FULL TITLE: Polysaccharide-specific memory B-cells predict protection against experimental human pneumococcal carriage

RUNNING TITLE: Memory B-cells predict protection against EHPC

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ABSTRACT

RATIONALE: We have previously demonstrated that experimental pneumococcal carriage enhances immunity and protects healthy adults against carriage reacquisition following re-challenge with a homologous strain. Here we have used a heterologous challenge model to investigate the role of naturally acquired pneumococcal protein and polysaccharide (PS)-specific immunity in protection against carriage acquisition.

METHODS: We identified healthy volunteers that were naturally colonised with pneumococcus and, following clearance of their natural carriage episode, challenged them with a heterologous 6B strain. In another cohort of volunteers we assessed 6BPS-specific, PspA-specific and PspC-specific IgG and IgA plasma and memory B-cell populations prior to and 7, 14 and 35 days following experimental pneumococcal inoculation.

RESULTS: Heterologous challenge with 6B resulted in 50% carriage among volunteers with previous natural pneumococcal carriage. Protection from carriage was associated with a high number of circulating 6BPS IgG-secreting memory B-cells at baseline. There were no associations between protection from carriage and baseline levels of 6BPS IgG in serum or nasal wash, PspA-specific or PspC-specific memory B-cells or plasma cells. In volunteers who did not develop carriage, the number of circulating 6BPS memory B-cells decreased and the number of 6BPS plasma cells 7 days post inoculation.

CONCLUSIONS: Our data indicate that naturally acquired polysaccharide-specific memory B-cells, but not levels of circulating IgG at time of pneumococcal exposure, are associated with protection against carriage acquisition.

INTRODUCTION

Pneumococcal carriage is a pre-requisite of infection and the primary reservoir for transmission (1, 2). Carriage is also an important immunising event in healthy adults and we have previously demonstrated that experimental carriage protects against reacquisition of carriage after reinoculation with the same strain (homologous) for up to eleven months following clearance of the first carriage episode (3).

Animal models have demonstrated that immunoglobulins play a role in host defence against carriage acquisition (4) and IL-17 secreting $CD4^+$ T-cells play a role in the clearance of carriage (5). While these immunological responses are well described in murine models, the immunological responses which mediate protection in humans have not been identified. We have previously observed that pneumococcal colonisation increases the level and functional capacity of pneumococcal-specific IgG and IgA in serum and nasal wash, and that colonisation also increases the number of pneumococcal-specific IL-17 secreting $CD4^+$ T-cells in the lung (3, 6, 7).

Epidemiological data supports the role of polysahccaride (PS)-mediated immunity in protection against carriage. Vaccination with pneumococcal conjugate vaccine (PCV) has reduced carriage of vaccine type strains (8), and has resulted in serotype replacement in vaccinated communities (9, 10). We have recently demonstrated the direct impact of PCV vaccination on pneumococcal carriage acquisition (11). In a double-blind randomised control trial, the rate of carriage acquisition, following challenge with 6B pneumococcus, was reduced by 78% in PCV vaccinated volunteers compared with control vaccinated volunteers. We have previously demonstrated that baseline levels of both PS-specific IgG and capacity for opsonphagocytic killing did not predict carriage outcome following intranasal pneumococcal inoculation (3, 6, 12).

PS-specific memory B-cells (B_{MEM}) are not present in the mucosal immune tissue of the human nasopharynx (13). This suggests that the localised stimulation and differentiation of B_{MEM} into plasma

cells (B_{PLAS}) is unlikely to occur *in situ* at the mucosa, and that PS-specific immunoglobulin from systemic circulation may instead mediate protection against carriage acquisition at the mucosal surface.

Here we identified healthy volunteers that were naturally colonised with pneumococcus and 9 to 16 months following clearance of their carriage episode challenged them with a heterologous pneumococcal serotype, resulting in a 50% rate of carriage. We have previously demonstrated 0% carriage reacquisition up to 11 months following a carriage episode when re-challenged with the same strain (3). We further assessed B-cell-mediated immunity among volunteers with no current pneumococcal carriage and an unknown history of previous pneumococcal carriage exposure. We observed that volunteers protected from carriage (carriage-negative) had similar levels of circulating IgG but higher number of PS-specific B_{MEM} than volunteers susceptible to carriage (carriage-positive). Responses to protein-based antigens were not associated with protection against carriage. Taken together these data suggest that naturally acquired PS-specific immunity plays a prominent role in mediating protection against acquisition of pneumococcal carriage in unvaccinated volunteers.

