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γδ T cells regulate mucosally induced tolerance in a dose-dependent fashion

Kohtaro Fujihashi[1], Taeko Dohi[2], Mi-Na Kweon[3], Jerry R. McGhee[2], Toshiya Koga[1], Max D. Cooper[2,4,5,6], Susumu Tonegawa[7] and **Hiroshi Kiyono[1,3]**

Departments of [1]Oral Biology, [2]Microbiology, [4]Medicine and [5]Pediatrics, Immunobiology Vaccine Center, and the [6]Howard Hughes Medical Institute, University of Alabama at Birmingham, Medical Center, Birmingham, AL 35294, USA

[7]Center for Cancer Research, Department of Biology, Howard Hughes Medical Institute and Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan

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Abstract

We used γδ TCR-deficient ($TCR\delta^{-/-}$) mice to examine the role of γδ T cells for induction of mucosal responses and systemic tolerance to high versus low doses of oral antigen. When either $TCR\delta^{-/-}$ or $TCR\delta^{+/+}$ mice were immunized orally with a high dose of ovalbumin (OVA) prior to parenteral challenge, systemic IgG and IgE antibody responses were markedly reduced in both types of mice, while mucosal IgA responses were reduced only in the $TCR\delta^{-/-}$ mice. Reduced T cell proliferative responses and delayed-type hypersensitivity were seen in $TCR\delta^{-/-}$ and $TCR\delta^{+/+}$ mice given the high dose of OVA. Antigen-induced T_h1 and T_h2 cytokine production by splenic $CD4^+$ T cells was severely inhibited in orally tolerized $TCR\delta^{-/-}$ and $TCR\delta^{+/+}$ mice. In contrast, while oral tolerance associated with increased levels of IL-10 synthesis was induced by a low dose of OVA in $TCR\delta^{+/+}$ mice, the $TCR\delta^{-/-}$ mice were not tolerized and failed to produce IL-10. Our findings indicate that γδ T cells play a significant immunoregulatory role in IL-10-mediated, low-dose oral tolerance induction, but are not essential participants in the induction of systemic tolerance to orally introduced antigens given in larger doses.

Introduction

Oral administration of proteins or haptens can induce a state of systemic unresponsiveness (1). Sulzberger and Chase established that oral administration of hapten inhibits contact hypersensitivity and this immunologic reaction still bears their name (2). In the early 1980s, this type of immunological response was dubbed oral tolerance and the concept used to refer specifically to immune responses elicited in mucosa-associated as opposed to systemic tissues (3). In general, oral administration of large amounts of antigen resulted in the induction of antigen-specific IgA responses in the mucosal compartment, whereas unresponsiveness to the same antigen was evident in the systemic immune system. Other recent studies have shown that nasal administration of proteins may also induce systemic unresponsiveness (4), leading mucosal immunologists to broaden the earlier term of oral tolerance to the more inclusive 'mucosally induced tolerance'.

Current evidence suggests that T lymphocytes are the major cell type involved in the induction of mucosally induced tolerance (1). Oral administration of myelin basic protein (MBP) induced transforming growth factor (TGF)-β-producing $CD8^+$ T cells that were capable of inducing unresponsiveness and inhibiting experimental autoimmune encephalomyelitis (5–7). Further, it was shown that experimental tracheal eosinophilia can be down-regulated by TGF-β-producing $CD4^+$ T cells (8). By contrast, $CD4^+$ T cells anergized by large doses of oral antigen feeding were implicated in the induction of antigen-specific systemic unresponsiveness (9). It has been suggested that T_h1-type cells are more sensitive to the induction of tolerance than T_h2-type cells (10,11). Thus, oral tolerance is possibly associated with selective down-regulation of T_h1 cells which are more sensitive to tolerance

Correspondence to: K. Fujihashi, Department of Oral Biology, Immunobiology Vaccine Center, University of Alabama at Birmingham, Medical Center, BBRB 761, Birmingham, AL 35294-2170, USA

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induction by T_h2 cells via their respective cytokines in the systemic immune compartment. However, recent studies have now shown that feeding high doses of ovalbumin (OVA) inhibited production of both T_h1 (IL-2 and IFN-γ) and T_h2 (IL-4, IL-5 and IL-10) cytokines, and resulted in the reduction of IFN-γ- and IL-4-dependent, antigen-specific IgG2a and IgG1 antibody responses respectively (9). These findings indicate that both T_h1 and T_h2 cell subsets are involved in the induction of oral tolerance.

