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Strong Differential Regulation of Serum and Mucosal IgA Responses as Revealed in CD28-Deficient Mice Using Cholera Toxin Adjuvant

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In this study, we show that costimulation required for mucosal IgA responses is strikingly different from that needed for systemic responses, including serum IgA. Following oral immunization with cholera toxin (CT) adjuvant we found that whereas CTLA4-Hy1 transgenic mice largely failed to respond, CD28−/− mice developed near normal gut mucosal IgA responses but poor serum Ab responses. The local IgA response was functional in that strong antitoxic protection developed in CT-immunized CD28−/− mice. This was in spite of the fact that no germinal centers (GC) were observed in the Peyer’s patches, spleen, or other peripheral lymph nodes. Moreover, significant somatic hypermutation was found in isolated IgA plasma cells from gut lamina propria of CD28−/− mice. Thus, differentiation to functional gut mucosal IgA responses against T cell-dependent Ags does not require signaling through CD28 and can be independent of GC formations and isotype-switching in Peyer’s patches. By contrast, serum IgA responses, similar to IgG-responses, are dependent on GC and CD28. However, both local and systemic responses are impaired in CTLA4-Hy1 transgenic mice, indicating that mucosal IgA responses are dependent on the B7-family ligands, but require signaling via CTLA4 or more likely a third related receptor. Therefore, T-B cell interactions leading to mucosal as opposed to serum IgA responses are uniquely regulated and appear to represent separate events. Although CT is known to strongly up-regulate B7-molecules, we have demonstrated that it acts as a potent mucosal adjuvant in the absence of CD28, suggesting that alternative costimulatory pathways are involved. The Journal of Immunology, 2003, 170: 55–63.

Protection against pathogenic microorganisms at mucosal surfaces is largely dependent on the production of secretory IgA (1). Gut mucosal IgA immunity is believed to be best stimulated by oral delivery of Ag (1, 2). However, most protein Ags given orally fail to stimulate immunity, but rather induce oral tolerance, unless appropriate adjuvants are coadministered (3). To improve and develop oral vaccines for mucosal immunization, a better knowledge about adjuvants and the regulatory mechanisms required for activation and differentiation of Ag-specific IgA B cells in the gut mucosal immune system is greatly needed.

It is widely held that induction of specific B cell responses, isotype-switch, somatic hypermutation, and memory development is largely dependent on germinal center (GC) formation in organized lymphoid tissues (4–6). Several cell-cell interactions have been found to be important for the formation of GC. Except for the first step of Ag-specific receptor binding, costimulation through CD80/86–CD28, OX40L–OX40, and B7RP-1-inducible costimulator (ICOS) as well as through CD40–CD40 ligand (CD40L) are critical events in the development of the GC reaction (7–11). Thus, mice that are deficient in any of these components lack or have impaired GC reactions in spleen or peripheral lymph nodes following immunization with T cell-dependent (TD) Ag (7, 8, 12, 13).

For a long time it has been considered a fact that Peyer’s patches (PP) are the inductive sites for gut mucosal IgA responses and a strict clonality between plasma cells in PP and the gut lamina propria (LP) has been documented (14, 15). It is thought that IgA B cell differentiation in PP is dependent on CD4+ T cell help and requires Th2 cytokines such as TGFβ for switch differentiation, while IL-10, and in particular, IL-5 and IL-6 are important for expansion of already committed IgA B cells. Also, signaling via CD40 on the B cell surface has been found to play an important role in IgA B cell differentiation (16).

We and others have reported that mucosal IgA responses, in several ways, appear to be differently regulated from systemic IgG responses. Thus, the induction of specific mucosal IgA immunity requires IL-4, whereas systemic IgG responses can develop in the absence of IL-4 (17). We have also found that IgA-differentiated B cells in the gut LP produce large quantities of total IgA despite the absence of GC formation in the PP, as seen in IL-4−/− (17), TNFRα−/− (18), and CD40−/− mice (E. Gärdbry, unpublished observations).

Studies that we undertook in CTLA4-Hy1 transgenic (Tg) mice that stably express the CTLA4-Hy1 protein which binds to CD80 or CD86 on the APC/B cell side, thereby blocking the costimulation pathway, revealed a paradox in the regulation of mucosal IgA responses. These mice exhibited GC formations in PP, but not in the spleen or peripheral lymph nodes. In contrast to the impaired

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total IgG levels in serum, total IgA production in the gut was normal compared with wild-type (WT) mice (19, 20). However, despite the seemingly unaffected IgA-inductive sites and the normal total IgA levels in these mice, they responded poorly to oral immunizations given together with cholera toxin (CT) adjuvant. Thus, the CTLA4-Hy1 Tg exhibited greatly impaired specific systemic as well as gut mucosal IgA responses following oral immunization, indicating that induction of gut immunity against TD Ags critically requires CD80/86 costimulation (21).

