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Research Paper

The Pneumococcal Alpha-Glycerophosphate Oxidase Enhances Nasopharyngeal Colonization through Binding to Host Glycoconjugates

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ABSTRACT

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen, causing a broad spectrum of diseases including otitis media, pneumonia, bacteraemia and meningitis. Here we examined the role of a potential pneumococcal meningitis vaccine antigen, alpha-glycerophosphate oxidase (*Sp*GlpO), in nasopharyngeal colonization. We found that serotype 4 and serotype 6A strains deficient in *Sp*GlpO have significantly reduced capacity to colonize the nasopharynx of mice, and were significantly defective in adherence to human nasopharyngeal carcinoma cells *in vitro*. We also demonstrate that intranasal immunization with recombinant *Sp*GlpO significantly protects mice against subsequent nasal colonization by wild type serotype 4 and serotype 6A strains. Furthermore, we show that *Sp*GlpO binds strongly to lacto/neolacto/ganglio host glycan structures containing the GlcNAcβ1-3Galβ disaccharide, suggesting that *Sp*GlpO enhances colonization of the nasopharynx through its binding to host glycoconjugates. We propose that *Sp*GlpO is a promising vaccine candidate against pneumococcal carriage, and warrants inclusion in a multi-component protein vaccine formulation that can provide robust, serotype-independent protection against all forms of pneumococcal disease.

1. Introduction

Streptococcus pneumoniae (the pneumococcus) continues to be responsible for major morbidity and mortality worldwide, causing a broad spectrum of diseases including otitis media (OM), pneumonia, bacteraemia and meningitis (WHO, 2014a, 2015). The prevalence of antibiotic-resistant pneumococci is increasing rapidly (WHO, 2014b), and available capsular-based vaccines are expensive and have major shortcomings with respect to immunogenicity, serotype replacement and/or strain coverage (Olarte et al., 2015). This has stimulated global efforts towards the development of cheaper, non-serotype-dependent vaccines based on conserved pneumococcal virulence proteins. However, protein antigens currently under consideration are being selected solely on the basis of their capacity to elicit protection in models of pneumonia and bacteremia, but their ability to protect against colonization of the nasopharynx, OM and meningitis is unproven (Ogunniyi and Paton, 2015).

Asymptomatic colonization of the nasopharynx almost invariably precedes disease; however, pneumococci vary in their capacity to colonize the nasopharynx, both in humans and in animal models. Certain serotypes and/or clonal groups are more often isolated from carriers, while others are more often isolated from sterile sites (Brueggemann et al., 2003; Sandgren et al., 2004; Sjostrom et al., 2006). In the context of rational pneumococcal protein vaccine design, an ideal vaccine formulation will reduce colonization as well as prevent invasive pneumococcal disease (Paton and Ogunniyi, 2011). A number of pneumococcal proteins have been shown to protect against colonization in addition to their protective capacities against invasive disease in animal models. Foremost among these are maltose/maltodextrin ABC transporter binding protein (MalX) (Moffitt et al., 2011), Neuraminidase A (NanA) (Tong et al., 2005), and pneumococcal surface proteins A and C (PspA and PspC) (Balachandran et al., 2002; Wu et al., 1997). Nevertheless, novel candidate proteins continue to be identified and appraised for inclusion in multi-component pneumococcal protein vaccines that are currently under development.

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In a series of investigations, we used *in vivo* gene expression and microarray analyses to identify the factors that trigger progression from colonization to invasive or meningeal infection in a murine model. One of these genes, *glpO*, encoding the pneumococcal alpha-glycerophosphate oxidase (herein designated *Sp*GlpO), was found to be upregulated in the brain *versus* blood of mice, and contributes significantly to the development of meningitis. Preliminary analysis also revealed that a pneumococcal *glpO* deletion mutant was significantly attenuated for nasopharyngeal colonization relative to the isogenic wild type (Mahdi et al., 2012). Given this important finding, we carried out a detailed analysis of the role of *Sp*GlpO in colonization, assessed its vaccine potential against pneumococcal carriage in a murine model, and examined its role in the host-pathogen interaction.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The pneumococcal strains used in this study were serotype 6A (WCH16), serotype 4 (WCH43), and their isogenic Δ*glpO* mutant derivatives (Table S1). Serotype-specific capsule production was confirmed by Quellung reaction, as described previously (Berry and Paton, 2000). Bacteria were grown statically at 37 °C in serum broth (SB; 10% heat-inactivated horse serum in nutrient broth) to $A_{600\ nm}$ of 0.16 (equivalent to approx. 5×10^7 CFU/ml).

2.2. Mice

Outbred 5- to 6-week-old female CD1 (Swiss) mice, obtained from the Laboratory Animal Services specific pathogen-free breeding facility of The University of Adelaide, were used in all experiments and were housed in the same facility for the entire duration of the experiments. Animals were provided with food and water *ad libitum*. The Animal Ethics Committee of The University of Adelaide approved all animal experiments (approval numbers S-2010-001 and S-2013-053). The study was conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition 2004 and 8th Edition 2013) and the South Australian Animal Welfare Act 1985. Animal experimentation were conducted in accordance with ARRIVE guidelines for animal research.

