 20 minutes, after the serum incubation. The AI was considered as the ratio between the OD treated with KSCN/OD without KSCN (Vermont et al. 2002). AI was classified as low if <30%, intermediate if 30–49% and high if ≥50% (Chackerian et al. 2001).

**Serum bactericidal assay (SBA)**

The serum-bactericidal assay was conducted using the Tilt method, as proposed by Borrow et al. (2003) and Cedré et al. (2012) with some modifications. This assay verifies the capacity of the immune sera to kill meningococci in vitro. The bactericidal activity is mediated by the opsonization of the bacteria by the antibodies followed by complement-mediated lysis (Borrow et al. 2003). SBA is the gold standard to test meningococcal vaccines, and titers ≥1/4 are considered protective (Granoff 2009).

The lyophilized C:2a:P1.5, the same strain used for immunization, was reactivated in blood agar and cultured at 35°C, 5% CO₂, overnight. Then, it was cultured in Tryptic-soy broth (TSB) agar supplemented with 5% of inactivated horse serum for 24 hours. One isolated colony was suspended in Hanks solution supplemented with 0.1% of glucose, 0.1% of bovine serum albumin, and 9 mM of Hepes and set for an OD 650 nm = 0.1. Finally, the working bacteria suspension was diluted at 1/250, to yield approximately 100 colonies, as previously tested in our laboratory. Pooled sera (pre-immune and immune sera collected at day 45) were inactivated at 56°C for 30 minutes. Two-fold dilutions of pooled sera, guinea pig sera as an exogenous complement source and working bacteria suspension were mixed on a sterile microplate (Silva et al. 2007). The assay controls were as follows: working bacteria suspension alone and working bacteria suspension plus inactivated immune sera and inactivated complement. The microplate was incubated at 35°C, 5% CO₂, for 1 hour. Then, 10 μL of each well was plaqued in TSB plates using the Tilt method. The plates were incubated at 35°C, 5% CO₂, for 24 hours, and colonies were counted. The bactericidal titer was considered as the reciprocal dilution yielding ≥50% reduction in colonies compared to the control (working bacteria suspension alone) (Borrow et al. 2003).

**Immunoblotting**

An SDS-PAGE was conducted as described above, using whole cell suspensions of *N. meningitidis* C:2a:P1.5 and W:2a:P1.5,2 strains. The content (approximately 3 × 10⁸ cells per strip) was transferred to a nitrocellulose membrane 0.45 μm (BioRad Laboratories). The strips were blocked with skimmed milk (La Serenissima) 5% for 2
hours, at room temperature (20-25°C). Pooled sera (pre-immune and immune sera collected at day 45) were incubated overnight, at 4°C. Anti-mouse IgG-γ chain (Sigma-Aldrich) diluted at 1:5,000 was incubated for 2 hours, at room-temperature (20-25°C). The enzymatic reaction was revealed using 5-bromo-4-chloro-3-indolyl-phosphate-nitro blue tetrazolium (BCIP/NBT) substrate (MabTech) for 30 minutes and stopped washing the strips with distilled water.

**Dot-blot**

1 µL of whole-cell suspensions (OD 650 nm = 0.1) of *N. meningitidis* strains we dotted in nitrocellulose membrane 0.45 µm (BioRad Laboratories). 22 strains were selected considering the main isolates of Brazil in recent years, which are described in Supplementary material #2 (Gorla et al. 2020). The membranes were blocked as described for Immunoblotting. Pooled sera (pre-immune and immune sera collected at day 45) diluted at 1:50 were incubated overnight, at 4°C. Anti-mouse IgG-γ chain (Kirkegaard & Perry Laboratories) diluted at 1:10,000 was incubated for 2 hours, at room temperature (20-25°C). The enzymatic reaction was revealed using 3-amino-9-ethylcarbazole (AEC) substrate (Sigma-Aldrich) for 20 minutes and stopped washing the strips with distilled water.

**Immunohistochemistry (IHC)**

Immunohistochemistry (IHC) was conducted at the Pathology Center of Adolfo Lutz Institute, São Paulo, SP, Brazil. Given that old age is associated with some immunological impairments (Cesta 2006), monoclonal antibodies (mAbs) were used to characterize macrophages, lymphocytes T, lymphocytes B, and activated lymphocytes in the spleen of elderly mice of each experimental group, as well as of naïve, adult mice, as control.

