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Noncovalent Association of Protein and Capsular Polysaccharide on Bacteria-Sized Latex Beads as a Model for Polysaccharide-Specific Humoral Immunity to Intact Gram-Positive Extracellular Bacteria

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Intact *Streptococcus pneumoniae* expressing type 14 capsular polysaccharide (PPS14) and type III *S. agalactiae* containing a PPS14 core capsule identical to PPS14 exhibit noncovalent associations of PPS14 and bacterial protein, in contrast to soluble covalent conjugates of these respective Ags. Both bacteria and conjugates induce murine PPS14-specific IgG responses dependent on CD4+ T cells. Further, secondary immunization with conjugate and *S. agalactiae*, although not *S. pneumoniae*, results in a boosted response. However, in contrast to conjugate, PPS14-specific IgG responses to bacteria lack affinity maturation use the 44.1-idiotype and are dependent on marginal zone B cells. To better understand the mechanism underlying this dichotomy, we developed a minimal model of intact bacteria in which PPS14 and pneumococcal surface protein A (PspA) were stably attached to 1 μm (bacteria-sized) latex beads, but not directly linked to each other, in contrast to PPS14–PspA conjugate. Beads coated simultaneously with PPS14+[PspA], similar to conjugate, induced in mice boosted PPS14-specific IgG secondary responses, dependent on T cells and ICOS-dependent costimulation, and in which priming could be achieved with PspA alone. In contrast to conjugate, but similar to intact bacteria, the primary PPS14-specific IgG response to beads coated simultaneously with PPS14+[PspA] peaked rapidly, with the secondary response highly enriched for the 44.1-idiotype and lacking affinity maturation. These results demonstrate that noncovalent association in a particle, of polysaccharide and protein, recapitulates essential immunologic characteristics of intact bacteria that are distinct from soluble covalent conjugates of these respective Ags. The *Journal of Immunology*, 2013, 191: 3254–3263.

Encapsulated Gram-positive bacteria, like *Streptococcus pneumoniae*, express a capsule formed by multiple copies of a single capsular polysaccharide (PS) covalently linked to the underlying cell wall peptidoglycan (1, 2). An array of different proteins are also attached to the cell wall through covalent and noncovalent links (3). Thus, PS and proteins are associated, but not directly linked, on the bacterial surface through the cell wall. Although there are some examples of bacterial pathogens expressing surface glycoproteins (4–6), the short glycan modifications usually function to decrease the immunogenicity of the protein or downmodulate host immunity (5). Gram-positive bacteria also contain multiple pathogen-associated molecular patterns (PAMPs), such as lipoteichoic acid and lipoproteins in the cell membrane and peptidoglycan in the cell wall (7–9). PAMPs, such as unmethylated CpG-containing DNA (10) and certain toxins (11), are also located intracellularly. These PAMPs can induce innate and subsequent adaptive immunity (12–14) through interactions with TLR (12), nucleotide-binding oligomerization domain-like receptors (15, 16), or surface lectins (17). Microbial pathogens can also express subcapsular components that suppress immunity (18–20). Thus, analyses of PS-specific Ig responses to intact bacteria are complicated by this structural and functional complexity.

Indeed, we recently showed that the composition of the bacterial subcapsular domain dramatically affects in vivo PS-specific Ig responses (21, 22). Thus, *S. pneumoniae* expressing type 14 capsular PS (PPS14) fails to induce PPS14-specific IgG responses after secondary immunization. In contrast, the strain COH1-11 of *S. agalactiae* type III (GBS-III) that expresses a PS structurally identical to PPS14 (23) induced highly boosted PPS14-specific IgG responses (22). PPS14-specific IgG responses to both bacteria share similar dependence on CD4+ T cells and marginal zone (MZ) B cells and a dominant use of the 44.1-idiotype (44.1-Id) with limited avidity maturation (24, 25). The mechanism underlying the differences in these two PPS14-specific IgG responses might reflect the presence, in the subcapsular domain, of particular immunostimulatory components in GBS and/or inhibitory components of *S. pneumoniae*.

The covalent linkage of a T cell–independent (TI) PS to a carrier protein, to create a soluble conjugate vaccine, converts the PS into...
a T cell–dependent (TD) Ag (26). These vaccines have had a major impact on the prevention of infections by encapsulated extracellular bacteria (27, 28). Conjugate vaccines of pneumococcal surface protein A (PspA) and PPS14 (PPS14-PspA), similar to intact GBS, induce highly boosted PPS14–specific IgG responses after secondary immunization in a TD and ICOS-dependent manner (22, 25). However, in contrast to intact bacteria, soluble PPS14–PspA conjugate induces PPS14–specific IgG responses derived from follicular B cells that undergo extensive affinity maturation and make minimal, if any, use of the 44.1-Id (24, 25, 29). Of note, the PPS14–specific IgG response to PPS14–PspA attached to aldehyde/sulfate latex beads is dependent on 44.1-Id+ MZ B cells, but nevertheless still exhibits extensive affinity maturation (24, 25). Thus, our data suggested that affinity maturation of the PPS14–specific IgG response requires the presence of a covalent linkage between the PS and the protein, absent in the intact bacteria. This covalent linkage allows for the binding of PS–associated peptide to MHC class II on the surface of the APC after processing (30). This results in CD4+ T cell responses specific for peptide conformations expressed only by the glycopeptide (31), combined saccharide–peptide motifs (32), or the saccharide component alone (33).

