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Structurally Identical Capsular Polysaccharide Expressed by Intact Group B *Streptococcus* versus *Streptococcus pneumoniae* Elicits Distinct Murine Polysaccharide-Specific IgG Responses In Vivo

Swadhinara Arjunaraja,*† Lawrence C. Paoletti,‡ and Clifford M. Snapper*

We previously reported distinct differences in the murine in vivo Ig polysaccharide (PS)-specific responses to intact *Streptococcus pneumoniae* compared with responses to *Neisseria meningitidis* and that in each case, the bacterial subcapsular domain markedly influences the Ig response to the associated PS. In light of potentially unique contributions of biochemically distinct capsular PS and/or their characteristic attachments to the underlying bacterium, it remains unresolved whether different bacterial subcapsular domains can exert differential effects on PS-specific Ig responses to distinct bacterial pathogens. In this report, we used a mutant strain of group B *Streptococcus* (*Streptococcus agalactiae*) type III (GBS-III) that expresses desialylated capsular polysaccharide of GBS-III, biochemically identical to capsular pneumococcal polysaccharide type 14 (PPS14) of *Streptococcus pneumoniae* (intact inactivated *Streptococcus pneumoniae*, capsular type 14, Pn14), directly to compare the in vivo PPS14-specific IgG responses to two distinct Gram-positive bacteria. Although both GBS-III and Pn14 elicited relatively rapid primary PPS14-specific IgG responses dependent on CD4^+^ T cells, B7-dependent costimulation, and CD40–CD40L interactions, only GBS-III induced a highly boosted ICOS-dependent PPS14-specific IgG response after secondary immunization. Of note, priming with Pn14 and boosting with GBS-III, although not isolated PPS14, elicited a similar boosted PPS14-specific IgG response that was dependent on CD4^+^ T cells during secondary immunization, indicating that Pn14 primes for memory but, unlike GBS-III, fails to elicit it. The inability of Pn14 to elicit a boosted PPS14-specific IgG response was overcome by coimmunization with unencapsulated GBS-III. Collectively, these data establish that structurally identical capsular PS expressed by two distinct Gram-positive extracellular bacteria can indeed elicit distinct PS-specific IgG responses in vivo. *The Journal of Immunology*, 2012, 188: 5238–5246.

Extracellular bacteria expressing polysaccharide (PS) capsules such as *Streptococcus pneumoniae*, *Streptococcus agalactiae* (group B *Streptococcus*), and *Neisseria meningitidis* are leading causes of morbidity and mortality due to sepsis, pneumonia, and meningitis (1–3). The PS capsule is a major virulence factor, and vaccines targeting these pathogens are based on immunization with isolated proteins and haptenated or nonhaptenated PS. During natural infections, however, the host encounters intact PS-encapsulated extracellular bacteria, in which proteins and PS are coexpressed in a single particulate structure along with multiple moieties like TLR, NLR, and scavenger receptor ligands that engage the innate immune system (4). Thus, the noncovalent coexpression of PS and proteins by the intact bacterium could potentially elicit bacterial peptide-specific CD4^+^ T cell help for PS-specific B cells, leading to the generation of enhanced PS-specific primary IgG responses and induction of memory. Indeed, an earlier study using intact, heat-killed *Streptococcus pyogenes* (group A *Streptococcus*) reported lower IgM and IgG3 anti-group A streptococcal carbohydrate responses in T cell-deficient mice (5). In this regard, our previous studies using intact inactivated *Streptococcus pneumoniae*, capsular type 14 (Pn14), a Gram-positive bacterium, provide strong evidence that intact PS-encapsulated extracellular bacteria may represent unique immunogens, exhibiting both T cell-independent and T cell-dependent characteristics of the associated PS-specific IgG response. Thus the primary pneumococcal polysaccharide type 14 (PPS14)-specific IgG response to intact Pn14 is dependent on CD4^+^ T cells, B7-dependent costimulation, and CD40–CD40L interaction and comprises all four IgG isotypes (T cell–dependent–like). However, the primary PPS14-specific IgG response devel-

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Abbreviations used in this article: CpG-ODN, CpG-containing oligodeoxynucleotide; GBS-III, group B *Streptococcus* (*Streptococcus agalactiae*) type III; IIIPS, capsular polysaccharide of GBS-III; MCPS, meningococcal type C polysaccharide; MenC, *Neisseria meningitidis*, serogroup C; Pn14, intact inactivated *Streptococcus pneumoniae*, capsular type 14; PPS14, pneumococcal polysaccharide type 14; PS, polysaccharide.
ops rapidly as an apoptosis-prone extrafollicular response that is more dependent on BCR signaling than the protein-specific IgG response. In addition, the PPS14-specific IgG response is ICOS-independent and fails to generate a boosted IgG response after secondary immunization (T cell-independent–like) (12–16).

