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*J Immunol* 2012; 188:569-577; Prepublished online 7 December 2011; doi: 10.4049/jimmunol.1101446

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The Nature of an In Vivo Anti-Capsular Polysaccharide Response Is Markedly Influenced by the Composition and/or Architecture of the Bacterial Subcapsular Domain

Swadhinayna Arjunaraja,*† Paola Massari,‡ Lee M. Wetzler,§ Andrew Lees,§ Jesus Colino,* and Clifford M. Snapper*

In vivo anti-polysaccharide Ig responses to isolated polysaccharide (PS) are T cell independent, rapid, and fail to generate memory. However, little is known regarding PS-specific Ig responses to intact Gram-positive and Gram-negative extracellular bacteria. We previously demonstrated that intact heat-killed Streptococcus pneumoniae, a Gram-positive bacterium, elicited a rapid primary pneumococcal capsular PS (PPS) response in mice that was dependent on CD4+ T cells, B7-dependent costimulation, and CD40–CD40L interactions. However, this response was ICOS independent and failed to generate a boosted PPS-specific secondary IgG response. In the current study, we analyzed the murine meningococcal type C PS (MCPS)-specific Ig response to i.p.-injected intact, heat-killed Neisseria meningitidis, serogroup C (MenC), a Gram-negative bacterium. In contrast to S. pneumoniae, the IgG anti-MCPS response to MenC exhibited delayed primary kinetics and was highly boosted after secondary immunization, whereas the IgG anti-MCPS response to isolated MCPS was rapid, without secondary boosting, and consisted of only IgG1 and IgG3, as opposed to all four IgG isotypes in response to intact MenC. The secondary, but not primary, IgG anti-MCPS response to MenC was dependent on CD4+ T cells, CD40L, CD28, and ICOS. The primary and secondary IgG anti-MCPS responses were lower in TLR4-defective (C3H/HeJ) but not TLR2−/− or MyD88−/− mice, but secondary boosting was still observed. Of interest, immunization of S. pneumoniae and MenC resulted in a boosted secondary IgG anti-PPS response to S. pneumoniae. Our data demonstrate that the nature of the in vivo anti-PS response is markedly influenced by the composition and/or architecture of the bacterial subcapsular domain. The Journal of Immunology, 2012, 188: 569–577.

Polysaccharide (PS)-encapsulated extracellular bacteria such as Streptococcus pneumoniae (Gram-positive [GP]) and Neisseria meningitidis (Gram-negative [GN]) are major sources of global morbidity and mortality among infants, the elderly, and the immunosuppressed (1). Adaptive immunity to extracellular bacteria is mediated largely by Abs specific for both protein and PS Ags (2). Protein and PS Ags are biochemically distinct and are processed differently by cells of the immune system. Unlike proteins, non-zwitterionic PSs fail to associate with MHC class II molecules (3, 4) and are unable to recruit cognate CD4+ T cell help for induction of anti-PS responses (5). However, PSs in contrast to proteins can deliver strong and sustained signals to specific B cells through multivalent membrane Ig cross-linking via repeating, identical structural units (6), which critically affects the nature of the B cell response to various second signals (7).

Thus, protein and PS Ags are classified as T cell-dependent (TD) and T cell-independent (TI) Ags, respectively. This central dogma is derived mostly from studies using purified protein and PS (5). However, covalent linkage of protein and PS to create a soluble conjugate vaccine converts the PS into a TD Ag, including the ability to generate PS-specific memory (8). Intact bacteria are complex particulate immunogens in which multiple protein and PS Ags and bacterial adjuvants are coexpressed. This raises the question of whether the PS expressed by intact bacteria also behaves like TD Ags, similar to those in conjugate vaccines. We previously demonstrated that the IgG anti-PS (capsular type 14 polysaccharide [PPS14]) response to intact, heat-killed Streptococcus pneumoniae, capsular type 14 (Pn14), a GP extracellular bacterium, is dependent on CD4+ T cells, B7-dependent costimulation, and CD40–CD40L interactions and comprises all four isotypes of IgG (as opposed to predominantly IgG3 and some IgG1 for isolated PS Ags) (9, 10), similar to that observed for the IgG anti-protein response. In contrast to the anti-protein response, the IgG anti-PPS14 response to intact Pn14 exhibits a rapid primary IgG response, dependent upon a shorter period of T cell help and B7-dependent costimulation, and fails to generate a boosted secondary response (11). Furthermore, the IgG anti-PPS14, in contrast to the IgG anti-protein response to S. pneumoniae, is ICOS independent, extracellular (10), and more apoptosis prone (12). Thus, PPS14 in the context of intact Pn14 combines certain features of both an isolated PS Ag and a PS–protein conjugate vaccine (11).

Studies on the anti-PPS14 response to intact Pn14 indicate that the bacterium can markedly influence the immunobiology of the expressed PS Ag. These studies, however, left unresolved whether the nature of the PPS14-specific Ig response to intact Pn14 was characteristic of intact PS-expressing extracellular bacterial subcapsular domains.