In this report, we use bacteria-sized latex beads, as a model for intact bacteria, to determine directly the immunologic consequences of noncovalent associations of PPS14 and PspA expressed on the bacterial surface by eliminating potential effects of bacterial subcapsular components. We demonstrate that beads coated simultaneously with PPS14 and PspA (PPS14+/PspA+ beads) induce highly boosted PPS14–specific IgG secondary responses dependent on T cells and ICOS-dependent costimulation, similar to both intact GBS–III and PPS14–PspA conjugate. However, in contrast to PPS14–PspA, but similar to intact GBS–III, PPS14+/PspA+ beads exhibit low-affinity maturation in the induced PPS14–specific IgG response. These studies elucidate the immunologic consequences of the noncovalent association of PS and protein on the bacterial surface and establish a minimal model for understanding the mechanism by which intact bacteria elicit PS–specific Ig responses.

Materials and Methods

Mice

Female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Female BALB/c athymic nude mice (CBByJ.Cg–tm1Mak; B6129P2-Icos/J), and control C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were used between 8 and 12 wk of age. The experiments in this study were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals (34) and approved by the Uniformed Services University of the Health Sciences Institutional Animal Use and Care Committee.

Bacterial strains and Ags

Strain R6-14 of S. pneumoniae is an isogenic transformant of strain R6 expressing PPS14 (25). Purified PPS14 was purchased from American Type Culture Collection (Manassas, VA). Protein contaminants in the PPS14 batches used were undetectable by bicinchoninic acid assay at 60˚C (Pierce, Rockford, IL) and were <0.08% of the total PPS14 weight. However, these purified PPS14 preparations contain a contaminating, unidentified TLR2 ligand(s) similar to the 23-valent pneumococcal polysaccharide vaccine (Pneumovax-23) (35). A covalent conjugate of recombinant pneumococcal surface protein A (PspA) and PPS14 (PPS14-PspA) was synthesized as described (25). The PspA in the conjugate contains the first 302 aa of the full-length PspA. The PPS14 to PspA ratio of PPS14-PspA is 1:24. The PPS14 expressed by R6-14 is serologically identical with that used for PPS14-PspA. A stimulatory 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) was synthetized as previously described (36).

Abs

Mouse IgG1 anti-PPS14 mAb (clone 44.1) and mouse IgG2a anti-PspA mAb (DC10-IA5) were gifts from Dr. Alex Lucas (Children’s Hospital Oakland Research Institute, Oakland, CA) and Dr. Rick Schuman (Ab and Immunossay Consultants, Rockville, MD), respectively. The preparation and characterization of the anti-Id 2B6.2 IgG1x mAb specific for an Id in or near the paratope of the PPS14–specific 44.1 mAb has been described previously (24). mAbs were oxidized with 10 mM metaperiodate and biotinylated using biotin-LC-hydrazone (Pierce, Rockford, IL) (37). The 44.1 mAb was labeled with Alexa Fluor 488–hydrazone (Invitrogen, Grand Island, NY) using a similar protocol. DC10-IA5 was labeled with Alexa Fluor 633–CFSE (Invitrogen). Rat IgG2a anti-mouse CD275 (B7RP-1) or ICOS ligand (clone HK5.3) and a rat IgG2a anti-TNP isotype control mAb (clone 2A3) were purchased from BioXCell (West Lebanon, NH).

Preparation of latex beads coated with PPS14

One hundred micrograms PPS14 in 0.1 M phosphate buffer (pH 6.5) was covalently linked to 107 surfactant-free aldehyde-sulfate latex beads (0.96 μm in diameter; Molecular Probes; Invitrogen) by incubation overnight in an orbital shaker. In most of the experiments, covalent attachment of PPS14 to the beads was stabilized by reduction with 200 μg cyanoborohydride (Sigma-Aldrich, St. Louis, MO) per 107 beads. Free binding sites in the latex beads were blocked using 0.1% glycine (Sigma-Aldrich) in PBS by incubation for 1 h at room temperature [beads coated with PPS14 and blocked with glycine (PPS14+Gly) beads]). The Gly solution was ultrafiltrated through 3 kDa ultrafiltration units (Amicon Ultra; Millipore, Billerica, MA) to remove possible protein contaminants and sterile filtered through 0.22-μm filters (Millipore). All manipulations during the coating of the beads were carried out under sterile conditions. Beads were washed five times with blocking buffer and resuspended in 0.05% Gly in PBS. Bead density after coupling was determined by densiometry at 630 nm. Beads coated only with Gly ([Gly]-beads) were produced using an identical protocol and used as a control.