After euthanasia, spleens were collected and fixed in buffered saline formaldehyde (pH 7.4) for 24 hours. The spleens were dehydrated with increasing alcohol concentrations and embedded in paraffin using an automatic tissue processor (TP 1020, Leica Microsystems) and an automatic embedding processor (EG1150C, Leica Microsystems). Then, spleens were sectioned into a microtome (Micron). Immunohistochemical reactions were performed manually. The tissue slides were immersed in xylol and rehydrated, decreasing alcohol concentrations. Epitope retrieval was conducted under heat (citric acid 10 mM, pH6), followed by overnight incubation with the primary antibodies anti-CD68 (KP1), and anti-CD4 (SP35), anti-CD79 (JCB117) and anti-CD25 (4C9) (Cell Marque) diluted at 1:2,000. Signal amplification and revealing of the IHC reaction were performed using the Complete Detection Kit with polymers conjugated with HRP and the substrate 3-3’-diaminobenzidine (DAB) (Spring Detection System SPD 12, Spring Biosciences). Counterstaining was performed with hematoxylin.

Given that there are a high number of immune cells in the spleen and their localization is not homogeneous throughout the organ (Cesta 2006), the IHC was evaluated using a semi-quantitative approach (Seidal et al. 2001). The whole spleens were observed at 100x magnification (Olympus B×40, Olympus LifeScience) and the expression of the CDs in the spleen regions (red pulp, germinal center, and periphery of the white pulp) was classified as 1+, 2+, 3+ and 4+. Representative figures of each level are available in Supplementary material #3.
**Enzyme-linked immunospot (ELISpot)**

The assay was performed using the kit Mouse IL-4 or IL-17A ELISpot PLUS (MabTech), following the manufacturer’s instructions. Briefly, microtiter plates pre-coated with anti-interleukin (IL)-4 or anti-IL-17 mAbs were washed five times with sterile PBS and blocked with Iscove’s medium-10% of fetal calf serum, at room temperature (20-25°C), for at least 30 minutes. Pooled splenocytes of each group (OMV+CTB, CTB, OMV+AH, OMV, and AH) were added at $1 \times 10^6$ cells/mL concentration. The cells were specific-stimulated with 50 μg/mL OMVs from strain C:2a:P1.5 or 25 μg/mL of polysaccharide C (PSC) from meningococci (Merck). As controls, cells were unspecific-stimulated with 10 μg/mL concanavalin A (ConA) (Sigma-Aldrich) or added without stimulus. The plates were incubated at 37°C, 5% CO₂, for 24 hours. Cells were removed from the plates by washing them five times with PBS; then, the plates were incubated with a PBS-EDTA 1 mM solution for 15 minutes, at 37°C, and washed again. Plates were incubated with biotinylated anti-IL-4 diluted at 1 μg/mL or anti-IL-17 diluted at 0.25 μg/mL for 2 hours, at room temperature (20-25°C). Later, they were incubated with alkaline-phosphatase (ALP)-streptavidin diluted at 1:1,000, for 1 hour at room temperature. Finally, the enzymatic reaction was developed with 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT-plus) until the appearance of the spots and the reaction was stopped washing the plate exhaustively with distilled water. After drying, the spots were counted using the AID ELISpot Reader v. 7.0 (Autoimmun Diagnostika GMBH, Germany). The duplicate mean was calculated, and the number of spots counted in control wells with no stimuli was subtracted. The results were expressed as IL-4 or IL-17 secretory cells per $10^6$ splenocytes/mL.

**Statistical analysis**

The results between the groups were compared using Kruskal-Wallis and Dunn’s Post-test. Analyses were conducted on GraphPad Prism 5 (GraphPad Softwares). $P$ values ≤0.05 were considered to be significant.

**Results**

**SDS-PAGE**

The electrophoretic profile of the OMVs is in Figure 1b. Antigens from 25 to 190 kDa were present in the OMVs.