The mucosal immune system possesses unique anatomical features and is composed of specialized subsets of lymphoid cells. Among the intestinal intraepithelial lymphocyte population, it is the $CD8^+$ T cell subset that is prevalent and γδ T cells comprise 30–50% of this population (12–15). Studies of TCR-deficient mice suggest an important role for the γδ T cells in the immune responses to intracellular bacteria and parasites (16–18). The γδ T cells appear to be necessary for maintenance of mucosal IgA responses in the presence of systemic unresponsiveness induced by oral immunization (19,20). Other studies have provided direct evidence for their influence on both IgA and IgE responses (4,21). These observations suggest that γδ T cells are a crucial functional component of the mucosal immune system. Indeed, recent reports indicate that γδ T cells are essential for the induction of both low- and high-dose oral tolerance (22,23), although the mechanisms for the immunoregulatory role of γδ T cells in the induction of systemic unresponsiveness to low and high antigen doses still remain to be elucidated.

In order to address these issues, we have compared the immune responses in $TCR\delta^{-/-}$ and in control mice given different doses of OVA orally prior to systemic challenge. The results indicate that systemic unresponsiveness to large doses of ingested OVA is unimpaired in $TCR\delta^{-/-}$ mice. In the case of low-dose tolerance, our data demonstrate that increased levels of IL-10 production are associated with the presence of γδ T cells for the induction of systemic unresponsiveness. In contrast, low doses of proteins fail to induce IL-10-mediated tolerance in $TCR\delta^{-/-}$ mice.

Methods

γδ T cell-deficient mice

The derivation and initial characterization of $TCR\delta^{-/-}$ mice have been described previously (24). $TCR\delta^{-/-}$ mice used in this study were maintained on a C57BL/6 ($H\text{-}2^b$) background. The δ chain-deficient and normal mice ($TCR\delta^{+/+}$) were maintained in Trexler isolators and remained pathogen-free. At 5–6 weeks of age, the mice were removed from the colony isolator unit, housed in micro-isolator cages in horizontal laminar flow cabinets, and provided sterile food and water *ad libitum*. The mice were between 7 and 10 weeks of age at the beginning of individual experiments.

Immunization

To establish systemic unresponsiveness to a high dose of oral antigen, mice were given 25 mg of OVA (Fraction V; Sigma, St Louis, MO) dissolved in 0.25 ml of PBS by gastric intubation. Control mice received PBS only. Seven days later, mice were immunized via the i.p. systemic route with 100 μg of OVA in 100 μl of complete Freund's adjuvant (OVA/CFA) (Difco, Detroit, MI). For induction of low-dose oral tolerance, mice were gavaged with 2.5 mg of OVA or hen egg white lysozyme (HEL; Sigma) in 0.25 ml of PBS on days 0, 2 and 5, then immunized s.c. on day 7 with OVA/CFA or 100 μg of HEL in 100 μl of CFA (HEL/CFA) (23). Antigen-specific T and B cell responses were determined 14 days after i.p. or 10 days after s.c. immunization. In some experiments, $TCR\delta^{+/+}$ mice were treated by i.p. injection of anti-mouse γδ mAb (GL3, 200 μg/mouse) or hamster IgG as a control (Jackson ImmunoResearch, West Grove PA) 3 days before oral administration of OVA or systemic immunization with OVA/CFA (23).

Delayed-type hypersensitivity (DTH) responses

Antigen-specific DTH responses were measured 7 and 14 days after systemic challenge by a standard protocol (25). Briefly, PBS (20 μl) containing 10 μg of antigen (OVA or HEL) was injected into the left ear pinna. Ear swelling was measured 24 h later with a dial thickness gauge (Ozaki, Tokyo, Japan). The DTH response was expressed as the increase of ear swelling after antigen (e.g. OVA or HEL) injection following subtraction of swelling in the control site.