Preparation of latex beads coated simultaneously with PPS14 and PspA

Latex beads initially coated with PPS14 alone were washed in 0.1 M phosphate buffer (pH 6.5) and incubated for 24 h with 8 μg PspA/107 latex beads. These conditions result in a similar ratio of PspA to PPS14 on beads as that found in the PPS14–PspA conjugate. However, incubation time and/or PspA concentration were altered in some experiments to change the amount of PspA attached per bead. Beads were then washed five times with 50 volumes PBS and resuspended in 0.05% Gly in PBS (PPS14+PspA beads). Beads coated only with PspA ([PspA] beads) were used as controls. Attachment of PspA to fresh beads was more efficient than PPS14-coated beads, further supporting that PspA is interacting directly with the bead and not with the PPS14, as PPS14 impairs rather than facilitates the attachment of PspA to the bead.

Preparation of latex beads coated with PPS14–PspA conjugate

Surfactant-free aldehyde-sulfate latex beads coated with PPS14–PspA were prepared as previously reported (25), with slight modifications. Fifty micrograms PPS14–PspA in 0.1 M phosphate buffer (pH 6.5) was covalently linked to 107 latex beads by incubation overnight in an orbital shaker (PPS14–PspA conjugate beads). Free binding sites in the latex beads were blocked by incubation for 1 h at room temperature with 0.1% Gly in PBS or maintained in PBS. Blocked beads were washed with 50 volumes of blocking buffer.

Quantitation of PspA, PPS14, and PPS14–PspA attached to beads

The content of PspA, PPS14, and PPS14–PspA conjugate attached to beads was determined by the ELISA methods previously described in detail (25). PspA content was determined by competitive inhibition ELISA using the mAb DC10-IA5, specific for PspA. PPS14 content was determined by a quantitative sandwich ELISA in which mAb 44.1 specific for PPS14 was used as both capture and detection Ab. The content of PPS14–PspA conjugate was determined as the average of the value obtained in two different quantitative assays: 1) a sandwich ELISA essentially identical with that used for the quantitation of PPS14, except for the use of PPS14–PspA conjugate as standard; and 2) a capture ELISA in which particles were captured with 5 μg/ml PspA–specific mAb DC10-IA5 and detected with 1 μg/ml biotinylated 44.1 (anti-PPS14 mAb).

Flow cytometric detection of Ag attached to latex beads

Beads (2.5 × 107) were incubated overnight at 4˚C with 5 μg 44.1 mAb labeled with Alexa Fluor 488, 1 μg DC10-IA5 labeled with Alexa Fluor 633, or a mixture of both in PBS plus 2% BSA (PBS-BSA). Bead fluo-
LATEX BEAD SURROGATES OF INTACT BACTERIA

Mouse immunizations and sera collection

Unless indicated, mice were immunized i.p. at day 0 and boosted on day 14. Latex beads were always washed in PBS immediately before immunization to remove any traces of Ag released free to the supernatant during storage. In most of the experiments, latex beads were injected mixed only with 25 μg stimulatory 30-mer CpG-ODN as adjuvant. However, in some experiments, 13 μg aluminum hydroxide (alum; Alhydrogel; Bengtsson Biosector, Fredriksund, Denmark) were included in the immunization mixture. All mixtures were incubated 1 h at room temperature before immunization. Sera were prepared from blood obtained through the tail vein.

Measurement of serum titers of PPS14- or PspA-specific Ig isotypes by ELISA

Immunon-4 HBX (Dynex Technologies Chantilly, VA) microtiter plates were coated with 0.5 μg/well PPS14 or PspA in PBS, overnight at 4°C. The plates were washed in PBS containing 0.05% Tween 20, and nonspecific binding was blocked with PBS-BSA. Threefold dilutions of the serum samples in PBS-BSA were added to the wells, and the plates were incubated overnight at 4°C. In most of the experiments, serum samples were diluted in PBS-BSA containing 20 μg/ml PBS22F and added to the wells. PBS22F is structurally unrelated to PPS14 and is known to be highly contaminated with cell wall C-PS. Nevertheless, PPS14-specific Ig titers were unaffected by serum preincubation with PBS22F, and there were no detectable serum titers of phosphorylcholine-specific Ig observed in PBS22F-coated ELISA plates in the absence of added PPS22F. A high-titer pooled antisera was included in every plate as control. The plates were incubated for 1 h at 37°C with polyclonal goat anti-mouse IgM, IgG (γ-chain), IgG1, IgG2a, IgG2b, or IgG3 conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) in PBS-BSA. Plates were washed five times with PBS containing 0.05% Tween 20, and the enzymatic reaction was measured at room temperature using p-nitrophenyl phosphate as substrate until absorbance for the standard wells reached predetermined values. Titers were expressed as the dilution of serum giving OD405 nm equal to 1.0. If the absorbances obtained were <1, the titer was extrapolated from the standard curve. Binding of the detected IgG and IgM to PPS14 was specific and completely inhibited by 10 μg/ml purified PPS14. For the quantitation of PPS14-specific IgG1 and IgG, serial dilutions of mouse IgG1 mAb 44.1 (150-0.03 ng/ml) were included in the corresponding plates. The standard curve obtained for 44.1 was used to convert OD405 nm into a final micrograms per milliliter concentration by using a four-parameter logistic regression method (38) with correction for the serum dilution. The specificity of 44.1 mAb has been extensively characterized (24, 39).

