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RESEARCH PAPER



# Identification of SP1683 as a pneumococcal protein that is protective against nasopharyngeal colonization

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**ABSTRACT**

Serotype-independent protein-based pneumococcal vaccines represent attractive alternatives to capsular polysaccharide-based vaccines. The aim of this study was to identify novel immunogenic proteins from *Streptococcus pneumoniae* that may be used in protein-based pneumococcal vaccine. An immunoproteomics approach and a humanized severe combined immunodeficient mouse model were used to identify *S. pneumoniae* proteins that are immunogenic for the human immune system. Among the several proteins identified, SP1683 was selected, recombinantly produced, and infection and colonization murine models were used to evaluate the capacity of SP1683 to elicit protective responses, in comparison to known pneumococcal immunogenic proteins (PhtD and detoxified pneumolysin, dPly). Immunisation with SP1683 elicited a weaker antibody response than immunisation with PhtD and did not provide protection in the model of invasive disease. However, similar to PhtD, it was able to significantly reduce colonization in the mouse model of nasopharyngeal carriage. Treatment with anti-IL17A and anti-IL17F antibodies abolished the protection against colonization elicited by SP1683 or PhtD + dPly, which indicated that the protection afforded in this model was Th17-dependent.

In conclusion, intranasal immunization with the pneumococcal protein SP1683 conferred IL17-dependent protection against nasopharyngeal carriage in mice, but systemic immunization did not protect against invasive disease. These results do not support the use of SP1683 as an isolated pneumococcal vaccine antigen. Nevertheless, SP1683 could be used as a first line of defence in formulations combining several proteins.

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**KEYWORDS**
IL-17; immunoproteomics; mouse model; pneumococcal protein; pneumococcal vaccine

## Introduction

*Streptococcus pneumoniae* is an encapsulated gram-positive bacterium that belongs to the commensal flora of the human upper respiratory tract. In 2008 it was estimated to have caused ~ 0.4 million children deaths worldwide.[1] *S. pneumoniae* can cause invasive diseases such as meningitis and sepsis and respiratory infections such as pneumonia and otitis media. Especially young children, the elderly and people with an underlying disease are vulnerable for infections with *S. pneumoniae*. Vaccination is an effective means to control pneumococcal disease.[1]

A 23-valent capsular polysaccharide (caps-PS)-based vaccine was made available in 1983.[2] The serotypes contained within this vaccine account for 90% of the serotypes causing serious pneumococcal disease in industrialized countries.[3] The vaccine induces serotype-specific antibodies which provide host protection by induction of opsonophagocytosis.[4] However, given the 90 different pneumococcal serotypes, a comprehensive caps-PS-based vaccine does not appear feasible. In addition, although caps-PS based vaccines can elicit antibody response in most healthy adults, they are ineffective in children less than two years of age and in immuno-compromised individuals.[5] Moreover, unconjugated caps-PS-based vaccines, due to their T-independent nature and thus the lack of T-cell help, are not able to induce B-cell immunological memory.[6]

To overcome these problems, vaccine manufacturers have developed pneumococcal conjugate vaccines in which pneumococcal caps-PS are covalently coupled to a protein carrier in order to elicit a T cell-dependent immune response.[7,8] Such vaccines have shown high level of effectiveness against pneumococcal diseases in children up to 5 years of age.[6,9] Nevertheless, in some countries in which conjugated vaccines are used, an increased prevalence of serotypes that are not contained in the vaccine (replacement phenomenon) was observed.[10] These observations have driven the search for new approaches, such as protein-based vaccines.[11] Indeed, broad and serotype-independent protection can be expected if highly conserved proteins are used as vaccine components.[12] In addition, protein vaccines should be simpler to manufacture and, therefore, potentially cheaper than conjugate vaccines.

Although a lot of information is available on immunogenic pneumococcal proteins, only few of them have been

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tested in clinical trials.[13,14] In the present study, we used an immuno-proteomics approach to identify proteins in *S. pneumoniae* that are immunogenic for the human immune system, hence possible pneumococcal vaccine candidates. One protein (SP1683) was selected for evaluation of its protective capacity after immunization in a colonization and an invasive disease model.

## Results

### Identification of immunogenic pneumococcal proteins

In a first step, we identified pneumococcal proteins that are immunogenic for the human immune system. For that, peripheral blood mononuclear cells (PBMCs) from three different adult blood donors were isolated and transferred to severe combined immunodeficient (SCID) mice. For each blood donor, four SCID/SCID mice were reconstituted with PBMCs and immunized with heat-inactivated *S. pneumoniae* serotype 3 (thus in total 12 mice reconstituted with PBMCs from three donors). Serum was collected two weeks after immunization.

Reactivity to caps-PS3 was confirmed by measurement of serotype-specific IgM and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) (data not shown). The serum was also used in Western blotting analyses after two-dimensional separation of an extract of *S. pneumoniae* serotype 3. Protein spots for which there was reactivity for each of the three different blood donors were excised and identified by mass spectrometry. The most important immunogenic pneumococcal proteins that we identified are listed in Table 1 and can be classified as histidine triad proteins, choline binding proteins, adhesins, proteins involved in the degradation of the extracellular matrix, transporters, stress proteins, proteins involved in various physiological processes, and hypothetical proteins.

