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# 4-1BB (CD137) Differentially Regulates Murine In Vivo Protein- and Polysaccharide-Specific Immunoglobulin Isotype Responses to *Streptococcus pneumoniae*

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**4-1BB (CD137) is induced on activated $CD4^+$ and $CD8^+$ T cells and delivers a costimulatory signal upon binding the 4-1BB ligand (4-1BBL) expressed on antigen-presenting cells. Induction of 4-1BB is dependent on activation via the T-cell receptor (TCR) and possibly CD28. It was previously demonstrated that both an in vivo protein (pneumococcal surface protein A [PspA])- and polysaccharide (phosphorylcholine [PC] determinant of teichoic acid)-specific immunoglobulin (Ig) isotype response to *Streptococcus pneumoniae* was dependent on $CD4^+$ $TCR\alpha\beta^+$ T cells and B7-dependent costimulation through CD28. We thus postulated that 4-1BB costimulation would also play a role in regulating the in vivo anti-PspA and anti-PC response to *S. pneumoniae*. We demonstrate that mice genetically deficient in 4-1BBL elicit a markedly reduced IgM and IgG anti-PC but normal primary and secondary IgG anti-PspA responses to *S. pneumoniae* relative to those for wild-type mice. However, injection of an agonistic anti-4-1BB monoclonal antibody (MAb), while having no significant effect on the anti-PC response, strongly inhibits the primary anti-PspA response, the generation of PspA-specific memory, and germinal center formation but does not induce a lasting state of tolerance. In contrast, anti-4-1BB MAb has no effect on the anti-PspA response when injected only at the time of secondary immunization. Delay of the addition of anti-4-1BB leads to progressively less inhibition of the primary response up to day 8. This inhibition is independent of $CD8^+$ T cells and is associated with the expansion of $CD4^+$ T cells with an activated phenotype, which is partly dependent on B7-dependent costimulation. These data are the first to suggest a stimulatory role for endogenous 4-1BB–4-1BBL interactions during a humoral immune response to a pathogen and further underscore significant differences in costimulation requirements for an in vivo protein- versus polysaccharide-specific Ig isotype response to an extracellular bacterium.**

B7-dependent costimulation of T-cell-receptor (TCR)-activated T cells via constitutively expressed CD28 is often a critical early event for the initial activation of naïve T cells (8). Upon activation, T cells upregulate other costimulatory molecules, which may then mediate the subsequent progression of the T-cell response. One such molecule is 4-1BB (CD137), a member of the tumor necrosis factor (TNF) receptor gene family. In the mouse, both 4-1BB and the 4-1BB ligand (4-1BBL, a member of the TNF gene family) are expressed on dendritic cells (DCs) (9, 33), 4-1BB is expressed on activated $CD4^+$ and $CD8^+$ T cells (22) and activated NK cells (20), and 4-1BBL is expressed on B cells and macrophages (12, 23). Triggering of T cells through 4-1BB can occur in both a CD28-dependent (11, 26) and CD28-independent (9, 5, 10, 15) manner and may depend on the strength of TCR signaling. Thus, 4-1BB can be upregulated on T cells via strong TCR signaling alone but requires CD28 costimulation at lower levels of TCR-mediated activation (11), consistent with the observation that 4-1BB mediates the costimulation of resting T cells upon activation with high, but not low, amounts of anti-CD3 monoclonal antibody (MAb) (26).

Agonistic anti-4-1BB MAb strongly costimulates the in vitro proliferation of murine splenic $CD8^+$ T cells, and, to a much lesser extent, $CD4^+$ T cells, that have been activated with anti-CD3 in the presence of antigen-presenting cells (APCs) (27). These data are consistent with the ability of anti-4-1BB to augment in vivo $CD8^+$-T-cell cytotoxicity in a number of model systems (11, 19, 27). Endogenous 4-1BB–4-1BBL interactions also appear to be important in $CD8^+$-T-cell responses, as illustrated by diminished antiviral cytotoxic lymphocyte (CTL) responses, skin allograft rejection, and graft-versus-host disease in 4-$1BBL^{-/-}$ mice (4, 10, 31, 32). In contrast, 4-$1BBL^{-/-}$ mice had no apparent defects in in vivo antigen-specific immunoglobulin (Ig) responses to vesicular stomatitis virus (VSV) (10), lymphocytic choriomeningitis virus (31), or influenza A virus (3), suggesting that 4-1BBL-dependent costimulation may play little, if any, physiologic role in humoral immunity. These data were confirmed in a more recent report on 4-$1BB^{-/-}$ mice demonstrating normal specific IgM and IgG responses to VSV and TNP-lipopolysaccharide, although two- to threefold reductions in specific IgG3 and IgG2a responses to keyhole limpet hemocyanin (KLH) without adjuvant were observed in mutant mice (17). However, a recent report indicated that agonistic anti-4-1BB MAb strongly inhibits in vivo

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T-cell-dependent, antigen-specific Ig responses to sheep red blood cells (SRBC) and human IgG but not the Ig response to the T-cell-independent type 2 antigen TNP-Ficoll (21). This anti-4-1BB-mediated inhibition was independent of $CD8^+$ T cells and associated with antigen-specific $CD4^+$-T-cell anergy.

Immunization of mice with either unencapsulated or encapsulated heat-killed, intact bacteria appears to represent a more physiologic approach to understanding the mechanisms of antimicrobial immunity than approaches which rely solely on the use of purified soluble antigens with or without adjuvant. Recent work from our laboratory has demonstrated both distinct and overlapping immune mechanisms underlying the induction of in vivo protein- versus polysaccharide-specific Ig isotype production in response to intact heat-killed *Streptococcus pneumoniae* (7, 34–36). In common, both types of Ig response (i.e., specific for the cell wall protein, pneumococcal surface protein A [PspA], and the phosphorylcholine [PC] determinant of the cell wall C polysaccharide [teichoic acid]) depend on $CD4^+$ $TCR\alpha\beta^+$ T cells and B7-2- and CD28-dependent costimulation and can be induced through the active participation of DCs (7, 34, 35). In contrast, the T-cell help for the anti-PC response develops more rapidly, is TCR nonspecific, does not require the cathepsin S-dependent peptide loading of major histocompatibility complex class II, and can occur in the absence of germinal center formation (36). In this regard, *S. pneumoniae*-pulsed DCs from major histocompatibility class $II^{-/-}$ mice can elicit IgG anti-PC, but not IgG anti-PspA, responses when transferred into wild-type, naïve mice in a T-cell- and B7-dependent manner (7). Despite T-cell help, there is little, if any, generation of memory for the anti-PC response whereas robust secondary responses for anti-PspA have been observed.

