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In Vivo Polysaccharide-Specific IgG Isotype Responses to Intact *Streptococcus pneumoniae* Are T Cell Dependent and Require CD40- and B7-Ligand Interactions

Zheng-Qi Wu,* Quirijn Vos,† Yi Shen,* Andrew Lees,‡ Samuel R. Wilson,‡ David E. Briles,¶ William C. Gause,§ James J. Mond,¶ and Clifford M. Snapper²*

In vivo Ig responses to soluble, haptenated polysaccharide (PS) Ags are T cell independent and do not require CD40 ligand (CD40L). However, little is known regarding the regulation of in vivo PS-specific Ig responses to intact bacteria. We immunized mice with a nonencapsulated, type 2 *Streptococcus pneumoniae* (R36A) and compared the parameters that regulated in vivo Ig isotype responses to the bacterial cell wall C-PS determinant, phosphorylcholine (PC), relative to Ig responses to the cell wall protein, pneumococcal surface protein A. Consistent with previous reports using soluble PS and protein Ags, the anti-PC and anti-pneumococcal surface protein A responses differed in that the anti-PC response was induced more rapidly, had a distinctive Ig profile, and failed to demonstrate boosting upon secondary challenge with R36A. However, in contrast to previous studies, the IgG anti-PC response was TCR-αβ⁺ T cell dependent, required CD40L, and was blocked by administration of CTLA4 Ig. The nature of the T cell help for the anti-PC response had distinct features in that it was only partially blocked by CTLA4 Ig and was dependent upon both CD4⁺ and CD8⁺ T cells. Surprisingly, whereas the IgM anti-PC response was largely T cell independent, a strong requirement for CD40L was still observed, suggesting the possibility of an in vivo T cell-independent source for CD40L-dependent help. These data suggest that the regulatory parameters that govern in vivo Ig responses to purified, soluble PS Ags may not adequately account for PS-specific Ig responses to intact bacteria. *The Journal of Immunology*, 1999, 163: 659–667.

Much of the humoral immunity to extracellular bacteria is conferred by polysaccharide (PS)-specific IgM, IgG, and/or IgA, which mediate opsono-phagocytosis and/or complement mediated lysis or prevent attachment to epithelial surfaces (for review, see Refs. 1, 2). Much of our knowledge of the parameters that regulate PS-specific humoral responses comes from studies using purified, soluble, often haptenated PS Ags or conjugate vaccines (for review, see Refs. 3, 4). These prior studies indicate that, unlike Ig responses to protein Ags, PS-specific IgM and IgG responses in vivo are T cell independent (TI) and do not require CD40-CD40 ligand (CD40L) interactions. This dichotomy appears to result from an inability of PS Ags to associate with MHC class II molecules on the surface of APCs leading to an inability of such Ags to recruit classical CD4⁺ T cell help (5). This can explain the relative inability of most PS Ags to induce germinal center formation, memory responses, and affinity maturation (6–8). Although in vivo Ig responses to soluble PS Ags do not have an absolute requirement for T cells, evidence for both positive and negative regulatory roles for T cells in such responses has been reported (3). Studies using an in vitro polyclonal model for PS-induced, membrane Ig-mediated B cell activation also suggest that extracellular bacteria may recruit non-T cell help for IgM and IgG PS responses, but does not exclude the further participation of T cells (9, 10).

Studies aimed at understanding the parameters that regulate PS-specific responses to bacterial challenge using soluble PS Ags may be misleading, because the immune system encounters PS Ags, not in isolation, but often in the context of the bacterial organisms that express them. Such organisms contain complex mixtures of proteins, lipids, as well as PS, which may alter PS-specific Ig responses in various ways. Further, the behavior, in vivo, of Ags expressed in a particulate vs soluble form may differ significantly (11). Finally, despite the overriding importance of PS-specific Ig responses for conferring protection against naturally occurring infections with extracellular bacteria, most isolated PS Ags are poorly immunogenic, if at all, suggesting that some form of help is required to elicit such responses in vivo. Indeed, vaccines designed to induce strong in vivo PS-specific humoral responses have relied on coupling soluble PS Ags to protein carriers (“conjugate” PS vaccines) to recruit T cell help (12, 13).