Inhibition ELISA to quantify serum PPS14-specific Ig expressing the 44.1-Id

The inhibition ELISA for the estimation of the serum content of 44.1-Id+ PPS14-specific IgG1, IgG, and IgM has been previously described (24). Briefly, inhibition mixtures prepared by mixing sera at predetermined dilutions with 10 μg/ml anti-Id 2B6.2 mAb and incubated for 24 h at 4°C were transferred to wells previously coated with 1 μg/ml PPS14 and blocked with PBS-BSA. 2B6.2 is specific for the 44.1-Id expressed by the PPS14-specific IgG1, IgG2a, and IgG2b mAbs (γ-chain), IgG1, IgG2a, IgG2b, or IgG3 conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) in PBS-BSA. Plates were washed five times with PBS containing 0.05% Tween 20, and the enzymatic reaction was measured at room temperature using p-nitrophenyl phosphate as substrate until absorbance for the standard wells reached predetermined values. Titers were expressed as the dilution of serum giving OD405 nm equal to 1.0. If the absorbances obtained were <1, the titer was extrapolated from the standard curve. Binding of the detected IgG and IgM to PPS14 was specific and completely inhibited by 10 μg/ml purified PPS14. For the quantitation of PPS14-specific IgG1 and IgG, serial dilutions of mouse IgG1 mAb 44.1 (150-0.03 ng/ml) were included in the corresponding plates. The standard curve obtained for 44.1 was used to convert OD405 nm into a final micrograms per milliliter concentration by using a four-parameter logistic regression method (38) with correction for the serum dilution. The specificity of 44.1 mAb has been extensively characterized (24, 39).

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Statistics

Data were expressed as geometric mean ± SEM of the individual serum samples. Significance between groups was determined by the Student t test. The p values < 0.05 were considered statistically significant.

Results

Latex beads can be simultaneously coated with PPS14 and a bacterial protein

The surface of S. pneumoniae is composed of a structured mixture of PS and bacterial proteins associated within the peptidoglycan cell wall, but not directly linked to each other. To reproduce, in a simple manner, this bacterial surface without the potential influence of other bacterial saccharide components, we covalently coated 0.96 μm in diameter aldehyde/sulfate latex beads with PPS14 alone or combined, within the same particle, with PspA (PPS14+[PspA] beads) (Fig. 1). This bead size was selected to approximate the size of an intact bacterium. As shown in Fig. 1A, PPS14+[PspA] beads contained both Ags, at concentrations similar to that in PPS14– PspA conjugate beads, a condition that allows for a direct comparison. Attachment to the bead was homogeneous and stable (Fig. 1A), with no significant bead aggregation observed by microscopy or FACS analysis (73–87% unstained beads were singlets). The content of free PspA was <0.05%, PPS14 <0.13%, and conjugate <0.06% of attached Ag. The linkage of PPS14 to the bead involved Schiff base formation, with only a few bonds to the bead per molecule of PPS14, as supported by the increased stability of the attachment of PPS14 by selective reduction with NaBH4 (Fig. 1B). Without reduction, PPS14 was continually released free during storage at 4°C, and by 5.1 mo, only 50% PPS14 remained attached (Fig. 1B). However, once reduced, the linkage to the bead was essentially permanent (>300 y to release 13% PPS14). Reactive aldehyde groups on beads, left unoccupied after coating, were blocked with Gly to prevent the undesired attachment of environmental proteins, which could create artifacts. Moreover, the very mild conditions used during coupling ensured that PspA did not conjugate to PPS14 when coupled to the same bead.