### Selection of an immunogenic pneumococcal protein

Out of the immunogenic pneumococcal proteins, a selection was made based on the following criteria: i) serotype-independent, ii) present on the bacterial surface, and iii) no homology with human proteins. Seven out of the 26 identified immunogenic proteins fulfilled these criteria: PsaA, ORF_0082, FBA, SP1683, SP1386, ManL, and SP0562. PsaA, ORF_0082 and FBA are adhesins helping the pneumococcus for the colonization of the upper airways. ManL, SP1386 and SP1683 are part of transporters important for the import of essential nutrients. SP0562 has been identified as a conserved hypothetical protein. PsaA has been evaluated in a clinical trial[15] and is part of a United States Patent Application (20090252756, Mizrachi-Nebenzahl, Yaffa, Beer Sheva, Israel). ORF_0082 has been reported not to be protective in animal study.[16] The polyamine transport protein PotD (SP1386) plays a role in pneumococcal pathogenesis and immunity.[17] Immunization with PotD (SP1386) protects CBA/N mice against systemic infection and nasopharyngeal colonization with *S. pneumoniae*.[18] The function of the hypothetical protein SP0562 is unknown. SP1683 and ManL are involved in sugar transport and have previously not been studied as vaccine candidates. We first focused on SP1683.

BLAST analysis revealed that SP1683 was conserved in a representative panel of 14 strains (serotypes 1, 2, 3, 4, 5, 6A, 6B, 10A, 11A, 14, 19A, 19F, 23F, and 35F) with more than 98% of identity. A signal peptide was predicted with a low score, but a

**Table 1.** Identification of pneumococcal proteins that are immunogenic in humans.

| Protein category | Proteins revealed by immuno-proteomics approach | Reference in mouse models | Reference in humans |
|---|---|---|---|
| Histidine triad proteins | Pneumococcal histidine protein A (PhpA, BHV-11) | P+[20,40] | CT [12] |
| Choline binding proteins | Pneumococcal surface protein A | P+[41] | D [42], I [43,44] |
| | Pneumococcal surface protein C | | CT,[12,45-47] I[48] |
| Adhesins | Pneumococcal surface adhesin A | P+[49] | D [51,52], I[53] |
| | ORF SP_0082 | NA | CT[12] |
| | Fructose bisphosphate aldolase | P+[50] | I [54], D [50] |
| Degradation ECM | Endo-$\beta$-N-acetylglucosaminidase | P+[55] | I [56] |
| | Zinc metalloprotease B | A[54] | NA |
| | Zinc metalloprotease C | NA | NA |
| | IgA1 protease | NA | I[57] |
| | Serine protease PrtA | P+[55] | I [55] |
| | $\alpha$-enolase | NA | I[58] |
| | Glyceraldehyde-3-phosphate dehydrogenase | P+[50] | D [50] |
| Transporters | ABC sugar transporter, sugar-binding protein (SP1683) | NA | NA |
| | ABC transporter (Maltose/maltodextrin) | NA | NA |
| | PTS system IIA component (mannose, ManL) | NA | NA |
| | ABC transporters for glutamine (*glnQ*) | NA | NA |
| | ABC transporters for spermidine/putrescine (*potD*) | P+[55] | NA |
| Stress proteins | DnaK | A[59] | D, I [56] |
| | GroEL | P−[60] | NA |
| | ClpP protease | A[61,62] | NA |
| Physiological processes | Pyruvate oxidase | NA | D[50] |
| | Phosphoglycerate kinase | NA | D[50] |
| | Pyruvate kinase | NA | NA |
| Hypothetical proteins | SP_1290 and SP_0562 | NA | NA |

A Antigenic in mouse models; P+ Protective in mouse models; P- Not protective in mouse models; D Detected in sera obtained from children attending day care centers and/or healthy adults; I Detected in sera obtained from infected individuals; CT proteins that are used in clinical trials; NA data not available.

PROSITE motif of "Prokaryotic membrane lipoprotein attachment site" was detected. Moreover, a "Bacterial extracellular solute-binding protein" domain ("SBP_bac_1", domain found in bacterial extracellular solute-binding protein family) was identified. Taken together, these observations indicate that SP1683 protein is probably found at the bacterial cell surface. No similarity with any human protein was found.

Hence, we selected SP1683 as a potential vaccine antigen candidate for it being serotype-independent, present on the bacterial surface and showing no homology with any human protein. A recombinant version of the protein was made. The identity of the recombinant protein was confirmed by matrix-assisted laser desorption ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry on two-dimensional gel electrophoresis (2D-GE) gel spots.[19] In addition, we could show by sodium dodecylsulphate polyacrylamide gel electrophoresis and immunoblotting that recombinant SP1683 was recognized by patients'sera and by serum from a SCID mouse transferred with human PBMCs after heat-inactivated *S. pneumoniae* serotype 3 vaccination. As the antibodies present in these human sera were elicited upon contact with the native SP1683, the results indicate that the recombinant SP1683 is probably immunologically similar to the native molecule.