Although earlier studies have shown that responses to PS on intact bacteria could be largely eliminated by both the xid/xid and nu/nu genotype (14, 15), no detailed studies have been published that describe the parameters that regulate PS vs protein-specific Ig isotype responses to an intact, extracellular bacterial organism. Thus, in this study we address these issues using the Gram-positive, nonencapsulated extracellular bacterium, *Streptococcus pneumoniae* type 2 (strain R36A), to study PS and protein-specific primary and secondary Ig isotype responses in vivo (16–19). We show that both PS- as well as protein-specific IgG responses require TCR-αβ⁺ T cell help and CD40- and B7-mediated costimulation. Surprisingly, despite their T...
cell dependence, the PS-specific IgG responses to R36A, unlike those to the protein Ag, and in further contrast to standard protein-PS conjugate vaccines, still demonstrate the rapid kinetics and lack of secondary boosting observed for soluble, purified PS Ags, as well as only a partial dependence on CD4+ T cells. Further, although a substantial PS-specific IgM response occurs in the absence of T cells, it is markedly reduced in mice lacking CD40L, suggesting a Tl role for CD40L in this response.

Materials and Methods

Mice

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME or National Cancer Institute, Gaithersburg, MD) were used as controls for the following mice: TCR-β-knockout (KO) (JR 2115), TCR-β-chain KO (JR 2120), TCR-βδ-chain KO (JR 2122), and MHC class II, locus Ma KO (JR 2248) (The Jackson Laboratory). B6129 F2 mice (The Jackson Laboratory) were used as controls for CD40L KO mice (JR 2428) (The Jackson Laboratory).

Reagents

Phosphorylcholine (PC) [6-(o-phosphorylcholine) hydroxyhexanoic acid (PC-CoH2)] was a generous gift from Dr. James Kenny (National Institute of Aging, National Institutes of Health, Baltimore, MD) and was prepared as described (20). PC was coupled to BSA using sulfo-N-hydroxysuccinimide and the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. Conjugates were dialyzed exhaustively. Phosphate content was determined as described (21). Protein content was determined using the Micro BCA assay (Pierce, Rockford, IL). Phosphate content was determined as described (21). Protein content was determined using the Micro BCA assay (Pierce, Rockford, IL). Protein content was determined as described (21). Protein content was determined using the Micro BCA assay (Pierce, Rockford, IL).

Preparation of, and immunization with, R36A

A nonencapsulated variant of type 2 S. pneumoniae (R36A) was grown in Todd Hewitt broth to mid-log phase and stored at -70°C. Rx1 and JY2004 are a variant of Rx1 in which the gene for the R36A protein-specific Ig isotype responses to an extracellular bacterium, we chose the PC determinant of the cell wall C-PS, and the cell wall protein, PspA, of the unencapsulated variant of type 2 S. pneumoniae (R36A) as representative target Ags (16–18). In initial studies, we tested the effect of varying the dose of heat-killed R36A on the level of induction of serum anti-PC and anti-PspA Ig isotypes. Doses of 1.0, 2.5, 5.0, and 10 × 107 CFU per mouse i.p. indicated that essentially maximal anti-PC and anti-PspA Ig responses could be obtained with 5 × 107 CFU per mouse, whereas 2.5 × 107 CFU per mouse generated a submaximal response (data not shown). Thus, in all subsequent experiments, a dose of 5 × 107 CFU of R36A i.p. per mouse was used. Although serum titers of all four IgG subclasses were often measured in each experiment, IgG subclass-specific differences in the different model systems studied were generally not observed. Thus, the figures illustrate representative data on the highly induced IgG subclasses observed in individual experiments.

Serum titers of different IgG isotypes specific for either PC or PspA were determined 7, 14, 21, and 28 days after a single immunization with R36A (Fig. 1). Serum IgM and IgG1 anti-PC titers reached maximal levels by day 7 after R36A immunization and remained relatively stable over the ensuing 3 wk. In contrast, relatively low serum titers of IgG anti-PspA were detected on day 7, whereas by day 14, maximal IgG anti-PspA titers were observed, rising ~10-fold relative to that seen on day 7. Serum anti-PspA titers then remained stable from day 14 to day 28. These kinetics were consistent with previous studies demonstrating more rapid kinetics of Ig isotype production in response to immunization with soluble PS relative to proteins.

