Differential Regulation of IgG Anti-Capsular Polysaccharide and Antiprotein Responses to Intact *Streptococcus pneumoniae* in the Presence of Cognate CD4$^+$ T Cell Help[1]

Abdul Q. Khan,* Andrew Lees,†‡ and Clifford M. Snapper[2]*

The relative lack of memory for IgG antipolysaccharide responses is believed to be secondary to the inability of polysaccharides to associate with MHC class II molecules and thus a failure to recruit cognate CD4$^+$ T cell help. However, little is known concerning the role of T cells and the generation of memory for antipolysaccharide Ig responses to intact extracellular bacteria. We used heat-killed, intact *Streptococcus pneumoniae*, capsular type 14 (Pn14), to evaluate the IgM and IgG responses specific for the capsular polysaccharide (PPS14), the phosphorylcholine determinant of the cell wall C-polysaccharide, and the cell wall protein, pneumococcal surface protein A (PspA). We demonstrate that the IgG (but not IgM), anti-PPS14, and anti-PspA responses to Pn14 are CD4$^+$ T cell dependent and TCR specific. Nevertheless, in contrast to the anti-PspA response, the IgG anti-PPS14 response shows no apparent memory, an accelerated kinetics of primary Ig induction, and a more rapid delivery of CD4$^+$ T cell help. In contrast, the IgG anti-phosphorylcholine response, although also dependent on CD4$^+$ T cells, is TCR nonspecific. We make similar observations using soluble conjugates of PPS14-PspA and C-polysaccharide-PspA. These data lead us to suggest that the central issue concerning the mechanisms underlying different functional outcomes for anti-bacterial IgG responses to capsular polysaccharide vs protein Ags is not necessarily based on the ability to recruit cognate CD4$^+$ T cell help, but perhaps on the nature of the B cell Ag receptor signaling that occurs and/or on the responding B cell subpopulations. *The Journal of Immunology*, 2004, 172: 532–539.

Infections with polysaccharide-encapsulated extracellular bacteria, such as *Streptococcus pneumoniae*, are a source of significant global morbidity and mortality (1–3). Adaptive immunity to extracellular bacteria is mediated largely by Ab specific for bacterial capsular polysaccharide, but Abs to cell wall polysaccharide and various protein Ags may also be protective (4). Studies using purified polysaccharide and protein Ags have demonstrated that induction of Ig in vivo is T cell-independent (TI)[3] and T cell-dependent (TD), respectively (5, 6). The inability of polysaccharides to associate with MHC class II molecules (7, 8), and hence their failure to facilitate cognate CD4$^+$ T cell help for Ig induction, may help to account for this dichotomy as well as for their relative inability to generate immunologic memory. However, polysaccharides, in contrast to proteins, can deliver strong and sustained signals to specific B cells through multivalent membrane Ig cross-linking via their repeating, identical monosaccharide moieties (9). The differential engagement of distinct B cell subpopulations by these two types of Ag may further account for distinct regulatory pathways in the elicitation of these specific Ig responses (10, 11).

Although extensive analyses of specific Ig responses to purified polysaccharide and protein Ags have contributed substantially to our understanding of how these distinct responses are regulated, far fewer analogous studies have been performed using intact extracellular bacteria as an immunogen. In addition to containing multiple adjuvanting moieties, including Toll-like receptor ligands important in stimulating the innate immune system (12), intact bacteria coexpress multiple protein and polysaccharide Ags within a single particulate structure. Although not yet demonstrated, the coexpression of protein with polysaccharide in the intact bacteria or within bacterial fragments could serve to recruit cognate CD4$^+$ T cell help for polysaccharide-specific B cells, similar to what has been demonstrated for soluble protein-polysaccharide conjugate vaccines (13–16). Furthermore, if CD4$^+$ T cells deliver cognate help for antipolysaccharide responses to intact bacteria, the question of whether these responses take on similar characteristics as those specific for bacterial proteins would be of particular interest.

To begin to address the above issues we initially studied the potential role of T cells in mediating help for an in vivo Ig response to the unencapsulated variant of *Streptococcus pneumoniae*, capsular type 2 (strain R36A) (17–19). Specifically, we immunized mice systemically with heat-killed R36A and measured the induction of both primary and secondary serum titers of IgM and/or IgG isotypes specific for the phosphorylcholine (PC) determinant of the cell wall C-polysaccharide (teichoic acid) and for the cell wall pneumococcal surface protein A (PspA). We demonstrated that whereas the IgM anti-PC response was TI, both the IgG anti-PC and IgG anti-PspA responses were dependent on TCR-α/β^+ CD4$^+$ T cells, B7-dependent costimulation, and CD40-CD40 ligand (CD40L) interactions. Nevertheless, in contrast to the IgG anti-PspA response, the IgG anti-PC response was more rapid,

Departments of *Pathology and †Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; and ‡Biosynexus, Gaithersburg, MD 20877

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[2] Address correspondence and reprint requests to Dr. Clifford M. Snapper, Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814. E-mail address: csnapper@usuhs.mil

[3] Abbreviations used in this paper: TI, T cell-independent; BCR, B cell Ag receptor; CD40L, CD40 ligand; C-PS, C-polysaccharide (teichoic acid); GBSIII, type III capsular polysaccharide from group B *Streptococcus*; KLH, keyhole limpet hemocyanin; mIg, membrane Ig; MZB, marginal zone B cell; PC, phosphorylcholine; Pn14, *Streptococcus pneumoniae*, capsular type 14; PPS14, capsular polysaccharide serotype 14; PspA, pneumococcal surface protein A; TD, T cell-dependent; TT, tetanus toxoid.

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was only modestly boosted on secondary immunization, and required a much shorter period of T cell help and B7-dependent costimulation, and most surprisingly, the T cell help was TCR nonspecific (18, 19).

The studies described above suggested distinct TD pathways for IgG antipolysaccharide vs antiprotein responses to intact *S. pneumoniae*. However, as PC is unusual in being both a self-Ag and a widely distributed environmental Ag that probably mediates continual priming of the host (20–22), it remains to be determined whether anti-PC responses to intact bacteria are representative of the more clinically relevant, serotype-specific, anti-capsular polysaccharide responses. In this study we address this question and extend our previous observations (19) by determining the kinetics of Ig induction, the role and nature of the T cell help, and the ability to achieve secondary boosting for in vivo IgM and/or IgG anti-capsular polysaccharide (PPS14) as well as for anti-PC and anti-PspA responses to intact heat-killed *S. pneumoniae*, capsular type 14 (Pn14), or soluble, covalent conjugates of C-polysaccharide (C-PS)-PspA and PPS14-PspA.