In contrast, our more recent studies using the Gram-negative extracellular bacterium Neisseria meningitidis type C (MenC) have shown that the primary IgG meningococcal type C polysaccharide (MCPS)-specific IgG response develops more slowly, with a highly boosted MCPS-specific IgG response after secondary immunization. The primary MCPS-specific IgG response is T cell-independent, whereas the boosted MCPS-specific response is dependent on CD4+ T cells, B7-dependent costimulation, and CD40–CD40L and ICOS–ICOSL interactions (17). Collectively, these studies using intact Pn14 and MenC demonstrate that the bacterial subcapsular domain markedly influences the Ig response to the associated PS, relative to that observed using isolated PS (18). However, they leave unresolved whether differences observed in PS-specific Ig responses between distinct intact extracellular bacteria reflect intrinsic differences in their biochemically unique capsular PS and/or the nature of the PS attachment to, or composition of, the underlying subcapsular bacterial domain.

In this regard, capsular PS expressed by Gram-positive bacteria are covalently attached to an underlying thick peptidoglycan cell wall to which a number of immunogenic proteins are also covalently linked (19, 20), whereas in Gram-negative bacteria, PS are covalently attached to the acyl glycerol moiety of the outer membrane that contains LPS and immunogenic proteins (21, 22). PS may also exhibit intrinsic features that potentially affect the elicited immune response. Thus, PPS14 can bind to SIGN-R1, a scavenger receptor present on marginal zone macrophages (23), and MCPS has unique immunomodulatory properties that can affect the MCPS-specific Ig response (24). In addition, the physical and chemical nature of the capsular PS such as the m.w. (25), charge (26), or sialic acid content (27) could also influence the induction of PS-specific Ig. In light of these potentially unique contributions of biochemically distinct capsular PS and/or unique bacterial attachments to the PS-specific Ig response to an intact bacterium, it remains of interest whether different bacterial subcapsular domains themselves can exert differential effects on PS-specific Ig responses between distinct bacteria.

In the current study, we used intact heat-killed group B Streptococcus (Streptococcus agalactiae) type III (GBS-III), a Gram-positive bacterium, initially to determine the nature of the murine in vivo capsular polysaccharide of GBS-III (IIIPS)-specific Ig response relative to that observed using isolated IIIPS Ag. Further, to assess directly the potential contribution of the subcapsular bacterial domain on the associated PS-specific responses between two distinct intact bacteria, we took advantage of the previous demonstration that the core IIIPS Ag of the IIIPS capsule expressed by GBS-III is biochemically identical to PPS14 expressed by Pn14, differing from the native IIIPS in lacking the terminal sialic acid (28). Thus, immunization with either conjugated or unconjugated IIIPS vaccines produces IgG that cross-reacts with PPS14 (29). In particular, we used a mutant GBS-III strain (COH1-11) that expresses the desialylated IIIPS that is identical to PPS14. In this manner, we were able to determine directly whether two distinct Gram-positive bacteria (Pn14 and GBS-III) expressing identical PS with similar attachments to the underlying subcapsular bacterial domain potentially elicit distinct PS-specific IgG responses in vivo. This report demonstrates that differences in underlying subcapsular bacterial domains can indeed differentially regulate PS-specific Ig responses in vivo.

Materials and Methods

**Mice**

BALB/c mice and athymic nude (BALB/c background) mice were purchased from the National Cancer Institute (Frederick, MD). Female mice were used between 7 and 10 wk of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, revised 1996) and were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

**Bacterial strains**

GBS-III (strain M781) was obtained from American Type Culture Collection (ATCC BAA-22; Manassas, VA). Another GBS strain, COH1, expressing the same capsular PS type III, and two isogenic COH1 mutants designated as COH1-13, lacking a capsule, and COH1-11, expressing the desialylated type III capsule that is similar to the PPS14 capsule of Pn14, were also used in this study (30). All three COH strains were a kind gift of Dr. Craig Rubens of Children’s Orthopedic Hospital (Seattle, WA). Pn14 (strain R614) was prepared as described previously (31). The isogenic non-encapsulated mutant of D39 (S. pneumoniae, capsular type 2; strain R63A) was provided by Dr. David Briles (University of Alabama at Birmingham, Birmingham, AL). Lysophilized or frozen stocks of bacteria were grown overnight on BBL blood agar plates (WYR International, Bridgewater, NJ). Isolated colonies on blood agar were grown in Todd-Hewitt broth media (BD Biosciences, San Jose, CA) to midlog phase, collected, and heat-killed by incubation at 65°C for 2 h or inactivated by overnight UV irradiation. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6, which corresponded to 107 CFU/ml. Bacteria were then aliquoted at 1010 CFU/ml and frozen at −20°C until their use as immunogens for mouse immunizations.

