Role of B7 Costimulatory Molecules in Mediating Systemic and Mucosal Antibody Responses to Attenuated *Salmonella enterica* Serovar Typhimurium and Its Cloned Antigen

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The purpose of the present study was to evaluate the ability of an attenuated *Salmonella enterica* serovar Typhimurium vaccine strain to up-regulate B7-1 and B7-2 on antigen-presenting cells and to examine the functional roles these costimulatory molecules play in mediating immune responses to *Salmonella* and to an expressed cloned antigen, the saliva-binding region (SBR) of antigen I/II. In vitro stimulation of B cells (B220⁺), macrophages (CD11b⁺), and dendritic cells (CD11c⁺) with *S. enterica* serovar Typhimurium induced an up-regulation of B7-2 and, especially, B7-1 expression. The in vivo functional roles of B7-1, B7-2, and B7-1/2 were evaluated in BALB/c wild-type and B7-1, B7-2, and B7-1/2 knockout (KO) mice following intranasal immunization with the *Salmonella* expressing the cloned SBR. Differential requirements for B7-1 and B7-2 were observed upon primary and secondary immunizations. Compared to wild-type controls, B7-1 and B7-2 KO mice had reduced mucosal and systemic anti-*Salmonella* antibody responses after a single immunization, while only B7-1 KO mice exhibited suppressed anti-*Salmonella* antibody responses following the second immunization. Mucosal and systemic antibody responses to SBR were reduced following the primary immunization, whereas a compensatory role for either B7-1 or B7-2 was observed after the second immunization. B7-1/2 double KO mice failed to induce detectable levels of mucosal or systemic immunoglobulin A (IgA) or IgG antibody responses to either *Salmonella* or SBR. These findings demonstrate that B7-1 and B7-2 can play distinct as well as redundant roles for mediating mucosal and systemic antibody responses, which are likely dependent upon the nature of the antigen.

Attenuated strains of bacteria, such as *Salmonella enterica* serovar Typhimurium, have received much attention as delivery systems due to their ability to target mucosal inductive sites (16). Previous studies have provided evidence for the efficacy of using attenuated recombinant *Salmonella* strains for augmenting immune responses to a variety of heterologously expressed antigens as well as inducing potent anti-*Salmonella* antibody responses in both the mucosal and the systemic compartments (4, 5, 7). Although these studies highlight the efficacy of using attenuated strains of *S. enterica* serovar Typhimurium as a mucosal delivery system, little is known regarding the underlying cellular mechanisms involved in the ability of the vector and of the cloned antigen in inducing T-cell-dependent immune responses.

Previous studies assessing naive CD4⁺ T-cell activation have provided evidence that two signals are required for optimal activation, which include a signal through the T-cell receptor–CD3 complex and a second costimulatory signal (1, 13). In this regard, the role of the B7-1 (CD80) and B7-2 (CD86) costimulatory molecules expressed on antigen-presenting cells (APC) in mediating CD4⁺ T-cell-dependent responses is well documented (1, 2, 8–10, 13, 18). While some studies comparing the relative contribution of B7-1 and B7-2 costimulatory molecules in the induction of immune responses have suggested that they appear to have compensatory roles (2, 11), several reports have suggested that B7-1 and B7-2 have nonredundant roles (10, 12). Indeed, the ability of several mucosal adjuvants to selectively up-regulate B7-1 or B7-2 levels on APC has been reported to directly affect the immunomodulatory properties to coadministered antigens (3, 14). Moreover, past studies have also provided evidence that B7-1 and B7-2 costimulatory molecules can play critical roles in the preferential development of Th helper 1 (Th1)- and Th2-type immune responses, respectively (10). Although it is presently unclear how B7-1 and B7-2 costimulatory molecules differentially exert their immunostimulatory effects, the expression and kinetics of B7-1 and B7-2 can vary depending on the amount and molecular nature of the stimulant. In this regard, B7-2 is normally more rapidly induced, whereas the expression levels of B7-1 typically persist longer (6). Furthermore, B7-1 and B7-2 have different binding affinities for their two reported ligands expressed on T cells, CD28, and cytotoxic T-lymphocyte antigen 4, in which CD28 supports positive signaling while cytotoxic T-lymphocyte antigen 4 immunoglobulin A (IgA) or IgG antibody responses to either *Salmonella* or SBR. These findings demonstrate that B7-1 and B7-2 can play distinct as well as redundant roles for mediating mucosal and systemic antibody responses, which are likely dependent upon the nature of the antigen.