PPS14+[PspA] beads induce boosted secondary PPS14-specific Ig responses in a TD manner, similar to conjugate vaccines

Using PPS14+[PspA] beads, we wished to determine whether noncovalent association of PS and protein, as found in intact bacteria, is sufficient to induce a TD PS-specific Ig response with induction of memory or whether covalent attachment, a feature of conjugate vaccines, is critical. We thus immunized athymic nude or control BALB/c mice with PPS14+[PspA] beads or PPS14–PspA beads in alum plus CpG-ODN, with similar boosting on day 14. A relatively low dose of alum was initially used in these experiments to facilitate close contact of CpG-ODN with the particles, but this was subsequently determined to be unnecessary (data not shown). We previously showed that the attachment of PPS14–PspA to beads not only preserves, but enhances its ability to induce a boosted secondary IgG anti-PPS14 response (25). As shown in Fig. 2, immunization with PPS14+[PspA] beads, similar to PPS14–PspA beads, induced highly boosted secondary IgG and IgM responses specific for both PPS14 and PspA in BALB/c mice, but not in syngeneic athymic nude mice, demonstrating both the TD nature of these responses and the induction of memory. In contrast, beads coated with PPS14 alone (PPS14+[Gly]) induced PPS14-specific IgG and IgM responses in BALB/c mice that were not statistically different to those induced in athymic nude mice (p > 0.45; Fig. 2), indicating their TI nature. Primary anti-PPS14 Ig responses to PPS14+[Gly] beads were not affected by sec-
ondary immunization. In addition, PPS14-specific IgG secondary responses to both bead-associated PPS14-PspA and PPS14+[PspA] exhibited a similar TD IgG isotype profile (Fig. 3A), although serum titers were significantly higher in response to bead-associated PPS14-PspA. In contrast, PPS14-specific IgG responses to PPS14+[Gly] beads were predominantly IgG1 and IgG3 in both BALB/c and athymic nude mice (Fig. 3A), similar to the IgG responses to free soluble PPS14, a classical TI Ag. Moreover, the levels of PPS14-specific Ig induced by PPS14+[PspA] beads were not significantly different from those induced by the same amount of free PPS14, as determined in a titration assay (data not shown). As expected, beads coated only with PspA ([PspA] beads) or Gly ([Gly] beads) did not induce detectable anti-PPS14 Ig responses (Fig. 2), indicating that latex beads by themselves do not induce PPS14 cross-reactive Igs. Moreover, the use of two different preparations of PPS14 or two different batches of latex beads did not affect the results obtained. Collectively, these results indicate that the presence of associated protein, and not the covalent attachment of PPS14 to the bead or the bead structure by itself, was critical for the TD induction of the boosted secondary PPS14-specific Ig responses to PPS14+[PspA] beads.

PPS14-PspA beads induce a more sustained, TD primary PPS14-specific IgG response relative to PPS14+[PspA] beads Despite similarities in the secondary PPS14-specific IgG response to PPS14-PspA and PPS14+[PspA] beads, noted above, key differences were observed for the primary response. Primary PPS14-specific IgM and IgG responses to PPS14+[PspA] beads peaked by day 7 postimmunization (Fig. 2), similar to intact S. pneumoniae (25), whereas the peak response to PPS14-PspA beads

**FIGURE 1.** Attachment of bacterial Ags to the surface of aldehyde/sulfate latex beads. (A) Single FACS scatter profile of aldehyde-sulfate latex beads, 0.96 μm in diameter, coated with the different Ags and Ag combinations indicated in the graphs and stained with Alexa Fluor 488–conjugated 44.1 (anti-PPS14) mAb and Alexa Fluor 633–conjugated DC-10IA5 (anti-PspA) mAb. The PspA, PPS14, or PPS14-PspA conjugate content per 10^6 beads and the amount of PPS14-PspA conjugate equivalents in PPS14+[PspA] beads, as determined by ELISA, is indicated in each of the representative preparations. For beads containing simultaneously PPS14 and PspA, the equivalent weight of PPS14-PspA conjugate is indicated. (B) Kinetics of release of the PPS14 attached to beads with stabilization of the PPS14 attachment (by reduction with cyanoborohydride [BH3CN]) or without stabilization. Beads coated with PPS14 were maintained at 4°C in PBS for the time indicated. The amount of PPS14 in the supernatant (Free) and attached to the beads (Beads) once collected by centrifugation was determined by ELISA and expressed as a percentage of the total amount of PPS14 initially attached to the beads.

