Polysaccharide-Specific Memory B Cells Predict Protection against Experimental Human Pneumococcal Carriage

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Abstract

Rationale: We have previously demonstrated that experimental pneumococcal carriage enhances immunity and protects healthy adults against carriage reacquisition after rechallenge with a homologous strain.

Objectives: To investigate the role of naturally acquired pneumococcal protein and polysaccharide (PS)-specific immunity in protection against carriage acquisition using a heterologous challenge model.

Methods: We identified healthy volunteers that were naturally colonized with pneumococcus and, after 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 before and 7, 14, and 35 days after experimental pneumococcal inoculation.

Measurements and Main 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 increased postinoculation.

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

Keywords: pneumococcal carriage; pneumococcal polysaccharide; memory B cells; plasma B cells; antibodies

Pneumococcal carriage is a prerequisite of infection and the primary reservoir for transmission (1, 2). Carriage is also an important immunizing 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 11 months after clearance of the first carriage episode (3).

Animal models have demonstrated that immunoglobulins play a role in host

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At a Glance Commentary

Scientific Knowledge on the

Subject: Animal models have demonstrated that immunoglobulins play a role in host defense against carriage acquisition. Using our experimental model of human pneumococcal carriage, we have demonstrated that preexisting levels of antibody to pneumococcal proteins and capsular polysaccharide were not associated with protection against pneumococcal colonization.

What This Study Adds to the

Field: Here we present data that demonstrate that naturally acquired polysaccharide-specific memory B cells to capsular polysaccharide of the inoculated strain are associated with protection against carriage acquisition.

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

Epidemiologic data support the role of polysaccharide (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 randomized control trial, the rate of carriage acquisition, after 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 opsonophagocytic killing did not predict

carriage outcome after 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 localized 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 colonized with pneumococcus and 9–16 months after 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 after a carriage episode when rechallenged 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.

Methods

Ethical Approval, Study Protocol, and Sampling

Eight healthy nonsmoking 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. Twenty-four healthy nonsmoking 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 $35,375 \pm 2,651$ cfu per naris of *Streptococcus pneumoniae* serotype 6B (strain BHN418) (Table 1) (14). In cohort B, each volunteer was inoculated with $61,944 \pm 4,603$ 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 before intranasal inoculation and then on Days 2, 7, and 14 after inoculation. Peripheral blood samples were obtained 5 days before intranasal inoculation and then 14 days after inoculation. In cohort B, nasal wash samples were obtained 5 days before intranasal inoculation and then on Days 2, 7, 14, 21, 28, and 35 after inoculation. Peripheral blood samples were obtained 5 days before intranasal inoculation and then on Days 7, 14, and 35 days after inoculation.

Antipneumococcal Capsular PS ELISA

Antipneumococcal capsular PS antibodies were determined using the World Health Organization internationally standardized method and reagents (16). For full details, see METHODS in the online supplement.

Opsonophagocytic Killing Assay

Opsonophagocytic killing assays were performed as previously described (3), with minor modifications (see METHODS in the online supplement).

Preparation of Enzyme-linked Immunospot Assay Plates

Wells of enzyme-linked immunospot assay (ELISPOT) plates (multiscreen IP 96-well filter plates; Merck Millipore, Billerica, MA) were coated with 100 μ l Dulbecco's phosphate-buffered saline^{−/−} (DPBS) supplemented with either anti-IgG (1:50; total IgG), anti-IgA (1:50; total IgA) 20 μ g/ml 6BPS, 2 μ g/ml PspA, 2 μ g/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. Twenty-four hours before use, ELISPOT plates were brought to room temperature and washed six times with DPBS. Plates were blocked with RPMI-1640

Table 1. Participants of Heterologous Challenge and Traditional Challenge Cohorts

Study	Number of Volunteers	Age (yr)	Sex (M:F)	Study Period	Dose (cfu/Naris)	Experimental Carriage Rate
Cohort A (heterologous challenge)	8	28 ± 14	4:4	February 2013–March 2013	35,375 ± 2,651	50% (4/8)
Cohort B (challenge)	24	23 ± 3	9:15	September 2012–November 2012	61,944 ± 4,603	42% (10/24)

supplemented with 2% bovine serum albumin for 2 hours. Plates were then washed six times with DPBS.

