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Mucosally Induced Systemic T Cell Unresponsiveness to Ovalbumin Requires CD40 Ligand-CD40 Interactions[1]

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CD40 ligand (CD40L) gene-disrupted ($CD40L^{-/-}$) mice were employed to examine the role of costimulatory signals via CD40L-CD40 interactions in mucosally induced tolerance. $CD40L^{-/-}$ and control ($CD40L^{+/+}$) mice of the same C57BL/6 × 129/J background were immunized orally with 25 mg of OVA before systemic challenge with OVA in CFA. While $CD40L^{+/+}$ mice showed reductions in Ag-specific T cell responses including delayed-type hypersensitivity (DTH) and proliferative responses, $CD40L^{-/-}$ mice underwent normal T cell responses. Further, cytokine analysis of splenic $CD4^+$ T cells showed that both Th1-type (e.g., IFN-γ and IL-2) and Th2-type (e.g., IL-4, IL-5, IL-6, and IL-10) responses were maintained in $CD40L^{-/-}$ mice orally immunized with OVA, whereas these cytokine responses in $CD40L^{+/+}$ mice were significantly reduced. In addition, splenic $CD4^+$ T cells from $CD40L^{-/-}$ mice orally immunized with OVA provided B cell help in Ag-specific Ab-forming cells when the cells were cultured with naive B cells in the presence of Ag and CD40L-transfected cell lines. In contrast, an identical culture condition containing splenic $CD4^+$ T cells from orally tolerized $CD40L^{+/+}$ mice did not exhibit helper activity. Taken together, these findings indicate that CD40L and CD40 interactions are essential for the induction of systemic T cell unresponsiveness to orally administered Ag. *The Journal of Immunology,* **1999, 162: 1904–1909.**

The CD40 ligand (CD40L),[3] which is a member of the TNF family of molecules, is preferentially expressed on activated $CD4^+$ T cells (1). The counter-receptor for this ligand is CD40, a member of the TNF receptor family that is expressed on APCs including B lymphocytes, dendritic cells, and macrophages (2, 3). The role of CD40L-CD40 interactions for the induction of humoral immunity was demonstrated by the multiple functional defects in CD40L gene-disrupted ($CD40L^{-/-}$) mice including a failure to form germinal centers (4), activate memory B cells, and induce Ig class switching (5). In addition, $CD40L^{-/-}$ mice are unable to elicit Th1-type responses, as reflected by low levels of IFN-γ and IL-12 production (6, 7).

The administration of repeated low doses or a high dose of soluble Ag via the mucosal route (e.g., oral or nasal) can result in the induction of IgA Ab responses in the mucosal compartment, with an otherwise unresponsiveness in the systemic immune compartment (8, 9). This phenomenon was originally termed oral tolerance (10); however, a more generalized term, mucosally induced tolerance, was recently introduced (9) because Ag-specific unresponsiveness can be induced by the nasal as well as oral route (11). It has been shown that the T cell compartment of the CD4 phenotype played important roles in the induction of systemic unresponsiveness to mucosally administered Ag (12, 13). For example, $CD4^+$ T cells anergized by a high dose of oral Ag were implicated in the induction of mucosally induced tolerance (14). In vitro treatment with anti-CD8 mAb during oral immunization with Ag did not influence reductions in Ag-specific Ab or T cell proliferative responses (15, 16). Finally, Ag-specific tolerance was induced in β_2 microglobulin gene-depleted mice given OVA orally (17).

Costimulatory molecules may be of importance in the induction of systemic unresponsiveness. For example, one recent study reported that selective blockade of B7-CTLA-4 interactions completely inhibited systemic tolerance induced by a high dose of Ag (18). Further, blockade of CD40L-CD40 interactions by anti-CD40L Ab results in tolerance to the hapten 2,4-dinitrofluorobenzene-induced, Th1-type of contact hypersensitivity (19). Furthermore, anergic Th1-type cells expressed reduced levels of CD40L expression (20). Despite these recent studies, the precise roles for costimulatory signals in the induction and maintenance of mucosally induced tolerance remain undefined.

In the present study, we assessed the role of CD40L-CD40 interactions for the regulation of T cell unresponsiveness in response to orally administered Ag using $CD40L^{-/-}$ mice. Our results show that CD40L-CD40 interactions are required for the induction of systemic T cell unresponsiveness, which follows when Ag is given by the oral route.