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what functional role the B7 costimulatory pathway plays in the generation of mucosal and systemic immune responses following mucosal immunization with an attenuated strain of *S. enterica* serovar Typhimurium, as well as how the different isoforms of B7 may be regulating immune responses to the vector and to the heterologously expressed antigen. Therefore, the purpose of the present study was to evaluate the ability of an *S. enterica* serovar Typhimurium *aroA* and *aroD* mutant to modulate the expression of B7-1 and B7-2 on APC and the functional significance of B7-1 and B7-2 in mediating mucosal and systemic antibody responses to *Salmonella* and its cloned antigen consisting of the saliva-binding region (SBR) of the *Streptococcus mutans* adhesin antigen I/II following intranasal (i.n.) immunization. Results are presented demonstrating that the *Salmonella* vector differentially up-regulates B7-1 and B7-2 on APC and that these costimulatory molecules mediate nonredundant and compensatory costimulatory signals in the establishment of mucosal and systemic antibody responses to an attenuated *Salmonella* vaccine strain and its cloned antigen, respectively.

**MATERIALS AND METHODS**

**Mice.** BALB/c wild-type (wt), B7-1 KO, B7-2 KO, and B7-1/2 double KO (DKO) mice were bred and maintained in an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham. The original breeder pairs of B7-1 KO, B7-2 KO, and B7-1/2 DKO BALB/c mice were obtained from Arlene Sharpe (Brigham and Women’s Hospital, Boston, Mass.), and mice were generated as previously described (2). Groups of 10- to 12-week-old female mice (six per group) were used in the present study. All studies were performed according to the National Institutes of Health guidelines, and protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Preparation of recombinant *S. enterica* serovar Typhimurium for immunization.** The *aroA* and *aroD* mutant *S. enterica* Typhimurium BRD509 expressing the cloned SBR of *Streptococcus mutans* under the control of the T7 promoter was used in the present study as previously described (4, 5). Cultures were grown in Luria-Bertani broth containing 50 μg of carbenicillin/ml and 50 μg of kanamycin/ml at 30°C with shaking (200 rpm). The bacteria were harvested by centrifugation (15 min at 4°C; 4,000 × g) and resuspended in phosphate-buffered saline (PBS) at a final concentration of 5 × 10^9 CFU/ml and used immediately for immunizations.

**Immunizations.** Groups of female BALB/c wt, B7-1 KO, B7-2 KO, and B7-1/2 DKO mice (six per group) were immunized on days 0 and 18 by i.n. administration of 10^9 CFU of *Salmonella* BRD509 in PBS in a total volume of 20 μl, applied equally to each nostril (10 μl/nostril) on days 0 and 18. An additional group of wt mice received 20 μl of PBS (10 μl/nostril) by the i.n. route and served as the sham-immunized control.

**Sample collections.** Plasma, saliva, and vaginal wash samples were collected from all mice on day 0 (preimmune samples) and at 10 days after primary and secondary immunizations. Blood was obtained from the retro-orbital plexus by using heparinized capillary tubes, and plasma was collected following centrifugation. Saliva samples were collected after the induction of saliva flow by intraoral injection with 5 μg of carbachol (Sigma Chemical Co., St. Louis, Mo.). Vaginal wash samples were collected by flushing the vagina twice with 60 μl of PBS. Individual plasma, saliva, and vaginal wash samples were stored at −70°C until assayed for antibody activity.

