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Intranasal vaccination with protein bodies elicit strong protection against *Streptococcus pneumoniae* colonization

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ABSTRACT

Protein bodies (PBs) are particles consisting of insoluble, aggregated proteins with potential as a vaccine formulation. PBs can contain high concentrations of antigen, are stable and relatively resistant to proteases, release antigen slowly and are cost-effective to manufacture. Yet, the capacity of PBs to provoke immune responses and protection in the upper respiratory tract, a major entry route of respiratory pathogens, is largely unknown.

In this study, we vaccinated mice intranasally with PBs comprising antigens from *Streptococcus pneumoniae* and evaluated the level of protection against nasopharyngeal colonization. PBs composed of the α-helical domain of pneumococcal surface protein A (PspAα) provided superior protection against colonization with *S. pneumoniae* compared to soluble PspAα. Immunization with soluble protein or PBs induced differences in antibody binding to pneumococci as well as a highly distinct antigen-specific nasal cytokine profile upon *in vivo* stimulation with inactivated *S. pneumoniae*. Moreover, immunization with PBs composed of conserved putative pneumococcal antigens reduced colonization by *S. pneumoniae* in mice, both as a single- and as a multi-antigen formulation.

In conclusion, PBs represent a vaccine formulation that elicits strong mucosal immune responses and protection. The versatility of this platform offers opportunities for development of next-generation vaccine formulations.

1. Introduction

Particle-based vaccine formulations offer several advantages over traditional subunit vaccines, including reduced antigen degradation, enduring antigen presenting cell (APC) stimulation by slow antigen release, and enhanced activation of APCs by higher local antigen concentrations and improved antigen uptake [1,2]. Insoluble protein aggregates – designated protein bodies (PBs) – are particles that, until now, have primarily been viewed as an undesirable by-product of recombinant protein production by bacteria [3–5]. Recently, we have optimized a broadly applicable short tag, which can be genetically fused to any antigen-encoding gene of interest, resulting in large quantities of PBs composed of the encoded fusion protein in a controlled fashion [6,7]. The generic applicability, combined with the potential for large scale production and relatively low manufacturing costs, make PBs an attractive delivery vehicle for a variety of biomedical applications, including vaccine development [3,4,8].

Earlier studies provided evidence that protein aggregates can successfully elicit immune responses [9–17]. PBs produced with the same platform as used in this study have previously been found to induce both CD4+ and CD8+ T cell responses following antigen presentation by murine dendritic cells *in vitro* [10]. It was shown that aggregated antigens are more potent in mounting an immune response leading to better protection than soluble antigens [9,16]. Immunization of mice with a *Mycobacterium tuberculosis* antigen formulated as protein aggregates without adjuvant conferred a higher level of protection against infection than the same antigen

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in soluble form administered together with an adjuvant [9]. Similarly, when compared to soluble antigen, mice immunized with protein aggregates of a *M. tuberculosis* fusion protein showed enhanced cellular and humoral immune responses [16].

The upper respiratory tract (URT) is the primary entry site of respiratory pathogens, from which they can spread to the lower airways leading to infection and disease. This process can be blocked efficiently when vaccination strategies induce effective mucosal immune responses [18]. *Streptococcus pneumoniae* is the most important global cause of lower respiratory tract infections in children, immune compromised individuals and the elderly [19,20]. Importantly, disease development is typically preceded by asymptomatic colonization of *S. pneumoniae* in the URT, which also facilitates transmission of the bacterium to susceptible individuals. Therefore, broadly protective and affordable immunization strategies providing protection against both colonization and disease are highly desired to replace or complement the currently available pneumococcal conjugate vaccines (PCVs) [21,22]. Protein-based vaccines have been examined as a promising alternative for polysaccharide-based PCVs [23,24].

In this study, we investigated the potency of PB-based formulations in establishing mucosal immune responses and protection against the pneumococcus in the URT, following intranasal administration in mice. We provide evidence that intranasally applied PBs in mice outperform the same antigen in soluble form with respect to protection against pneumococcal colonization. Analysis of the nasal cytokine profile upon *in vivo* stimulation with *S. pneumoniae* identified significant differences between mice immunized with a soluble or particulate antigen. Considering the versatility of this technology, PBs have significant potential as a (mucosal) vaccine platform.

2. Materials and methods

2.1. Ethics statement

Mice experiments performed for this study were approved by the Radboudumc Committee for Animal Ethics (AVD103002017904 and AVD103002016454) and conducted according to the Dutch legislation.

2.2. Bacterial strains and growth conditions

For all experiments, *S. pneumoniae* strains were cultured in Todd-Hewitt broth (BD, Maryland, USA) supplemented with yeast extract (CONDA, Madrid, Spain) (THY) under static conditions at 37 °C, 5% CO_2 to an optical density $(OD)_{620}$ of 0.25. Subsequently, glycerol stocks were stored at −80 °C until usage. For mouse infection experiments, TIGR4 (serotype 4), and Pneumococcal Bacteraemia Collection Nijmegen (PBCN) isolates 0231 (serotype 4) and 0460 (serotype 18C) were used. To assess antibody binding to pneumococci, TIGR4, TIGR4Δ*pspA,* TIGR4Δ*cps* and TIGR4Δ*cps*Δ*pspA* were used. For the C3 deposition experiments, TIGR4 and TIGR4Δ*cps* were used. For *in vivo* stimulation experiments, TIGR4 and TIGR4Δ*pspA* were used, which were heat-killed (30 min, 65 °C) prior to storage at −80 °C.

2.3. Soluble PspAα protein production and purification

Escherichia coli BL21(DE3) cells harboring a pET16b expression plasmid, pET16b-nHis-PspAα (Supplementary Methods), were grown in Lysogeny Broth (10 g/L of tryptone (Oxoid, Hampshire, United Kingdom), 5 g/L of yeast extract (Oxoid) and 10 g/L of NaCl (Sigma-Aldrich, Missouri, USA)) supplemented with 2 g/L of glucose (Merck, Darmstadt, Germany) containing 2 g/L glucose (Merck, Darmstadt, Germany) and kanamycin (50 μg/ml, Sigma-Aldrich) to early log phase. Protein expression was induced by the addition of isopropyl-β-D thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the cells were incubated for a further 2.5 h. After centrifugation, the cells containing pellet was resuspended in buffer A (50 mM NaPO4, 300 mM NaCl [pH 7.4]), and phenylmethylsulfonyl fluoride (PMSF) was added to a concentration of 1 mM. The cells were disrupted by two passages through a One Shot cell disruptor (Constant Systems Ltd., Daventry, UK) at 1.2×10^8 Pa. Cell debris and membranes were removed by centrifugation at 10,000*g* and 293,000*g*, respectively, at 4 °C. The His_6-tagged protein was isolated from the cleared lysate using Talon Superflow medium (GE Healthcare Life Sciences, Massachusetts, USA) according to the manufacturer's instructions. The eluate was dialyzed overnight at 4 °C against 500 volumes of PBS (pH 7.4). After dialysis, glycerol was added to 15%, and aliquots were stored at −80 °C. The concentration of the antigen was determined by comparison with a BSA standard on Coomassie-stained SDS-PAGE gel. Before use the protein solution was diluted with PBS containing 15% glycerol, typically to a concentration of 2.5 mg/mL.