We used the potent adjuvant and immunogen CT that enhances humoral immunity and is necessary for induction of mucosal IgA responses against protein Ags. However, the mechanisms behind its adjuvanticity is not fully understood. Both direct effects on the B cell as well as indirect effects via enhancement of CD4+ T cell priming has been observed (22). One possible effect of CT is the up-regulation of CD80/86 on APC/B cells that could lead to enhanced activation of CD4+ T cells via costimulation through CD28 (23, 24). However, we found that CT was able to enhance humoral responses despite the block in the costimulatory pathway in CTLA4-Hy1 Tg mice, suggesting that other important mechanisms were stimulated by CT (21). Alternatively, it could be a result of incomplete blocking of the rapid up-regulation of CD80 and CD86 after exposure to CT in CTLA4-Hy1 Tg mice. Nonetheless, apart from CD28-signaling, which is required for induction of high-level IL-2 production, clonal proliferation, and the prevention of anergy, an expanding family of alternative costimulatory molecules have been shown to play an important role in different stages of T cell activation/differentiation and in promoting development of effector functions (25–27).

CD28-deficient mice were found to lack GC in the spleen and peripheral lymph nodes and poorly responded to TD Ag, with defects in isotype switching and no evidence of somatic hypermutation in clonally expanded B cells (12). The aim of the present study in CD28−/− mice was to investigate whether the paradoxical results in the CTLA4-Hy1 Tg mice were truly CD28-dependent or due to an incomplete blocking of CD80/86 in the gut-associated lymphoid tissues (GALT). Moreover, the seemingly poor relationship between the presence of GC in PP and specific IgA B cells in the gut LP observed in previous studies was reanalyzed and the dependence on CD28 for somatic hypermutation and development of functional protective IgA immunity was studied. Finally, we examined the adjuvant ability of CT in mice lacking CD28-signaling properties.

Materials and Methods

Animals

CD28−/− (28), CTLA4-Hy1 Tg mice (20), and C57BL/6 WT mice were bred under pathogen-free conditions using microisolator cages and sterile work benches at the Department of Medical Microbiology and Immunology (University of Göteborg, Sweden). Breeding pairs were kindly provided by T. Mak (Toronto, Ontario, Canada) (CD28/H9253); University of Birmingham, U.K.) (CTLA4-H11002). The genotype of the H11002 mice was confirmed by PCR using appropriate primers, as previously described (19, 28). All experiments were performed with sex-matched, 8–12-wk-old mice. All animal studies have been approved by the Institutional Review Board.

Immunizations

Mice were given three oral immunizations at 10 days apart with keyhole limpet hemocyanin (KLH) (Sigma-Aldrich, St. Louis, MO) at 2.0 mg/dose in the presence or absence of 10 μg/dose of CT (List Biological Laboratories, Campbell, CA) (29). Six to 8 days following the final immunization, mice were sacrificed and the immune responses were analyzed. I.p. immunizations were performed twice at 10 days apart with DNP-KLH (Boehringer Mannheim, Indianapolis, IN) at 200 μg/dose in the absence or presence of 1 μg/dose CT (List Biological Laboratories).

Preparation of lymphoid cells

LP lymphocytes were prepared as described (30). Briefly, the small intestines were removed and the PF were carefully excised and discarded. The intestines were opened up, cut in small pieces, and washed three times in calcium- and magnesium-free HBSS (CMF-HBSS; Life Technologies, Paisley, U.K.), supplemented with 25 mM EDTA (Merck, Darmstadt, Germany) and 10% heat-inactivated horse serum (Life Technologies). After each incubation, the supernatant containing the sloughed epithelial cells and the intraepithelial lymphocytes were discarded. After four incubations, the epithelium was removed and the supernatants were clear. To block any remaining EDTA activity, the tissue pieces were incubated for 15 min with RPMI 1640 (Flow Laboratories, Irvine, U.K.) containing 25 mM HEPES and 10% inactivated horse serum. Next, the tissue pieces were incubated three times for 60 min with collagenase (120 IU/ml; Sigma-Aldrich) dissolved in RPMI 1640 containing 25 mM HEPES and 20% inactivated horse serum (Life Technologies). After each incubation, the suspension of cells were centrifuged, washed, and centrifuged and stored in IMDM containing 10% FCS. Spleen lymphoid cells were prepared by teasing the tissue through a nylon screen. RBCs were lysed by osmotic shock and single-cell suspensions were prepared and washed twice in CMF-HBSS (Life Technologies) containing 10% FCS (Life Technologies).