Antigen-specific T cell responses

The spleen was removed aseptically and single cell suspensions prepared in RPMI 1640 (Cellgro Mediatech, Washington, DC) containing HEPES buffer, non-essential amino acids, sodium pyruvate, L-glutamine, penicillin/streptomycin and gentamycin (incomplete medium) (21). $CD4^+$ T cells were purified by the MACS system (Stefen Milteny, Bergish-Gladbach, Germany) as previously described (21). Cells were passed through the magnetized column following incubation with biotin-labeled anti-CD4 mAb (GK 1.5) and streptavidin-conjugated microbeads. The isolated $CD4^+$ T cells were >95% pure and >99% viable. To evaluate OVA or HEL responses, $CD4^+$ T cells were cultured with 1 mg/ml of OVA or HEL in the presence of feeder cells for 4 days. During the last 18 h of incubation, 0.5 μCi of [^{3}H]thymidine was added before the cells were harvested and the amount of incorporation determined by scintillation counting. Control cultures were without antigen stimulation. In other experiments, lymphocytes isolated from spleen or intestinal epithelium of $TCR\delta^{+/+}$ mice fed low doses of OVA after 4 days of systemic challenge were stained with FITC-conjugated anti-mouse γδ mAb (GL3; PharMingen, San Diego, CA) and then purified by flow cytometry (Becton Dickinson, Oxnard, CA). The purified γδ T cells (2.5×10^5–1×10^6) were cultured with $CD4^+$ T cells (2×10^6) isolated from $TCR\delta^{-/-}$ mice fed low doses of OVA in the presence of 1 mg of OVA and feeder cells for 7 or 14 days. In some experiments, a double-chamber culture system (Becton Dickinson, Franklin Lakes, NJ) was employed. The purified γδ T cells (2.5×10^5–1×10^6) were added to the upper well of the double chamber with $CD4^+$ T cells, feeder cells and OVA as described above.

Cytokine-specific ELISA and ELISPOT assays

Levels of cytokines in culture supernatants were measured by an ELISA, while the number of cytokine-secreting cells in $CD4^+$ T cell fractions was determined by cytokine-specific

ELISPOT assays. The details of the ELISA and ELISPOT assays for IFN-γ, IL-2, IL-4, IL-5, IL-6 and IL-10 have been described previously (25–27). Combinations of anti-IFN-γ: R4-6A2 and XMG 1.2 mAb; anti-IL-2: JES6-1A12 and JES6-5H4 mAb; anti-IL-4: BVD4-1D11 and BVD6-24G2 mAb; anti-IL-5: TRFK-5 and TRFK-4 mAb; anti-IL-6: MP5-20F3 and MP5-32C11 mAb; and anti-IL-10: JES5-2A5 and JES5-16E3 mAb were used as coating and detection antibodies respectively. A mouse IL-10 immunoassay kit (Quantikine M; R & D systems, Minneapolis, MN) was used to detect IL-10 in the culture supernatants. The levels of antigen-specific cytokine production were calculated by subtracting the results of control cultures (e.g. without antigen stimulation) from those of antigen-stimulated cultures. This ELISA was capable of detecting 0.8 ng/ml of IFN-γ, 0.4 U/ml of IL-2, 25 pg/ml of IL-4, 0.8 U/ml of IL-5, 200 pg/ml of IL-6 and 4 pg/ml of IL-10.

Antibody assays

Antibody titers in serum and fecal extracts were determined by an ELISA (21,28). Falcon Microtest assay plates (Becton Dickinson) were coated with an optimal concentration of OVA or HEL (100 μl of 1 mg/ml) in PBS. Endpoint titers were expressed as the last dilution yielding an optical density at 414 nm (OD_{414}) > 0.1 U above negative control values after a 15 min incubation. Total IgE levels were determined by ELISA using mouse IgE mAb (clone 27-74; PharMingen) as the standard. Rat anti-mouse IgE mAb (clone R35-72; PharMingen) and biotinylated rat anti-mouse IgE mAb (clone R35-92; PharMingen) were employed respectively as coating and detection antibodies. OVA-specific serum IgE was detected by a modified IgE-capture luminometric assay (28). Light development was carried out in a Dynetech ML-3000 luminometer by injection of Ca^{2+} buffer (50 mM Tris, 20 mM calcium acetate, pH 7.5). Endpoint titers were determined as the dilution of each sample showing a 2-fold higher level of luminometric units above background.

Enumeration of antibody-forming cells (AFC)

Spleens were aseptically removed and single-cell suspensions prepared (21). Mononuclear cells in the lamina propria were isolated after removal of Peyer's patches from the small intestine by the use of a combination of enzymatic dissociation and discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden) (21,28). Mononuclear cells in the interface between the 40 and 75% layers were removed, washed, and resuspended in RPMI 1640 containing 10% FCS. An ELISPOT assay was employed to detect cells producing IgM, IgG and IgA antibodies (21). Ninety-six well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with 1 mg/ml of OVA (100 μl/well) in order to detect anti-OVA specific AFC (28).