Materials and Methods

$CD40L^{-/-}$ mice

The generation and initial characterization of $CD40L^{-/-}$ mice have been described previously (4, 5). $CD40L^{-/-}$ mice used throughout this study were kindly obtained from Dr. Charles R. Maliszewski at Immunex Research and Development Corporation (Seattle, WA) and maintained as random hybrids on a C57BL/6 × 129/J background. $CD40L^{-/-}$ and $CD40L^{+/+}$ mice were genotyped by a standard PCR method. All mice were

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[3] Abbreviations used in this paper: AFCs, Ab-forming cells; CD40L, CD40 ligand; DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; HEL, hen egg white lysozome; PP, Peyer's patch.

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maintained in flexible Trexler isolators and have remained pathogen-Ab negative. At 5–6 wk of age, the mice were removed from the colony isolator unit, housed in microisolator cages in horizontal laminar flow cabinets, and provided sterile food and water ad libitum. The mice were between 7 and 10 wk of age at the beginning of individual experiments.

Immunization

To induce systemic unresponsiveness to the chosen Ag OVA (Fraction V; Sigma, St. Louis, MO), a standard oral administration protocol was used in this study (9). In some experiments, hen egg white lysozome (HEL; Sigma) was also used as an additional model Ag. Mice were given 25 mg of Ag dissolved in 0.25 ml of PBS by gastric intubation. Control mice received oral PBS only. Seven days later, mice were immunized via the s.c. route with 100 μg of OVA or HEL emulsified in 100 μl of CFA (Difco Laboratories, Detroit, MI). The mice were sacrificed 14 days after the s.c. immunization to examine Ag-specific T cell responses.

Measurement of delayed-type hypersensitivity (DTH) responses

A standard protocol for the measurement of DTH responses was employed in this study (9). Briefly, 20 μg of OVA or HEL in 20 μl of PBS was injected into the left ear pinna, and the right ear pinna received PBS as a control. Ear swelling was measured 24 h later with a dial thickness gauge (Ozaki Manufacturing, Tokyo, Japan). The DTH responses were expressed as the increase of ear swelling after Ag injection following subtraction of swelling in the control site.

OVA-induced splenic CD4$^+$ T cell proliferation

The spleen was removed aseptically and single-cell suspensions were prepared in RPMI 1640 (Cellgro Mediatech, Washington, DC) containing HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). CD4$^+$ T cells were purified by the magnetic activated cell sorter system (Miltenyl Biotec, Sunnyvale, CA) as described previously (21). Briefly, cells were incubated in a nylon wool column (Polysciences, Warrington, PA) to remove B cells and macrophages. Enriched T cell populations were then incubated with biotinylated anti-CD4 (GK 1.5) followed by streptavidin-conjugated microbeads and passed through the magnetized column. The purified T cell fractions were >95% CD4$^+$ and were >99% viable. Cells were resuspended in complete medium (RPMI 1640 containing 10% heat-inactivated FBS). Purified CD4$^+$ cells (4×10^6 cells/ml) were cultured with or without 1 mg/ml of OVA or HEL in the presence of T cell-depleted irradiated (3000 rads) splenic APCs from naive F_2 (C57BL/6 × 129/J) mice in 96-well or 24-well tissue culture plates (Corning Glass Works, Corning, NY) for 4 days at 37°C in a moist atmosphere of 5% CO_2 in air. To measure Ag-specific T cell proliferation, 0.5 μCi of tritiated [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added for the final 18 h of incubation. The cells were harvested, and the amount of [^{3}H]thymidine incorporation was determined by scintillation counting. In some experiments, culture supernatants were harvested after 2 and 4 days of the incubation and were then subjected to cytokine-specific ELISA. For cytokine-specific mRNA analysis, CD4$^+$ T cells were harvested after 2 days of incubation and were then subjected to cytokine-specific semiquantitative RT-PCR assays.

Semiquantitative analysis of cytokine-specific mRNA

For evaluation of cytokine-specific mRNA levels in OVA-stimulated CD4$^+$ T cells, a semiquantitative RT-PCR was employed (22). Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure and 25 μg/ml of extracted RNA was subjected to reverse transcriptase reaction using Superscript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD). The cDNA from 5 ng of RNA was used for each cytokine-specific PCR. For the amplification of cDNA, 35 cycles of reaction programmed as 1 min at 95°C and 1 min at 60°C was performed. The PCR product was quantitated by capillary electrophoresis with the laser-induced fluorescence detection system (CE-LIF, LIF-P/ACE; Beckman Instruments, Fullerton, CA) as described previously (22). The fluorescence content of each cytokine-specific RT-PCR product was expressed as the peak area of relative fluorescence light units. The level of amplified cytokine cDNA was normalized to the corresponding β-actin levels as 100%.

