Conjugation of PspA4Pro with Capsular Streptococcus pneumoniae Polysaccharide Serotype 14 Does Not Reduce the Induction of Cross-Reactive Antibodies

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ABSTRACT Current pneumococcal vaccines are composed of bacterial polysaccharides as antigens, plain or conjugated to carrier proteins. While efficacious against vaccine serotypes, epidemiologic data show an increasing incidence of infections caused by nonvaccine serotypes of Streptococcus pneumoniae. The use of pneumococcal surface protein A (PspA) as a carrier protein in a conjugate vaccine could help prevent serotype replacement by increasing vaccine coverage and reducing selective pressure of S. pneumoniae serotypes. PspA is present in all pneumococcal strains, is highly immunogenic, and is known to induce protective antibodies. Based on its sequence, PspA has been classified into three families and six clades. A PspA fragment derived from family 2, clade 4 (PspA4Pro), was shown to generate antibodies with a broad range of cross-reactivity, across clades and families. Here, PspA4Pro was modified and conjugated to capsular polysaccharide serotype 14 (PS14). We investigated the impact of conjugation on the immune response induced to PspA4Pro and PS14. Mice immunized with the PS14-mPspA4Pro conjugate produced higher titers of anti-PS14 antibodies than the animals that received coadministered antigens. The conjugate induced antibodies with opsonophagocytic activity against PS14-carrying strains, as well as against a panel of strains bearing PspAs from five clades (encompassing families 1 and 2) bearing a non-PS14 serotype. Furthermore, mice immunized with PS14-mPspA4Pro were protected against nasal colonization with a nonrelated S. pneumoniae strain bearing PspA from clade 1, serotype 6B. These results demonstrate that the cross-reactivity mediated by PspA4Pro is retained following conjugation, supporting the use of PspA4 as a carrier protein in order to enhance pneumococcal vaccine coverage and encourage its further investigation as a candidate in future vaccine designs.

KEYWORDS conjugate vaccine, Pneumococcus, PspA, cross-reactivity, pneumococcus

Streptococcus pneumoniae is an important worldwide pathogen that can cause several diseases, such as pneumonia, meningitis, acute otitis media, and bacteremia (1). The most efficient prevention of pneumococcal diseases is achieved by vaccination. So far, all commercialized pneumococcal vaccines use the capsular polysaccharide (CPS) as the antigen, plain or conjugated to a carrier protein. Plain CPS induces T-cell-independent immune responses and therefore fails to elicit immunological memory and to confer protection in children under 2 years old (2, 3). When CPS is covalently linked to a protein, it can engage T helper cells, resulting in long-lived immunity even in children under 2 years of age (4, 5).
Although antibodies to CPS confer protection against disease, this protection is serotype specific and therefore limited to those included in the vaccine. There are more than 90 serotypes described in *S. pneumoniae* (6, 7), and each CPS has a unique chemical structure. Polyvalent polysaccharide conjugate vaccines (PCVs) have been shown to effectively reduce invasive pneumococcal disease (IPD) due to vaccine serotypes (8). However, the introduction of PCVs has been followed by an increase in disease rates caused by serotypes not included in their formulation, also known as serotype replacement (9, 10). In order to circumvent this limitation, we have been evaluating pneumococcal surface protein A (PspA) as a carrier protein in a pneumococcal conjugate in order to broaden the vaccine coverage and possibly reduce serotype replacement (11–13).

PspA is a surface protein that is highly immunogenic and expressed by all clinically important capsular serotypes of *S. pneumoniae* (14). It is associated with pneumococcal virulence, and its ability to inhibit complement deposition on the bacterial surface is one of its well-studied functions (15–19). PspA and other pneumococcal proteins, such as pneumococcal surface antigen A (PsaA), pneumococcal choline-binding protein A (PcpA), a polyamine binding protein, PotD, and pneumolysin (Ply) and its detoxified forms (20), have been studied as protein vaccine candidates.

Based on the sequence variations of its N-terminal region, PspA can be grouped into three families that are divided in six clades, distributed as follows: clades 1 and 2 belong to family 1, clades 3, 4, and 5 belong to family 2, and clade 6 belongs to family 3 (21). More than 90% of clinical isolates are from families 1 and 2 (22, 23).

