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The factor H-binding fragment of PspC as a vaccine antigen for the induction of protective humoral immunity against experimental pneumococcal sepsis

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ABSTRACT

Pneumococcal surface protein C (PspC) is a major virulence factor of *Streptococcus pneumoniae* and interferes with complement activity by binding complement factor H (fH). In this study, protection against experimental sepsis caused by pneumococci carrying different PspC variants was evaluated by immunisation with the fH-binding fragment of PspC. The mechanisms of protection mediated by antibodies to PspC were also studied. Mice were immunised with a PspC fragment ($PspC_{39–261}$) from the type 3 strain HB565 and infected intravenously with either strain HB565 (homologous challenge), or strains D39 and TIGR4 (heterologous challenge). Immunisation with $PspC_{39–261}$ elicited high titers (>300,000) of PspC-specific serum IgG and conferred protection from challenge with HB565. In contrast, cross-protection was either limited or absent in vaccinated animals infected with D39 and TIGR4, respectively. To correlate protection with reactivity and function of PspC antibodies, pooled sera from vaccinated mice were tested in IgG binding and complement deposition experiments. IgG antibodies efficiently bound to HB565, while binding was lower with D39 and absent with TIGR4. In the presence of mouse post-immune sera, C3 deposition was increased onto HB565, while no effect was observed with D39 and TIGR4. Antibody cross-reactivity and complement deposition progressively declined with reduced amino acid identity between PspC variants. Antibodies to PspC were also found to interfere with fH binding to HB565. Finally, *in vitro* and *ex vivo* phagocytosis assays demonstrated that PspC-specific antibodies promoted opsonophagocytic killing of bacteria.

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1. Introduction

Streptococcus pneumoniae is a major human pathogen, causing otitis media, community-acquired pneumonia, meningitis and sepsis. Host protection against pneumococcal infection is largely mediated by complement- and/or antibody-dependent phagocytosis. Both the alternative (AP) and classical (CP) pathways of complement are essential for immunity to *S. pneumoniae* [1–5]. Factor H (fH) is a fluid-phase regulator of the AP and has a key function in protection of host cells from complement attack [6,7]. FH inhibits binding of factor B (fB) to C3b, acts as a cofactor for factor I (fI)-mediated cleavage of C3b into inactive iC3b, and accelerates the decay of the AP C3 convertase [8]. Thereby, binding of fH represents a strategy used by microbial pathogens, including *S. pneumoniae*, to evade complement activation.

Current pneumococcal vaccines use the capsular polysaccharides as the main constituent. This has led to the design of polyvalent formulas due to the presence of more than 90 different capsular serotypes, as well as conjugation to protein carriers in order to be effective also in young children [9]. Nonetheless, as protection provided by conjugate vaccines is limited to the serotypes included, investigators have been looking into pneumococcal proteins as an alternative to develop new vaccines that would provide broader protection in all age groups [10,11]. Several pneumococcal proteins have been shown to be immunogenic and offer protection against challenge in experimental disease models, including pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), pneumococcal surface adhesin A (PsaA), pneumolysin, the histidine triad proteins, the iron transporters PiuA and PiaA, and the pilus proteins (for reviews see [10–12]).

Abbreviations: PspC, pneumococcal surface protein C; fH, complement factor H; CP, complement classical pathway; AP, complement alternative pathway; PRR, proline-rich region; cfu, colony forming units.

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Table 1
S. pneumoniae strains.

Strain	Capsular serotype	Features[a]	PspC allelic type[b]	Reference
HB565	3	w.t.	PspC11.4	[39,58]
FP33	Rough (3)	*cps*-	PspC11.4	[39]
PF20	3	*pspC*-	–	[20]
D39	2	w.t.	PspC3.1	[39,59]
FP22	Rough (2)	*cps*-	PspC3.1	[39]
FP30	2	*pspC*-	–	[20]
TIGR4	4	w.t.	PspC3.4	[60]
FP23	Rough (4)	*cps*-	PspC3.4	[40]
FP28	4	*pspC*-	–	[20,21]

[a] *cps*-, unencapsulated derivative; *pspC*-, PspC deficient mutant.
[b] PspC allelic types are as described by Iannelli et al. [15].

PspC, also designated as CbpA, Hic, PbcA, and SpsA [13–18] contributes to the virulence of *S. pneumoniae* [18–24] and is considered a promising vaccine antigen [14,25–28]. Sequence comparison of the *pspC* locus from 43 pneumococcal isolates demonstrated that the protein is highly polymorphic [15]. PspC allelic variants can be divided into 2 major groups based on cell wall attachment (choline-binding domain or LPXTG motif), and into 11 subgroups based on organisation of structural/functional modules [15]. PspC has several functions, including binding of complement fH [17,29–31]. FH binding interferes with the AP and results in reduced C3b deposition onto the pneumococcal surface, thereby inhibiting opsonophagocytosis [19]. FH binding has also been demonstrated to occur *in vivo* during sepsis in mice [32]. Pneumococcal clinical isolates vary in their capability to interact with human fH [33,34], and fH binding has been mapped in some PspC allelic types [17,30,31,35,36]. Recently, it was shown that certain pneumococcal serotypes also bind the complement inhibitor C4bp in a PspC allele-dependent manner [37]. Immunisation with PspC protects mice against pneumococcal carriage [27] and sepsis [14,26].

The proline rich regions (PRRs) of PspC and PspA, which are conserved among different alleles, have been used as vaccine antigens to elicit cross-protective antibodies against pneumococcal infection [14,38]. In this work, we have investigated the potential of a different portion of PspC, the fH-binding fragment, to induce protective immunity in mice challenged intravenously with pneumococcal strains carrying different PspC variants. We have also analysed the mechanisms of protection mediated by antibodies to PspC using different assays, such as complement deposition, antibody binding, fH binding, opsonophagocytosis and killing.

2. Materials and methods

2.1. Pneumococcal strains and growth conditions

S. pneumoniae wild type and isogenic mutant strains used in this study are listed in Table 1. Unencapsulated and PspC-deficient mutants were obtained by allelic replacement of the entire capsule locus or the *pspC* gene with an antibiotic resistance marker, respectively. Construction of mutants has previously been described [20,39,40]. Bacteria were cultured at 37 °C in Todd-Hewitt broth (Difco, Detroit, MI, USA) supplemented with 0.5% yeast extract (THY) or Tryptic Soy broth (TSB, Difco) until mid-logarithmic phase and frozen at −80 °C with 10% glycerol. Solid media were prepared by addition of 1.5% agar and 3% defibrinated horse blood (Biotec Snc, Grosseto, Italy) to TSB. Colony forming units (cfu) counts were performed on blood agar plates incubated for 18–24 h at 37 °C with 5% CO_2.

2.2. Mouse sepsis model

Eight-week old CBA/Jico mice were obtained from Charles River (Charles River Italia, Italy). Experiments were conducted according to institutional and national guidelines. Animals were exposed to an infrared lamp (200 W) for 2–3 min to promote vasodilation, and then 200 μl of bacterial suspension was injected into the tail vein, as previously described [20,41]. Mice were monitored for 10 days, and the survival time of each mouse was recorded.