Cytokine-specific ELISA

Cytokine levels of culture supernatants were measured by an ELISA as previously described (9). In brief, the immunoplates (Nunc, Naperville, IL) were coated with anti-IFN-γ (R4–6A2), anti-IL-2 (JES6–1A12), anti-IL-4 (BVD4–1D11), anti-IL-5 (TRFK-5), anti-IL-6 (MP5–20F3), or anti-IL-10 (JES5–2A5) mAbs (PharMingen, San Diego, CA). After blocking with 3% BSA in PBS, samples and serial two-fold dilutions of standards were added to duplicate wells and incubated overnight at 4°C. The wells were washed and incubated with biotinylated anti-IFN-γ (XMG1.2), anti-IL-2 (JES6–5H4), anti-IL-4 (BVD6–24G2), anti-IL-5 (TRFK-4), anti-IL-6 (MP5–32C11), or anti-IL-10 (JES5–16E5) mAbs, respectively. After incubation, peroxidase-labeled anti-biotin mAb (Vector Laboratories, Burlingame, CA) was added and developed with 1.1 mM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 0.1 M citrate-phosphate buffer, pH 4.2, containing 0.01% H_2O_2. Standard curves were generated using mouse rIFN-γ, rIL-2, rIL-4, rIL-5, rIL-6 (Genzyme, Cambridge, MA), or rIL-10 (PharMingen).

Th cell assay for B cell responses

To assess Th cell activity in the CD4$^+$ T cell subset from CD40L$^{-/-}$ mice given OVA orally, a modified in vitro B cell culture system was employed (23). In some experiments, murine L cells stably transfected with plasmid expressing CD40L were used as a source for costimulatory signal provided via the ligand (24). Purified splenic CD4$^+$ T cells (2.5×10^6 cells/ml) from CD40L$^{-/-}$ mice orally immunized with OVA or PBS were cocultured with or without CD40L-expressing L cells (irradiated at 10,000 rads; 1×10^5 cells/ml) in the presence of naive B cells (2.5×10^6 cells/ml), feeder cells, and OVA (1 mg/ml) for 5 days at 37°C with 5% CO_2 in air. Nonadherent cells were harvested and then assessed for IgM, IgG, and IgA Ab-forming cell (AFC) responses with an OVA-specific enzyme-linked immunospot (ELISPOT) assay. In brief, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with OVA (1 mg/ml) overnight at 4°C and blocked with PBS containing 3% BSA. After addition of appropriate dilutions of cells in RPMI 1640 containing 10% FCS, the plates were incubated for 4 h at 37°C with 5% CO_2 in air. OVA-specific IgM, IgG, and IgA AFC were detected with peroxidase-labeled anti-mouse μ-, γ-, and α-chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) and visualized by adding 3-amino-9-ethylcarbazole in 0.1 M sodium acetate buffer (Moss, Pasadena, MA). Spots representing AFCs were counted with the aid of a stereo microscope.

Statistics

The data are expressed as the mean ± SEM and compared using an unpaired Mann-Whitney *U* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) for Macintosh computers and were considered to be statistically significant if p values were <0.05.

Results

Lack of induction of T cell tolerance in CD40L$^{-/-}$ mice

To investigate the potential role of CD40L-CD40 interactions for the development of mucosally induced tolerance, CD40L$^{-/-}$ and CD40L$^{+/+}$ mice were given 25 mg of OVA or PBS orally and then examined for DTH responses by measuring OVA-induced ear swelling (Fig. 1). Both CD40L$^{+/+}$ and CD40L$^{-/-}$ mice given PBS showed significant ear swelling (50–70 μm). As expected, the levels of OVA-specific DTH responses were significantly reduced in CD40L$^{+/+}$ mice given oral OVA in comparison to those mice given oral PBS. Conversely, no reductions of OVA-specific DTH responses were observed in CD40L$^{-/-}$ mice given oral OVA. Similar results were obtained when HEL was used as Ag (Fig. 1).