Statistics

The significance of the difference (e.g. *P* values) between groups was evaluated by the Mann–Whitney *U*-test using a Statview II program designed for Macintosh computers.

Results

Systemic B cell unresponsiveness in TCRδ$^{-/-}$ mice immunized orally with a large dose of OVA

In order to investigate the potential role of γδ T cells in oral tolerance induction, we initially examined the effects of TCRδ gene disruption on the efficiency with which oral administration of OVA induces unresponsiveness. When OVA-specific antibody levels in serum of TCRδ$^{-/-}$ mice and control mice of the same background (TCRδ$^{+/+}$; C57BL/6) were examined, both groups of mice given 25 mg of OVA orally had significantly reduced IgG responses following i.p. challenge when compared with mice given PBS only (Fig. 1A, $P < 0.004$). Similar results were obtained when mice were challenged by the s.c. route (data not shown). On the other hand, reduced levels of fecal IgA antibodies were seen in TCRδ$^{-/-}$ mice given OVA orally when compared with fecal IgA levels in TCRδ$^{+/+}$ mice fed the same antigen (Fig. 1B, $P < 0.003$). These findings were confirmed at the single-cell level by using the ELISPOT assay. Prior oral administration of OVA also significantly inhibited splenic IgG responses in TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice to subsequent parenteral immunization (Fig. 2A, $P < 0.001$). The small intestinal lamina propria in both TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice contained OVA-specific IgA-producing cells, although the number of these was significantly lower in TCRδ$^{-/-}$ mice than in normal controls (Fig. 2B, $P < 0.001$). Oral administration of a large dose of protein antigen thus elicits reduced

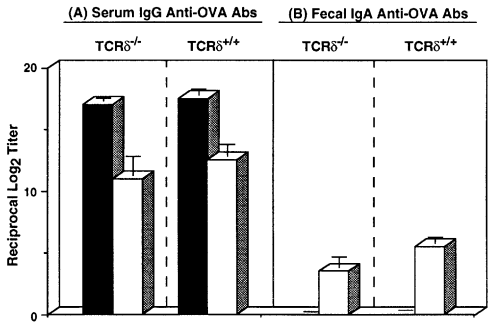

Fig. 1. OVA-specific (A) IgG responses in serum and (B) IgA levels in fecal extracts from TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice orally administered with 25 mg of OVA (□) or PBS (■). Both groups of mice were challenged by the i.p. route with 100 μg of OVA in 100 μl of CFA 7 days after feeding. Fourteen days after i.p. challenge, serum and fecal extract samples were collected and examined for OVA-specific IgG and IgA responses respectively. The results represent the mean values ± SEM for 12 mice in each experimental group.

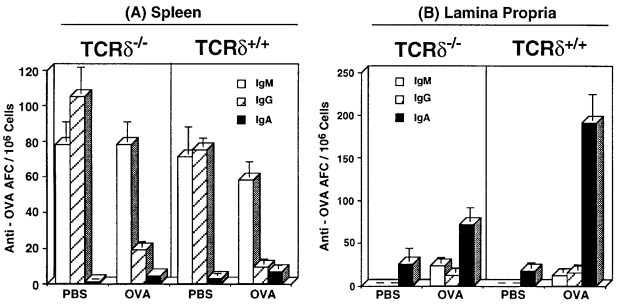

Fig. 2. Analysis of OVA-specific AFC in mice pre-fed with OVA or PBS before i.p. immunization with OVA plus CFA. Mononuclear cells isolated from the (A) spleen and (B) lamina propria of the small intestine were examined in an OVA-specific ELISPOT assay to determine the numbers of IgM (□), IgG (▨) and IgA (■) AFC. The results represent the mean values ± SEM for 12 mice in each experimental group.

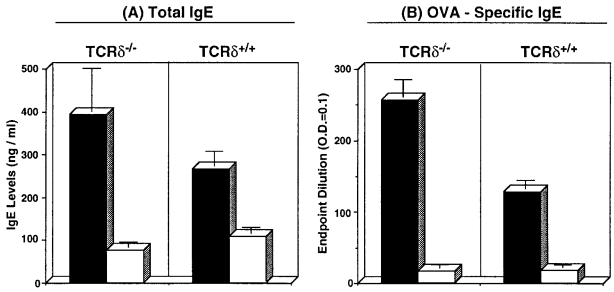

Fig. 3. IgE levels and OVA-specific IgE responses in TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice pre-fed OVA. Serum samples of TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice fed with 25 mg of OVA (□) or PBS (■) were obtained 14 days after i.p. immunization as described in the legend of Fig. 1. (A) Total IgE levels were determined by ELISA and (B) OVA-specific IgE antibodies were detected by a modified IgE luminometry assay. The results represent the mean values ± SEM for 12 mice in each experimental group.