2.3. Immunisation and challenge studies

Mice were immunised with a recombinantly expressed and purified fragment of PspC ($PspC_{39–261}$) from the HB565 strain (originally described as protein Hic) [17]. $PspC_{39–261}$ corresponds to amino acids 39–261 and includes the fH-binding domain [17]. Genebank accession number is AF252857. Mice were immunised subcutaneously (s.c.) with 10 μg of $PspC_{39–261}$ with complete Freund's adjuvant (priming at day 0) and received two boosters (at days 21 and 35) with 5 μg of protein with incomplete Freund's adjuvant in a final volume of 100 μl. Control mice were given complete and incomplete Freund's adjuvant in phosphate-buffered saline (PBS).

For protection experiments, animals (n = 4–12/group) were divided in eight groups, four of which were immunised with $PspC_{39–261}$ and four served as controls. On day 50 after priming, mice were challenged intravenously (i.v.) with four doses (10^5, 10^6, 10^7, or 10^8 cfu/mouse) of the homologous strain HB565. For cross-protection, mice that had been immunised with $PspC_{39–261}$ from strain HB565 were infected by the i.v. route with the heterologous strains D39 and TIGR4. Eight groups of mice (four immunised and four control; n = 4–8/group) were challenged with D39 (10^4, 10^5, 10^6, or 10^7 cfu/mouse), whereas four groups of animals (two immunised and two control; n = 6/group) were challenged with TIGR4 (10^6 or 10^7 cfu/mouse).

2.4. Serum samples

Blood samples were collected from mice at days 0 (pre-immune), 21, 35 and 49 after priming from the sub-mandibular vein. Samples were centrifuged (2000 × *g*, 15 min at 37 °C), and sera were recovered and frozen at −80 °C until use. As a source of human complement factors, serum samples were obtained from unvaccinated healthy volunteers according to institutional guidelines. Single-use aliquots were prepared and conserved at −80 °C until use.

2.5. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Determination of serum titers of PspC-specific IgG in the sera of immunised mice was performed by ELISA as previously described [42]. Sera from immunised animals were tested individually, while those from controls were pooled. Serum samples were initially diluted 1:20 and titrated in twofold dilutions in microtiter plates coated with $PspC_{39–261}$ (1 μg/ml). After incubation for 3 h, plates were treated with alkaline-phosphatase-conjugated goat anti-mouse IgG (1:1500, Southern Biotechnology Associates, Birmingham, AL, USA) and developed by adding p-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine pH = 10, Sigma–Aldrich). Absorbance at 405 nm was recorded using a 340 ATC reader (SLT Labinstrument, Austria). ELISA titer was defined as the reciprocal of the highest serum dilution with $OD_{405} \geq 0.2$ after subtracting the background value.

2.6. Antibody binding assays

Binding of anti-PspC antibodies to *S. pneumoniae* was assessed by flow cytometry adapting a previously described protocol [43]. Briefly, bacterial stocks (~2×10^7 cfu/sample) were thawed,

washed in PBS and blocked in PBS–1% BSA for 30 min at 37 °C. Bacteria were incubated with mouse sera (diluted 1:30 in PBS–BSA) for 1 h at 4 °C in a final volume of 50 μl. Samples were washed twice and suspended in 100 μl of PBS containing a 1:64 dilution of FITC-conjugated anti-mouse IgG (Sigma–Aldrich). After incubation at 37 °C for 30 min, bacteria were washed twice and resuspended in 300 μl of 1% paraformaldehyde in PBS for flow cytometry. Two control samples were added by incubating bacteria with the serum from control mice or with PBS–BSA. Flow cytometry analysis was conducted on a FACScalibur instrument (Becton Dickinson Immunocytometry System, San Josè, CA, USA) with a CellQuest software (Becton Dickinson).

2.7. C3 deposition assays

Bacterial stocks (~2×10^7 cfu/sample) were thawed, washed, and blocked in PBS–1% BSA for 30 min at 37 °C. Samples were treated for 30 min at 37 °C with a 1:30 dilution of sera from either immunised or control mice in Gelatin Veronal Buffer (GVB^{2+} with Ca^{2+} and Mg^{2+}, Sigma–Aldrich) containing 10 mM EDTA to block all complement pathways. Bacteria resuspended in GVB^{2+}/EDTA were used as a background control. After washing 3 times, samples were incubated for 20 min at 37 °C with 50% of either normal mouse sera (NMS) or normal human sera (NHS) in a total volume of 10 μl of GVB^{2+} or GVB^{2+} with 10 mM EGTA (to block the classical and lectin pathways). Bacteria were washed twice in ice-cold GVB^{2+}/EDTA and suspended in a 1:100 dilution of FITC-conjugated goat anti-mouse or anti-human C3 IgG (Sigma–Aldrich). After incubation on ice for 30 min, bacterial cells were washed twice and resuspended in 300 μl of 1% paraformaldehyde in PBS for flow cytometry analysis.

2.8. FH binding assays

The interaction between fH and PspC was examined by flow cytometry. Bacterial stocks (~2×10^7 cfu/sample) were thawed, washed, and incubated for 1 h at 37 °C with 10% NHS in GVB^{2+}. Samples were washed twice and incubated for 30 min at 37 °C with a 1:100 dilution of goat anti-human fH IgG (Complement Technology, Inc., Tyler, TX, USA) in GVB^{2+}. After washing, samples were incubated for 30 min at 37 °C with FITC-conjugated rabbit anti-goat IgG (1:160; Sigma–Aldrich). Bacteria were washed again and resuspended in 300 μl of 1% paraformaldehyde in PBS. Controls were constituted by samples incubated in GVB^{2+}. For competitive fH binding assays, pneumococci were incubated for 1 h at 37 °C with 10% NHS and two-fold serial dilutions (starting from 1:8) of either pooled pre-immune (control) or post-immune mouse sera (containing anti-PspC IgG) in a total volume of 100 μl of GVB^{2+}. Samples were treated with primary and secondary antibodies and analysed by flow cytometry as described above. The fH inhibition titer was defined as the reciprocal of the highest serum dilution where differences in fH binding between samples treated with pre-immune or post-immune mouse sera were statistically significant.

2.9. Opsonophagocytosis and killing assays

For opsonophagocytosis, effector cells were the mouse monocyte/macrophage J774A.1 cell line (DSMZ, Braunschweig, Germany; [44]). Cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma–Aldrich) and antibiotics (streptomycin 10 mg/ml and penicillin 10,000 U/ml, Sigma–Aldrich). The assay was modified from a recently described protocol [45]. Briefly, bacterial stocks were thawed, washed, heat-killed at 65 °C for 1 h, and labelled by incubation for 30 min at 37 °C with 5 μM of Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA/SE, Sigma–Aldrich). After labelling, pneumococci (~4×10^6 cfu) were washed three times and resuspended in 30 μl of $HBSS^{2+}$ (Hank's Balance Salt Solution with Ca^{2+}, Mg^{2+}; Invitrogen, Milano, Italy) containing 0.2% BSA and sera from vaccinated mice at two concentrations (1% and 10%). As no exogenous source of complement (*i.e.*, baby rabbit serum) was added, the complement supply was from the sera of vaccinated mice. Bacteria were opsonized for 30 min at 37 °C with gentle shaking. Negative controls included samples incubated with $HBSS^{2+}$. Approximately 4×10^5 J774 cells were added to bacteria (moi = 10:1) in a total volume of 160 μl, and samples were incubated at 37 °C for 45 min with gentle shaking. Samples were spun at 300 × *g* for 10 min at 4 °C, washed with $HBSS^{w/o}$ (Hank's Balance Salt Solution without Ca^{2+} and Mg^{2+}, Invitrogen) containing 0.2% BSA, and resuspended in 300 μl of 1% paraformaldehyde in PBS for flow cytometry . For the *ex vivo* killing assay, strain FP33 (~1×10^6 cfu/sample) was thawed, washed and incubated at 37 °C for 30 min with a 1:10 dilution of either pre-immune mouse sera or pooled sera from vaccinated animal in a final volume of 50 μl. Heparinised fresh blood was obtained by cardiac puncture from 8-week old female BALB/c mice (Charles River) and immediately transferred at 37 °C. One hundred μl of blood (containing ~2×10^5 neutrophils/monocytes) was added to previously opsonised bacteria (moi = 5:1) in a total volume of 150 μl and incubated in a rotating shaker at 37 °C for 2 h. Samples were placed on ice for 5 min, and then 10 μl were diluted and plated onto blood agar plates.