No studies have yet addressed a potential role for 4-1BB–4-1BBL in an in vivo protein- and polysaccharide-specific Ig isotype response to an intact extracellular bacteria. In light of earlier studies demonstrating distinct forms of B7-dependent T-cell help for the in vivo IgG anti-PspA versus IgG anti-PC responses to *S. pneumoniae*, we postulated a potential and perhaps differential role for 4-1BB–4-1BBL in regulating these two responses. Utilizing both $4\text{-}1BBL^{-/-}$ mice (10) and an agonistic anti-4-1BB MAb (27), we confirmed this hypothesis. We demonstrate, for the first time, a stimulatory role for endogenous 4-1BB–4-1BBL interactions in the in vivo polysaccharide-specific Ig isotype response to intact *S. pneumoniae* and further demonstrate a marked inhibitory effect of an agonistic anti-4-1BB MAb on the antiprotein response. These latter results both confirm and significantly extend an earlier report on the effects of anti-4-1BB MAb on in vivo T-cell-dependent and -independent Ig responses to soluble and particulate antigens (21).

## MATERIALS AND METHODS

**Mice.** Female C57BL/6 mice were obtained from the National Cancer Institute (Frederick, Md.) and used at 7 to 10 weeks of age. $4\text{-}1BBL^{-/-}$ mice backcrossed seven times onto C57BL/6 mice were generated as previously described (10). The experiments in this study were conducted according to the principles set fourth by the National Institutes of Health (21a).

**Reagents.** PC-KLH, a kind gift of Andrew Lees (Biosynexus, Inc., Rockville, Md.), was synthesized as described previously (34). Recombinant pneumococcal surface protein A (PspA) was expressed in *Saccharomyces cerevisiae* BJ3505 as a $His_6$-tagged fusion protein and purified by Ni-nitrilotriacetic acid affinity chromatography (34). The expressed protein includes amino acids 4 to 299 of the mature protein. Rat IgG2a anti-mouse 4-1BB MAb (clone 3H3) was prepared as previously described (27). Rat IgG2b anti-mouse CD8 MAb (clone 2.43), hamster IgG anti-mouse B7-1 MAb (clone 16-10A1) (24), and rat IgG2a anti-mouse B7-2 MAb (clone GL1) (14) were purified from ascites by ammonium sulfate precipitation followed by passage over a protein G column. Purified hamster IgG and rat IgG were purchased from Accurate Chemical and Scientific Corporation (Westbury, N.Y.). Unless otherwise indicated, all MAbs injected into mice were first diluted in phosphate-buffered saline (PBS) and then injected intravenously typically 1 day before immunization with R36A. The following doses of MAb were injected: 0.3 mg of anti-4-1BB/mouse, 0.5 mg of anti-B7-1/mouse, 0.5 mg of anti-B7-2/mouse, or 1 mg of anti-CD8/mouse. Doses of control rat IgG and hamster IgG were equivalent to the corresponding doses of experimental MAbs.

**Preparation of and immunization with R36A.** A nonencapsulated variant (strain R36A) of virulent *S. pneumoniae* capsular type 2 (strain D39) (2) 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 to two characteristic colonies were selected and suspended in 200 ml of Todd-Hewitt broth and placed in a shaker water bath at 37°C for 4 to 6 h until an optical density (absorbance at 650 nm) of 0.6 was achieved as measured by a spectrophotometer (Spectronic 100; Bausch & Lomb, Rochester, N.Y.). 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 $10^{10}$ CFU/ml, was divided into aliquots and frozen at −70°C until used for immunization. Mice were immunized intraperitoneally with $10^8$ CFU of R36A in 250 μl of PBS. Samples for measurement of anti-PC and anti-PspA antibody titers in serum were prepared from blood obtained through the tail vein.

**Measurement of serum antigen-specific Ig isotype titers.** Immulon 2 plates were coated with PC-KLH (5 μg/ml), and Immulon 4 plates were coated with PspA (5 μg/ml) in 1× PBS for 1 h at 37°C or overnight at 4°C. Plates were then blocked with blocking buffer (1× PBS, 0.5% bovine serum albumin) at 37°C for 30 min or at 4°C overnight. Threefold dilutions of serum Ig samples in blocking buffer were then added, starting at a 1/50 serum dilution. After a 1-h incubation at 37°C, plates were washed three times with PBT (1× PBS, 0.1% Tween 20). Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG3, IgG1, IgG2b, and IgG2a antibodies (200-ng/ml final concentration in blocking buffer) were then added, and plates were incubated for 37°C for 1 h. Plates were washed five times with PBT. Substrate (4-methylumbelliferyl phosphate) was then added (50 μg/ml, 50 μl/well), and fluorescence was read on a MicroFLUOR (Dynatech Laboratories, Inc., Chantilly, Va.) enzyme-linked immunosorbent assay (ELISA) reader. Serum Ig titers were calculated as follows. A standard curve was generated by using threefold dilutions of a positive serum Ig sample, starting with an initial 1/50 serum dilution. The signal (in fluorescence units) from the most dilute sample of the standard curve that was still above background was randomly assigned a titer of 1, and all signals from consecutively smaller dilute samples were assigned the numbers 3, 9, 27, etc. For each experimental sample, a dilution that generated a signal within the linear part of the standard curve was chosen. The value extrapolated from the standard curve was then multiplied by the inverse of that dilution in order to generate the final inverse titer.

**Quantitation of germinal centers by immunohistochemistry.** Spleens were removed from unimmunized mice and from mice 14 days after intraperitoneal immunization with R36A. Sections (8 μm) were cut, fixed in acetone for 10 min, and stored at −70°C. Germinal center B cells were stained with 15 μg of fluorescein isothiocyanate (FITC)-peanut agglutinin (ICN, Costa Mesa, Calif.)/ml for 1 h at room temperature. Slides were washed with PBS, allowed to dry, and then covered with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, Ala.) for analysis by fluorescence microscopy with a Zeiss Axiophot fluorescence microscope.

**Flow cytometric analysis.** All steps were performed on ice. Fc receptors were specifically blocked with 2.5 μg of anti-CD16/CD32 MAb/ml/$10^6$ cells (clone 2.4G2; BD Pharmingen) in PBS containing 1% fetal calf serum staining buffer for 45 min before the staining and also during the staining. Cells were stained by incubation for 30 min with FITC- or phycoerythrin (PE)-conjugated MAbs (BD Pharmingen) specific for CD4 (clone GK1.5), CD8α (clone 53-6.7), B220 (CD45R) (clone RA3-6B2), CD62L (L-selectin) (clone Mel-14), CD69 (very early activation antigen) (clone H1.2F3), and CD25 (interleukin 2Rα [IL-2Rα], p55) (clone PC61). Irrelevant isotype and species-matched MAbs were used as staining controls. Cells were analyzed on an EPICS XL-MCL (Beckman Coulter, Miami, Fla.) apparatus. Dead cells and debris were excluded from analysis by gating on the appropriate forward and side scatter profiles.