Abbreviations used in this article: CpG-ODN, CpG-containing oligodeoxynucleotide; GN, Gram-negative; GP, Gram-positive; MCPS, meningococcal type C polysaccharide; MCPPS–TT, conjugate of MCPS and tetanus toxoid; MenC, intact Neisseria meningitidis, serogroup C; Pn14, Streptococcus pneumoniae, capsular type 14; PPS, pneumococcal capsular polysaccharide; PPS14, capsular type 14 polysaccharide; PS, polysaccharide; PspA, pneumococcal surface protein A; TD, T cell-dependent; TI, T cell-independent; WT, wild-type.

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Received for publication May 17, 2011. Accepted for publication November 9, 2011.

This work was supported by the National Institutes of Health (Grant 2R01-AI49192 to C.M.S.) and by the Uniformed Services University of the Health Sciences Dean’s Research and Education Endowment Fund (to C.M.S.).

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Abbreviations used in this article: CpG-ODN, CpG-containing oligodeoxynucleotide; GN, Gram-negative; GP, Gram-positive; MCPS, meningococcal type C polysaccharide; MCPPS–TT, conjugate of MCPS and tetanus toxoid; MenC, intact Neisseria meningitidis, serogroup C; Pn14, Streptococcus pneumoniae, capsular type 14; PPS, pneumococcal capsular polysaccharide; PPS14, capsular type 14 polysaccharide; PS, polysaccharide; PspA, pneumococcal surface protein A; TD, T cell-dependent; TI, T cell-independent; WT, wild-type.

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bacteria in general or perhaps represented a characteristic feature of PPS14, the underlying structure and/or composition of intact S. pneumoniae, or perhaps a more general dichotomy between GP and GN bacteria. Thus PPS14, among several other pathogen-derived substances, can bind to SIGN-R1, a scavenger receptor present on marginal zone macrophages (13). Capsular PS may additionally vary based on m.w. (14), charge characteristics (15), sialic acid content (16), or unique immunomodulatory properties (17), which may influence the nature of the associated immune response. Further, bacteria may express components within the cell wall, such as phosphorylcholine expressed by S. pneumoniae, and other pathogens, which may inhibit immunity (18).

In addition to the above considerations, the structure of intact GP and GN extracellular bacteria are significantly different, and these differences may influence the nature of the anti-PS response to the intact bacteria. Thus, capsular PS expressed by GP bacteria are covalently linked to a thick, underlying cell wall peptidoglycan to which a number of proteins are also covalently attached (19, 20). Capsular PS expressed by GN bacteria, which express a thin peptidoglycan cell wall, is attached through a labile covalent linkage to the acyl glycerol moiety of the outer membrane. The outer membrane is known to have multiple immunomodulatory properties, in part due to the presence of porin proteins (TLR2 ligand) and LPS (TLR4 ligand) (21, 22). Notably, immunization of mice with either 1) crude outer membrane complexes from intact Neisseria meningitidis, serogroup C (MenC) (containing both LPS and capsular PS), 2) purified complexes (lacking LPS), or 3) outer membrane complexes (lacking meningococcal type C polysaccharide (MCPS) all resulted in significant boosting of the IgG anti-MCPS response after secondary immunization (23).

In addition, mice primed systemically with live MenC exhibited a highly boosted IgG anti-MCPS response after secondary infection (24). Collectively, these data suggest that the nature of the PS-specific Ig response to intact S. pneumoniae and N. meningitidis may differ.

In this report, we used intact, heat-killed MenC to determine how the biochemically complex bacterial particle influences the nature of the anti-MCPS response relative to that elicited by isolated, soluble MCPS. In addition, these experiments were performed in a manner analogous to those previously conducted using intact heat-killed Phn14 to determine whether bacteria comprising different structures may influence the Ig anti-PS responses in distinct ways. We demonstrate that the anti-MCPS response to intact MenC is strikingly different from that elicited by isolated MCPS and is also different from the anti-PPS14 response to intact Phn14. We conclude that the nature of the in vivo anti-PS response is markedly influenced by the composition and/or architecture of the subcapsular domain.

Materials and Methods

**Mice**

CD28−/− mice (C57BL/6 background; B6.129S2-CD28tm1Mak/J; cat. no. 002666), ICOS−/− mice (C57BL/6 background; B6.129P2-Icos−/−; cat. no. 004589), C3H/HeJ mice (cat. no. 000659), C3H/HeJ bald mice (cat. no. 000655), TLR2−/− mice (C57BL/6 background; B6.129-Tlr2−/−; cat. no. 004650), and MyD88−/− mice (C57BL/6 background; B6.129-Tlr2−/−; MyD88−/−; cat. no. 130659) were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6, BALB/c, 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**

MenC (strain M1883; ATCC 53414) was obtained from American Type Culture Collection (Manassas, VA). The encapsulated MenC strain FAM18 C+ and its isogenic unencapsulated variant strain FAM18 C− was a kind gift of Dr. Mustafa Akkoyunlu (Food and Drug Administration, Bethesda, MD) (25). Lyophilized or frozen stocks of bacteria were grown overnight on BBL blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Brain Heart Infusion 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 to PBS to give an absorbance reading at 650 nm of 0.1, which corresponds with 109 CFU/ml. Bacteria were then aliquoted at 1010 CFU/ml and frozen at −20°C until their use as immunogen for mouse immunizations. Phn14 (strain R614) was prepared as described previously (26).