### Lethal challenge

Next, we evaluated whether immunization with SP1683 is effective to protect against invasive disease in a murine invasive pneumococcal infection model. We used polyhistidine triad protein D (PhtD) as a control protein, as this protein has already been shown to be protective in animal models.[20-22] Mice were immunized with adjuvanted PhtD or SP1683. On day 27, anti-PhtD and anti-SP1683 geometric mean IgG antibody concentrations were 2215 $\mu$g/mL and 122 $\mu$g/mL, respectively. After challenge with the *S. pneumoniae* 4/CDC strain, mouse survival was recorded over the following 10 days (Figure 1). The results indicated that immunization with PhtD allowed the survival of 14/20 mice (70%), as seen after 10 days, while all animals in the control group had already died after five days. When immunized with SP1683, only 2/20 mice (10%) survived the challenge with 4/CDC strain. Challenge with 3/43 strain gave similar results (Figure 2). When immunized with PhtD, the survival rate after 10 days was 75% (15/20 mice). It was 5% (1/20 mice) for both SP1683 and control groups. Mean anti-PhtD and anti-SP1683 IgG concentrations in this second challenge experiment were 1391 $\mu$g/mL and 94.5 $\mu$g/mL, respectively.

### Nasopharyngeal colonisation

Next, we evaluated whether immunization with SP1683 was effective to protect against nasopharyngeal colonization. Again, PhtD was used as a control protein. Mice were immunized with PhtD or SP1683 through the intranasal route in the presence of *E. coli* heat-labile toxin (LT) and humoral immune responses were evaluated 13 days after the third immunization. In the PhtD group, all animals seroconverted with average anti-PhtD IgG antibody concentration being 352 [95%CI: 265-469] $\mu$g/mL. In the SP1683 group, all animals seroconverted with average anti-SP1683 IgG concentration being 59.1 [95%CI: 37-94] $\mu$g/mL. To assess the protective activity of PhtD and SP1683 immunization against nasopharyngeal carriage, mice were challenged intranasally with the 2D39 strain. As can be seen in Figure 3, immunizations with PhtD ($P < 0.001$ against control on Day 6) and with SP1683 ($P < 0.05$ against control on Day 6) both protected against 2D39 nasopharyngeal colonisation, but the protection afforded by PhtD was higher than that afforded by SP1683. In another experiment, anti-PhtD and anti-SP1683 antibody concentrations were 331 [95%CI: 165-661] $\mu$g/mL and 54.7 [95%CI: 36-83] $\mu$g/mL, respectively. After challenge with 6B/CDC

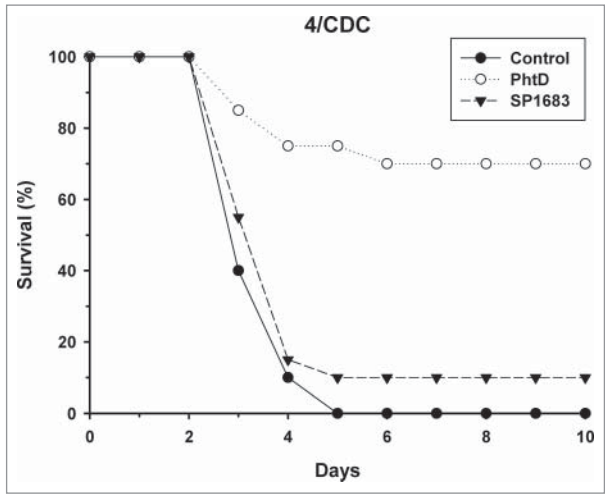


**Figure 1.** Mouse survival upon intranasal challenge with pneumococcal strain 4/CDC. Mice (n = 20/group) were immunized twice intramuscularly at a two-week interval with AS01 alone (control), 3 $\mu$g PhtD or SP1683 adjuvanted with AS01. Fourteen days after the second injection, mice were challenged intranasally with $5 \times 10^6$ cfu of *S. pneumoniae* type 4/CDC. The mortality was recorded during 10 days.