**FIGURE 2.** PPS14+[PspA] beads induce PPS14-specific Ig responses in a TD manner. BALB/c and BALB/c athymic nude mice (n = 7) were immunized on days 0 and 14 (arrows) with ≥2 × 10^9 latex beads, coated with the Ag combinations indicated, in alum + CpG-ODN. Each dose of the corresponding beads contained ~250 ng of serologically active PPS14 and/or the equivalent of 425 ng of total PPS14-PspA conjugate. Beads used in this experiment are the same shown in Fig. 1A. Serum titers of PPS14- and PspA-specific IgG and IgM were determined by ELISA. Values are expressed as geometric mean ± SEM. *p < 0.05 (between the responses elicited in BALB/c versus athymic nude mice), **p < 0.05 (titer^−1 relative to the previous bleeding of each mouse group).
Figure 3. All IgG subclasses are boosted in a TD manner during the PPS14- and PspA-specific secondary responses to PPS14+[PspA] beads. (A) Titers of PPS14-specific IgG isotypes in sera of BALB/c and BALB/c athymic nude mice (n = 7), 14 d after a primary immunization (Primary) and 20 d after a secondary immunization (Secondary) with 2 × 10^6 latex beads, coated with the Ag combinations indicated, in alum + CpG-ODN. For IgG1 determinations, a scale in weight units is included as a reference. (B) PspA-specific IgG isotype titers in selected BALB/c mice sera from several independent experiments chosen for having a similar content of PspA-specific IgG. Sera were collected 14 d after a primary immunization (Primary) and 7 d after the secondary immunization (Secondary). Values are expressed as geometric mean ± SEM. * p < 0.05 (titer −1 in primary relative to secondary response), # p < 0.05 (between the responses elicited in BALB/c versus athymic nude mice for each bleeding and group), ^ p < 0.05 (between responses in BALB/c and athymic nude mice for each bleeding and group), ^p 0.05 (titer between responses elicited in BALB/c and athymic nude mice for each bleeding and group). 

ICOS-dependent costimulation is critical for the induction of primary and secondary PPS14-specific IgG responses to PPS14+[PspA] beads

The induction of memory typically requires ICOS-dependent costimulation of CD4+ T cells, which promotes T follicular helper cell differentiation and the germinal center reaction (41, 42). We thus wished to determine a potential role for ICOS in the TD-induced secondary PPS14-specific IgG response to PPS14+[PspA] beads. ICOS−/− and C57BL/6 wild-type control mice were immunized i.p. with beads coated with PPS14+[PspA], PPS14-PspA, or PPS14+[Gly], containing identical amounts of PPS14 and PspA. Beads were admixed only with CpG-ODN, in the absence of alum, in this experiment and thereafter, because alum was determined to have no effect on the CpG-ODN-adjuvanted anti-PPS14 IgG responses induced by any type of bead (data not shown).

As illustrated in Figure 4A, the primary and secondary PPS14-specific IgG responses to PPS14+[PspA] and PPS14-PspA beads were markedly reduced in ICOS−/− mice, whereas the responses to PPS14+[Gly] were unaffected compared with control C57BL/6 mice. ICOS−/− mice exhibited a more complete reduction in the primary PPS14-specific IgG response to PPS14-PspA relative to PPS14+[PspA] beads, despite comparable inhibition in the PspA-specific IgG responses (Fig. 4A). Similar results were obtained in mice injected with 1 mg of a neutralizing anti-CD275 mAb (anti-ICOS ligand) 24 h before primary immunization with PPS14+[PspA] beads (Fig. 4B). Thus, ICOS-dependent T cell costimulation is required for the induction of optimal primary and boosted secondary PPS14-specific IgG responses to beads containing both PspA and PPS14, regardless of the presence of a covalent linkage between these two Ags. The ICOS dependence of these boosted PPS14-specific IgG responses strongly suggests that they depend upon germinal center formation. ICOS−/− mice immunized with intact S. pneumoniae or free PPS14-PspA exhibit essentially complete abrogation in germinal center formation (43).

Secondary PPS14-specific IgG and IgG1 responses to PPS14+[PspA] beads are highly enriched in the expression of the 44.1-Id

We previously demonstrated that the 44.1-Id, absent in the repertoire of natural PPS14-specific Igs, dominates the PPS14-specific IgG, but not IgM, response to intact S. pneumoniae—expressing PPS14 in BALB/c mice (24) (Fig. 5). In distinct contrast, PPS14-specific IgG responses to soluble, free PPS14-PspA exhibit minimal usage of the 44.1-Id, although significant 44.1-Id expression is elicited in response to PPS14-PspA attached to latex particles (24) (Fig. 5). These data indicate a role for particulation in the expression of this Id. In this regard, we now observe that the degree of 44.1-Id use in the PPS14-specific IgM, IgG1, and IgG responses to PPS14+[PspA] beads is largely similar to that observed using PPS14-PspA beads, with a higher proportion of 44.1-Id usage in the secondary IgG or IgG1 responses, relative to the primary. In contrast, the proportion of 44.1-Id anti-PPS14 IgM...
remained mostly unchanged \((p = 0.55)\). Notably, 44.1-Id usage in the secondary PPS14-specific IgG or IgG1 responses to PPS14+[Gly] beads, which fail to recruit CD4+ T cells, were not significantly increased relative to the primary and thus remained a minor contribution to the overall PPS14-specific response (Fig. 5). Similarly, in BALB/c nude mice, the titer and 44.1-Id contribution to the PPS14-specific IgG secondary responses to PPS14+[PspA] beads \((p = 0.44; 9.8 \pm 4.2\%)\) were similar to those of the primary (data not shown). Collectively, these data indicate that the recruitment of 44.1-Id+ B cell clones, likely of MZ B cell origin, by particulate forms of PPS14 requires the recruitment of T cell help during the primary response to PspA in the particle, but without the need for direct covalent attachment between PspA and PPS14.