**Reagents**

Purified IIIPS was isolated and purified from strain M781 (32). The conjugates IIIPS–HSA and IIIPS–rAlp3 were made respectively, as previously described (29, 33). Purified PPS14 was purchased from American Type Culture Collection. Rat IgG2b anti-mouse CD4 mAb (clone GK1.5) was purified from ascites by ammonium sulfate precipitation and passage over a protein G column. Purified polyclonal rat IgG was purchased from Sigma (St. Louis, MO). Hamster IgG anti-mouse CD40L mAb (clone MR1), polyclonal hamster IgG, rat IgG2a anti-mouse CD25 mAb (clone HK5.3), rat IgG2a isootype control (clone 2A3), hamster IgG2 anti-mouse CD80 (B7-1) mAb (clone 16-10A1), and rat IgG2a anti-mouse CD86 (B7-2) mAb (clone GL-1) were purchased from BioXcell (West Lebanon, NH). Alum (Alhydrogel 2%) was obtained from Brentnag Biosector (Frederikssund, Denmark). A stimulatory 30mer CpG-containing oligodeoxynucleotide (Cpg-ODN) was synthesized as previously described (34).

**Preparation of group B Streptococcus whole protein extract**

Fifty milliliters of live unencapsulated GBS-III (strain COH1-13) bacteria were centrifuged at 3000 rpm for 20 min, and the supernatant fluid was discarded. About 5 ml of B-PER bacterial protein extraction reagent from Pierce (Rockford, IL) was added to the bacterial pellet (1:10 ratio), mixed well by shaking, and incubated at room temperature for 15 min. The treated bacterial culture was again centrifuged at 3000 rpm for 10 min. The supernatant fluid containing the soluble proteins was obtained, and the concentration was determined by the bicinchoninic acid assay.

**Immunizations**

Mice were immunized with 1 × 107 CFU heat-killed or UV-inactivated GBS-III in saline (2 × 107 CFU) or 10 μg purified IIIPS in saline or 1 μg IIIPS–rAlp3 adsorbed on 13 μg of alum mixed with 25 μg CpG-ODN. Serum samples for measurement of anti-protein and anti-PS Ig isotype titers by ELISA were prepared from blood obtained through the tail vein.

**ELISA**

For measurement of serum titers of IIIPS-specific Ig, Immunulon 4 ELISA plates were coated with IIIPS–HSA (1 μg/ml, 100 μl/well) in PBS. To measure the serum titers of whole group B Streptococcus protein-specific Ig, the plates were coated with group B Streptococcus protein extract (30 μg/ml, 50 μl/well). Plates were then washed three times with PBS plus 0.1% Tween 20 and were blocked with PBS plus 1/5% BSA for 2 h at 37°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in

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PBS plus 1% BSA were incubated overnight at 4°C, and plates were then washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG, IgG3, IgG1, IgG2b, or IgG2a Abs (200 ng/ml final concentration) in PBS plus 1% BSA were then added, and plates were incubated at 37°C for 1 h. Plates were washed three times with PBS plus 0.1% Tween 20. Substrate (p-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in TM buffer [1 M Tris, 0.3 mM MgCl₂ (pH 9.8)] was then added for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Helsinki, Finland). Serum titers of PPS14-specific Ig were measured as described previously (13).

Statistics

Serum Ig isotype titers were expressed as geometric means ± SEM of the individual serum Ig isotype titers. Significance was determined by the Student t test, and p values of ≤0.05 were considered statistically significant. All experiments were performed at least twice.

Results

Intact GBS-III elicits a boosted secondary IIIPS-specific IgG response similar to a IIIPS conjugate vaccine

We previously demonstrated that the IgG PPS14-specific IgG response to intact Pn14 is distinct from that of a PPS14 conjugate vaccine in that Pn14 elicits more rapid primary kinetics and fails to boost serum titers of PPS14-specific IgG after secondary immunization, despite the primary response being CD4⁺ T-cell dependent (12, 14). As discussed above, we wished to compare the PS-specific Ig response to Pn14 with that elicited by another intact Gram-positive bacterium, GBS-III. We began by comparing the in vivo IIIPS-specific Ig response to intact GBS-III relative to that induced by a IIIPS–rAlp3 conjugate vaccine (33). Alps (α-like proteins) are a family of related surface-anchored proteins with large numbers of repeat units that are expressed by most GBS strains, with Alp3 expressed on most isolates of group B Streptococcus type 5 and type 8 (35).