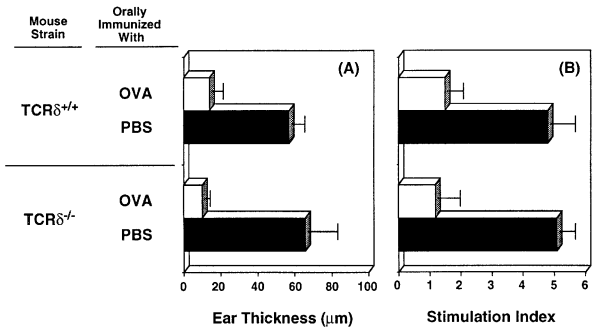

Fig. 4. Effects of the oral administration of OVA on (A) DTH and (B) T cell proliferative responses in TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice. (A) Thirteen days after i.p. immunization, PBS (20 μl) containing 10 μg of OVA was injected into the left ear pinna and the control right ear pinna was injected with PBS. The DTH response was expressed as the increase of ear swelling after OVA injection over the level of swelling in the control. (B) Fourteen days after i.p. immunization, splenic CD4$^+$ T cells were cultured with or without OVA for 4 days. The stimulation index was determined as c.p.m. of wells with antigen/wells without antigen (controls). The levels of [^{3}H]thymidine incorporation for each control well was between 500 and 1000 c.p.m. The results represent the mean values ± SEM from three separate experiments (triplicate wells/experiment).

antigen-specific B cell responses in both systemic and mucosal compartments of TCRδ$^{-/-}$ mice.

When the serum levels of IgE were examined in TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice fed either OVA or PBS, the TCRδ$^{+/+}$ mice that ingested OVA (25 mg) showed lower total IgE levels than did those given PBS (Fig. 3A, $P < 0.004$). IgE levels were similarly diminished in TCRδ$^{-/-}$ mice given OVA orally (Fig. 3A, $P < 0.002$). However, OVA-specific IgE antibody responses were comparably reduced in the TCRδ$^{-/-}$ and control mice fed OVA prior to systemic immunization (Fig. 3B, $P < 0.002$). Systemic IgE responses to protein antigens can therefore be inhibited in TCRδ$^{-/-}$ as well as in TCRδ$^{+/+}$ mice by prior oral administration of a protein antigen, at least in relatively high doses.

Oral OVA (high-dose) administration diminishes DTH and T cell proliferative responses in TCRδ$^{-/-}$ mice

We next determined whether or not oral administration of a protein antigen in relatively high doses similarly reduces subsequent DTH responsiveness in TCRδ$^{-/-}$ mice. Twenty-four hours after intradermal OVA challenge, DTH responses were determined by measuring the ear swelling reaction in systemically immunized mice. Both TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice given PBS orally and then immunized parenterally showed significant ear swelling (50–60 μm) when the challenge dose of OVA (10 μg) was injected into the pinna. DTH responses were reduced comparably in both groups of mice given 25 mg of OVA via the oral route prior to systemic immunization (Fig. 4A, $P < 0.02$). Having established that DTH responses were compromised in TCRδ$^{-/-}$ mice fed a relatively high dose of OVA, we examined the proliferative response of their T cells to OVA. Splenic CD4$^+$ T cells were cultured for 4 days with antigen-presenting cells (APC) in the presence of OVA. Relatively low levels of T cell proliferation were observed for splenic CD4$^+$ T cells from OVA-tolerized TCRδ$^{-/-}$ mice and TCRδ$^{+/+}$ mice, whereas relatively vigorous proliferative responses were observed for splenic CD4$^+$ T cells from control TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice given saline orally (Fig. 4B). The systemic hyporesponsiveness induced by the oral administration of a large dose of OVA is thus evident at the T cell level in TCRδ$^{-/-}$ mice.