Secondary immunization with R36A leads to significant boosting of serum IgG anti-PspA, but not IgG anti-PC titers

In the next set of experiments, we determined the profile of PC- and PspA-specific serum titers of IgM and the IgG subclass response in response to both primary and secondary immunization with R36A. Significant induction of PC-specific IgM and all four IgG subclasses were observed after primary and secondary immunization with R36A (Fig. 2). The relative titers of the four PC-specific IgG...
subclasses after primary or secondary immunization were IgG3 = IgG2b > IgG1 > IgG2a. In contrast, little if any induction of IgM anti-PspA was detected after either primary or secondary immunization with R36A, whereas induction of all four IgG subclasses specific for PspA were observed. Although the relative levels of PspA-specific IgG1, IgG2b, and IgG2a varied from experiment to experiment, the titers of PspA-specific IgG3 were always substantially lower. Secondary IgM anti-PC responses were typically up to 2- to 3-fold higher than primary IgM anti-PC responses after rechallenge with R36A 14 days after primary immunization, whereas no significant boosting of the IgG anti-PC response was observed after rechallenge with R36A. In contrast, specific Ab to PspA was boosted by 8- to 10-fold following the second injection of bacteria. In a separate experiment, mice were boosted with R36A 42 days after initial immunization (data not shown). Primary PC- and PspA-specific titers remained relatively high over the 42-day period following initial immunization, decreasing only modestly over time after their peak induction on day 7 or day 14, respectively. Once again, secondary IgM anti-PC titers were 2- to 3-fold higher than those observed for the primary response, whereas primary and secondary IgG anti-PC titers were similar. In contrast, secondary IgG anti-PspA titers were over 10-fold higher than those observed in the primary immunization. Thus, in regards to the kinetics of Ig induction, Ig isotype profile, and the elicitation of significantly boosted Ig responses upon secondary immunization, the anti-PC and anti-PspA responses resemble those responses observed previously for soluble PS and protein Ags, respectively.

Both the IgG anti-PC and IgG anti-PspA responses are dependent upon TCR-\(\alpha\beta^+\), but not TCR-\(\gamma\delta^-\), T cells

The Ig isotype responses to soluble PS Ags are considered largely TI, whereas Ig responses to soluble proteins typically have a strong dependence upon CD4+ TCR-\(\alpha\beta^+\) T cells. Nevertheless, earlier studies suggested a possible role for T cells in influencing the quantitative and/or qualitative outcome of anti-PS responses (3), and more recently a role for TCR-\(\gamma\delta^+\) T cells in anti-bacterial immunity, through recognition of nonprotein Ags, has been demonstrated (31, 32).

We wished to compare the potential role of TCR-\(\alpha\beta^+\) and/or TCR-\(\gamma\delta^+\) T cells in regulating the anti-PC, in addition to the anti-PspA, responses induced by R36A. We employed mice made genetically deficient in either the TCR-\(\beta\)-chain (TCR-\(\beta^-/-\)), TCR-\(\delta\)-chain (TCR-\(\delta^-/-\)), or doubly deficient in both the TCR-\(\beta\)- and TCR-\(\delta\)-chains (TCR-\(\beta^-/-\) TCR-\(\delta^-/-\)).

FIGURE 1. Kinetics of induction of primary PC- and PspA-specific serum Ig isotype titers in response to R36A immunization. Five C57BL/6 mice were immunized with R36A and titers of PC- and PspA-specific Ig isotypes were determined, by ELISA, from serial serum samples. Values represent mean ± SEM. Representative data from one of two experiments.

FIGURE 2. Induction of primary and secondary PC- and PspA-specific serum Ig isotype titers in response to R36A immunization. Five C57BL/6 mice were each immunized with R36A on day 0 and then on day 14. Titers of PC- and PspA-specific Ig isotypes were determined, by ELISA, from serum samples. Values represent mean ± SEM. Representative data from one of six experiments is shown.
TCR-δ-chain (TCR-β×δ⁻/⁻) (33, 34). These mice have an absolute lack in TCR-αβ⁺, TCR-γδ⁺, or all T cells, respectively. Primary and secondary IgM anti-PC responses to R36A were either largely unaffected or at most reduced by 2- to 3-fold in TCR-β⁻/⁻ and TCR-β×δ⁻/⁻ mice, but never reduced to any degree in TCR-δ⁻/⁻ mice (Fig. 3 and data not shown). In contrast, 6- to 10-fold reductions in serum IgG anti-PC titers were consistently observed in TCR-β⁻/⁻ and TCR-β×δ⁻/⁻ mice, whereas in TCR-δ⁻/⁻ mice IgG anti-PC responses were only modestly reduced. Whereas some induction of IgG anti-PC was still observed in TCR-β⁻/⁻ and TCR-β×δ⁻/⁻ mice, IgG anti-PspA responses in such mice were consistently undetectable. In contrast, IgG anti-PspA responses were at most only modestly reduced in TCR-δ⁻/⁻ mice. Thus, both the IgG anti-PC and IgG anti-PspA responses to R36A demonstrate a strong dependence upon TCR-αβ⁺ T cells.