2.3. Mixed Infection Experiments

For *in vivo* competition experiments, mutant and wild type bacteria were mixed at an input ratio of 1:1, and groups of mice were challenged intranasally (i.n.) under anesthesia with 50 μl mixed bacterial suspension containing approx. 2.5×10^6 CFU each strain in SB. At 48 and 72 h post-challenge, 14–16 mice from each mixed inoculum experiment were sacrificed, and the bacterial load for each strain in the nasopharynx was quantitated as described previously (Ogunniyi et al., 2012). A 40 μl aliquot of each sample was serially diluted in SB and plated on blood agar and blood agar with a selective antibiotic marker (spectinomycin resistance) carried by the Δ*glpO* deletion mutant to determine the ratio of mutant to wild type bacteria. Competitive indices were calculated as the ratio of mutant to wild type bacteria recovered, adjusted by the input ratio. Differences in colonization between wild type and mutant were analyzed by one-sample *t*-test (two-tailed). A *P* value of <0.05 was considered statistically significant.

2.4. Adherence Assays

Adherence of pneumococci to human nasopharyngeal carcinoma epithelial (Detroit 562) cells (ATCC® CCL 138™) was assayed essentially as described previously (Mahdi et al., 2012). Cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM, and Ham's F-12 medium (Gibco; Cat No: 11320–033), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 100 U per ml penicillin-streptomycin. Strains were grown in C + Y medium (Lacks and Hotchkiss, 1960) supplemented with 1% glycerol (instead of glucose) for maximal *Sp*GlpO expression and diluted to approximately 2×10^6 CFU/ml in serum-free medium (without antibiotics). 1 ml aliquots of bacteria were inoculated in triplicate onto washed confluent monolayers (approx. 2×10^5 cells) in 24-well tissue culture trays and incubated at 37 °C in 5% CO_2 for 2 h. In addition, aliquots of wild type and mutant bacteria were treated before inoculation with 2.5 μl of high titer (1:50,000) polyclonal mouse anti-*Sp*GlpO serum. Following adherence, cell monolayers were washed 3 times with PBS and released and resuspended using 100 μl of 0.25% Trypsin-EDTA (Gibco; Cat No: 25200056) for 2 min after which 400 μl of 0.025% Triton X 100 (Sigma; Cat No: T8787) was added for 5 min to lyse Detroit 562 cells and release bacteria. Samples were resuspended by repeated pipetting and adherent bacteria were quantiated by serial dilution and plating on BA overnight at 37 °C in 5% CO_2. Assays were performed in triplicate in two independent experiments. Differences between means were analyzed using the unpaired Student's *t*-test (two-tailed).

2.5. Glycan Array

Glycan arrays were produced from a library consisting of 367 diverse glycans with and without one of three spacers (sp2, sp3 or sp4) (Blixt et al., 2004) made up of two previously described glycan libraries (Arndt et al., 2011; Huflejt et al., 2009). Glycans were either amine functionalized with spacers sp2, sp3 or sp4 as previously described (Blixt et al., 2004) or without spacers as previously published (Day et al., 2009). Glycan arrays were printed as previously published (Waespy et al., 2015). Glycan array experiments were performed and analyzed as previously described (Shewell et al., 2014) using 2 μg of purified *Sp*GlpO per array.

2.6. Surface Plasmon Resonance (SPR) Analyses

SPR analyses were performed using a Biacore T100 System (GE Healthcare Life Sciences) at 25 °C at a flow rate of 30 μl per min. Purified *Sp*GlpO was diluted to 50 μg per ml in PBS and loaded on the appropriate flow cells of a Ni^{2+} NTA sensor chip with 5 min contact time. Flow cell 1 in each run contained an unrelated His-tagged protein, which was tested to ensure it did not bind glycans, and was used as a reference. Glycans analyzed for interactions with *Sp*GlpO were serially diluted from 200 μM to 0.32 μM in PBS and analyzed as previously described (Shewell et al., 2014).

2.7. Inhibition of Adherence by Sugars

Inhibition of adherence of wild type pneumococci and their isogenic Δ*glpO* mutant derivatives to Detroit 562 cells by asialo-GM1 (aGM1; Cat No: GLY102, Elicityl OligoTech) trisaccharide, lacto-*N*-neotetraose tetrasaccharide (LNnT; Cat No: GLY021, Elicityl OligoTech) and lacto-*N*-tetraose tetrasaccharide (LNT; Cat No: GLY010, Elicityl OligoTech) was carried out as follows. Each bacterial strain (2×10^5 CFU) was pre-incubated with 560 μM of each sugar (in duplicate) for 2 h at 37 °C in 5% CO_2 before addition to duplicate Detroit 562 cell monolayers in 24 well plates containing 2×10^4 cells in antibiotic-free 1:1 DMEM and Ham's F-12 medium + 10% FCS). Trays were incubated for a further 2 h at 37 °C in 5% CO_2 after which monolayers were washed 3 times in PBS and treated with 25 μl of 0.25% Trypsin for 2 min and then 100 μl of 0.025% Triton X-100 for 5 min. Samples were then plated on BA and incubated overnight at 37 °C in 5% CO_2 for bacterial enumeration. Assays were performed in duplicate in two independent experiments. Differences between means were analyzed using the unpaired Student's *t*-test (two-tailed).