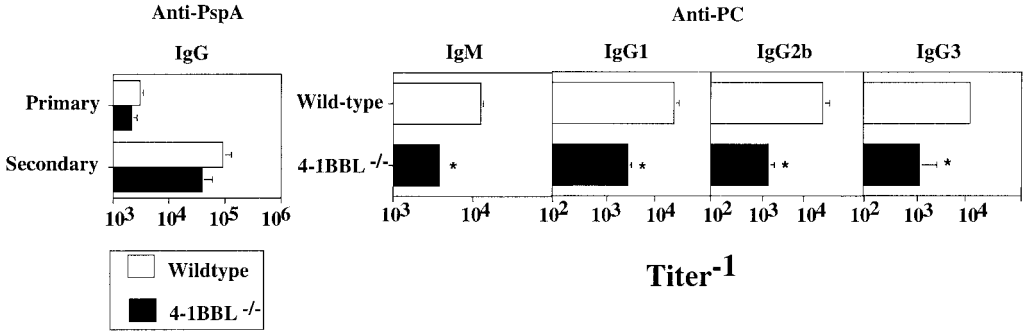


FIG. 1. The anti-PC response to R36A is reduced in 4-1BBL$^{-/-}$ mice. 4-1BBL$^{-/-}$ and wild-type mice (seven per group) were immunized with $10^8$ CFU of R36A each and boosted 14 days later with $10^8$ CFU of R36A. Sera were obtained on days 7 (anti-PC), 14 (primary anti-PspA), and 21 (secondary anti-PspA) for the determination of titers of anti-PC and/or anti-PspA Ig isotypes by ELISA. Data are representative of two similar experiments. *, $P \leq 0.05$.

**Statistics.** Data are expressed as the arithmetic means of Ig titers of individual samples in serum ± the standard errors of the means. Differences between treatment groups were considered significant at $P < 0.05$ by using Student's *t* test.

## RESULTS

**The in vivo IgM and IgG anti-PC, but not IgG anti-PspA, response to R36A is strongly reduced in 4-1BBL$^{-/-}$ mice.** Both the in vivo IgG anti-PC and IgG anti-PspA responses to *S. pneumoniae* were dependent on CD4$^+$ TCR$\alpha\beta^+$ T cells and B7-dependent, CD28-mediated costimulation (34, 35) and could be elicited in a T-cell- and B7-dependent manner by the adoptive transfer of *S. pneumoniae*-pulsed DCs (7). In light of the presence in the mouse of both 4-1BB and 4-1BBL on DCs (9, 33), 4-1BB on activated CD4$^+$ T cells (22), and 4-1BBL on B cells (23), we initially wished to determine whether endogenous 4-1BB–4-1BBL interactions played a role in the in vivo anti-PC and anti-PspA responses to *S. pneumoniae*. We thus immunized 4-1BBL$^{-/-}$ (10) and wild-type mice with the nonencapsulated variant of *S. pneumoniae*, capsular type 2 (strain R36A) (2) and then boosted them with R36A 14 days later. Sera were collected on days 14 and 21 for measurement of IgG anti-PspA titers at the peak of the primary and secondary responses, respectively, and on day 7 for peak serum IgM and IgG anti-PC titers (34, 35). Preimmunization levels of anti-PC or anti-PspA were low or undetectable, respectively, with no differences observed between 4-1BBL$^{-/-}$ and wild-type mice (data not shown). As illustrated in Fig. 1, both the primary and secondary IgG anti-PspA responses in 4-1BBL$^{-/-}$ mice were comparable to those seen in wild-type mice. In contrast, both the IgM and IgG anti-PC responses were strongly reduced in 4-1BBL$^{-/-}$ mice. Similar reductions were seen for PC-specific IgM and for all the IgG isotypes. These data are the first to demonstrate a stimulatory role for endogenous 4-1BB–4-1BBL interactions in an in vivo humoral response to a pathogen and contrast with previous studies in 4-1BBL$^{-/-}$ mice showing no defect in in vivo antigen-specific Ig responses to VSV (10), lymphocytic choriomeningitis virus (31), or influenza A virus (3).

**Agonistic anti-4-1BB MAb, independent of CD8$^+$ T cells, strongly inhibits the in vivo primary IgG anti-PspA response and inhibits the generation of PspA-specific memory but has no effect on the anti-PC response to R36A.** Agonistic anti-4-1BB MAb strongly costimulates the in vitro proliferation of murine splenic CD8$^+$ T cells, and, to a much lesser extent, CD4$^+$ T cells, that have been activated with anti-CD3 in the presence of APCs (27). These data are consistent with the ability of anti-4-1BB to augment in vivo CD8$^+$-T-cell cytotoxicity in a number of model systems (11, 19, 27) but contrast with a recent report that anti-4-1BB MAb inhibits in vivo T-cell-dependent, antigen-specific Ig responses to SRBC and human IgG but not the Ig response to the T-cell-independent type 2 antigen TNP-Ficoll (21). The ability of anti-4-1BB MAb to modulate in vivo protein- and polysaccharide-specific Ig isotype production in response to an extracellular bacterium has not been tested. Thus, we immunized mice with R36A in the presence of agonistic anti-4-1BB MAb and obtained sera 7 and 14 days after immunization for the determination of primary titers of IgM and IgG anti-PC and of IgG anti-PspA in serum, respectively. Injection of control rat IgG as well as control hamster IgG (see Fig. 5 through 7) in R36A-immunized mice had no effect relative to that for mice immunized with R36A alone (data not shown). As illustrated in Fig. 2A, anti-4-1BB MAb had no effect on the PC-specific IgM or IgG isotype response to R36A in contrast to the stimulatory role of endogenous 4-1BB–4-1BBL interactions in the anti-PC response (Fig. 1). In contrast, anti-4-1BB MAb strongly inhibited the primary IgG anti-PspA response (Fig. 2B) despite there being no effect of endogenous 4-1BB–4-1BBL on this response (Fig. 1). These data confirm that agonistic anti-4-1BB MAb is not acting as a blocking antibody in vivo.