2.10. Statistical analysis

ELISA titers were expressed as geometric mean (GM) ± standard error of the mean (SEM). Differences in mouse survival between immunised and control groups were analysed by the Fisher's exact test (percent survival) and the Mann–Whitney *U* test (time-to-death). LD_{50} was calculated by using the method of Reed and Muench [46] and Probit analysis with 95% confidence interval [47]. To combine the percentage of positive bacteria and the binding intensity, data on IgG binding, C3 deposition and fH binding were represented as fluorescence index (FI) ± SEM. The FI was calculated by multiplying the percentage of positive events with the geometric mean fluorescence intensity (MFI), as previously described [34]. Differences in complement deposition, fH competitive binding, opsonophagocytosis and killing were analysed using the Student's unpaired *t* test (for comparison of two groups). *P* values <0.05 were considered statistically significant.

3. Results

In this study mice were immunised using a 222-aa long fragment from the N-terminal portion of PspC ($PspC_{39–261}$) from the type 3 HB565 strain as the vaccine antigen. This fragment was previously shown to contain the fH-binding domain of PspC of HB565 [17]. Priming with $PspC_{39–261}$ induced PspC-specific serum IgG in most mice, but variability of ELISA titers was relatively high (Fig. 1). After the second boost, titers of antibodies to PspC were high in all vaccinated mice and reached mean values greater than 300,000 (Fig. 1). Animals were challenged i.v. with HB565 (homologous challenge), and with the type 2 D39 strain or the type 4 TIGR4 strain (heterologous challenge). PspC is a highly allelomorphic protein in pneumococcal isolates [15], and this versatility is shown by D39 and TIGR4 having allelic variants with different homology to PspC from HB565. In particular, the $PspC_{39–261}$ fragment has an overall amino acid identity of 50% and 30% with the corresponding regions of the PspC proteins of D39 and TIGR4, respectively.

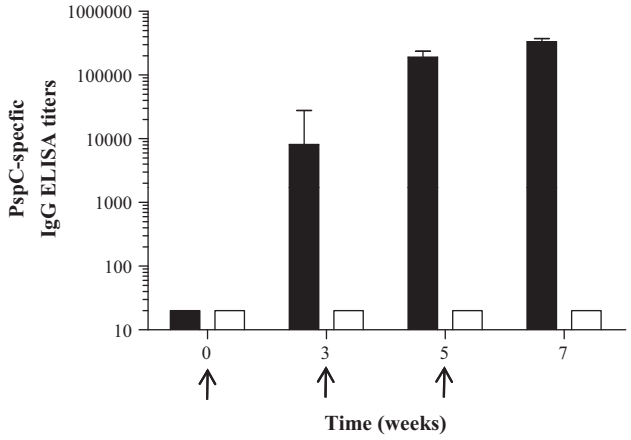

Fig. 1. ELISA titers of PspC-specific total serum IgG after immunisation with $PspC_{39–261}$. CBA/J mice (total $n = 70$) were inoculated s.c. with $PspC_{39–261}$ from the type 3 HB565 strain (priming at day 0 and boosters at days 21 and 35). Control mice (total $n = 61$) were given PBS. Serum samples were collected from animals at days 0 (pre-immune), 21 (wk 3), 35 (wk 5), and 49 (wk 7). The presence of PspC-specific IgG in sera was assessed by ELISA in microtiter plates coated with $PspC_{39–261}$. Sera from immunised mice (closed histograms) were tested individually (results are expressed as GM ± SEM), while those from controls (open histograms) were pooled. Antibody titers are expressed as the reciprocal of the highest serum dilution with $OD_{405} \geq 0.2$ after subtraction of the background value. Arrows indicate the times of inoculations.

3.1. *Immunisation with $PspC_{39–261}$ of HB565 confers protection from sepsis (homologous challenge)*

Mice immunised with $PspC_{39–261}$ were challenged i.v. with different doses (10^5–10^8 cfu/mouse) of HB565. Increased survival of immunised animals was observed at all doses. The LD_{50} of HB565 for vaccinated animals was 1.2×10^7 cfu/mouse, with a 69-fold increase over control mice (1.75×10^5 cfu/mouse). Median time-to-death was also higher in immunised animals compared to controls, and differences were statistically significant at the two intermediate challenge doses of 10^6 cfu ($P = 0.0032$) and 10^7 cfu ($P = 0.048$) (Fig. 2A).

3.2. *Cross-protection from heterologous challenge*

Mice immunised with $PspC_{39–261}$ of HB565 were challenged i.v. with strains D39 and TIGR4. All mice died due to infection with D39, except for 1 animal (16% survival) in the vaccine group challenged with 10^4 cfu. Nonetheless, a statistically significant increase in median time-to-death was observed in immunised mice infected with D39 at 10^5, 10^6, and 10^7 cfu (10^5, $P = 0.045$; 10^6, $P = 0.03$; 10^7, $P = 0.016$) (Fig. 2B). The limited cross-protection observed for D39 was not found in mice infected with TIGR4 (Fig. 2C). As the D39 variant of PspC shows a higher degree of similarity to the immunogen than PspC of TIGR4, the cross-protection results are consistent with the different levels of amino acid identity among the PspC allelic variants.

3.3. *Differential binding of PspC-specific antibodies to the three challenge strains*

To correlate protection from challenge by different pneumococcal strains with strain-specific reactivity of anti-PspC antibodies, pooled sera from vaccinated mice were assessed for binding to HB565, D39 and TIGR4 by flow cytometry. Serum IgG from mice immunised with $PspC_{39–261}$ of strain HB565 efficiently bound to the surface of HB565. Binding was lower to D39, and no binding was observed to TIGR4, as shown by the levels of both MFI (Fig. 3A) and FI (Fig. 3B). When the assay was repeated with the isogenic PspC-deficient strains of HB565, D39 and TIGR4, no IgG binding

