B7 Requirements for Primary and Secondary Protein- and Polysaccharide-Specific Ig Isotype Responses to *Streptococcus pneumoniae*[1,2]

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The requirements for B7 costimulation during an in vivo humoral response to an intact extracellular bacteria have not been reported. In this study we immunized mice with *Streptococcus pneumoniae* (R36A) to determine the B7 requirements for induction of Ig, specific for two determinants on R36A, the phosphorylcholine (PC) determinant of C-polysaccharide and pneumococcal surface protein A (PspA). We show that the primary anti-PspA response, the development of PspA-specific memory, and the induction of the secondary anti-PspA response in primed mice were completely dependent upon B7 costimulation. Of note, costimulation was required only briefly after the secondary immunization compared with after the primary immunization for optimal induction of Ig. Blockade of B7 costimulation at the time of secondary immunization also completely abrogated the established state of memory, but did not induce tolerance. In contrast to the anti-PspA response, the primary anti-PC response involved only a very short period of B7 costimulation. Whereas B7-2 alone was required for induction of the primary anti-PspA and anti-PC responses, a redundant role for B7-1 and B7-2 was noted for the PspA-specific secondary response. CTLA4Ig blocked both the anti-PC and anti-PspA responses equally well over a wide range of bacterial doses. These studies demonstrate a critical, but variable, role for B7-dependent costimulation during an Ig response to an extracellular bacteria. *The Journal of Immunology,* **2000, 165: 6840–6848.**

Immunity to extracellular bacteria is mostly conferred through induction of Ig specific for bacterial protein and polysaccharide determinants (1, 2). In vivo immunization protocols using purified protein and polysaccharide Ags indicate that anti-protein responses are strictly T cell dependent, whereas anti-polysaccharide responses occur in the absence of T cells (3, 4). This dichotomy appears to be due in large part to the inability of polysaccharides to associate with MHC class II molecules on APCs (5). Nevertheless, both positive and negative regulation of anti-polysaccharide responses by noncognate forms of T cell help have been suggested (3).

Much less is known regarding the parameters that regulate in vivo protein and polysaccharide-specific Ig responses to intact extracellular bacteria. The particulate nature of bacteria (6), their expression of a variety of immunomodulating protein, lipid, and polysaccharide moieties, and their ability to interact with host structures (1, 7–9), suggest potential differences in the regulatory pathways that may govern Ag-specific humoral responses to intact bacteria vs those to isolated, soluble Ags. In this regard we have been investigating the parameters that regulate in vivo protein-specific vs polysaccharide-specific murine Ig isotype responses to an intact extracellular bacterium, the nonencapsulated type 2 variant of *Streptococcus pneumoniae* (R36A) (10–14). Ig isotype responses to the cell wall protein, pneumococcal surface protein A (PspA)[4] and to the phosphorylcholine (PC) determinant of the cell wall C-polysaccharide have been used as models. We recently reported that optimal induction of both anti-PspA and anti-PC responses to R36A in vivo required TCR-$\alpha\beta^+$, $CD4^+$ T cells and B7 ligand-dependent costimulation (10). Of note, boosting of previously primed mice, while inducing a memory IgG anti-PspA response, failed to elicit a memory IgG anti-PC response. Recent data from our laboratory (Z.-Q. Wu et al., manuscript in preparation) further indicate that the T cell help for the anti-PC response is mechanistically distinct from that regulating the anti-PspA response and thus could show unique features regarding the requirement for costimulation.

Numerous studies have indicated at least a two-signal model for T cell activation, which includes signaling through the TCR-CD3 complex combined with a costimulatory signal (15–17). APC-TCR/ligand pairs implicated in mediating such T cell costimulation include B7/CD28, 4-1BBL/41-BB, LFA-1/ICAM-1, heat-stable Ag and its ligand, OX40L/OX40, CD70/CD27, and TNF-related activation-induced cytokine-R/TNF-related activation-induced cytokine (18). Of these, B7 ligand-dependent costimulation generally appears to have the most dominant role. Specifically, B7-1 and B7-2, expressed on APCs such as dendritic cells, macrophages, and B cells, bind to CD28 and CTLA4 on T cells. CD28 mediates a positive signal, whereas most studies suggest that a negative signal is transmitted by CTLA4 (19). A number of issues concerning B7 ligand-dependent

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[4] Abbreviations used in this paper: PspA, pneumococcal surface protein A; PC, phosphorylcholine; PBT, 1× PBS, 1% BSA, and 0.1% Tween 20.

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costimulation continue to command intense research interest. These include the relative role of B7-1 vs B7-2 in CD28 vs CTLA4 signaling and Th subset determination, the role of Ag dose in overcoming costimulation requirements, and the relative role and kinetics of costimulation in primary vs memory responses and in the induction of tolerance. In this regard the complexity of the B7 ligand-dependent system is underscored by the differing results obtained in response to these questions, dependent upon the nature of the model system under study and the experimental conditions used. With these questions in mind, we undertook a detailed analysis of the in vivo costimulation requirements for both protein- and polysaccharide-specific humoral immunity in response to R36A to clarify the role of cellular interactions in the Ig response to an extracellular bacteria.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or National Cancer Institute (Bethesda, MD). CD28$^{-/-}$ mice were obtained from The Jackson Laboratory. Mice were used at 7–10 wk of age and were maintained in a pathogen-free environment.