**IgG, IgG1 and IgG2a titers**

Figure 2 shows the individual levels of IgG titers and the comparisons between groups. Adjuvants were needed to increase IgG titers. OMV+CTB was the only group statistically superior to pre-immune control since the first dose ($p < .05$), also, IgG titers increased after the booster dose ($p < .01$) and were maintained in the middle and old ages ($p < .05$ for both). OMV+AH needed a booster dose to achieve higher antibody titers. Forty-five days after the beginning of immunization, it was higher than pre-immune and AH controls ($p < .001$ and $p < .05$, respectively). After 180 days, IgG titers
decreased, but it was still statistically superior to controls (\(p < .01\) considering pre-immune and \(p < .05\) considering AH). The groups immunized with OMVs alone did not achieve statistical superiority, even though they presented higher titers than pre-immune sera.

The IgG1 and IgG2a titers were assessed in sera collected 45 days after the immunization (Figure 3). Some individuals presented higher IgG2a titers, but there was no statistical significance. On the other hand, IgG1 titers of the OMV+CTB group were higher than pre-immune and CTB (\(p < .05\) for both), as well as OMV+AH, which was superior to pre-immune and AH (\(p < .05\) for both). The higher ratios of IgG1/IgG2a suggest a Th2-type immune response modulated by adjuvants.

**Figure 2.** IgG titers were assessed in sera collected (a) 15, (b) 45, (c) 280 and (d) 475 days after immunization. Only adjuvanted groups were statistically superior to controls, although the group OMV presented higher titers. OMV+CTB maintained its superiority in all time points analyzed, whereas OMV +AH was superior after receiving a booster dose and until middle-age. AH: aluminium hydroxide; CTB: cholera toxin subunit B; OMV: outer membrane vesicles; *: \(p < .05\), **: \(p < .01\), ***: \(p < .001\).
Table 1 presents the mean AI of each group at different time points. All the groups presented high avidity indexes 45 days after the beginning of the immunization. In OMV+CTB and OMV+AH groups, avidity increased until 280 days, and at the last sampling, 475 days after the immunization, it was stable in the OMV+AH group and decreased approximately 10% in the OMV+CTB group. For the OMV group, the avidity index decreased in the later collections. However, the overall classification was high for all groups and all-time points assessed and no statistical difference was observed.

Table 2 presents the bactericidal titers of sera collected 45 days after the immunization. There was no bactericidal activity in the assay controls. Pre-immune sera and adjuvants alone did not present bactericidal activity, as expected. Only adjuvanted groups presented bactericidal activity against the C:2a:P1.5 strain: OMV+CTB was bactericidal until 1/8 dilution, while OMV+AH was bactericidal until 1/32 dilution. The group immunized with OMVs alone did not present bactericidal activity.

**Avidity index and bactericidal activity**

Table 1 presents the mean AI of each group at different time points.

All the groups presented high avidity indexes 45 days after the beginning of the immunization. In OMV+CTB and OMV+AH groups, avidity increased until 280 days, and at the last sampling, 475 days after the immunization, it was stable in the OMV+AH group and decreased approximately 10% in the OMV+CTB group. For the OMV group, the avidity index decreased in the later collections. However, the overall classification was high for all groups and all-time points assessed and no statistical difference was observed.

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**Table 1. Avidity index of immune sera collected during adult, middle and elderly age.**

| Group         | 45 days | 280 days | 475 days |
|---------------|---------|----------|----------|
| OMV+CTB       | 72.5% (±13.7) | 90.7% (±8.1) | 81.1% (±11.8) |
| OMV+AH        | 73.9% (±13.7) | 91.8% (±5.9) | 90.1% (±7.3) |
| OMV           | 79.4% (±17.7) | 52.1% (±13.4) | 68.4% (±38.4) |

Mean Avidity Index (%) immune groups at different time points. All indexes were considered high (≥50%). The parenthesis shows the standard deviation of individual AI values. AH: aluminium hydroxide; CTB: cholera toxin subunit B; OMV: outer membrane vesicles.

**Table 2. Bactericidal activity of immune sera.**

| Group         | Bactericidal titer |
|---------------|--------------------|
| OMV+CTB       | 1/8                |
| OMV+AH        | 1/32               |
| OMV           |                     |

Bactericidal titer of pooled sera collected 45 days after the beginning of immunization. AH: aluminium hydroxide; CTB: cholera toxin subunit B; IN: intranasal; IM: intramuscular; OMV: outer membrane vesicles.
Figure 4. Immunoblotting performed with (a) C:2a:P1.5 and (b) W:2a:P1.5,2 strains. All groups (OMV+CTB, OMV+AH and OMV) recognized bands of approximately 80 and 46 kDa, the molecular weights related to Tbp and Porin A, respectively. The OMV+CTB sera also recognized bands of high molecular weight (>100 kDa) of W:2a:P1.5,2 strains. AH: aluminium hydroxide; CTB: cholera toxin subunit B; OMV: outer membrane vesicles.