To further address that T cell unresponsiveness was not induced by either oral OVA or HEL in CD40L$^{-/-}$ mice, Ag-specific T cell proliferative responses were assessed (Fig. 1). CD4$^+$ T cells isolated from spleen of CD40L$^{+/+}$ mice orally immunized with OVA or HEL showed significantly lower Ag-specific proliferative responses than did those of CD40L$^{+/+}$ mice given oral PBS. In contrast, identical levels of Ag-specific T cell proliferative responses were seen in CD40L$^{-/-}$ mice given oral Ags or PBS.

Th1- and Th2-type cytokine responses are not down-regulated in CD40L$^{-/-}$ mice by prior oral administration of a high dose of OVA

Because the levels of Ag-specific DTH and T cell proliferative responses were identical between CD40L$^{-/-}$ mice given Ag or

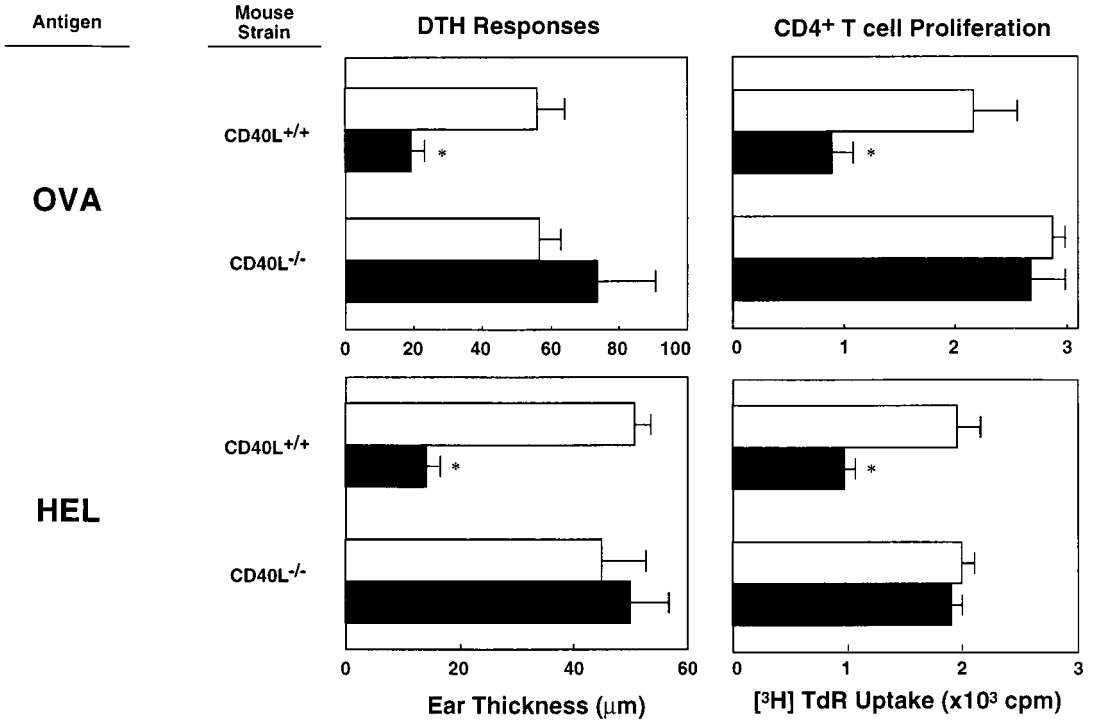

FIGURE 1. Analysis of Ag-specific DTH and T cell proliferative responses in CD40L$^{+/+}$ and CD40L$^{-/-}$ mice orally immunized with PBS (□) or Ag (OVA or HEL) (■) followed by s.c. immunization with OVA or HEL in CFA. The details of the protocol are described in *Material and Methods*. The results of T cell proliferative responses were expressed as cpm of the OVA- or HEL-stimulated mice minus cpm of the medium-only cultures. Baseline values for splenic CD4$^+$ T cell proliferation in medium only were always below 300 cpm. No detectable DTH or CD4$^+$ T cell proliferative responses were seen in mice fed Ag or PBS without systemic immunization. The results expressed are the mean ± SEM of three separate experiments containing five mice per group. (*, $p < 0.05$ vs PBS orally immunized group).