**Enzyme-linked immunosorbent assay.** The levels of SBR- and *Salmonella*-specific antibodies in the various samples were quantified by enzyme-linked immunosorbent assay. Briefly, MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) coated with purified recombinant SBR (2.5 μg/ml) or formalin-killed *Salmonella* BRD509 (5 × 10^8 CFU/ml) diluted in borate-buffered saline over night at 4°C were used for the detection of anti-SBR or anti-*Salmonella* antibodies, respectively. The generation of recombinant SBR and of formalin-killed *Salmonella* BRD509 has been described previously (4, 5). Total IgA levels in the saliva and vaginal washes were determined by coating plates with goat anti-mouse IgA antibodies (0.25 μg/ml; Southern Biotechnology Associates, Birmingham, Ala.). Serial twofold dilutions of plasma, saliva, and vaginal washes were added in duplicate, and plates were incubated overnight at 4°C. Plates were then washed with PBS containing 0.1% Tween and incubated at room temperature (4 h) with the appropriate peroxidase-conjugated goat anti-mouse Ig isotype-specific reagent (Southern Biotechnology). After washing, the plates were developed with o-phenylenediamine and hydrogen peroxide for 20 min and the optical density was measured at 490 nm. The levels of antibodies and of total Ig were calculated by interpolation on calibration curves generated by using a mouse Ig reference serum (ICN Biomedicals, Aurora, Ohio).

**FACS analysis.** Splenocytes and cervical lymph nodes (superficial) from BALB/c wt mice were aseptically removed (three per group) and homogenized by using a sterile, stainless steel wire mesh (60-μm pore size). The spleen cells were suspended in an ammonium chloride-potassium bicarbonate buffer for 5 min at room temperature to lyse the red blood cells. The spleen cells were washed with PBS, resuspended in RPMI 1640 (Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM l-glutamine, 20 mM HEPES, and 1 mM sodium pyruvate, and cultured in 24-well plates or polypropylene tubes. Freshly grown *Salmonella* BRD509 lacking *aroA* and *aroD* and expressing SBR was added to wells at different multiplicities of infection (MOI). Following incubation for either 24 or 48 h, cells were harvested and washed in ice-cold PBS. Cells were stained with annexin V-allophycocyanin (Molecular Probes, Eugene, Ore.) to exclude apoptotic cells. The cells were then stained with phycoerythrin-conjugated anti-mouse B7-1, fluorescein isothiocyanate-conjugated anti-mouse B7-2, and biotin-conjugated anti-mouse CD11b, CD11c, or CD220 (eBioscience, San Diego, Calif.). The cells were then washed three times in fluorescence-activated cell sorter (FACS) buffer (3% fetal bovine serum and 0.1% sodium azide in PBS) and resuspended in FACS buffer containing streptavidin-peridinin chlorophyll protein (Streptavidin-PerCp; BD Pharmingen, San Diego, Calif.) for 10 min at 4°C. Following washing, the cells were analyzed by using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, Calif.) and Cell Quest Software (BD Immunocytometry Systems). The mean fluorescence intensities (MFIs) of B7-1 and B7-2 were determined by gating on cells exhibiting forward and side scatter properties consistent with live cells and staining positive for peridinin chlorophyll protein.

**Statistical analysis.** The significances of differences between groups were calculated by analysis of variance and the Tukey multiple-comparison test with the InStat program (GraphPad Software, San Diego, Calif.). Differences between groups were considered significant when *P* values were <0.05.

**RESULTS**

Attenuated *S. enterica* serovar Typhimurium differentially up-regulates B7-1 and B7-2 on B cells, macrophages, and dendritic cells. In order to determine the ability of the *Salmonella* BRD509 expressing SBR to up-regulate B7-1 and B7-2 on different APC, murine spleen cells were cocultured in the presence or absence of various MOI of *Salmonella* for a period of 24 or 48 h. The *Salmonella* induced an up-regulation of both B7-1 and B7-2 on B cells (B220^+^) (Fig. 1A and B), and the intensity of B7-1 expression at 24 h was significantly higher than that of B7-2 (*P* < 0.05), except when the MOI was greater than or equal to 1:10 (Fig. 1A). The general profile of B7-1 observed at 48 h (Fig. 1B) was similar to that seen at 24 h (Fig. 1A) in that the expression of B7-1 was significantly higher than that of B7-2 (*P* < 0.05). A slight but insignificant increase in the MFI of B7-2 expression on B cells was observed following a 48-h incubation with *Salmonella*.