Plasma Cell Detection

Peripheral blood mononuclear cells were isolated using Histopaque-1077 (Sigma-Aldrich, St Louis, MO), according to the manufacturer's instructions (*see METHODS* in the online supplement). Cells were seeded in ELISPOT plates in 100- μ l 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 16 hours at 37°C in 5% CO₂. 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 2 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, Raleigh, NC) for 2 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 1 hour. For detection of both IgG and IgA, plates were washed six times with 0.05% PBS-Tween and incubated with 100 μ l *p*-nitrophenyl phosphate (Sigma-Aldrich). Plates were washed six times with distilled H₂O and then stored in the absence of light.

Memory B-Cell Detection

A total of 2.5×10^6 cells/ml were seeded in six-well plates, in complete medium supplemented with 1:2,500 *Staphylococcus aureus* Cowan strain, 1:3,000 pokeweed mitogen (Sigma-Aldrich), and 1:40 CpG oligonucleotide (ODN-2006; TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT; InvivoGEN, San Diego, CA). Plates were incubated for 7 days at 37°C in 5% CO₂. Cells were harvested, washed in complete medium, and the cell concentration

determined using an Improved Neubauer Haemocytometer (CamLab, Cambridge, UK). Cells were then seeded in ELISPOT plates in 100- μ l 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 16 hours at 37°C in 5% CO₂. Plates were washed six times with 0.05% PBS-Tween and incubated with 1:2,000 anti-human IgG-alkaline phosphatase for 2 hours. Plates were washed six times with 0.05% PBS-Tween and incubated with 100 μ l *p*-nitrophenyl phosphate (Sigma-Aldrich). Plates were washed six times with distilled H₂O and then stored in the absence of light.

ELISPOT Counting

ELISPOT data were acquired using an AID ELISPOT reader (ELR02) and the data analyzed using AID ELISPOT software version 3.3 (Cadama Medical, Oxford, UK). Identical settings were used for the analysis of all wells. All plates were manually verified and any artifacts 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 version 22 (IBM, Armonk, NY)

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* less than or equal to 0.05. Multiple comparisons were made within carriage-positive and carriage-negative groups using paired Student's *t* tests with manual Bonferroni correction (significance level $\alpha = 0.05$ with number of tests *n* = 3); differences were considered significant at *P* less than 0.0166.

Results

Natural Episodes of Pneumococcal Carriage Do Not Protect against Heterologous Challenge

To determine the role of naturally acquired serotype-specific immunity in protection against carriage acquisition we identified a cohort of eight healthy volunteers (cohort A) that were naturally colonized with pneumococcus and, after clearance, experimentally challenged them with a heterologous strain.

We observed that heterologous inoculation with 6B pneumococcus 9–16 months after clearance of natural carriage resulted in 50% carriage (Tables 1 and 2).

Table 2. Preexisting Serotypes and Carriage Status of Heterologous Challenge Cohort (Cohort A)

Subject	Serotype	Carriage	Time between Carriage Episode and Rechallenge (mo)
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

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 uncolonized volunteers were inoculated with doses of 6B pneumococcus ranging between 40,000 and 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], 1.0 $\mu\text{g/ml}$ [0.6–1.6] vs. 0.9 $\mu\text{g/ml}$ [0.5–1.4]). In carriage-positive volunteers, postinoculation levels of 6BPS IgG were comparable at Day 7 (1.1 $\mu\text{g/ml}$ [0.6–2.0]), but were significantly higher at Day 14 (1.3 $\mu\text{g/ml}$ [0.8–3.8]; $P=0.001$) and Day 35 (2.5 $\mu\text{g/ml}$ [1.2–5.8]; $P=0.001$) than at baseline. In carriage-negative volunteers levels of 6BPS IgG in serum after inoculation were comparable with those at baseline. Levels of 6BPS IgG in nasal wash at baseline were similar in carriage-positive volunteers and carriage-negative volunteers (Figure 1B) (1.0 ng/ml [0.8–2.4] vs. 0.7 ng/ml [0.6–0.8]). In both groups levels of 6BPS IgG in nasal wash after inoculation were comparable with those at baseline.