BALB/c mice were immunized i.p. with either UV-inactivated GBS-III (strain M781) in saline or IIIPS–rAlp3 conjugate (1 μg/mouse) in alum plus CpG-ODN and boosted 28 d later in a similar manner. The primary IIIPS-specific IgG response to intact M781 exhibits relatively rapid kinetics of induction with peak titers observed by day 7 (Fig. 1A), similar to that elicited by IIIPS–rAlp3 (Fig. 1B). Although the degree of induction of serum titers of rAlp3-specific IgG in response to conjugate was similar to that of IIIPS-specific IgG by day 7, rAlp3-specific IgG titers continued to rise significantly with peak titers observed by day 21 (Fig. 1B). Further, M781, in addition to IIIPS–rAlp3, elicits a secondary IIIPS-specific IgG response that is >10-fold higher than the primary, similar to the level of boosting of the secondary rAlp3-specific IgG response to the conjugate. The primary IIIPS-specific IgM response to M781 also peaked early (day 7) with modest, but transient, secondary boosting. Thus, intact GBS-III, like Pn14, generates a relatively rapid primary PS-specific IgG response but, in contrast to that previously reported for Pn14, elicits a highly boosted secondary PS-specific IgG response, similar to that observed for a conjugate vaccine.

The IIIPS-specific IgG responses to intact GBS-III versus isolated IIIPS are distinct

Isolated nonzwitterionic PS are unable to recruit CD4⁺ T cell help and thus typically induce primarily IgM, with relatively minor IgG responses after primary immunization. These PS also fail to induce IgG memory upon secondary challenge (7), although IgM memory responses to these Ags have been observed (36). In this regard, we directly compare the IIIPS-specific IgG response to isolated IIIPS versus intact GBS-III to determine the impact of the bacterial subcapsular domain on the associated PS-specific Ig re-
IIIPS demonstrates a primary and secondary PPS14-specific IgG response to both intact COH1 and COH1-11 that is similar to that observed for IIIPS-specific IgG. Indeed, preincubating COH1- and COH1-11–induced immune sera with PPS14 largely abrogates the detection of IIIPS-specific IgG using the IIIPS-specific ELISA (data not shown). Thus, these data indicate that the majority of the IIIPS-specific IgG are directed toward the type III core Ag that is identical to PPS14. The COH1-13 bacterial strain that lacks capsular PS fails to induce any detectable serum titers of IIIPS-specific or PPS14-specific IgG, thus establishing the specificity of the ELISA assay for the capsular PS.

The primary and secondary IIIPS- and PPS14-specific IgG responses to GBS-III are dependent upon CD4+ T cell help and CD40L-dependent costimulation

Whereas the PS-specific Ig responses to most isolated PS are T cell-independent, studies using intact Pn14 demonstrated dependence on CD4+ T cells, as well as CD40L, for the primary PPS14-specific IgG response (12, 14). Further, studies using intact MenC have shown that the secondary, but not primary, MCPS-specific IgG response is also dependent on CD4+ T cells and CD40L (17). In this regard, we wished to determine the role of CD4+ T cells and CD40L in the induction of the IIIPS-specific and PPS14-specific IgG responses to intact GBS-III. To determine this, BALB/c mice were injected with an anti-CD4+ T cell-depleting mAb (clone GK1.5) or control polyclonal rat IgG 24 h before immunization with heat-inactivated GBS-III (strain M781). Flow cytometric analysis confirmed that 95% of the CD4+ T cells were specifically depleted after 24 h in the anti-CD4 mAb-injected group. Mice were boosted on day 14 in the absence of anti-CD4 mAb or control polyclonal rat IgG. In addition, athymic nude mice (BALB/c background), which are markedly deficient in T cells, were also immunized at the same time with M781 and boosted similarly on day 14. As illustrated in Fig. 4A, the primary IIIPS-specific and PPS14-specific IgG responses to M781 in mice injected with control rat IgG peaked by day 7 with a >10-fold boost after secondary immunization. In contrast, both the anti-CD4 mAb-injected mice and athymic nude mice showed a nearly complete abrogation of the primary and secondary IIIPS- and PPS14-specific IgG responses compared with the control group, establishing their critical dependence on CD4+ T cells.