Materials and Methods

Mice

H-Y$\alpha^{-/-}$ mice, a gift from Dr. O. Kanagawa (Washington University School of Medicine, St. Louis, MO) were created through introduction of the H-Y (male Ag) TCR transgene into TCR$\alpha^{-/-}$ mice as previously described (23) and were bred in our animal facility. Female 129B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used as controls for H-Y$\alpha^{-/-}$ mice. Female athymic nude and control BALB/c mice were obtained from the National Cancer Institute (Gaithersburg, MD). Mice were used at 7–10 wk of age and were maintained in a pathogen-free environment at U.S. Uniformed Health Services (Bethesda, MD). The experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare (National Institutes of Health document 78-23).

Reagents

Recombinant PspA was expressed in *Saccharomyces cerevisiae* BJ3505 as a His^6-tagged fusion protein and was purified by Ni-NTA affinity chromatography (17). PC-keyhole limpet hemocyanin (PC-KLH) was synthesized as described previously (17). The resulting conjugate had a substitution degree of 19 PC/KLH molecules. Purified *S. pneumoniae* capsular PPS14 was purchased from American Type Culture Collection (Manassas, VA). Purified *S. pneumoniae* cell wall C-PS was purchased from Statens Serum Institute (Copenhagen, Denmark). Rat IgG2b anti-mouse CD4 mAb (GK1.5) was purified from ascites by ammonium sulfate precipitation and passage over a protein G column. Purified rat IgG was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY).

Preparation and immunization of Pn14

A frozen stock of Pn14, was thawed and subcultured on BBL premade blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies in blood agar were grown in Todd Hewitt broth (BD Biosciences, Sparks, MD) to mid-log phase, collected, and heat-killed by incubation at 60°C for 1 h. 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 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} CFU/ml and frozen at −80°C until their use as Ag for mouse immunizations. Mice were immunized i.p. with 2×10^8 CFU of heat-killed bacteria in 250 μl of PBS. Serum samples for measurement of anti-PPS14, anti-PC, and anti-PspA Ab titers were prepared from blood obtained through the tail vein.

Preparation of C-PS-PspA and PPS14-PspA conjugates

Preparation of NH_2-C-PS. Ten milligrams of C-PS was made up in 1 ml of H_2O and left to solubilize at room temperature overnight. C-PS was functionalized with amines as previously described (24). In brief, 35 μl of 1-cyano-4 dimethyl aminopyridine tetrafluoroborate (100 mg/ml in acetonitrile; Research Organics, Cleveland, OH) was added to 5 mg (0.5 ml) of C-PS. The pH was maintained at 9.0 by the addition of 0.2 M triethylamine over 2 min. At 2.5 min, 0.5 ml of hexanediamine, adjusted to pH 9.3 with sodium bicarbonate, was added. After 2-h incubation at room temperature, the solution was dialyzed exhaustively into water. Primary amines were determined using trinitrobenzenesulfonic acid. The C-PS concentration was determined using a resorcinol/sulfuric acid assay (25). The product contained ~16 amines/100 kDa of C-PS.

Preparation of C-PS-PspA. Maleimide was added to C-PS as follows: 100 μl of 0.75 M HEPES and 10 mM EDTA, pH 7.3, was added to NH_2-C-PS (1.3 ml, ~ 5 mg) and 5 mg of *N*-(γ-maleimidobutyryloxy)succinimide ester (BioAffinity Systems, Roscoe, IL) in 178 μl of *N*-methylpyrrolidone was added. After 1 h, unreacted reagent was removed by repeated washings using am Ultrafree 4 (30 kDa cutoff; Millipore, Bedford, MA) device and 10 mM MES, 0.15 M NaCl, and 2 mM EDTA, pH 6.5 (MES buffer). The final volume was 400 μl. PspA was labeled with thiols as follows. PspA (2.3 mg in 300 μl of 0.1 M HEPES, pH 8) was reacted with 8 μl of 0.1 M *N*-succinimidyl 3-(2-pyridyldithio)propionate (BioAffinity Systems) for 1 h. The pH was reduced to 5 by the addition of 1 M sodium acetate, pH 5, and then the mixture was incubated with 25 mM DTT for 30 min. The protein was desalted on a P6DG column (Bio-Rad, Hercules, CA) equilibrated in MES buffer and concentrated to 400 μl using an Ultrafree 4 (10 kDa cutoff) device (Millipore). The maleimide-C-PS and the thiolated PspA were combined, and the pH was raised to 7.3. After an overnight incubation at 4°C, the reaction was quenched by first making the solution 0.2 mM ME and then 10 mM iodoacetamide. The conjugate was obtained by fractionation on a 1 × 60-cm S300HR column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS. The void volume fraction was pooled and sterile-filtered using a Millipore GV device. The conjugate was determined to contain approximately 2 mg of PspA/mg C-PS.

Preparation of PPS14-PspA. NH_2-PPS14 prepared as described (24), was briefly sonicated to reduce its viscosity and was filtered using a 0.45-μm pore size filter. Maleimide was added as described for C-PS. PspA at 16 mg/ml was labeled with an 8-fold molar excess of *N*-succinimidyl 3-(2-pyridyldithio)propionate. After 1 h, the thiol was deprotected in 25 mM DTT at pH 5, desalted, and concentrated. The maleimide-PPS14 and thiolated PspA were combined (1.5 mg PspA/mg Pn14) and reacted at pH 7.3. After quenching with ME and iodoacetamide, the conjugate was fractionated by gel filtration, and the void volume was pooled. The conjugate was sterile-filtered and was determined to contain 1.2 mg of PspA/mg of PPS14.