Reagents

PC (6-(*O*-phosphorylcholine) hydroxyhexanoic acid) was a generous gift from Dr. James Kenny (National Institute of Aging, National Institutes of Health, Baltimore, MD) and was prepared as previously described (20). PC was coupled to BSA as described previously (10). Recombinant PspA was expressed as previously described (21) and purified using the QIAexpressionist system (Qiagen, Chatsworth, CA). The expressed protein includes aa 4–299 of the mature protein. Murine CTLA4Ig and a control fusion protein, L6, were prepared as previously described (22). Hamster IgG anti-mouse B7-1 mAb (16-10A1) (23) and rat IgG2a anti-mouse B7-2 mAb (GL1) (24) were purified from culture supernatant using a protein G column. Rat IgG2a anti-*Escherichia coli* β-galactosidase (GL117) was purified from ascites by ammonium sulfate precipitation, followed by protein G purification, and was used as a control. Purified polyclonal hamster IgG (whole molecule) was obtained from Pierce (Rockford, IL) and was used as a control.

Preparation of, and immunization with R36A

A nonencapsulated variant of type 2 *Streptococcus pneumoniae* (R36A) was grown in Todd Hewitt broth to mid-log phase and stored at −70°C. For immunization, frozen bacteria were thawed and subcultured on blood agar plates. One or two characteristic colonies were selected and suspended in 200 ml of Todd Hewitt broth, placed in a shaker water bath at 37°C for 4–6 h until an OD (absorbance at 650 nm) of 0.6 was achieved as measured by a spectrophotometer (Spectronic 100, Bausch & Lomb, Rochester, NY). The 200-ml preparation of R36A was then heat-killed by incubation in a 60°C water bath for 10 h (1 h/20 ml). Sterility was confirmed by culture. This bacterial stock containing 1×10^9 CFU/ml was aliquoted and frozen at −70°C until used for immunization. Mice were immunized i.p. with various doses (CFU) of heat-killed bacteria in 250 μl of PBS. Serum samples for measurement of anti-PC and anti-PspA Ab titers were prepared from blood obtained through the tail vein.

Measurement of serum titers of anti-PC Ig isotypes by ELISA

Immulon-2 plates were coated with PC-BSA (5 μg/ml, 50 μl/well) in PBS for 1 h at 37°C or overnight at 4°C. Plates were then blocked with PBT (1× PBS, 1% BSA, and 0.1% Tween 20) at 37°C for 30 min or 4°C overnight. Three-fold dilutions of serum samples in PBT were then added starting at a 1/50 or 1/100 serum dilution. After 1-h incubation at 37°C plates were washed three times with PBS and 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG3, IgG1, IgG2b, and IgG2a Abs (200 ng/ml final concentration in PBT) were then added, and plates were incubated for 37°C for 1 h. Plates were washed five times with PBS and 0.1% Tween 20. Substrate (4-methylumbiliferyl phosphate) was then added (50 μg/ml, 50 μl/well), and fluorescence was read on a MicroFLUOR ELISA reader (Dynatech, Chantilly, VA).

Measurement of serum titers of anti-PspA Ig isotypes by ELISA

Immulon 4 plates were coated with recombinant PspA (gift from Luba Grinberg, Uniformed Services University of the Health Sciences) at 5 μg/ml (50 μl/well) in PBS. After overnight incubation at 4°C, plates were washed three times with PBS and 0.1% Tween 20 and were blocked with PBS and 1% BSA at 4°C for 4 h or overnight. Diluted serum samples (see anti-PC ELISA) were then added, and plates were incubated overnight at 4°C. Plates were washed three times, alkaline phosphatase-conjugated polyclonal goat anti-mouse Ig isotype Abs were added (see anti-PC ELISA) for 2 h at room temperature, and plates were then washed three times. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma, St. Louis, MO) at 1 mg/ml in 1 M Tris and 0.3 mM $MgCl_2$, pH 9.8, was then added for about 30 min at room temperature for color development. Color was read at an absorbance of 450 nm on a Titer-Tek Multiskan Plus (MK II) ELISA reader (Labsystems, Helsinki, Finland).

Statistics

Data are expressed as the arithmetic mean of Ig titers of individual serum samples ± SEM. Differences between treatment groups were considered significant at $p < 0.05$ using Student's *t* test.

Results

The B7 ligand-dependent costimulating activity for IgG anti-PspA and anti-PC responses is due entirely to CD28

In a previous study we demonstrated that CTLA4Ig, injected at the time of primary immunization with R36A, completely abolished the primary IgG anti-PspA response and reduced the IgG anti-PC response by >6-fold, with no significant effect on IgM anti-PC (10). CTLA4Ig, through binding to B7-1 and B7-2, blocks B7 interactions with both CD28 and CTLA4 (22). Both the IgG anti-PspA and anti-PC responses, but not the IgM anti-PC response, were dependent upon TCR-$\alpha\beta^+$, CD4$^+$ T cells. Although most studies indicated that CD28 and CTLA4 deliver positive and negative signals to the T cell, respectively (25), some reports also suggested a T cell stimulatory effect resulting from CTLA4 ligation (26, 27). Thus, we used CD28$^{-/-}$ mice (28) to determine the relative role of CD28 in costimulating IgG anti-PspA and anti-PC responses. CD28$^{-/-}$ and control mice were immunized i.p. with heat-killed R36A, and serum was obtained 7 and 14 days after immunization. Our previous study indicated that the primary anti-PC response peaked on day 7, whereas the primary anti-PspA response was optimal on day 14 (10). Since no significant IgG subclass-specific effects were observed in the current study, representative IgG isotypes that showed relatively higher responses are illustrated. As demonstrated in Table I, IgG anti-PspA responses were essentially abolished in CD28$^{-/-}$ mice, whereas 4- to 8-fold reductions in IgG anti-PC subclass responses were observed. No significant reduction in the IgM anti-PC response was seen in CD28$^{-/-}$ mice relative to controls. These data are quantitatively similar to those we previously obtained using CTLA4Ig (10) and suggest that the B7 ligand-dependent costimulating activity for IgG anti-PspA and anti-PC responses is due entirely to CD28.