On the basis of a 7.5-day half-life of the anti-4-1BB MAb (21), we waited 6 weeks after primary immunization with R36A plus anti-4-1BB MAb before boosting mice with R36A alone in order to assess the effect of anti-4-1BB MAb on the generation of PspA-specific memory during the primary response. As a control, we demonstrated that the injection of anti-4-1BB MAb in the absence of R36A, followed by primary R36A immunization 6 weeks later, had no effect on the primary anti-PspA response (data not shown), confirming the lack of functional anti-4-1BB MAb in vivo 6 weeks after its injection. As illustrated in Fig. 2B, boosting of mice previously immunized with R36A plus anti-4-1BB MAb resulted in an IgG anti-PspA response no greater than that normally seen after primary immunization with R36A alone, indicating that PspA-specific memory was completely abolished by the anti-4-1BB MAb that was present during the primary response. Fol-

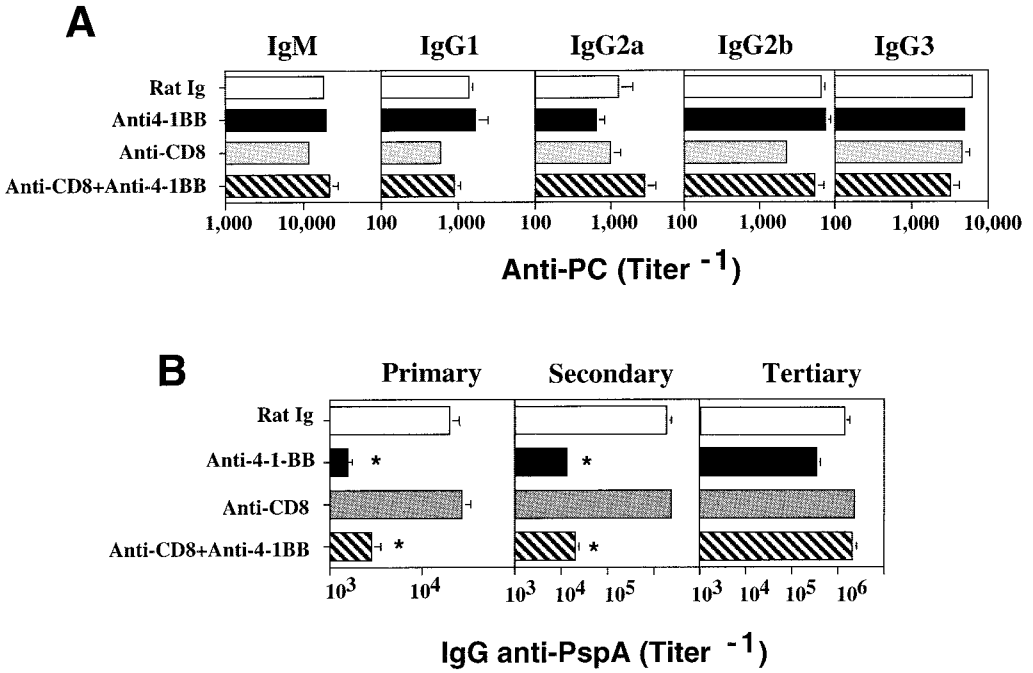


FIG. 2. Anti-4-1BB MAb inhibits the primary anti-PspA response and the generation of PspA-specific memory independent of $CD8^+$ T cells. Mice (five per group) were immunized with R36A in combination with anti-CD8 and/or anti-4-1BB MAb and/or control rat IgG. Mice were boosted with R36A alone 6 weeks after primary immunization and then boosted again 2 weeks later. Sera were obtained on days 7 (anti-PC) and 14 (primary anti-PspA) and at 8 weeks (secondary anti-PspA) and 10 weeks (tertiary anti-PspA) for the determination of titers of anti-PC and/or anti-PspA Ig isotypes by ELISA. Data are representative of two similar experiments. *, $P \leq 0.05$ relative to that for rat Ig.

lowing a second boost 2 weeks later (Fig. 2B), the IgG anti-PspA response in mice initially injected with anti-4-1BB MAb reached normal secondary levels. These data indicated that anti-4-1BB MAb injected at the time of primary immunization did not lead to long-lasting tolerance in that, once anti-4-1BB MAb was cleared, a normal primary and secondary response could be obtained.

Although agonistic anti-4-1BB MAb strongly activates $CD8^+$ T cells in vitro and in vivo (11, 19, 27), depletion of $CD8^+$ T cells with anti-CD8 MAb at the time of treatment with R36A plus anti-4-1BB MAb had no additional effect on the anti-PC or anti-PspA responses in these mice (Fig. 2A and B), indicating no role for $CD8^+$ T cells in the anti-4-1BB-mediated inhibition of the PspA-specific Ig response. The ability of anti-4-1BB MAb to inhibit the induction of PspA-specific memory was consistent with the ability of anti-4-1BB MAb to inhibit splenic germinal center (GC) formation in response to R36A (Fig. 3). Injection of control rat IgG in R36A-immunized mice had no effect on GC formation relative to that for mice immunized with R36A alone (data not shown).

**Delay of addition of anti-4-1BB leads to progressively less inhibition of the primary IgG anti-PspA response up to day 8.** It was previously demonstrated that cytotoxic T lymphocyte antigen (CTLA) 4Ig inhibits the primary IgG anti-PspA response and the generation of PspA-specific memory when injected as late as 4 days, but not 6 days, after primary immunization (35). In light of the constitutive expression of CD28 on T cells versus the requirement to induce T cells to express 4-1BB, we wished to determine the time after primary immunization with R36A when anti-4-1BB MAb, relative to that for CTLA4Ig, could inhibit primary anti-PspA responses and the generation of PspA-specific memory. As illustrated in Fig. 4, anti-4-1BB MAb significantly inhibited the primary IgG anti-PspA response to R36A when injected as late as 8 days after R36A immunization. Maximal inhibition occurred when anti-4-1BB MAb was injected at the time of immunization, and the level of inhibition diminished thereafter in a time-dependent manner. Secondary challenge with R36A alone 6 weeks after primary immunization, when anti-4-1BB MAb had been cleared, revealed that the generation of memory was also inhibited with the same kinetics as that seen for the primary anti-PspA response. A tertiary immunization revealed that, once mice initially injected with anti-4-1BB MAb were fully

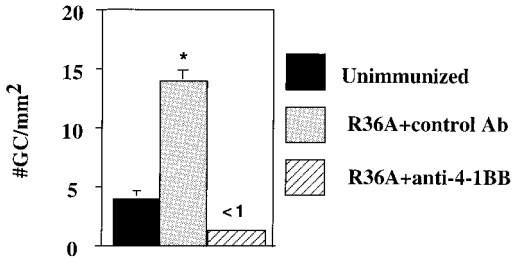


FIG. 3. Anti-4-1BB MAb inhibits the formation of GCs in response to R36A. Mice (three per group) were immunized with R36A in the presence of anti-4-1BB MAb or control rat IgG. Spleens were removed 14 days after immunization for the analysis of GC formation relative to that in spleens from three unimmunized mice. *, $P \leq 0.05$ relative to that for unimmunized mice.