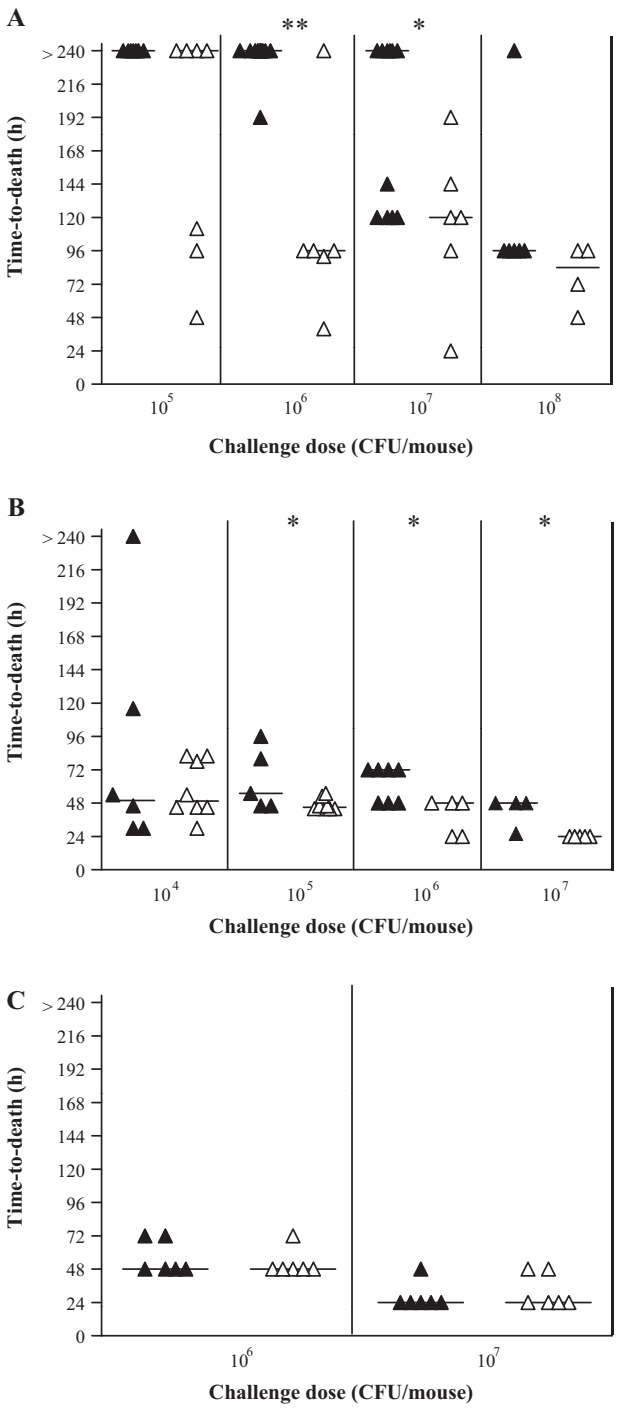

Fig. 2. Homologous and heterologous challenge. (A) Homologous challenge. Eight groups of CBA/J mice ($n = 4$–12) were immunised with $PspC_{39–261}$ from the type 3 HB565 strain (closed triangles) or injected with PBS (open triangles) and then challenged i.v. with HB565 (from 10^5 to 10^8 cfu/mouse). (B) Heterologous challenge with D39. Eight groups of CBA/J mice ($n = 4$–8) were injected with either $PspC_{39–261}$ from strain HB565 (closed triangles) or PBS (open triangles) and challenged i.v. with four doses (from 10^4 to 10^7 cfu/mouse) of strain D39. (C) Heterologous challenge with TIGR4. Four groups of mice ($n = 6$) were injected with either $PspC_{39–261}$ from the HB565 strain (closed triangles) or PBS (open triangles), and infected i.v. with 10^6 cfu and 10^7 cfu of strain TIGR4. In all panels, time-to-death of individual mice at different doses and the median time-to-death (bars) of each group are shown. Asterisks indicate statistical significance (Mann–Whitney *U* test; $^*P < 0.05$, $^{**}P < 0.01$).

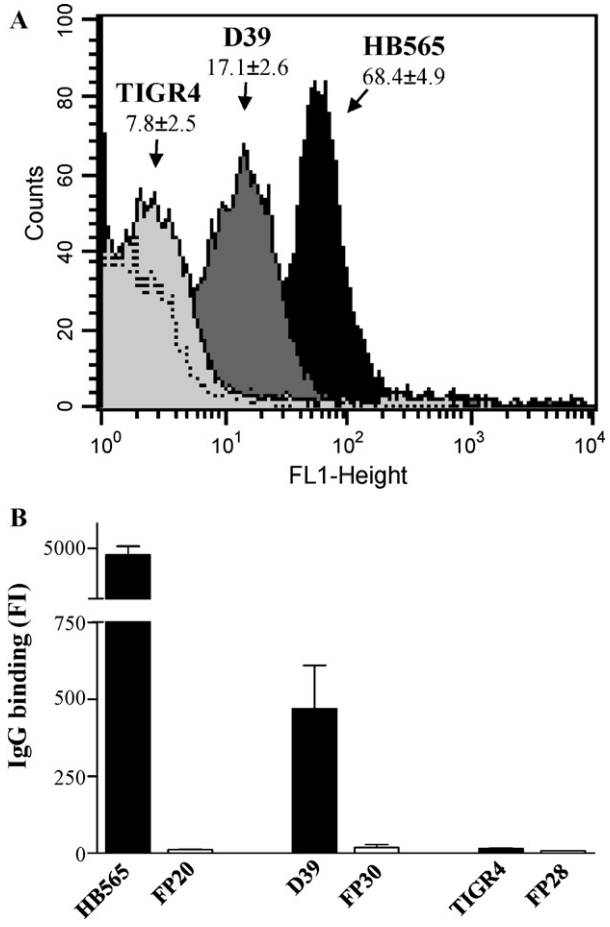

Fig. 3. Binding of anti-PspC antibodies to *S. pneumoniae* strains. Sera of mice immunised with $PspC_{39–261}$ were tested for binding to pneumococcal strains HB565, D39, TIGR4 and their corresponding isogenic PspC-deficient mutants FP20, FP30, and FP28 by flow cytometry. Bacteria were incubated with a 1:30 dilution of pooled mouse sera and then treated with FITC-conjugated anti-mouse IgG. (A) Binding of PspC-specific IgG to HB565 (black histogram), D39 (dark grey histogram), and TIGR4 (light grey histogram) is expressed as MFI ± SEM of 3–6 independent experiments. No binding was observed with control bacteria incubated with pre-immune mouse sera (dotted line histogram). (B) IgG binding to pneumococcal isogenic pairs HB565/FP20, D39/FP30 and TIGR4/FP28 is represented as FI ± SEM (3–6 experiments) after subtraction of the background represented by bacteria incubated with PBS–BSA.

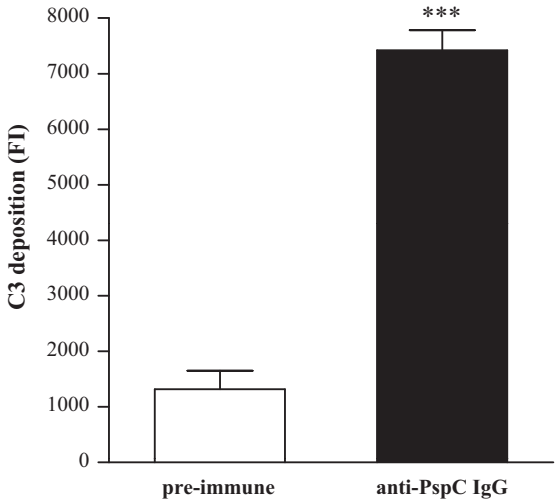

Fig. 4. C3 deposition onto HB565 in the presence of anti-PspC antibodies. HB565 was pretreated with a 1:30 dilution of pooled sera from either control (white column, pre-immune) or immunised (black column, anti-PspC IgG) mice in GVB^{2+} with EDTA. Samples were washed, incubated with 50% NMS and then treated with FITC-conjugated goat anti-mouse C3 IgG. Results are represented as FI ± SEM of 3–4 independent experiments. Statistically significant differences are shown by asterisks (Student's unpaired *t* test; $^{***}P < 0.0001$). Control samples were represented by bacteria resuspended with GVB^{2+}/EDTA, and C3 deposition was 2317 ± 408.

was found with the corresponding mutants FP20, FP30 and FP28, confirming that the reactivity observed with flow cytometry was entirely due to PspC-specific antibodies (Fig. 3B). The limited antibody cross-reactivity, which progressively weakens with reduced amino acid identity between the PspC variants, correlates with the cross-protection data.