PBS by the oral route, it was important to assess cytokine synthesis by Ag-specific splenic CD4$^+$ T cells isolated from orally treated CD40L$^{-/-}$ or CD40L$^{+/+}$ mice. Levels of Th1-type (IFN-γ and IL-2) and Th2-type (IL-4, IL-5, IL-6, and IL-10) cytokines were significantly reduced in culture supernatants of splenic CD4$^+$ T cells from CD40L$^{+/+}$ mice given oral OVA when compared with those of mice given oral PBS (Fig. 2). These findings are consistent with the fact that decreased T cell proliferative responses were also seen in CD40L$^{+/+}$ mice orally immunized with OVA. In contrast, similar levels of Th1- and Th2-type cytokine responses were noted in CD40L$^{-/-}$ mice given oral OVA or PBS. Further, it is important to point out that the levels of IL-2 synthesis, where low production is an indicator of T cell anergy (25, 26), was maintained in both groups of CD40L$^{-/-}$ mice (Fig. 2; 7.7 ± 0.2 vs 8.5 ± 0.3 U/ml).

To address the levels of Th1- and Th2-type cytokine-specific responses at the molecular level, a semiquantitative RT-PCR analysis was employed. Decreased levels of Th1- and Th2-type cytokine-specific mRNA were noted in CD40L$^{+/+}$ mice orally immunized with OVA when compared with those of mice given oral PBS. In contrast, similar levels of Th1- and Th2-type cytokine-specific mRNA were noted in CD40L$^{-/-}$ mice fed either OVA or PBS (Fig. 2). Taken together, these findings indicate that T cell unresponsiveness, which is normally induced by a high dose of oral Ag, was not established in mice deficient in the CD40L-CD40 signaling pathway.

CD4$^+$ T cells from orally treated CD40L$^{-/-}$ mice provide B cell help in the presence of CD40L transfectants

To further confirm the presence of intact T cell functions in CD40L$^{-/-}$ mice after oral administration of a high dose of OVA, Th cell functions for B cell responses were evaluated. Splenic CD4$^+$ T cells isolated from CD40L$^{-/-}$ mice were cocultured with CD40L transfectants in the presence of OVA, APCs, and naive B cells for 5 days. OVA-specific AFCs were assessed by the ELISPOT assay. When appropriate costimulatory signals through CD40L-CD40 were substituted by CD40L transfectants, CD4$^+$ T cells from CD40L$^{-/-}$ mice given oral OVA were able to provide efficient Th cell function for the induction of OVA-specific IgM and IgG AFCs. In contrast, splenic CD4$^+$ T cells from CD40L$^{+/+}$ mice given oral OVA only provided low levels of B cell help with OVA-specific IgM and IgG AFC responses when compared with CD40L$^{+/+}$ mice given oral PBS ($p < 0.05$, Table I). Lower numbers of OVA-specific IgM and IgG AFC were detected in the cultures containing CD4$^+$ T cells from CD40L$^{-/-}$ mice without CD40L transfectants or with control murine L cells that did not express CD40L. In addition, very low numbers of Ag-specific IgM and IgG AFC were seen in wells that did not contain CD4$^+$ T cells (Table I). These data indicate that not only Ag-induced DTH, T cell proliferative, and cytokine responses, but also Ag-specific Th cell functions for B cell responses were maintained in CD40L-deficient mice even after oral administration of a high dose of Ag.

Discussion

To explore a possible role for costimulatory signals transduced by the CD40L-CD40 interactions for induction of mucosally induced tolerance, CD40L$^{-/-}$ and CD40L$^{+/+}$ mice were given a high dose of OVA by the oral route. As one might expect, oral administration of 25 mg of OVA to CD40L$^{+/+}$ mice resulted in the induction of