OVA-specific T_h1 and T_h2 cytokine responses of splenic CD4$^+$ T cells

Since T cell unresponsiveness was induced by oral administration of OVA to TCRδ$^{-/-}$ as well as in control mice, it was of interest to evaluate the T_h1 and T_h2 cytokine responses in orally tolerized mice. Purified CD4$^+$ T cells from the spleens of TCRδ$^{-/-}$ mice previously fed OVA or PBS were incubated with or without 1 mg of OVA in the presence of autologous APC. Four days later, when the culture supernatants were harvested and examined by cytokine-specific ELISA, antigen-specific T_h1 (IFN-γ and IL-2) and T_h2 (IL-4, IL-5, IL-6 and IL-10) cell responses were significantly reduced in TCRδ$^{+/+}$ mice given OVA (Table 1). A virtually identical profile of down-regulation in cytokine synthesis was seen in the TCRδ$^{-/-}$ mice following oral administration of OVA. To confirm these findings at the cellular level, the number of T_h1 and T_h2 cytokine-producing cells was determined by cytokine-specific ELISPOT assays (Table 2). Splenic CD4$^+$ T cells from TCRδ$^{-/-}$ mice systemically immunized with OVA in CFA included significant numbers of IFN-γ-producing T_h1 type cells as well as IL-4-, IL-5-, IL-6- and IL-10-secreting T_h2-type cells 4 days after *in vitro* antigen stimulation (Table 2). Conversely, when TCRδ$^{-/-}$ mice received a tolerizing dose of OVA before parenteral challenge, the numbers of antigen-specific cytokine-producing cells were greatly reduced for both T_h1 and T_h2 types of cytokines, and similar results were obtained for the TCRδ$^{+/+}$ control mice (Table 2). These data indicate that T_h1 and T_h2 types of systemic responses are comparably down-regulated in TCRδ$^{-/-}$ and control mice by prior oral administration of a large dose of protein antigen.

Low doses of oral OVA fail to induce systemic unresponsiveness in TCRδ$^{-/-}$ mice

Since repeated oral administration of relatively low doses of antigen can elicit systemic tolerance, the role of γδ T cells

Table 1. Splenic CD4$^+$ T_h1 and T_h2 cytokine synthesis in TCR$\delta^{-/-}$ mice orally tolerized with OVA[a]

Mouse strain	Orally immunized with	T_h1 type[b]		T_h2 type[b]			
		IFN-γ (ng/ml)	IL-2 (U/ml)	IL-4 (pg/ml)	IL-5 (U/ml)	IL-6 (ng/ml)	IL-10 (pg/ml)
TCR$\delta^{+/+}$							
	PBS	7.7 ± 0.4[c]	1.1 ± 0.1	135.6 ± 15.0	91.4 ± 7.1	2.7 ± 0.2	1071.8 ± 276.0
	OVA	2.6 ± 0.2	0.4 ± 0.1	26.6 ± 7.9	24.1 ± 2.8	1.5 ± 0.1	221.4 ± 12.9
TCR$\delta^{-/-}$							
	PBS	5.4 ± 0.3	1.0 ± 0.1	91.4 ± 12.3	66.9 ± 5.3	2.8 ± 0.2	754.8 ± 79.4
	OVA	0.6 ± 0.1	0.3 ± 0.1	15.8 ± 6.9	ND[d]	0.8 ± 0.1	150.1 ± 67.1

[a]Splenic CD4$^+$ T cells (2×10^6/ml) from each group of mice were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and irradiated splenic feeder cells (4×10^6/well).
[b]Culture supernatants were harvested following 4 days (2 days for IL-2) of incubation and analyzed by the respective cytokine-specific ELISA.
[c]Results represent values (mean ± SEM) from three separate experiments.
[d]Not detected.

Table 2. The frequencies of splenic CD4$^+$ T_h1 and T_h2 cytokine producing cells from TCR$\delta^{-/-}$ mice orally tolerized with OVA[a]

Mouse strain	Orally immunized with	No. of cytokine-producing cells/10^6 CD4$^+$ T cells[b]					
		T_h1 type		T_h2 type			
		IFN-γ	IL-2	IL-4	IL-5	IL-6	IL-10
TCR$\delta^{+/+}$							
	PBS	85 ± 15[c]	36 ± 17	278 ± 97	120 ± 10	295 ± 60	85 ± 15
	OVA	0	0	100 ± 15	65 ± 12	0	0
TCR$\delta^{-/-}$							
	PBS	115 ± 16	0	320 ± 74	80 ± 14	245 ± 31	85 ± 12
	OVA	30 ± 1	0	0	0	0	0