Differential effects of anti-B7-1 and anti-B7-2 mAbs on primary anti-PC and primary and secondary anti-PspA responses

A number of studies have indicated a dominant role for B7-2 in mediating T cell-dependent immune responses (29, 30). This may be due to both the constitutive expression (31, 32) and the more rapid kinetics (31, 33–35) of B7-2 induction relative to B7-1. Nevertheless, other studies employing different model systems have demonstrated redundant roles for B7-1 and B7-2 in costimulating T cell-dependent immune responses (36, 37). Thus, we used blocking anti-B7-1 and anti-B7-2 mAbs, injected at the time of primary immunization, to determine their effects on the primary IgG anti-PspA and anti-PC response to R36A. As illustrated in Fig. 1*A*, neither anti-B7-1 nor anti-B7-2 mAbs had any significant effect on the primary IgM anti-PC response as reported previously (38). In contrast, anti-B7-2, but not anti-B7-1, mAb significantly inhibited

Table I. *Immunization of CD28$^{-/-}$ vs wild-type mice with R36A for induction of anti-PC and anti-PspA responses*[a]

	Anti-PC Titers (Day 7)			Anti-PspA Titers (Day 14)	
	IgM	IgG2b	IgG3	IgG1	IgG2b
Control	37,400 ± 3,500	4,800 ± 850	16,000 ± 3,300	1,040 ± 266	3,380 ± 483
CD28$^{-/-}$	26,350 ± 3,850	600 ± 125*	4,550 ± 700*	55 ± 4*	111 ± 22*

[a] CD28$^{-/-}$ and wild-type control mice (five per group) were immunized i.p. with R36A (5×10^7 CFU/mouse). Sera were collected on day 7 (anti-PC) and day 14 (anti-PspA) after immunization. Values are expressed as the arithmetic mean ± SEM,
*, $p < 0.05$.

the primary IgG anti-PC (Fig. 1*B*) and the primary IgG anti-PspA response (Fig. 1*C*).

The role of B7 ligand-dependent costimulation for establishment of immunological memory and for the elicitation of memory effector function is still unresolved, although a number of studies suggest a decreased requirement of memory cells for B7 costimulation or a redundant role for heat-stable Ag (39–43). Additionally, some studies show that blocking B7 ligand interactions in vivo alone induces anergy (44, 45), whereas other studies argue against this view (46, 47). In this regard we wished to elucidate the requirement for B7 costimulation in establishing and eliciting memory for *S. pneumoniae*. Mice were primed with R36A in the presence of anti-B7 mAbs or control mAbs, and then boosted 6 wk later (Fig. 1*C*). The time of boosting was selected to allow for clearance of the anti-B7 mAbs. Injection of anti-B7-2, but not anti-B7-1, mAb at the time of primary immunization with R36A completely blocked the memory IgG anti-PspA response. However, boosting with R36A did induce an IgG anti-PspA response that was at the level of the primary response. These data indicate that blocking costimulation during the primary anti-PspA response also inhibits the generation of memory, but does not induce tolerance to R36A. This is consistent with additional data demonstrating the ability of CTLA4Ig (data not shown) or anti-B7-2 mAb (38) to completely inhibit the formation of germinal centers (48) in response to R36A.

We next determined the role of B7-1 and B7-2 in mediating costimulation during a secondary IgG anti-PspA response. Thus, anti-B7-1 and/or anti-B7-2 mAbs were injected at the time of boosting of mice that were previously immunized with R36A alone and thus were allowed to develop memory. Neither anti-B7-1 mAb nor anti-B7-2 mAb alone significantly affected the elicitation of the memory IgG anti-PspA response (Fig. 1*D*). However, the combination of anti-B7-1 and anti-B7-2 mAbs was markedly inhibitory. Thus, the generation of memory effector function also required costimulation in this model system. However, unlike the primary IgG anti-PspA response and the development of memory, which was strictly dependent upon B7-2, redundant roles for B7-1 and B7-2 are observed for induction of memory effector function.

The requirement for costimulation is independent of R36A dose

Previous studies have suggested that the requirement for B7 ligand-dependent costimulation might be obviated under conditions of strong and/or persistent TCR stimulation (49–51). To further study this issue we immunized mice with a wide dose range (125-fold) of R36A (5×10^6 to 6.25×10^8 CFU/mouse) in the presence of either CTLA4Ig or control L6. After 6 wk, when CTLA4Ig had been cleared, mice were boosted with R36A at the same dose as that used for primary immunization. Sera were then analyzed for both primary and memory IgG anti-PspA responses and for the primary IgG anti-PC response. The IgG anti-PspA and IgG anti-PC responses showed a marked dependence on the dose of R36A (Fig. 2). CTLA4Ig, injected at the time of primary immunization, abolished both the primary IgG anti-PspA response as well as the memory response to boosting at all doses of R36A; boosting with R36A under these conditions induced an IgG anti-PspA response at the level of the primary response, indicating that tolerance was not induced at any dose of R36A (also see Fig. 1). Finally, the primary IgG anti-PC response was strongly reduced by CTLA4Ig to the same degree with all doses of R36A. Thus, the requirement for costimulation for both the primary and memory IgG anti-PspA and primary IgG anti-PC responses does not appear to depend on the overall level of immune stimulation by R36A. Whether the doses of R36A employed specifically induced different levels of T cell stimulation was not, however, formally demonstrated in this study.