2.4. Production of PB-based vaccine formulations

For production of the various PBs, DNA inserts encoding the used antigens (sequences in Supplementary Methods) flanked on either side by triple repeats of the TorA signal sequence (PspAα and Green Fluorescent Protein (GFP)) [6], or a truncated (Δ29-36) derivative thereof (PnrA, DiiA, SP1690 and AliA) [7] were cloned under *tet*-promoter control in pASK-IBA3 (IBA GmbH, Göttingen, Germany). To isolate PBs, *E. coli* K-12 strain TOP10F' (Invitrogen, Massachusetts, USA) cells harbouring one of the pIBA plasmids were grown in Lysogeny Broth supplemented with 2 g/L of glucose (Merck) and 100 mg/L of ampicillin (Sigma-Aldrich) from an OD_{600} of 0.05 to an OD_{600} of approximately 0.4 at 37 °C with aeration. Protein expression was induced by the addition of anhydrotetracycline to a final concentration of 0.2 mg/L. After three hours of further incubation, the cells were collected by centrifugation. The cells were resuspended in Lysis buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 10 μg/mL lysozyme) to an OD_{600} of 20, incubated at 37 °C for one hour and lysed using a Branson Sonifier 250 (Branson Ultrasonics, Connecticut, USA). The resulting lysate was subjected to centrifugation (15,000*g*, 15 min) to sediment the PBs. To remove contaminants, the pelleted PB material was subjected to a number of consecutive washing steps: The PBs were resuspended (2x concentrated) in 10 mM Tris–HCl pH 8.0, 1 mM EDTA using tip sonication, after which the suspension was mixed with an equal volume of Triton Wash Buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 2% Triton X-100) and incubated at room temperature for 1 h. PBs were collected by centrifugation (15,000*g*, 15 min) and resuspended (2x concentrated) in 10 mM Tris–HCl pH 8.0 using tip sonication. After addition of an equal volume of Urea/Salt Wash buffer (10 mM Tris–HCl pH 8.0, 2 M Urea, 2 M NaCl) the suspension was incubated at room temperature for 1 h. PBs were again collected by centrifugation (15,000*g*, 15 min), resuspended (1x concentrated) in PBS using sonication, again sedimented by centrifugation (15,000*g*, 15 min), and finally resuspended in a small volume (typically ∼ 100× more concentrated) of PBS containing 15% glycerol and stored at −80 °C. The concentration of the antigen is determined by comparison with a BSA standard on Coomassie-stained SDS-PAGE gel. Before use the PB suspension was diluted with PBS containing 15% glycerol, typically to a concentration of 2.5 mg/mL.

2.5. Particle visualization by negative stain – Transmission electron microscopy (NS-TEM)

Of each PB sample, 3 μL (∼7.5 μg) was incubated for 1 min on a glow discharged grid (Cu, 100 mesh with carbon-coated formvar

film) and excess sample was blotted off. Grids were negative stained with 0.5% uranyl acetate (UA) in MQ, by rinsing twice with 0.5% UA followed by 1 min incubation with 0.5% UA and subsequent blotting and overnight air drying. Grids were analysed using a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) operating at 200 kV.

2.6. Mouse immunization

For all mouse experiments, 7 week-old female C57Bl/6J mice were obtained from Charles River in France or Germany. Upon arrival, mice were randomized, using Random.org sequence generator, in groups of 3, 4 or 5 over individually ventilated cages. Prior to inclusion in the experiments, mice had at least one week to acclimate. Mice were allocated to control and experimental groups randomly, the investigators were not blinded. Sterile water and food were provided *ad libitum*, and mice were monitored daily.

Mice were intranasally immunized three times under inhalation anaesthesia (isoflurane) with a two weeks interval. Vaccine formulations consisted of PBs, soluble antigen with or without 4 μg Cholera Toxin subunit B (CTB, Sigma-Aldrich) per vaccine dose in a total volume of 5 μL in PBS. Prevnar was administered intramuscularly, without anaesthesia, at 1/15th of the human dose using the same immunization scheme as for intranasally vaccinated mice. Two weeks post third vaccination, blood was collected from the tail vein for further analysis.

2.7. Mouse colonization model

Three weeks after the third vaccination, mice were intranasally challenged with 1×10^6 colony forming units (CFU) *S. pneumoniae* TIGR4, PBCN0231 or PBCN0460 in 5–10 μL in PBS under inhalation anaesthesia (isoflurane). Three or seven days post-infection, mice were euthanized, and nasal tissue was harvested, homogenized, and used for plating on blood agar plates to determine the bacterial load. Remaining homogenized nasal tissue was snap frozen and stored for further analysis. After incubating (37 °C, 5% CO_2) the blood agar plates overnight, the nasal bacterial load was quantified.

2.8. In vivo stimulation model

Three weeks after the third vaccination, mice were intranasally stimulated with 1×10^6 CFU heat-killed *S. pneumoniae* TIGR4 or TIGR4Δ*pspA* in 5 μL PBS under inhalation anaesthesia (isoflurane). 2, 4, 6 or 24 h post-stimulation, mice were euthanized, and nasal tissue was harvested. Homogenized tissue was directly snap-frozen in liquid nitrogen for further analysis.

2.9. Cytokine analysis

Cytokine levels in supernatant of nasal homogenates were determined using the mouse Bio-Plex Pro assay (Bio-Rad, California, USA) including the detection of mouse IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12-p70, IL-13, IL-17A, KC, and TNF-α. The assay was performed according to the manufacturers protocol. Data acquisition was performed on the FlexMap-3D System (Luminex, 's-Hertogenbosch,

The Netherlands) using xPONENT software (Luminex) and data analysis was performed using Bio-Plex manager software (Bio-Rad).