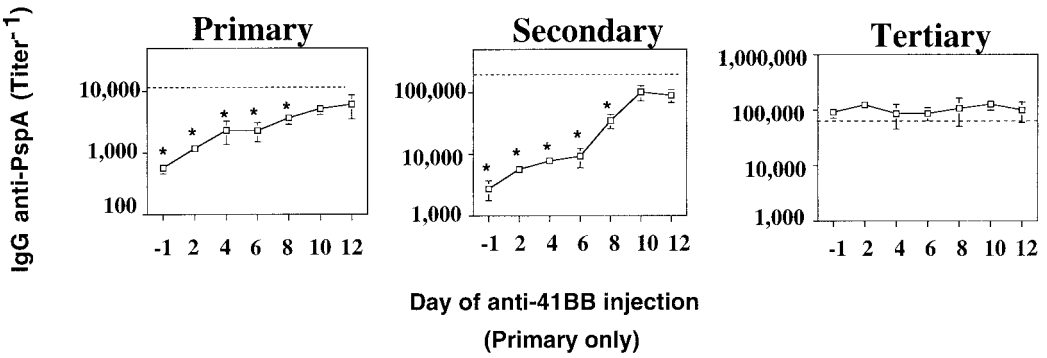


FIG. 4. Anti-4-1BB-mediated inhibition of the primary anti-PspA response and the generation of PspA memory decreases steadily with a delay of injection of MAb up to day 8. Separate groups of mice (five each) were immunized with R36A. Anti-4-1BB MAb was injected into different groups of mice on different days after R36A immunization as indicated. Mice were boosted with R36A 6 weeks after primary immunization and boosted again 2 weeks later. Sera were obtained on day 14 (primary anti-PspA) and at 8 weeks (secondary anti-PspA) and 10 weeks (tertiary anti-PspA) for the determination of titers of IgG anti-PspA by ELISA. Data are representative of two similar experiments. *, $P \leq 0.05$ relative to that for mice treated with rat IgG (broken lines).

primed, normal secondary responses could be obtained, indicating that at no time point of anti-4-1BB MAb injection was their evidence for the long-lasting induction of tolerance or enhancement of Ig responses. These data contrasted with the earlier observation that CTLA4Ig significantly inhibited a primary anti-PspA response and PspA-specific memory when injected up to 4 days, but not 6 days, after R36A immunization (35). Thus, in anti-4-1BB MAb-injected mice, 4-1BB can mediate its inhibitory actions at a later time after primary R36A immunization relative to the time frame for CD28-induced costimulation.

**Anti-4-1BB MAb does not inhibit the secondary IgG anti-PspA response in R36A-primed mice.** In separate experiments, we further tested whether anti-4-1BB MAb, like CTLA4Ig (35), inhibited the secondary anti-PspA response in R36A-primed mice when injected at the time of secondary immunization with R36A. As illustrated in Table 1, anti-4-1BB MAb injected 6 weeks after R36A immunization failed to inhibit the secondary anti-PspA response to R36A despite its ability to inhibit the primary anti-PspA response and PspA-specific memory when injected at the time of primary R36A immunization. This contrasted with the previous observation that CTLA4Ig, when injected into R36A-primed mice at the time of secondary immunization with R36A, completely inhibited the secondary anti-PspA response (35).

**Anti-4-1BB MAb enhances in vivo splenic $CD4^+$- or $CD8^+$-T-cell expansion in a completely or partly B7-dependent manner, respectively.** A previous report demonstrated through adoptive transfer studies that the anti-4-1BB-mediated inhibition of the T-cell-dependent, in vivo Ig response to SRBC and to human IgG was associated with the induction of $CD4^+$-T-cell anergy and not dependent upon $CD8^+$ T cells (21). Our data likewise indicated that the anti-4-1BB-mediated inhibition of the R36A-induced anti-PspA response was not mediated by $CD8^+$ T cells (Fig. 2), suggesting that the injection of anti-4-1BB MAb also induced an inhibition of $CD4^+$-T-cell help during this response. Since the anti-PspA response is dependent on $CD4^+$ T cells, we especially wished to assess the effect of anti-4-1BB MAb on $CD4^+$-T-cell function in R36A-immunized mice. Further, we wished to determine whether the effects of anti-4-1BB MAb on T cells was dependent on B7-dependent costimulation, given previous reports indicating a role for CD28-mediated costimulation for the induction of 4-1BB on TCR-activated T cells (11, 26). Thus, mice were immunized with R36A in the presence of anti-4-1BB MAb and/or anti-B7-1 plus anti-B7-2 MAbs or of control Ig and spleen cells were isolated 14 days later. The total average numbers of nucleated cells obtained per spleen from each group 14 days after immunization were as follows: unimmunized, $2.9 \times 10^7$ cells; R36A plus control Ig, $4.3 \times 10^7$; R36A plus anti-4-1BB MAb, $8.7 \times 10^7$; R36A plus anti-B7-1 and anti-B7-2 MAbs, $4.9 \times 10^7$; R36A, anti-4-1BB MAb, and anti-B7-1 and anti-B7-2 MAbs, $8.3 \times 10^7$. As illustrated in Fig. 5, mice immunized with R36A plus control Ig had significant absolute increases in the total number of $CD4^+$ and $CD8^+$ T cells and of B cells per spleen relative to those for unimmunized mice. This R36A-mediated expansion was unaffected by the injection of anti-B7-1 plus anti-B7-2 MAbs. Mice immunized with R36A plus anti-4-1BB MAb showed further significant expansions of both $CD4^+$ and $CD8^+$ T cells and B cells relative to mice immunized with R36A plus control Ig or with R36A plus anti-B7-1 and anti-B7-2 MAbs. However, anti-B7-1 plus anti-B7-2 MAbs significantly inhibited the anti-4-1BB-mediated expansion of $CD4^+$ T cells but did not significantly

TABLE 1. Anti-4-1BB MAb does not inhibit the secondary anti-PspA response when injected at the time of boosting of R36A-primed mice[a]

| Group | Day(s) of injection after immunization with: | | Serum IgG anti-PspA titer (mean ± SEM) on day: | |
|---|---|---|---|---|
| | Anti-4-1BB | R36A | 21 | 56 |
| Rat IgG | −1 | 0, 42 | 5,620 ± 1,590 | 93,100 ± 17,200 |
| Anti-4-1BB | −1 | 0, 42 | 950 ± 170 | 1,125 ± 116 |
| Rat IgG | 41 | 0, 42 | 1,470 ± 547 | 94,400 ± 45,100 |
| Anti-4-1BB | 41 | 0, 42 | 3,390 ± 610 | 65,700 ± 23,900 |

[a] Groups of five mice each were immunized with $10^8$ CFU of R36A/mouse in the presence of either rat IgG or anti-4-1BB MAb (0.3 mg/mouse) injected at the time of either the primary or secondary immunization, and sera were obtained as indicated for the determination of titers of IgG anti-PspA by ELISA.