Differential costimulation kinetics for primary vs memory IgG anti-PspA responses and for primary IgG anti-PspA vs primary IgG anti-PC responses

Little is known regarding the time period after immunization when costimulation is required for optimal induction of a primary Ig response, the formation of memory, and the induction of memory effector function following secondary immunization. Further, based on preliminary data (Z.-Q. Wu and C. M. Snapper, manuscript in preparation) that the nature of the T cell help for IgG anti-PspA and PC responses is mechanistically distinct, it was possible that different time frames for the required costimulatory activity would exist between these two responses. In this regard we first wished to determine the kinetics of the primary anti-PC response and the primary and secondary anti-PspA responses, and the formation of PspA-specific memory. Thus, either naive or primed mice were immunized with R36A, and serum samples were obtained on various days thereafter for measurement of anti-PC and/or anti-PspA titers. Fig. 3*A* demonstrates that the primary anti-PC response peaked 6 days after immunization, whereas the primary anti-PspA response was maximal on day 10, consistent with the more rapid kinetics of a classical polysaccharide- vs protein-specific Ig response (3, 4). Fig. 3*B* indicated that the secondary anti-PspA response was more rapid than the primary response, peaking on day 5, consistent with classical observations of more rapid memory vs primary Ig responses to TD Ags. In an additional experiment we wished to determine at what point after primary immunization with R36A, PspA-specific memory developed. To accomplish this, naive mice were immunized with R36A, then boosted on varying days thereafter with R36A. In each of the boosted groups serum samples were obtained 6 days after boosting. For comparison of the primary vs secondary anti-PspA responses, sera obtained on equivalent days after primary immunization were directly compared, i.e., between groups that received only primary immunization and those that also were boosted at a given time after the primary immunization. Our standard dose of 2×10^8 CFU of R36A was administered for both the primary immunization and the boost. Importantly, for the sake of directly comparing the primary vs secondary anti-PspA responses, primary

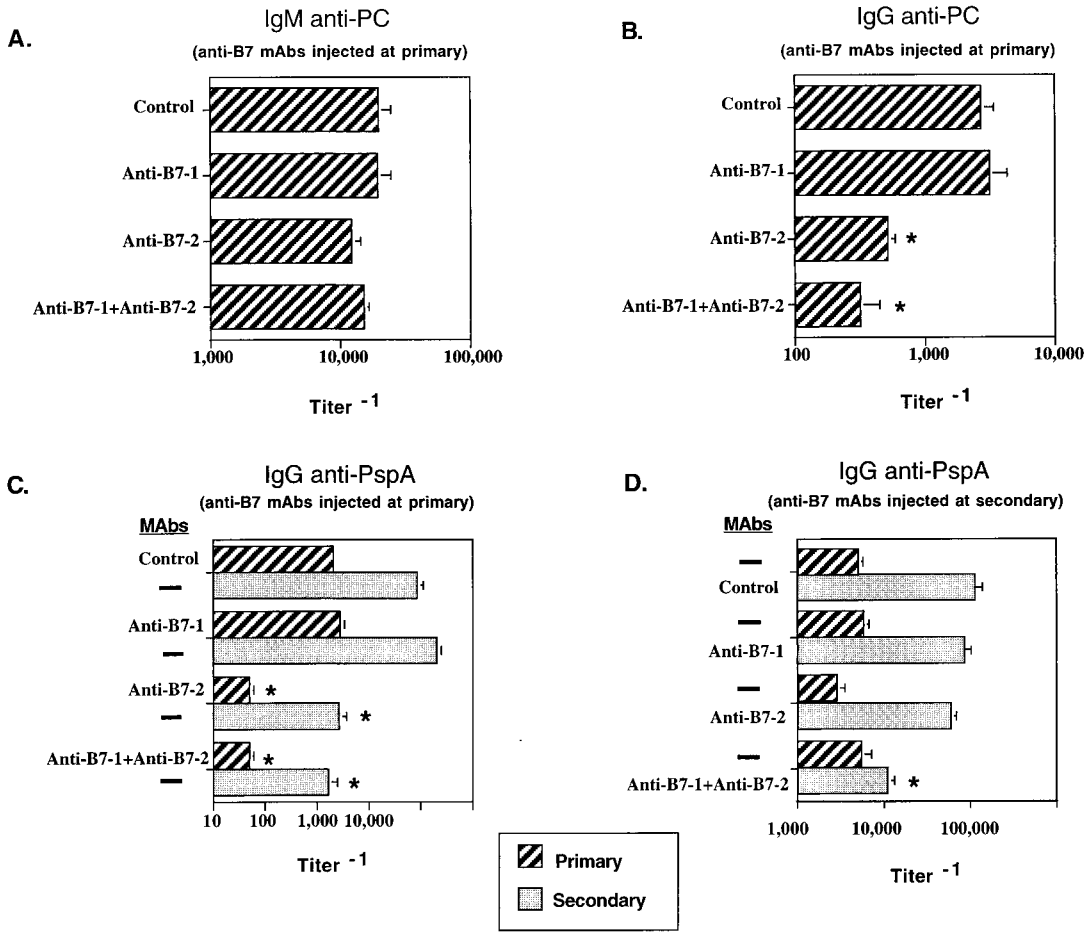

FIGURE 1. Roles of B7-1 and B7-2 in the anti-PC and anti-PspA responses to R36A. *A—C*, Mice were injected i.v. with 0.3 mg of anti-B7-1 mAb and/or 0.3 mg of anti-B7-2 mAb. Control mAbs consisted of equal amounts of hamster IgG (for anti-B7-1) and/or rat IgG (for anti-B7-2). Twenty-four hours later mice were injected i.p. with R36A (5×10^7 CFU/mouse). Six weeks after R36A immunization, mice were boosted with R36A alone (5×10^7 CFU/mouse). Sera were collected 2 wk after primary immunization with R36A (primary), and 1 wk after boosting with R36A (secondary) for determination of anti-PC and anti-PspA titers. Values represent arithmetic mean ± SEM of five mice per group. *, $p < 0.05$. *D*, Mice were injected i.p. with R36A alone (5×10^7 CFU/mouse). Six weeks later primed mice were injected i.v. with 0.3 mg of anti-B7-1 mAb and/or 0.3 mg of anti-B7-2 mAb, followed 24 h later by boosting with R36A (5×10^7 CFU/mouse). Values represent the arithmetic mean ± SEM of five mice per group. *, $p < 0.05$.

immunization with 4×10^8 CFU of R36A did not induce an anti-PspA response that was significantly higher than that observed using 2×10^8 CFU (data not shown). As illustrated in Fig. 3*C*, PspA-specific memory appeared to be fully developed by 8–10 days after primary immunization. Thus, the primary anti-PspA response and the formation of PspA-specific memory appear to follow very similar kinetics.