Immunoblotting and Dot-blot

Figure 4 shows the immunoblotting reaction using the homologous strain (C:2a:P1.5) and a heterologous strain (W:2a:P1.5,2). The immune sera of all groups (OMV+CTB, OMV +AH and OMV) recognized bands of approximately 80 and 46 kDa, which are possibly related to Transferrin-binding protein and PorA, respectively (Pollard and Frasch 2001). OMV+CTB sera recognized bands of high molecular weight of the heterologous strains as well.

In Dot-blot, OMV+CTB sera recognized 14 out of 22 strains, OMV+AH recognized 11/22 and OMV, 10/22. Figure 5 represents the results of Dot-blot, showing the serotypes and subtypes of the isolates recognized by immune sera.

The recognition of more than one band in immunoblotting and of strains presenting different phenotypic characterizations suggests that there is more than one subcapsular antigen modulating the immune response.

Cellular characterization and immune response

The immunohistochemistry assay characterized immune cells in the spleen of elderly mice. Immunized groups did not express more cells, which would be suggestive of immune activation (Table 3). However, it is not expected to happen 475 days after the immunization. Thus, elderly mice did not present lower cellular semi-quantification compared with adult mice. Representative figures for each marker, for each group, are available in Supplementary material #4.
The functional responses of the splenocytes were assessed using the ELISpot technique. The groups OMV+CTB and OMV+AH secreted more cytokines than the control groups. The higher secretion of IL-4, regardless of the antigenic stimuli, corroborates the results of IgG isotypes, pointing to a Th2 pattern of the immune response. IL-17 secreting cells were rarer, however, the OMV+CTB group, immunized by IN/IM routes, secreted slightly higher levels of this cytokine. PSC was able to stimulate cytokine secretion, suggesting that the OMVs also lead to a polysaccharide response. The ELISpot results are represented in Figure 6.

**Figure 5.** Serogroups, serotypes and subtypes recognized by immune sera in dot-blot performed with *N. meningitidis* strains, representatives of Brazilian isolates. Pre-immune sera did not recognize the strains, while (a) OMV+CTB, (b) OMV+AH and (c) OMV sera recognized isolates presenting different phenotypes. AH: aluminium hydroxide; CTB: cholera toxin subunit B; OMV: outer membrane vesicles.

**Table 3.** Semi-quantitative immunohistochemistry characterization of mice’s spleens.

|        | CD68+ | CD4+ | CD79+ | CD25+ |
|--------|-------|------|-------|-------|
|        | RP    | Perip| GC    | RP    | Perip| GC    | RP    | Perip | GC    |
| OMV+CTB| 3+    | 1+   | 1+    | 3+    | 3+   | 1+    | 3+    | 3+    | 1+    |
| CTB    | 4+    | 2+   | 1+    | 4+    | 4+   | 2+    | 4+    | 4+    | 2+    |
| OMV+AH | 3+    | 1+   | 1+    | 2+    | 2+   | 1+    | 3+    | 3+    | 1+    |
| AH     | 3+    | 2+   | 1+    | 3+    | 3+   | 1+    | 3+    | 3+    | 1+    |
| OMV    | 4+    | 2+   | 1+    | 4+    | 4+   | 2+    | 4+    | 4+    | 1+    |
| Naïve  | 3+    | 2+   | 1+    | 2+    | 2+   | 1+    | 3+    | 3+    | 2+    |
|        |       |      |       |       |      |       |       |      |       |

AH: aluminium hydroxide; CTB: cholera toxin subunit B; GC: germinal centre; OMV: outer membrane vesicles; RP: red pulp; Perip: periphery.
We could not verify the statistical difference (Kruskal–Wallis test), possibly due to the low \( n \), since the test was performed using replicates of pooled spleens. However, immune groups, especially OMV+CTB and OMV+AH, presented higher cytokine quantifications. After OMV stimulation, IL-4 secretion of OMV+CTB was almost 5x higher than CTB and for OMV+AH, 124x higher than AH. OMV+CTB group secreted 3x more IL-17 than CTB, and OMV+AH secreted the double of IL-17 of AH. Upon PSC stimuli, OMV+CTB secreted 10x more IL-4 than CTB, and OMV+AH, 33x more IL-4 than AH. Both control groups did not secrete IL-17 after PSC stimulation, while the counting of OMV+CTB and OMV+AH were, respectively, 28 and 10 spots. OMV alone secreted more cytokines than adjuvant controls, but, generally, spot counting was less than adjuvanted groups.