**Reagents**

A covalent conjugate of MCPS and tetanus toxoid (MCPS–TT) was prepared as previously described (9). Purified MCPS was obtained from the Serum Institute of India (Pune, India). Purified PPS14 was purchased from an American Type Culture Collection. Rat IgG2b anti-mouse CD40L mAb (clone MR1), polyclonal hamster IgG, rat IgG2a anti-mouse CD275 mAb (HK5.3), and rat IgG2a isotype control (clone 2A3) were purchased from BioXcell (West Lebanon, NH). Alum (Alhydrogel 2%) was obtained from Brenntag Biosector (Frederikssund, Denmark). A stimulatory 30mer CpG-containing oligodeoxynucleotide (CpG-ODN) was synthesized as previously described (27).

**Purification of native PorB protein from N. meningitidis**

Neisseria meningitidis serogroup B (strain H44/76) (28) was grown on gonococcal-agar plates containing 1% (v/v) Isosvitalex overnight at 37°C in a humidified incubator with 5% (v/v) CO2. The next day, colonies were inoculated in liquid gonococcal medium and grown to exponential phase as previously described (29). After overnight expansion of the cultures, the bacterial suspension was centrifuged at 3900 g for 15 min at 4°C, and the total protein content was extracted with the CaCl2-Zwittgenert method. PorB was purified using an AktaPrime protein chromatography machine as previously described (29). Briefly, the protein suspension was first subjected to ion-exchange chromatography on two DEAE/carbosymethyl columns in tandem, followed by gel filtration chromatography on a Sephacryl S-300 column and finally a Matrix Cellufine Sulfate column for removal of endotoxin traces.

**Preparation of MenC whole protein extract**

Uncapsulated FAM18 C− bacteria were centrifuged at 3000 rpm for 20 min, and the supernatant was discarded. The 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 and again centrifuged at 3000 rpm for 10 min. The supernatant containing the soluble proteins was obtained, and the concentration was determined by the BCA assay (Pierce).

**Production and purification of recombinant pneumococcal surface protein A**

Recombinant pneumococcal surface protein A (PspA) was expressed in *Saccharomyces cerevisiae* BJ3505. The supernatant was then passed through a Q FF column (Amersham Pharmacia, Piscataway, NJ) and eluted with 0.2 M NaCl solution containing 20 mM Tris pH 9. Eluted PspA was then added to a Phenyl HP column (Amersham Pharmacia) equilibrated with 40 mM phosphate buffer containing 1.3 M ammonium sulfate, pH 7. PspA was then eluted from this column using a 1.3 to 0.4 M ammonium sulfate gradient. Pooled PspA was dialyzed against 20 mM sodium acetate, pH 4.7 solution, and loaded into an S15 column (Amersham Pharmacia). PspA was then eluted with an 0.3 M NaCl solution containing 20 mM sodium acetate. Eluted PspA was dialyzed and concentrated and found to be 95% pure by Coomassie staining.

**Immunization**

Depending on the experiment, groups of seven mice were immunized with 2 × 108 CFU heat-killed or UV-inactivated MenC in saline (i.p. or i.v.), 5 × 107 CFU live MenC (i.p.), 10 μg purified MCPS in saline (i.p.), or 1 μg

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MCPS–TT adsorbed on 13 μg alum mixed with 25 μg CpG-ODN (i.p.). Serum samples were prepared at different time points from blood obtained through the tail vein.

**ELISA**

For measurement of serum titers of MCPS-specific Ig, Immulon 4 ELISA plates were precoated with poly-l-lysine (Sigma) (5 μg/ml, 100 μl/well) in PBS for 1 h at 37°C. The plates were then washed three times with PBS plus 0.1% Tween 20 and then coated overnight at 4°C with purified MCPS (10 μg/ml, 100 μl/well) in PBS. To measure the serum titers of PorB- or whole-protein-specific Ig, the plates were directly coated with PorB or whole protein extract (2 μg/ml, 50 μl/well). Plates were then washed three times with PBS plus 0.1% Tween 20 and then blocked with PBS plus 1.0% BSA for 1 h at 37°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 1.0% 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, 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 and then coated overnight at 4˚C with purified MCPS whole protein extract (2 × 10⁸ CFU/mouse) in saline or MCPS–TT conjugate vaccine. We immunized BALB/c mice i.p. with either UV-inactivated MenC (strain M1883) (2 × 10⁸ CFU/mouse) in saline or 1 μg MCPS–TT in alum plus CpG-ODN and boosted i.p. with the same dose on day 28. Serum titers of Ag-specific IgM and IgG were measured by ELISA. *p < 0.05 (significance between secondary titers relative to peak primary titers). B. BALB/c mice (seven per group) were immunized i.p. with 2 × 10⁸ CFU heat-inactivated intact FAM18 C+ (encapsulated MenC) or FAM18 C− (unencapsulated MenC) and boosted i.p. with the same dose on day 21. Serum titers of Ag-specific IgG were determined by ELISA. *p < 0.05 (significance between FAM18 C+ and FAM18 C−).