2.10. Antibody binding to pneumococci

Antibody binding to pneumococci was performed based on a previously described method [25], with several adjustments. Bacteria were thawed, pelleted (5 min, 16,000 g) and resuspended in PBS with 0.075% catalase (Sigma-Aldrich) and 5 mM carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich) for labelling (30 min, 37 °C, 220 rpm). Bacteria were washed three times and resuspended in PBS with 2% BSA at a concentration of 2×10^8 CFU/mL. 10 μL bacteria was mixed with 10 μL serum collected 2 weeks after the third immunization or 10 μL homogenized nasal tissue collected at termination in a sterile 96 wells round bottom plate and incubated (30 min, 37 °C, 220 rpm). Nasal tissue was used undiluted, serum was diluted 1:1000 (IgG, IgG1, IgG2c) or 1:50 (IgA, IgM) in PBS with 2% BSA. After incubation, bacteria were washed twice with PBS and stained with fluorescently labelled antibodies (30 min, 4 °C) diluted in PBS with 2% BSA: 1:200 goat anti-mouse IgA PE (Southern Biotech, Alabama, USA, 1040–09), 1:100 goat anti-mouse IgG AF647 (Southern Biotech, 1030–31), 1:200 goat anti-mouse IgG1 AF647 (Southern Biotech, 1070–31), 1:100 goat anti-mouse IgG2c PE (Southern Biotech, 1079–09) and 1:100 goat anti-mouse IgM PE (Southern Biotech, 1020–09). Measurement of IgG1 (660+\-20 nm) and IgG2c (575 ± 26 nm) on pneumococci (530 ± 30 nm) was multiplexed. Similarly, IgG (660+\-20 nm) and IgA (575 ± 26 nm) were combined in case the same serum/nasal tissue dilution could be used to detect both antibody classes. IgM (575 ± 26 nm) binding to pneumococci was measured separately. Bacteria were washed once and fixed in 2% paraformaldehyde in PBS (20 min, room temperature). Bacteria are diluted 10-fold in PBS and used directly for flow cytometric analysis on a LSR II machine (BD biosciences, Vianen, The Netherlands). 20,000 CFSE positive events were recorded and analysed using Flow JoX version 10.2. Groups were compared based on the mean fluorescent intensity (MFI) in arbitrary units (AU) for each of the antibody isotypes or subclasses. Bacteria incubated with PBS and pooled samples from CTB immunized mice were included as negative controls. To evaluate potential antigen-independent recognition of *S. pneumoniae*, incubations with TIGR4Δ*pspA* were included in parallel for all samples.

2.11. C3 deposition on pneumococci

Mouse complement C3 deposition was performed similarly as described above for antibody binding to pneumococci, with a few adjustments. Bacteria were thawed, pelleted (5 min, 16,000 g) and resuspended in PBS with 0.075% catalase (Sigma-Aldrich) and 5 mM Pacific Blue™ Succinimidyl ester (PBSE, Invitrogen) for labelling (30 min, 37 °C, 220 rpm). Bacteria were washed three times and resuspended in Hanks' Balanced Salt Solution (HBSS, Gibco, Amarillo, Texas) without phenol red containing Ca^{2+}/Mg^{2+} + 0.1% gelatin (HBSS3+), at a concentration of 2×10^8 CFU/mL. 10 μL bacteria was mixed with 10 μL 1:50 diluted serum collected 2 weeks after the third immunization and 1% final concentration of C57BL6 mouse Complement (mC) source (Innovative Research, Novi, Michigan) in HBSS3 + in a sterile 96 wells round bottom plate and incubated (30 min, 37 °C, 220 rpm). A 1:50 dilution of serum was selected as this dilution enabled detectable levels of IgG as well as IgM binding to the bacterial surface. Bacteria were washed once and fixed in 2% paraformaldehyde in PBS (20 min, room temperature). Subsequently, bacteria were stained with goat anti-mouse C3-FITC (525 ± 40 nm) (MPbio, Eschwege, Germany; 0855500) 1:800 diluted in PBS with 2% BSA (15 min, room temperature). Bacteria were washed once, diluted 10-fold in PBS, and used directly for flow cytometric analysis on a CytoFLEX (Beckman Coulter, Woerden, Netherlands). 20,000 PBSE positive events were recorded and analysed using Flow JoX version 10.2. Groups were compared based on the MFI in AU. As negative controls, heat-inactivated mC (20 min, 56 °C) or HBSS only are used.

2.12. Statistics

Group sizes in animal vaccination experiments with as primary read-out the nasal bacterial load, ranged between n = 8 and n = 12, with the main control group in each experiment being 1.5- or 2-fold larger, to improve statistical power when performing multiple comparisons. Values used for sample size calculations are, standard deviation: 0.75–1.25 log10 CFU, true difference of means: 1.0–1.8 log10 CFU, alpha: 0.05, power: 0.8. In case a mouse developed symptoms of invasive pneumococcal disease following challenge (humane endpoint), this mouse was excluded from all subsequent analyses (criterium established *a priori*). Mucosal cytokine analysis was performed using n = 3 (time series pilot) and n = 4 mice, as minimal number of animals required for statistical analysis.

PCA and effect size analysis on nasal cytokine levels were performed using R (R Core Team, 2016). To estimate the difference in cytokine production between HKSpWT and HKSpΔ*pspA* stimulation conditions, cytokine values were first log10 transformed to render a normal distribution. A linear model was constructed per cytokine with vaccine and infection conditions as well as their interaction as covariates. In R notation: Cytokine ~ Vaccine + Condition + Vaccine : Condition. The difference of least square means of HKSpWT and HKSpΔ*pspA* knockout stimulation conditions was extracted using the 'lsmeans' package in R for each vaccine condition (Tukey-adjusted comparisons). Differences between soluble PspAα and PB PspAα vaccine conditions for the HKSpWT - HKSpΔ*pspA* comparison (the difference of differences) were extracted from the estimate of the interaction term.

Other statistical analyses were all performed using GraphPad Prism version 5.03 (GraphPad Software, California, USA). Details on the statistical parameters used can be found in the figure legends. Analysis of bacterial loads and cytokine levels were performed on log10 transformed data to render normal distribution. $p < 0.05$ was considered significant. For access to raw data contact the corresponding author.

3. Results

3.1. PB production and characterization using pneumococcal model antigen PspAα

To investigate the potency of PBs in establishing protection in the URT, we selected a well-studied model antigen of *S. pneumoniae*, i.e. pneumococcal surface protein A (PspA). PspA has been shown to protect against pneumococcal colonization and disease following mucosal or parenteral vaccination when administered soluble or as a particle-based formulation [26–28]. We previously showed that the N-terminal, surface-exposed, α-helical coiled-coil domain of PspA, referred to as the PspAα fragment, is highly immunogenic and induces strong protection against pneumococcal colonization when presented on *Salmonella* outer membrane vesicles (OMVs) [26,29].

In a previous study, we showed that fusion of a PB-formation tag based on the signal sequence of *torA* from *E. coli* (i.e. PB-tag) to the N- or C-terminus of the normally soluble TrxA and MBP proteins results in PB formation [6,7]. Here, we generated PBs of PspAα by a genetic fusion of the α domain of *pspA* to the PB-tag. Upon high expression conditions in *E. coli*, the PB-tag-PspAα fusion protein formed insoluble aggregates, as was shown previously for other constructs [6,7]. PBs were subsequently isolated by disruption of *E. coli* cells followed by low-speed centrifugation. The PBs in the pellet were then sequentially washed with Triton X-100 to remove excess membrane material including LPS, then with urea to break low-affinity protein interactions, and finally with a high concentration of NaCl to break potential electrostatic protein interactions. The purified PspAα PBs were analysed by SDS-PAGE (Fig. 1a). NS-TEM analysis revealed that the PBs displayed a rough surface geometry (Fig. 1b).