MATERIALS AND METHODS

Ethical approval, study protocol and sampling

8 healthy non-smoking adult volunteers aged between 18 and 60 years, who had a confirmed previous natural carriage episode, and who had no close contact with risk individuals were recruited to cohort A. 24 healthy non-smoking adults aged between 18 and 60 years, who had no current pneumococcal carriage and an unknown history of pneumococcal exposure, and who had no close contact with at-risk individuals were recruited to cohort B. Written informed consent was obtained from all volunteers. Both studies were approved by the National Research Ethics Service (11/NW/0592) and were sponsored by the Royal Liverpool and Broadgreen University Hospitals Trust.

In cohort A, each volunteer was inoculated with 35375 ± 2651 colony-forming units (cfu) per naris of *S. pneumoniae* serotype 6B (strain BHN418) (14) (Table 1). In cohort B, each volunteer was inoculated with 61944 ± 4603 cfu/naris of *S. pneumoniae* serotype 6B (Table 1). Bacterial preparation and inoculation, as well as nasal wash sampling and carriage detection were performed as previously described (15). In cohort A, nasal wash samples were obtained 5 days prior to intranasal inoculation and then on days 2, 7 and 14 following inoculation. Peripheral blood samples were obtained 5 days prior to intranasal inoculation and then 14 days following inoculation. In cohort B, nasal wash samples were obtained 5 days prior to intranasal inoculation and then on days 2, 7, 14, 21, 28 and 35 following inoculation. Peripheral blood samples were obtained 5 days prior to intranasal inoculation and then on days 7, 14 and 35 days following inoculation.

Anti-pneumococcal capsular polysaccharide ELISA

Anti-pneumococcal capsular polysaccharide (PS) antibodies were determined using the World Health Organisation internationally standardised method and reagents (16). For full details, please refer to Supplementary Materials and Methods.

Opsonophagocytic killing assay (OPKA)

OPKA assays were performed as previously described(3), with minor modifications. For full details, please refer to Supplementary Materials and Methods.

Preparation of Enzyme-linked immunosorbent spot (ELISpot) plates

Wells of ELISpot plates (multiscreen IP 96-well filter plates; Millipore) were coated with 100µL Dulbecco's $PBS^{-/-}$ (DPBS) supplemented with either anti-IgG (1:50; total IgG), anti-IgA (1:50; total IgA) 20µg/mL 6BPS, 2µg/mL PspA, 2µg/mL PspC, influenza (1:50; positive control, unpublished data) or unsupplemented DPBS (negative control). Coated plates were stored at 4˚C for up to 48 hours. 24 hours prior to use, ELISpot plates were brought to room temperature and washed six times with DPBS. Plates were blocked with RPMI-1640 supplemented with 2% BSA for two hours. Plates were then washed six times with DPBS.

Plasma cell (B_{PLAS}) detection

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque®-1077 (Sigma-Aldrich), according to the manufacturers instructions. For full details, please refer to Supplementary Materials and Methods. Cells were seeded in ELISpot plates in 100µL volumes, in complete medium, in triplicate, at a concentration of either 5×10^6 cells/mL (6BPS, PspA, PspC) or 5×10^5 cells/mL (total IgG, total IgA, positive control, negative control). Plates were incubated for sixteen hours at 37°C in 5% CO_2. For detection of IgG, plates were washed six times with 0.05% PBS-tween and incubated with 1:2,000 anti-human-IgG-alkaline-phosphatase (Sigma-Aldrich) for two hours. For detection of IgA, plates were washed six times with 0.05% PBS-tween and incubated with 1:2,000 anti-human-IgA (AbD Serotec) for two hours. Plates were then washed a further six times with 0.05% PBS-tween and incubated with 1:2,000 streptavidin:alkaline phosphatase (AbD Serotec) for one hour. For detection of both IgG and IgA, plates were washed six times with 0.05% PBS-tween and incubated with 100µL

p-nitrophenyl phosphate (*p*NPP; Sigma-Aldrich). Plates were washed six times with distilled H_2O and then stored in the absence of light.