Measurement of serum Ig isotype titers of anti-PC, anti-PPS14, and anti-PspA

ELISA plates were coated with 5 μg/ml (50 μl/well) of either PC-KLH (Immulon 2 plates; Dynex Technologies, Chantilly, VA), PPS14 (Immulon 2 or 4), or PspA (Immulon 4) in PBS for 1 h at 37°C or overnight at 4°C. Plates were washed three times with PBS and 0.1% Tween 20 and were blocked with PBS and 1% BSA for 30 min at 37°C or overnight at 4°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS and 0.05% Tween 20 were then added for 1 h at 37°C or overnight at 4°C, and plates were washed three times with PBS and 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM or IgG Abs (200 ng/ml final concentration in PBS and 0.05% Tween 20) were then added, and plates were incubated at 37°C for 1 h. Plates were washed five times with PBS and 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma-Aldrich, St. Louis, MO) at 1 mg/ml in TM buffer (1 M Tris and 0.3 mM $MgCl_2$, pH 9.8) was then added for 30 min at room temperature for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Ospoo, Finland).

Statistics

Data were expressed as the arithmetic mean ± SEM of the individual titer. Levels of significance of the differences between groups were determined by Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Distinct primary kinetics and boosting of anti-PPS14 and anti-PC vs anti-PspA responses to intact Pn14

In a previous study we demonstrated that the primary in vivo IgG anti-PC response, in distinct contrast to that for IgG anti-PspA, developed more rapidly and demonstrated either no or relatively moderate boosting upon secondary immunization in response to immunization with the heat-killed, unencapsulated variant of *S. pneumoniae*, capsular type 2 (strain R36A) (19). Nevertheless, both responses were dependent on $CD4^+TCR\alpha/\beta^+$ T cells, B7-2-dependent costimulation, and CD40/CD40L interactions (17–19).

Surprisingly, in contrast to the T cell help for IgG anti-PspA responses, that for IgG anti-PC was TCR nonspecific and delivered more rapidly (19). In light of the distinct nature of the PC determinant of the cell wall teichoic acid, this study left unresolved whether the more clinically relevant anticapsular polysaccharide response to an intact extracellular bacteria was regulated in a similar manner to that observed for anti-PC.

Thus, we immunized mice i.p. with heat-killed intact Pn14 and determined the IgM and/or IgG responses specific for PPS14, PC, and PspA after both primary and secondary immunizations. As illustrated in Fig. 1*A*, a significant increase in serum titers of IgM anti-PC and IgM anti-PPS14 were observed as early as day 3 after primary immunization and peaked on day 5, whereas the IgG anti-PC and IgG anti-PPS14 responses showed somewhat more delayed kinetics, with little specific IgG observed on day 3, but optimal titers also seen on day 5. In distinct contrast, the primary IgG anti-PspA response developed more slowly, with peak serum titers observed on day 11. The kinetics of the anti-PC and anti-PspA responses to Pn14 were essentially identical with that observed previously for R36A (17, 19). Upon secondary immunization, the IgG anti-PspA response was consistently boosted by >20-fold relative to primary serum titers (Fig. 1B). In contrast, the IgM and IgG anti-PPS14 responses variably showed either no boosting or up to 3-fold boosting relative to the primary response. More consistent boosting of the IgM and IgG anti-PC responses were observed, but, again, were typically no >3- to 4-fold. These data demonstrate that in contrast to the anti-PspA response, which behaves like a classical TD antiprotein response, the kinetics and boosting characteristics of the anti-PC and anti-PPS14 responses are more similar to those observed for purified polysaccharide Ags.

IgG anti-PPS14, anti-PC, and anti-PspA, but not IgM anti-PPS14 and anti-PC, responses to intact Pn14 are dependent on CD4$^+$ T cells and CD40L-dependent costimulation

We next determined the role of T cells in the protein- and polysaccharide-specific IgM and IgG responses to intact Pn14. Athymic nude or control BALB/c mice were immunized i.p. with heat-killed Pn14, and sera were obtained on days 7 and 14. As illustrated in Fig. 2*A*, the IgM anti-PPS14 and anti-PC responses were T cell independent. In contrast, induction of optimal IgG responses specific for PPS14, PC, and PspA was strongly dependent on T cells. Complementary studies, in which anti-CD4 mAb was injected into wild-type mice before Pn14 immunization to deplete CD4$^+$ T cells, showed a similar strong dependence of all three IgG, but not IgM, responses on CD4$^+$ T cell help (Fig. 2*B*). In light of the critical importance of CD40/CD40L interactions for mediating in vivo TD Ig responses to soluble protein Ags, but not for soluble TI-2 polysaccharide Ags, we injected a blocking anti-CD40L mAb (MR1) into wild-type mice before Pn14 immunization. Consistent with a requirement for CD4$^+$ T cells, IgG anti-PPS14, anti-PC, and anti-PspA responses, but not IgM anti-PPS14 or anti-PC responses, were strongly inhibited by anti-CD40L mAb. Thus, in response to intact bacteria IgG, but not IgM, responses specific for both a cell wall and capsular polysaccharide as well as a cell wall protein are strongly dependent on CD4$^+$ T cells and CD40L-dependent costimulation, unlike responses observed for purified soluble polysaccharide Ags (5).

CD4$^+$ T cell help for induction of IgG anti-PC and IgG anti-PPS14 responses to intact Pn14 is delivered rapidly relative to that for the IgG anti-PspA response

In a previous study we demonstrated that the CD4$^+$ T cell help for the IgG anti-PC response to intact R36A was delivered more rapidly than that for the IgG anti-PspA response (19). In the next experiment we wished to determine the period during which CD4$^+$ T cells were required after immunization with intact Pn14 for induction of the IgG anti-PPS14 response relative to IgG anti-PC and IgG anti-PspA. Anti-CD4 mAb was injected into separate groups of immunized mice on day 0, 1, 2, 3, or 4 after Pn14 injection. A control rat IgG was injected into immunized mice on day 0, and these mice were considered to represent the maximal response. Sera were collected on day 7 for peak titers of anti-PC and anti-PPS14 and on day 14 for peak titers of anti-PspA. Anti-CD4 mAb had a significant, although modest, inhibitory effect (20–30% inhibition) on the IgM anti-PC and anti-PPS14 response when injected on day 0, 1, or 2 (Fig. 3). In contrast anti-CD4 mAb was