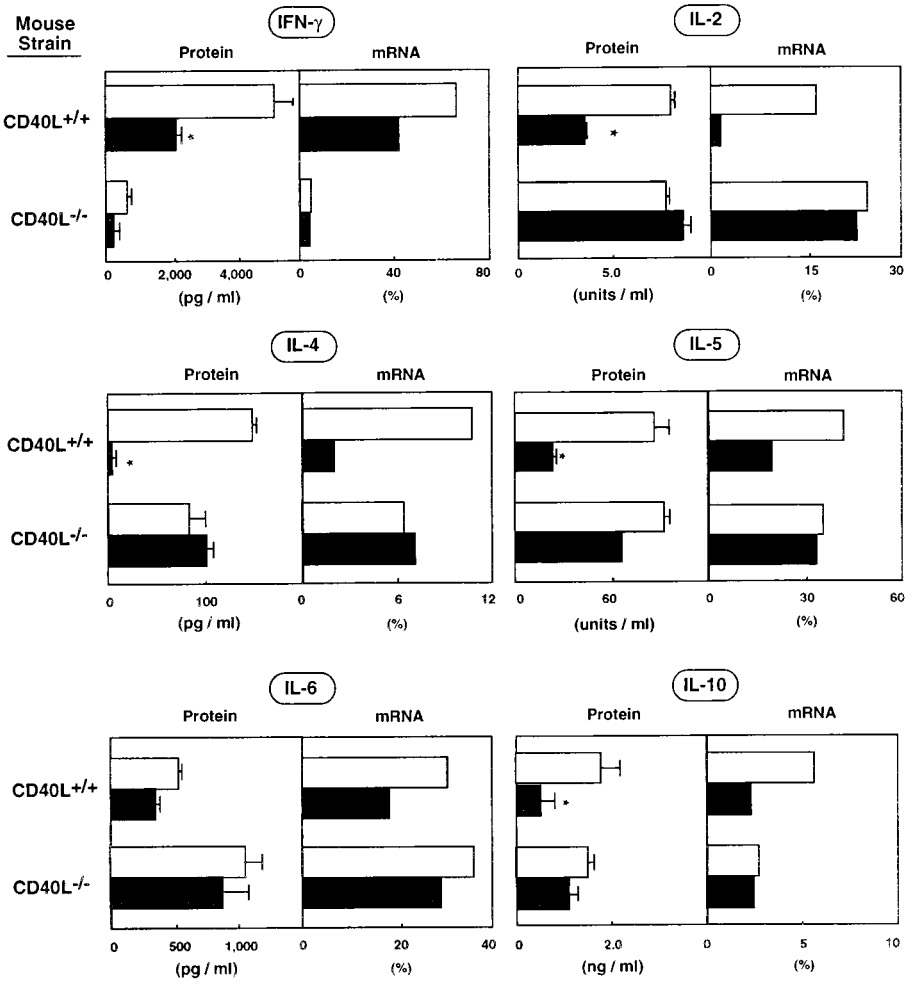

FIGURE 2. Profiles of Th1-type (IFN-γ and IL-2) and Th2-type (IL-4, IL-5, IL-6, and IL-10) cytokines secreted and mRNA expressed by CD40L$^{+/+}$ and CD40L$^{-/-}$ mice orally administered with PBS (□) or OVA (■) followed by s.c. immunization with OVA in CFA. Splenic CD4$^+$ T cells were cultured with or without 1 mg/ml of OVA in the presence of irradiated APCs. Culture supernatants were harvested after 2 (for IL-2) or 4 days (for IFN-γ, IL-4, IL-5, IL-6, and IL-10) of culture and were then analyzed by the respective cytokine-specific ELISA. Nonadherent cells were harvested after 2 days of culture and were then subjected to extraction of RNA. The levels of mRNA were expressed as the relative peak area based on the value of β-actin (β-actin = 100). No detectable cytokine responses were seen in mice fed OVA or PBS without systemic immunization. Results are expressed as the mean ± SEM and show one example from three separate experiments with similar results containing five mice per group. (*, $p < 0.05$ vs PBS orally immunized group).

systemic T cell unresponsiveness, where OVA-specific DTH and proliferative responses as well as Th1- and Th2-type cytokine synthesis were reduced in splenic CD4$^+$ T cells when compared with those of mice given PBS only. In contrast, when the same experimental protocol was performed in CD40L$^{-/-}$ mice, Ag-specific systemic T cell unresponsiveness was not observed after oral administration of OVA. A similar finding was noted when HEL was used as Ag. Further, our results indicate that splenic CD4$^+$ T cells from CD40L$^{-/-}$ mice orally immunized with OVA were capable of supporting Ag-specific B cell responses in vitro through costimulatory signals provided by CD40L-transfected cells. These findings indicate that the costimulatory signals transduced via the CD40L-CD40 interaction serve an important role in the induction of systemic T cell unresponsiveness following oral administration of a high dose of protein Ag.