In the next set of experiments groups of five mice each were given a single injection of CTLA4Ig at different times (0–12 days) after primary immunization with R36A, and sera were obtained 14 and 21 days after the initial R36A injection for determination of serum IgG anti-PspA and anti-PC titers. L6, injected at the time of R36A immunization, was used as a control. Six weeks after R36A immunization, when CTLA4Ig levels were cleared (see Table II), mice were boosted with R36A, and serum was collected 1 and 2 wk later for determination of secondary serum IgG anti-PspA titers. As illustrated in Fig. 4*A*, injection of CTLA4Ig on day 0, 2, or 4 following primary R36A immunization induced significant inhibition of the primary IgG anti-PspA response, whereas CTLA4Ig given on day 6 and thereafter had no significant effect, relative to control, on serum IgG anti-PspA titers. In contrast, whereas CTLA4Ig strongly inhibited the primary IgG anti-PC response when injected at the time of R36A immunization, it had no significant effect when injected on day 2 or thereafter (Fig. 4*B*).

The time during which costimulation was required for optimal induction of the primary IgG anti-PspA response was similar to that required for the generation of memory. Specifically, administration of CTLA4Ig on day 0, 2, or 4 after primary immunization with R36A resulted in a markedly reduced memory IgG anti-PspA response upon boosting with R36A 6 wk after the primary immunization, whereas CTLA4Ig given on day 6 and thereafter had no significant effect (Fig. 4*A*). To determine the kinetics of costimulation during a memory IgG anti-PspA response, a second series of experiments was conducted in which mice were first primed with R36A alone, then boosted with R36A 6 wk later in the presence of either control L6 (day 0) or CTLA4Ig given at different times after the secondary immunization (days 0–6). In striking contrast to what was observed for the primary IgG anti-PspA response and the development of memory (Fig. 4*A*), CTLA4Ig inhibited the memory IgG anti-PspA response when injected at the time of boosting with R36A, but not 1 day later or thereafter (Fig. 4*C*). Thus, costimulation is required over a much earlier time frame, after immunization, for induction of the primary anti-PC and elicitation of

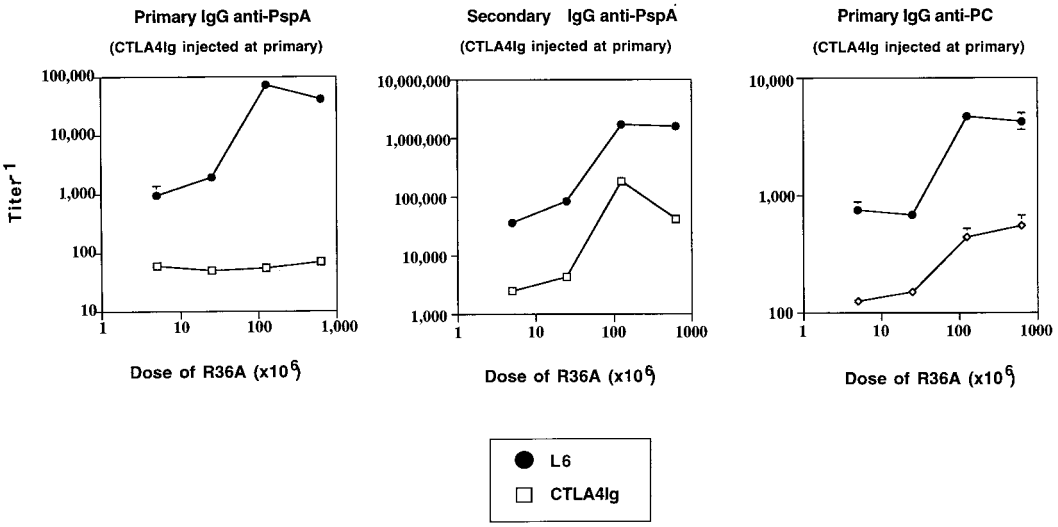

FIGURE 2. Role of R36A dose in costimulation dependence of anti-PC and anti-PspA response. Mice were injected i.v. with 0.2 mg of either CTLA4Ig or control L6, followed 24 h later by i.p. injection with four different doses of R36A (5×10^6, 25×10^6, 125×10^6, and 625×10^6 CFU/mouse). Six weeks later, mice were boosted with R36A alone with the same dose as that used in the primary immunization. Sera were collected 2 wk after primary immunization with R36A (primary) and 1 wk after boosting with R36A (secondary) for determination of anti-PC and anti-PspA titers. Values represent the arithmetic mean ± SEM of five mice per group.

memory anti-PspA responses relative to that observed for the primary anti-PspA response and the development of memory.