3.4. Mouse anti-PspC antibodies enhance C3 deposition on HB565

To analyse the impact of anti-PspC antibodies on C3 deposition, HB565, D39, and TIGR4 were treated with pooled mouse sera in the presence of EDTA (to block complement activation during antibody binding) and then incubated with NMS (as a complement source). C3 deposition was 6-fold higher when HB565 was incubated with sera from immunised mice compared to pre-immune serum ($P < 0.0001$) (Fig. 4). When the complement CP was inhibited by EGTA, C3 deposition diminished considerably but was not totally eliminated as in the case of samples treated with EDTA (data not shown). C3 deposition onto D39 and TIGR4 was very low, and no differences were found between samples incubated with pre-immune or post-immune mouse sera (data not shown). Similarly, levels of C3 deposition were negligible with the PspC-deficient pneumococcal mutants (data not shown). Experiments were repeated with NHS as a source of complement factors. Results were comparable to those obtained using NMS, as deposition was high onto HB565 (FI = 5690 ± 750) and approximately 6-fold and 14-fold lower onto D39 and TIGR4, respectively. In the presence of anti-PspC antibodies, a 1.78-fold increase of FI (FI = 10,129 ± 1058; $P = 0.007$) was recorded for HB565, while deposition onto D39 and TIGR4 was not augmented (data not shown). The modest increase of human C3 deposition on HB565 with post-immune mouse sera could be due to the higher background (presence of anti-capsular antibodies) or a degree of incompatibility between murine antibodies and human complement.

These data show that CP activation is crucial for specific antibody-mediated C3 deposition, although it is likely that AP amplification also contributes to total C3 deposition.

3.5. Antibodies to PspC interfere with fH binding to HB565

A flow cytometry assay was used to study the interference of PspC-specific antibodies with fH binding to the pneumococcal surface. In the absence of serum from immunised animals, binding of human fH to HB565 was 4.3- and 8.4-fold higher (in FI) as compared to TIGR4 and D39, respectively (Fig. 5A). Addition of a 1:10 dilution of post-immune mouse sera inhibited fH binding to HB565 (3.2-fold decrease in MFI) (Fig. 5B), while no effect was observed with D39 (Fig. 5C) and TIGR4 (Fig. 5D). The fH binding inhibitory activity of pooled sera from immunised animals was then titrated against pooled sera of control mice. A statistically significant inhibition was observed at serum dilutions ranging from 1:8 to 1:256, suggesting that the fH binding inhibition titer was 256 (data not shown).

3.6. Sera from vaccinated animals enhance opsonophagocytosis of strains HB565 and FP33

To investigate whether serum antibodies from animals immunised with $PspC_{39–261}$ promote opsonophagocytosis of *S. pneumoniae* by murine macrophages, a flow cytometry assay was

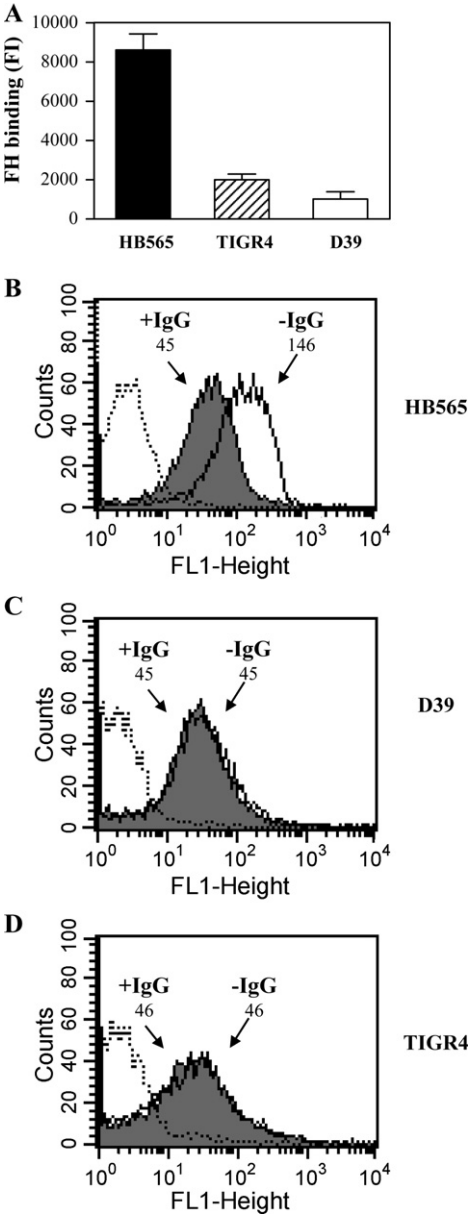

Fig. 5. FH binding by *S. pneumoniae* in the presence or absence of anti-PspC antibodies. Strains HB565, D39 and TIGR4 were incubated with 10% NHS from unvaccinated donors. Results of fH binding are expressed as FI ± SEM of 4–8 independent experiments (A). For competitive fH binding, strains HB565 (B), D39 (C) and TIGR4 (D) were incubated with NHS in the presence (grey histograms) or absence (solid line histograms) of 10% pooled sera from mice immunised with $PspC_{39-261}$. Bacteria incubated with GVB^{2+} were used as controls (dashed line histograms). Binding to fH was assessed by flow cytometry using goat anti-human fH IgG followed by FITC-conjugated anti-goat IgG. A representative experiment is shown. In each panel, arrows indicate MFI of fH binding with (+IgG) or without (−IgG) post-immune mouse sera.