Memory B-cell (B_{MEM}) detection

2.5×10^6 cells/mL were seeded in 6-well plates, in complete medium supplemented with 1:2,500 *Staphylococcus aureus* Cowan strain (SAC), 1:3,000 pokeweed mitogen (PWM; Sigma-Aldrich), and 1:40 CpG oligonucleotide (ODN-2006; TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT; InvivoGEN). Plates were incubated for seven days at 37°C in 5% CO_2. Cells were harvested, washed in complete medium and the cell concentration determined using an Improved Neubauer Haemocytometer (CamLab). Cells were then seeded in ELISpot plates in 100µL volumes, in complete medium, in triplicate, at a concentration of either 5×10^6 cells/mL (6BPS, PspA, PspC) or 5×10^5 cells/mL (total IgG, positive control, negative control). Plates were incubated for sixteen hours at 37°C in 5% CO_2. Plates were washed six times with 0.05% PBS-tween and incubated with 1:2,000 anti-human-IgG-alkaline-phosphatase for two hours. Plates were washed six times with 0.05% PBS-tween and incubated with 100µL *p*NPP (Sigma-Aldrich). Plates were washed six times with distilled H_2O and then stored in the absence of light.

ELISpot counting

ELISpot data were acquired using an AID ELISpot Reader (ELR02) and the data analysed using AID ELISpot Software v3.3 (Cadama Medical). Identical settings were used for the analysis of all wells. All plates were manually verified and any artefacts removed by an operator who was blinded for the ELISpot condition, the carriage status of each volunteer and the sampling time point.

Statistical analyses

All statistical analyses were performed using SPSS v22 (IBM) and all *P* values are two-tailed. Where appropriate, data were logarithmically transformed for purposes of statistical analyses. Comparisons were made between carriage-positive and carriage-negative groups using unpaired *t* tests;

differences were considered significant at $P \leq 0.05$. Multiple comparisons were made within carriage-positive and carriage-negative groups using paired *t* tests with manual Bonferroni correction (significance level $\alpha=0.05$ with number of tests n=3); differences were considered significant at $P<0.0166$.

RESULTS

Natural episodes of pneumococcal carriage do not protect against heterologous challenge

In order to determine the role of naturally acquired serotype-specific immunity in protection against carriage acquisition we identified a cohort of 8 healthy volunteers (cohort A) that were naturally colonised with pneumococcus and, following clearance, experimentally challenged them with a heterologous strain.

We observed that heterologous inoculation with 6B pneumococcus 9 to 16 months following clearance of natural carriage resulted in 50% carriage (Table 1 and 2). In another cohort of 24 volunteers (cohort B), with no current pneumococcal carriage and unknown history of pneumococcal exposure, inoculation with 6B pneumococcus resulted in 42% carriage (Table 1).

This is consistent with the 40-60% carriage rates observed in our previous studies where uncolonised volunteers were inoculated with doses of 6B pneumococcus ranging between 40,000-130,000 cfu/naris (3).

Levels of 6BPS IgG and IgA in serum and nasal wash are not associated with protection against carriage acquisition

We assessed levels of pneumococcal antibody and B-cells in cohort B. Levels of 6BPS IgG in serum at baseline (day -5) were similar in carriage-positive volunteers (those that were susceptible to carriage) and carriage-negative volunteers (those that were protected against carriage) (Figure 1A; median [interquartile range (IQR)]: 1.0μg/mL [0.6-1.6] versus 0.9μg/mL [0.5-1.4]). In carriage-positive volunteers, post inoculation levels of 6BPS IgG were comparable at day 7 (1.1μg/mL [0.6-2.0]), but were significantly higher at day 14 (1.3μg/mL [0.8-3.8]; *P*=0.001) and day 35 (2.5μg/mL [1.2-5.8]; *P*=0.001) than at baseline. In carriage-negative volunteers levels of 6BPS IgG in serum following inoculation were comparable to those at baseline. Levels of 6BPS IgG in nasal wash at baseline were similar in carriage-positive volunteers and carriage-negative volunteers (Figure 1B;

1.0ng/mL [0.8-2.4] versus 0.7ng/mL [0.6-0.8]). In both groups levels of 6BPS IgG in nasal wash following inoculation were comparable to those at baseline.