CTLA4Ig injected at the time of secondary immunization results in the abrogation of the PspA-specific memory state

Earlier in this paper (Figs. 1 and 4), we demonstrated that blocking costimulation at the time of secondary immunization with R36A completely inhibited the memory IgG anti-PspA response. In a final set of experiments we wished to determine whether this abrogation of the secondary IgG anti-PspA also impacted on the status of the memory immune cell population. To accomplish this, mice were first primed with R36A alone, boosted 6 wk later with R36A in the presence of CTLA4Ig, reboosted 6 wk thereafter with R36A alone, then given a final R36A immunization 6 wk after that. In each case, serum titers of IgG anti-PspA were determined 7–14 days after each immunization. A series of control groups was also established, as indicated and discussed below (Table II). Mice given CTLA4Ig at the time of secondary immunization with R36A failed to elicit a memory IgG anti-PspA response (Table II, groups 3 and 4). Immunization of these mice with a third dose of R36A 6 wk after the secondary dose failed to elicit an IgG anti-PspA response any higher than that observed for the primary. These data strongly suggested that the PspA-specific memory state had been abrogated in these mice. Nevertheless, a memory IgG anti-PspA response was elicited in these mice upon a fourth immunization with R36A, indicating that once the mice were reprimed (third immunization), a memory response could be elicited upon further boosting (Table II, groups 3 and 4). In contrast, tertiary immunization of mice that were given CTLA4Ig at the time of the primary immunization led to a normal memory IgG anti-PspA response (Table II, groups 1 and 2). As a control, we demonstrated that after priming with R36A, a memory response could still be elicited even when mice were boosted as late as 84 days later (Table II, group 7). This indicated that the failure to elicit a memory response in group 4 upon tertiary immunization on day 84 was due to the CTLA4Ig injected at the time of the secondary immunization and not to a natural loss of the memory state by this time point. Further, elicitation of a normal primary IgG anti-PspA response by immunization with R36A 6 wk after mice were injected with CTLA4Ig alone (Table II, groups 5 and 6, indicated that 6 wk was sufficient for effective clearance of CTLA4Ig.

Discussion

In this report we have examined the role of B7 ligand interactions during the primary and secondary immune responses to a protein and polysaccharide Ag following immunization with intact *Streptococcus pneumoniae*. Our studies provide new insights into the function of these molecules in regulating immune responsiveness to bacterial Ags. They demonstrate that 1) the requirements for B7 costimulation are independent of Ag dose; 2) during the primary response, effector and memory cell development require CD28 and B7-2, but not B7-1, whereas a redundant role for B7-1 and B7-2 is observed for the secondary response; 3) during the secondary response, both memory cell activation and maintenance of the memory pool require B7 interactions; and 4) the temporal window required for B7 signaling varies between the primary and secondary responses and with the type of Ag.

B7 blockade effected a degree of inhibition of the primary IgG anti-PSPA response that was the same over a 125-fold dose range of R36A; this dose range encompassed up to 100-fold differences in PspA-specific Ig titers. These findings stand in contrast to previous studies that suggested that strong TCR signaling, secondary to the dose of Ag and/or the degree of TCR affinity for the peptide/MHC complex, is associated with a decreased requirement for costimulatory molecules (49–52). These studies relied in part on cell culture systems to assess the relationship between Ag dose and B7 ligand requirements. It is possible that a sufficiently high density and/or affinity of PspA-derived peptide/MHC complexes was not achievable in our in vivo system to override the B7 dependence of this response. In this regard it would be of value to study the Ig response to other proteins expressed by R36A. Nevertheless, our finding that the anti-PspA response requires B7-mediated costimulation independent of the Ag dose is more consistent with the idea that CD28 transduces signals to the T cell that are qualitatively different from those that are delivered upon TCR engagement of peptide-MHC complexes (53, 54).

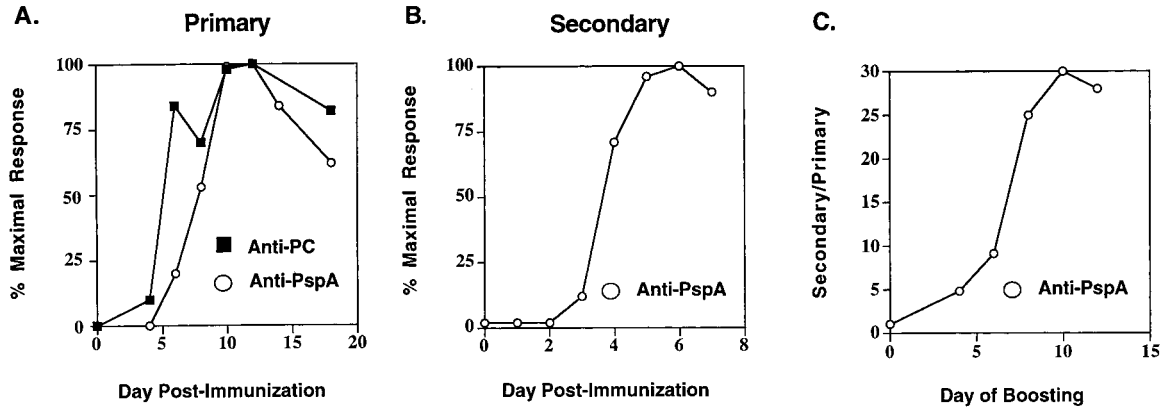

FIGURE 3. Kinetics of primary anti-PC and anti-PspA responses, generation of PspA-specific memory, and secondary anti-PspA response. *A*, Mice were immunized i.p. with R36A (2×10^8 CFU/mouse). Sera were collected every other day following immunization from several similar cohorts of five mice each (days 4–18). *B*, Mice were immunized i.p. with R36A (2×10^8 CFU/mouse). Two weeks later, mice were boosted with R36A (2×10^8 CFU/mouse), and sera were collected each day following the boost from several similar cohorts of five mice each (days 0–7). *C*, Mice were immunized i.p. with R36A (2×10^8 CFU/mouse), then boosted at different times after primary immunization (days 4, 6, 8, 10, and 12), and sera were collected 6 days following each boost for determination of secondary anti-PspA titers. These titers were compared with primary anti-PspA titers from sera obtained after an equivalent total number of days following primary immunization, but without boosting. All data points are calculated from the arithmetic mean of five mice per group.