**Discussion**

Our study compared the immunization of A/Sn mice with low antigenic doses of OMVs from *N. meningitidis* via IN/IM or IM/IM routes, respectively, adjuvanted by CTB or AH. Meningococcal vaccines based on OMVs technology controlled epidemics in New Zealand, Norway, and Cuba (Biolchi et al. 2020a). In fact, the OMVs are well known for their immunogenicity and suitability for both parenteral and mucosal administration. Thus, it is an accessible technology, especially for low-income and developing countries (Balhuizen et al. 2021). Here, not only OMVs were immunogenic but also led to persistent immune responses, showing how this platform is still relevant.
Mucosal immunization usually elicits lower antibody titers, due to mucosal barriers and tolerogenic immune response of these tissues, while parenteral administration elicits higher antibodies titers, given the delivery of the full dose at a sterile site (Zimmermann and Curtis 2019). In our investigation, the IN/IM administration elicited IgG titers as high as the IM/IM administration (Figure 3). Other studies reported robust antibody response to mucosal (Dalseg et al. 1999) or mucosal/parenteral (Reyes et al. 2020) delivery of OMVs. Of note, the mucosal administration can confer local immunity, mediated by secretory IgA, which is interesting to neutralize pathogens following their first contact with the host (Lavelle and Ward 2021). Unfortunately, we could not verify the mucosal immune response in this study.

Although some individuals from the OMV group presented higher antibody titers, only the OMV+CTB and OMV+AH groups reached a statistical difference. In IMD, higher antibody titers are beneficial, improving the opsonization and mediating the complement-activation, which is the main mechanism to kill meningococci. Considering that, the use of adjuvants benefited the immunization strategies (Granoff et al. 1998; Pollard and Frasch 2001).

The persistence of the immune response following vaccination is desired. Here, the OMV +CTB and OMV+AH groups maintained statistically higher IgG titers until middle age, if compared with control – and, in the case of OMV+CTB, until old age (Figure 3). Aluminum and nanoparticle-based adjuvants induced persistent humoral response to N. meningitidis (Trzewikoswki de Lima et al. 2020) and B. anthracis (Kelly et al. 2021) antigens in previous studies. Our results contribute to those, showing how adjuvants improve the long-term immune response, even when low antigenic doses are administered.

Another interesting characteristic of adjuvants is to modulate the response, so more adequate mechanisms of immunity are elicited. It can be pointed out by IgG isotypes (Shah et al. 2017). IL-2 and IFN-Y, Th1 cytokines, support the IgG2a class switch; IL-4, a Th2 cytokine, induces IgG1 (Mosmann et al. 1989). Here, the IgG1 isotype was statistically higher in OMV+CTB and OMV+AH groups, while there was no difference considering IgG2a titers (Figure 3). Along with the higher IgG1/IgG2a ratio of these groups, it points to a Th2 immune response. AH is described as an inducer of Th2 immunity (He et al. 2015; Holmgren et al. 1993). CTB can induce a mixed pattern of immune response (Stratmann 2015); however, IN immunization adjuvanted by CTB induced a strong Th1 response for a varicella vaccine (Sasaki et al. 2003), a Th2 response for Staphylococcus (Arlian and Tinker 2011) and a mixed Th1/Th2 response to Bordetella pertussis (Olivera et al. 2014). We hypothesize that the antigen nature could also play a role in formulations containing CTB. While AH is already used in meningococcal vaccines, our evidence supports the use of CTB as another suitable adjuvant for it, provided that a Th2-biased immune response is ideal for meningococcal protection (Pollard and Frasch 2001). The OMV group presented a balanced IgG1/IgG2a ratio, which could be interpreted as a balanced Th1/Th2 response. The protein nature of OMVs can induce IgG2a and a Th1 response (Nimmerjahn and Ravetch 2005); however, the low titers of these groups can impair the analysis.