Furthermore, a recombinant PspA fragment from family 1 (clade 2), rPspA2, has been tested in humans in a phase I clinical trial and shown to be safe and immunogenic (24). Antibodies induced by immunization with rPspA2 in this trial were demonstrated to passively protect mice against invasive pneumococcal challenge with strains of several PspA clades, highlighting the potential of PspA to induce cross-protection (25).

The extension of immunological cross-reactivity induced by different PspA molecules within and between clades, however, remains controversial. It has been reported that the cross-reactivity of antibodies is greater between PspA molecules from homologous families (24, 26). Goulart et al. (27) expressed different PspA molecules from family 1, five from clade 1 and five from clade 2, and only two from each clade showed high cross-reactivity with bacteria bearing heterologous PspAs. Recombinant PspA hybrid proteins comprising fragments from families 1 and 2 (PspA clades 2 and 4, PspA clades 2 and 5, and PspA clades 3 and 2) were tested for cross-reactivity, and the broader protective effect was obtained by immunization with PspA clades 3 and 2 (28). Furthermore, there seems to be greater cross-reactivity between PspAs from family 1 than between those from family 2 (26, 27). These apparently discordant results can be attributed to the specific PspA sequence of each molecule analyzed.

On the other hand, Moreno et al. (29) showed that immunization with PspA4 and PspA5 (both from family 2) elicited functional antibodies with broad cross-reactivity and were able to induce protection against intranasal challenge with strains bearing PspA clades 2 and 5. They also analyzed the contribution of the proline and nonproline (nonPro) regions to the cross-reactivity of PspA4. Using a PspA4Pro fragment containing the N-terminal α-helical region plus the first block of prolines, but lacking the nonPro region and the second block of prolines, they were able to demonstrate that the exclusion of the nonPro region of PspA4 induced antibodies with a higher capacity to bind to PspA4 in strains that lack this region.

Here we investigated the conjugation of capsular polysaccharide serotype 14 to PspA4Pro to verify whether it would affect the previously described cross-reactivity. To evaluate this, sera of mice immunized with the PS14-mPspA4Pro conjugate or with the free components were tested regarding the levels of antibodies produced, and their protective activity was measured by opsonophagocytic assay and nasal colonization challenge.
RESULTS

Characterization of the PS14-mPspA4Pro conjugate. The PS14-mPspA4Pro conjugate was synthetized as described previously (12). The polysaccharide hydrolysis with HCl resulted in PS14 molecules of approximately 25 kDa. Reaction between hydrolyzed PS14 and NaIO₄ generated 7 aldehydes per PS14 molecule. These aldehyde groups were reacted with adipic acid dihydrazide (ADH). Previously, rPspA4Pro was treated with formaldehyde in order to avoid intramolecular reactions. The final product, PS14-mPspA4Pro conjugate, was purified by gel filtration chromatography (Superose 12 prep grade). The protein/PS ratio in the purified conjugate was 2:1 (wt/wt).

Immunogenicity of PS14-mPspA4Pro conjugate. The anti-PS14 and anti-PspA4 IgG titers induced by the conjugate and coadministered groups were measured by enzyme-linked immunosorbent assay (ELISA) and are shown in Fig. 1. As expected, the conjugation of PS14 to mPspA4Pro resulted in a higher induction of anti-PS14 IgG than that provided by the free PS14 (Fig. 1A). Although conjugation to the PS14 seemed to slightly reduce the levels of anti-PspA4Pro IgG antibodies by approximately 0.5 log compared to those induced by free mPspA4Pro (Fig. 1B), both conjugated and free mPspA4Pro (Co-adm) were shown to induce high titers of anti-PspA4Pro IgG antibodies in mice (Fig. 1B).