The induction of CD40L on activated CD4+ T cells is critical in mediating T cell-dependent humoral immune responses, including induction of isotype switched Abs, germinal center formation, and the generation of memory, through delivery of costimulatory signals.
signals via CD40 on APC (37). Preventing CD40–CD40L interactions in vivo through the use of blocking Abs largely abolishes T cell-dependent humoral immunity (38). To determine the role for CD40L costimulation on the IIIPS- and PPS14-specific IgG responses to intact GBS-III, BALB/c mice were injected with a blocking anti-CD40L mAb (clone MR1) or polyclonal hamster IgG as a control 24 h before immunization with intact heat-killed GBS-III (strain COH1). Mice were boosted in a similar fashion on day 14 in the absence of blocking anti-CD40L mAb or control IgG. As shown in Fig. 4B, both the primary and secondary IIIPS-specific and PPS14-specific IgG responses were significantly inhibited compared with the control (primary, 4.6- to 7.0-fold reduction; secondary, 3.8- to 7.9-fold reduction). Of interest, blocking of CD40L did not prevent boosting of the IgG responses to either IIIPS or PPS14 after secondary immunization, although the serum titers were significantly reduced compared with the control. Collectively, these data demonstrate that the primary and boosted secondary PS-specific IgG responses to intact GBS-III require CD44+ T cell help as well as CD40L-dependent costimulation.

B7- and ICOS-dependent costimulation is required for optimal induction of PS-specific IgG responses to intact GBS-III

CD28 constitutively expressed on CD4+ T cells binds to CD80/CD86 (B7-1/B7-2) expressed on APCs, and this interaction is critical for the initiation of CD4+ T cell activation (39, 40). We previously demonstrated that the primary PPS14-specific IgG response to intact Pn14 and the secondary, but not primary, MCPS-specific IgG response to MenC is dependent on CD28 (13). In this regard, we wished to determine the role of CD28-mediated signaling in the PS-specific IgG response to intact GBS-III. Therefore, mice were injected with blocking anti-CD80 (clone 16-10A1) and anti-CD86 (clone GL-1) mAbs or polyclonal hamster and rat IgG as a control 24 h before immunization with intact GBS-III (strain COH1), with secondary immunization on day 14. As illustrated in Fig. 5A, the primary and secondary IIIPS- and PPS14-specific IgG responses to COH1 were markedly inhibited in the presence of blocking mAbs compared with the control (primary, 8.2- to 17.3-fold reduction; secondary, 14.9- to 133-fold reduction).

ICOS, a member of the CD28 family, induced on CD4+ T cells upon TCR cross-linking and CD28-mediated signaling, binds to ICOSL expressed on APC (39, 41). ICOS is the key regulator of germinal center formation and immunological memory (42, 43). Our earlier studies using Pn14 demonstrated that the PPS14-specific IgG response is ICOS-independent, whereas the secondary, but not primary, MCPS-specific IgG response to MenC required ICOS-costimulation. In light of the boosted secondary PS-specific IgG responses to GBS-III, we wished to determine whether it was ICOS-dependent. Therefore, we injected BALB/c mice with anti-ICOSL mAb (clone HK5.3) or control polyclonal hamster IgG 24 h before immunization with intact GBS-III (strain COH1). Mice were boosted on day 14 in the absence of anti-ICOSL mAb or control Ab. As shown in Fig. 5B, the secondary, but not primary, IIIPS-specific IgG and PPS14-specific IgG responses to M781 were significantly, although not completely, inhibited in anti-ICOSL mAb-injected mice (3.2- to 3.3-fold reduction), whereas a near-complete abrogation of the secondary protein-specific IgG response was observed. Thus, these data demonstrate that B7-dependent, T cell costimulation is critical for induction of primary and boosted secondary IIIPS-specific and PPS14-specific IgG responses to intact GBS-III. Further, the importance of ICOSL-dependent costimulation for the boosted PS-specific IgG response to GBS-III supports the notion that GBS-III induces PS-specific IgG memory, likely dependent on GC formation.

FIGURE 5. B7- and ICOS-dependent costimulation is required for optimal induction of PS-specific IgG responses to intact GBS-III. (A) BALB/c mice (seven per group) were injected i.p. with either blocking hamster anti-CD80 mAb (B7-1) (clone 16-10A1) and rat anti-CD86 mAb (B7-2) (clone GL-1) or control polyclonal hamster and rat IgG (0.5 mg/mouse) 24 h before i.p. immunization with 1 × 109 CFU GBS-III (strain COH1). Mice were boosted similarly on day 14 in the absence of Abs. Serum titers of IIIPS- and PPS14-specific IgG were determined by ELISA. *p < 0.05 (significance between control versus anti-CD80 and anti-CD86 mAb-injected mice). (B) BALB/c mice (seven per group) were injected i.p. with either blocking anti-ICOSL mAb (clone HK5.3) or control rat IgG2a mAb (clone GL-1) or control polyclonal rat Ab. As shown in Fig. 5A, the primary and secondary IIIPS- and PPS14-specific IgG responses to COH1 were markedly inhibited in the presence of blocking mAbs compared with the control (primary, 8.2- to 17.3-fold reduction; secondary, 14.9- to 133-fold reduction).