Levels of 6BPS IgA in serum at baseline were higher in carriage-positive volunteers than in carriage-negative volunteers (Figure S1A; 0.5µg/mL [0.4-0.6] versus 0.3µg/mL [0.2-0.4]; *P*=0.001). In carriage-positive volunteers, post inoculation levels of 6BPS IgA were comparable at day 7 (0.5µg/mL [0.4-0.6]), significantly higher day 14 (0.8µg/mL [0.5-1.9]; *P*=0.005) but were not significantly higher at day 35 (0.7µg/mL [0.5-1.5]) than at baseline. In carriage-negative volunteers levels of 6BPS IgA in serum following inoculation were comparable to those at baseline. Levels of 6BPS IgA in nasal wash were similar in carriage-positive volunteers and carriage-negative volunteers between both groups at baseline (Figure S1B; 18.3ng/mL [12.0-45.0] versus 14.0ng/mL [6.9-16.4]) and were unchanged following challenge.

We assessed whether the capcity of antibodies in mediating phagocytosis was associated with protection. No difference was between carriage-negative volunteers compared with carriage-positive volunteers at baseline (Figure S2).

High baseline number of 6BPS IgG-secreting B_{MEM} is associated with protection against carriage acquisition

At baseline, the number of 6BPS IgG-secreting B_{MEM} in carriage-positive volunteers was approximately three-times lower than that in carriage-negative volunteers (Figure 2A; 12.3 [9.4-25.4] versus 32.1 [14.9-86.4]; *P*=0.042). Post inoculation, in carriage-positive volunteers, the numbers of 6BPS IgG-secreting B_{MEM} at day 7 (15.0 [4.4-46.8]), day 14 (15.6 [5.4-24.9]) and day 35 (7.0 [5.6-30.0]) was comparable to those at baseline. In carriage-negative volunteers, the number of 6BPS IgG-secreting B_{MEM} was not significantly lower at day 7 (8.3 [5.7-16.4]) or day 14 (11.8 [8.4-26.7]; *P*=0.03) but was significantly lower at day 35 (5.9 [3.4-11.4]; *P*=0.012) compared to baseline.

At baseline, the number of 6BPS IgG-secreting B_{PLAS} was not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure 2B; 3.0 [1.8-37.8] versus 5.3 [0.5-14.9]). Post inoculation, in carriage-positive volunteers, the number of 6BPS IgG-secreting B_{PLAS} were not significantly different to those at baseline. In carriage-negative volunteers, the number of 6BPS IgG-secreting B_{PLAS} was approximately four-times higher at day 7 (83.5 [45.6-135.3]; *P*=0.006) compared to baseline, returning to a comparable level at day 14 (3.3 [1.0-9.0]) and day 35 (7.3 [2.5-13.3]).

Baseline number of PspA and PspC IgG-secreting B_{MEM} are not associated with protection against carriage acquisition

At baseline, the number of PspA IgG-secreting B_{MEM} was not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure 3A). In both groups numbers of PspA IgG-secreting B_{MEM} following inoculation were comparable to those at baseline.

At baseline, the number of PspA IgG-secreting B_{PLAS} was not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure 3B; 25.5 [9.8-36.2] versus 13.8 [4.5-71.8]). Post inoculation, in carriage-positive volunteers, the number of PspA IgG-secreting B_{PLAS} was not significantly higher at day 7 (50.7 [31.0-84.7]) or day 14 (79.2 [25.8-141.6]) but was significantly higher at day 35 (74.8 [67.7-79.6]; *P*=0.005) compared to baseline. In carriage-negative volunteers the numbers following inoculation were comparable to those at baseline.

At baseline, the number of PspC IgG-secreting B_{MEM} was not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure 4A). In both groups the numbers following inoculation were comparable to those at baseline.

At baseline, the number of PspC IgG-secreting B_{PLAS} was not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure 4B; 3.2 [1.7-40.6] versus 9.5 [4.1-15.1]). Post inoculation, in carriage-positive and carriage-negative volunteers, the number of PspC IgG-secreting B_{PLAS} were not significantly different to those at baseline.