Opsonophagocytic assay. The functionality of anti-PspA4 and anti-PS14 antibodies was measured by their opsonophagocytic activity. First we investigated whether sera from mice immunized with conjugate PS14-mPspA4Pro or with mPspA4Pro coadministered with PS14 and a heterologous PS (non-PS14) (Table 1). Sera from mice immunized with the conjugate reduced the survival of the three strains of pneumococci tested, while sera from mice immunized with coadministered mPspA4Pro and PS14 significantly diminished the number of CFU recovered in two of the three strains tested (Fig. 2). Interestingly, the level of opsonophagocytic activity differed between the conjugated and coadministered groups according to the strain. In one case, the number of CFU recovered was significantly reduced in the conjugated group in comparison with the group immunized with mPspA4Pro coadministered with PS14 (strain P166, serotype 6A), and in the two cases involving serotype 19F strains, the numbers of CFU were either comparable (strain P101) or the sera from mice immunized with free mPspA4Pro coadministered with PS14 was shown to reduce the survival of strain P40 more efficiently (Fig. 2).

We next sought to characterize the opsonophagocytic activity against heterologous strains from the same family 2: PspA clades 3 and 5. Sera from mice immunized either with the conjugate or with the coadministered components were able to significantly...
reduce the numbers of CFU recovered in the four strains tested (Fig. 3 and 4). Conjugation maintained opsonophagocytic activity against strains Tigr4 (PspA3, serotype 4), ATCC 6303 (PspA5, serotype 3), and P865 (PspA5, serotype 23 F) and, surprisingly, increased activity against P1148 (PspA3, serotype 6B), in comparison to sera from mice immunized with free mPspA4Pro coadministered with PS14 (Fig. 3 and 4).

Opsonophagocytosis activity was also evaluated in strains from PspA family 1, PspA clades 1 and 2. The sera from conjugate-immunized mice showed significant opsonophagocytic activity in all strains tested bearing PspA clades 1 and 2 (Fig. 5 and 6). The reduction in survival was comparable to that obtained by sera from mice immunized with free mPspA4Pro coadministered with PS14 for all strains. Strains tested were from serotypes 6A, 1, 2, and 3; strain RM200 is a nonencapsulated strain (Fig. 5 and 6).

Finally, we determined the opsonophagocytosis activity against homologous PS strains (PS14 strains) bearing a heterologous PspA (PspA1, strain 245/00) or a homologous PspA (PspA4, strain P1140). The sera from conjugate-immunized mice showed significant opsonophagocytic activity in both strains tested, demonstrating the functionality of anti-PS14 antibodies after the conjugation process (Fig. 7). Additionally, it is noteworthy that when using a strain bearing homologous PS and PspA in the op-

| Strain | Serotype | PspA clade | Source |
|--------|----------|------------|--------|
| A66.1  | 3        | 2          | UAB    |
| ATCC 6303 | 3      | 5          | IAL    |
| D39    | 2        | 2          | UAB    |
| P40    | 19F      | 4          | UFG    |
| P101   | 19F      | 4          | UFG    |
| P105   | 6A       | 1          | UFG    |
| P166   | 6A       | 4          | UFG    |
| P865   | 23F      | 5          | UFG    |
| P1079  | 1        | 1          | UFG    |
| P1148  | 6B       | 3          | UFG    |
| RM200  | —        | 2          | BCHHMS |
| Tigr4  | 4        | 3          | UAB    |
| 0603   | 6B       | 1          | BCHHMS |
| 245/00 | 14       | 1          | IAL    |
| P1140  | 14       | 4          | UFG    |

aUAB, University of Alabama at Birmingham, Birmingham, AL, USA; UFG, Universidade Federal de Goiás, Goiânia, Brazil; IAL, Instituto Adolpho Lutz, São Paulo, Brazil; BCHHMS, Boston Children’s Hospital, Harvard Medical School, Boston, MA, USA.

bNonencapsulated.