A boosted secondary PPS14-specific IgG response is elicited by GBS-III in Pn14-primed mice

We demonstrated previously that intact Pn14 fails to generate a boosted PPS14-specific IgG response upon secondary immunization, despite the dependence of the primary response on CD44+ T cells. Although these data suggested that Pn14 failed to induce memory for PPS14-specific IgG, an alternative possibility was that Pn14 induced a state of memory but was unable to elicit a boosted memory response upon secondary immunization, in contrast to GBS-III. Therefore, we sought to determine whether priming of mice with Pn14 (strain R614) or GBS-III (strain COH1-11) followed alternatively by secondary immunization on day 14 with COH1-11 or R614, respectively, would elicit a boosted PPS14-specific IgG response. We used strain COH1-11 because it expresses the identical PPS14 capsule as R614. As illustrated in Fig. 6A, primary and secondary immunization with R614, in contrast to COH1-11, failed to elicit a boosted PPS14-specific IgG secondary response, as previously observed. Of note, primary immunization with R614 followed by secondary immunization with COH1-11 resulted in a boosted PPS14-specific IgG response similar to that observed using COH1-11 for both immunizations (p < 0.001). These data thus indicate that R614 can induce memory for PPS14-specific IgG but fails to elicit a boosted secondary response in R614-primed mice. Of interest, mice primed with COH1-11 followed by secondary immunization with R614 showed a more modest boost in the secondary PPS14-specific
strain COH1-13 to preclude a PPS14-specific contribution of GBS-III to the PPS14-specific IgG response. As shown in Fig. 6B, coimmunization with R614 and COH1-13 resulted in a significant boost in the secondary PPS14-specific IgG response, although significantly lower than in mice immunized only with the encapsulated COH1-11 strain. Mice immunized with COH1-11 in the presence of unencapsulated Pn14 (strain R36A) also elicited a significantly boosted secondary PPS14-specific IgG response similar to mice coimmunized with R614 plus COH1-13, but significantly lower than mice immunized with COH1-11 alone. Collectively, these data suggest that the absence of a boosted secondary PPS14-specific IgG response to Pn14 may reflect an inhibitory effect mediated by this bacterium during secondary immunization of Pn14-primed mice and that this might be overcome by stimulation by innate immune mechanisms.

The boosted secondary PPS14-specific IgG response to GBS-III requires GBS-III–associated PPS14 and CD4+ T cells during secondary immunization

The ability of COH1-11 to boost the secondary PPS14-specific IgG response in COH1-11– or R614-primed mice left unresolved whether boosting during secondary immunization simply required BCR cross-linking of PPS14-specific B cells in the absence of a putative inhibitory influence of R614, appropriate nonspecific innate stimulation, or both, and whether this effect required CD4+ T cells during secondary immunization. To test this, we immunized mice with heat-inactivated COH1-11 or R614 followed by secondary immunization with isolated PPS14 on day 14. As a control group, mice were immunized with isolated PPS14 for both the primary and secondary. In addition, to determine the BCR specificity of the secondary PPS14-specific IgG response, mice were primed with COH1-11 or R614 and boosted with their unencapsulated variants, COH1-13 and R36A, respectively. Neither isolated PPS14 nor nonspecific stimulation by unencapsulated bacteria were able to elicit a boosted PPS14-specific IgG response in either COH1-11– or R614-primed mice (Fig. 7A). Additionally, as illustrated in Fig. 7B, mice primed with COH1-11 alone followed by secondary immunization with COH1-11 in the presence of depleting anti-CD4 mAb failed to elicit a boosted PPS14-specific IgG response relative to mice receiving control Ab. Collectively, these data suggest that boosting of the secondary PPS14-specific IgG response in GBS-III–immunized mice requires both PPS14-specific BCR engagement and CD4+ T cells, the latter likely triggered via recognition of bacterial-derived pep-
tides in association with MHC class II. These data also suggest that APC processing of intact Pn14 and GBS-III might generate cross-reactive peptides for the generation of CD4+ T cell help.