Baseline number of PS IgG-secreting B_{MEM} are not associated with levels of PS IgG in serum

Since we observed that a high baseline number of 6BPS IgG-secreting B_{MEM} was associated with protection, and that the number of IgG B_{PLAS} were increased following inoculation in carriage negative volunteers, we analysed whether the number of 6BPS IgG-secreting B_{MEM} at baseline or the number of 6BPS IgG-secreting B_{PLAS} at day 7 were associated with increased levels of 6BPS IgG in serum post inoculation.

Unadjusted linear regression analysis revealed that the baseline number of 6BPS IgG-secreting B_{MEM} did not correlate with the area under the curve (AUC) of 6BPS IgG in serum (data not shown). There was also no correlation between the increase in 6BPS IgG-secreting B_{PLAS} at day 7 and the AUC of 6BPS IgG or the absolute number of 6BPS IgG in serum at any time point (data not shown).

No correlation was observed between the increase in 6BPS IgG B_{PLAS} at any time point or AUC of 6BPS IgG and colonisation intensity (density and duration; AUC) over the 35 day study period (data not shown).

Baseline number of 6BPS, PspA and PspC IgA-secreting B_{MEM} or B_{PLAS} are not associated with protection against carriage acquisition

At baseline, the number of 6BPS IgA-secreting B_{MEM} and B_{PLAS} were not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure S3A). In both groups the numbers of 6BPS IgA-secreting B_{MEM} and B_{PLAS} following inoculation were comparable to those at baseline.

At baseline, the number of PspA IgA-secreting B_{MEM} and B_{PLAS} were not significantly different in carriage-positive volunteers compared with carriage-negative volunteers (Figure S4A; 2.7 [1.5-4.7] versus 3.9 [1.4-4.4] and Figure S2B; 26.9 [5.0-47.5] versus 6.1 [4.2-10.6]). Post-incoulation, in carriage-positive and carriage-negative volunteers, the number of PspA IgA-secreting B_{PLAS} were not significantly different to those at baseline.

At baseline, the number of PspC IgA-secreting B_{MEM} was not different in carriage-positive volunteers compared with carriage-negative volunteers (Figure S5A). In both groups the numbers following inoculation were comparable to those at baseline.

At baseline, the number of PspC IgA-secreting B_{PLAS} was higher in carriage-positive volunteers than in carriage-negative volunteers (Figure S5B; 46.5 [13.3-103.8] versus 4.1 [3.0-9.4]; *P*=0.025). Post-inoculation, in carriage-positive and carriage-negative volunteers, the number of PspC IgA-secreting B_{PLAS} were not significantly different to those at baseline.

DISCUSSION

Using our experimental human pneumococcal carriage model and heterologous challenge of previously colonized volunteers we have demonstrated that PS-specific, but not protein-specific, B-cell mediated immunity is associated with protection against carriage acquisition. Specifically, we observed that a high number of PS IgG-secreting B_{MEM} rather than PS6B IgG levels was associated with protection against carriage.

It is well established that antibody-mediated immune responses targeting serotype-specific polysaccharides protect against pneumococcal disease (17). Serotype replacement, which has been observed following the introduction of PCV, highlights the role of PS-mediated immunity in protection against carriage (8, 11). We have previously demonstrated 0% carriage acquisition following re-challenge with a homologous strain up to 11 months following clearance of the first carriage episode (3). Here we demonstrate that heterologous challenge results in a 50% rate of carriage, which is similar to the carriage rates observed in our previous studies in which volunteers with no current pneumococcal carriage and an unknown history of previous pneumococcal exposure were challenged with a 6B strain (3, 11). This supports the hypothesis that PS-specific immunity plays an important role in protection against carriage.

Following on from heterologous challenge experiments, we assessed B-cell mediated PS-specific immunity in another cohort of 24 volunteers with no current pneumococcal carriage and an unknown history of previous pneumococcal exposure. There was no association between the functional capacity of serum antibody, nor number of PspA- or PspC B_{MEM} or B_{PLAS} and protection against carriage. We did, however, observe that a high number of circulating PS IgG-secreting B_{MEM} at baseline was associated with protection against experimental carriage acquisition.