FIG 2 Opsonophagocytic activity of sera from mice immunized with conjugate PS14-mPspA4Pro on pneumococcal strains bearing PspA clade 4. Pneumococcal strains P101, P40, and P166, bearing PspA from clade 4, were incubated with the pooled sera of mice (10 animals/group) immunized with the PS14-mPspA4Pro conjugate, mPspA4Pro coadministered with PS14 (Co-adm), or Al(OH)₃ in PBS (Alum) plus a complement source (NMS). Al(OH)₃ was used as an adjuvant in all groups. The opsonized pneumococci were incubated with peritoneal cells and plated on blood agar plates. The numbers of CFU recovered after 18 h were compared by one-way ANOVA with Tukey’s multiple-comparison test. The results are representative of an experiment performed in triplicate. The lines represent the means ± SEM, and asterisks indicate statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001). ns, nonsignificant differences.
sonophagocytic assay (strain P1140), conjugation led to a significantly lower number of CFU recovered compared to that of the coadministered group (free mPspA4 and PS14) and conjugate groups (Fig. 7).

Colonization challenge. To determine the protective effect of the PS14-mPspA4Pro conjugate against a nonrelated pneumococcal strain (non-PspA4, non-PS14), we immunized mice subcutaneously (s.c.) with the conjugate or coadministered antigens, in a three-dose scheme, and challenged them intranasally using pneumococcus type 6B strain 0603 bearing PspA from clade 1 (10^7 CFU). The number of CFU recovered was determined in the mouse nasal washes after 7 days of challenge. As shown in Fig. 8, immunization with the PS14-mPspA4Pro conjugate significantly protected the animals against pneumococcal colonization, with protection at levels similar to those observed for the animals that received the coadministered antigens PS14 and mPspA4Pro.

DISCUSSION

The use of pneumococcal surface proteins as an alternative to heterologous proteins, such as CRM197, or tetanus toxoid as a carrier for CPS, would be a way to improve...
pneumococcal vaccine coverage. PspA has been shown to be an efficient carrier for capsular polysaccharide, as well as an antigen able to induce protective antibodies when conjugated (11–13, 30). PspA is one of the pneumococcal surface proteins described as a protein vaccine candidate, and the main reason is its capacity to induce protective antibodies (25, 28, 31). PspA is a mosaic protein with a variable domain in its N-terminal sequence that induces antibodies cross-reacting within proteins from the same family (21) but to a lesser extent across families; this is a restriction for a vaccine candidate. Different approaches have been used in order to circumvent this limitation, such as the combination of PspAs from different clades and families (28, 32).

On the other hand, the antiserum induced against rPspA clade 4 has been described as broadly cross-reactive, recognizing pneumococcal strains containing PspAs of all clades from families 1 and 2 (33). The functionality of the anti-rPspA4 antibodies was analyzed as to the capacity to bind to and mediate complement deposition on intact bacteria in vitro, and mice immunized with rPspA4 and rPspA5 were protected against intranasal lethal challenge with pneumococcal strains (A66.1 and ATCC 6303) bearing PspAs from families 1 and 2 (29). We here evaluated the functionality and immuno-

FIG 5 Opsonophagocytic activity of sera from mice immunized with conjugate PS14-mPspA4Pro on pneumococcal strains bearing PspA clade 1. Pneumococcal strains P105 and P1079, bearing PspA from clade 1, were incubated with the pooled sera of mice (10 animals/group) immunized with mPspA4Pro coadministered with PS14 (Co-adm), the PS14-mPspA4Pro conjugate, or Al(OH)3 in PBS (Alum) plus a complement source (NMS). Al(OH)3 was used as an adjuvant in all groups. The opsonized pneumococci were incubated with peritoneal cells and plated on blood agar plates. The numbers of CFU recovered after 18 h were compared by one-way ANOVA with Tukey’s multiple-comparison test. The results are representative of an experiment performed in triplicate. The lines represent the means ± SEM, and asterisks indicate statistically significant differences (*, P ≤ 0.05; ***, P ≤ 0.001). ns, nonsignificant differences.