The unexpected T cell dependence of the IgG anti-PC response could, in theory, reflect an unusual characteristic of the B cell subpopulation that responds to PC-containing Ags, and not the property of the immunogen itself. Thus, for example, earlier studies have implicated the B-1 B cell in Ab responses to PC-containing Ags (35). To test this hypothesis, we challenged TCR-β×δ⁻/⁻ or TCR-β⁻/⁻ mice with soluble PC-Ficoll or purified C-PS, respectively, and measured primary IgM and IgG anti-PC responses. Serum titers of both IgM and IgG anti-PC Ig in control vs TCR-β×δ⁻/⁻ or TCR-β⁻/⁻ mice were essentially comparable (Fig. 4), as were the more classical IgM and IgG anti-trinitrophenyl responses to trinitrophenyl-Ficoll (data not shown). Thus, the data suggest that the T cell dependence of the IgG anti-PC response to R36A cannot be explained simply by some unusual property of PC-specific B cells, and more likely reflects the nature of the immunogen.

PspA is not critical for mediating T cell help for the anti-PC response

Noncovalent linkage of PspA to the PC moiety on the C-PS of S. pneumoniae has been described (36). This suggested the possibility that PC-specific B cells might present PspA peptides to T cells upon internalization and processing of R36A or shed Ag. Similarly, it was also possible that PC-specific B cells used other R36A-derived proteins to recruit T cell help. To test these hypotheses, wild-type mice were challenged with Rx1 (a variant of

FIGURE 3. Induction of both PC- and PspA-specific IgG is inhibited in TCR-β⁻/⁻ and TCR-β×δ⁻/⁻, but not TCR-δ⁻/⁻ mice. C57BL/6, TCR-β⁻/⁻, TCR-δ⁻/⁻, and TCR-βδ⁻/⁻ mice (five mice each) were immunized with R36A on day 0 and day 14. Titters of PC- and PspA-specific Ig isotypes were determined, by ELISA, from serial serum samples. (PC, primary day 7; secondary day 21; PspA, primary day 14, secondary day 21). Values represent mean ± SEM. Representative data from one of three experiments is shown.

FIGURE 4. Induction of PC-specific Ig isotypes in response to PC-Ficoll or soluble C-PS is normal in TCR-δ⁻/⁻ mice. Five C57BL/6 mice were immunized with PC-Ficoll (50 µg per mouse i.p.) or soluble C-PS (0.5 µg per mouse i.p.). Titters of PC-specific Ig isotypes were determined by ELISA from sera obtained 7 and 14 days after immunization. Representative values from day 7 serum samples are shown as mean ± SEM. For PC-Ficoll, similar data from one of three experiments is shown. For C-PS, a single experiment is shown.
R36A), Rx1 subjected to treatment with pepsin, or a mutant Rx1 in which the PspA gene was purposefully deleted (29), and anti-PC and anti-PspA responses were compared. As illustrated in Fig. 5, whereas IgG anti-PspA responses to either PspA-deficient Rx1 or pepsinized Rx1 were completely abrogated, serum titers of IgM and IgG anti-PC were essentially unaffected. These data rule out a critical role for PspA and other pepsin-sensitive surface proteins in mediating help for anti-PC responses. Because it is unlikely that pepsin treatment eliminates cytoplasmic proteins present within Rx1, a role for bacterial protein, in general, in recruiting T cell help for the anti-PC response has not been ruled out.