Ligated loop test

For evaluation of protection against CT-induced diarrhoea/fuid loss, the method described by Lange and Holmgren (31) was used. Briefly, the abdomen was opened under light ether anesthesia, and a 6- to 8-cm loop was ligated in the middle part of the small intestine. CT (List Biological Laboratories) 2.5 μg in 0.2 ml of PBS, was injected into the loop, and the abdomen was closed. After 4 h the mice were sacrificed, whereafter the loop with its fluid content was weighed and its length was determined. Unimmunized mice of both strains were equally sensitive to CT injected in ligated small intestinal loops. Values for fluid accumulation in the ligated loops, reflecting the degree of immune protection, were expressed as the weight per length ratio in milligrams per centimeter ± SD of 5–7 mice per group.

ELISPOT assay

LP lymphocytes were analyzed for Ag-specific or total IgA Ab production at the single-cell level (spot-forming cell; SFC) using the ELISPOT assay (32). For the Ag-specific analyses, petri dishes were coated with KLH (Sigma-Aldrich) at 100 μg/ml or 3 nmol/ml ganglioside GM1 (Sigma-Aldrich) followed by 3 μg/ml CT (List Biological Laboratories). Lymphocytes in complete IMDM were added at 1 × 106 cells/ml, for 30 min in 0.2 ml of PBS, and 400 μl/well, in duplicates to coated petri dishes after blocking with 0.2% BSA/PBS. Cells were allowed to incubate for 2.5–4 h at 37°C. Petri dish SFC were visualized using a two-step procedure involving a first incubation at 4°C overnight with goat anti-mouse IgG (Cappel; Organon Teknika, West Chester, PA) followed by a second HRP-labeled rabbit anti-goat Ig (DAKO, Glostrup, Denmark) both at 1/200 dilution for 2 h at room temperature. Total Ig SFC in splenic cells were detected by sequential incubations of HRP-labeled rabbit anti-mouse Ig (DAKO) followed by HRP-labeled anti-rabbit Ig (DAKO). The SFC reaction was visualized by adding the HRP substrate: paraphenylendiamine at 0.5 mg/ml and 0.01% H2O2 in 0.01% agar in PBS.

Intestinal lavage samples

Intestinal secretions for Ab determinations were collected as described in detail (33). Briefly, the small intestines were taken out, rinsed in PBS, and carefully injected with 1.8 ml of a protease inhibitor solution consisting of 0.1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich), 50 mM EDTA (Sigma-Aldrich), and 1 mM PMSF (Boehringer Mannheim) in PBS. After incubation for 10 min at room temperature, the intestinal content was transferred to a test tube, vigorously vortexed, sonicated, centrifuged, and transferred to a microfuge tube with PMSF at a final concentration of 1 mM and 5% FCS. The supernatants were stored at −70°C until analyzed.

Serum and gut lavage ELISA

Total or specific Ig concentrations were determined by ELISA (33, 34). Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with unlabeled rabbit anti-mouse IgG1, IgG2a, IgG3, IgM
(Southern Biotechnology Associates, Birmingham, AL), or purified monoclonal IgA (BD PharMingen, San Diego, CA) Abs at 5 μg/ml. For Ab-specific Ab, plates were coated with KLH (Sigma-Aldrich) at 100 μg/ml or GM1 ganglioside (Sigma-Aldrich) at 0.5 nmol/ml followed by CT (List Biological Laboratories) at 0.5 μg/ml. Serum was diluted at 1/100 or 1/200 and incubated at 4°C for 2 or 4 h, respectively, and washed three times in corresponding subwells and incubated overnight at 4°C. The following day, alkaline phosphatase-conjugated isotype-specific rabbit anti-mouse Abs (Southern Biotechnology Associates) at 1/300 dilution were added to the wells. Nitrophenyl-phosphatase substrates tablets (Sigma-Aldrich) at 1 mg/ml in ethanolamine buffer (pH 9.6) were used to visualize the alkaline phosphatase-labeled Abs. The enzymatic reactions were read at 405 nm using a Titertek Multiscan MS spectrophotometer (Labsystems, Stockholm, Sweden). Ab titers were defined as the interpolated OD reading giving rise to an absorbance of 0.4 above background, which consistently gave OD readings on the linear part of the curve. Titers were given as log10 titers ≥ SD.