FIG 6 Opsonophagocytic activity of sera from mice immunized with conjugate PS14-mPspA4Pro on pneumococcal strains bearing PspA clade 2. Pneumococcal strains A66.1, D39, and RM200, bearing PspA from clade 2, were incubated with the pooled sera of mice (10 animals/group) immunized with mPspA4Pro coadministered with PS14 (Co-adm), the PS14-mPspA4Pro conjugate, or Al(OH)3 in PBS (Alum) plus a complement source (NMS). Al(OH)3 was used as an adjuvant in all groups. The opsonized pneumococci were incubated with peritoneal cells and plated on blood agar plates. The numbers of CFU recovered after 18 h were compared by one-way ANOVA with Tukey’s multiple-comparison test. The results are representative of an experiment performed in triplicate. The lines represent the means ± SEM, and asterisks indicate statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001). ns, nonsignificant differences.
logical cross-reactivity of the antibodies induced by the s.c. immunization of BALB/c mice with mPspA4Pro conjugated to PS14. Polysaccharide as a thymus-independent antigen induces low-avidity antibodies, mainly of the IgM type, and does not induce immunological memory. When covalently linked to a protein, PS becomes a T-cell-dependent antigen inducing long-term immunity in young children (4). Therefore, the increased anti-PS14 IgG after conjugation with mPspA4Pro indicates that the reaction was successful. Our results showed that after conjugation, the titer of anti-rPspA4 was lower than that induced by the free mPspA4Pro coadministered with PS14. This indicates that the conjugation may have changed the protein epitope profile. Considering these results, it is essential to evaluate whether the decrease in the anti-rPspA antibody level could affect the functionality of these antibodies.

Opsonophagocytic activity is a means of evaluating the functional activity of the antibodies in vitro and is dependent on the antibody binding and complement deposition that lead to killing and clearance. The opsonophagocytic activities exhibited by anti-PspA4Pro antibodies in the sera of mice immunized with the PS14-mPspA4Pro

FIG 7 Opsonophagocytic activity of sera from mice immunized with conjugate PS14-mPspA4Pro on pneumococcal strains of serotype 14. Pneumococcal strains 245/00 and P1140 (serotype 14), bearing PspA from clade 1 and clade 4, were incubated with the pooled sera of mice (10 animals/group) immunized with mPspA4Pro coadministered with PS14 (Co-adm), the PS14-mPspA4Pro conjugate, or Al(OH)₃ in PBS (Alum) plus a complement source (NMS). Al(OH)₃ was used as an adjuvant in all groups. The opsonized pneumococci were incubated with peritoneal cells and plated on blood agar plates. The numbers of CFU recovered after 18 h were compared by one-way ANOVA with Tukey’s multiple-comparison test. The results are representative of an experiment performed in triplicate. The lines represent the means ± SEM, and asterisks indicate statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001). ns, nonsignificant differences.

FIG 8 Colonization challenge. Groups of 10 BALB/c mice immunized subcutaneously with the PS14-mPspA4Pro conjugate, PS14 and mPspA4Pro (Co-adm), or Al(OH)₃ in PBS (Alum) were challenged intranasally 7 days after the third immunization with pneumococcal type 6B strain 0603 (1 × 10⁷ CFU), bearing PspA from clade 1. Al(OH)₃, was used as an adjuvant in all groups. The nasal wash was collected after 7 days, diluted, and plated on blood agar plate. Each dot represents the CFU recovered from one mouse. A horizontal line denotes the median for each group. Data were compared by one-way ANOVA with Tukey’s multiple-comparison test. Asterisks indicate statistically significant differences (**, P ≤ 0.01; ***, P ≤ 0.001). ns, nonsignificant differences.
conjugate were evaluated using 14 pneumococcal strains carrying PspAs from clade 1 to clade 5 and a variety of serotypes. Despite the lower antibody levels induced by the conjugate, these antibodies were shown to be functional against all strains tested comprising PspAs from both families, including clades 1 to 5 and serotypes 1, 2, 3, 4, 6A, 6B, 19F, and 23F. The reduction in the number of CFU recovered obtained with the sera from the conjugate was either comparable to or greater than that obtained with mPspA4Pro coadministered with PS14. The only exception was found in strain P40 (PspA clade 4, serotype 19F).

It is also very important to observe that the anti-PS14 antibodies generated by immunization with the conjugate are functional, as can be seen in the opsonophagocytosis assay in the PS14 strains tested (245/1000 and P1140). PS14 antibodies from mice immunized with conjugated PS14-mPspA4Pro showed opsonophagocytic activity that was significantly higher than that from nonconjugated PS. In the case of testing a strain bearing a homologous PspA4 (P1140), it was possible to see the effect of PspA4, as the reduction in the number of CFU recovered was greater than that in strain 245/00, which expresses PspA1.