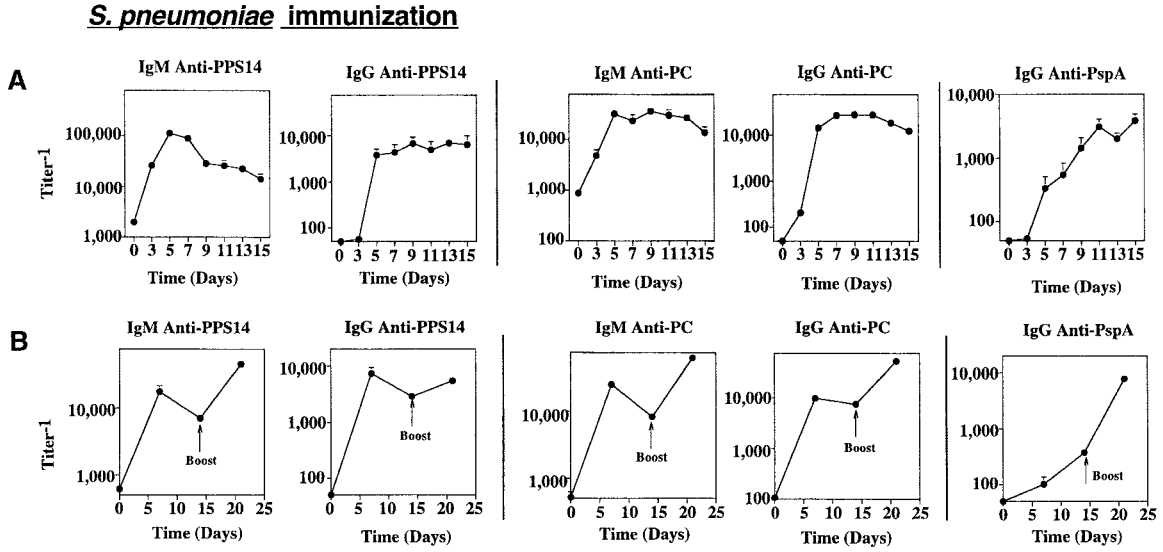

FIGURE 1. The IgG anti-PPS14 and IgG anti-PC responses are more rapid than the IgG anti-PspA response to intact Pn14 and show little, if any, generation of memory. *A*, Eight BALB/c mice were injected i.p with 2×10^8 CFU of heat-killed Pn14. Sera were obtained on different days for determination of Ag-specific Ig isotype titers by ELISA. *B*, Eight BALB/c mice were each immunized with Pn14 on day 0 and then on day 14. Sera were obtained on different days for determination of Ag-specific Ig isotype titers by ELISA. Values for both *A* and *B* represent the arithmetic mean ± SEM, and each is representative of three independent experiments.

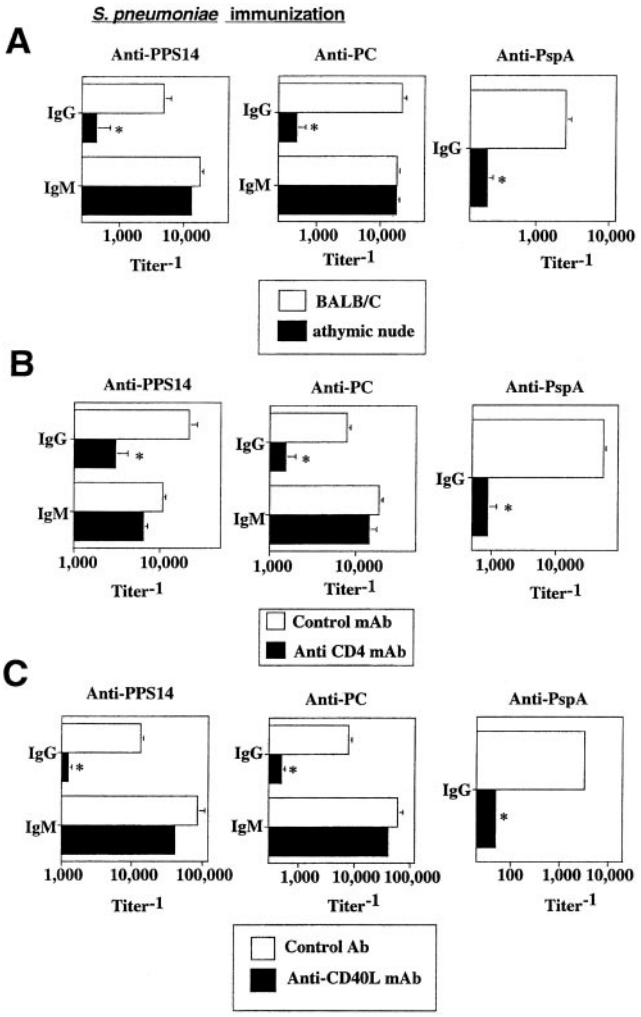

FIGURE 2. Dependence of IgG anti-PC, IgG anti-PPS14, and IgG anti-PspA responses to intact Pn14 on $CD4^+$ T cells and CD40L. *A,* Athymic nude and control BALB/c mice (eight mice per group) were injected i.p. with 2×10^8 CFU of heat-killed Pn14. Sera were obtained on day 7 (peak anti-PPS14 and anti-PC) and day 14 (peak anti-PspA) for determination of Ag-specific Ig isotype titers by ELISA. Values represent the arithmetic mean ± SEM and are representative of three independent experiments. *B,* BALB/c mice (six mice per group) were injected i.p. with 1 mg of either anti-CD4 mAb or control rat IgG. Twenty-four hours later, mice were injected i.p. with Pn14, and sera were analyzed as described in *A.* One of two representative experiments is shown. *C,* BALB/c mice (six per group) were injected i.p with 0.3 mg of anti-CD40L mAb (MR-1) or the same amount of control hamster IgG. Twenty-four hours later, mice were injected i.p. with Pn14, and sera were analyzed as described in *A.* One of three representative experiments is shown. ∗, $p \leq 0.05$.

strongly inhibitory for the IgG anti-PC and IgG anti-PPS14 responses when injected on day 0 (95% inhibition), with progressively less inhibition seen on days 1 (80%) and 2 (40–60%). No effect of the anti-CD4 mAb was observed when it was injected on day 3 or 4. In contrast, anti-CD4 mAb strongly inhibited the IgG anti-PspA response even when injected 4 days after immunization (85% inhibition). Thus, the kinetics of $CD4^+$ T cell help are relatively rapid and are similar for both the IgG anti-PC and IgG anti-PPS14 responses compared with those for IgG anti-PspA.