**Immunohistochemistry**

Frozen sections (5 μm) of gut mucosa from naive or immunized mice were prepared on microslides using a cryostat (model 1720; Leitz, Wetzlar, Germany). The sections were stored at −70°C and transferred directly to 50% acetone in H2O for 30 s, followed by fixation in 100% acetone for 5 min after which they were washed in PBS twice for 5 min. After washing, the sections were incubated with horse serum 20% in PBS for 15 min in a humidified box at room temperature to avoid unspecific binding to the tissue section. Detection of GC reactions and IgA containing cells in PP or mesenteric lymph nodes (MLN) was achieved by single or double labeling with FITC-conjugated peanut (Arachis hypogaea) hemagglutinin (PNA; Sigma-Aldrich) and/or biotin-labeled rat anti-mouse IgA mAb (BD Pharmingen) followed by Texas Red-conjugated streptavidin (Sera-Lab, Sussex, U.K.). The slides were mounted in Fluorescent mounting media (DAKO), evaluated, and photographed using a Leica DMLD (Leica, Wetzlar, Germany).

**Mutational analysis**

LP lymphocytes from WT and CD28−/− mice were prepared as above and used for purification of IgA+ plasma cells by MACS enrichment using biotinylated goat anti-IgA Abs (Southern Biotechnology Associates) and streptavidin-coated beads. The highly enriched gut IgA+ plasma cells were ≥95% pure, as assessed by FACS analysis and contained no other surface Ig+ cells (i.e., <1%). Single-cell suspensions of C57BL/6 splenocytes were prepared as above and B220 (RA3-6B2) positive cells were enriched by MACS to 98% purity as determined by FACS. DNA from the IgA+ B220+ populations was prepared using TRizol (Life Technologies) and used as a template for the subsequent PCR. Total mutational load in IgA+ B220+ cells from either WT or CD28−/− mice was compared using the strategy developed by Jolly et al. (35), analyzing intronic sequences from the J558 VH family. The splenic B220+ cells served as a negative control for the experiment. Amplifications were performed using a polymerase mix containing a proofreading activity (Expand High Fidelity System; Roche, Basel, Switzerland). Amplified material was blunt end cloned into pGEM-3Z cut with SmaI, sequenced, and analyzed using the computer software VectorNTI (Informax, Bethesda, MD).

**Statistical analysis**

We used Student’s t test for independent samples for analysis of significance.

**Results**

**Unaltered total gut IgA production despite lack of GC in the PP**

Our previous studies in CTLA4-H1 Tg mice showed a paradox in the regulation of gut mucosal IgA immunity (21). We observed that the block in CD80/86 costimulation resulted in severely impaired specific mucosal and serum IgA responses following oral immunizations, despite seemingly normal IgA-inductive sites in PP and normal levels of total gut and serum IgA (21). In this report, we have extended our studies to CD28−/− mice. In contrast to CTLA4-H1 Tg mice, naive CD28−/− mice exhibited no GC in PP and greatly reduced levels of serum IgA (Fig. 1). Despite this, CD28−/− mice had almost normal levels of total gut IgA in lavage and IgA+ plasma cells in the LP (Fig. 1). Serum IgM was unaltered, whereas IgG1 and IgG2a Abs were substantially reduced compared with WT mice (Fig. 1). Thus, IgA production in the gut LP can be independent of CD28 signaling, whereas serum IgA appears to be dependent on this pathway, suggesting a differential regulation of mucosal and systemic IgA responses. Furthermore, GC in the PP were not required for IgA production in the gut LP, indicating that the mucosal IgA B cell progeny can be derived from sites other than PP.

**Strong gut mucosal IgA responses to oral immunization in CD28-deficient mice**

Next, we analyzed the IgA response to an oral immunization with KLH and CT adjuvant in CD28−/− mice and compared the result with that of CTLA4-H1 Tg and WT mice. Unexpectedly, and in contrast to the poor IgA response in CTLA4-H1 Tg mice, CD28−/− mice demonstrated near normal gut mucosal IgA SFC responses to both KLH and CT (Fig. 2). Particularly, the response to the strong mucosal immunogen CT differed significantly between CD28−/− and CTLA4-H1 Tg mice, with >95% reduced, or even undetectable, anti-CT IgA SFC activity in the latter strain, while the specific gut IgA LP response in CD28−/− mice was not significantly different from that of WT mice (Fig. 2). Therefore, the ability to respond to KLH or CT after oral immunization appeared to be independent of CD28 signaling, but blocked by CTLA4-H1 protein, as seen in the Tg animals. Moreover, we found no correlation between a specific IgA response in the gut LP and the presence or absence of GC and IgA+ B cells in the PP, since the CTLA4-H1 Tg mice had GC in PP, but could not respond (21), whereas immunized CD28−/− mice had no GC in PP or MLN (data not shown), and responded normally to an oral immunization (Fig. 2E). Of note, also other peripheral lymph nodes and the spleen were lacking GC formations following both oral and systemic immunizations, which agrees well with earlier reports using CD28−/− mice (12).