Pneumococcal disease is generally initiated by bacterial colonization of the nasopharynx mucosa. Protection against nasal carriage is crucial for herd immunity to pneumococcal disease and would have a direct effect on the reduction of invasive disease. In our study, subcutaneous immunization with free or conjugated mPspA4Pro was able to reduce the bacterial load in colonized mice. It has been described that cellular rather than humoral immunity promotes the clearance of pneumococcal nasal colonization requiring CD4+ cells, where monocytes seem directly related to the bacterial clearance and interleukin 17A (IL-17A) is critical to the recruitment of phagocytic cells (34, 35). Therefore, we could speculate that the induction of cellular immune responses would be similar for the free and conjugated mPspA4Pro, since mice immunized with either vaccine were equally protected from nasal challenge.

On a whole, our results show that the conjugation of mPspA4Pro with PS14 provides the expected IgG increase against the polysaccharide moiety, indicating that the conjugation was effective in modifying it into a T-cell-dependent antigen. In addition, the anti-PS14 antibodies are functional and reduced the number of CFU in the opsonophagocytic killing assay. Moreover, the PS14-mPspA4Pro conjugate retains the antigenic properties of the mPspA4Pro molecule, as the antisera generated against the conjugate has opsonophagocytic properties comparable to those of the free protein in a large panel of strains and shows comparable protection against colonization. These results reinforce the potential of using highly immunogenic pneumococcal proteins as carriers for capsular polysaccharide in order to broaden their immunogenic cross-reactivity.

MATERIALS AND METHODS

The nontagged recombinant fragment of PspA from clade 4 (PspA4Pro) and capsular polysaccharide from S. pneumoniae serotype 14 were produced and purified at Departamento de Engenharia Quimica (UFSCar) and Centro de Biotecnologia (Instituto Butantan) as described previously (36, 37). Adipic acid dihydrazide (ADH) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) were from Sigma-Aldrich. All other reagents were analytical grade.

**PS14-mPspA4Pro conjugation.** PS14-mPspA4Pro conjugation was performed as described earlier (12). Briefly, PS14 (5 mg ml⁻¹) was hydrolyzed by 0.5 M HCl at 80°C for 30 min in a reflux system, followed by the addition of NaOH (pH 7.5). The hydrolyzed PS14 was oxidized with NaIO₄ (10 mM) in phosphate-buffered saline (PBS), pH 7.5, for 30 min in the dark at room temperature. The reaction was stopped by glycerol (10 eq) and diafiltered using a 5-kDa cutoff membrane (Pellicon XL; Millipore). Modified PS14 (10 mg ml⁻¹) was incubated with mPspA4Pro (20 mg ml⁻¹) previously reacted with formaldehyde and DMT-MM (0.05 M) in PBS buffer, pH 7.5, for 24 h and quenched by ammonium acetate 0.1 M. The product was purified by Superose 12 prep grade gel filtration chromatography in an HR 16/50 column (GE Healthcare) using PBS, pH 7.5, as the mobile phase (0.5 ml min⁻¹).

**Analytical methods.** PS14 was quantified by the phenol sulfuric acid method (38). PS14 from the ATCC was used as the standard. The extension of oxidation was estimated by the colorimetric method using bicinchoninic acid (BCA) (39). The extension of the reaction of ADH with lysine ε-aminogroups was...
Estimated by the TNBS method (40) using ADH as the standard. The molar size of the hydrolyzed PS14 was determined by gel filtration chromatography, with Sephacryl S-500 resin packed in a XK16/100 column (GE Healthcare), using PBS, pH 7.5, as the mobile phase at a flow rate of 1 ml min⁻¹. The column was calibrated with dextrins (Sigma-Aldrich) of known sizes (2,000 kDa, 410 kDa, 150 kDa, 50 kDa, 25 kDa, and 12 kDa).