### Results

**The primary and secondary IgG anti-MCPS responses to intact MenC are similar to that elicited by a meningococcal conjugate vaccine**

We previously demonstrated that the anti-PPS14 response to intact heat-killed Pn14, a GP bacterium, is distinct from that elicited by a soluble covalent conjugate of PPS14 and PspA (PPS14–PspA), in that the former exhibited rapid primary kinetics (peak day 6) with no secondary boosting, despite the CD4+ T cell dependence of the IgG anti-PPS14 responses to both Pn14 and PPS14–PspA (11). Based on the distinct subcapsular composition and/or structure of GP and GN bacteria, we hypothesized that the PPS14-specific IgG response to Pn14 might be immunologically different from a PS-specific IgG response induced by a GN bacterium. In this regard, we chose to study the nature of the anti-MCPS response to intact MenC, a GN bacterium, in comparison with that elicited by a meningococcal conjugate vaccine. We immunized BALB/c mice i.p. with either UV-inactivated MenC (strain M1883) (2 × 10⁸ CFU/mouse) in saline or MCPS–TT conjugate (1 μg/mouse) in alum plus CpG-ODN and boosted the mice 28 d later in a similar fashion. As illustrated in Fig. 1A, there was minimal induction of serum IgM anti-MCPS in the primary response to M1883, with a 4-fold significant boost in serum titers after secondary immunization. In contrast, MCPS–TT elicited a significant though transient primary induction of IgM anti-MCPS and no boosting after secondary immunization. The primary IgG anti-MCPS responses to both M1883 and MCPS–TT were relatively modest and peaked on day 21 (M1883) or day 14 (MCPS–TT) (Fig. 1A). In contrast to IgM, the secondary IgG anti-MCPS responses to both M1883 and MCPS–TT were highly and rapidly boosted. The two MCPS-specific IgG responses were similar in kinetics and boosting to the associated anti-protein responses (PorB for M1883, TT for MCPS–TT). Similar primary and secondary IgM and IgG anti-MCPS responses were observed in response to 5 × 10⁸ CFU/mouse of live M1883 (injected i.p.) or 2 × 10⁸ CFU/mouse of heat-killed M1883 (injected i.v.) (data not shown) or i.p. (all experiments below). Immunization i.p. with heat-killed M1883 at 2 × 10⁸ CFU/mouse versus 1 × 10⁸ CFU/mouse gave similar results (data not shown).

The specificity of the MCPS-specific ELISA was established using a different strain of encapsulated MenC (FAM18 C+) versus its unencapsulated isogenic mutant (FAM18 C−) (25) (Fig. 1B). Thus, FAM18 C− failed to elicit a detectable IgG anti-MCPS response, in contrast to FAM18 C+, the latter exhibiting detectable primary and highly boosted secondary IgG anti-MCPS responses, whereas both strains induced comparable IgG anti-PorB responses. Collectively, these data demonstrate that the kinetics and boosting characteristics of the IgG anti-MCPS response to intact MenC is similar to that of a corresponding conjugate vaccine but distinct from the previously reported IgG anti-PPS14 response to intact Pn14. In addition, the weak IgM anti-MCPS response to M1883 contrasts with the previously reported high-titer IgM anti-PPS14 response to Pn14.

**The MCPS-specific IgM and IgG isotype responses to isolated MCPS and intact M1883 are distinct**

Isolated PS are poorly immunogenic, elicit Ig responses largely of the IgM isotype, with smaller amounts of IgG3, and sometimes IgG1, and fail to show boosted IgG anti-PS responses after secondary immunization (5). To determine the effect of M1883 on the associated anti-MCPS response, we directly compared the MCPS-specific IgM and IgG isotype responses to isolated, soluble MCPS...
(10 μg/mouse in saline) versus heat-killed, intact M1883 (2 × 10⁸ CFU/mouse) both injected i.p., followed by a similar secondary immunization on day 21. As illustrated in Fig. 2, the primary IgM anti-MCPS response to purified MCPS peaked by day 7 and failed to show a boosted secondary response, whereas the primary IgM anti-MCPS response to intact M1883 peaked by day 14 at somewhat lower peak titers than that observed for isolated MCPS, and also failed to show a boosted secondary response. The primary peak titers of MCPS-specific IgG were somewhat higher for mice immunized with M1883 versus MCPS (3-fold, p < 0.05), although the latter response peaked earlier (Fig. 2). In contrast, M1883 induced a 15-fold boost in IgG anti-MCPS serum titers after secondary immunization relative to the primary, whereas MCPS failed to induce any significant boost. Thus, M1883 overall elicited 42-fold higher secondary serum titers of IgG anti-MCPS relative to secondary titers after MCPS immunization. The IgG isotypes elicited by isolated MCPS were IgG3 and IgG1, with no detectable IgG2b or IgG2a, whereas intact M1883 induced all four IgG isotypes (Fig. 2). Collectively, these data demonstrate that the intact bacterium markedly changes the nature of the associated MCPS-specific Ig response relative to that observed with isolated MCPS.