T cell help for both IgG anti-PPS14 and PspA is TCR specific, in contrast to that observed for IgG anti-PC

In a previous study we used H-Y$\alpha^{-/-}$ mice (23) to demonstrate that whereas $CD4^+$ T cell help for the IgG anti-PspA response to

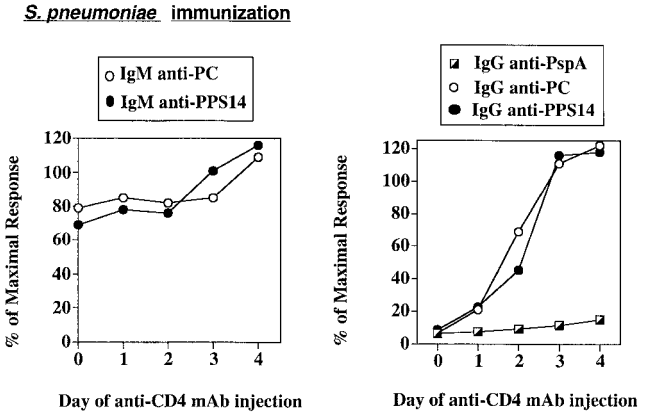

FIGURE 3. More rapid delivery of $CD4^+$ T cell help for anti-PPS14 and anti-PC vs anti-PspA responses to intact Pn14. Different groups of BALB/c mice (eight mice per group) were immunized i.p. with 2×10^8 CFU of heat-killed Pn14 with either 1 mg of control rat IgG on day 0 or with 1 mg of anti-CD4 mAb on day 0, 1, 2, 3, or 4. Sera were obtained on day 7 (peak anti-PPS14 and anti-PC) and day 14 (peak anti-PspA) for determination of Ag-specific Ig isotype titers by ELISA. Values represent the arithmetic mean ± SEM. One experiment was performed.

R36A was TCR specific, that for the IgG anti-PC response was TCR nonspecific (19). In particular, as all TCR transgenic mice exhibit variable degrees of endogenous TCR gene rearrangements leading to a T cell repertoire with multiple specificities, we used a TCR transgenic mouse (specific for the male, H-Y Ag in association with MHC class I) that had previously been crossed with mice genetically deficient in TCR-α (designated H-Y$\alpha^{-/-}$). These mice expressed no endogenous TCR, possessed relatively normal numbers of $CD4^+$ and $CD8^+$ T cells, and had normal numbers of B cells. $CD8^+$ T cells in H-Y$\alpha^{-/-}$ mice expressed specificity for H-Y, whereas $CD4^+$ T cells were Id negative, but did express TCR-β, perhaps in association with another, undetermined protein (23). Importantly, although both the Id-positive and -negative T cells from H-Y$\alpha^{-/-}$ mice both responded to Con A, they failed to respond to a conventional protein Ag in adjuvant. This observation is consistent with other reports using nontransgenic TCR-$\alpha^{-/-}$ mice, which also demonstrate complete abrogation of Ig responses to conventional protein Ags.

Using H-Y$\alpha^{-/-}$ mice, we next determined whether the IgG anti-PPS14 response to heat-killed Pn14 required specific T cells. H-Y$\alpha^{-/-}$ and control 129B6 mice were immunized i.p. with heat-killed Pn14, and sera were obtained on days 7 and 14. As illustrated in Fig. 4, H-Y$\alpha^{-/-}$ and control mice showed essentially equivalent IgM anti-PPS14, IgM anti-PC, as well as IgG anti-PC responses. In contrast, the IgG anti-PPS14 and IgG anti-PspA responses were strongly reduced in H-Y$\alpha^{-/-}$ mice relative to controls, indicating a requirement for specific T cells for both the IgG anti-PPS14 and anti-PspA, but not anti-PC, responses.

Specific IgM and/or IgG responses to soluble conjugates of PPS14-PspA and C-PS-PspA are regulated in a manner similar to that observed for heat-killed, intact Pn14

These data support our initial hypothesis (26) that an in vivo IgG antipolysaccharide response to an intact bacteria, but not to a soluble, purified polysaccharide Ag, would be augmented by endogenous $CD4^+$ T cells. This hypothesis was based on the idea that coexpression of protein with polysaccharide within the intact bacteria would allow for recruitment of T cell help in a manner similar to that observed for soluble polysaccharide-protein conjugate vaccines. Consistent with this hypothesis is our current observation

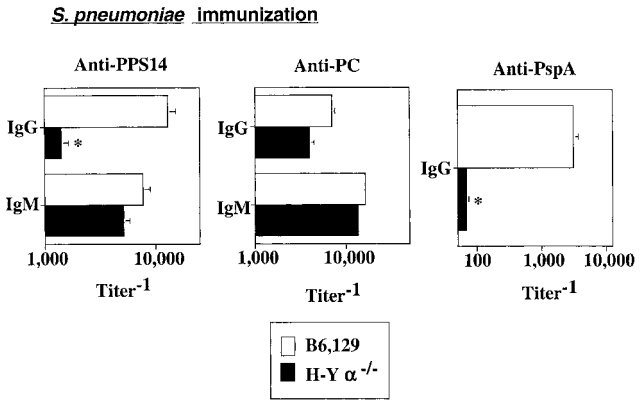

FIGURE 4. The T cell help for the IgG anti-PPS14 and IgG anti-PspA, but not the IgG anti-PC response to intact *S. pneumoniae*, is TCR-specific. H-Y$\alpha^{-/-}$ and control B6,129 mice (eight mice per group) were immunized i.p. with 2×10^8 CFU of heat-killed Pn14. Sera were obtained on day 7 (anti-PPS14 and anti-PC) and day 14 (anti-PspA) for determination of Ag-specific Ig isotype titers by ELISA. Values represent the arithmetic mean ± SEM. *, $p \leq 0.05$. One of three representative experiments is shown.

that an optimal IgG anti-PPS14 response to intact Pn14 is indeed dependent on specific $CD4^+$ T cell help, but not with our finding that the $CD4^+$ T cell help for the IgG anti-PC response is TCR nonspecific. One potential explanation is that whereas PPS14-specific B cells bind and internalize Pn14-derived PPS14 in association with bacterial protein, PC-specific B cells do not. Alternatively, it is possible that the two responses are mediated by distinct B cell subsets that differ physiologically in their ability to engage in cognate interactions with T cells. To begin to address these possibilities and to compare Ig responses to intact bacteria with those of soluble, bacterial-derived polysaccharide-protein conjugates, we directly conjugated PspA to both purified PPS14 and C-PS and used them as immunogens to determine the kinetics, boosting, and potential role of specific T cells for the resulting specific IgM and/or IgG responses.