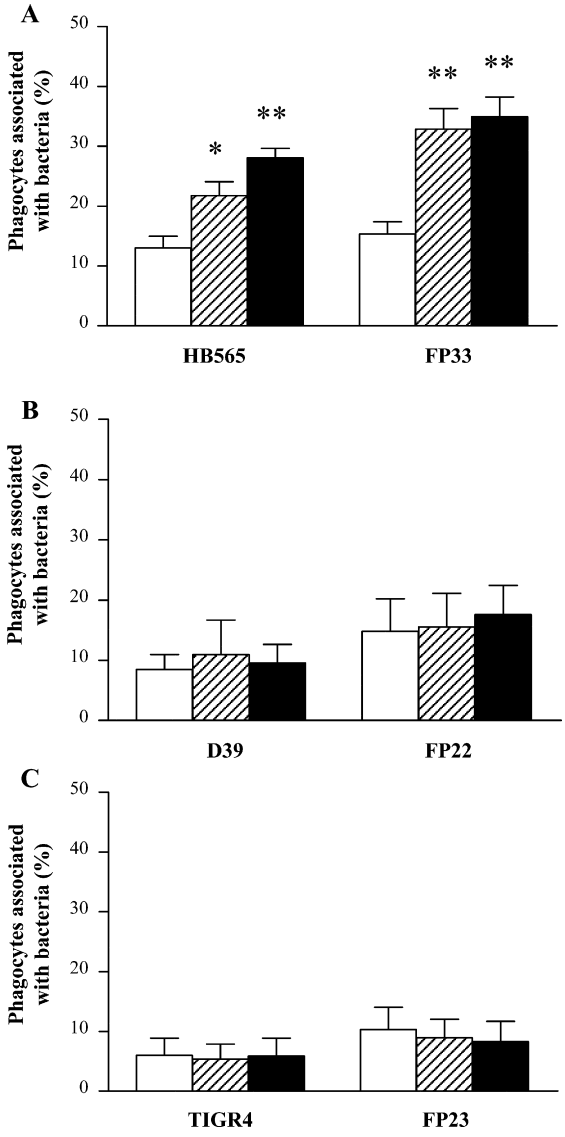

Fig. 6. Opsonophagocytosis of *S. pneumoniae* in the presence of anti-PspC antibodies. Strains HB565 (A), D39 (B), and TIGR4 (C) and their corresponding unencapsulated derivatives FP33 (A), FP22 (B) and FP23 (C) were labelled with CFDA/SE, opsonised with 1% (hatched columns) or 10% (black columns) sera from immunised animals, and incubated with J774 cells (moi = 10:1) in $HBSS^{2+}$. Controls were in $HBSS^{2+}$ (white columns). Phagocytes were washed and subjected to flow cytometry. Results of 4 independent experiments are represented as percentage association (±SEM) of phagocytes with bacteria. In the presence of pre-immune serum, percent of phagocytosis was 11.3 ± 1.4. Asterisks indicate statistical significance (Student's unpaired *t* test; $^{*}P < 0.05$, $^{**}P < 0.01$).

carried out to measure the percentage of phagocytes associated with labelled bacteria. Two different concentrations (1% and 10%) of serum from immunised mice were used for each bacterial strain. A statistically significant enhancement of bacteria-macrophage association in the presence of PspC-specific antibodies was found for HB565 and its unencapsulated derivative FP33 even when using the lowest concentration of mouse post-immune serum (Fig. 6A). No effect was observed with D39, TIGR4, and their corresponding unencapsulated mutants FP22 and FP23 (Fig. 6B, C).

To assess the bactericidal properties of anti-PspC antibodies, an *ex vivo* opsonophagocytic killing assay was set up by incubating the FP33 strain with fresh mouse blood as a source of neutrophils and monocytes. When pneumococci had been pre-opsonised with 10% pooled sera from immunised mice, the number of cfu recovered was 30% lower than that obtained from bacterial samples treated with pre-immune mouse serum. This difference was statistically significant ($P = 0.032$) (Fig. 7).

4. Discussion

Escape from complement attack is an important mechanism used by microbes to evade innate immunity. Bacterial pathogens have developed several strategies to elude complement activation, including avoiding immune recognition, inhibiting opsonisation, degrading complement components, or recruiting complement inhibitors onto the bacterial surface [48]. Bacterial structures

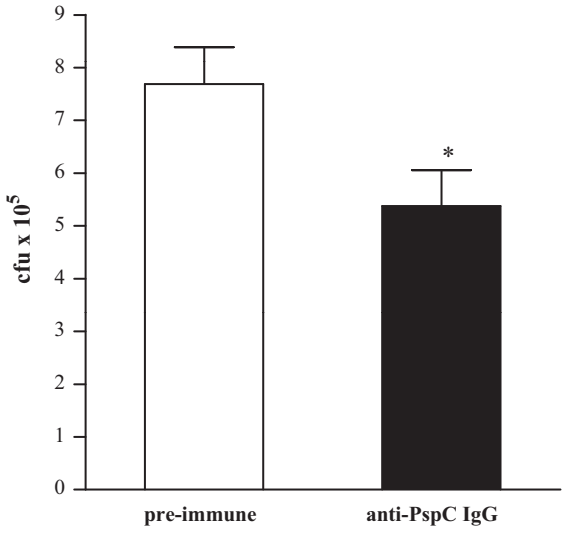

Fig. 7. Opsonophagocytic killing of *S. pneumoniae* in the presence of anti-PspC antibodies. Strain FP33 was incubated with 10% of either pre-immune mouse serum (white column) or pooled sera from mice immunised with $PspC_{39–261}$ (black column), and then mixed with 100 μl of fresh mouse blood (moi = 5:1). Samples were incubated at 37 °C for 2 h, and then cfu counts were determined. Results are shown as the mean (±SEM) of viable cfu counts from 2 independent experiments. The asterisk indicates statistical significance (Student's unpaired *t* test; $^*P < 0.05$).

recruiting complement regulatory proteins are attractive vaccine candidates because vaccine efficacy may be enhanced by virulence impairment [49]. Several complement inhibitors are being considered as potential vaccine antigens, such as the M protein of *Streptococcus pyogenes*, the β protein of *Streptococcus agalactiae*, the GNA1870 protein of *Neisseria meningitidis*, and also PspC of *S. pneumoniae* [49]. All these proteins counter complement attack by the same mechanism, namely the binding of fH which leads to inhibition of complement AP.

In this study, we have explored the vaccine potential of the fH-binding fragment of PspC by analysing cross-protection against sepsis caused by three pneumococcal strains carrying different PspC allelic variants and by examining the properties of antibodies elicited to PspC. We chose strain HB565 because its PspC protein was the first pneumococcal protein being identified as capable of binding to fH [17] and because levels of fH binding by HB565 are comparatively high [34; this work]. D39 and TIGR4 were selected because they are genome reference strains used by many research groups working on *S. pneumoniae*. Results obtained showed that: (i) the fH-binding fragment of PspC ($PspC_{39–261}$) from *S. pneumoniae* HB565 is highly immunogenic in mice and confers protective immunity against homologous challenge, (ii) the levels of cross-protection are consistent with the degree of amino acid identity between the homologous fragments of the different PspC alleles, (iii) the modest cross-reactivity of PspC-specific antibodies shown by flow cytometry correlates with *in vivo* cross-protection results, (iv) antibodies to PspC enhance complement deposition onto HB565 mainly through the complement CP, (v) antibodies compete for fH binding to PspC of HB565, thereby interfering with the complement AP, (vi) PspC-specific antibodies enhance opsonophagocytosis of HB565 and its unencapsulated derivative FP33 by mouse phagocytes.

Different fragments of other PspC alleles have previously been reported as "protective antigens" in mouse challenge studies by homologous pneumococcal strains [14,26,28]. Immunisation with PspC proteins from both D39 [26] and TIGR4 [50] conferred protection against murine sepsis from homologous challenge. Cross-reactive antibodies directed against the PRRs present in both PspC and PspA seem to be responsible for the cross-protection observed in some studies [14,38]. In our case, we can rule out PRR-mediated cross-reactivity and cross-protection, as the $PspC_{39–261}$ fragment used for immunisation does not contain the PRR.