The avidity index measures the strength of the antigen/antibody interactions (Correa et al. 2021). In this study, all groups presented high avidity indexes until old age (Table 1). There is a correlation between high AI and bactericidal activity against meningococci. High-avidity antibodies would bind with greater strength to the antigens, improving the opsonization for complement-mediated lysis (Granoff et al. 1998). In our experience, OMVs
detoxified from LPS do not induce high-avidity IgG when administrated alone, a characteristic improved by adjuvant use (Gaspar et al. 2013; Trzewikoswki de Lima and De Gaspari 2019). Unexpectedly, in this study, the OMVs administrated alone induced high AI. It suggests high immunogenicity of the strain used, despite the low antibody titer, which could be explained by the low antigenic doses. Studies following virus vaccines describe high avidity antibodies even when IgG titers are low (Sasaki et al. 2011; Stiasny et al. 2012).

The sera of adjuvanted groups (OMV+CTB and OMV+AH) were bactericidal, presenting SBA titers >1/4 (Granoff 2009). Even though OMVs alone induced antibodies with elevated avidity, the sera were not bactericidal (Table 2). This discrepancy in this study could be explained by the low IgG titers of groups immunized with OMVs alone, which would fail to opsonize bacteria for complement lysis adequately (Weynants et al. 2007). Given that bactericidal antibodies are correlated with protection against IMD (Pollard and Frasch 2001), we reaffirm the importance of adjuvants to improve the immune response to low antigenic doses of OMVs. Note that bactericidal titers might have been underestimated because of the complement source. Guinea-pig serum provided lower bactericidal titers than human serum before (Silva et al. 2007). Another possibility is the influence of the mouse strain used, given that isogenic mice can present lower SBA responses than outbred mice (Arunachalam et al. 2022). Unfortunately, there are only a few studies comparing the differences of mouse strains in SBA, which limits this discussion. Indeed, the importance of standardizing the complement source in SBA was highlighted before (Brookes et al. 2013).

The dominance of the Porins in the OMVs may limit the cross-reactivity between different strains (Pizza et al. 2020), so it is important to verify the antigens recognized. The bands of 80 and 46 kDa suggest the recognition of Tbp and the Porin A by the immune sera (Figure 4) (Pollard and Frasch 2001). While the Porins are more specific, Tbps present limited heterogeneity between strains and are important virulence factors. Anti-Tbp antibodies are bactericidal even at low concentrations, impair meningococcal survival and confer cross-reactivity (Danve et al. 1993; Ferreirós et al. 2002). The OMV+CTB group also recognized high molecular weight antigens (>100 kDa) in the Immunoblotting of the heterologous strains. This difference could be related to different antigenic expression by the W:2a:P1.5,2 strain; however, it is an interesting result. Like Tbp, high molecular weight proteins, such as NadA, are known to confer cross-reactivity against meningococcal strains (Comanducci et al. 2002). The intensity of the bands suggests that the OMV+CTB group could potentially recognize different isolates.

Thus, strains presenting completely different phenotypes were recognized by immune sera in Dot-blot (Figure 5). From those, it should be emphasized that several MenB strains were recognized and this is, along with MenC, the main serogroup circulating in our country. The C:23:P1.14,6 strain, the main isolate in Brazil in past years, was recognized by all immune sera (Gorla et al. 2020). Strains expressing the serotype 4,7 were recognized, as well. This phenotype was characterized in virulent strains, which caused epidemics before (Sacchi et al. 1998). Thus, the serotype 19.1, frequently recognized, has been expressed among B and Y isolates recently (Gorla et al. 2020). Finally, the antibodies recognized W:2a: P1.5,2 antigens in Immunoblotting and Dot-blot, and this is a relevant strain considering the emergence of serogroup W (Gorla et al. 2020; Lemos et al. 2010; Weidlich et al. 2008).

Although the recognition of antigens is not a correlate of protection, like the SBA, it is interesting to highlight that more antigens were recognized. Even though N. meningitidis strains are highly heterogenous, antigens which contribute to virulence might be
maintained among different isolates (Urwin et al. 2004). Thus, when different antigens are recognized, it improves the opsonization of the pathogen for complement-mediated lysis (Weynants et al. 2007). Taken together, these observations suggest that multi-antigenic preparations are interesting for meningococcal vaccines.