To prove that the specific gut mucosal IgA response was dependent on a CTLA4-H1-blockable costimulatory pathway and independent of CD28 signaling, we extended our analysis to include CD28−/− mice that did or did not express the CTLA4-H1 Tg. By cross and intercross breeding we obtained CTLA4-H1 Tg positive or negative CD28−/− mice that were given oral immunizations with CT. We found that the presence of the CTLA4-H1 Tg greatly impaired the gut IgA anti-CT SFC response also in CD28−/− mice (Fig. 2F). Thus, this result corroborated the data in WT mice, and clearly demonstrated the presence of an alternative, CD28-independent, costimulatory pathway in the gut mucosal immune system.

**Evidence of a differential regulation of local mucosal and systemic IgA immunity**

Whereas the local IgA response, especially that against CT, was clearly independent of the CD28-signaling pathway, we investigated whether this was also the case for systemic responses following oral or i.p. immunizations with CT. However, contrary to the gut IgA responses, both CD28−/− and CTLA4-H1 Tg mice exhibited poor or no specific SFC responses in the spleen following either oral or i.p. immunizations, as illustrated in Fig. 3. Likewise, serum anti-CT Ab responses, including IgA, were also dramatically impaired after oral (Fig. 4) or i.p. immunizations (data not shown). Thus, CD28 signaling was clearly required for systemic immunity, including serum IgA, whereas mucosal IgA responses appeared to be independent of signaling through the B7-CD28 pathway.
**FIGURE 1.** Intact gut mucosal IgA production while serum IgA levels are low in CD28<sup>−/−</sup> mice. The total IgA concentration in serum (A) and in gut lavage (B) was determined in CD28<sup>−/−</sup> (◻) or WT (◼) mice. A. For comparison total serum IgG1, IgG2a, and IgM were determined in both strains. The bars represent mean values and are given as log<sub>10</sub> titers (SEM). B. The frequency of IgA<sup>+</sup> cells/visual field with 4–5 villi was 28 ± 4 in CD28<sup>−/−</sup> and 32 ± 5 in WT mice. Frozen sections of gut tissue from WT (D) or CD28<sup>−/−</sup> (E) mice were double-labeled with PNA-FITC (green) and anti-IgA Texas Red Abs (red), showing the complete absence of GC and IgA<sup>+</sup> cells in PP of CD28<sup>−/−</sup> mice. Frozen sections are representative of four separate experiments given similar results with 3–4 mice in each group. *, Significance, p < 0.05.

**Gut LP IgA<sup>+</sup> cells from CD28<sup>−/−</sup> mice are modified by somatic hypermutations**

Given the ability to raise near normal specific gut IgA responses in CD28<sup>−/−</sup> mice, despite the complete absence of GC in the PP or MLN, we analyzed whether gut IgA LP plasma cells were modified by somatic hypermutations following oral immunizations. After extensive cell sorting using MACS technology to obtain highly pure IgA<sup>+</sup> plasma cells (36) from the gut LP of immunized WT and CD28<sup>−/−</sup> mice, we used the strategy developed by Jolly et al. (35) to analyze the presence or absence of hypermutation. This method uses the fact that mutations are found extending into the J-C intron. Thus, by using a degenerate V gene primer, recognizing all V genes of the J558 V gene family, together with a primer hybridizing in the intronic enhancer region, a global analysis, irrespective of specific V gene usage, can be performed. By this approach we found that IgA<sup>+</sup> plasma cells in the LP of the CD28<sup>−/−</sup> mice carried modifications by somatic hypermutation (Fig. 5). Although the frequency of mutation (1/300 bp) was reduced compared with the WT mice (1/80 bp), the mutational load of the CD28<sup>−/−</sup> plasma cells were clearly above the background PCR error rate, as shown by the simultaneous analysis of naive B cells (1/2500). Thus, despite the absence of GC, gut IgA<sup>+</sup> plasma cells in CD28<sup>−/−</sup> mice were modified by somatic hypermutation, a characteristic of B cells undergoing maturation processes normally associated with clonal expansion and differentiation in GC in the GALT in WT mice.