Among volunteers protected against carriage, the number of circulating PS IgG-secreting B_{MEM} was dramatically reduced and the number of PS-specific IgG-secreting B_{PLAS} increased 7 days post

289 inoculation. Expansion of the PS IgG-secreting B_{PLAS} population was relatively short-lived and
290 returned to baseline levels 14 days following inoculation. These data are consistent with a rapid
291 differentiation of PS IgG-secreting B_{MEM} into short-lived B_{PLAS}. A similar effect has been observed and
292 implicated in immunity to malaria (21).

293 Among carriage-negative volunteers, the increased number of circulating PS IgG-secreting B_{PLAS} was
294 not accompanied by increased levels of PS IgG in serum or nasal wash. For this reason we
295 hypothesise that antibodies produced by these plasma cells bind to the inoculated bacteria in the
296 nasopharynx, facilitating bacterial agglutination, impairing bacterial adherence and leading to
297 protection from colonisation acquisition. In carriage-positive volunteers, levels of PS IgG and IgA in
298 serum were higher at day 14 than at baseline – this response is likely generated through
299 immunisation by colonisation rather than an acute response to inoculation. We observed that
300 baseline levels of PS IgA in serum were higher in volunteers that were susceptible to colonisation.
301 We have previously observed a similar association between susceptibility and increased levels of IgG
302 to LytC, PcsB SP0609, SPR0057 and SPR2021 (3). This may be an indication of an underlying
303 suceptability to pneumocccal carriage, with elevated serum immunoglobulin indicative of repeated
304 exposure.

305 While it is well established that repeated exposure to pneumococci is required for the maintenance
306 of IgG-secreting B_{MEM} populations, more recently it has been suggested that exposure during life also
307 enhances the adult immune response to PCV (22). A similar effect has been observed in the UK,
308 where lack of *Haemophilus influenzae* type B carriage led to an increased incidence of disease
309 among children who received a single dose of Hib vaccine(23).

310 Based on data presented here, we postulate that: [1] pneumococcal nasal inoculation results in the
311 rapid expansion of pre-existing PS-specific B_{MEM} into short-lived IgG-secreting B_{PLAS}. Expansion of B_{PLAS}
312 results in increased PS-specific antibody production; these antibodies bind to the inoculated bacteria
313 at the nasopharynx resulting in bacterial agglutination and protection from colonisation acquisition.

[2] Although pneumococcal colonisation is immunising, adsorption of antibody by bacteria in the nasopharynx may prevent the detection of increased antibody production promoted by exposure to pneumococcus. [3] Only if colonisation is established B_{PLAS} continue to circulate in high number, continued stimulation and antibody production eventually leads to the detection of increased PS-specific serum IgG as the bacteria are cleared from the nasopharynx. We are currently investigating the impact of nasal antibody-mediated agglutination and its role in protection against pneumococcal carriage acquisition in PCV vaccinated volunteers.

IgA plays a prominant role in defence at the mucosal surface; it is involved in controlling invasive pneumococcal diseases (24) and the regulation of *S. pneumoniae* colonisation in the nasal cavity of mice (25). We did not observe an association between the number in 6BPS IgA-secreting B-cell populations and protection against carriage acquisition, indicating that the peripheral assessment of IgA-secreting populations may not reflect the activity of IgA populations at the mucosa. IgA1 accounts for approximately 90% of all IgA found in peripheral blood and the upper respiratory tract (18, 19). *Streptococcus pneumoniae* is able to cleave IgA1, via IgA1 protease, a mechanism that facilitates pneumococcal adherence to the epithelium and abrogates the protective effects of IgA (20). As a result, we hypothesise that protection against colonisation was associated with PS IgG-secreting B_{MEM} populations rather than PS IgA-secreting B_{MEM} populations due to the advantage conferred through the generation of IgG rather than IgA based responses.

The strength of this study is the use of a unique experimental human pneumococcal challenge model with multiple assessments of B-cell populations in blood, and antibodies in serum and in nasal wash samples; this model allows us to study carriage and its associated immunological responses with a known exposure time point. One weakness of this study is that our results propose an important mechanism of protection but do not exclude the possibility that other immune cell populations, or responses targeting alternate antigens may also contribute towards protection against carriage acquisition.