3.2. Intranasal immunization with PspAα PBs confers protection against pneumococcal colonization

To explore the potency of PBs as a vaccine formulation, we used the PspAα PBs to intranasally vaccinate mice three times with a two-week interval. A dose range from 1 to 50 μg PspAα PBs per vaccination, adjuvanted with CTB [30], was tested. For comparison, groups of mice vaccinated with PBS, CTB, soluble PspAα, PspAα PBs without CTB, or PBs comprised of the unrelated GFP with CTB were included. Three weeks after the third vaccination, mice were challenged with 1×10^6 CFU of *S. pneumoniae* strain TIGR4, a challenge strain that is widely used in animal experiments [26,31,32]. At three days post-challenge, the nasal bacterial load was determined (Fig. 2a). When comparing each of the groups that received a CTB-adjuvanted formulation to the CTB only group, a statistically significant reduction of the bacterial load was observed for mice vaccinated with 50 μg, 10 μg, or 3 μg of PspAα PBs, of which immunization with 10 μg PspAα PBs resulted in the strongest decrease (45-fold) (Fig. 2a). Immunization with an equimolar amount of soluble PspAα (5 μg) adjuvanted with CTB did not significantly protect against pneumococcal colonization. In addition, neither PspAα PBs without adjuvant, nor GFP PBs with CTB reduced the nasal bacterial load.

Since the expression and accessibility of PspA on the bacterial surface may vary between clinical strains and serotypes, we repeated the experiment using additional challenge strains. To this end, we selected two isolates from a pneumococcal bacteraemia cohort that contained a PspAα amino acid sequence identical to that of TIGR4: PBCN0231 (serotype 4) and PBCN0460 (serotype 18C). For this experiment, mice were either vaccinated intranasally with 10 μg PspAα PBs, vaccinated intramuscularly with 13-valent PCV (Prevnar) or left untreated. The bacterial load in the murine nose was determined at three- or seven-days post-challenge with 1×10^6 CFU (Fig. 2b, c). PB PspAα immunized mice challenged with either PBCN0231 or PBCN0460 showed a strong reduction in bacterial load compared to unvaccinated mice, both at three days and seven days post-challenge (Fig. 2b, c). In contrast, vaccination with Prevnar did not result in a reduction of pneumococcal colonization (Fig. 2b, c), as has been reported previously [33,34].

3.3. Differences in antibody binding to pneumococci following intranasal immunization with PspAα soluble or PBs

To gain insight into the mechanisms underlying the protection induced by PspAα PBs, we investigated the antibody response in PB-vaccinated mice and compared this to soluble PspAα immunized mice. Recognition of pneumococci by vaccine-induced antibodies was measured by *in vitro* binding to TIGR4 and an isogenic *pspA* knock-out strain (TIGR4Δ*pspA*) using flow cytometry (Fig. 3, Fig. S1). Binding of mucosal antibodies to pneumococci was determined using homogenized nasal tissue collected three days post-infection. Systemic antibody binding to pneumococci was determined in serum collected two weeks after the third immunization. A moderately elevated mean fluorescent intensity (MFI), expressed as arbitrary units (AU), of mucosal IgA bound to pneumococci was detected for PB PspAα immunized mice compared to soluble PspAα immunized mice ($p = 0.0520$), a similar trend was found for serum IgA. Binding of serum-derived IgM and IgG1 was higher for mice immunized with the soluble antigen when compared to PB immunized mice ($p = 0.0005$ and $p = 0.0024$,

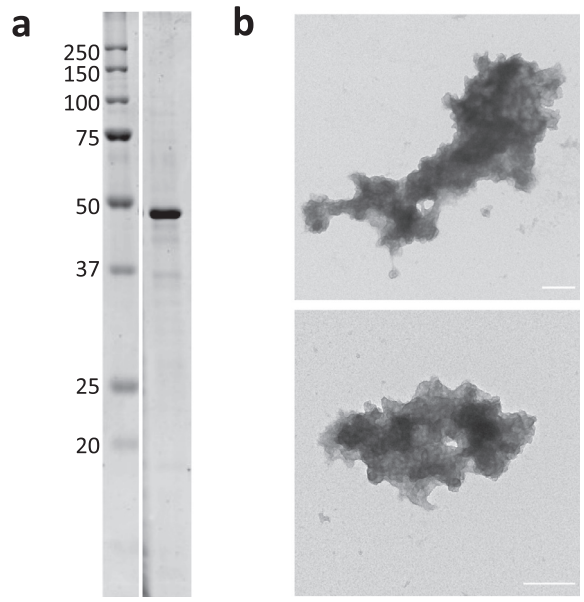


Fig. 1. Characterization of PspAα PBs. PspAα PBs were characterized by SDS-PAGE with Coomassie staining (**a**), and negative-stain transmission electron microscopy (NS-TEM) (**b**). **a**) Purified PBs were analysed by SDS-PAGE (PspAα fusion protein: 49 kDa). **b**) Two examples of NS-TEM pictures of purified PspAα PBs are shown. White scale bars indicate 200 nm.

respectively). Except for IgM, binding of mucosal or systemic antibodies to the bacterial surface revealed similar patterns.

As pneumococci downregulate their capsule during colonization to allow nutrient uptake and adhesion to the mucosal surface [35], we also measured antibody binding to unencapsulated pneumococci (Fig. S2). Results were overall highly similar compared to those obtained using the encapsulated strain. Statistical significance was only reached for the increased nasal IgG1 binding measured in samples from soluble PspAα immunized mice ($p = 0.0392$). Of note, no correlation was found between antibody binding to pneumococci and the nasal bacterial load measured three days post-infection (data not shown). In addition, no antibody binding was detected using nasal tissue or serum of CTB immunized mice, or when using *pspA* knockout strains (Figs. S1 and S2), suggesting that bacterial recognition by antibodies is highly antigen-specific. Taken together, immunization with soluble PspAα or PB PspAα induces differences in antibody binding to pneumococci by IgA, IgM and IgG1. To study whether these differences in antibody binding have distinct functional consequences that could potentially contribute to differences in protection, we assessed the capacity of serum-derived antibodies from immunized mice to induce complement C3 deposition on the bacterial surface in presence of an external complement source. No differences in C3 deposition were found when sera of PB PspAα or soluble PspAα immunized mice were used (Fig. S3a, b), indicating that the antibodies induced by vaccination with these formulations have similar complement-activating capacity.

3.4. PspAα PB vaccinated mice display a distinct mucosal cytokine profile following intranasal stimulation with S. pneumoniae

To examine whether differences in the local cytokine response correlate with protection, we conducted a separate study in which we vaccinated mice again with either soluble or PB PspAα, both adjuvanted with CTB. Since the *in vivo* immune response following infection may not only be influenced by the initial challenge dose but also by subsequent differences in bacterial replication, we intranasally administered heat-killed *S. pneumoniae* expressing *pspA* (wild-type; HKSpWT). To determine whether these responses were antigen-specific or more reflective of an early innate response to *S. pneumoniae*, we also stimulated mice with an isogenic *pspA* knock-out strain (HKSpΔ*pspA*). Following intranasal stimulation, nasal tissue was harvested after 4 h, homogenized, and used for multiplex measurement of IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12-p70, IL-13, IL-17A, KC and TNF-α. The 4 h time point was selected based on the outcome of an initial optimization experiment (Fig. S4).