**CD4⁺ T cells are required for the induction of the secondary but not primary IgG anti-MCPS response to intact M1883**

Whereas the Ig responses to isolated PS are largely TI, our previous studies using intact Pn14 demonstrated that the rapid primary IgG anti-PPS14 response was dependent on CD4⁺ T cells (11). In light of the CD4⁺ T cell dependence of the primary and boosted secondary IgG anti-MCPS response to intact M1883, we were interested in determining their relative CD4⁺ T cell dependence. To determine this, we injected M1883 into T cell-deficient athymic nude mice or BALB/c mice acutely depleted of CD4⁺ T cells by a single injection of a depleting rat IgG anti-mouse CD4 mAb (clone GK1.5) given 24 h prior to primary immunization (Fig. 3). Collectively, these data demonstrate that the intact bacterium markedly changes the nature of the associated MCPS-specific Ig response relative to that observed with isolated MCPS.

ICOS is a member of the CD28 family that is induced on CD4⁺ T cells upon TCR cross-linking and CD28-mediated signaling (30, 31). CD28, which is expressed constitutively on CD4⁺ T cells, is critical for initiation of CD4⁺ T cell activation, whereas ICOS plays a role in the subsequent T cell effector response such as the germinal center reaction and the generation of memory (32, 33). We previously demonstrated that the CD4⁺ TD primary IgG anti-PPS14 response to Pn14 is CD28/B7-2–dependent but ICOS-independent (10). In light of the CD4⁺ T cell dependence of the boosted secondary IgG anti-MCPS response to M1883, we wished to determine whether it was CD28- and/or ICOS-dependent. Thus, mice genetically deficient in CD28 (CD28−/−), ICOS (ICOS−/−), and strain-matched C57BL/6 wild-type (WT) mice were immunized i.p. with M1883 and boosted 3 wk later. As illustrated in Fig. 4, M1883-immunized WT mice elicited a detectable primary IgG anti-MCPS and anti-PorB response and, after secondary immunization, significant boosting in both MCPS- and PorB-specific IgG serum titers. The primary IgG anti-MCPS responses in CD28−/− and ICOS−/− mice were similar to that observed in WT control mice, consistent with the TI nature of the response (Fig. 3), whereas the CD4⁺ TD secondary IgG anti-MCPS responses were essentially abrogated in both mutant mouse strains (Fig. 4). In contrast, both the primary and secondary IgG anti-PorB responses...
were markedly inhibited in both CD28−/− and ICOS−/− mice. Similar data were obtained using neutralizing anti-ICOSL mAb (HK.5.3) versus control polyclonal hamster IgG injected into mice 1 d before immunization with M1883 (data not shown).

The interaction of CD40 on APC with CD40L, induced on CD4+ T cells after TCR/CD28-mediated activation, is critical for the induction of the TD primary Ig response, germinal center reaction, and the development of memory in response to immunization with protein Ags, or for the anti-PS response to conjugate vaccines (34, 35). A role for endogenous CD40L has also been reported in stimulating Ig responses to some but not all PS Ags (36–39). We previously demonstrated that the primary CD4+ TD IgG anti-PS14 response to S. pneumoniae was CD40L− as well as CD28-dependent (9, 10). We thus wished to determine whether CD40L-dependent costimulation was critical for the primary TI and/or TD IgG anti-MCPS response to M1883. Thus, we injected BALB/c mice i.p. with a single dose of blocking hamster IgG anti-mouse CD40L mAb (MR1) 1 d before M1883 immunization, using polyclonal hamster IgG as a control, with a secondary immunization 21 d later, using M1883 alone. As shown in Fig. 5, there was no difference in the primary anti-MCPS IgG response between the control and the MR1-injected group, whereas the secondary IgG anti-MCPS response in the MR1-injected group was markedly inhibited compared with control mice. In contrast, both the primary and secondary IgG anti-PorB responses were significantly reduced in the MR1-treated mice compared with controls. Collectively, these data demonstrate that the TD secondary, though not TI primary, IgG anti-MCPS response to intact M1883 is dependent on CD28+, ICOS-, and CD40L-dependent costimulation. This is in contrast to the TD primary IgG anti-PS14 response to Pn14, which is CD28 and CD40L dependent but ICOS independent.