Data presented here has important implications for the future of pneumococcal vaccine development, as it is likely that PS-mediated immunity is required for high levels of protection against carriage acquisition. Several protein-based vaccines are currently in development and it will be important to assess whether these vaccines will impact carriage both directly, in immunised volunteers, and indirectly, via a herd effect, as observed for PCV vaccines (8, 11, 26). The role of T-cell responses in the control of carriage density and in clearance of carriage suggests that protein-based vaccines capable of inducing this type of immunity may offer several advantages over currently licensed vaccines, facilitating control of carriage intensity (density and duration), whilst maintaining the opportunity for immunisation through natural carriage acquisition (5, 27, 28). Specifically, it is possible that protein-based vaccines could prevent pneumonia and invasive pneumococcal disease while maintaining the opportunity for further immunisation during natural episodes of carriage. We have previously demonstrated the protective effect of PCV-13 on pneumococcal carriage (11). We have now demonstrated that PS-mediated naturally acquired immunity is a key mediator of protection against carriage acquisition.

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Figure 1: Kinetics of polysaccharide 6B IgG. IgG specific ELISAs in serum (**A**) and nasal wash (**B**) were performed against pneumococcal 6B polysaccharide (PS6B). Levels of antibody were determined using serum and nasal wash samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Nasal wash samples were obtained prior (day -5) and post inoculation (day 7, day 2, day 14 and day 35). Serum samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35). The box in the upper right section shows data collected prior to (day -5) and post (day 14) challenge for those volunteers who underwent heterologous challenge (cohort A). Horizontal bars represent median values and bars represent the interquartile range.

Figure 2. Kinetics of polysaccharide 6B IgG-producing B-cell populations. ELISpots were performed against pneumococcal polysaccharide 6B (PS6B). The number of IgG-producing memory B-cells (B_{MEM}) (**A**) and plasma cells (B_{PLAS}) (**B**) were determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35) from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values and bars represent the interquartile range.

Figure 3. Kinetics of PspA IgG-producing B-cell populations. ELISpots were performed against PspA. The number of IgG-producing memory B-cells (B_{MEM}) (**A**) and plasma cells (B_{PLAS}) (**B**) were determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35) from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values and bars represent the interquartile range.

Figure 4. Kinetics of PspC IgG-producing B-cell populations. ELISpots were performed against PspC. The number of IgG-producing memory B-cells (B_{MEM}) (**A**) and plasma cells (B_{PLAS}) (**B**) were determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers.

Samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35) from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values and bars represent the interquartile range.

Supplementary Figure 1. Kinetics of polysaccharide 6B IgA. IgA specific ELISAs in serum (**A**) and nasal wash (**B**) were performed against pneumococcal 6B polysaccharide (PS6B). Levels of antibody were determined using serum and nasal wash samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Nasal wash samples were obtained prior (day -5) and post inoculation (day 7, day 2, day 14 and day 35). Serum samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35). The box in the upper right section shows data collected prior to (day -5) and post (day 14) challenge for those volunteers who underwent heterologous challenge (cohort A). Horizontal bars represent median values and bars represent the interquartile range.

Supplementary Figure 2. Opsonophagocytic activity of serum antibodies at baseline against 6B. The functional capacity of antibody against *Streptococcus pneumoniae* 6B was determined using heat-inactivated serum samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. The percentage of killing is shown for 3 (1 in 3, 1 in 9 and 1 in 27) . Horizontal bars represent median values and bars represent the interquartile range.

Supplementary Figure 3. Kinetics of polysaccharide 6B IgA-producing B-cell populations. ELISpots were performed against pneumococcal polysaccharide 6B (6BPS). The number of IgA-producing memory B-cells (B_{MEM}) (**A**) and plasma cells (B_{PLAS}) (**B**) were determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35) from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values and bars represent the interquartile range.

Supplementary Figure 4. Kinetics of PspA IgA-producing B-cell populations. ELISpots were performed against PspA. The number of IgA-producing memory B-cells (B_{MEM}) (**A**) and plasma cells (B_{PLAS}) (**B**) were determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35) from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values and bars represent the interquartile range.