2.8. Cloning, Expression and Purification of Recombinant His_6-tagged Proteins

A series of N- and C-terminal His_6-tagged truncated fragments of *Sp*GlpO were constructed by PCR-amplification from WCH43 DNA and cloning into pQE31. The position of each fragment was guided by the Coils prediction algorithm (Lupas et al., 1991). In addition, the NanA open reading frame from *S. pneumoniae* D39 (excluding the LPXTG anchoring motif at the C-terminus) was amplified using primers *nanA* F (5′-GATTGTAGGATCCGTGGTATTTGGAACG-3′) and *nanA* R (5′-GTGCCTGCAGAGCAAGAGGAGCTTTG-3′), incorporating *Bam*HI and *Pst*I restriction sites (underlined), respectively. The PCR product was digested with the restriction enzymes and cloned into the corresponding restriction sites in pQE30. Each recombinant plasmid construct was then used to transform *E. coli* BL21 (DE3) *lpxM*$^-$ strain (Cognet et al., 2003). High-level expression of His_6- tagged NanA, *Sp*GlpO and *Sp*GlpO adjunct proteins was achieved by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; ThermoFisher Scientific, Cat No: 15529019) for 3 h at 37 °C. The cloning and expression of His_6-tagged MalX as well as PspA and PspC protein fragments has been described previously (Ogunniyi et al., 2012; Ogunniyi et al., 2001; Yother and Briles, 1992). Each recombinant protein or fragment was then purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Cat No: 30210), as described previously (Ogunniyi et al., 2007). Each purified protein was judged to be >98% pure by SDS-PAGE and Coomassie blue staining (not shown).

2.9. Immunization Studies

For i.n. immunization-challenge, female CD1 mice (12–14 per group) were immunized with purified *Sp*GlpO, *Sp*GlpO adjunct, MalX, NanA, PspA fragment or PspC fragment, formulated with *E. coli* heat-labile toxin (LTB) adjuvant. Each mouse received three doses of 10 μg of each antigen with 2 μg of LTB at 10-day intervals (LTB was omitted for the last immunization). Non-immunized mice received an identical course of saline plus LTB. One week after the last immunization, approximately 75 μl saliva was collected from each mouse after intraperitoneal injection of 200 μg of pilocarpine-HCl (Sigma; Cat No: P6503), and approximately 120 μl serum was obtained from individual mice by sub-mandibular bleeding 3 days later. Two weeks after the third immunization, mice were challenged i.n. with approx. 5×10^6 CFU (in 20 μl) of either wild type WCH16 or WCH43 by microtip instillation into both nares without anesthesia. At 72 h post-challenge, mice were euthanized and bacteria from the nasopharynx (nasal wash and nasal tissue) were enumerated as described previously (McAllister et al., 2011). Differences in nasopharyngeal colonization between groups were analyzed using the unpaired *t*-test (two-tailed). A *P* value of <0.05 was considered statistically significant.

2.10. Immunogenicity ELISA

For this assay, 0.045 μM of each protein or protein fragment was used to coat Nunc Maxisorb trays (Thermo Scientific; Cat No: M9410-1CS). Antigen-specific ELISA IgG titers were determined for pooled sera from each group and expressed as the reciprocal of the dilution giving 50% of the highest absorbance reading above the background at $A_{405\ nm}$. Total salivary IgA levels were determined per 50 μl of pooled saliva per group at $A405_{\ nm}$. Differences in salivary IgA levels between groups were analyzed by one-way ANOVA using Dunnett's multiple comparisons test. A *P* value of <0.05 was considered statistically significant.

The potential contribution of a T-cell-mediated immune response was also investigated by antigen restimulation of immune spleen cells. For this assay, splenocytes from 3 mice from LTB, *Sp*GlpO, *Sp*GlpO adjunct or MalX-immunized group were harvested and concentrations of IFN-γ, interleukin (IL)-6, IL-10 and IL-17A were measured in supernatants of antigen-stimulated splenocytes at 72 h by sandwich ELISA, using mouse ELISA Ready-SET-Go kits (eBioscience; Cat Nos 88-7314-88, 88-7064-88, 88-7104-88, 88-7371-88, respectively), essentially as described recently (Babb et al., 2016). Concanavalin A was used as a control, and concentrations of cytokines in supernatants were determined relative to the standard curve generated using cytokine standards provided by the manufacturer.

3. Results

3.1. A ΔglpO Mutant is Significantly Attenuated for Adherence

To examine the contribution of *Sp*GlpO to nasopharyngeal colonization, we carried out competition experiments in which groups of mice were challenged i.n. with approximately equal numbers of wild type *S. pneumoniae* strains WCH16 (serotype 6A) or WCH43 (serotype 4) and their respective Δ*glpO* mutant derivatives. This enabled evaluation of subtle differences between wild type and mutant by calculating the competitive index for each mutant in the nasopharynx at 48 and 72 h post-infection. We found that the Δ*glpO* mutant was significantly outcompeted by wild type in the nasopharynx at both time points in both strains, with the WCH16Δ*glpO* mutant substantially out-competed (>200 fold) by the wild type for adherence (Fig. 1).