The IgG anti-PspA response is dependent upon CD4\(^+\) T cells, whereas both CD4\(^+\) and CD8\(^+\) T cells are required for an optimal IgG anti-PC response

Both CD4\(^+\) and CD8\(^+\) T cells may become activated during bacterial infections and each subset is potentially capable of delivering B cell help for Ig production through CD40L expression and/or elaboration of cytokines, although CD8\(^+\) T cells are believed to be far less effective in this regard (37). To test a role for one or both of these subsets in the anti-PC and anti-PspA responses to R36A, we acutely depleted CD4\(^+\) and/or CD8\(^+\) T cells in normal mice using cytotoxic anti-CD4 and anti-CD8 mAbs 1 day before challenge with R36A. Normal rat IgG was used as a control. Flow cytometric analysis 3 days after R36A immunization confirmed >98% specific depletion of CD4\(^+\) and/or CD8\(^+\) cells, respectively (data not shown). The IgG anti-PspA response was essentially abrogated by anti-CD4 mAb treatment, whereas treatment with anti-CD8 mAb exerted a reproducibly small but not statistically significant effect (Fig. 6). In contrast, treatment with anti-CD4 or anti-CD8 mAbs alone each led to a reproducibly small, although not statistically significant, reduction in IgG anti-PC titers, with no change in IgM. However, combined treatment with anti-CD4 and anti-CD8 mAbs led to a highly significant reduction in IgG, but not

![Figure 5](https://via.placeholder.com/150)

**FIGURE 5.** Induction of PspA- but not PC-specific Ig isotypes is inhibited in response to pepsinized R36A or PspA-deficient Rx1 vs control bacteria. Mice were immunized with pepsinized or control R36A or PspA-deficient or control Rx1. Titers of PC- and PspA-specific Ig isotypes were determined, by ELISA, from serial serum samples. (PC, primary day 7; PspA, primary day 14). Values represent mean ± SEM. Representative data from one of two experiments is shown. Response to R36A (shown) was similar to that for Rx1.

![Figure 6](https://via.placeholder.com/150)

**FIGURE 6.** Induction of PspA-specific IgG is CD4\(^+\) T cell dependent whereas induction of PC-specific IgG is dependent upon both CD4\(^+\) and CD8\(^+\) T cells. Groups of six C57BL6 mice were treated with 0.5 mg of either control rat IgG, anti-CD4 mAb, anti-CD8 mAb, or both anti-CD4 plus anti-CD8 mAbs 1 day before immunization with R36A. A single spleen from each group was obtained, 3 days after R36A immunization, for flow cytometric analysis of percentage of CD4\(^+\) and CD8\(^+\) T cells. Sera was obtained from the remaining five mice in each group for PC (day 7) or PspA-specific (day 14) Ig isotype titers. Values represent mean ± SEM. Representative data from one of two experiments is shown. Significance (p value) was determined by Student’s t test.
IgM, anti-PC titers, similar to that observed in TCR-β−/− or TCR-β×δ−/− mice. Furthermore, only a partial, although significant, reduction in IgG anti-PC titers were observed in mice made genetically deficient in MHC class II expression (data not shown), which have a selective deficiency in CD4+ T cells (38).

Both IgM and IgG anti-PC and IgG anti-PspA responses are deficient in CD40L KO mice

Induction of CD40L expression on activated T cells is believed to play a key role in mediating both humoral and cell-mediated immune responses through engagement of CD40 on B cells, macrophages and/or dendritic cells (39, 40). Of interest, other cells may also express CD40L but the physiologic role of this expression is unknown (39, 40). In vivo IgM and IgG responses to purified haptenated PS Ags are normal in CD40−/− or CD40L−/−KO mice (42) or mice treated with a blocking anti-CD40L mAb (43), whereas responses to immunization with protein in adjuvant are abrogated. Nevertheless, our observation, above, that T cells play a role in the IgG anti-PC response prompted us to determine a potential role for CD40L in mediating this effect. Thus, CD40L−/− and control mice were immunized with R36A and both primary and secondary serum anti-PC and anti-PspA Ig titers were measured. A marked reduction in primary and secondary serum IgM anti-PC titers were consistently seen in CD40L−/− mice relative to controls, in contrast to the more modest reduction or no change at all observed in TCR-β−/− or TCR-β×δ−/− mice (Fig. 7). Further, primary and secondary serum titers of IgG anti-PC Abs were nearly undetectable in CD40L−/− mice, a reduction even more profound than that observed in TCR-β−/− mice. Similarly, IgG anti-PspA responses in CD40L−/−KO mice were undetectable in both the primary and secondary responses. Thus, in contrast to what has been observed using soluble, purified PS Ags, the PS-specific IgG response to R36A is strongly dependent upon CD40L. Further, the quantitative differences in induction of IgM anti-PC, and to a lesser extent IgG anti-PC, observed between CD40L−/− and TCR-β−/− or TCR-β×δ−/− mice suggest a possible TI role for CD40L in these responses.