Levels of 6BPS IgA in serum at baseline were higher in carriage-positive volunteers than in carriage-negative volunteers (see Figure E1A in the online supplement) (0.5 $\mu\text{g/ml}$ [0.4–0.6] vs. 0.3 $\mu\text{g/ml}$ [0.2–0.4]; $P=0.001$). In carriage-positive volunteers, postinoculation levels of 6BPS IgA were comparable at Day 7 (0.5 $\mu\text{g/ml}$ [0.4–0.6]), significantly higher Day 14 (0.8 $\mu\text{g/ml}$ [0.5–1.9]; $P=0.005$), but were not significantly higher at Day 35 (0.7 $\mu\text{g/ml}$ [0.5–1.5]) than at baseline. In carriage-negative volunteers levels of 6BPS IgA in serum after inoculation were comparable with those at baseline. Levels of 6BPS IgA in nasal wash were

Figure 1. Kinetics of polysaccharide 6B (PS6B) IgG. IgG-specific ELISAs in serum (A) and nasal wash (B) were performed against pneumococcal 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 before (Day –5) and after (Days 2, 7, 14, and 35) inoculation. Serum samples were obtained before (Day –5) and after (Days 7, 14, and 35) inoculation. The inset at the top right shows data collected before (Day –5) and after (Day 14) challenge for those volunteers who underwent heterologous challenge (cohort A). Horizontal bars represent median values, and error bars represent the interquartile range.

similar in carriage-positive volunteers and carriage-negative volunteers between both groups at baseline (see Figure E1B) (18.3 ng/ml [12.0–45.0] vs. 14.0 ng/ml [6.9–16.4]) and were unchanged after challenge.

We assessed whether the capacity of antibodies in mediating phagocytosis was associated with protection. No difference was seen between carriage-

negative volunteers compared with carriage-positive volunteers at baseline (see Figure E2).

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] vs. 32.1 [14.9–86.4]; $P = 0.042$). Postinoculation, 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 with 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 with 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] vs. 5.3 [0.5–14.9]). Postinoculation, in carriage-positive volunteers, the number of 6BPS IgG-secreting B_{PLAS} was not significantly different than 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 with 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} after inoculation were comparable with 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] vs. 13.8 [4.5–71.8]). Postinoculation, 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 with baseline. In carriage-negative volunteers the numbers after inoculation were comparable with 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 after inoculation were comparable with 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] vs. 9.5 [4.1–15.1]). Postinoculation, in carriage-positive and carriage-negative volunteers, the number of PspC IgG-secreting B_{PLAS}

Figure 2. Kinetics of polysaccharide 6B (PS6B) IgG-producing B-cell populations. Enzyme-linked immunospot assays were performed against pneumococcal PS6B. The number of IgG-producing memory B cells (B_{MEM}) (A) and plasma cells (B_{PLAS}) (B) was determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained before (Day -5) and after (Days 7, 14, and 35) inoculation from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values, and error bars represent the interquartile range. PBMCs = peripheral blood mononuclear cells.

Figure 3. Kinetics of PspA IgG-producing B-cell populations. Enzyme-linked immunospot assays were performed against PspA. The number of IgG-producing memory B cells (B_{MEM}) (A) and plasma cells (B_{PLAS}) (B) was determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained before (Day -5) and after (Days 7, 14, and 35) inoculation from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values, and error bars represent the interquartile range. PBMCs = peripheral blood mononuclear cells.

was not significantly different than those at baseline.

Baseline Number of PS IgG-Secreting B_{MEM} Are Not Associated with Levels of PS IgG in Serum

Because 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} was increased after

inoculation in carriage-negative volunteers, we analyzed whether the number of 6BPS IgG-secreting B_{MEM} at baseline or the number of 6BPS IgG-secreting B_{PLAS} at Day 7 was associated with increased levels of 6BPS IgG in serum postinoculation.

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 colonization 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} was not different in carriage-positive volunteers compared with carriage-negative volunteers (see Figure E3A). In both groups the numbers of 6BPS IgA-secreting B_{MEM} and B_{PLAS} after inoculation were comparable with those at baseline.

At baseline, the number of PspA IgA-secreting B_{MEM} and B_{PLAS} was not significantly different in carriage-positive volunteers compared with carriage-negative volunteers (see Figure E4A) (2.7 [1.5–4.7] vs. 3.9 [1.4–4.4]) (see Figure E2B) (26.9 [5.0–47.5] vs. 6.1 [4.2–10.6]). Postinoculation, in carriage-positive and carriage-negative volunteers, the number of PspA IgA-secreting B_{PLAS} was not significantly different than 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 (see Figure E5A). In both groups, the numbers after inoculation were comparable with those at baseline.