In order to complement the *in vivo* observations, the ability of WCH16 and WCH43 and their otherwise isogenic Δ*glpO* mutant counterparts to adhere to human nasopharyngeal carcinoma epithelial (Detroit 562) cells was assessed. In this assay, the Δ*glpO* mutant of WCH16 had significantly reduced adherence to Detroit 562 cells relative to wild type ($P < 0.001$) (Fig. 2a); adherence was also attenuated when wild type bacteria were pre-incubated with anti-*Sp*GlpO serum ($P < 0.0001$). Similarly (albeit to a lesser extent), the Δ*glpO* mutant of WCH43 had significantly reduced adherence to Detroit 562 cells relative to wild type ($P < 0.01$) as did wild type WCH43 pre-incubated with polyclonal anti-*Sp*GlpO serum ($P < 0.05$) (Fig. 2b).

3.2. SpGlpO Interacts With Glycans

Given the clear indication for a role for *Sp*GlpO in nasopharyngeal colonization in the mouse carriage and *in vitro* adherence studies, we

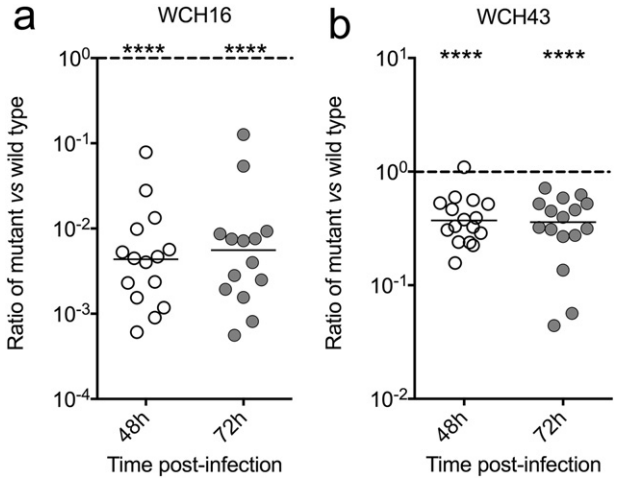

Fig. 1. Competition between wild type and their isogenic Δ*glpO* mutant derivatives for nasopharyngeal colonization. Results are expressed as the ratio of *S. pneumoniae* WCH16 (serotype 6A), WCH43 (serotype 4), and their isogenic Δ*glpO* mutant derivatives in the nasopharynx of mice at 48 h and 72 h post-infection. (a), WCH16 *vs* WCH16Δ*glpO* mutant; (b), WCH43 *vs* WCH43Δ*glpO* mutant. 14–16 female CD1 mice were infected i.n. with equal numbers (approx. 2.5×10^6 CFU each) of WT and mutant in each experiment. Each datum point represents the ratio of recovered mutant bacteria to wild type. The horizontal broken line represents a 1:1 ratio of recovered mutant bacteria. The horizontal solid line denotes the geometric mean value of the ratio of recovered mutant bacteria for each comparison (**** $P < 0.0001$; one sample *t*-test; two-tailed).

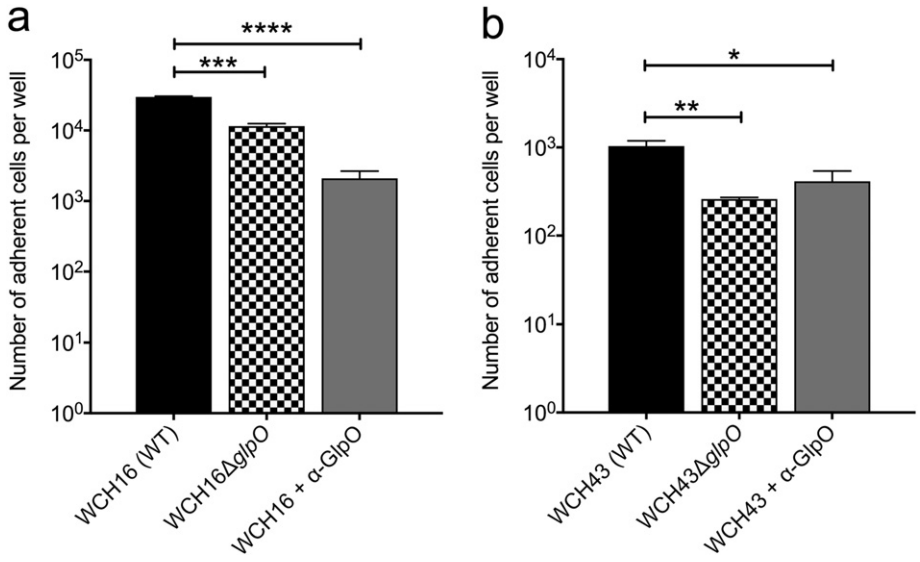

Fig. 2. Adherence to Detroit 562 nasopharyngeal carcinoma epithelial cells. Adherence of wild type WCH16 (a), WCH43 (b) and their isogenic Δ*glpO* mutant derivatives to Detroit 562 monolayers was assayed, with and without pre-incubation with anti-SpGlpO serum, as described in Materials and Methods. The number of adherent bacteria for each treatment was determined in triplicate. Data (mean ± SEM) were analyzed using Student's *t*-test (two-tailed). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