An analysis of B7-1 and B7-2 expression on macrophages (CD11b^+^ cells) following stimulation with *Salmonella* revealed a two- to threefold higher level of expression of B7-1 than B7-2 at both 24 and 48 h (*P* < 0.05) (Fig. 1C and D). The incubation of spleen cells with *Salmonella* at an MOI exceeding 1:10 resulted in reductions in the MFIs of B7-1 and B7-2 to nearly baseline (unstimulated) levels (Fig. 1C and D). The B7-1 and B7-2 expression profiles observed following stimulation of dendritic cells (CD11c^+^) were similar to those seen for macrophages, in that B7-1 expression levels were significantly higher than B7-2 levels in the *Salmonella*-stimulated CD11c^+^ cells.
These results demonstrate that the MOI, type of APC, and kinetics of expression are important variables influencing the levels of B7-1 and B7-2 following stimulation with attenuated *Salmonella enterica* serovar Typhimurium. Moreover, a predominance of the expression of B7-1 compared to that of B7-2 was observed at both 24 and 48 h following stimulation of all APC populations with the *Salmonella* vaccine strain.

**FIG. 1.** Effects of *Salmonella* on B7-1 and B7-2 expression on APC. Murine spleen cells were incubated in the absence or presence of various numbers of *Salmonella* BRD509 for 24 or 48 h. Cells were initially stained with annexin V-allophycocyanin to exclude apoptotic cells. Expression profiles show the MFIs of B7-1 (filled circles) and B7-2 (open circles) expression on B220+ (A and B), CD11b+ (C and D), CD11c+ (E and F) cells, 24 h (A, C, and E) or 48 h (B, D, and F) after stimulation with the *Salmonella* vaccine strain. Results are representative of three separate experiments.
mice exhibited significantly lower plasma IgG anti-Salmonella responses than wt mice (P < 0.05), whereas the levels of plasma IgG anti-Salmonella antibody activity in B7-2 KO mice were similar to those observed for wt mice (Fig. 2B). No plasma IgG anti-Salmonella response was detected in the B7-1/2 DKO mice, even after a second immunization. These results demonstrate that B7-1, but not B7-2, was required for the induction of wt levels of IgG anti-Salmonella antibody activity after two immunizations. Furthermore, our results demonstrate that the requirements for B7-1 or B7-2 for the induction of plasma IgG anti-Salmonella responses vary based on a primary or secondary immunization with an attenuated Salmonella strain.

To investigate if a lack in either B7-1 or B7-2 modulates Th1- and Th2-associated immune responses to the Salmonella vector, we assessed the levels of anti-Salmonella IgG1, IgG2a, and IgG2b antibodies following the secondary immunization. B7-1 KO mice had significantly lower IgG1 (P < 0.001) (Fig. 3A), IgG2a (P < 0.05) (Fig. 3B), and IgG2b (P < 0.001) (Fig. 3C) anti-Salmonella antibody levels compared to those observed with wt mice. An analysis of IgG subclass responses to Salmonella in B7-2 KO mice revealed lower IgG1 (P < 0.001) and IgG2b (P < 0.05) levels, whereas the mean level of IgG2a anti-Salmonella antibody activity was higher but not significantly different from that observed with wt mice. No IgG1, IgG2a, or IgG2b anti-Salmonella antibody activity was observed with B7-1/2 DKO mice (Fig. 3). These results suggest that B7-1 influences both Th1- and Th2-associated IgG subclass responses to Salmonella, while the lack of B7-2 appears to selectively suppress Th2-type IgG subclass responses.