Principal Component Analysis (PCA) showed that the cytokine profile of PB PspAα vaccinated mice stimulated with HKSpWT was highly distinct from all other groups (Fig. 4a, Fig. S5). This was visualised by a shift along the x-axis corresponding to principal component 1, which accounts for approximately 65% of the variation in the dataset. Mice vaccinated with soluble PspAα and stimulated with HKSpWT also showed a unique cytokine profile deviating from all other groups, based on the separate cluster formed by these mice (Fig. 4a). In contrast, no shifts were observed in the PCA following stimulation with HKSpΔ*pspA*, implying that the observed differences in cytokine responses are antigen-specific (Fig. 4a).

We found that nasal cytokine levels of mice vaccinated with PB PspAα and stimulated with HKSpWT were significantly lower than in the other groups, except for IL-17A and KC (Fig. 4b).

The antigen-mediated effect on the cytokine response (effect size) was analysed by comparing the cytokine response following stimulation with HKSpWT and HKSpΔ*pspA* within each vaccination group (Fig. 4c). PB vaccinated mice showed a significant antigen-mediated effect on the levels of all cytokines, except IL-17A and KC, whereas a significant effect for soluble PspAα vaccinated mice was only found for KC, IL-6 and IL-12-p70 (Fig. 4c). Additionally, significant differences between the effect sizes of PB PspAα and soluble PspAα vaccinated mice were observed for all cytokines, except IL-17A, KC and IL-4 (Fig. 4c, Fig. S6). Thus, the cytokine profile of PB vaccinated mice is impacted by the presence of the PspAα antigen during the stimulation and this effect size is, for the majority of the cytokines, statistically different from the effect size determined for soluble PspAα vaccinated mice.

3.5. Vaccination with PBs composed of conserved antigens confers protection against pneumococcal colonization

To evaluate whether the PB platform is suitable for proteins other than the highly variable PspA protein, we also examined PB formulations of four highly conserved, putative pneumococcal vaccine antigens: nucleoside binding protein PnrA, oligopeptide binding protein AliA, dimorphic cell wall protein DiiA, and carbohydrate binding protein SP1690 [36–41]. Purified PB formulations were analysed by SDS-PAGE (Fig. S7a, c, e, g). Subsequent analysis by NS-TEM demonstrated that the particles have a rough surface geometry, as was found for PspAα PBs, however to a slightly different extent depending on the protein (Fig. S7b, d, f, h).

The protective capacity of these PB variants was determined following intranasal vaccination of mice with 10 μg PBs composed of PnrA, DiiA, SP1690, AliA, or a cocktail of all four PBs (40 μg in total), all adjuvanted with CTB. Mice vaccinated with PB PnrA or the PB cocktail showed a strong and statistically significant reduction in pneumococcal colonization compared to the CTB control group (PnrA: 36-fold, $p = 0.0097$; cocktail: 49-fold, $p = 0.0043$) (Fig. 5. Although not statically significant, immunization with AliA PBs also resulted in a moderately decreased colonization level compared to the CTB-only group (15-fold, $p = 0.0940$). In contrast, mice immunized with DiiA or SP1690 PBs showed roughly similar colonization levels compared to CTB vaccinated mice. Taken together, these results demonstrate that PB-based formulations of various