evaluated the molecular mechanism(s) by which it contributes to the host-pathogen interaction. The data obtained in the colonization studies raised the possibility that there is a receptor for *Sp*GlpO on host mucosal surfaces. Using glycan array analysis, we investigated the glycan binding specificities of purified *Sp*GlpO on an array comprising 367 distinct glycans. The *Sp*GlpO bound to 57 of the 367 structures on the array, mostly lactose/neolactose/ganglioside structures with a range of modifications including sulfation, sialylation and fucosylation (Table S2). The *Sp*GlpO also bound to mannobiose structures and *N*-acetylglucosamine and the glycosaminoglycan hyaluronic acid.

To validate the glycan array results and characterize the interaction of *Sp*GlpO with glycans, we performed surface plasmon resonance (SPR) analysis with the core structures recognized. The interactions with *Sp*GlpO and glycans were strongest with the lacto/neolacto/ganglio structures, particularly asialo-GM1 (aGM1) trisaccharide, lacto-*N*-neotetraose (LNnT) and lacto-*N*-tetraose (LNT) tetrasaccharides, with interactions in the low micromolar range, while interactions with mannose and N-Acetylglucosamine were weaker (>100 μM) affinity (Table 1; Fig. S1). Pre-incubation of wild type WCH16 with 560 μM aGM1 or LNnT significantly reduced adherence to Detroit 562 cells ($P < 0.05$ in both cases). However, when WCH16Δ*glpO* cells were pre-incubated with the sugars, the level of adherence was not significantly affected (Fig. 3a), demonstrating a specific role for *Sp*GlpO-glycan interactions in adherence to nasopharyngeal epithelial surfaces. Pre-incubation of wild type WCH43 with aGM1 also significantly inhibited adherence ($P < 0.01$), but this did not occur when WCH43Δ*glpO* cells were tested. There was, however, a small but significant increase in adherence of WCH43Δ*glpO* cells pre-incubated with LNnT (Fig. 3b).

Table 1
Affinity of *S. pneumoniae* GlpO for a range of glycans.

Name	Structure	K_D
asialoGM1 (aGM1)	Galβ1-3GalNAcβ1-4Galβ1-4Glc	15.1 μM ± 1.3
Lacto-*N*-neotetraose (LNnT)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	5.1 μM ± 0.45
Lacto-*N*-tetraose (LNT)	Galβ1-3GlcNAcα1-3Galβ1-4Glc	6.3 μM ± 0.78
α1-2 mannobiose	Manα1-2Man	279.6 μM ± 36
N-acetylglucosamine (GlcNAc)	GlcNAc	154 μM ± 27

3.3. GlpO Modeling

To date, attempts to crystallize *Sp*GlpO in both the full-length and truncated forms have been unsuccessful. However, based on the available structure of GlpO from a *Streptococcus* sp. that has a 62% amino acid identity to *Sp*GlpO (PDB ID 2RGO) (Colussi et al., 2008), a 3-dimensional model of *Sp*GlpO was obtained with 100% confidence using PHYRE2 (Kelley and Sternberg, 2009). The GlpO model incorporated all amino acid residues with the exception of a 54 residue insert (amino acids 362–415) that is associated with the absent flexible surface region of the *Streptococcus* sp. GlpO structure and thus was unable to be accurately modeled.

The GlpO model along with SwissDock (Grosdidier et al., 2011a, b) was used to predict the binding sites of the aGM1 trisaccharide, as well as LNnT and LNT tetrasaccharides, with the binding modes consisting of the top 5 most favorable energies assessed (Fig. 4). One region stood out as a potential binding site candidate where 10 of the top 15 sites among the three ligands were favored. This potential binding site encompasses mostly loop and linker regions in a deep cleft that appears to fit multiple sugar residues, with the reducing carbohydrate hydroxyl residue at position C1 protruding to the solvent to accommodate additional sugars that would be required to attach the oligosaccharide to the host cell *via* a protein or lipid.

3.4. Immunization of Mice With SpGlpO Protects Against Nasopharyngeal Colonization

Given the findings of the *in vivo* and *in vitro* assays with the Δ*glpO* mutant, we examined by i.n immunization-challenge experiments if *Sp*GlpO can protect against nasopharyngeal colonization, using recombinant MalX, NanA, PspA and PspC as comparators. These proteins have been shown previously to protect against colonization in various animal models (Balachandran et al., 2002; Moffitt et al., 2011; Tong et al., 2005; Wu et al., 1997).