Wild-type mice were immunized i.p. with optimal or suboptimal doses of either PPS14-PspA (Fig. 5*A*) or C-PS-PspA (Fig. 5*B*) without adjuvant and were boosted 14 days later. Similar to what we observed for intact Pn14, the primary serum titers for IgM and IgG anti-PPS14 and anti-PC in response to the respective conjugates reached maximal levels with more rapid kinetics than those observed in each case for the IgG anti-PspA response. Likewise, upon secondary immunization, substantial boosting of the IgG anti-PspA response was observed for both optimal and suboptimal doses of each conjugate. In contrast, neither IgM nor IgG anti-PPS14 and anti-PC responses showed significant boosting, with the exception of a moderate boost of the IgG anti-PPS14 response using a suboptimal dose of conjugate.

Immunization of athymic nude vs wild-type BALB/c mice with PPS14-PspA or C-PS-PspA demonstrated a strong T cell dependence for the IgG anti-PPS14 and anti-PC responses, respectively, in addition to the IgG anti-PspA response, whereas IgM responses were T cell independent (Fig. 6*A*). In contrast, both the IgM and IgG anti-PPS14 and anti-PC responses to purified, unconjugated PPS14 and C-PS, respectively, were T cell independent as expected. Furthermore, optimal IgG anti-PPS14, anti-PC, and anti-PspA responses in wild-type mice were dependent on $CD4^+$ T cells, whereas injection of anti-CD4 mAb had no effect on the specific IgM response to either conjugate (Fig. 6*B*). Surprisingly, but similar to what we observed using intact Pn14, immunization of H-Y$\alpha^{-/-}$ mice with PPS14-PspA or C-PS-PspA demonstrated a requirement for specific T cells for induction of optimal anti-

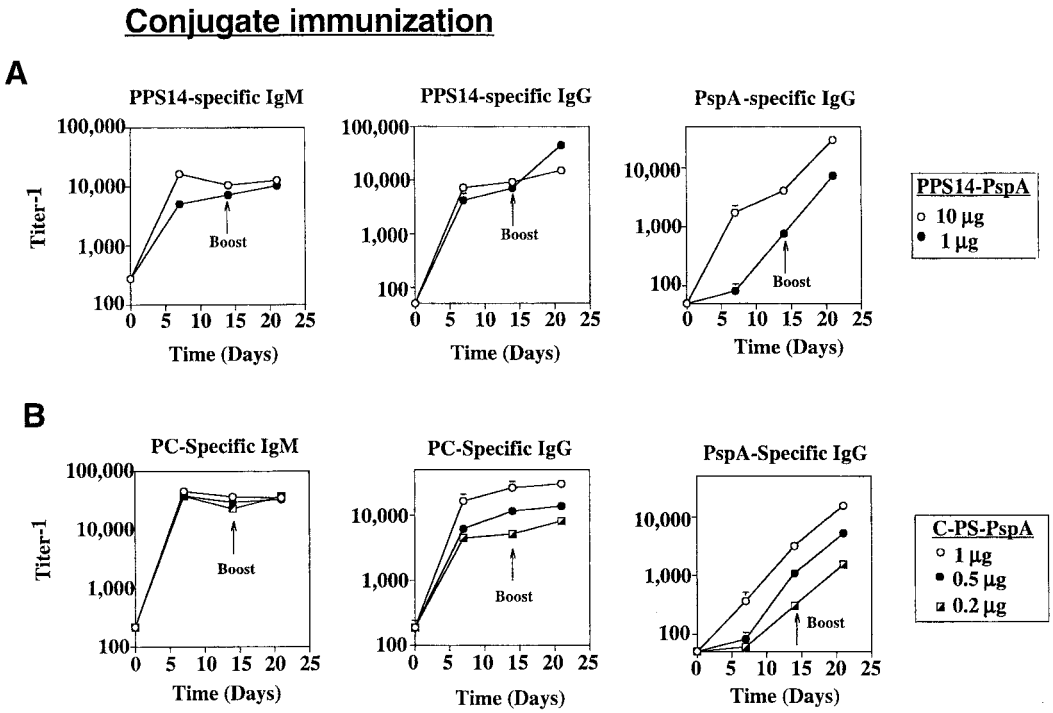

FIGURE 5. The IgG anti-PPS14 or anti-PC responses to soluble PPS14-PspA or C-PS-PspA conjugates, respectively, are more rapid than in vivo IgG anti-PspA responses and, unlike IgG anti-PspA responses, show little, if any, generation of memory. *A*, Eight BALB/c mice per group were each immunized i.p. with either 10 or 1.0 μg of PPS14-PspA in saline, then boosted on day 14 with the same primary immunization dose. Serum titers of PPS14- and PspA-specific Ig isotypes (days 0, 7, 14, and 21) were determined by ELISA. *B*, Eight BALB/c mice per group were each immunized i.p. with 1.0, 0.5, or 0.2 μg of C-PS-PspA in saline, then boosted on day 14 with the same primary immunization dose. Serum titers of PC- and PspA-specific Ig isotypes (days 0, 7, 14, and 21) were determined by ELISA. Values represent the arithmetic mean ± SEM. *A* and *B* are each representative of two experiments.