Our studies further demonstrated that the development of PspA-specific memory also required B7-dependent costimulation independent of Ag dose, since blocking B7 costimulation during the primary response also inhibited the subsequent memory response to R36A, which was conducted in the absence of CTLA4Ig. Specifically, following primary immunization with R36A in the presence of anti-B7-2 mAb, secondary immunization with R36A only led to an anti-PspA response quantitatively similar to a normal primary response. If the formation of memory had been unaffected by anti-B7-2 mAb given at the time of primary immunization, we would have expected the dramatically higher titers of anti-PspA elicited from the normal memory pool following secondary immunization with R36A. This would have been observed independently of the differing, but much lower, primary anti-PspA titers seen in the presence or the absence of anti-B7-2 mAb treatment. Our findings are consistent with previous studies using SRBC immunization that showed a requirement for B7 interactions for the development of memory (42), but contrast with the findings of more recent studies suggesting that heat-stable Ag can substitute for CD28 for induction of the memory state (43, 55). Some studies show that blocking B7 ligand interactions in vivo alone induces anergy (44, 56, 57), whereas other studies argue against this view (46, 47). In this regard, although the blocking of B7 interactions at the time of primary immunization with R36A blocked both the primary anti-PspA response and the generation of PspA-specific memory, tolerance was not observed, since secondary immunization induced serum anti-PspA titers that were equivalent to what was observed for a normal primary.

Although most studies indicated that CD28 and CTLA4 deliver positive and negative signals to the T cell, respectively (25), some reports also suggested a T cell stimulatory effect resulting from CTLA4 ligation (26, 27). Further, the role of CD28 in mediating B7-dependent stimulation during an in vivo immune response has been somewhat controversial (58–61). Our findings that the anti-PspA response is abrogated in $CD28^{-/-}$ mice are consistent with the general model that CD28 provides the positive costimulatory signal. However, this is in contrast with other studies that demonstrate an intact in vivo T cell-dependent type 2 cytokine response in $CD28^{-/-}$ mice challenged with the protozoa, *Leishmania major* (58), or the nematode parasite, *Heligmosomoides polygyrus* (59–61), despite the ability of CTLA4Ig or anti-B7 mAbs to block these responses in wild-type mice. In the case of *H. polygyrus*, the type 2 response is also blocked in B7-1/B7-2 double-knockout mice (M. Ekkens et al., manuscript in preparation). Reports of a positive signaling role for CTLA4 (26, 27) could be relevant to these latter studies. Collectively, these studies demonstrate marked differences in the requirement for CD28 during B7-dependent in vivo responses, indicating the importance of examining costimulatory molecule requirements for different immunogens.

B7-1 and B7-2 may also show distinct differences in their influence on T cell effector function during responses to different Ags (16). Both may be expressed on macrophages, B cells, and

Table II. *Role of B7 ligand blockade during primary or secondary response to R36A on the subsequent primary and/or memory anti-PspA response*[a]

	Time (day)	Time (day)		Serum IgG Anti-PspA Titers		
Group	CTLA4Ig/L6	R36A	Day 14	Day 64	Day 98	Day 133
1	L6,–1	0, 42, 84, 126	3,300	93,900	105,000	73,500
2	CTLA4Ig,–1	0, 42, 84, 126	<50	3,650	97,600	144,000
3	L6,41	0, 42, 84, 126	2,900	60,000	60,000	96,970
4	CTLA4Ig,41	0, 42, 84, 126	2,900	2,900	3,050	131,900
5	L6,–1	42, 84, 126	–	3,400	70,000	131,000
6	CTLA4Ig,–1	42, 84, 126	–	4,600	124,000	96,700
7	–	0, 84, 126	2,000	1,750	58,900	ND

[a] Mice were immunized i.p. with R36A (5×10^7 CFU/mouse) on the days indicated. CTLA4Ig and L6 (0.2 mg/mouse) were injected i.v. on the days indicated. Sera were collected on the days indicated for measurement of anti-PspA titers. Values represent arithmetic means ± SEM of five groups of mice each.