Table I. *Comparison of splenic CD4$^+$ Th cell activity from mice given either PBS or OVA by the oral route*[a]

Source of CD4$^+$ T Cells	Oral Administration	CD40L-Transfected Murine L Cells	No. of OVA-Specific AFCs/Culture	
			IgG	IgM
CD40L$^{+/+}$	PBS	−	37 ± 12*	225 ± 60†
	OVA	−	7 ± 1*	101 ± 8†
	PBS	+	36 ± 6*	215 ± 45†
	OVA	+	10 ± 2*	92 ± 14†
CD40L$^{-/-}$	PBS	−	3 ± 1‡	48 ± 16‡
	OVA	−	6 ± 2‡	64 ± 3‡
	PBS	+	45 ± 13‡	244 ± 72‡
	OVA	+	37 ± 16‡	243 ± 59‡
None		−	7 ± 1	27 ± 6
		+	6 ± 2	23 ± 5

[a] Splenic CD4$^+$ T cells (2.0×10^6/ml) were cultured with or without murine L cells expressing CD40L in the presence of naive B cells (2.5×10^6/ml), feeder cells, and OVA (1 mg/ml). After cultivation for 5 days, nonadherent cells were harvested and OVA-specific AFC were determined using the ELISPOT assay. No AFC were detected in mice fed OVA or PBS without systemic immunization. Results are expressed as the values (mean ± SEM) from four separate experiments containing five mice/group/experiment. *, $p < 0.01$; †, $p < 0.05$; ‡, $p < 0.03$.

It is now generally agreed that mucosally induced tolerance is established and maintained at the level of T cells (13, 14). Two distinct forms of T cell unresponsiveness, e.g., active suppression and clonal deletion and/or anergy, which were induced by low and high doses of mucosally administered Ag, respectively, have been proposed as major mechanisms for the induction of mucosally induced tolerance (8, 13, 26). Repeated administration of low doses of Ag induced active suppression that was mediated by the production of suppressive cytokines such as TGF-β, IL-4, and IL-10 (8). In contrast, a high dose of Ag elicited clonal deletion (13) and/or anergy, which was characterized by the absence of T cell proliferation and decreased IL-2 synthesis as well as IL-2R expression (25, 26, 27). Oral administration of a high dose of OVA induced diminished levels of OVA-specific proliferative and cytokine responses including IL-2 synthesis by splenic CD4$^+$ T cells in CD40L$^{+/+}$ mice, suggesting that systemic T cell unresponsiveness is mediated by clonal anergy. Further, the fact that feeding of OVA failed to reduce T cell proliferation and cytokine synthesis in CD40L$^{-/-}$ mice indicates that CD40L-CD40

interactions play a critical role in the induction of systemic T cell unresponsiveness.

It has been reported that T cell anergy could be induced when T cells were activated without adequate costimulatory signals normally provided by APCs (18, 28). Interestingly, however, our findings showed that T cell unresponsiveness could not be induced in the absence of costimulatory signals resulting from CD40L-CD40 interactions. These results are consistent with other findings that CD4$^+$ T cell proliferative responses were maintained in CD40L$^{-/-}$ mice after priming with myelin basic protein in CFA (29). Thus, CD40L-CD40 interactions could be critical for induction of T cell unresponsiveness. In this regard, previous studies have demonstrated that CD40L and CD40 interactions are involved in the regulation of CD80/CD86 expression by APCs (30). In addition, the interaction of CD80/CD86 and CTLA-4 provide negative signals for the inhibition of activated T cells (31, 32). One possible mechanism is that the lack of a CD40L and CD40 interaction leads to the subsequent loss of negative signals generated by interaction of CD80/CD86 with CTLA-4 in CD40L$^{-/-}$ mice. To support this view, a recent study showed abrogation of high-dose mucosally induced tolerance in mice treated with CTLA-4-Ig fusion protein (18). Our results do suggest that the interactions of CD40L and CD40 are necessary for the expression of CD80/CD86 molecules that affect T cell activation or unresponsiveness; however, the relationship between T cell activation and unresponsiveness as determined by CD28 or CTLA-4 engagement of CD80/CD86 molecules remains to be defined.