Discussion
To determine whether the subcapsular bacterial domain of distinct PS-encapsulated extracellular bacteria can differentially affect the nature of an in vivo PS-specific response, it was necessary to study two distinct bacteria with biochemically identical PS having similar bacterial attachments. In this regard, we made use of inactivated intact GBS-III, a Gram-positive extracellular bacterium that expresses a capsule containing a core PS (i.e., GBS-III capsular PS without the terminal sialic acid) identical to that of the capsular PS of Pn14. We also used an isogenic mutant of GBS-III that expresses the core PS alone. In vivo, IIIPS- and PPS14-specific IgM and IgG responses to intact inactivated GBS-III were compared with those elicited by isolated IIIPS, as well as to intact inactivated Pn14, another Gram-positive extracellular bacterium, using the i.p. route of immunization. The use of inactivated, as opposed to viable, intact bacteria was to focus selectively on the properties of these pathogens as complex particulate immunogens, with the understanding that bacterial metabolism and alternate routes of immunization will add additional layers of complexity to the conclusions being drawn.

These data, combined with our previous observations using intact Pn14 and MenC (18), lend further support to the proposal that the bacterial subcapsular domain converts a normally T cell-independent PS-specific Ig response to one that is dependent on CD4+ T cells, including a requirement for B7-dependent, and potentially ICOS-dependent, costimulation, and CD40–CD40L interactions. The stronger reduction of the PP514- and IIIPS-specific IgG responses to group B Streptococcus that occurred in the absence of T cells or in the presence of blocking anti-CD80/86 mAbs, in contrast to that resulting from blockade of CD40L or ICOSL, may reflect either incomplete blockade by the mAbs used in the latter responses or additional helper activities of CD4+ T cells independent of CD40 or ICOS costimulation. However, a more absolute requirement may exist for B7-dependent costimulation for the early activation of T cells. The CD4+ T cell help during the primary response to the Gram-positive bacteria, GBS-III and Pn14, induces a relatively rapid and robust PS-specific IgG response comprising multiple IgG isotypes (44) (Fig. 2). In contrast, the primary PS-specific IgG response to the Gram-negative bacterium, MenC, develops more gradually and is T cell-independent (17). Whether this represents a more general dichotomy between Gram-positive and Gram-negative bacteria remains to be determined. However, induction of PS-specific IgG memory, a process dependent on CD4+ T cells, can occur after primary immunization with all three bacteria although only MenC and GBS-III elicit a boosted PS-specific IgG response after secondary immunization (17) (Fig. 6). Thus, recruitment of CD4+ T cells may potentially enhance PS-specific adaptive immunity to these pathogens, although as discussed below, the subcapsular domain of S. pneumoniae also appears to contain a structure that is immunosuppressive.

The immunologic properties of PS expressed by intact bacteria are thus distinct from those observed using classical T cell-independent Ags (7), which in isolation are divorced from the more complex context likely observed during natural infections. In particular, the inability of isolated IIIPS to induce a detectable IIIPS-specific IgG response in vivo (Fig. 2) is consistent with our earlier in vitro studies demonstrating a requirement for a second signal in addition to multivalent BCR cross-linking for induction of Ig secretion and class switching in response to PS (9). The requisite second signal may comprise a TLR ligand, various inflammatory cytokines, and/or BAFF/TACI, collectively recruited into the PS-specific Ig response by the subcapsular bacterial domain in the presence of innate immune cells (9, 45, 46). The degree to which natural purified bacterial PS induce IgG response in vivo may depend on the degree of contamination of the PS preparation with activating bacterial cell wall structures. In this report, isolated PPS14, in contrast to IIIPS, induced a detectable, though relatively modest, PPS14-specific IgG response. However, we previously demonstrated that this PPS14-specific IgG response was critically dependent upon a contaminating TLR2 ligand, likely derived from the S. pneumoniae cell wall (47).

The dependence of the boosted secondary IIIPS- and PPS14-specific IgG responses to GBS-III on CD4+ T cells, CD40–CD40L interaction, and B7- and ICOS-dependent costimulation (Figs. 4, 5) suggests that PS-specific memory B cells and/or bacterial peptide-specific memory CD4+ T cells may be generated within a germinal center reaction in response to intact GBS-III. In this context, the ability of GBS-III to trigger a boosted PPS14-specific IgG response in Pn14-primed mice, which is dependent on CD4+ T cells (Figs. 6, 7), may indicate that these two different pathogens share cross-reactive CD4+ T cell epitopes. This observation is not unprecedented in nature as memory T cells that are specific for one virus can become activated during infection with an unrelated heterologous virus (48). Of interest, we observe no evidence for cross-reactive protein-specific B cell epitopes between GBS-III and Pn14, in that detectable serum titers of protein-specific IgG, using whole protein extract from the corresponding uncapsulated bacteria for ELISA, are only observed for the homologous bacteria (data not shown). Alternatively, it is possible that CD4+ T cells that mediate help for PS-specific IgG responses to intact bacteria are largely specific for PS, as recently illustrated using a conjugate vaccine of IIIPS and carrier protein (49). Nevertheless, the attachment of PS to MHC class II was via carrier-derived peptide covalently linked to the PS. Thus, PS-specific IgG memory responses to conjugate vaccines appear to require the covalent attachment of PS and protein (49–52).