At baseline, the number of PspC IgA-secreting B_{PLAS} was higher in carriage-positive volunteers than in carriage-negative volunteers (see Figure E5B) (46.5 [13.3–103.8] vs. 4.1 [3.0–9.4]; $P = 0.025$). Postinoculation, in carriage-positive and carriage-negative volunteers, the numbers of PspC IgA-secreting B_{PLAS} were not significantly different from those at baseline.

Discussion

Using our experimental human pneumococcal carriage model and

Figure 4. Kinetics of PspC IgG-producing B-cell populations. Enzyme-linked immunospot assays were performed against PspC. The number of IgG-producing memory B cells (B_{MEM}) (A) and plasma cells (B_{PLAS}) (B) was determined using blood samples from carriage-positive (carriage +) and carriage-negative (carriage -) volunteers. Samples were obtained before (Day -5) and after (Days 7, 14, and 35) inoculation from healthy volunteers experimentally inoculated with *Streptococcus pneumoniae* 6B. Horizontal bars represent median values, and error bars represent the interquartile range. PBMCs = peripheral blood mononuclear cells.

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 PSs protect against

pneumococcal disease (17). Serotype replacement, which has been observed after the introduction of PCV, highlights the role of PS-mediated immunity in protection against carriage (8, 11). We have previously demonstrated 0% carriage acquisition after rechallenge with a homologous strain up to 11 months after 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, or 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 postinoculation. Expansion of the PS IgG-secreting B_{PLAS} population was relatively short-lived and returned to baseline levels 14 days after inoculation. These data are consistent with a rapid differentiation of PS IgG-secreting B_{MEM} into short-lived B_{PLAS} . A similar effect has been observed and implicated in immunity to malaria (18).

Among carriage-negative volunteers, the increased number of circulating PS IgG-secreting B_{PLAS} was not accompanied by increased levels of PS IgG in serum or nasal wash. For this reason we hypothesize that antibodies produced by these plasma cells bind to the inoculated bacteria in the nasopharynx, facilitating bacterial agglutination, impairing bacterial adherence, and leading to protection from colonization acquisition. In carriage-positive volunteers, levels of PS IgG and IgA in serum were higher at Day 14 than at baseline; this response is likely generated through immunization by colonization rather than an acute response to inoculation. We observed that baseline levels of PS IgA in serum were higher in volunteers that were susceptible to colonization. We have previously observed a similar association between susceptibility and increased levels of IgG to LytC, PcsB SP0609, SPR0057, and SPR2021 (3). This

may be an indication of an underlying susceptibility to pneumococcal carriage, with elevated serum immunoglobulin indicative of repeated exposure.

Although it is well established that repeated exposure to pneumococci is required for the maintenance of IgG-secreting B_{MEM} populations, more recently it has been suggested that exposure during life also enhances the adult immune response to PCV (19). A similar effect has been observed in the United Kingdom, where lack of *Haemophilus influenzae* type B carriage led to an increased incidence of disease among children who received a single dose of Hib vaccine (20).

Based on data presented here, we postulate the following. (1) Pneumococcal nasal inoculation results in the rapid expansion of preexisting PS-specific B_{MEM} into short-lived IgG-secreting B_{PLAS}. Expansion of B_{PLAS} results in increased PS-specific antibody production; these antibodies bind to the inoculated bacteria at the nasopharynx resulting in bacterial agglutination and protection from colonization acquisition. (2) Although pneumococcal colonization is immunizing, adsorption of antibody by bacteria in the nasopharynx may prevent the detection of increased antibody production promoted by exposure to pneumococcus. (3) Only if colonization 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 prominent role in defense at the mucosal surface; it is involved in controlling invasive pneumococcal diseases (21) and the regulation of *S. pneumoniae* colonization in the nasal cavity of mice (22). 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 (23, 24). *S. 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 (25). As a result, we hypothesize that protection against colonization was associated with PS IgG-secreting B_{MEM} populations rather than PS IgA-secreting B_{MEM} populations because of 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 immunologic 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 toward protection against carriage acquisition.

Data presented here have important implications for the future of pneumococcal vaccine development, because 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 is important to assess whether these vaccines will impact carriage both directly, in immunized 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), while maintaining the opportunity for immunization 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 immunization 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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