Supplementary Figure 5. Kinetics of PspC IgA-producing B-cell populations. ELISpots were performed against PspC. The number of IgA-producing memory B-cells (B_{MEM}) (**A**) and plasma cells (B_{PLAS}) (**B**) were determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained prior (day -5) and post inoculation (day 7, day 14 and day 35) from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values and bars represent the interquartile range.

501 **Table 1: Participants of heterologous challenge and traditional challenge cohorts**
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Study	Number of Volunteers	Age (years)	Sex (M:F)	Study Period	Dose (cfu/naris)	Experimental Carriage Rate
Cohort A (heterologous challenge)	8	28 ± 14	4:4	Feb 2013 - Mar 2013	35375 ± 2651	50% (4/8)
Cohort B (challenge)	24	23 ± 3	9:15	Sep 2012- Nov 2012	61944 ± 4603	42% (10/24)

503 **Table 2: Pre-existing serotypes and carriage status of heterologous challenge cohort (cohort A)**

Subject	Serotype	Carriage	Time between carriage episode and re-challenge (months)
1	6	No	14
2	3	No	11
3	33	No	9
4	3	No	13
5	33	Yes	16
6	19	Yes	11
7	15	Yes	14
8	33	Yes	11

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SUPPLEMENTARY MATERIALS AND METHODS

Anti-pneumococcal capsular polysaccharide ELISA

Anti-pneumococcal capsular polysaccharide (PS) antibodies were determined by using the WHO internationally standardised method and reagents (16). Briefly, 96-well ELISA plates were coated using 5µg/mL of purified 6BPS (Statens Serum Institute) for 5 hours at 37°C. Wells were blocked with 10% foetal bovine serum in PBS (PBS-F) for 1 hour at 37°C. Plates were washed 3 times with PBS containing 0.05% Tween-20 between each step. Samples were diluted in PBS-F containing 10µg/mL CWPS Multi (Statens Serum Institute) and incubated for 30 minutes at 37°C. 89-SF5 reference serum received from U.S. Food and Drug Administration was used as a standard. Diluted/adsorbed samples were then transferred to pre-coated plates and incubated overnight at 4°C. Antibody detection was performed as described for anti-pneumococcal protein ELISA. All samples were run in triplicate in four dilutions, and samples with a CV of greater than 15% were repeated. Results are expressed as µg/mL calculated using the assigned IgG concentrations in reference serum 89-SF5.

Opsonophagocytic killing assay (OPKA)

Heat-inactivated serum samples were diluted 1:3, 1:9 and 1:27 in opsonisation buffer B (OBB). A control standard was created using heat-inactivated 89SF serum diluted 1:3, 1:9, 1:27, 1:81, 1:243, 1:729, 1:2,187 and 1/6561 in OBB. Serum and control samples were then added to the wells of a 96-well U-bottom plate (Costar). *Streptococcus pneumoniae* 6B were washed, resuspended in Hanks' Balanced Salt Solution $(HBSS)^{+/+}$, and added to wells, as required, at concentration of 1×10^3 cfu/well. Plates were then incubated at 37°C for 20 minutes with shaking (300 r.p.m). 10% complement and 1×10^5 THP- 1 cells were added separately to wells, as required (multiplicity of infection 100:1 [cells:bacteria]). Plates were then incubated at 37°C for 60 minutes with shaking (300 r.p.m).

Control wells were included which contained THP-1 cells, bacteria and complement, without a source of antibody. Additional control wells containing heat-inactivated complement were also included.

Following incubation, plates were placed on ice for 10 minutes. 10µl of the suspension from each reaction was then plated in triplicate onto horse blood agar plates (Oxoid). Plates were then incubated overnight at 37°C with 5% CO_2. The serum killing index was determined by comparing cfu recovered from serum treated wells with that from control wells.

Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood samples were collected in lithium heparin Vacutainers™ (BD Biosciences). Samples were diluted with an equal volume of Dulbecco's $PBS^{-/-}$ (DPBS; Invitrogen) and PBMCs isolated by differential centrifugation using Histopaque®-1077 (Sigma-Aldrich). Cells were washed twice in DPBS, re-suspended in complete medium (RPMI-1640 supplemented with 10% FBS and 2% 200mM L-glutamine; Invitrogen), and the cell concentration determined using an Improved Neubauer Haemocytometer (CamLab). Samples were stored on ice for use in downstream applications.