In order to determine the immunodominant fragment of *Sp*GlpO, we initially characterized the B-cell regions of *Sp*GlpO by constructing a series of N- and C-terminal fragments of *Sp*GlpO and assessing their immunogenicity by ELISA, leading to the construction of the *Sp*GlpO adjunct (Fig. S2). ELISA analysis of saliva samples from mice immunized with *Sp*GlpO, *Sp*GlpO adjunct, NanA, PspA and PspC mice showed

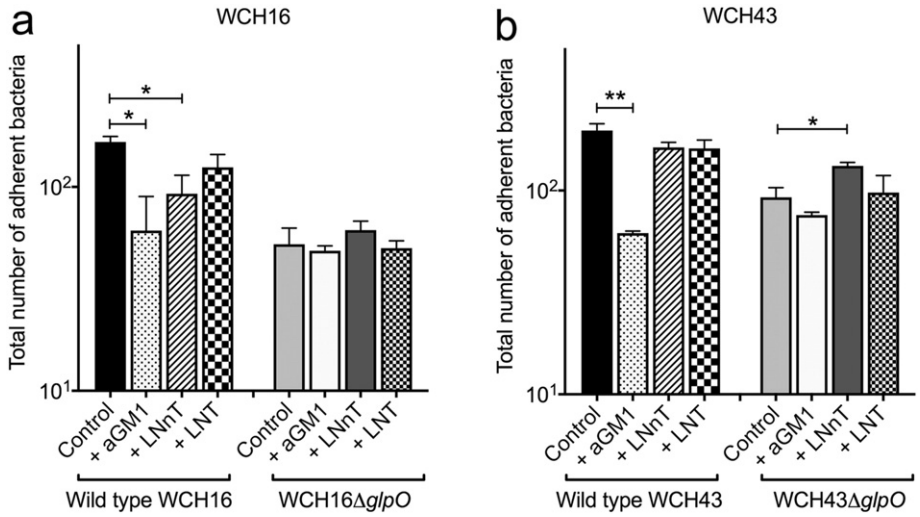

Fig. 3. Inhibition of adherence of pneumococci by asialo-GM1 (aGM1) trisaccharide, lacto-*N*-neotetraose (LNnT) and lacto-*N*-tetraose (LNT) tetrasaccharides. Wild type WCH16 (a), WCH43 (b) and their isogenic Δ*glpO* mutant derivatives were pre-incubated with 560 μM of each sugar (in duplicate) for 2 h at 37 °C in 5% CO_2 before addition to duplicate Detroit 562 cell monolayers. The number of adherent bacteria for each treatment was determined using 2 biological replicates for each treatment. Data (mean ± SEM) were analyzed using Student's *t*-test (two-tailed). NS, Not significant; $^*P < 0.05$; $^{**}P < 0.01$.

negligible IgA detection, with only MalX-immunized mice eliciting a strong IgA response (Fig. S3). However, there was a significant total IgG response from mice immunized with all the antigens (not shown). Furthermore, higher interferon-gamma (IFN-γ) and interleukin (IL)-17A (but not IL-6 or IL-10) responses were obtained in supernatants of *Sp*GlpO, *Sp*GlpO adjunct and MalX-stimulated splenocytes relative to *E. coli* heat-labile toxin B subunit (LTB)-stimulated splenocytes (data not shown). Importantly, mice immunized with *Sp*GlpO were significantly protected against i.n. challenge with either WCH16 or WCH43 compared to the placebo group, as judged by reduction in CFU counts of bacteria harvested from the nasopharynx. Protection against colonization was similar to (and in some cases better than) that achieved by immunization with MalX, NanA, PspA or PspC, antigens known to play a role in colonization (Fig. 5).