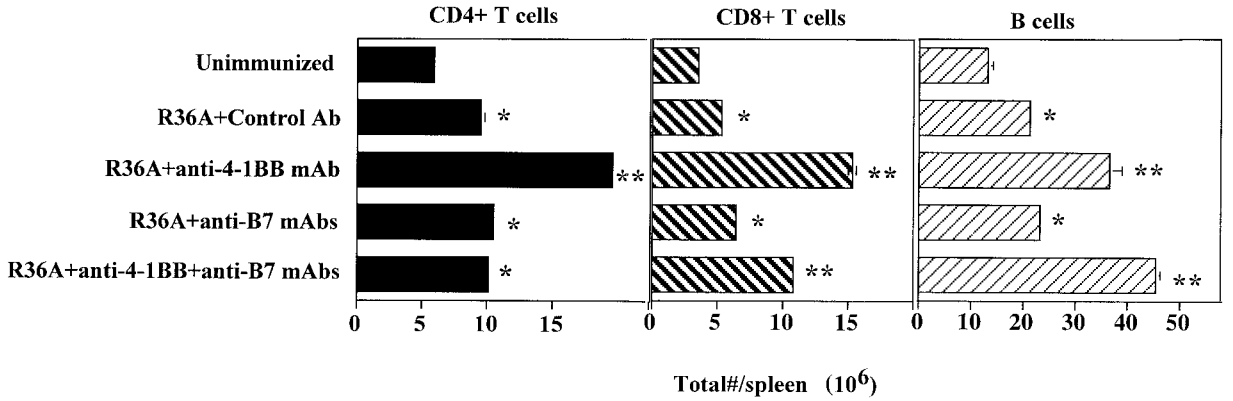


FIG. 5. Anti-4-1BB MAb induces the expansion of splenic *CD4*$^+$ and CD8$^+$ T cells in a B7-dependent manner. Mice (five per group) were injected with R36A with control rat IgG plus hamster IgG or with anti-4-1BB MAb and/or anti-B7-1 plus anti-B7-2 MAbs. Spleens were removed 14 days after immunization, and red blood cell-lysed spleen cells from each group were combined and stained in triplicate as indicated with either PE–anti-CD4, PE–anti-CD8, or PE–anti-B220 (B cells) MAb for flow cytometry. Average total number of CD4$^+$ and CD8$^+$ T cells and B cells per spleen was determined by multiplying average total number of red blood cell-lysed spleen cells (see text) with the percentage in each population. Data are representative of two similar experiments. *, $P \leq 0.05$ relative to that for unimmunized mice; **, $P \leq 0.05$ relative to that for mice immunized with R36A plus anti-B7 MAbs.

affect the numbers of CD8$^+$ T cells or B cells. These data indicate that anti-4-1BB MAb induces an expansion of CD4$^+$ and CD8$^+$ T cells in R36A-immunized mice, which, at least for CD4$^+$ T cells, is B7 dependent.

**Anti-4-1BB MAb induces CD4$^+$- and CD8$^+$-T-cell activation.** Splenic CD4$^+$ and CD8$^+$ T cells from mice immunized with R36A plus control MAb or with R36A plus anti-B7-1 and anti-B7-2 MAbs showed no detectable evidence of activation on day 14 after immunization relative to that for unimmunized mice, as assessed by the cell surface expression of CD69, CD62L (clone Mel-14), or CD25 (IL-2R$\alpha$) (Fig. 6). However, mice immunized with R36A plus anti-4-1BB MAb had substantially greater percentages of CD4$^+$ and CD8$^+$ T cells that were CD69$^+$, a somewhat comparable decrease in the percentages of CD4$^+$ and CD8$^+$ T cells expressing CD62L, and no changes in the percentages of CD25$^+$ T cells relative to those for unimmunized mice or mice immunized with R36A plus control Ig or with R36A plus anti-B7-1 and anti-B7-2 MAbs. Thus, anti-4-1BB MAb induced a fraction of CD4$^+$ and CD8$^+$ T cells to express an activated phenotype (CD69$^{hi}$ CD62L$^{lo}$) in R36A-immunized mice in addition to enhancing the total number of CD4$^+$- and CD8$^+$-T-cell numbers. Anti-B7-1 plus anti-B7-2 MAbs partially inhibited the anti-4-1BB-induced activated phenotype of CD4$^+$ T cells but had no apparent effect on CD8$^+$ T cells. Collectively, the data suggest that, although anti-4-1BB MAb induces CD4$^+$-T-cell activation and expansion in vivo that depends on B7-dependent costimulation, this is associated with a striking inhibition of the primary anti-PspA response, the generation of PspA-specific memory, and the associated germinal center reaction, all CD4$^+$-T-cell-dependent events.

**Anti-4-1BB MAb does not reverse the anti-B7-mediated inhibition of the anti-PspA response in R36A-immmunized mice.** In a last set of studies, we wished to determine whether anti-4-1BB MAb could substitute for CD28 in delivering a costimulatory signal for generating the IgG anti-PspA response to R36A despite its inhibitory activity in the presence of CD28-mediated costimulation. Mice were immunized as described above (Fig. 5 and 6), and serum was obtained 7 and 12 days after immunization for determination of IgG anti-PspA titers. As illustrated in Fig. 7, injection of anti-4-1BB MAb did not reverse the anti-B7-mediated inhibition of the R36A-induced IgG anti-PspA response.

## DISCUSSION

Previous studies on in vivo antigen-specific Ig responses to a number of viruses in 4-1BBL$^{-/-}$ mice failed to demonstrate a role for endogenous 4-1BB–4-1BBL interactions in regulating Ig production (3, 10, 31). However, a recent study demonstrated a two- to threefold reduction in serum anti-KLH titers, only of the IgG3 and IgG2a isotypes, in 4-1BB$^{-/-}$ mice immunized with KLH in the absence of adjuvant (17). This latter study suggested a possible role for 4-1BB in stimulating gamma interferon production, inasmuch as gamma interferon is a switch factor for IgG3 (29) and IgG2a (28). 4-1BB$^{-/-}$ mice, nevertheless, demonstrated normal specific IgM and IgG responses to VSV. In contrast, we demonstrate that 4-1BBL$^{-/-}$ mice exhibited strong reductions in R36A-induced titers of both PC-specific IgM and IgG in serum in an IgG isotype-nonselective manner, without any alteration in the IgG anti-PspA response relative to that for wild-type mice. Although T-cell help is required for an optimal IgG anti-PC response, the IgM anti-PC response is T cell independent (34). Thus, at least some of the effect(s) of 4-1BB–4-1BBL interactions on the anti-PC response may not be mediated through alterations in T-cell function. Although these data suggest that 4-1BB–4-1BBL interactions play a role in promoting the induction of PC-specific Ig during the immune response to *S. pneumoniae*, the possibility that this effect results from a developmental alteration, perhaps in PC-specific B-cell precursors, cannot be ruled out.