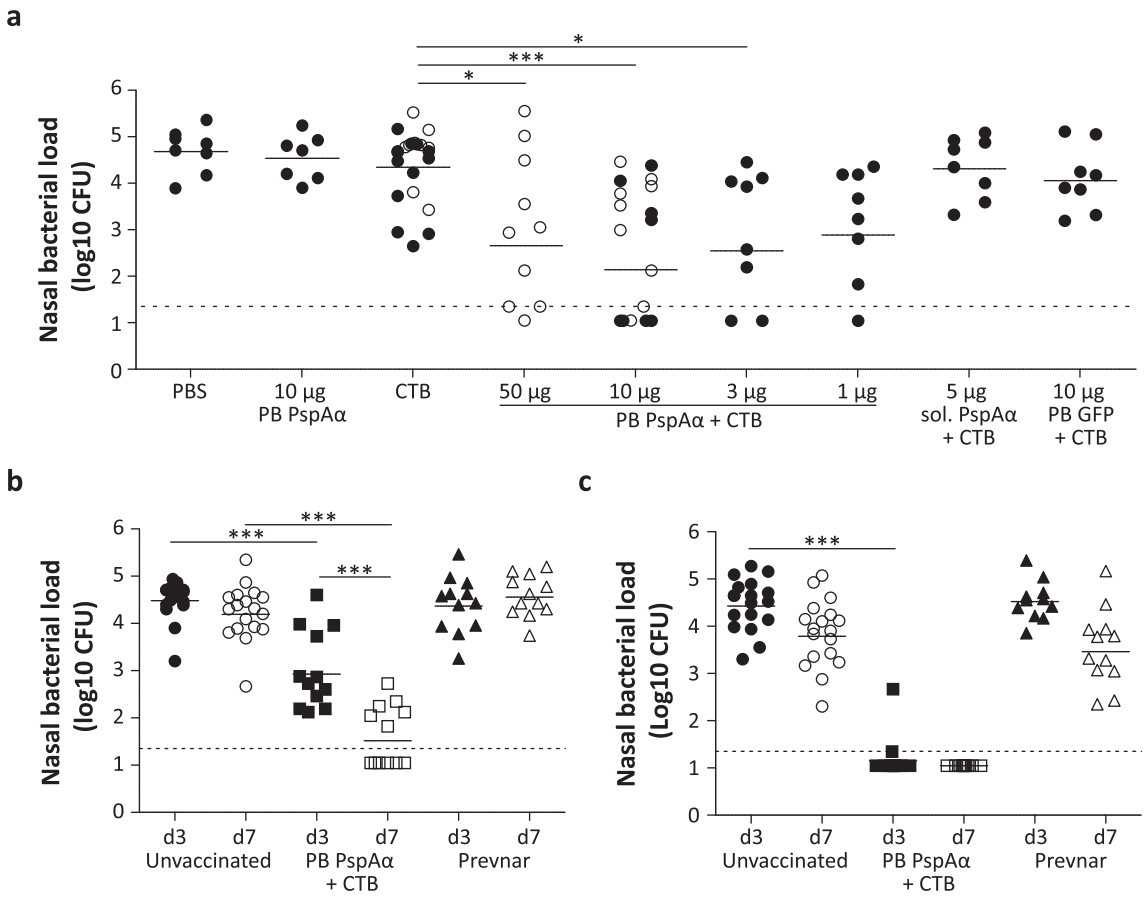


Fig. 2. Intranasally administered PspAα PBs confer protection against colonization by *S. pneumoniae*. **a**) Mice were intranasally vaccinated three times with PBS (n = 8), CTB (n = 22), various dosages of PspAα PBs + CTB (50 µg (n = 10), 10 µg (n = 18), 3 µg (n = 8), 1 µg (n = 8)), 10 µg PspAα PBs (n = 7), 5 µg soluble PspAα + CTB (n = 8) or 10 µg Green Fluorescent Protein (GPF) PBs + CTB (n = 8). Ten µg PspAα PBs is equivalent to 5 µg of soluble PspAα based on the number of PspAα molecules/dose. Mice were challenged with 1×10^6 CFU *S. pneumoniae* strain TIGR4 and the nasal bacterial load was assessed 3 days post-challenge. Data was obtained from two separate animal experiments, indicated by open and closed circles. Statistical analysis was performed using a one-way ANOVA with Dunnett Post-Hoc testing and 95% confidence intervals on log10 transformed data, using CTB vaccinated mice as control group. In 10 µg PspAα PBs group 1 mouse was excluded because of pneumococcal disease development. **b**, **c**) Mice were vaccinated three times, either intranasally with 10 µg PB PspAα + CTB (n = 11/12) or intramuscularly with 1/15th of the human Prevnar dose (n = 11/12), and compared to unvaccinated mice (n = 18). Mice were challenged with 1×10^6 CFU *S. pneumoniae* strain PBCN0231 (**b**) or PBCN0460 (**c**) and the nasal bacterial load was assessed 3- or 7-days post-challenge. Statistical analysis was performed using a two-sided T-test with 95% confidence intervals on log10 transformed data, except for PB PspAα + CTB at day 7 in (**c**) because no bacteria were detected. In 10 µg PspAα PBs + CTB group and Prevnar group both 1 mouse was excluded because of pneumococcal disease development. * p-value < 0.05, *** p-value < 0.001. Dashed lines indicate detection limit (22 CFU/animal), symbols represent individual mice with the geometric mean indicated by a horizontal line.

conserved pneumococcal proteins are able to reduce pneumococcal colonization following intranasal administration.

4. Discussion

Improvements in nanomedicine-based research have encouraged the development of highly effective particle-based vaccine formulations over the past decade [1,2]. However, the applicability of these particle-based vaccines is often low due to complicated designs, high manufacturing costs or difficulties with scaling up the production process [42]. Here, we explored the use of insoluble protein aggregates – which are easy to produce at low costs – as a platform for mucosal vaccination. We demonstrate that PB-based formulations provide superior protection against URT infection of mice with *S. pneumoniae* as compared to antigen administration in soluble form. This observation was associated with differences in antibody binding to pneumococci as well as a highly distinct mucosal cytokine profile in response to *in vivo* stimulation. Moreover, we show that this PB-strategy is applicable to a variety of pneumococcal antigens.

The improved efficacy observed following intranasal vaccination with PspAα PBs when compared to vaccination with the corresponding soluble antigen is in line with a study of Ahmad *et al.* [9]. In this study, subcutaneous immunization with aggregated unadjuvanted antigen Ag85 protected significantly better against infection with *M. tuberculosis* in mice than immunization with adjuvanted soluble Ag85 [9]. The lack of protection against colonization by unadjuvanted PspAα PBs in our study might be explained by differences in vaccination route, as the nasal mucosa is considered to be a more tolerant environment compared to parenteral sites [43].

While PspAα PBs provided better protection than soluble PspAα, this did not correlate with differences in mucosal or systemic antibody binding to pneumococci on a single mouse level and could not be linked to differences in complement C3 deposition on the bacterial surface. The relatively high binding of nasal tissue-derived IgA to pneumococci when compared to serum, which is also slightly elevated in PB PspAα immunized mice, suggests that mainly secretory IgA is produced, which has previously been shown to play an important role in mucosal protection [44]. Interestingly, we observed differences in IgG1 and IgG2c/IgG2a distribution between mice vaccinated with aggregated or soluble PspAα, which has also been described by others [9,16]. These differences could be the result of differences in total levels of