In this work, we have shown that levels of cross-protection are consistent with the degree of amino acid identity between the vaccine antigen and the PspC variants produced by the heterologous strains. Previous studies on PspA also showed that cross-protection among strains carrying different PspA alleles depended upon similarity between the immunising PspA fragment and the protein produced by the challenge strain [51–53]. Both strains used here for the heterologous challenge were more virulent than HB565. A challenge dose of 10^5 cfu of TIGR4 or D39 killed all control animals in 48 h, while 100% killing by HB565 occurred only with 10^7 cfu and was delayed considerably (median time-to-death = 120 h). In addition, previous results showed that PspC is necessary for full virulence, but its effect is less pronounced in D39 compared to HB565 (19-fold versus 160-fold virulence attenuation of isogenic *pspC* mutants, respectively) [20]. These differences in virulence may also contribute to explain the limited cross-protection observed upon vaccination with $PspC_{39–261}$ from HB565, since PspC-specific antibodies may not have been sufficient to prevent a fatal outcome by the more virulent strains D39 and TIGR4. In addition, other factors such as the capsular serotype or the genetic background may have affected the outcome of infection, as previously reported [5,54]. Future studies with genetically manipulated strains expressing the same PspC allele in different genetic backgrounds, and/or the use of a larger number of challenge strains with different PspC types may clarify the picture.

Deposition of both mouse and human C3 onto the surface of HB565 was significantly enhanced by the sera of vaccinated mice, demonstrating that complement activation occurred by PspC-specific antibodies. Deposition assays with MgEGTA and MgEDTA showed that the CP is the dominant pathway triggered by antibodies to PspC. Ren et al. also demonstrated that the effect of PspA antibodies on complement activation is through the CP [3]. PspC antibodies also showed moderate bactericidal properties (30% reduction of viable counts) against the unencapsulated derivative of HB565 in an *ex vivo* opsonophagocytic killing assay using fresh mouse blood. This finding is in agreement with recently published data showing that anti-PspA antibodies also promoted similar rates (30–40%) of killing of *S. pneumoniae* by peritoneal phagocytes [55]. Binding of fH varies between pneumococcal isolates [33,34] and it has been mapped in some PspC allelic types [17,30,31,35,36]. $PspC_{39–261}$ contains three putative fH-binding sites [35], and fH binding by HB565 is higher than that of D39 and TIGR4 ([34]; this work). PspC-specific antibodies neither enhanced C3 deposition nor interfered with fH binding in D39 and TIGR4, in accordance with the limited cross-protection observed. In contrast, fH binding by HB565 was significantly diminished by the addition of antibodies to PspC. Thus, in mice challenged with HB565, PspC antibodies may act by two complement-mediated mechanisms: (i) directly, by activating complement through the CP, and (ii) indirectly, by interfering with PspC capability of binding fH, which leads to reduced inactivation of C3b through the AP. As a result, opsonophagocytosis of HB565 is enhanced, and this may lead to increased mouse survival. This notion, however, relies upon the assumption that PspC binds murine fH, and that this contributes to pneumococcal virulence in the mouse model. Different research groups have reported conflicting results, and the binding of mouse fH to PspC was described to be lower [32] or absent [56] in comparison with human fH. However, virulence analysis of PspC mutants in C3-deficient mice demonstrated that PspC plays a role in evading complement attack in the murine host, suggesting that the interaction of PspC with fH is not human-specific [21]. An experimental approach that may help clarifying this picture would be the use of an infection model with transgenic mice for human fH [57].

This work corroborates the notion that PspC should be used for the development of protein-based pneumococcal vaccines, possibly in combination with other protein antigens [28,50]. As for the current pneumococcal vaccines which are based on multiple capsular polysaccharides, strategies need to be devised to address the remarkable genetic variability of this surface protein. Screening of PspC allelic variants and fragments for their capability to induce immune responses *in vivo* will help to determine the immunogenic and protective features of PspC, contributing to the development of effective protein-based vaccines against the pneumococcus.

5. Conclusions

The aim of this work is to investigate the vaccine potential of the fH-binding fragment of the PspC protein of *S. pneumoniae*. The fragment was protective against homologous challenge and partially protective against heterologous challenge in a mouse model of sepsis. In addition, anti-PspC antibodies were found to activate the complement classical pathway, improve opsonophagocytosis and inhibit fH binding to the bacterial surface. These features indicate that PspC could be incorporated into a vaccine formulation with other pneumococcal proteins and administered successfully to all age groups in order to protect against pneumococcal disease.