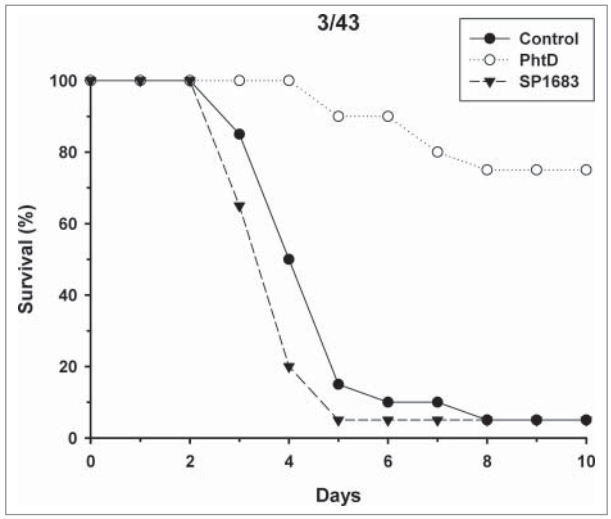


**Figure 2.** Mouse survival upon intranasal challenge with pneumococcal strain 3/43. Mice (n = 20/group) were immunized twice intramuscularly at a two-week interval with AS01 alone (control), 3 $\mu$g PhtD or SP1683 adjuvanted with AS01. Fourteen days after the second injection, mice were challenged intranasally with $1 \times 10^5$ cfu of *S. pneumoniae* type 3/43. The mortality was recorded during 10 days.

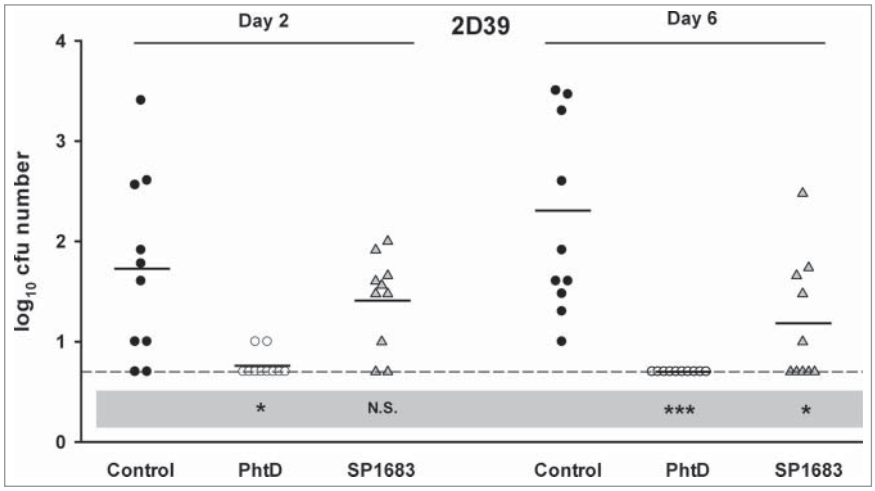


**Figure 3.** Vaccine efficacy in a *S. pneumoniae* nasopharyngeal colonization model. Mice were immunized intranasally with adjuvanted PhtD, SP1683 or adjuvant alone (control) before they were intranasally challenged with pneumococcal strain 2D39. Bacterial colonies in nasal washings were counted on Day 2 and Day 6 post-challenge and expressed as log10 mean cfu. Each dot represents a mouse. Dashed lines indicate the limit of detection; Black horizontal bars are means. Statistical analyses were carried out per day with ANOVA. All significant differences, compared with the control, are shown. *, $P < 0.05$; ***, $P < 0.001$; N.S., not significant.

strain, both groups performed in a similar way ($P < 0.01$ against control on Day 3) (Figure 4).

### *Protection from nasopharyngeal colonization by S. pneumoniae is IL17-dependent*

IL17 has been implicated in adaptive immunity to colonization by *S. pneumoniae*.[23,24] The role of IL17 in the protection elicited by the immunizations was analysed by treating mice previously immunized with PhtD + detoxified pneumolysin (dPly) or SP1683 with anti-IL17 antibody to inhibit the IL17 signalling pathway. Mice were immunized with PhtD/dPly or SP1683, both adjuvanted with LT, through the intranasal route. On Day 41, seroconversion was 100% in all groups. Anti-PhtD, anti-dPly and anti-SP1683 IgG concentrations were 131 [95%CI: 83-208] $\mu$g/mL, 530 [95%CI: 418-673] and 453 [95%CI: 340-604] $\mu$g/mL, respectively, for the groups that were not treated with anti-IL17 antibody, and 156 [95%CI: 97-250] $\mu$g/mL, 505 [95%CI: 396-644] and 442 [95%CI: 296-659] $\mu$g/mL, respectively, for the groups that were treated with anti-IL17 antibody. Results showed that anti-IL17 antibody treatment at the time of 2D/39 challenge was able to inhibit the immune response against the combined PhtD and dPly formulation and against SP1683, allowing colonization of the nasopharynx (Figure 5).