IgG anti-PC and IgG anti-PspA responses to R36A are differentially reduced in mice treated with CTLA4 Ig

Many, although not all, reports have indicated a key role for T cell costimulation through CD28 in the generation of optimal effector T cell function (44, 45). In contrast, a predominantly inhibitory role for CTLA4 has been reported. Initiation of T cell signaling through CD28 and CTLA4 is accomplished through binding of these molecules to B7-1 and/or B7-2, expressed on APCs, such as B cells, macrophages, and dendritic cells. Because both the IgG anti-PC and IgG anti-PspA responses were found to be T cell dependent, we determined whether either or both of these responses required such costimulation for their development. Thus, mice were given a single injection of the chimeric fusion protein, CTLA4 Ig, or a control fusion protein (L6), 1 day before immunization with R36A. CTLA4 Ig blocks both CD28 and CTLA4 signaling by binding to both B7-1 and B7-2 on APCs (25). Treatment with CTLA4 Ig had no effect on the primary or secondary IgM anti-PC response (Fig. 8). In contrast, CTLA4 Ig treatment led to a significant reduction in serum IgG anti-PC titers 7 days after R36A immunization, although titers increased significantly over the ensuing 2 wk, reaching normal levels for IgG3 anti-PC on day 14, but still significantly reduced for IgG2b anti-PC. In contrast, a more absolute and sustained reduction in serum IgG anti-PspA titers was observed consequent to CTLA4 Ig treatment. Thus, the IgG anti-PC and IgG anti-PspA responses show differential requirements for costimulation through CD28 and/or CTLA4.

Discussion

The vast majority of studies addressing the mechanism of anti-PS immune responses used purified soluble PS Ags, oftentimes purposefully haptenated to facilitate detection of the induced Ig (3, 4). However, little is known concerning the parameters that mediate induction of anti-PS Ig isotypes in response to immunization with an intact bacterial organism. Thus, in this report we studied the mechanism(s) underlying the in vivo anti-PC (anti-PS) vs anti-PspA (anti-protein) Ig isotype response to an unencapsulated S. pneumoniae.
pneumoniae type 2 (R36A). In contrast to previous studies using soluble PS Ags, we demonstrate a striking dependence of both the anti-PS and anti-protein IgG responses on TCR-αβ⁺ T cells, CD40L, and B7-CD28/CTLA4 costimulation. Whereas IgM anti-PS responses were relatively independent of T cells and B7-CD28/CTLA4 costimulation, a strong dependence on CD40L for the anti-PS response was nevertheless observed.

Previous studies, mostly using athymic nude mice, strongly suggested that T cells were not required to generate either IgM or IgG anti-PS responses to soluble Ags (3, 4). Likewise, no role for CD40/CD40L interactions were observed for Ig responses to soluble PS Ags when using anti-CD40 mAb to block CD40-CD40L interactions, or when immunizing CD40 or CD40L KO mice (41–43), despite the ability of at least some PS Ags to induce CD40L on T cells (46). Finally, transgenic mice overexpressing a soluble, chimeric CTLA4 Ig fusion protein, which effectively abrogates B7-CD28/CTLA4 interactions, demonstrated a normal Ig response to a haptenated PS but a markedly defective Ig response to a TD Ag (47). Collectively, these data argued against a role for classical, cognate T cell interactions in mediating anti-PS responses. This is consistent with the inability of soluble PS Ags to associate with MHC class II molecules (5). Nevertheless, many studies have provided evidence for the ability of T cells to exert either positive or negative effects on otherwise TI anti-PS Ig responses (3). However, the vast majority of these studies also used soluble PS Ags. The mechanisms underlying these T cell-mediated effects that were observed are largely unknown. Potential mechanisms for nonclassical T cell effects on anti-PS responses have previously been described in detail (3).