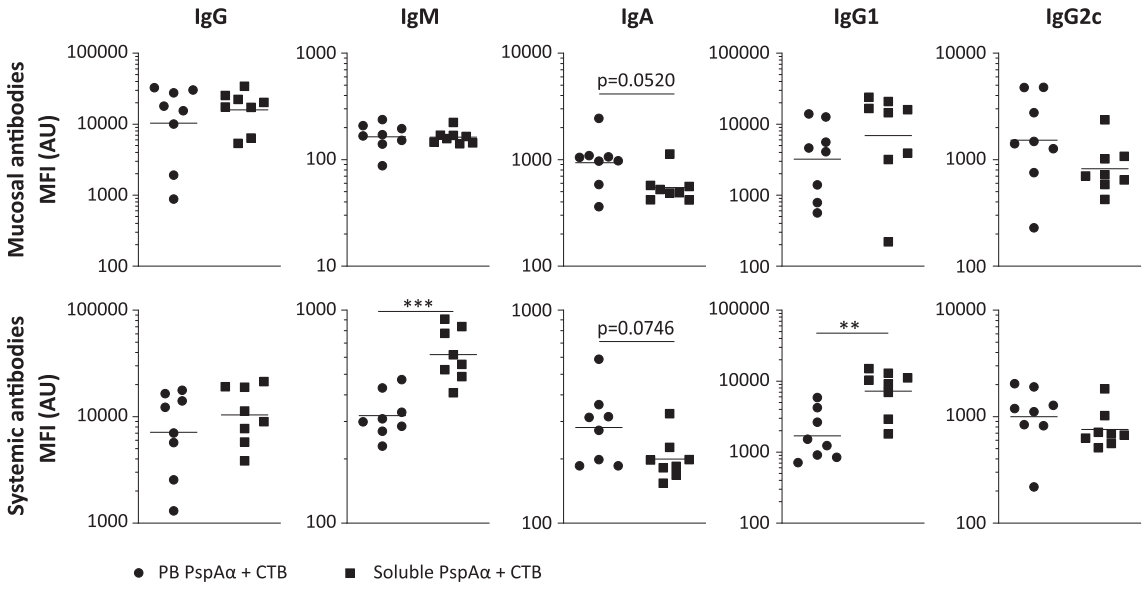


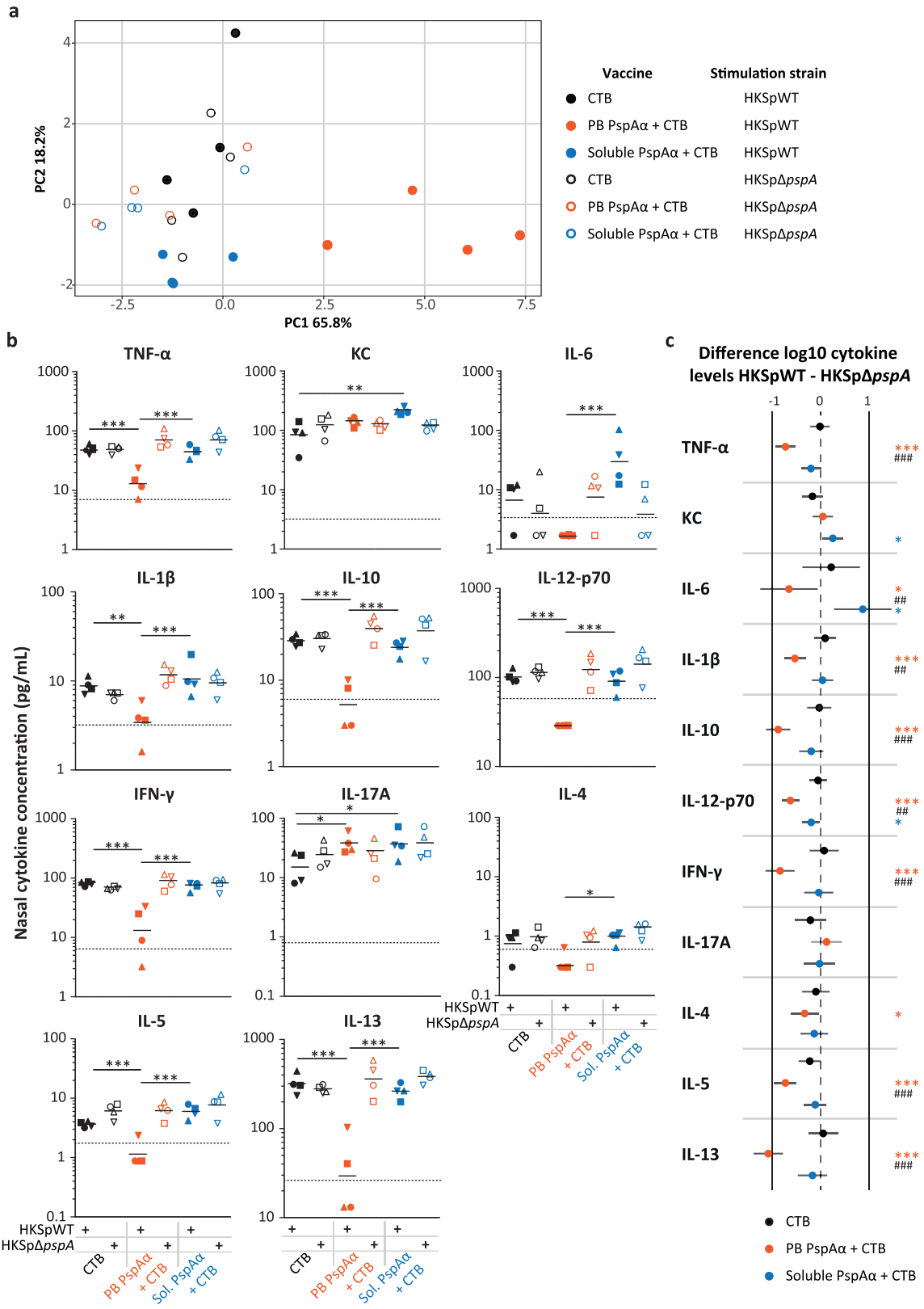
Fig. 3. Differential antibody binding to *S. pneumoniae* between soluble or PB PspAα immunized mice. Mice were intranasally vaccinated three times with 10 μg PB PspAα + CTB (n = 8; circles) or 5 μg soluble PspAα + CTB (n = 8; squares). Serum was collected two weeks after the third vaccination. Three weeks after the third vaccination, mice were challenged with 1×10^6 CFU *S. pneumoniae* strain TIGR4 and nasal tissue was collected 3 days post-challenge. Binding of antibodies (IgG, IgM, IgA, IgG1, IgG2c) present in serum (systemic) and nasal tissue (mucosal) to encapsulated TIGR4 was measured by flow cytometry. Nasal tissue was used undiluted, serum samples were diluted 1:1000 (IgG, IgG1, IgG2c) or 1:50 (IgM, IgA). Data is presented as mean fluorescent intensity (MFI) in arbitrary units (AU). Groups were compared by a two-sided T-test. ** $p < 0.01$; *** $p < 0.001$. Symbols represent individual mice with the geometric mean indicated by a horizontal line.

anti-PspA antibodies and/or differences in capacity to recognize the native antigen on the bacterial surface. Of note, as a large proportion of the proteins in the PB formulation has most likely not attained its native conformation, it is conceivable that PB induced antibodies primarily recognize linear epitopes within PspAα. However, it is also possible that certain PspAα protein domains are properly folded, and/or that proteins released from the PB structure attain their native conformation [5,8,11].

Besides minor differences in antibody binding to pneumococci following immunization with PB or soluble PspAα, we also observed a distinct mucosal cytokine profile after intranasal stimulation with non-replicating pneumococci. In PB vaccinated mice we observed reduced levels of most of the cytokines tested, including cytokines associated with both an innate and an adaptive immune response. Although cytokine levels in PB PspAα vaccinated mice were reduced in comparison to the soluble PspAα vaccinated animals, it remains to be determined how these levels relate to the levels before re-stimulation. Pre-challenge IL-6 levels have previously been shown to differ between unvaccinated mice and mice intranasally immunized with soluble PspA adjuvanted with whole cell pertussis vaccine, possibly as a consequence of increased infiltration of immune cells [45]. Importantly, the measured cytokine responses are a snapshot and the net result of the interplay between the innate and adaptive immune system, for which different vaccine formulations might display different kinetics. We speculate that a fast and strong adaptive immune response in PBvaccinated mice may have downregulated the pro-inflammatory response to intranasal challenge. Such a response may have facilitated improved clearance compared to soluble PspAα vaccinated mice. Importantly, the PB-dependent cytokine responses were antigen-specific since no differences were observed between soluble and PB PspAα immunized mice upon *in vivo* stimulation with the *pspA* knock-out strain.

Protection against colonization induced by protein antigen-based vaccine formulations – including PspA – has been associated with production of CD4 T-cell dependent IL-17 [29]. IL-17 has been shown to play an important role in clearance of pneumococcal colonization in mice [46]. It was therefore surprising that no differences in IL-17 levels were found between the vaccination groups, even though clear differences were detected with regards to levels of protection. Possibly, the timing of cytokine measurement might have played a role in this observation. The improved protection and the effective T cell memory induced when aggregating antigens, as shown previously [10], can possibly be explained by a variety of features. First of all, the higher local antigen density may have facilitated a higher antigen quantity per APC, which could have enabled a prolonged or higher level of antigen presentation [1,47,48]. In addition, their relative resistance to proteases, which are abundant at mucosal surfaces, may facilitate prolonged APC stimulation [49,50]. Thirdly, the uptake of PB particles by APCs and the transport across the epithelial barrier is probably more efficient than that of soluble proteins. This might be further aided by repetitive epitope display by the PB, consequently impacting the transport of the antigen to lymphoid organs [9]. Also, the activation of different intracellular signalling pathways following interaction with a soluble protein or a particulate antigen could have contributed to a more effective immune response [48,51]. Considering the important role of particle size and shape in the type and magnitude of an immune response [47,51], it is tempting to speculate that PB structure may also play a role in immunogenicity and protection.