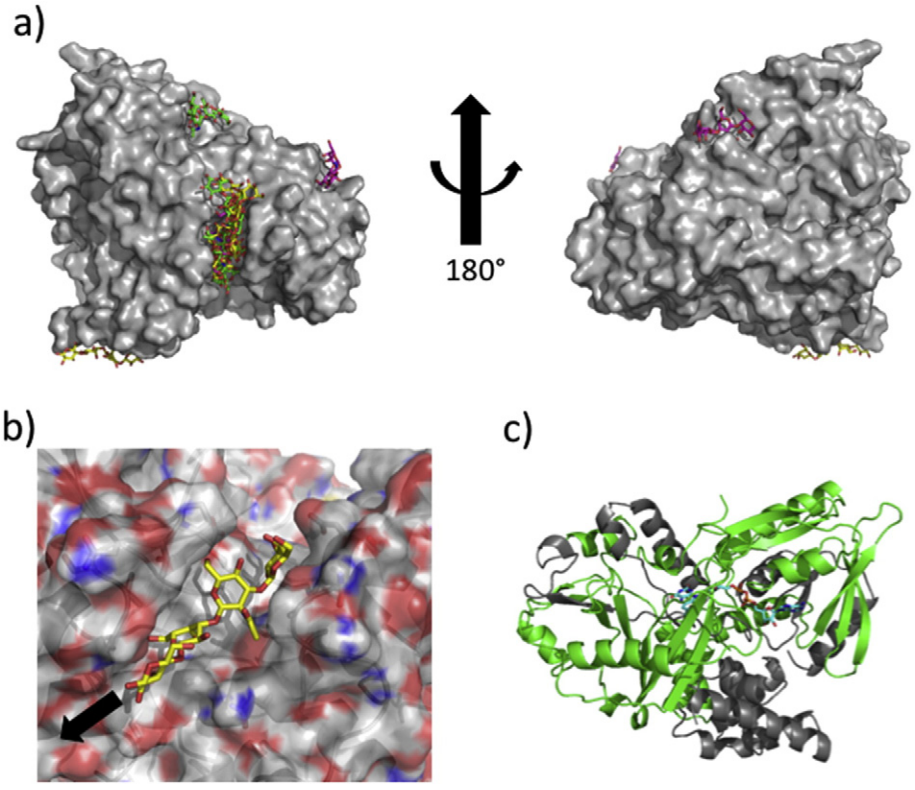

Fig. 4. (a) SwissDock experiments with the GlpO model and its ligands (LNT in yellow, LNnT in green, and asialo-GM1 in magenta). *Sp*GlpO is shown as a surface representation and the carbohydrate ligands as sticks. (b) A predicted binding mode for LNT with a surface representation of the GlpO model. The arrow indicates where additional sugar residues would be on a longer oligosaccharide from a host cell receptor. Red and blue sticks and surface representations correspond to oxygen and nitrogen, respectively. (c) Truncations for the *Sp*GlpO adjunct. Cartoon representation of *Sp*GlpO with the *Sp*GlpO adjunct shown in green and the truncations shown in grey. FAD was modeled into the active site and is shown as sticks. Oxygen, nitrogen, and phosphate are colored red, blue, and orange.

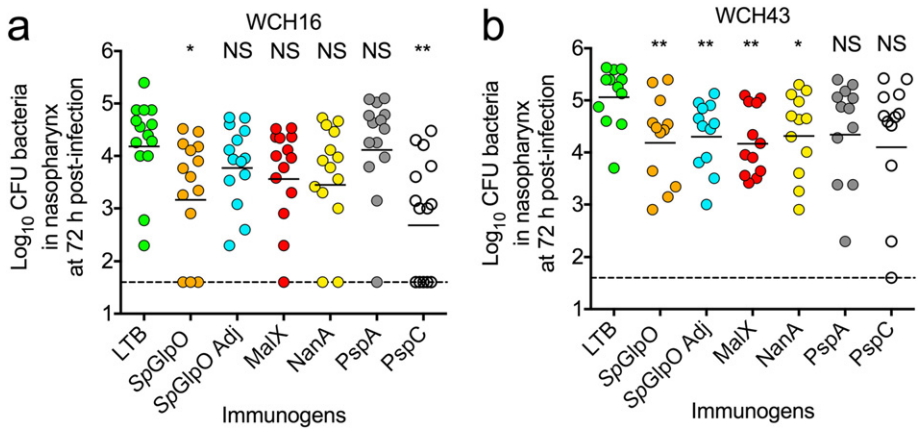

Fig. 5. Mucosal immunization and challenge. The abilities of wild type WCH16 (a) and WCH43 (b) to colonize the nasopharynx of female CDI mice (12–14 per group) after intranasal immunization with various recombinant pneumococcal proteins, as detailed in Materials and Methods. The *E. coli* heat-labile toxin (LTB) was used as the adjuvant. Mice were challenged with pneumococci two weeks after the third immunization and were sacrificed 72 h after challenge to enumerate colonizing bacteria. Differences in levels of nasopharyngeal colonization between control LTB immunization alone and vaccine groups were analyzed by unpaired *t*-test (two-tailed). NS, Not significant; $^{*}P < 0.05$; $^{**}P < 0.01$. The horizontal solid line denotes the geometric mean counts of recovered bacteria for each group. The horizontal broken line represents limit of detection of bacteria (40 CFU).

4. Discussion

In this work we carried out a detailed molecular analysis of *Sp*GlpO, examined its role in the host-pathogen interaction during nasopharygeal colonization, and also assessed its vaccine potential against colonization. Our results suggest that *Sp*GlpO, a conserved protein expressed by all pneumococcal strains, plays a significant role not only in meningitis, as we have previously described (Mahdi et al., 2012), but also in adherence and subsequent colonization of the nasopharynx through binding of specific host glycans. We also show that intranasal immunization with recombinant *Sp*GlpO significantly protects mice against subsequent nasal carriage at levels similar to that seen with other protein vaccine candidates, especially MalX (Moffitt et al., 2011). A recent study also demonstrated significant protection against pneumonia and sepsis using a combination of *Sp*GlpO and PncO (a bacteriocin ABC transporter transmembrane protein) (Li et al., 2016), indicating that *Sp*GlpO has promise as a pan-serotype vaccine candidate.

It has been established that pneumococcal adherence factors differentially bind to specific epithelial cell receptors on mammalian cells and tissues (Andersson et al., 1981; Svanborg Eden et al., 1984), with specific binding to glycoconjugate receptors containing the GlcNAcβ1-3Galβ disaccharide on nasopharyngeal cells (Andersson et al., 1983; Tong et al., 1999) and the GalNAcβ1-4Gal disaccharide on lung and vascular endothelial cells (Cundell and Tuomanen, 1994; Krivan et al., 1988). There has been evidence that *S. pneumonaie* binds to GalNAcβ1-4Gal through the phosphoryl choline residue of the *C*-polysaccharide, a major surface component of the pneumococcus (Sundberg-Kovamees et al., 1996). However, to our knowledge the pneumococcal binding partner for GlcNAcβ1-3Gal has yet to be identified.