Even though the polysaccharide-conjugated vaccines are highly effective for serogroups A, C, W and Y (Pizza et al. 2020), it is important to consider the capsule-switch of meningococci strains for future vaccine improvements (Tzeng et al. 2016). Concerning the protein antigens, a reduced incidence of *N. gonorrhoeae* infection was observed after OMV vaccination (Petousis-Harris et al. 2017), and immune sera of people immunized with Bexsero® (GlaxoSmithKline) (composed by NadA, NHBA and fHbp proteins, plus OMVs) killed a diversity of meningococci strains (Biolchi et al. 2020a, 2020b). Such evidence supports the idea that subcapsular antigens might be relevant in meningococci vaccine development.

The cellular populations were localized at the spleens’ regions as expected: the majority of macrophages (CD68+) were found in the red pulp, lymphocytes T (CD4+), B (CD79+) and activated lymphocytes (CD25+) were centered at the periphery of the white pulp (Cesta 2006). The IHC did not point to higher cellular density, suggestive of immune activation, in spleens of immunized mice compared with controls (Table 3 and Supplementary material #4). This is not surprising, provided that the literature describes that the peak of the immune response, followed by increased cellularity in the spleens, occurs from hours to a few days, depending on the immunization route and mice lineage (Biozzi et al. 1968, 1972). Considering immunization with antigens from meningococci, splenocytes collected 7, 14, 28, or 42 days after immunization did not present differences in cellular levels, but functional assays revealed antibody secretion (Luijlx 2006). Similarly, our results show that specific antigenic stimuli lead to cytokine secretion in ELISpot, even when there are no differences in cellular characterization (Figure 6).

The OMV+CTB and OMV+AH groups presented higher quantities of IL-4 and IL-17 secreting cells. Such cytokines were chosen considering that they would be beneficial to preventing meningococcal disease, supporting the humoral (IL-4) and mucosal (IL-17) responses (Pollard and Frasch 2001; Poolman 2020).

The IL-4 secretion corroborates the IgG isotypes results, suggesting that the adjuvants modulated a Th2 response. Thus, the immunization of mice with NadA and NspA, proteins from the outer membrane, resulted in high IL-4 levels and bactericidal activity in Ying et al. (2014) and Hou et al. (2019). The role of Th17 response in IMD prevention is not completely elucidated. However, some studies observed increased levels of IL-17 and bactericidal activity, leading the authors to suggest that this cytokine would activate the immune response by inflammation (González-Miró et al. 2018; Hou et al. 2019). Thus, there is a report of mice immunized via IN route with a meningococcal protein presenting increased IL-17 levels and survival after a meningococcal challenge (Li et al. 2021).

Splenocytes are stimulated with PSC secreted cytokines, especially IL-4 (Figure 6). The polysaccharide present in OMV content seemed to guarantee *T*-dependent immune responses and, therefore, immunologic memory (Sun et al. 2019). The polysaccharide is an important antigen to prevent IMD caused by serogroup C (Pollard and Frasch 2001). So, we can hypothesize that a serogroup C immunization using OMVs could confer cross-reactivity with other meningococci C strains.
Conclusion

We verified that OMVs are immunogenic and induce bactericidal antibodies even in low antigenic doses, as long as administered with adjuvants. A high molecular weight protein was recognized by immune sera, along with PorA, and strains presenting different phenotypes were recognized as well. Similar to AH, CTB elevated antibody titers, which were maintained at different mice’s ages, modulated a Th2 type of immune response and conferred immunologic memory. All this suggests that it could be an adjuvant option for meningococcal vaccines. PSC elicited a cellular response, which might improve cross-reactivity. Finally, OMVs are still relevant for immunization against meningococci, especially when adjuvants are used.

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Authors’ contribution

All authors contributed with discussions of the results, reviewed the final manuscript and agreed with its submission. AIP: Investigation, methodology, data curation and analysis, writing original manuscript. VAC: Investigation, methodology, data analysis. CSC: Investigation, methodology, data analysis. EDG: Conceptualization, investigation, methodology, data curation and analysis, funding acquisition, supervision.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability

Data are available upon reasonable request to the correspondent author (Elizabeth De Gaspari).

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