The PPS14-specific IgG response to PPS14+[PspA] beads undergoes minimal avidity maturation, similar to intact bacteria, but in contrast to PPS14-PspA beads

We previously demonstrated that PPS14-specific IgG responses to free, soluble, or bead-associated PPS14-PspA undergo strong avidity maturation throughout both the primary and secondary responses \((24)\) (Fig. 6). In contrast, the avidity of PPS14-specific
IgG secondary responses to intact PPS14-expressing S. pneumoniae or S. agalactiae were unaffected or even reduced relative to the primary response (24) (Fig. 6). We now show that, although early (day 7) in the primary response, PPS14-specific IgG induced in response to PPS14+[PspA] beads shows similar avidity to that induced by PPS14-PspA beads ($p = 0.43$), the avidity in the latter response increases modestly by day 14 ($p = 0.01$). Whereas avidity maturation during the secondary response to PPS14-PspA beads increased substantially, a decrease after secondary immunization with PPS14-PspA beads ($p = 0.00004$) was observed, indistinguishable to that observed in response to intact S. pneumoniae type 14 (Fig. 6) or the PPS14-expressing COH1-11 strain of GBS-III (24). These data indicate that a direct covalent link between PPS14 and PspA, as found in the conjugate vaccine but not in PPS14+[PspA] beads or intact bacteria, is critical for avidity maturation of the PPS14-specific IgG response, although not necessary for recruitment of CD4+ T cell help or secondary boosting.

**Beads coated only with PspA prime for the PPS14-specific IgG response to PPS14+[PspA] beads**

PS-specific IgG responses to conjugate vaccines typically exhibit the carrier effect. Specifically, preimmunization with a protein, the carrier, primes carrier-specific CD4+ T cells for an enhanced IgG response specific for a hapten that is covalently attached to the same protein (28, 44). Thus, we tested whether PPS14-specific IgG responses to PPS14+[PspA] beads exhibit this effect. BALB/c mice preimmunized 14 d earlier with [PspA] beads were then immunized with PPS14+[PspA] beads and boosted in a similar fashion 17 d later. Naïve mice immunized and boosted 17 d apart with PPS14+[PspA] or [PspA] beads were used as controls. As shown in Fig. 7, both PspA- and PPS14-specific IgG responses to a primary immunization with PPS14+[PspA] beads were highly boosted in mice previously immunized with [PspA] beads, compared with the primary responses in naïve mice. In contrast, preimmunization with PPS14+[Gly] beads, or beads coated with autologous mouse albumin did not affect the PPS14-specific IgG responses to primary or secondary immunization with PPS14+[PspA] beads (data not shown), indicating that the booster effect is derived from the response to PspA and not to the beads themselves or to PPS14. The carrier effect extended to the PPS14-specific responses to a secondary immunization with PPS14+[PspA] beads, which were enhanced in mice preimmunized with [PspA] beads relative to controls (Fig. 7). Similarly enhanced PPS14-specific IgG responses were observed in naive mice after a third immunization with PPS14+[PspA] beads, 2 mo later, but still without showing any significant increase in avidity maturation (data not shown). Thus, the PPS14-specific IgG response to PPS14+[PspA] beads exhibited a carrier effect associated with PspA priming, similar to conjugate vaccines. This further supports the notion that noncovalent association of PspA and PPS14 within a particle is sufficient for recruiting CD4+ T cell help for a PPS14-specific IgG response, although insufficient for inducing avidity maturation.

**Discussion**

These results demonstrate that noncovalent association of PPS14 and PspA attached to the surface of bacteria-sized aldehyde/sulfate latex beads recapitulate the essential immunologic characteristics of those Ags in the intact bacterium. Thus, the primary PPS14-specific IgG response to PPS14+[PspA] beads was rapid, peaks on or before day 7, with a highly boosted, TD secondary IgG response, comprising all IgG subclasses and abundantly enriched in the usage of the 44.1-Id, but with minimal avidity maturation. Optimal primary and secondary PPS14-specific IgG responses also required ICOS-dependent costimulation, strongly suggesting a key role for the germinal center reaction. These characteristics of the PPS14-specific Ig response to PPS14+[PspA] beads are essentially identical to those displayed in response to intact GBS-expressing PPS14 (22, 24).