[a]Splenic CD4$^+$ T cells (2×10^6/ml) from each group of mice were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and irradiated splenic feeder cells (4×10^6/well).
[b]Non-adherent cells were harvested following 4 days of incubation and analyzed by the respective cytokine-specific ELISPOT assays.
[c]Results represent values (mean ± SEM) from three separate experiments.

was assessed in this form of oral tolerance. Both TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice were fed three doses of 2.5 mg of OVA prior to challenge by the parenteral (s.c.) route. TCR$\delta^{+/+}$ mice showed lower anti-OVA IgG antibody responses in serum than did mice given oral PBS (Fig. 5A, $P < 0.02$). Splenic IgG AFC were also reduced in the TCR$\delta^{+/+}$ mice (Fig. 5B, $P < 0.004$). In contrast, no differences in serum IgG anti-OVA antibody or splenic IgG AFC responses were observed between control and oral feeding groups of TCR$\delta^{-/-}$ mice.

OVA-specific T cell responses were also assessed in TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice given small oral doses of antigen. Oral administration of small doses of OVA prior to systemic challenge resulted in diminished ear swelling or DTH ($P < 0.004$) in TCR$\delta^{+/+}$ mice but not in TCR$\delta^{-/-}$ mice (Fig. 6A). A similar pattern was revealed by analysis of the CD4$^+$ T cell proliferative responses. Thus, splenic CD4$^+$ T cells from TCR$\delta^{-/-}$ mice fed either PBS or small doses of OVA responded with significant proliferation after OVA stimulation, while the CD4$^+$ T cell proliferative responses were significantly reduced in OVA pre-fed TCR$\delta^{+/+}$ mice (Fig. 6B, $P < 0.02$). Collectively, these results suggest that the γδ T cells are essential for the

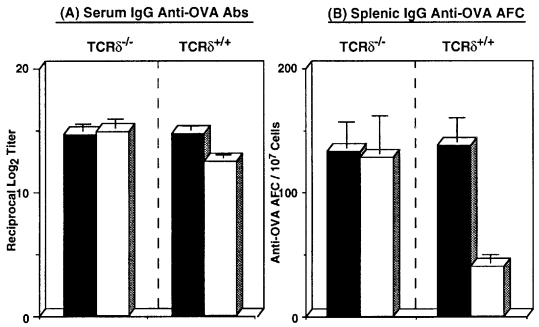

Fig. 5. OVA-specific IgG responses in (A) serum and (B) spleen from TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice fed 2.5 mg of OVA (□) or PBS (■) at days 0, 2 and 5. Both groups of mice were challenged by the s.c. route with 100 μg of OVA in 100 μl of CFA at day 7. Ten days after s.c. challenge, serum samples were collected and examined for OVA-specific IgG responses. Mononuclear cells isolated from spleen were also used in an OVA-specific ELISPOT assay to determine the numbers of IgG AFC. The results represent the mean values ± SEM for 12 mice in each experimental group.

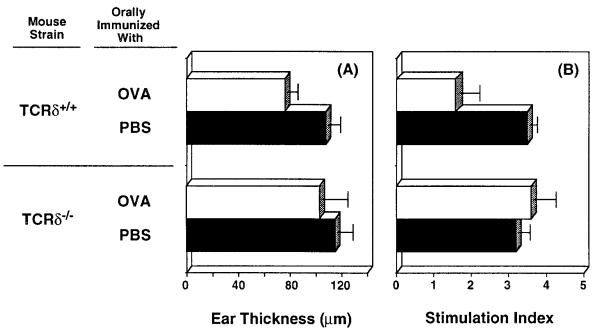

Fig. 6. The effects of oral administration of OVA on (A) DTH and (B) T cell proliferative responses in TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice. Ten days after s.c. immunization, DTH responses were determined as described in the legend of Fig. 4 and splenic CD4$^+$ T cells were cultured with or without OVA for 4 days. The stimulation index was determined as described in the legend of Fig. 4. The levels of [^{3}H]thymidine incorporation for each control well was between 500 and 1000 c.p.m. The results represent the mean values ± SEM from two separate experiments (triplicate wells/experiment).