Because bacterial organisms contain both protein and PS Ags, one might have anticipated that anti-PS responses to R36A would be similar to that observed for anti-PS responses to soluble, protein-PS conjugate vaccines, which are also T cell dependent (48). However, our studies indicate at least three distinct differences between whole bacteria and protein-PS conjugates. Firstly, whereas conjugation of protein to a soluble PS Ag mediates the generation of PS-specific IgG memory responses upon rechallenge with the conjugate vaccine or even unconjugated PS Ag, no such PS-specific IgG memory response was observed using R36A, despite its T cell dependence. Secondly, conjugate vaccines demonstrate the same delayed kinetics for the anti-PS response as that observed for the Ig response to classical soluble protein Ags, whereas the anti-PS response to R36A showed the more classic rapid kinetics of Ig responses to soluble PS Ags. Thirdly, recent in vivo studies from our laboratories using a Pn14-tetanus toxoid conjugate vaccine showed a complete dependence of the anti-Pn14 response on CD4⁺ T cells, whereas the anti-PS response to R36A was only partially dependent on such cells (J.J.M., unpublished observation). The ability of a bacterial organism to regulate humoral responses in a manner distinct from a conjugate vaccine or soluble PS Ag may reflect a number of fundamental differences between these immunogens, including their respective particulate vs soluble nature, the presence in bacteria of complex mixtures of immunomodulating lipids, proteins, and PS, including adjuvanting moieties, as well as the organized nature of the bacterial immunogen itself.

PC is a common environmental Ag (49–51), and thus it is likely that mice are already primed for anti-PC responses before purposeful immunization. Therefore, to test the generality of our conclusions, additional studies of responses to other PS Ags, expressed by intact bacteria, that mice have not likely encountered before immunization are warranted. Additionally, potential differences in the parameters for generating humoral responses to live vs dead (52), and unencapsulated vs encapsulated bacteria (53) need to be explored.

The nature of the T cell help for the anti-PS response to R36A has not yet been clarified but the dependence of this response on CD40L and B7-CD28/CTLA4 costimulation suggests a role for cognate T cell-APC interactions, perhaps mediated by bacterial...
protein. Although PspA is noncovalently linked to the PC determinant of the cell wall C-PS of R36A, and anti-PspA Abs comprise a substantial proportion of the protein-specific Ig made in response to R36A (17, 18, 36), we did not observe any changes in PC-specific Ig titers when utilizing PspA-deficient or pepsinized R36A, despite complete abrogation of the anti-PspA response in both instances. Although, anti-PC titers were unaffected by pepsinization of R36A, we cannot rule out a role for residual R36A-derived protein and peptides that are likely to be present even after pepsin treatment.

The T cell help for the anti-PC and anti-PspA responses may be at least partly distinct. Firstly, differences in the requirement for B7-CD28/costimulation are seen between the anti-PC and anti-PspA responses in that anti-PC titers, which are initially depressed, rise significantly over time following a single injection of CTLA4 Ig, whereas anti-PspA titers remain profoundly suppressed over a similar time course. Secondly, the anti-PC response depends upon both CD4+ and CD8+ T cells, whereas depletion of CD4+ T cells alone leads to abrogation of the anti-PspA response. Likewise, we observed that the anti-PC response is only partly reduced in MHC class II-deficient mice, whereas the anti-PspA response in these mice is undetectable (data not shown). In this regard, previous in vitro studies have demonstrated the ability of CD8+, as well as CD4+ T cells, to mediate B cell help, both through expression of membrane CD40L and the secretion of cytokines, although CD8+ cells are less effective based on their cytotoxicity and weak expression of CD40L (37). The possibility that nonclassical T cell help additionally, or exclusively, plays a role in the anti-PC response cannot be ruled out and, as discussed above, may involve one or more distinct mechanisms (3).

The observation of a normal or modestly reduced IgM anti-PC response to R36A in TCR-β−/− or TCR-β−/−×δ−/− mice but a strongly reduced IgM anti-PC response in CD40L−/− mice suggests an in vivo TI role for CD40L. The observation that non-T cells, including B cells, NK cells, dendritic cells, mast cells, and eosinophils, can express functional CD40L (39, 40) provides a theoretical foundation for this hypothesis. For example, the capacity for bacteria to activate mast cells is well known, and mast cells have been shown to be capable of delivering B cell help through CD40L and cytokine expression (54). CD40L expressed on B cells has also been shown to exert autocrine and/or paracrine effects on B cell function (55). In addition, CD40L may serve to activate CD40-expressing dendritic cells (56), which have recently been shown to have potent B cell helper function (57), in addition to presenting Ag efficiently.

In conclusion, the use of an intact bacterial organism to study an anti-PS response has in part confirmed some earlier notions concerning in vivo Ig responses to soluble PS Ags with respect to kinetics, Ig isotype profile, and the generation of B cell memory. However, the strong dependence of the anti-PS response on T cells and CD40- and B7-ligand-mediated costimulation indicates that anti-PS responses to bacteria may be more complex than what has been suggested by in vivo studies using purified soluble PS Ags.

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