However, intact S. pneumoniae induces PPS14-specific IgG responses that diverge from this basic pattern in two major ways. Thus, it induces a PPS14-specific IgG response that, although dependent on CD4+ T cells, is ICOS independent and fails to generate a boosted IgG response upon secondary immunization (43, 45). These deviations from this model strongly suggest the presence of an immunosuppressive component(s) in the subcapsular domain of S. pneumoniae. This effect is likely direct and not mediated by CD25+ regulatory T cells (46). However, a deficiency in the expression of SLAM-associated protein, which impairs the ability of CD4+ T cells to stably interact with cognate B cells early during the immune response (47), results in the complete abrogation of the primary and secondary PPS14-specific IgG responses to S. pneumoniae (43). Thus, this putative inhibitory effect of S. pneumoniae does not prevent early B–T cell cognate interactions, but likely impacts on later events involved in the subsequent generation of a secondary PPS14-specific IgG response. This inhibitory property of intact S. pneumoniae may also be responsible for its ability to markedly suppress TD IgG responses to coimmunized soluble proteins, a property not observed for intact GBS (48). Thus, the distinct nature of the PS-specific IgG responses to intact S. pneumoniae versus GBS underscores the importance of the subcapsular domain in determining the outcome of the humoral response to the associated capsular PS.

The induction of T cell help for generating the PPS14-specific Ig response to PPS14+[PspA] beads is due to the bead-associated PspA. Thus, beads coated only with PspA prime for the boosted PPS14-specific IgG response upon subsequent immunization with PPS14+[PspA] beads. Immunization with PspA, as with other
proteins, results in germinal center formation, accounting for the ICOS dependence of the PPS14-specific IgG response. In contrast, it is highly unlikely that CD4+ T cell help for the PPS14-specific IgG response is induced by bead-associated PPS14. Thus, priming with beads coated only with PPS14 had no effect on the PPS14-specific IgG response to a subsequent immunization with PPS14+[PspA] beads or with beads coated simultaneously with PspA and PPS14 (PPS14+[PspA] beads) admixed with Cpg-ODN without alun. *p < 0.05 (each group relative to the responses of naive mice to the immunization with PPS14+[PspA] beads at days 0 and 17), *p < 0.05 (titer -1 relative to the previous bleeding in each mouse group).

**FIGURE 7.** Effect of priming with [PspA] beads and PPS14+[PspA] beads in the PspA- and PPS14-specific IgG responses to PPS14+[PspA] beads. Groups of five naive BALB/c mice (PBS) and mice previously immunized with 2 × 106 beads coated only with PspA ([PspA] beads; 167 ng/dose) 14 d earlier (d–14), were immunized at days 0 and 17 with 174 ng of PspA in the form of beads coated only with PspA ([PspA] beads) or with beads coated simultaneously with PspA and PPS14 (PPS14+[PspA] beads) admixed with Cpg-ODN without alun. *p < 0.05 (each group relative to the responses of naive mice to the immunization with PPS14+[PspA] beads at days 0 and 17), *p < 0.05 (titer -1 relative to the previous bleeding in each mouse group).
in response to a known biological warfare agent. This could be achieved with a single immunization of bead-associated target Ag and carrier protein in individuals previously exposed to the carrier, although repetitive immunizations would likely further increase the level of immunity. The selective targeting of MZ B cells by Ags in a particulate form may also select for broadly neutralizing cross-reactive Abs that might be of value for highly mutating viruses. Although other groups have proposed the use of PS entrapped in biodegradable particles as a vehicle to maintain a sustained release of Ag (57, 58) and the ability of PS encapsulated in cross-linked BSA particles to induce the release of IL-8, TNF-α, and IL-1β (57), to our knowledge, this is the first report to study the adaptive responses to noncovalent associations of PS and protein on the surface of inert particles.

Our bead-based approach to vaccination has numerous advantages. Noncovalent associations of protein and PS on the surface of latex beads can be produced without the need for the use of organic solvents, complex methods of conjugation, or chromatographic separation. Instead, they can be produced using centrifugation or filtration as the only method for separation of uncoupled material. Moreover, preassembled beads coated with each serotype-specific PS could be stored to be later combined with the proper carrier protein. The lack of avidity maturation of the PPS14-specific IgG secondary responses to PPS14+[PsaP] beads, a characteristic in common with the IgG responses to intact bacteria, may not be a serious drawback for their application as a vaccine, because PS-specific IgG responses to intact S. pneumoniae, of similar avidity, are highly protective (59). Furthermore, in contrast to free soluble PPS14-PsaP conjugate, PPS14+[PsaP] beads induce rapid increases and secondary boosting in the circulating levels of both PPS14-specific IgG as well as IgM. PS-specific IgM responses can also be fully protective against pneumococcal infection (60). In summary, our data on the in vivo immunogenic properties of bead-associated PPS14 and PsaP suggest a simple model for better understanding PS-specific IgG responses to intact bacteria as well as a novel approach to vaccination.

Disclosures

The authors have no financial conflicts of interest.

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