Several effects of 4-1BB or 4-1BBL on non-T cells could serve to augment anti-PC responses to R36A. Thus, ligation of 4-1BB expressed on murine bone marrow-derived DCs and freshly isolated splenic DCs stimulates the secretion of IL-6

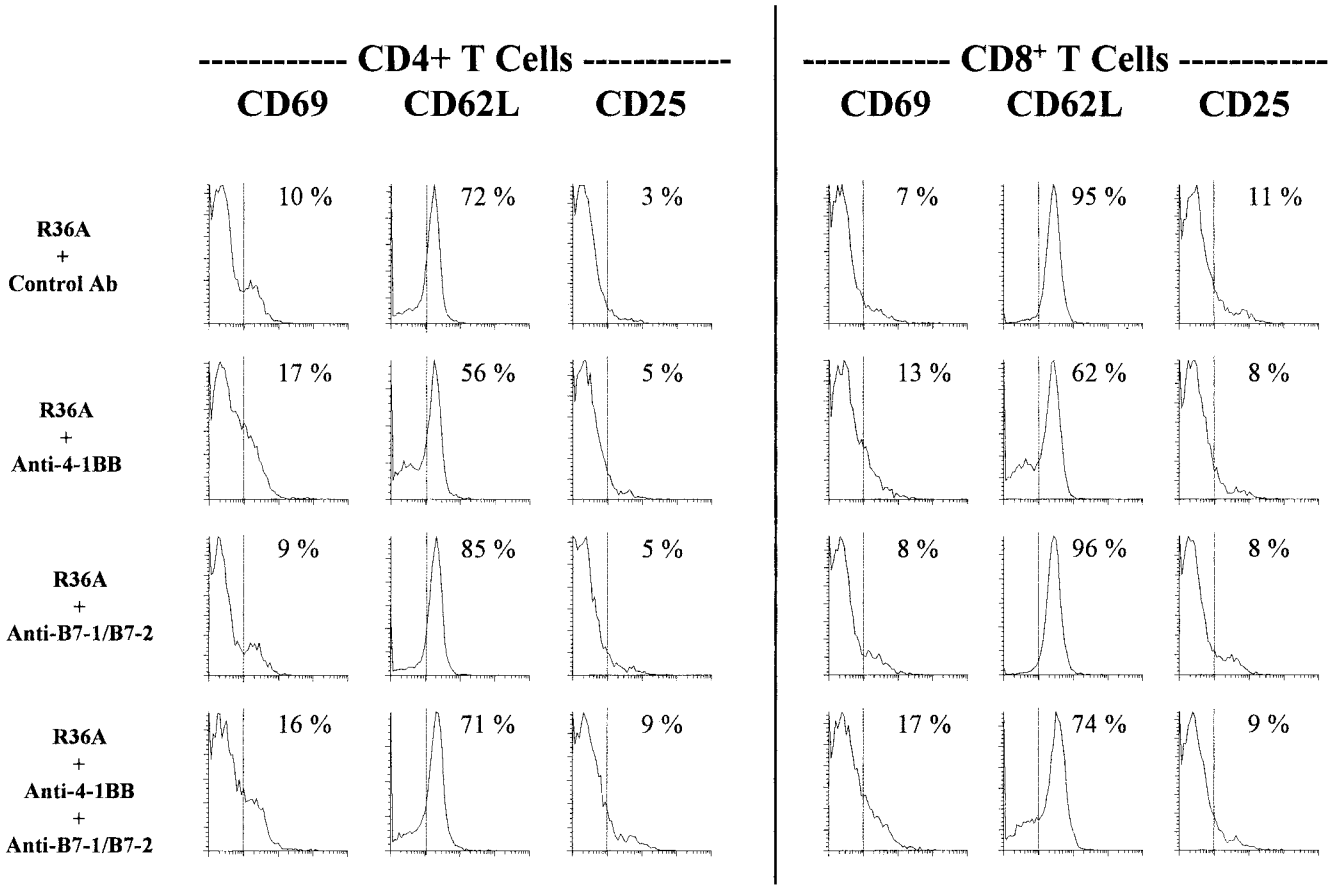


FIG. 6. Anti-4-1BB MAb stimulates $CD4^+$- and $CD8^+$-T-cell activation in a B7-dependent manner. Mice were immunized and spleen cells were prepared for flow cytometry as described in the legend to Fig. 5. Spleen cells were stained with FITC–anti-CD69, FITC–anti-CD62L, or FITC–anti-CD25 with either PE–anti-CD4 or PE–anti-CD8. Cells were gated on positive staining for CD4 or CD8, and single histograms were generated for CD69, CD62L, and CD25 expression. Data are representative of two similar experiments.

and IL-12 (33). Furthermore, cross-linking of Ig on murine splenic B cells upregulates 4-1BBL expression, which can then mediate a costimulatory signal for proliferation of the anti-Ig-activated B cells (23). Cross-linking of 4-1BBL on human monocytes stimulates the release of proinflammatory cytokines such as IL-6, IL-8, and TNF-α; inhibits the release of the anti-inflammatory cytokine IL-10; and upregulates the cell surface expression of intracellular adhesion molecule 1 (CD54) (18). In this regard, DCs can directly deliver helper signals to activated B cells (6) and, recently, DCs demonstrated an active role in inducing an in vivo anti-PC response to R36A (7). Further, we demonstrated that endogenous proinflammatory cytokines stimulate anti-PC responses to R36A whereas endogenous IL-10 is inhibitory (16). Finally, the presence of repeating PC moieties on the teichoic acid expressed by R36A will induce multivalent membrane Ig cross-linking of PC-specific B cells and likely lead to the upregulation of 4-1BBL. Thus, the described effects of 4-1BB or 4-1BBL on DCs, B cells, and monocytes could all potentially contribute to an augmented anti-PC response to R36A in vivo.