However, in bacteria, it does not appear that capsular PS is directly linked to protein via a covalent bond (19, 20). In this regard, in a previous study using intact vaccinia virus, CD4+ T cell help for an in vivo IgG response to a specific viral protein was induced only by CD4+ T cells with the same protein specificity as the B cell (53). This requirement for intramolecular help would thus be satisfied by a soluble PS–protein conjugate vaccine, but not by an intact bacterium. In addition, BCR-mediated Ag uptake by B cells involves endocytic vesicles that are only 50–150 nm in diameter and thus would be predicted to exclude intact bacteria (53). However, a single study demonstrated BCR-mediated uptake of intact Salmonella typhimurium by human B cells with subsequent display of MHC class II–peptide complexes and primary CD4+ T cell activation (54). Alternatively, PS-specific B cells could potentially acquire bacterial protein subsequent to processing by macrophages and/or dendritic cells (55, 56). Given these considerations, it is possible that intact bacteria elicit a T cell-dependent response that produces a stable expansion of unmutated PS-specific B cells, lacking properties of bona fide memory B cells. The requirement for CD4+ T cell help could be cognate at the level of initial dendritic cell priming but noncognate for B cells, or perhaps entirely noncognate in nature. In any event, our data indicate that BCR cross-linking is necessary but not sufficient for triggering B cells from GBS-III–primed mice for a boosted secondary PS-specific IgG response, as neither secondary immunization with isolated PPS14 nor uncapsulated GBS-III can mediate this effect (Fig. 7). In addition, CD4+ T cells are also...
required during secondary immunization to affect the PS-specific IgG booster response (Fig. 7).

Although the majority of PPS14-specific IgG elicited in response to either Pn14 or GBS-III shares the same dominant idiotype (J. Colino, L. Duke, S. Arjunaraja, Q. Chen, L. Liu, A.H. Lucas, and C.M. Snapper, submitted for publication), Pn14 fails to elicit a boosted secondary PPS14-specific IgG response in Pn14-primed mice and induces a blunted booster response in GBS-III–primed mice (Fig. 6). Further, coimmunization with GBS-III and unencapsulated S. pneumoniae also leads to a lower boosted PPS14-specific IgG response relative to immunization with GBS-III alone (Fig. 6). In light of our previous report that S. pneumoniae inhibits T cell-dependent IgG responses to coimmunized soluble Ags independent of capsule expression (57), these data suggest that S. pneumoniae expresses an immunosuppressive structure in its subcapsular domain that is not present in the GBS-III strains used in this study. This does not appear to be secondary to the generation of CD25+ regulatory T cells (58). One possible candidate is phosphorylcoline, a haptenic moiety covalently attached to the S. pneumoniae cell wall teichoic acid and membrane lipoteichoic acid (4). Thus, previous studies demonstrated the ability of a secreted filarial protein (ES-62) to mediate immunosuppression through a mechanism that is dependent on ES-62 expression of phosphorylcoline and the triggering of IL-10 secretion (59). Extracellular bacteria, in addition to S. pneumoniae, such as Pseudomonas aeruginosa, N. meningitidis, Neisseria gonorrhoeae, and Haemophilus influenzae, can also express phosphorylcoline on their LPS or pili in a regulated manner (60–62), but the immunologic consequences of this remain largely unexplored. In light of the above, the ability of R614 partially to boost the PPS14-specific IgG response in COH1-11–primed, but not R614-primed, mice may in some way be related to the high levels of serum phosphorylcoline-specific IgM and IgG, which can bind to R614, at the time of secondary immunization that were observed in mice primed with R614 but not COH1-11. The ability of unencapsulated GBS-III partially to restore the boosted PPS14-specific IgG response when coimmunized with Pn14 (Fig. 6) suggests that innate immune activation mediated by the GBS-III subcapsular domain can overcome this potential S. pneumoniae-mediated suppressive effect. In this regard, inactivated GBS is a significantly more potent activator of macrophages and monocyties than inactivated S. pneumoniae, inducing higher levels of TNF-α (63, 64).

Disclosures

The authors have no financial conflicts of interest.

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