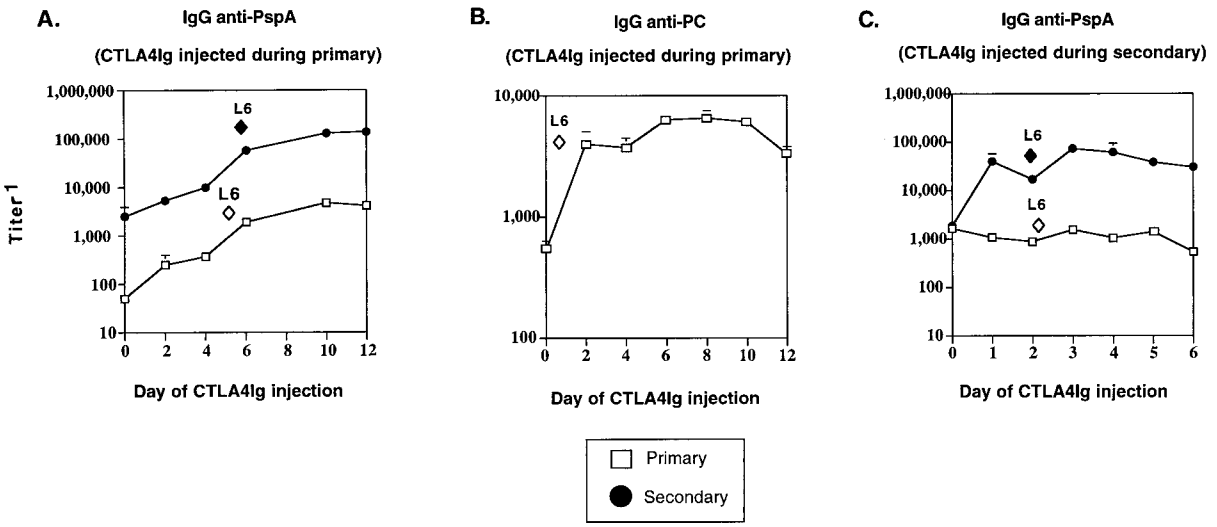

FIGURE 4. Effect of blocking with CTLA4Ig, at different times following primary or secondary immunization with R36A on the primary anti-PC and primary and secondary anti-PspA responses. *A* and *B*, Mice were injected i.p. with R36A (2×10^8 CFU/mouse). CTLA4Ig (0.2 mg/mouse i.v.) was injected into different groups of five mice each on day 0, 2, 4, 6, 10, or 12 days after immunization. Sera were collected 14 days after R36A immunization for determination of primary anti-PC and anti-PspA titers. L6, given at the time of R36A immunization, was used as a control. *C*, Mice were immunized i.p. with R36A (2×10^8 CFU/mouse). Two weeks later sera were obtained (primary anti-PspA titers), then mice were boosted with R36A (2×10^8 CFU/mouse), and CTLA4Ig was injected into different groups of five mice each 1, 2, 3, 4, 5, or 6 days after the boost. Sera were collected 7 days after the boost for determination of secondary anti-PspA titers. L6, given at the time of secondary R36A immunization, was used as a control. Values represent the arithmetic mean ± SEM of five mice per group.

dendritic cells, but B7-2 is up-regulated earlier and shows higher expression than B7-1 during the primary response (31–35). Our findings demonstrate a selective requirement for B7-2 during the primary anti-PspA response, consistent with its early up-regulation and in general agreement with several earlier studies in other model systems (29, 30). The more permissive dependence of the secondary response on either B7-1 or B7-2 suggests that B7-1 may be more rapidly up-regulated or constitutively expressed during the secondary response or, alternatively, that memory T cells may have different costimulatory molecule requirements than naive T cells in response to the same immunogen.

Our findings that CTLA4Ig inhibited the secondary anti-PspA response when given only at the time of boosting with R36A demonstrates that activation of pre-existing memory cells in this system also requires B7 costimulation. In contrast, previous studies of the immune response to SRBCs (42) and the nematode parasite, *H. polygyrus* (41), demonstrated that the secondary immune response was refractory to administration of B7 antagonists. In vitro studies have also shown reduced B7-dependent costimulatory requirements for memory T cells (39, 40). It is possible that these other immunogens evoke more potent memory responses, which trigger sufficient T cell signaling through the TCR and perhaps other costimulatory molecules to circumvent a need for B7 costimulation. Thus, disparities in the costimulatory molecule requirements during secondary responses are observed depending on the model system.

B7-dependent costimulation was required for a much shorter duration (within 1 day) during the secondary anti-PspA response, relative to that required for the primary anti-PspA response (3–4 days), suggesting that memory cells can differentiate more quickly to a B7-independent activation state compared with naive cells during the primary response. This is consistent with our kinetic studies showing that induction of maximal titers of anti-PspA following secondary immunization occurred earlier (4–5 days) than primary PspA-specific titers (8–10 days). Thus, although B7 costimulation is required for both responses, there is a markedly reduced temporal requirement for costimulatory signals during the secondary response to R36A. Recent studies have suggested that memory cells enter the cell cycle and differentiate upon Ag stimulation much more rapidly than do naive cells (62), providing a possible mechanism for the shorter duration of costimulatory molecule requirements. Of interest, the development of PspA-specific memory during the primary response required the same duration of costimulation as did the primary anti-PspA response itself, suggesting that both effector and memory cells developed in tandem from naive precursors. This is supported by our kinetic studies demonstrating similar kinetics between induction of the primary anti-PspA response and development of PspA-specific memory. These data argue against the idea that memory cells develop from effector cells (63).

Our studies also indicate that blocking B7 costimulation during the secondary response abrogated the functional memory population that had differentiated during priming, since a normal secondary response was still blocked after a third immunization with R36A alone (see Table II, groups 3 and 4). These studies suggest that during a secondary response, Ag exposure in the absence of B7 costimulation either deletes or functionally inactivates the memory cell pool so that it also cannot respond following subsequent challenge. During the third immunization, priming and memory cell development recurred, since the subsequent fourth immunization did induce a normal "secondary" response.

As mentioned earlier, the anti-PC response, like that for PspA, is dependent upon $CD4^+$ TCR-$\alpha\beta^+$ T cells, although PC-specific memory does not develop. Both the primary anti-PC and anti-PspA responses require B7-2 and CD28, but not B7-1. In contrast, a redundant role for B7-1 and B7-2 was observed for both the in vivo primary and secondary anti-polysaccharide responses to a glycoconjugate vaccine consisting of group B streptococcal capsular polysaccharide conjugated to tetanus toxoid (64). Additionally, the in vivo T cell-mediated, idiotype-specific suppression of

the anti-$\alpha(1\rightarrow3)$dextran Ig response involves an interaction of B7-1 and CD28 (65). Consistent with the more rapid kinetics of the primary anti-PC (peak on day 6) vs the primary anti-PspA (peak on day 10) response, costimulation for an optimal anti-PC response is required over a shorter duration (within 1 day vs 4–6 days for the primary anti-PspA response). In this regard, recent studies from our laboratory (Z.-Q. Wu et al., manuscript in preparation) suggest that the T cell help for the anti-PC response, in contrast to that for anti-PspA, is TCR nonspecific and acts at an earlier time during the immune response.

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