Endogenous TLR4, but not TLR2 or MyD88, signaling is critical for induction of peak primary and secondary serum titers of MCPS-specific IgG in response to M1883 but is not critical for secondary boosting

LPS, a component of the outer membranes of GN bacteria, is a ligand for TLR4 and a potent activator of the innate immune system (40). We thus wished to determine whether endogenous TLR4 signaling affected the humoral immune response to M1883 and might account for the differences observed with the IgG anti-PS14 response to Pn14. We previously observed no role for TLR4 in the IgG anti-PS14 response to Pn14 (data not shown), although genetic deficiency of TLR2 resulted in a significant reduction in IgG3, IgG2b, and IgG2a but not IgG1 PPS14-specific serum titers (41). C3H/HeJ (Tlr4−/−) mice that carry a spontaneous mutation in the TLR4 gene and C3H/HeOuJ control mice were immunized i.p. with M1883 and boosted on days 21 and 35. As illustrated in Fig. 6A, both the primary, secondary, and tertiary IgG anti-MCPS responses in C3H/HeJ mice were significantly reduced, though not eliminated relative to control mice. Of note, the absence of TLR4 signaling did not prevent a secondary boost in the IgG anti-MCPS response. In contrast, there were no significant differences in the primary, secondary, or tertiary protein-specific IgG serum titers between the two groups (data not shown).

Further analysis of serum titers of IgM and IgG isotypes after secondary immunization indicated that TLR4 deficiency significantly reduced MCPS-specific IgM and IgG2a (Fig. 6A). Thus, endogenous TLR4 signaling plays a significant role in the magnitude of both the TI and TD components of the IgG anti-MCPS response but is not critical for the generation of memory. Of note, in additional experiments using TLR2−/− (Fig. 6B) and MyD88−/− (Fig. 6C) mice, no significant reductions in serum titers of MCPS-specific IgM or IgG were observed, although a significant 4-fold reduction in the IgG2b anti-MCPS titer was noted in MyD88−/− relative to control C57BL/6 mice.

**Coimmunization of MenC and Pn14 promotes secondary boosting of the IgG anti-PS14 response to Pn14**

The inability of Pn14 to induce a boosted secondary IgG anti-PS14 response remains unexplained. One possibility is that Pn14 fails to provide adequate innate signaling and/or inhibits the induction of the secondary, but not primary, IgG anti-MCPS response to M1883. BALB/c mice (seven per group) were injected i.p. with either anti-CD40L mAb (clone MR1) or polyclonal hamster IgG (“Control”) (0.3 mg per mouse). Mice were then immunized i.p. 1 d later with 2 × 10⁴ CFU heat-inactivated intact M1883 in saline and boosted 21 d later. Serum titers of Ag-specific IgG were determined by ELISA. *p < 0.05 (significance between “Control” versus anti-CD40L-injected mice).
M1883 had no effect on the IgG anti-PspA response to R614 (Fig. 7). Conversely, with the exception of a modest increase in the primary IgG anti-MCPS response to M1883 on day 14, coimmunization with R614 had no other significant effects on either the IgG anti-MCPS or IgG anti-PorB response. Isolated MCPS has been reported to increase expression of CD86 and MHC class II on murine B cells (17), suggesting a possible role in augmenting adaptive immunity. In this regard, in an additional experiment, coimmunization of R614 with either FAM18 C+ or FAM18 C− similarly boosted the secondary IgG anti-PPS14 response (Fig. 7B) indicating that the boosting effect of MenC is not secondary to the presence of the MCPS capsule. Thus, these data suggest

FIGURE 7. Coimmunization of MenC and Pn14 promotes secondary boosting of the IgG anti-PPS14 response to Pn14. BALB/c mice (seven per group) were immunized i.p. with $2 \times 10^8$ CFU heat-inactivated intact M1883 in saline and boosted on days 21 and 35. Serum titers of MCPS-specific IgM, IgG, and IgG isotypes were determined by ELISA from sera obtained on the indicated days. *p < 0.05 (significance between mice immunized with single bacteria versus combination).

FIGURE 6. Endogenous TLR4, but not TLR2 or MyD88, signaling is critical for induction of peak primary and secondary serum titers of MCPS-specific IgG in response to M1883 but is not critical for secondary boosting. C3H/HeJ (Tlr4−/−) and C3H/HeOuJ (control mice) (A) or TLR2−/− (B) or MyD88−/− (C) mice (same group of control C57BL/6 mice used for both B and C) (seven per group) were immunized i.p. with $2 \times 10^8$ CFU heat-inactivated intact M1883 in saline and boosted on days 21 and 35. Serum titers of MCPS-specific IgM, IgG, and IgG isotypes were determined by ELISA from sera obtained on the indicated days. *p < 0.05 (significance between C3H/HeOuJ versus C3H/HeJ in A and significance between C57BL/6 versus Myd88−/− in C).
that the failure of *S. pneumoniae* to induce a boosted secondary IgG response may reflect either subcritical innate signaling by *S. pneumoniae* or an inhibitory effect that can be overcome by MenC.