Our results revealed that the levels of IFN-γ synthesis from OVA-stimulated splenic CD4$^+$ T cells were significantly lower in CD40L$^{-/-}$ mice than those of the same genetic background. In this regard, it was shown that CD40L-CD40 interactions are more critical for the generation of Th1- than Th2-type responses. Thus, these costimulatory interactions are associated with IL-12 secretion by both macrophages and dendritic cells for the subsequent promotion of IFN-γ production by Th1 cells (7, 33). In fact, recent studies also demonstrated that the inability of CD40L$^{-/-}$ mice to control a parasite infection was due to impaired IL-12 production (6, 34). In support of this, our separate study demonstrated that the levels of Th1-type (e.g., IFN-γ and IL-2) but not Th2-type (e.g., IL-4, IL-5, IL-6, and IL-10) cytokines were significantly lower in anti-CD3 Ab-stimulated splenic CD4$^+$ T cells isolated from naive CD40L$^{-/-}$ mice than from control mice (unpublished data). Additionally, recent studies have suggested that IFN-γ plays a central role for induction of mucosally induced tolerance (9). In support of this notion, initial priming of Th1-type responses (e.g., IFN-γ synthesis) could occur before mucosally induced tolerance is established (35). Moreover, repeated oral feeding of high doses of OVA to OVA TCR-transgenic mice resulted in a IFN-γ-dominant immune response in Peyer's patches (PP) (36). Based upon these reports, a potentially interesting experiment would be treatment of CD40L$^{-/-}$ mice with exogenous IFN-γ, which may restore systemic T cell unresponsiveness. However, it also remains possible that this cytokine treatment may fail totally, because an appropriate Th1 induction pathway for oral tolerance may not be triggered due to the lack of organized PP, an important inductive site for the mucosal immune system in CD40L$^{-/-}$ mice. Alternatively, IFN-γ treatment may enhance MHC class II expression by intestinal epithelial cells, which leads to active Ag presentation for Ag-specific immune responses instead of tolerance induction. Indeed, it has been shown that enhanced MHC class II expression by intestinal epithelial cells does occur in IFN-γ-treated mice with subsequent abrogation of mucosally induced tolerance (37). Despite this likelihood, our present findings suggest that another explanation for the lack of mucosally induced tolerance in the CD40L$^{-/-}$ mice fed a high dose of Ag could be their impaired ability to induce Th1-type responses.

The other possible explanation for the lack of T cell unresponsiveness to orally ingested Ag in CD40L$^{-/-}$ mice is that these mice do not form distinct geminal centers in PP (4). In this regard, it has been shown that oral immunization induced Ag-specific immune responses in both systemic and mucosal compartments, and these immune responses were strongly associated with Ag uptake and processing pathways in PP (38). Thus, it remains possible that induction of systemic unresponsiveness to orally administered Ag requires the presence of normal immunocompetent cells in PP. To support this view, it has been shown that oral tolerance to a high dose of Ag was due to clonal deletion of T cells in the PP (13). Thus, it will be important to determine whether lack of T cell tolerance in CD40L$^{-/-}$ mice is due to the absence of actual CD40L-CD40 interactions or to a loss of germinal center formation that occurred subsequent to the disruption of the CD40L gene. To address this important issue, we are currently investigating mucosally induced tolerance in mice treated with a lymphotoxin β receptor Ig fusion protein in utero because this treatment resulted in the absence of PP but an otherwise normal lymph node development (39).

It has been shown that TGF-β is a key cytokine for the induction of low-dose oral tolerance (8). Evidence from studies in OVA-transgenic mice has revealed increased production of this cytokine in PP of mice with high-dose oral tolerance (36, 40). However, a recent study in TGF-β1$^{-/-}$ mice demonstrated that this cytokine was not essential for the induction of low- or high-dose oral tolerance (41). To further address a potential role for TGF-β in mucosally induced tolerance, it will be interesting to assess the levels of TGF-β produced in CD40L$^{-/-}$ mice fed a high dose of Ag. According to recent findings, which showed that reduced IFN-γ or IL-12 synthesis resulted in the enhancement of TGF-β production (36, 42), it can be predicted that increased TGF-β production would most likely occur in CD40L$^{-/-}$ mice, because these mice manifest an impairment of Th1-type responses. Studies along these lines are currently under investigation in our laboratory.

In summary, the present study showed that the absence of CD40L-CD40 interactions prevented the development of systemic T cell unresponsiveness which is normally induced by a high oral dose of Ag. Although the precise mechanisms for the role of CD40L-CD40 signal pathways in the induction of mucosally induced tolerance needs to be further investigated, our study suggests that regulatory signals transduced by CD40L-CD40 play an important role in the induction and maintenance of mucosally induced tolerance.

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