Functional requirements of B7-1, B7-2, and B7-1/2 for systemic antibody responses to the cloned antigen expressed by S. enterica serovar Typhimurium. We next investigated the roles of B7-1 and B7-2 in systemic antibody responses to the cloned antigen, SBR, following one or two immunizations. Following the first immunization, B7-2 KO mice had significantly lower levels of plasma IgG anti-SBR antibodies than those observed with wt mice (P < 0.05) (Fig. 4A). Moreover, while the mean level of IgG anti-SBR antibody activity was lower in B7-1 KO mice than that seen with wt mice, the difference was not statistically significant (Fig. 4A). Following the second immunization, B7-1 KO mice had a similar level of IgG anti-SBR antibody activity as that seen with the wt mice (Fig. 4B). Although the mean level of IgG anti-SBR activity observed for B7-2 KO mice was approximately 30% lower than for wt mice following the second immunization, the difference was not significantly different (Fig. 4). B7-1/2 DKO mice failed to produce any significant plasma IgG anti-SBR responses following the first (Fig. 4A) or second (Fig. 4B) immunization, compared to sham-immunized controls.

The relative contribution of B7-1 and B7-2 in modulating the IgG subclass responses to SBR was also evaluated after the second immunization. No significant difference was observed between the B7-1 KO and wt mice in the levels of anti-SBR IgG1, IgG2a, or IgG2b antibody responses (Fig. 5). In contrast, B7-2 KO mice exhibited significantly lower IgG1 (P < 0.05) (Fig. 5A) and IgG2b (P < 0.05) (Fig. 5B) anti-SBR responses, but not IgG2a anti-SBR responses, than wt mice. These results suggest that B7-1 and B7-2 are compensatory for Th1-associated IgG subclass responses, whereas B7-2 is important for the

FIG. 2. Effect of B7-1 and B7-2 on plasma IgG responses to Salmonella following mucosal immunizations. BALB/c mice (six per group) were immunized on days 0 and 18 by the i.n. route with 5 × 10⁹ CFU of Salmonella BRD509 expressing SBR. Salmonella-specific plasma IgG responses were analyzed 10 days after primary (A) and secondary (B) immunizations. Results are the arithmetic means ± standard errors of the mean (SEM) of results for six mice per group. The significances of differences between results for the wt and other groups of mice are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Sham, wt control mice receiving i.n. PBS only.

Typhimurium. The contributions of the B7-1 and B7-2 co-stimulatory molecules in the generation of systemic antibody responses to the attenuated Salmonella BRD509 vaccine strain following primary and secondary i.n. immunizations were determined by using wt, B7-1 KO, B7-2 KO, and B7-1/2 DKO mice. Mice deficient in either B7-1 or B7-2 had significantly lower levels of Salmonella-specific IgG antibody activity than wt mice following the primary immunization (P < 0.01) (Fig. 2A). Essentially no increase in the level of IgG anti-Salmonella antibody activity was observed with B7-1/2 DKO mice compared to the sham-immunized controls. These results indicate a critical role for both B7-1 and B7-2 in the induction of systemic immune responses following a single i.n. immunization with an attenuated S. enterica serovar Typhimurium strain.

In contrast to the results obtained after a single immunization with the Salmonella BRD509, a different response pattern was seen following a second immunization (Fig. 2B). B7-1 KO
generation of Th2-associated IgG subclass immune responses to the cloned antigen SBR.

Functional requirements of B7-1, B7-2, and B7-1/2 in mucosal antibody responses to both the cloned antigen and *Salmonella enterica* serovar *Typhimurium*. In order to determine the requirements for B7-1 and B7-2 for mucosal responses to the *Salmonella* and its heterologous cloned antigen, we next examined the influence of these costimulatory molecules in generating mucosal IgA responses in salivary and vaginal secretions of immunized mice. No detectable salivary or vaginal IgA anti-*Salmonella* antibodies were observed after the primary immunization in any immunized group (data not shown). After the second immunization, B7-1 KO mice had significantly lower levels of salivary (*P* < 0.001) (Fig. 6A) and vaginal (*P* < 0.05) (Fig. 6B) IgA anti-*Salmonella* antibody activity than wt mice. No significant difference was observed between B7-2 KO and wt mice in the levels of salivary (Fig. 6A)
and vaginal (Fig. 6B) IgA anti-\textit{Salmonella} antibody activity. In B7-1/2 DKO mice, no detectable salivary or vaginal IgA responses to \textit{Salmonella} following mucosal immunizations. Taken together, these data indicate that B7-1 plays a nonredundant role for inducing IgA anti-\textit{Salmonella} antibody responses and cannot be completely compensated for by the presence of B7-2.