**Discussion**

The inability of non-zwitterionic capsular PS to associate with MHC class II molecules for presentation to CD4⁺ T cells (3, 4) results in a limited capacity of these Ags to elicit PS-specific IgG isotypes, GC formation, and IgG-specific memory (5). However, isolated PS have been reported to induce TI IgM-specific memory that is downregulated by PS-specific IgG (42). Covalent attachment of an immunogenic protein to an isolated PS results in the induction of a TD PS-specific IgG memory response (43). This likely occurs through specific uptake of PS–protein conjugate by PS-specific B cells with subsequent presentation of protein peptide/MHC class II to CD4⁺ T cells (35), although CD4⁺ T cells specific for the PS and combined PS–protein components are also elicited (44). Capsular PS expressed by extracellular bacteria is associated indirectly with immunogenic proteins, through covalent attachment to cell wall peptidoglycan (GP bacteria) (19, 20) or the acyl glycerol moiety of the outer membrane (45). This suggests the possibility that PS-specific Ig responses to intact bacteria might behave in a manner similar to that elicited by conjugate vaccines. Indeed, somatically mutated PS-specific IgG is elicited in response to encapsulated bacteria, suggesting a role for T cells in this process (46, 47). However, 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 (48). Nevertheless, 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 (49).

Our previous studies on the in vivo IgG anti-PPS14 response to intact, heat-killed Pn14 indicated that the bacteria significantly altered the nature of the associated PPS14-specific IgG response relative to that elicited by isolated PPS14. The IgG anti-PPS14 response to intact Pn14 was similar to that elicited by a TD conjugate vaccine, in that both responses were dependent on CD4⁺ T cells, B7-dependent costimulation, and CD40–CD40L interactions, with elicitation of all four IgG isotypes (9, 10). However, in contrast to conjugate vaccines, the IgG anti-PPS14 response to Pn14 was relatively rapid, failed to induce a boosted secondary response, and was ICOS-independent, similar to that elicited by TI isolated PS Ags. Nevertheless, Pn14 elicited a protein-specific (i.e., PspA) IgG response that was classically TD (9, 10). The basis for this unique immunologic behavior of intact Pn14 is a matter of ongoing investigation (26).

In this report, we wished to determine whether the nature of the PPS14–specific Ig response to intact Pn14 was characteristic of intact PS-expressing extracellular bacteria in general or perhaps represented either a characteristic feature of PPS14, the underlying structure and/or composition of intact *S. pneumoniae* or perhaps a more general dichotomy between GP and GN bacteria. To begin this address of this question, we studied the nature of the MCPS-specific Ig response to intact MenC, a GN bacterium, and compared this response to that elicited by an MCPS–TT conjugate or isolated MCPS and to our previously published data on Pn14. In this report, we demonstrate the following: 1) Intact MenC elicits an IgG anti-MCPS response that, similar to the MCPS–TT conjugate, shows prolonged primary kinetics of development and significant boosting upon secondary immunization. This is in distinct contrast to the IgG anti-PPS14 response to intact Pn14 that showed rapid primary kinetics of induction and no secondary boosting. The IgG anti-PorB and total IgG anti-MenC protein response to MenC shows similar kinetics and boosting to that of the IgG anti-MCPS response. 2) In contrast to MenC, the IgG anti-MCPS response to isolated MCPS shows rapid primary kinetics of induction, no boosting upon secondary immunization, and expression of IgG3 and IgG1 in contrast to all four IgG isotypes elicited by MenC, indicating a marked effect of the intact bacterium on the associated MCPS-specific IgG response. The absence of secondary boosting observed using isolated MCPS is consistent with early studies using other soluble PS Ags (50, 51). 3) Surprisingly, the primary IgG anti-MCPS response to intact MenC is TI, although the boosted secondary response, similar to both the primary and secondary IgG anti-PorB response, is dependent on CD4⁺ T cells and requires CD28, ICOS, and CD40L. This is in contrast to the primary IgG anti-PPS14 response to Pn14 that was dependent on CD4⁺ T cells, CD28, and CD40L, although not on ICOS. 4) Both the TI and TD IgG anti-MCPS response to MenC is significantly reduced in TLR4-defective (C3H/HeJ) but not TLR2⁻/⁻ or MyD88⁻/⁻ mice, although boosting of the IgG anti-MCPS response is still observed after secondary MenC immunization. 5) Immunization of MenC and Pn14 results in a boosting of the IgG anti-PPS14 response to Pn14, independent of expression by MenC of MCPS.

These data, and our previously published reports, demonstrate distinct differences in the nature of the PS-specific Ig responses to intact Pn14 versus MenC. Although it is clear from these data that the intact bacterium significantly alters the nature of the PS-specific Ig response to the associated PS Ag, it remains unresolved whether the differences between intact Pn14 and MenC reflect a more general dichotomy between GP and GN bacteria, the biochemical nature of the specific expressed PS Ag and its interaction with the immune system, or unique features in the composition and/or structure of one or both of these bacteria, not manifested by other bacterial species. In this regard, isolated MCPS and type V capsular PS from *Streptococcus agalactiae*, but not (4-hydroxy-3-nitrophenyl)acetyl–Ficoll, were shown to inhibit a number of B cell functions mediated by BAFF and APRIL (17). Additionally, MCPS enhanced the expression of CD86 and MHC class II on B cells. Other bacterial PSs, such as C-polysaccharide and PPS1 of *S. pneumoniae*, *Staphylococcus aureus* capsular polysaccharides types 5 and 8, and PS-A of *Bacteroides fragilis*, are zwitterionic on the basis of expression of both positive and negative charges and can stimulate CD4⁺ T cells through association with MHC class II (15). Additional differences between capsular PS such as the ability to bind to SIGN-R1, a scavenger receptor on splenic marginal zone macrophages (13, 52), capacity to fix complement (53), or expression of terminal sialic acid residues that interact with the inhibitory CD22 receptor on B cells (16), may also have potential effects on PS-specific Ig responses to intact bacteria. Thus, a determination of how differences in bacterial composition and structure may potentially impact the specific Ig response to an expressed capsular PS will require study of distinct bacteria with biochemically identical PS capsules.