**Animals and immunizations.** All in vivo experiments were approved by the Instituto Butantan Animal Care and Use Committee under license numbers CEUAIB 1129/13 and 5084030615. Inbred 8- to 10-week-old female BALB/c mice were immunized subcutaneously (s.c.) with the PS14-mPspA4Pro conjugate (2.5 µg PS14 and 5 µg mPspA4Pro/dose). PS14 at 2.5 µg plus mPspA4Pro at 5 µg (coadministered) and PBS were used as controls. Al(OH)₃ (50 µg/dose) was used as an adjuvant in all groups. The animals (10 mice per group) were subjected to a three-dose immunization scheme (at 0, 14, and 28 days), and blood samples were collected at day 42 via the retro-orbital plexus.

**ELISA.** Anti-PS14 and anti-mPspA4Pro antibodies were determined by conventional ELISA as follows. ELISA 96-well microtiter plates (Nunc MaxiSorp) were coated with 50 µg/well of PS14 or 1 µg/well of rPspA4Pro in PBS (pH 7.2) overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS and 10% skim milk for 1 h at 37°C. The plates were washed with PBS-T and incubated with serial dilutions of serum from individual mice in PBS and 1% bovine serum albumin for 2 h at 37°C for anti-PS14 or 1 h at 37°C for anti-PspA, and the plates were washed with PBS-T. Peroxidase-conjugated polyclonal goat anti-mouse IgG (1:5,000) was then added, and the plates were incubated at 37°C for 2 h (PS14) or 1 h (PspA). The plates were washed three times with PBS-T, and the substrate o-phenylenediamine dihydrochloride in citrate buffer (pH 5.0) with 0.5 µl ml⁻¹ of 30% hydrogen peroxide was added for 15 min in the dark. The enzyme reaction was quenched by adding 4 M H₂SO₄. The plates were read at 492 nm on a Multiskan EX ELISA reader (Labsystems Uniscience). Titters were calculated by using the dilution resulting in an absorbance value of 0.1 at 492 nm.

**Opsonophagocytic assay.** The modified opsonophagocytic assays were performed as described by Goulart et al. (27). The *S. pneumoniae* strains shown in Table 1 were grown in Todd-Hewitt broth supplemented with yeast extract (THY) to an optical density at 600 nm (OD₆₀₀ nm) of 0.4 to 0.5, which corresponds to approximately 1 × 10⁸ CFU/ml, and centrifuged at 4,000 × g for 3 min. The pellets were washed once with PBS and resuspended in Hanks’ buffer (Invitrogen) containing 0.1% gelatin. Aliquots containing 2.5 × 10⁶ CFU were incubated with heat-inactivated pooled sera in the test at a final dilution of 1:100, in triplicate, for 30 min at 37°C. Sera from mice that received saline plus Al(OH)₃ were used as a control for all the assays. The samples were incubated with 10% normal mouse serum (NMS) diluted in Hanks' buffer containing 0.1% gelatin (opsono-buffer) at 37°C for 30 min. After that, the samples were washed once with PBS and incubated with 4 × 10⁶ peritoneal cells diluted in opsono-buffer at 37°C for 30 min with shaking (200 rpm). The reaction was stopped by incubation on ice for 5 min. Tenfold dilutions of the samples were plated on blood agar plates in triplicate. The plates were incubated at 37°C, in 5% CO₂, and the pneumococcal CFU recovered were counted after 18 h of incubation at 37°C.

**Colonization challenge.** The colonization assay was based on the protocol described by Malley et al. (41). Pneumococcal strain 0603 (serotype 6B, PspA clade 1) was inoculated (1 × 10⁷ CFU/animal in 10 μl of cold PBS) into the nostrils of previously immunized mice. On day 7 after the colonization challenge, the animals were euthanized (with 1.25 mg/kg of pentobarbital sodium), they were sacrificed and their peritoneal cavities were washed with 5 ml of cold PBS.

**Statistical analysis.** Tukey’s multiple-comparison test was performed for all the experiments, followed by the one-way analysis of variance (ANOVA). For all comparisons, a P value of <0.05 was considered to represent statistical significance.

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