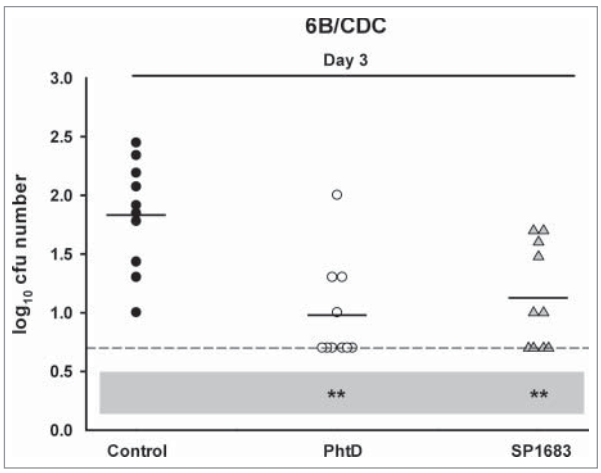


**Figure 4.** Vaccine efficacy in a *S. pneumoniae* nasopharyngeal colonization model. Mice were immunized intranasally with adjuvanted PhtD, SP1683 or adjuvant alone (control) before they were intranasally challenged with pneumococcal strain 6B/CDC. Bacterial colonies in nasal washings were counted on Day 3 post-challenge and expressed as log10 mean cfu. Each dot represents a mouse. Dashed lines indicate the limit of detection; Black horizontal bars are means. Statistical analyses were carried out with ANOVA. All significant differences, compared with the control, are shown. **, $P < 0.01$.

## Discussion

We have identified several immunogenic pneumococcal proteins by using a humanized SCID/SCID mouse model. Many of them had previously been identified as immunogenic in humans with other methods (see Table 1), which supports our immuno-proteomics approach. Other pneumococcal proteins identified in this study have previously been reported to be immunogenic or protective in different mouse models, or have been described to contribute to pneumococcal virulence in mice (see Table 1). However, we have also identified new immunogenic proteins, such as SP1683, PTS system IIA component (maltose/maltodextrin, ManL), pyruvate kinase, and hypothetical proteins (SP1290 and SP0562). Selected out of the newly identified immunogenic bacterial proteins, SP1683 fulfilled the following criteria: i) potentially serotype-independent, based on the analysis of 14 strains ii) likely present on the bacterial surface, based on genetic sequence data iii) no homology with human proteins, and iv) not previously evaluated as a vaccine candidate.

SP1683 is one of the components of the ABC transporter specific for sialic acid (referred to as satABC).[25] SP1683 has been predicted to be the sialic acid-binding component, while the two other proteins of satABC, SP1682 and SP1681 were predicted to be permease components.[25] Free sialic acid is not readily available in the host upper airways but is a common terminal modification on N- and O-linked

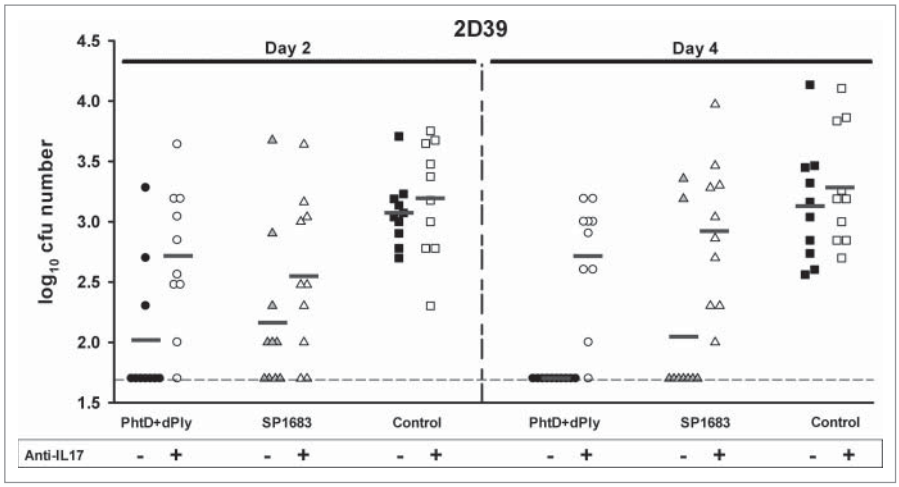


**Figure 5.** Vaccine efficacy in a *S. pneumoniae* nasopharyngeal colonization model. Mice were immunized intranasally with adjuvanted PhtD/dPly, SP1683 or adjuvant alone (control) on Days 0, 14 and 28 before they were intranasally challenged with pneumococcal strain 2D39 on Day 42. Mice were treated or not with anti-IL17A and anti-IL17F antibodies on Days 41, 43 and 45. Bacterial colonies in nasal washings were counted on Day 2 and 4 post-challenge and expressed as log10 mean cfu. Each dot represents a mouse. Dashed lines indicate the limit of detection. Dark grey horizontal bars are means. Statistical analyses were carried out with ANOVA2. Groups were compared to each other over time. Groups SP1683 and PhtD/dPly versus control group: $P < 0.001$. Group PhtD/dPly versus group Phtd/dPly + anti-IL17: $P < 0.001$. Group SP1683 versus SP1683 + anti-IL17, $P = 0.053$.

human glycoproteins that can be cleaved by the pneumococcal exoglycosidases (sialidases).[25] Sialic acid is an important source of carbohydrates for the bacterium so that it was shown that the import of sialic acid by satABC contributes to pneumococcal colonization.[25] This was further demonstrated when intranasal inoculation of mutant strains deficient in satABC led to reduced colonization compared with wild strain.[25] These data reinforced our choice of evaluating SP1683 as vaccine antigen.