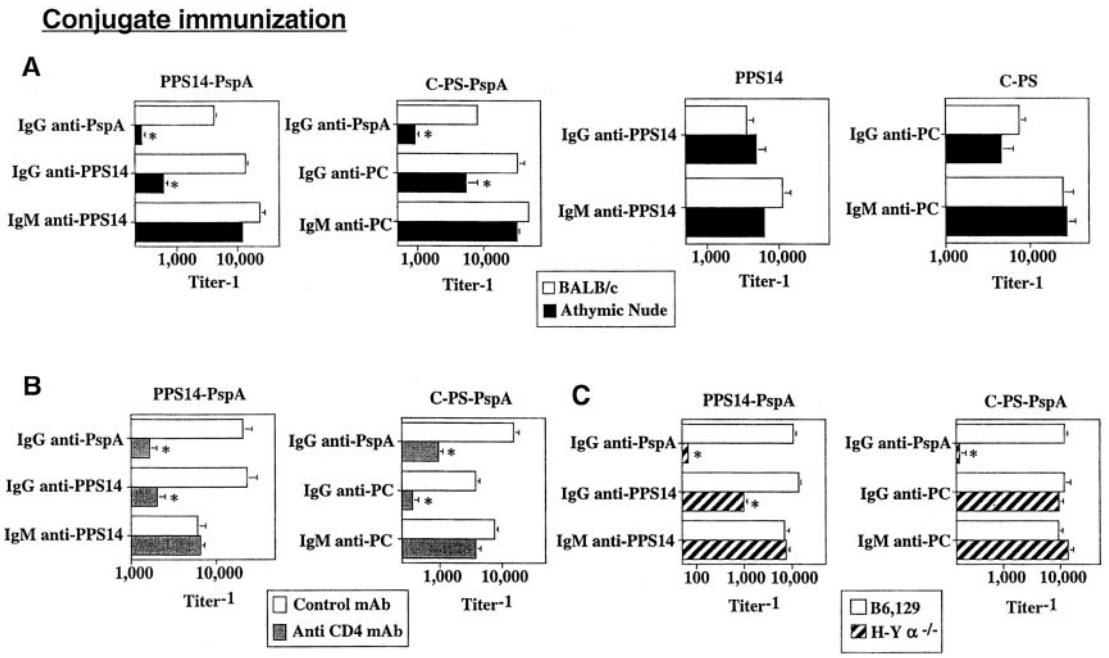

FIGURE 6. The IgG anti-PPS14, IgG anti-PspA, and IgG anti-PC responses to soluble conjugates are all dependent upon $CD4^+$ T cell help, but specific T cell help is observed only for PPS14 and PspA. *A,* Athymic nude and control BALB/c mice (eight mice per group) were injected i.p. with soluble PPS14-Pspa, C-PS-PspA, PPS14, or C-PS in saline (1.0 μg/mouse). Sera were obtained on day 7 (anti-PPS14 and anti-PC) and day 14 (anti-PspA) for determination of Ag-specific Ig isotype titers by ELISA. Values represent the arithmetic mean ± SEM. One of two representative experiments performed is shown. *B,* BALB/c mice (six mice per group) were injected i.p. with 1 mg of anti-CD4 or 1 mg of control rat IgG. Twenty-four hours later, mice were injected i.p. with soluble PPS14-PspA or C-PS-PspA (1.0 μg/mouse). One experiment was performed. *C,* H-Y$\alpha^{-/-}$ and control B6,129 mice (eight mice per group) were injected with soluble PPS14-Pspa or C-PS-PspA (1.0 μg/mouse). Sera were obtained on day 7 (anti-PPS14 and anti-PC) and day 14 (anti-PspA) for determination of Ag-specific Ig isotype titers by ELISA. Values represent the arithmetic mean ± SEM. One of two representative experiments is shown. *, $p \leq 0.05$.

PPS14 and anti-PspA responses, but not for an optimal IgG anti-PC response (Fig. 6*C*). Collectively, these data highlight key similarities between the regulation of a specific Ig isotype response to an intact bacteria and soluble conjugates of bacterial polysaccharide and protein and further delineate distinct pathways of T cell-dependent induction of specific IgG that depend on the nature of the Ag.

Discussion

To further clarify the parameters that mediate microbial-induced antipolysaccharide and antiprotein responses, we studied the primary kinetics, secondary boosting, and role of specific $CD4^+$ T cells in an Ig isotype response to heat-killed, intact Pn14 and compared these responses to Pn14-derived soluble conjugates of PPS14-PspA and C-PS-PspA. These data strongly suggest the following. 1) Recruitment of specific vs nonspecific T cell help for the IgG anti-PPS14 vs IgG anti-PC response, respectively, is not explained by differential association of the polysaccharides with other bacterial constituents. On this basis we suggest alternative possibilities that may depend upon the nature of the Ag and/or intrinsic functional differences in the responding B cells. 2) IgG antipolysaccharide responses to intact bacteria may be regulated by $CD4^+$ T cells in a manner similar to that observed for soluble, bacterial-derived protein-polysaccharide conjugates. 3) Antipolysaccharide and antiprotein responses exhibit distinct features even after the conversion of the polysaccharide into a TD Ag, both physiologically via association with protein within an intact bacteria, as well as through chemical coupling with an immunogenic carrier protein.

Although endogenous $CD4^+$ T cells augment both IgG anti-PPS14 and IgG anti-PC, as well as anti-PspA responses to intact Pn14, the data using H-Y$\alpha^{-/-}$ mice strongly suggest that the T cell help is largely TCR specific for induction of PPS14- and PspA-specific IgG, but, as we demonstrated previously using the unencapsulated *S. pneumoniae*, capsular type 2 strain R36A (19), the T cell help is TCR nonspecific for induction of PC-specific IgG. Covalent linkage of the same immunogenic protein carrier (PspA) to purified PPS14 and C-PS also results in recruitment of $CD4^+$ T cell help that is TCR specific and nonspecific, respectively, despite the fact that in both cases the IgG anti-PspA responses are comparably induced and boosted upon secondary immunization. These data suggest that the differences observed in recruitment of T cell help for the different polysaccharide-specific IgG responses are not related to the manner in which PPS14 and C-PS associate with other bacterial constituents, but, rather, may reflect intrinsic differences in the immunostimulatory properties of these two polysaccharides and/or the B cell subsets that respond to them.

In this regard, whereas PPS14 is a neutral polysaccharide, C-PS is zwitterionic (27). Zwitterionic polysaccharides, which contain a balance of both negative and positive charges, have been shown to activate T cells via association with MHC on the APC (28), although few details on the mechanism of this interaction are available, including TCR specificity requirements. In addition, anti-PC responses to intact *S. pneumoniae* have been shown to arise from both B1 and marginal zone B cells (MZB), which upon bacterial encounter undergo rapid migration from the marginal zone into the splenic red pulp, where they differentiate into plasma cells (29). MZB and B1 cells have been shown to be functionally and phenotypically distinct from follicular B cells (30–32). Whether this characteristic functional response pattern of B1 and MZB precludes PC-specific B cells from engaging in productive cognate T

cell interactions and/or developing into germinal center cells capable of becoming memory B cells remains to be determined. Additionally, PC is both a self and environmental Ag, and thus PC-specific B cells are also likely to be primed before intentional immunization (33, 34). Importantly, the nature of the B cell subsets responding to capsular polysaccharides and the degree to which the responding B cells are preprimed, is largely unknown, but differences relative to PC-specific B cells might account for their distinct interactions with $CD4^+$ T cells.