An important finding of the present study is the suitability of the PB platform for multiple conserved pneumococcal proteins. The development of a broadly protective, protein-based, vaccine against *S. pneumoniae* represents a promising approach to eliminate the emergence of non-PCV serotypes in pneumococcal colonization and disease [21,22,24]. PBs consisting of PnrA were able to significantly reduce the pneumococcal load in the murine nose. Previously, PnrA was shown to be important for bacterial virulence, and immunization with this protein conferred protection against pneumococcal colonization and disease [36,52], underscoring the relevance of PnrA as a vaccine antigen. Moreover, combining PnrA PBs with PBs consisting of AliA, DiiA, and SP1690, which have previously been shown to protect against pneumococcal colonization



when administered as soluble antigen [37–41], resulted in the largest reduction in colonization compared to adjuvant-only vaccinated mice. This observation illustrates exciting opportunities for a future multi-protein, PB-based pneumococcal vaccine. By the development of vaccines that reduce colonization, as shown in this study, the chance that pneumococci migrate from the nasopharynx to distant sites will be limited and subsequent disease development can be prevented. Future studies will therefore investigate whether PB-based vaccines can also induce immunity and protection in the lungs and protect against invasive disease.

In conclusion, this study provides insight into the potency of PB-based vaccine formulations in enabling mucosal immunity

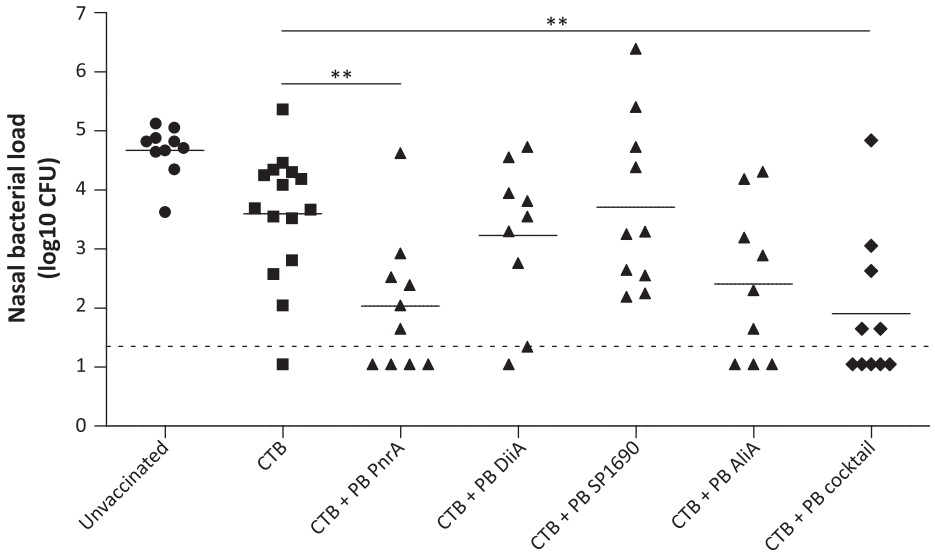


Fig. 5. PBs composed of conserved pneumococcal antigens can protect against colonization in mice. Mice were vaccinated intranasally three times with 10 μg of PBs of a single antigen (n = 9 or 10/group), or with a cocktail of the four PBs (10 μg of each PB variant, n = 10), all adjuvanted with CTB. CTB vaccinated mice (n = 15) and unvaccinated mice (n = 10) were included as controls. Mice were challenged with 1×10^6 CFU PBCN0231 and the nasal bacterial load was determined 3 days post-challenge. Statistical analysis was performed using a one-way ANOVA with Dunnett Post-Hoc testing and 95% confidence intervals on log10 transformed data, using CTB vaccinated mice as control group. In CTB + PB DiiA and CTB + PB AliA groups 1 mouse was excluded because of pneumococcal disease development. ** p-value < 0.01. Dashed lines indicate detection limit (22 CFU/animal), symbols represent individual mice with the geometric mean of each group indicated by a horizontal line.

and protection against *S. pneumoniae* in the URT of mice, upon intranasal administration. The PB vaccination approach seems very promising for broad application as we have shown that multiple different pneumococcal proteins can be formulated as PBs inducing strong immunogenicity. More importantly, this study has provided evidence that antigens formulated as PBs can induce superior protection as compared to soluble antigen. Therefore, PBs represent a low-cost, safe, easy to produce, and versatile platform for the development of vaccines.

Author contributions

L.F.B., J.D.L., D.A.D., M.I.J., J.L. designed the experiments; L.F.B., J.D.L., H.B.B.S., F.J.O., R.M. performed the experiments; L.F.B. and J.G. performed the data analysis; W.S.P.J., L.N., D.A.D., M.I.J., I.J., J.L. were involved in supervision. All authors have approved the final article.

Data availability

The data that support the findings of this study are available from the corresponding author on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: L.F.B., J.D.L., H.B.B.S., W.S.P.J., J.L. and M.I.J. are co-inventors on a patent application describing the presented work. H.B.B.S., W.S.P.J. and J.L. are associated to the company Abera Bioscience AB that aims to develop pneumococcal vaccines. The remaining authors declare no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.10.006.

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Fig. 4. Highly distinct nasal cytokine profile in mice vaccinated with PB or soluble PspAα following *in vivo* stimulation with *S. pneumoniae*. Mice were intranasally vaccinated three times with CTB (n = 8, black), 10 μg PspAα PBs + CTB (n = 8, orange) or 5 μg soluble PspAα + CTB (n = 8, blue), with a two-week interval. Three weeks after the last vaccination mice were intranasally stimulated (1×10^6 CFU) with either heat-killed TIGR4 wild type (HKSpWT; n = 4 per group; closed symbols), or heat-killed TIGR4Δ*pspA* (HKSpΔ*pspA*; n = 4 per group; open symbols). Nasal tissue was harvested 4 h post-stimulation and cytokines (IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12-p70, IL-13, IL-17A, KC and TNF-α) were measured in homogenized nasal tissue using a multiplex bead-based assay. a) Principal component analysis of all cytokines measured using log10 transformed values. PC1, Principal component 1; PC2, principal component 2. b) Absolute nasal cytokine levels. Dashed lines indicate detection limit (IL-1β 3.2 pg/mL; IFN-γ 6.4 pg/mL; IL-6 3.4 pg/mL; IL-4 0.6 pg/mL; IL-5 1.8 pg/mL; IL-10 6.0 pg/mL; TNF-α 7.0 pg/mL; IL-13 26.2 pg/mL; KC 3.2 pg/mL; IL-17A 0.8 pg/mL; IL-12-p70 58.0 pg/mL), symbols represent individual mice with the geometric mean indicated by a horizontal line. Statistical analysis was performed using a two-way ANOVA with Bonferroni correction on log10 transformed cytokine levels. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001. c) Effect size analysis of the antigen-mediated cytokine response was performed by calculating the differences in estimated bootstrap means between the HKSpWT and HKSpΔ*pspA* condition per vaccine formulation, determined following linear regression analysis on log10 transformed data. Dots represent difference in estimated means, estimated 95% confidence levels are indicated by horizontal lines. * p-value < 0.05; *** p-value < 0.001. Differences of the HKSpWT - HKSpΔ*pspA* comparison between PB PspAα + CTB and soluble PspAα + CTB vaccinated mice (differences of differences) were extracted from the estimate of the interaction term (Fig. S6). ## p-value < 0.01; ### p-value < 0.001.

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