We first evaluated the protective capacity of anti-SP1683 immune responses in mice by using both an invasive pneumococcal infection model and a colonization model. The histidine triad protein PhtD[20,22,26] was used as a positive control antigen. PhtD is a promising pneumococcal vaccine antigen candidate at the forefront of protein-based vaccine development, currently evaluated in humans in combination with dPly, another vaccine antigen candidate.[27,28] Our data confirmed previous data showing that immunization with PhtD (i) elicits a strong antibody response, (ii) controls nasopharyngeal colonisation, and (iii) protects against lethal challenge. In contrast, systemic immunization with SP1683 elicited a weak antibody response and did not protect against lethal challenge. Absence of protection may be due to the low level of elicited antibodies or to non-functional antibodies, which was not investigated further.

In another mouse model, using the intranasal route of administration, we showed that immunization with SP1683 was able to protect against nasopharyngeal colonization, although anti-SP1683 IgG levels were again lower than those induced by immunization with PhtD. This result was important, as bacterial colonization of the nasopharynx is a necessary step before *S. pneumoniae* infection takes place, hence protection against colonization would help to contain pneumococcal spread and limit or impede pneumococcal disease.[29] In our study, some animals were still colonized by the pathogen at the latest time points investigated; indicating that a longer time may have been needed for these animals to clear the infection. Or, although all mice seroconverted after immunization, it may be that some simply are not able to clear the colonization. However, this observation does not question the validity of the experiment, as sterilizing immunity is not a goal in such type of experiments. A significant reduction in bacterial load at a given time point is sufficient to conclude about the protective effect of the injected antigen. Indeed, the clinical relevance of an incomplete protection against colonization in a mouse model is unknown, considering the differences between a challenge in mice with a defined and certainly exaggerated amount of bacterium and the occurrence of a natural pneumococcal infection in human.

In mice, there is evidence that Th17 $CD4^+$ T cells play an essential role in the development of natural protective immunity against pneumococcal nasopharyngeal colonization.[30] More recently, Moffitt et al.[31] identified a number of Th17 antigens from killed whole cell vaccines that were protective against serotype 6B nasopharyngeal colonization as mucosal vaccines. Th17 $CD4^+$ cell-mediated protection against nasopharyngeal colonization may occur as a consequence of IL17A-induced recruitment of monocyte/macrophage to the site of colonization and potentiation of pneumococcal killing by innate cells.[24,32] In our model, depletion of IL17 abolished the protection against colonization that is otherwise induced by SP1683 or PhtD+dPly immunization, suggesting an important role of IL17 in protein-mediated immunity against pneumococcal nasopharyngeal colonization. Considering the importance of nasopharyngeal colonization as a precursor to non-invasive and invasive pneumococcal disease,[29] a Th17-mediated first line defence is considered crucial to impede or stop pneumococcal infections. Therefore, generating vaccine-induced, protective IL17A immune responses that prevent nasopharyngeal colonization is desirable (reviewed in ref[33]).

In summary, in our experimental conditions, SP1683 did not fulfil all requirements to be considered as a potential isolated pneumococcal vaccine antigen. Nevertheless, we showed

that it can elicit IL17-dependent protection against nasopharyngeal colonization, in the same way as the previously described PhtD, and as such could be used as a first line of defence in combined immunizations.

## Materials & methods

### Transferring PBMCs to SCID mice

Peripheral blood buffy coat from healthy blood donors was obtained from the Blood Transfusion Centre of the Red Cross Leuven. Human PBMCs were prepared by density gradient centrifugation on Lymphoprep (Axis-shield Poc AS) and analyzed by flow cytometry (BD Biosciences). One day before transferring human PBMCs, the SCID/SCID mice received TM$\beta$1 by intraperitoneal (i.p.) injection. TM$\beta$1 is a rat monoclonal antibody recognizing the mouse IL-2 receptor beta-chain, aiming to improve the survival and functionality of the transplant.[34] PBMCs were dissolved in phosphate-buffered saline and injected i.p. into the SCID/SCID mice ($7 \times 10^7$ cells/mouse). At the same time, the mice were immunized i.p. with $2 \times 10^8$ CFU heat-killed *S. pneumoniae* serotype 3. Fourteen days later, blood was drawn by heart puncture in isoflurane-anesthetized mice. Mice were euthanized by cervical dislocation after isoflurane inhalation.