Despite a strong dependence on $CD4^+$ T cells for induction of polysaccharide- and protein-specific IgG (although not IgM) responses to both intact bacteria and soluble conjugates, the more rapid kinetics and relative lack of boosting observed for the polysaccharide-specific IgG responses suggest that the immunologic property of the TD Ag may depend on whether it is a polysaccharide or protein. These properties were observed independently of the strain of mouse used (BALB/c, 129B6, or C57BL/6). In this regard, we demonstrate that $CD4^+$ T cell help for both IgG anti-PC and anti-PPS14 responses is delivered more rapidly than that observed for the IgG anti-PspA response. The modest, although significant, reduction observed in IgM anti-PC and IgM anti-PPS14 after depletion of $CD4^+$ T cells (Fig. 3) was consistent with the trend observed in Fig. 2, although this latter reduction in IgM was not statistically significant. This seeming discrepancy may be explained by the modest nature of the effect combined with intrinsic mouse-to-mouse variation. The data nevertheless indicate that, unlike the IgG response, the IgM response is largely T cell independent. Polysaccharides, in contrast to proteins, express repeating, identical antigenic epitopes that can effect multivalent cross-linking of membrane Ig (mIg) on the specific B cell. We and others previously demonstrated that multivalent mIg-mediated signaling by itself delivers a powerful stimulus for B cell proliferation (9) and in concert with additional stimuli can mediate Ig secretion and class switching (35). Hence, early and potent signaling through B cell Ag receptor (BCR) in response to specific B cell recognition of a polysaccharide Ag may accelerate the kinetics of Ig induction relative to those observed for antiprotein responses that probably depend more heavily on CD40 signaling upon cognate interaction with specific T cells (36). Additionally, combined signaling via mIg and CD40 induces functional synergy (37–40), which may further serve to accelerate antipolysaccharide responses. In this regard, we show in this study that anti-CD40L mAb inhibits both the IgG anti-PC and anti-PPS14 as well as the IgG anti-PspA response to intact Pn14. Similarly, it was previously demonstrated that injection of anti-CD40L mAb into mice immunized with intact heat-killed *S. pneumoniae*, capsular type 6B inhibited the subsequent Ig response specific for PspA and Cps6B, although, in contrast to our study, it did not inhibit C-PS (largely PC)-specific Ig (41). However, in this latter study specific IgM and IgG were not separately measured.

The relative lack of boosting for IgG anti-PC and especially IgG anti-PPS14 responses to intact Pn14, as well as to optimal doses of the soluble conjugates also distinguished antipolysaccharide from antiprotein responses. The failure to generate substantial immunologic memory despite $CD4^+$ T cell help could reflect in part the rapidity of the antipolysaccharide response and the shortened period during which $CD4^+$ T cells deliver help. Additionally, B cell subsets with distinct patterns of chemokine responsiveness (42–44) may exhibit differing migratory patterns and cell-cell interactions within the secondary lymphoid organ, perhaps leading to exclusion from the developing germinal center. Studies using BCR-transgenic mice have demonstrated that relatively strong and repetitive mIg-mediated signals favor induction of B cell tolerance or deletion in the absence of additional help (32, 33) and thus may adversely impact on the generation of long-lived memory B cells in the presence of limiting helper signals. Further, strong signaling through BCR could potentially favor the rapid differentiation of B cells into plasma cells as opposed to memory B cells. Thus, differences in BCR signaling in response to polysaccharide vs protein Ags may contribute to limited memory cell generation for physiologic antipolysaccharide responses and not, as previously thought, simply be due to a lack of cognate $CD4^+$ T cell help.

Our data on the requirement for TCR-specific $CD4^+$ T cell help for the anti-PPS14 response to the PPS14-PspA conjugate is consistent with a previous, more detailed analysis using a conjugate consisting of type III capsular polysaccharide from group B *Streptococcus* (GBSIII) linked to tetanus toxoid (TT), in which the IgG anti-GBSIII response was shown to depend on MHC class II-TCR, B7-CD28 and CD40-CD40L interactions (45), similar to what we previously demonstrated using intact *S. pneumoniae* (17–19) and further drawing parallels between intact bacteria and soluble conjugates in the regulation of antipolysaccharide responses. However, in contrast to our data, the GBSIII-TT conjugate showed strong boosting of the anti-GBSIII response on repeated immunization, slower kinetics of the primary anti-GBSIII response relative to that for TT, and a role for endogenous T cells in augmenting the IgM anti-GBSIII response. An adoptive transfer study in mice using the GBSIII-TT conjugate confirmed that memory for the anti-GBSIII response can be transferred using spleen cells from primed mice (46).

The potential ability of various conjugate vaccines, unlike isolated polysaccharides, to show boosted IgG antipolysaccharide responses upon secondary and tertiary immunization has been studied extensively with mixed results. Several, although not all, studies in mice have shown strong boosting of antipolysaccharide responses to conjugates (47–49). Likewise, studies in humans have shown boosting in infants, although boosting responses are generally absent in adults (15, 50, 51). Thus, these studies on induction of memory for antipolysaccharide responses to conjugates collectively paint a somewhat complicated picture that may reflect the antigenic composition of the conjugate (including both polysaccharide and carrier protein and the associated B cell repertoire), the manner in which the conjugate was made, the ratio of protein to polysaccharide, the dosages used, and whether the host has been previously primed by environmental exposure and/or cross-reactive Ags (52–54).

Collectively our data suggest the possibility that IgG anti-capsular polysaccharide responses to intact bacteria may typically be $CD4^+$ T cell dependent, secondary to the coexpression of protein with polysaccharide Ags within the intact bacteria or within bacterial fragments. In this respect, these responses would resemble those elicited by conjugate vaccines as opposed to isolated polysaccharide Ags. The more central issue concerning the mechanisms underlying different functional outcomes for physiologic IgG responses to polysaccharide vs protein Ags may not necessarily lie, as previously thought, on the relative ability to recruit cognate $CD4^+$ T cell help, but perhaps more importantly on the nature of the BCR signaling that occurs and/or on the responding B cell subpopulations.

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