Examination of the SBR-specific mucosal antibody responses in wt and B7 KO mice revealed that B7-1 KO and B7-2 KO mice were able to generate both salivary (Fig. 7A) and vaginal (Fig. 7B) IgA responses to SBR that were not signifi-

FIG. 5. Role of B7-1 and B7-2 in the induction of plasma IgG subclass responses specific for the cloned SBR expressed by \textit{Salmonella} following mucosal immunizations. SBR-specific plasma IgG1 (A), IgG2a (B), and IgG2b (C) responses 10 days following the secondary immunization of mice on day 18 with $5 \times 10^5$ CFU of \textit{Salmonella} BRD509 expressing SBR. Results represent the arithmetic mean \pm SEM of results for six mice per group. The significances of differences between results for the wt and other groups of mice are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Sham, wt control mice receiving i.n. PBS only.

FIG. 6. Requirements of B7-1 and B7-2 for the induction of mucosal IgA responses specific for \textit{Salmonella} following mucosal immunizations. \textit{Salmonella}-specific salivary (A) and vaginal (B) IgA responses 10 days following the second immunization of mice on day 18 with $5 \times 10^5$ CFU of \textit{Salmonella} BRD509 expressing SBR. Results represent the arithmetic mean \pm SEM of results for six mice per group. The significances of differences between results for the wt and other groups of mice are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Sham, wt control mice receiving i.n. PBS only.

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cantly different from those seen with wt mice. In contrast, B7-1/2 DKO mice exhibited no detectable salivary or vaginal IgA anti-SBR antibody activity. These results suggest that B7-1 and B7-2 can compensate for the generation of mucosal salivary and vaginal IgA responses to the cloned antigen in mucosal secretions.

DISCUSSION

The present study provides evidence for the importance of B7-1 and B7-2 costimulatory molecules in mediating both mucosal and systemic antibody responses after mucosal immunization with attenuated *Salmonella* serovar Typhimurium. Interestingly, while the humoral immune responses against SBR and *Salmonella* were strictly dependent upon the presence of B7 costimulatory molecules, the roles of the two isoforms of B7 differed in the ability to mediate antibody responses to the attenuated *Salmonella* and its cloned antigen.

Previous studies have shown that the B7-CD28 signaling pathway is critical for the formation of germinal centers and isotype switching (2). Moreover, studies assessing the individual roles of B7-1 and B7-2 costimulatory molecules have demonstrated that both the route of immunization and the type of adjuvant used can play a critical role in influencing antigen-specific humoral immune responses following parenteral immunization (2). Borriello et al. (2) have shown that B7-2 KO mice exhibited a significant reduction in T-dependent immune responses when immunized by the intraperitoneal route with antigen and the adjuvant alum. However, similar humoral responses were observed in B7-2 KO mice and wt mice following subcutaneous immunization (2). Additionally, when B7-2 KO mice were immunized with complete Freund’s adjuvant either by the intraperitoneal or footpad route, no difference in the antibody response was observed between wt and B7-2 KO mice (2). Thus, these past findings demonstrate that the functional role of B7-2 in mediating humoral immune responses appears to be dependent upon both the route of immunization and the type of adjuvant used. In contrast, other previous studies have shown that B7-1 KO mice exhibit humoral immune responses similar to those of wt control mice, regardless of the adjuvant or parenteral route used for immunization (2). The present study extends these findings by demonstrating that upon mucosal immunization, both B7-1 and B7-2 are required to mount wt levels of antibody responses to both the attenuated *Salmonella* and its cloned antigen after the primary immunization. However, the strict dependency of both isoforms of B7 needed to mediate antibody responses to *Salmonella* and SBR differed after the secondary immunization. Specifically, while B7-1 and B7-2 appeared to function in a redundant manner in potentiating mucosal and systemic anti-SBR antibody responses after the secondary immunization, B7-1 KO mice exhibited a significant reduction in mucosal IgA and systemic IgG anti-*Salmonella* responses compared to wt mice. Taken together, these findings demonstrate that the route of immunization and type of adjuvant are not the only critical factors in determining the role of the B7 isoforms, but that the nature of the antigen also appears to be a key determinant in defining the functional ability of B7-1 and B7-2 to mediate humoral immune responses in both the systemic and the mucosal compartments.