The boosting of the IgG anti-MCPS response after secondary immunization with intact MenC and its dependence on CD4⁺ T cells, CD40L, CD28, and especially ICOS (30) strongly suggests that MCPS-specific B cells enter into a germinal center reaction in which MCPS–specific memory B cells are generated. This further suggests that MCPS-specific B cells engage in cognate interactions with CD4⁺ T cells that are specific for bacterial protein and that association, although not direct covalent attachment, of capsular PS and protein within the bacterial particulate is critical. It remains to be determined whether PS-specific B cells can directly internalize intact bacteria for presentation of bacterial
protein to CD4+ T cells or perhaps obtain bacterial protein indirectly after uptake, transport, and/or processing of intact bacteria by other immune cells, including dendritic cells and macrophages (54). Of note, the primary TI IgG anti-MCPS response to MenC was independent of CD40L. Previous reports have indicated a role for CD40L–CD40 interactions in the PPS-specific IgM and IgG response to isolated PPS (36), although not to TNP–Ficoll (39). The reasons for these differences remain unexplained.

Previous studies from our laboratory using an in vitro polyclonal model for multivalent membrane Ig cross-linking in response to PS Ags (i.e., multiple dextran-conjugated anti-IgD Abs) indicated that multivalent membrane Ig cross-linking alone, or in concert with CD40-mediated activation, induced vigorous proliferation but no Ig secretion or isotype switching by highly purified B cells (7). However, addition of a number of cytokines or various TLR ligands such as bacterial lipoproteins (TLR2), Neisseria porins (TLR2), unmethylated CpG-containing oligodeoxynucleotides (TLR9), or LPS (TLR4) to dextran-conjugated anti-IgD Ab-activated B cell cultures induced substantial Ig secretion and isotype switching (7). On this basis, we proposed that during natural infections with PS-encapsulated bacteria, the associated bacterial TLR ligands will play an especially critical role in eliciting the TI component of the anti-PS response, either through direct activation of B cells and/or via cytokine induction through innate cell stimulation (7). Our data (Fig 6) demonstrating an important role for TLR4 in enhancing the magnitude of the primary TI anti-MCPS response to intact MenC supports this hypothesis. TLR4 signaling in response to MenC is likely mediated by lipooligosaccharide (55). In this regard, the capacity of at least some isolated PS Ags to induce Ig secretory responses in vivo may reflect the presence of contaminating TLR ligands remaining after PS purification from the intact bacteria (56). Whether this is true for the IgM and IgG anti-MCPS to isolated MCPS remains to be determined.

TLR4-defective mice also exhibited a reduced secondary TD MCPS-specific IgG response to MenC, with the reduction in IgG due to selective abrogation of the IgG2a response. The capacity of LPS potently to induce IL-12–dependent IFN-γ (57), a switch factor for the IgG2a subclass (58), likely accounts for this effect.

In addition to LPS, IFN-γ has also been shown to stimulate maturation of B cells into IgM-secreting cells (59), perhaps accounting for the reduction in the IgM anti-MCPS as well. In this regard, a recent report demonstrated the ability of isolated MCPS to inhibit IFN-γ–mediated dendritic cells’ release of BAFF (17), a mediator of B cell maturation and class switching to IgG. Perhaps the stimulating effects of intact MenC override this inhibitory property of isolated MCPS. Nevertheless, TLR4-defective mice exhibited a boosted MCPS-specific secondary IgG response to MenC, despite reduced serum titers relative to WT controls, and no changes in the more prolonged kinetics of primary MCPS-specific IgG induction. Thus, differences in TLR signaling between Pn14 and MenC per se are unlikely to account for the distinct PS-specific IgG responses between the two bacteria. In this regard, the demonstration that coimmunization of Pn14 with MenC results in a boosted secondary IgG anti-PPS14 response to Pn14 suggests the possibility that nonviable Pn14 exerts an inhibitory influence on the PPS14-specific secondary IgG response that is at least partially overcome by innate signaling in response to MenC. The comparable IgM and IgG anti-MCPS responses to MenC, observed in MyD88−/− versus WT mice, despite reduced responses in TLR4-defective mice, suggest a possible role for TLR4-dependent TRIF signaling (60). Finally, although MenC expresses porins that are TLR2 ligands and signal in a MyD88-dependent manner (22), no significant differences in Ig responses to MenC were observed in TLR2−/− versus WT mice.

Disclosures

The authors have no financial conflicts of interest.

References