### 2D-GE and Western blotting

A *S. pneumoniae* serotype 3 extract was prepared by sonication as described by Encheva et al.[35] Non-protein impurities from the *S. pneumoniae* serotype 3 extract were removed by using the 2-D clean-Up kit. Immobilized pH gradient (IPG) strips ranging from pH 4 to 7 were rehydrated overnight using 250 $\mu$g of the *S. pneumoniae* serotype 3 protein extract. After one dimensional isoelectric focusing on a Multiphor II Electrophoresis System according to the manufacturer's instructions, the IPG strips were equilibrated in solution I [0.05 M Tris-HCl, pH 6.8 containing 6 M urea, 35 mM SDS, 30% (v/v) glycerol and 0.25% (w/v) dithiotreitol] and solution II [0.05 M Tris-HCl, pH 6.8 containing 6 M urea, 35 mM SDS, 30% (v/v) glycerol, 0.45% (v/v) iodoacetamide and bromophenol blue] for 15 min each. Equilibrated strips were loaded on a 12.5% SDS polyacrylamide gel for separation in the second dimension, according to the manufacturer's instructions. After run, the proteins were either transferred on a polyvinylidene difluoride membrane by electroblotting using a NovaBlot apparatus or visualized by Coomassie Blue staining. Membranes were consecutively treated with 5% (w/v) bovine serum albumin for 1 h, humanized SCID mice serum (dilution 1:250) overnight, goat anti-human IgG antibodies (dilution 1:5000) for 1 h, and horseradish peroxidase-conjugated rabbit anti-goat IgG antibodies (dilution 1:5000) for 45 min. All antibodies were diluted in Tris saline buffer (TSB) containing 10 mM Tris-HCl, 150 mM NaCl and 0.1% Triton; pH 7.6. Intermittent washing steps were performed in TSB ($3 \times 10$ min). Finally, 0.7 mM 3.3'diamino-benzidinetetrahydrochloride containing 0.1% $H_2O_2$ in TBS was added as a substrate to detect protein-antibody interactions and incubated for 5 min. Coomassie Blue-stained gels were used to excise the corresponding visualized spots. 2D-GE and Western blotting experiments were performed in triplet to confirm the identity of the excised protein spots.

### Protein identification by mass spectrometry

Gel spots containing the protein of interest were washed with HPLC-grade water, dried in a SpeedVac (Savant) and digested overnight at 37°C with 10 $\mu$l of 25 ng/$\mu$l trypsin (sequence grade) in 200 mM ammonium bicarbonate. The resulting peptide mixture was cleaned up through C18 resin and analyzed by MALDI-TOF/TOF mass spectrometry in the presence of $\alpha$-cyano-4-hydroxycinnamic acid in an Applied Biosystems 4800 Proteomics Analyzer. Protein identifications were executed with the MASCOT (Matrix Science) search engine using Comprehensive Microbial Resource (CMR) (http://cmr.jcvi.org) *S. pneumoniae* databases.

### Bioinformatics

All identified pneumococcal protein vaccine candidates were looked up in the "Comprehensive Microbial Resource" (CMR) (http://cmr.jcvi.org) database. This database contains information on genes of *S. pneumoniae* serotype 4, R6 and G54. The "National Centre for Biotechnology Information" (NCBI) Gene databank was also consulted. The Expert Protein Analysis System (ExPASy) (http;//www.expasy.org) was used to obtain information on the protein structure. The "Protein Family Database" (Pfam) (http://pfam.sanger.ac.uk) was used to document the functional domains, the conserved binding domains, and the membrane anchoring domains.

To determine the SP1683 gene (strain ATCC BAA-334 / TIGR4) conservation across serotypes, the Basic Local Alignment Tool (BLAST) from the National Centre for Biotechnology information (NCBI) and the Universal Protein Resource Uniprot were used. Fourteen serotypes (1, 2, 3, 4, 5, 6A, 6B, 10A, 11A, 14, 19A, 19F, 23F, and 35F) were analyzed.

To predict the localization of SP1683, the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) has been used for signal peptide prediction. Moreover, a research of functional domain/motifs using the "Protein Family Database" (Pfam) (http://pfam.xfam.org/) and PROSITE (http://prosite.expasy.org/) was also performed on SP1683.

### Antigens

Pneumococcal SP1683 protein was recombinantly produced as follows: ORF1683 (from TIGR4 strain) was cloned into pDest17 vector via Gateway system (Invitrogen) and transfected to Krx *Escherichia coli*. After induction of expression by isopropyl $\beta$-D-1-thiogalactopyranoside (1 mM), *E. coli* were cultured for 6 h at 37°C. The recombinant protein was harvested by centrifugation (4°C, 10 min, 8000 $\times$ *g*) and purified via its polyhistidine tail through a nickel-nitrilotriacetic acid purification system (Invitrogen), following the instructions of the manufacturer.

PhtD.[22,36] was cloned from the N4 strain (serotype 4) and produced in *E. coli*. The protein was purified from bacterial lysate through multiple chromatography steps.

Pneumolysin was cloned from the 6B 493/73 strain, produced in *E. coli* and also purified from bacterial lysate through multiple chromatography steps. Further, pneumolysin was detoxified by formaldehyde treatment to obtain dPly.[37] Detoxification was ascertained by the absence of residual hemolytic activity *in vitro*, the absence of local reactogenicity after intramuscular injection in rats and the absence of *in vivo* toxicity after intranasal challenge in mice.