**Gut IgA responses in the CD28<sup>−/−</sup> mice are functionally normal**

Somatic hypermutations in CD28<sup>−/−</sup> gut IgA<sup>+</sup> cells were clearly found, but whether these mutations also represented differentiation and maturation of specific IgA B cell clones following oral immunization was unclear. Therefore, we orally immunized CD28<sup>−/−</sup> and WT mice with CT and subsequently subjected them to an intestinal ligated loop test to assess whether their gut IgA responses were functional and could neutralize toxin in an in vivo challenge model (31). As illustrated in Fig. 6, we found strong and comparable antitoxic protection in both CD28<sup>−/−</sup> and WT mice, indicating that gut IgA B cell clones in CD28<sup>−/−</sup> mice could both expand and mature to produce functionally relevant mucosal IgA Abs. Immunized mice from both strains resisted an intestinal challenge with CT and showed >75% protection compared with unimmunized control mice (Fig. 6).

**The adjuvant effect of CT can be independent of CD28 signaling**

In previous studies we have demonstrated that CT dramatically increases expression of both CD80 and CD86 on B cells (24) and others have reported similar findings in macrophages and dendritic cells (23, 37). Accordingly, it has been proposed that CT exerts much of its adjuvant function through enhancing costimulation,
and especially signaling through the B7-CD28 pathway (24, 38, 39). However, in the present study, we unexpectedly found that CT greatly augmented mucosal IgA anti-KLH responses in CD28−/− mice and that the IgA responses appeared to be functionally normal. Extending the analysis, we determined the enhancing effect of admixed CT on specific serum Ab responses following i.p immunizations with DNP-KLH. We found consistently increased DNP-specific serum titers when CT adjuvant was used (Fig. 7). As expected, responses to DNP-KLH of all isotypes were much lower in CD28−/− compared with WT mice, but the enhancing effect of CT was comparable (Fig. 7 A). Calculated as a relative change, CD28−/− and WT mice had comparable increases in serum specific titers, as a function of CT adjuvant (Fig. 7B). On average the specific serum titers were 70–90% higher in CT adjuvant-treated as opposed to nontreated mice. Thus, CT exerted both mucosal and systemic adjuvant function in the complete absence of CD28. The difference in responsiveness to mucosal as opposed to systemic immunizations with protein Ag rather reflected the degree of dependence on CD28, with mucosal IgA being relatively independent, whereas systemic/serum IgA responses are highly dependent on signaling through the CD28 pathway.

**Discussion**

The present study in CD28−/− and CTLA4-Hy1 Tg mice was undertaken to investigate the growing complexity of costimulation required for priming of immune responses at mucosal sites and for production of gut mucosal IgA in particular (40). The findings reported in this study provide new information on IgA B cell differentiation that help explain seemingly conflicting observations, made by us and other groups in various gene knockout models (17, 21, 41, 42). More specifically, a paradox in previous studies in CTLA4-Hy1-Tg-, IL-4-, and CD19-deficient mice is that systemic immunity, i.e., IgG production, was found dramatically impaired, although total gut IgA levels were largely unaltered. Moreover, whereas GC reactions traditionally have been tightly linked to systemic IgG B cell differentiation and affinity maturation, gut mucosal IgA responses showed poor correlation to the absence or presence of GC in PP in these models.

Although PP are considered prime inductive sites for gut IgA immunity, one possibility to accommodate some of the conflicting findings would be to consider also MLN and/or the recently described small isolated lymphoid follicles in the small intestine as important inductive sites for gut mucosal IgA (43–45). In addition,
the importance of these inductive sites for IgA responses against TD as opposed to T cell-independent (TI) Ags may differ (11, 21, 46, 47). Thus, in the absence of a specific response to oral immunization, the GC reactions in PP observed in CTLA4-Hy1-Tg and CD19/H/H mice would, according to such a model, reflect responses against the commensal flora of bacteria, mostly TI type of Ags, but reveal little about the ability or inability to respond to an oral immunization with TD Ags.

The present study clarifies several important issues with regard to the regulation of mucosal and systemic humoral immune responses against TD Ags. Firstly, CD28−/− mice demonstrate that in contrast to systemic IgG responses, gut IgA responses do not require GC formation and can be independent of signaling through the B7-CD28 pathway. Secondly, functional, i.e., fully protective, mucosal IgA Abs can develop in the complete absence of GC in the GALT or elsewhere. In addition, somatic hypermutations, commonly associated with the affinity maturation of an immune response, are found in gut IgA plasma cells independently of GC signaling.

**FIGURE 3.** Impaired systemic responses in CD28−/− and CTLA4-Hy1-Tg mice after oral as well as systemic immunizations. Mice were immunized orally (A and B) or i.p (C and D) with CT. Specific systemic responses in the spleen were recorded following a booster immunization. CT-specific responses are given as mean splenic total anti-CT SFC activity in CTLA4-Hy1-Tg or CD28−/− mice (□) or WT (■) mice, as indicated. The SFC activity is expressed as mean SFC values per 10⁷ cells ± SD from three pairs of mice per group. This is one representative experiment of four. *, Significance, p < 0.05.