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References

[1] Hosea SW, Brown EJ, Frank MM. The critical role of complement in experimental pneumococcal sepsis. J Infect Dis 1980;142:903–9.
[2] Brown JS, Hussell T, Gilliland SM, Holden DW, Paton JC, Ehrenstein MR, et al. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. Proc Natl Acad Sci USA 2002;99:16969–74.
[3] Ren B, Szalai A, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004;72:114–22.
[4] Kerr A, Paterson GK, Riboldi-Tunnicliffe A, Mitchell TJ. Innate immune defense against pneumococcal pneumonia requires complement component C3. Infect Immun 2005;73:4245–52.
[5] Hyams C, Yuste J, Bax K, Camberlein E, Weiser JN, Brown JS. *Streptococcus pneumoniae* resistance to complement-mediated immunity is dependent on the capsular serotype. Infect Immun 2010;78:716–25.
[6] Pangburn MK. Host recognition and target differentiation by factor H, a regulator of the alternative pathway of complement. Immunopharmacology 2000;49:149–57.
[7] Zipfel PF, Hallström T, Hammerschmidt S, Skerka C. The complement fitness factor H: role in human diseases and for immune escape of pathogens, like pneumococci. Vaccine 2008;26S:I67–74.
[8] Walport MJ. Complement. First of two parts. New Eng J Med 2001;344:1058–66.
[9] Siber G, Klugman KP, Mäkelä HP. Pneumococcal vaccines: the impact of conjugate vaccine. Washington, DC, USA: ASM Press; 2008.
[10] Briles DE, Hollingshead SK, Brooks-Walter A, Nabors GS, Ferguson L, Schilling M, et al. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. Vaccine 2000;18:1707–11.
[11] Swiatlo E, Ware D. Novel vaccine strategies with protein antigens of *Streptococcus pneumoniae*. FEMS Immunol Med Microbiol 2003;38:1–7.
[12] Barocchi MA, Censini S, Rappuoli R. Vaccines in the era of genomics: the pneumococcal challenge. Vaccine 2007;25:2963–73.
[13] Hammerschmidt S, Tilling MP, Wolff S, Vaerman JP, Chhatwal GS. Species-specific binding of human secretory component to SpsA protein of *Streptococcus pneumoniae* via a hexapeptide motif. Mol Microbiol 2000;36:726–36.
[14] Brooks-Walter A, Briles DE, Hollingshead SK. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect Immun 1999;67:6533–42.
[15] Iannelli F, Oggioni MR, Pozzi G. Allelic variation in the highly polymorphic locus *pspC* of *Streptococcus pneumoniae*. Gene 2002;284:63–71.
[16] Cheng Q, Finkel D, Hostetter MK. Novel purification scheme and functions for a C3-binding protein from *Streptococcus pneumoniae*. Biochemistry 2000;39:5450–7.
[17] Janulczyk R, Iannelli F, Sjöholm AG, Pozzi G, Björck L. Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function. J Biol Chem 2000;275:37257–63.
[18] Rosenow C, Ryan P, Weiser JN, Johnson S, Fontan P, Ortqvist A, et al. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. Mol Microbiol 1997;25:819–29.
[19] Jarva H, Janulczyk R, Hellwage J, Zipfel PF, Björck L, Meri S. *Streptococcus pneumoniae* evades complement attack and opsonophagocytosis by expressing the *pspC* locus-encoded Hic protein that binds to short consensus repeats 8–11 of factor H. J Immunol 2002;168:1886–94.
[20] Iannelli F, Chiavolini D, Ricci S, Oggioni MR, Pozzi G. Pneumococcal surface protein C contributes to sepsis caused by *Streptococcus pneumoniae* in mice. Infect Immun 2004;72:3077–80.
[21] Kerr AR, Paterson GK, McCluskey J, Iannelli F, Oggioni MR, Pozzi G, et al. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. Infect Immun 2006;74:5319–24.
[22] Ogunniyi DA, Le Messurier KS, Graham RM, Watt JM, Briles DE, Stroeher UH, et al. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. Infect Immun 2007;75:1843–51.
[23] Peppoloni S, Colombari B, Neglia R, Quaglino D, Iannelli F, Oggioni MR, et al. The lack of pneumococcal surface protein C (PspC) increases the susceptibility of *Streptococcus pneumoniae* to the killing by microglia. Med Microbiol Immunol 2007;195:21–8.
[24] Quin LR, Moore III QC, McDaniel LS. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. Infect Immun 2007;75:2067–70.
[25] Briles DE, Hollingshead SK, Swiatlo E, Brooks-Walter A, Szalai A, Virolainen A, et al. PspA and PspC: their potential for use as pneumococcal vaccines. Microb Drug Resist 1997;3:401–8.
[26] Ogunniyi DA, Woodrow MC, Poolman JT, Paton JC. Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. Infect Immun 2001;69:5997–6003.
[27] Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. Infect Immun 2002;70:2526–34.
[28] Ogunniyi DA, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect Immun 2007;75:350–7.
[29] Dave S, Brooks-Walter A, Pangburn MK, McDaniel LS. PspC, a pneumococcal surface protein, binds human factor H. Infect Immun 2001;69:3435–7.
[30] Dave S, Carmicle S, Hammerschmidt S, Pangburn MK, Mc Daniel LS. Dual roles of PspC, a surface protein of *Streptococcus pneumoniae*, in binding human secretory IgA and factor H. J Immunol 2004;173:471–7.
[31] Lu L, Ma Y, Zhang JR. *Streptococcus pneumoniae* recruits complement factor H through the amino terminus of CbpA. J Biol Chem 2006;281:15464–74.
[32] Quin LR, Carmicle S, Dave S, Pangburn MK, Evenhuis JP, Mc Daniel LS. *In vivo* binding of complement regulator factor H by *Streptococcus pneumoniae*. J Infect Dis 2005;192:1996–2003.
[33] Quin LR, Onwubiko C, Carmicle S, McDaniel LS. Interaction of clinical isolates of *Streptococcus pneumoniae* with human complement factor H. FEMS Microbiol Lett 2006;264:98–103.
[34] Yuste J, Khandavilli S, Ansari N, Muttardi K, Ismail L, Hyams C, et al. The effects of PspC on complement-mediated immunity to *Streptococcus pneumoniae* vary with strain background and capsular serotype. Infect Immun 2010;78:283–92.
[35] Jarva H, Hellwage J, Jokiranta TS, Lehtinen MJ, Zipfel PF, Meri S. The group B streptococcal β and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion. J Immunol 2004;172:3111–8.
[36] Hammerschmidt S, Agarwal V, Kunert A, Haelbich S, Skerka C, Zipfel PF. The host immune regulator factor H interacts via two contact sites with the PspC protein of *Streptococcus pneumoniae* and mediates adhesion to host epithelial cells. J Immunol 2007;178:5848–58.
[37] Dieudonnè-Vatran A, Krentz S, Blom AM, Meri S, Henriques-Normark B, Riesbeck K, et al. Clinical isolates of *Streptococcus pneumoniae* bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. J Immunol 2009;182:7865–77.
[38] Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, et al. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infect Immun 2010;78:2163–72.
[39] Pearce BJ, Iannelli F, Pozzi G. Construction of new unencapsulated (rough) strains of *Streptococcus pneumoniae*. Res Microbiol 2002;153:243–7.
[40] Ali F, Lee ME, Iannelli F, Pozzi G, Mitchell TJ, Read RC, et al. *Streptococcus pneumoniae*-associated human macrophage apoptosis after bacterial internalizazion via complement and Fc-gamma receptors correlates with intracellular bacterial load. J Infect Dis 2003;188:1119–31.
[41] Chiavolini D, Pozzi G, Ricci S. Animal models of *Streptococcus pneumoniae* disease. Clin Microbiol Rev 2008;21:666–85.
[42] Ricci S, Medaglini D, Rush CM, Marcello A, Peppoloni S, Manganelli R, et al. Immunogenicity of the B monomer of the *Escherichia coli* heat-labile

toxin expressed on the surface of *Streptococcus gordonii*. Infect Immun 2000;68:760–6.
[43] Jomaa H, Yuste J, Paton JC, Jones C, Dougan G, Brown JS. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun 2005;73:6852–9.
[44] Ralph P, Moore MA, Nilsson K. Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines. J Exp Med 1976;143: 1528–33.
[45] Tian H, Weber S, Thorkildson P, Kozel TR, Pirofski LA. Efficacy of opsonic and nonopsonic serotype 3 pneumococcal capsular polysaccharide-specific monoclonal antibodies against intranasal challenge with *Streptococcus pneumoniae* in mice. Infect Immun 2009;77:1502–13.
[46] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938;27:493–7.
[47] Finney DJ.Probit analysis. 3rd ed. London, UK: Cambridge University Press; 1971.
[48] Blom AM, Hallström T, Riesbeck K. Complement evasion strategies of pathogens-acquisition of inhibitors and beyond. Mol Immunol 2009;46:2808–17.
[49] Meri S, Jordens M, Jarva H. Microbial complement inhibitors as vaccines. Vaccine 2008;26S:I113–7.
[50] Cao J, Chen D, Xu W, Chen T, Xu S, Luo J, et al. Enhanced protection against pneumococcal infection elicited by immunization with the combination of PspA, PspC, and ClpP. Vaccine 2007;25:4996–5005.
[51] Roche H, Ren B, McDaniel LS, Håkansson A, Briles DE. Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae*. Infect Immun 2003;71:4498–505.
[52] Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes AP, Ren B, et al. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. Infect Immun 2007;75:5930–8.
[53] Moreno AT, Oliveira MLS, Ferreira DM, Ho PL, Darrieux M, Leite LC, et al. Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. Clin Vaccine Immunol 2010;17:439–46.
[54] Melin M, Trzcinski K, Meri S, Kaythy H, Vakevainen M. The capsular serotype of *Streptococcus pneumoniae* is more important than the genetic background for resistance to complement. Infect Immun 2010;78:5262–70.
[55] Goulart C, Darrieux M, Rodriguez D, Pimenta FC, Brandileone MC, de Andrade AL, et al. Selection of a family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. Vaccine 2011;29:1634–42.
[56] Lu L, Ma Z, Jokiranta TS, Whitney AR, DeLeo FR, Zhang J-R. Species-specific interaction of *Streptococcus pneumoniae* with human complement factor H. J Immunol 2008;181:7138–46.
[57] Beernink PT, Shaughnessy J, Braga EM, Liu Q, Rice PA, Ram S, et al. A meningococcal factor H binding protein mutant that eliminates factor H binding enhances protective antibody responses to vaccination. J Immunol 2011;186:3606–14.
[58] Bernheimer HP, Wermundsen IE. Unstable binary capsulated transformants in *Pneumococcus*. J Bacteriol 1969;98:1073–9.
[59] Lanie JA, Ng WL, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ, et al. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J Bacteriol 2007;189:38–51.
[60] Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, et al. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science 2001;293:498–506.