We found that *Sp*GlpO interacts with a wide range of host glycans with the strongest binding to those that are commonly found as O-linked glycans of protein glycosylation and as lactoceramides/gangliosides. In addition, asialo-GM1 contains the core GalNAcβ1-4Gal suggesting that *Sp*GlpO is an alternative binding partner for these gangliosides, with this interaction proving important in mediating pneumococcal adherence to nasopharyngeal cells. Given these findings, we propose that *Sp*GlpO enhances pneumococcal colonization of the nasopharynx through its direct binding to host glycoconjugates. In this context, the use of oligosaccharides to block pneumococcal adherence to mucosal surfaces presents an attractive prospect for therapy and disease prevention, as demonstrated for LNnT using a rabbit model of pneumonia and bacteremia and an infant rat model of colonization (Idanpaan-Heikkila et al., 1997).

GlcNAcβ1-3Gal is a core structure of gangliosides, which are predominantly found on neuronal surfaces. Previous work has demonstrated a ganglioside-mediated colonization of the nasopharynx and brain by pneumococci (van Ginkel et al., 2003). We previously reported that an *Sp*GlpO mutant showed decreased adherence to human brain microvascular endothelial cells (Mahdi et al., 2012), although a detailed mechanism for this was not determined. This study suggests that the binding of *Sp*GlpO to different gangliosides, like asialo-GM1, could facilitate attachment to brain cells, providing an alternative mechanism for pneumococcal attachment to brain tissue. Interestingly, this would suggest an additional role for *Sp*GlpO, along with its hydrogen peroxide producing cytotoxic effects, in the pathogenesis of pneumococcal meningitis. Moreover, the binding of *Sp*GlpO to gangliosides opens up the likelihood that such interactions play a major role in the pathogenesis of otitis media, as was previously shown in chinchilla tracheal epithelia (Tong et al., 1999), and in gerbils where CNS infection occurred after pneumococcal otitis media (Muffat-Joly et al., 1994).

Immune-mediated clearance of pneumococci from the nasopharynx involves both antibody-dependent and antibody-independent mechanisms (Goldblatt et al., 2005; Malley et al., 2007; van Rossum et al., 2005). Antibody-independent clearance is thought to involve an IL-17A-mediated T-cell response, resulting in the recruitment of neutrophils to the site of infection and subsequent clearance of colonization (Lu et al., 2008; Malley et al., 2006). Consistent with these reports, intranasal immunization of mice with *Sp*GlpO or *Sp*GlpO-adjunct elicited significant total serum IgG, negligible salivary IgA, and more IFN-γ and IL-17A responses compared with LTB controls. The finding that immunization with *Sp*GlpO did not completely block colonization could be advantageous in the context of reducing colonization to levels that significantly impacts on overall pneumococcal translocation to deeper host tissues, while intra- and inter-species competition in the nasopharynx is maintained at asymptomatic levels. This is also likely to result in reduction in transmission from carriers to new hosts. Our previous work with WCH16 and WCH43 showed that stable colonization of the nasopharynx of CD1 (Swiss) mice was firmly established over a 48- to 96-h period, with optimal and uniform colonization at 72 h post-infection (Mahdi et al., 2012). This finding led us to choose 72 h after challenge as the ideal time-point for mouse sacrifice to determine the level of nasopharyngeal colonization.

In summary, we provide evidence for a direct contribution of *Sp*GlpO to colonization of the nasopharynx through its binding to host glycoconjugates, and show that intranasal immunization of mice with *Sp*GlpO elicited significant protection against subsequent nasal colonization. We conclude that *Sp*GlpO warrants consideration for inclusion

in an optimal, multi-component protein vaccine formulation that can provide robust, serotype-independent protection against nasopharyngeal colonization and all forms of invasive pneumococcal disease.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

Conceptualization, L.K.M., M.A.H., C.J.D, J.T., L.E.H., M.P.J., D.L.G., A.W·P, J.C.P. and A.D.O.; Methodology, L.K.M., M.A.H., C.J.D, J.T., L.E.H., and A.D.O.; Investigation, L.K.M., M.A.H., C.J.D, J.T., L.E.H., and A.D.O.; Writing – Original Draft, L.K.M., M.A.H., C.J.D, J.T., L.E.H., D.L.G., and A.D.O.; Writing – Review & Editing, L.K.M., M.A.H., C.J.D, J.T., L.E.H., M.P.J., D.L.G., A.W·P, J.C.P. and A.D.O.; Funding Acquisition, L.K.M., M.P.J., D.L.G., J.C.P. and A.D.O.; Resources, J.T., L.E.H., M.P.J., D.L.G., A.W·P, J.C.P. and A.D.O.

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Appendix A. Supplementary data

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