**FIGURE 4.** Strongly impaired serum Ab responses to oral immunizations with CT in CD28−/− mice. Anti-CT serum isotype-specific titers are given as mean log₁₀ titers ± SD for six mice per group and three identical experiments. *, Significance, p < 0.05.

**FIGURE 5.** IgA+ plasma cells from gut LP of CD28−/− mice display modifications by somatic hypermutation in the absence of PP GC. Analysis of the levels of somatic hypermutation in IgA+ LP plasma cells from either WT or CD28−/− mice. DNA were prepared from MACS-enriched cells and used for amplification of Ig intronic sequences using a proofreading polymerase. Purified splenic B cells served as a negative control for the PCR. Each diagram represents the analysis of a 240-bp fragment from 30 individual clones and shows the number of mutations per sequence. The average frequency of mutation is indicated below each diagram.
giving similar results. The data are representative of two identical experiments. Fluid accumulation in response to challenge with CT, 2.5 μg/loop, was determined and compared with that observed in CT-challenged loops of unimmunized control mice. Values are given in milligrams per centimeter.

**FIGURE 6.** Intact antitoxic protection in gut mucosa following oral immunization with CT in CD28−/− mice. We determined functional antitoxic protective immunity in gut-ligated loops of perorally CT-immunized CD28−/− or WT mice. Three oral immunizations with 10 μg/dose of CT were given and 7 days following the final dose, mice were analyzed for antitoxic protection against CT-induced diarrhea by the ligated loop test. Fluid accumulation in response to challenge with CT, 2.5 μg/loop, was determined and compared with that observed in CT-challenged loops of unimmunized control mice. Values are given in milligrams per centimeter.

![unimmunized](unimmunized.png)  ![immunized](immunized.png)

As seen in this study as antitoxic protection, in the total absence GC and IgA switch differentiation in the PP in CD28−/− mice. Therefore, the question is where such events could take place in the GALT. Findings in lymphotixin β Ig-treated mice, which lack PP but have intact MLN, seemed to indicate that the MLN could be the alternative site for induction of TD Ag-specific gut IgA responses in the GALT (43). However, the gut antigenic IgA response and the lack of GC in MLN of immunized CD28−/− mice argues for additional sites where IgA switch differentiation and maturation can take place. A recent study has demonstrated that the gut mucosal LP can be the site for switch differentiation of B220IgM+ B cells to B220IgA+ (46). As there are no clear GC in the gut LP, the microenvironment itself may provide GC-like conditions, which was indicated by the strong IgA switch-promoting influence of gut stromal cells (46). Whether somatic hypermutation was induced in the gut LP B220IgM+ B cells was not investigated in that study, but the stromal cell-induced IgA switching was independent of CD40-CD40L interactions, suggesting TI Ag-stimulated responses and few, if any, somatic hypermutations (46). We found clear evidence of somatic hypermutation in isolated IgA+ plasma cells, which argues for a T cell CD40L-dependent process and thus, may not result from stromal cell interactions (46). It is yet to be identified how and where these gut LP IgA+ cells had undergone isotype switching and somatic hypermutation. Although we failed to detect GC in the gut LP of the CD28−/− mice (our unpublished observations), we cannot rule out that PNA+ cell clusters dispersed in the tissue may have escaped our microscopic inspection (44). An RNA-editing enzyme, present in GC B cells, was recently described by Muratmatsu et al. (48), and when the encoding gene, AID, was mutated, abrogation of somatic hypermutation was observed. Investigating the transcriptional activity of this gene in gut LP may be a possible strategy. In any case, the CD28−/− mice could generate a functional antitoxic IgA response following oral immunization with CT. This must be considered highly surprising since anti-CT IgA responses have been found strictly T cell- and CD40-CD40L-dependent (22). Furthermore, systemic IgG responses undergo affinity maturation based on successful somatic hypermutation (49). It is unlikely that antitoxic protective IgA responses are generated in the absence of affinity maturation; therefore, our findings of somatic hypermutations in gut IgA+ cells in CD28−/− mice probably also reflect affinity maturation of anti-CT IgA B cell clones.