In contrast to our observations of reduced serum IgM and IgG anti-PC titers and a normal IgG anti-PspA response to R36A in $4\text{-}1BBL^{-/-}$ mice, injection of an agonistic anti-4-1BB MAb had no effect on the anti-PC response but strongly inhibited the primary IgG anti-PspA response and the generation of PspA-specific memory. Although the basis for the divergent effects seen in $4\text{-}1BBL^{-/-}$ mice and the use of agonistic anti-4-1BB MAb are unknown, the data collectively in-

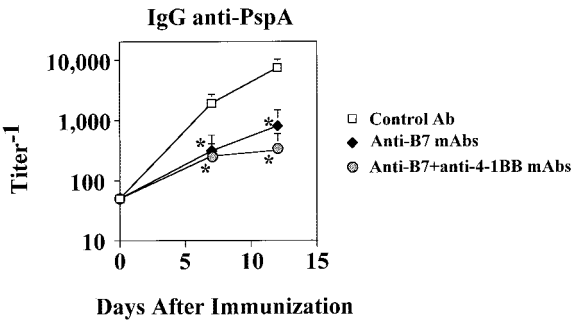


FIG. 7. Inhibition of the anti-PspA response to R36A is no different in mice injected with anti-B7 MAbs or anti-B7 MAbs plus anti-4-1BB MAb. Mice (five per group) were immunized with R36A with control rat IgG plus hamster IgG or anti-4-1BB MAb or with anti-B7-1 and anti-B7-2 MAbs plus anti-4-1BB MAb. Sera was obtained 14 days after immunization for the determination of IgG anti-PspA titers by ELISA. Data are representative of two similar experiments. *, $P \leq 0.05$ relative to that for mice treated with control antibody (Control Ab).

dicate that the effects of agonistic anti-4-1BB MAb in vivo were not mediated simply by some theoretical blocking function but likely rather through the induction of cell signaling. It has been demonstrated that agonistic anti-4-1BB MAb strongly costimulates the in vitro proliferation of $CD8^+$ T cells and, to a lesser extent, $CD4^+$ T cells, that have been activated with anti-CD3 in the presence of APCs (27). These data are consistent with the ability of anti-4-1BB to augment in vivo $CD8^+$-T-cell cytotoxicity in a number of model systems (11, 19, 27) but are somewhat surprising in light of our data and those of Mittler et al. (21), which demonstrate an inhibitory effect of anti-4-1BB MAb on an in vivo humoral response either to a pathogen or to soluble or particulate antigens, respectively.

Mittler et al. previously demonstrated that agonistic anti-4-1BB MAb injected during primary immunization with the T-cell-dependent (TD) antigens, SRBC, or human IgG inhibited both primary and secondary induction of specific Ig. This inhibition was similarly observed in β2-microglobulin-deficient mice, which lack $CD8^+$ T cells, and was associated with the long-term anergy of T cells but not B cells. In contrast, anti-4-1BB MAb had no effect on the Ig responses to the TI-2 antigen TNP-Ficoll. Our data, using agonistic anti-4-1BB MAb during in vivo Ig responses to an intact pathogen, confirm certain key observations of Mittler et al. as well as significantly extend these findings in a number of ways. Specifically, we show the following. (i) Anti-4-1BB MAb, independent of $CD8^+$ T cells, inhibits the primary anti-PspA response and the generation of PspA-specific memory when coinjected with R36A into naïve mice but has no effect on the secondary anti-PspA response when injected into primed mice at the time of secondary immunization. (ii) In contrast to Mittler et al., who used an adoptive transfer approach, we observed directly in vivo that anti-4-1BB MAb does not induce long-lasting anergy in that the boosting of mice immunized with R36A plus anti-4-1BB MAb leads to the reestablishment of normal primary and secondary anti-PspA responses when the anti-4-1BB MAb has been cleared from the circulation. The reasons for this difference may lie in the use by Mittler et al. of SCID mice in their adoptive transfer study, which did not allow for the generation of new T cells, whereas we tested anti-4-1BB MAb directly in wild-type mice, where new T cells were continuously generated. (iii) Anti-4-1BB-mediated suppression of the anti-PspA response is associated with a marked inhibition of the germinal center reaction. (iv) Delay of the addition of anti-4-1BB leads to progressively less inhibition of the primary IgG anti-PspA response up to day 8. (v) Anti-4-1BB-mediated suppression of the anti-PspA response is associated with splenic $CD4^+$- and $CD8^+$-T-cell expansion and activation as well as B-cell expansion. (vi) Especially for $CD4^+$ T cells, this is B7 dependent.

The mechanism underlying the anti-4-1BB-mediated suppression of the anti-PspA response to R36A is presently unknown. Delay of the addition of anti-4-1BB MAb led to steadily less inhibition of the anti-PspA response up to day 8 after R36A immunization. Thus, anti-4-1BB-mediated suppression can occur early after R36A immunization, suggesting a potential effect at the level of DCs which can express 4-1BB. However, ligation of 4-1BB on DCs in vitro led to the induction of IL-6 and IL-12 and the enhancement of APC function (33), effects which would likely promote, not suppress, an anti-PspA response (16). The partial inhibitory effects of delayed anti-4-1BB MAb injection also suggest a potential direct effect of the MAb on $CD4^+$ T cells. The B7 dependence of the anti-4-1BB-mediated induction of $CD4^+$-T-cell activation and expansion is consistent with the notion that anti-4-1BB MAb was acting directly on $CD4^+$ T cells, since B7-dependent signaling was likely required for both initial activation as well as subsequent 4-1BB induction on the T cells (11, 26). It is possible that the strong and sustained signaling of $CD4^+$ T cells by anti-4-1BB MAb resulted in some undefined impairment in their ability to mediate the primary anti-PspA response and the generation of PspA-specific memory, although the anti-4-1BB-mediated expansion of B cells that we observed might reflect polyclonal B-cell proliferation secondary to the increased $CD4^+$-T-cell activation. The inability of anti-4-1BB MAb to inhibit the anti-PC response, which is also dependent on $CD4^+$ T cells, may reflect the more rapid delivery and/or the qualitatively distinct nature of the T-cell help for the anti-PC versus the anti-PspA response (36). Similarly, the apparently more rapid and, perhaps, potent delivery of T-cell help for induction of the secondary anti-PspA response (35) may explain the inability of anti-4-1BB MAb to inhibit anti-PspA responses when injected at the time of secondary R36A immunization, in part since 4-1BB must be induced on the $CD4^+$ T cell. Anti-4-1BB MAb can also induce NK cell activation (20), and NK cells can potentially inhibit B-cell function through the release of transforming growth factor β (13) and perhaps through the expression of Fas ligand (1, 25).

In summary, these data implicate endogenous 4-1BB–4-1BBL interactions in stimulating an in vivo antipolysaccharide, though not antiprotein, response to an intact extracellular bacteria and further underscore distinct pathways leading to humoral immunity based on the nature of the antigen expressed by the intact pathogen (30). Furthermore, the potent inhibitory effect of agonistic anti-4-1BB MAb on the primary antiprotein, but not antipolysaccharide, Ig response and the generation of protein-specific memory both confirm and extend the data of Mittler et al. (21) pointing to the potential use of this MAb as a therapeutic tool for down-modulating pathogenic antibody responses in vivo.

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