Past studies assessing the humoral immune response to both wt and attenuated strains of *Salmonella* have shown that signaling via CD28 was absolutely required to mediate T-dependent anti-*Salmonella* antibody responses (15, 17). The present study extends these observations by providing evidence that both the mucosal and systemic humoral immune responses to *Salmonella* are completely dependent on B7 costimulatory molecules after mucosal immunization. Moreover, while the present study demonstrates that both B7-1 KO and B7-2 KO mice were able to mount detectable levels of *Salmonella*-specific antibody responses, a direct comparison between B7-1 KO
and B7-2 KO mice revealed that mice lacking B7-1 exhibited a significant reduction in both mucosal and systemic antibody responses compared to wt or B7-2 KO mice. Moreover, analysis of B7-1 and B7-2 expression on B cells, macrophages, and dendritic cells stimulated with *Salmonella* demonstrated a prominent increase in the levels of expression of B7-1 compared to those of B7-2. These findings suggest that the ability of *Salmonella* to preferentially increase B7-1 expression on APC, in part, subsequently dictates the magnitude of the humoral immune response to *Salmonella* in a B7-1-dependent manner. Indeed, past studies assessing the functional role of the B7-1 and B7-2 costimulatory molecules in mediating the immunogenic properties of mucosally applied antigens have shown a strong correlation between the ability of the antigen to selectively up-regulate B7-1 or B7-2 on APC and the preferential role the up-regulated costimulatory molecule plays in mediating systemic and mucosal antibody responses to the antigen (3, 14).

The role of B7 costimulatory molecules in influencing Th-associated humoral responses is well documented (10–13,18). However, the functional ability of B7-1 or B7-2 to selectively influence Th2 (IgG1) and Th1 (IgG2a)-associated antibody responses remains an unresolved issue. Initial studies assessing the in vivo role of B7-1 and B7-2 in mediating Th1- and Th2-type antibody responses demonstrated a preferential role for B7-1 and B7-2 in activating Th1- and Th2-type humoral responses, respectively (10). However, other studies have suggested that B7-1 and B7-2 molecules exhibit redundant or overlapping roles for the generation of IgG subclass responses (11). It was subsequently shown with B7-2 KO mice that B7-2 could influence both Th1- and Th2-dependent IgG subclass responses (2). Our present findings regarding the IgG1 and IgG2a antibody responses to the attenuated *Salmonella* and its cloned antigen in B7-1 KO and B7-2 KO mice demonstrate no strict dependency on these costimulatory molecules for selectively dictating Th1- or Th2-associated humoral responses. In this regard, analysis of IgG1 and IgG2a anti-*Salmonella* responses revealed that mice lacking B7-1 had significantly lower levels of IgG1 and IgG2a than wt mice. These results indicate that B7-1 could influence the induction of both Th1- and Th2-type antibody responses. In contrast, an assessment of T-dependent IgG subclass responses in B7-2 KO mice demonstrated that while the levels of anti-*Salmonella* and anti-SBR IgG2a were similar to those observed with wt mice, the levels of IgG1 against either antigen were significantly reduced. These findings demonstrate that while B7-1 mediates both Th1- and Th2-type IgG subclass responses to *Salmonella*, B7-2 preferentially affects the levels of IgG1 against *Salmonella* and its cloned antigen.

In summary, we provide evidence for a strict dependency on the B7 costimulatory molecules in mediating mucosal and systemic antibody responses to both the attenuated *Salmonella* and its expressed cloned antigen. Additionally, our findings demonstrate that the functional ability of B7-1 and B7-2 to mediate unique as well as redundant roles in potentiating humoral immune responses to mucosally applied antigens can differ depending upon the nature of the antigen. This study provides new insight into the cellular mechanisms mediating humoral immune responses following mucosal immunization.

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