The fact that protein Ags such as CT and KLH could stimulate a gut IgA response in CD28−/− mice, but failed to do so in CTLA4-Hy1 Tg mice, argues for an alternative costimulatory pathway operating at the gut mucosal level. This costimulatory pathway appears to be unique, or at least essential, for the mucosal immune system. Hence, serum IgA responses were dramatically reduced, whereas mucosal IgA responses were largely intact following oral immunizations. The alternative pathway was clearly susceptible to blockade with the CTLA4-Hy1 fusion protein, as evidenced by the poor or no specific gut IgA response seen after oral immunization in the WT or CD28−/− mice expressing the CTLA4-Hy1 Tg. This suggests that the GALT hosts a CD28-independent pathway that requires CD80/86. By inference, this means signaling via CTLA4 or a yet undefined receptor on the T cell that binds to a B7 type ligand (40). Although some previous studies has ascribed an up-regulatory role of CTLA-4 in TGFβ1 secretion, most investigations have shown CTLA-4 to be involved with down-regulation of T cell responses, even with regard to TGFβ1 secretion by CD4+ T cells (50, 51). It awaits to be proven whether CTLA-4 or, as evidenced in recent studies, there exist alternative signaling pathways, which could explain what appears to be a unique costimulatory pathway in the GALT. Such a costimulatory pathway could mediate signaling via membrane receptors on the T cells, such as ICOS, LFA-1, OX40, CD27, or other hitherto unidentified receptors involved in different stages of T cell activation (25–27). At the systemic level, alternative pathways appear not to compensate for the lack of the CD28 pathway, while they are clearly redundant to CD28 for gut mucosal IgA responses, as seen in the present study. In particular, ICOS has been found important for Th2 differentiation and CD40-mediated isotype switching, which are considered prerequisites for mucosal IgA responses (9, 10, 16, 52). These data clearly argue that ICOS is important for T-B cell interactions in general, but may be particularly critical for the development of mucosal immunity. This is supported by Gonzalo et al. (53), who found that ICOS signaling is critical for IgE production and Th2-effector functions at mucosal sites. Nevertheless, information about ICOS-regulated events do not explain why we could block the mucosal IgA response with CTLA4-Hy1 protein, since this protein is not known to block interactions with ICOS (A. Sharpe, unpublished observations). Of
note, the Ag as well as the adjuvant system itself may differentially affect/depend on CD28 relative to ICOS signaling, as demonstrated by several recent studies (40). Moreover, the specific roles of these costimulatory pathways for priming as opposed elicitation of effector type of responses may differ (40). Nonetheless, a study in mice lacking both CD28 and CTLA4 clearly indicated the existence of an additional receptor for B7 molecules, which was blocked by CTLA4-H/H92531 protein involved in CD4 T cell priming (54). This receptor could provide a potent costimulatory pathway at mucosal sites and could be particularly important for CT adjuvant function (22). Future studies using CD28/CTLA4, ICOS, or B7RP-1-deficient mice will specifically address this question in the context of the various adjuvant systems.

When CT is used as an adjuvant together with soluble protein Ags in WT mice, it induces strong specific gut IgA and systemic IgG responses (22). The main mechanism for CT’s adjuvant function still remains to be defined, although previous studies from a large number of laboratories have indicated that CT acts via the APC. CT has been found to up-regulate surface expression of CD80 and CD86 on the APC (24, 38, 39). This observation suggested that CT might enhance immune responses via an increased priming of Ag-specific CD4+ T cells. However, our recent investigations using the CTLA4-Hyl Tg and now the CD28-deficient mice dispute that costimulation via these factors are key elements of the adjuvant function of CT. Although the induction of specific gut mucosal IgA immunity was impaired in the CTLA4-Hyl Tg mice, the adjuvant function at the systemic level was intact in those mice (21). Thus, the absence of CD80/86 costimulation or CD28 did not reduce the enhancing effect of CT on humoral immune responses. Rather CT was found to enhance TD IgG responses to the same degree in CTLA4-Hyl Tg as in WT mice, suggesting other mechanisms for CT’s immunoenhancing effects (21). The present findings of an almost intact adjuvant function in CD28−/− mice rule out the simple explanation that CT functioned in the CTLA4-H/H92531 Tg mice because of an incomplete blockade of CD80/86. Whereas costimulation involving CD80/86 and CD28 appears to be dispensable, we recently found that CD40L expression is required for an adjuvant effect of CT (47). It is possible that CT adjuvant affects several costimulatory pathways, and in the absence of CD28 signaling other pathways are available for modulation. This can be by means of up-regulation of the heat-stable Ag, OX40, B7RP-1, B7-H1, 4-1BB, CD54, CD83, or other yet undefined ligands on the APC (11, 25, 27, 37, 55, 56). Further investigations are warranted to disclose the mechanism of CT’s adjuvant effect and particularly knowledge of which genes are regulated by CT exposure of APC would greatly benefit the field of holotoxin vaccine adjuvant research.

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References