### ELISA

Briefly, 96-well microtiter plates (Maxisorp, Nunc, Denmark) were coated for 2 h at 37°C with PhtD (1 $\mu$g/mL), SP1683 (1 $\mu$g/mL) or native Ply (8 $\mu$g/mL) in phosphate-buffered saline (PBS). After washing, serial two-fold dilutions of tested samples and one calibrated reference serum (in PBS-Tween-20 0.05%, PBST) were added to the wells. After incubation for 30 min at room temperature (RT) and washing, peroxidase-labelled anti-mouse IgG antibodies (Jackson 115-035-003) were added at a dilution of 1/2500 for 30 min incubation at RT. Plates were washed and then revealed by the addition of O-phenylenediamine dihydrochloride in citrate buffer 0.1 M (pH 4.5), in the presence of hydrogen peroxide. The reaction was stopped after 15 min by the addition of HCl 1 N and the optical density was read at 490 nm (620 nm for the reference filter) in a microtiter plate reader. The individual IgG concentrations (expressed as $\mu$g/ml) were calculated by the 4-parameter method using the Soft Max Pro software.

Anti-caps-PS antibodies were detected as previously described.[38]

### Lethal challenge model with type 4/CDC and 3/43 strains

MF1 female mice (4 weeks-old; n = 20/group) were immunized intramuscularly on days 0 and 14 with 2 doses of 3 $\mu$g PhtD or SP1683 formulated with AS01 (GSK, Rixensart, Belgium), which is a liposome-based Adjuvant System containing 3-*O*-desacyl-4'- monophosphoryl lipid A (MPL; GSK), and QS-21 (*Quillaja saponaria* Molina, fraction 21; licenced by GSK from Antigenics LLC, a wholly owned subsidiary of Agenus Inc, a Delaware, USA corporation). Control mice were injected with AS01 alone. Sera were taken on day 27 (13 days after last immunisation) and two serum pools/group were made to determine humoral responses. On day 28, mice were challenged intranasally with $5 \times 10^6$ cfu of *S. pneumoniae* type 4/CDC (DS2382-94)[39] or $1 \times 10^5$ cfu of *S. pneumoniae* type 3/43. Mortality was recorded during 10 days.

### Nasopharyngeal colonization model with type 6B/CDC and 2D39 strains

Four weeks-old Balb/c mice (n = 10/group) were intranasally immunized on days 0, 14 and 28 with 2.5 $\mu$g of PhtD or SP1683 protein, adjuvanted with LT (except at the third immunization). Control animals received LT alone. Sera were collected on Day 41. The mice were challenged on day 42 with $2 \times 10^5$ cfu of the *S. pneumoniae* type 6B/CDC or type 2D39 strain, both heterologous for PhtD and SP1683. The bacterial load was measured in nasal washes collected 2 and 6 days post-challenge (with a total of 10 mice per group and per time-point, N = 30) for the *S. pneumoniae* type 2D39 strain and 3 days post-challenge (with a total of 10 mice per group, N = 30) for the type 6B/CDC. For that, the log10 weighted mean number of CFU/10 $\mu$l was determined by counting the colonies grown on Columbia agar 5% horse blood plates after plating 10 $\mu$l of 4 ten-fold serial dilutions of the washing. The arithmetic mean of the log10 weighted mean number of CFU/10 $\mu$l and the standard deviations were calculated for each group.

### IL17 depletion experiment in the mouse nasopharyngeal colonization model

Four weeks-old Balb/c mice were intranasally immunized on days 0, 14 and 28 with 3 $\mu$g of PhtD/dPly (used as a positive control) or SP1683, adjuvanted with LT except at the third immunization. Control animals received LT alone. The mice were challenged on day 42 with $2 \times 10^5$ cfu of the *S. pneumoniae* type 2D39 strain, heterologous for PhtD, dPly and SP1683. Mice were treated or not with 100 $\mu$g anti-IL17A and 100 $\mu$g anti-IL17F monoclonal antibodies given through the intraperitoneal route on days 41, 43 and 45. The bacterial load was measured in nasal washings collected 2 and 4 days post-challenge. Anti-IL17A and anti-IL17F antibodies were obtained from the Ludwig Institute for Cancer Research, Brussels.

## Disclaimer

Animal studies were carried out at GSK (Rixensart, Belgium) or at the KU Leuven in accordance with European Directive 2010/63, and the GSK Policy on the Care, Welfare and Treatment of Animals. Approval for the study was granted by the respective local ethics committees.

## Disclosure of potential conflicts of interest

All authors have declared the following interests: PH, CY and FG are, or were at the time of the study, employees of the GSK group of companies. PH and FG report ownership of GSK shares and/or restricted GSK shares. PH, CY and FG are listed as inventors on patents owned by the GSK group of companies. LM, GW, XB, RD, EW and TW report no financial conflicts of interest.

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## Author contributions

LM, PH, FG, XB were involved in the conception and design of the study. LM, TW, PH, GW, RD, EW, CY acquired the data. LM, PH, TW, GW, TD, FG and XB analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

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