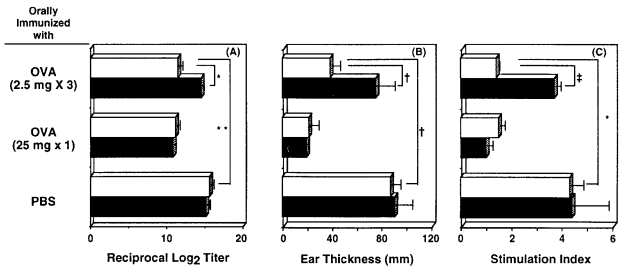

Fig. 7. The effects of anti-γδ mAb treatment on the induction of oral tolerance. (A) OVA-specific IgG antibody responses in serum from mice treated with anti-γδ mAb (■) or hamster IgG (□) fed either 2.5 mg or 25 mg of OVA. The groups of mice were then challenged by the i.p. or s.c. route with 100 μg of OVA in 100 μl of CFA. After systemic challenge, serum samples were collected and examined for OVA-specific IgG antibody responses. The effects of oral administration of OVA on (B) DTH and (C) T cell proliferative responses in anti-γδ mAb (■) or hamster IgG (□) treated mice. DTH responses were determined as described in the legend to Fig. 4 and splenic CD4$^+$ T cells were cultured with or without OVA for 4 days. The stimulation index was determined as described in the legend to Fig. 4. The levels of [^{3}H]thymidine incorporation for each control well was between 500 and 1000 c.p.m. The results represent the mean values ± SEM from two separate experiments (triplicate wells/experiment). $^*P < 0.03$, $^{**}P < 0.01$, $^\dagger P < 0.04$, $^\ddagger P < 0.02$.

induction of mucosally induced tolerance established by low doses of oral antigen.

Similar results were also obtained when HEL was used as antigen. TCR$\delta^{+/+}$ mice fed relatively low doses of HEL showed significant reductions in anti-HEL IgG antibody responses in serum when compared with mice fed PBS (reciprocal $\log_2$ titer, HEL fed: 8.2 ± 0.2 versus PBS fed: 14.4 ± 0.5, $P < 0.001$). On the other hand, TCR$\delta^{-/-}$ mice fed low doses of HEL had no reductions in HEL-specific IgG antibody responses (reciprocal $\log_2$ titer, HEL fed: 14.2 ± 0.6 versus. PBS fed: 14.5 ± 0.5). These results, indicating a lack of mucosally induced low-dose tolerance in TCR$\delta^{-/-}$ mice, were confirmed by the observation of normal DTH and T cell proliferative responses in these mice (data not shown). These findings indicate the absence of low-dose oral tolerance in TCR$\delta^{-/-}$ mice is antigen-specific.

Dose-dependent mucosally induced tolerance in anti-γδ mAb-treated TCRδ$^{+/+}$ mice

In order to examine whether or not dose-dependent mucosally induced tolerance is related to ontogenetic abnormalities in TCR$\delta^{-/-}$ mice, a series of experiments were performed with TCR$\delta^{+/+}$ mice treated with anti-γδ mAb. The anti-γδ treated TCR$\delta^{+/+}$ mice fed a high dose of OVA prior to systemic immunization showed reduced IgG antibody responses when compared with anti-γδ treated TCR$\delta^{+/+}$ mice given oral PBS only (Fig. 7A, $P < 0.02$). In contrast, when anti-γδ treated TCR$\delta^{+/+}$ mice were fed low doses of OVA, no reduction of the anti-OVA IgG antibody response was seen. A similar pattern of responses was noted at the T cell level. Thus, anti-γδ treated TCR$\delta^{+/+}$ mice fed low doses of OVA resulted in the maintenance of DTH and T cell proliferative responses after systemic challenge with OVA/CFA. On the other hand, these T cell responses were markedly reduced in anti-γδ treated TCR$\delta^{+/+}$ mice given a high oral dose of OVA (Fig. 7B and C, $P < 0.004$ and $P < 0.02$ respectively). These results confirmed the dose-dependent γδ T cell requirement for mucosally induced tolerance observed in TCR$\delta^{-/-}$ mice.

Elevated levels of IL-10 in TCRδ$^{+/+}$ but not TCRδ$^{-/-}$ mice given low oral doses of OVA

The T_h1 and T_h2 cytokine responses in TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice given small doses of OVA orally were assessed by culturing CD4$^+$ T cells for 4 days followed by measurement of cytokines in the supernatants. The major difference observed in cytokine responses of TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice involved IL-10 production. Although none of the experimental groups produced IL-10 without *in vitro* antigen stimulation, splenic CD4$^+$ T cells from TCR$\delta^{+/+}$ mice fed multiple small doses of OVA prior to systemic immunization produced elevated levels of IL-10 when compared with TCR$\delta^{+/+}$ mice given PBS only (Table 3, $P < 0.03$). In contrast, CD4$^+$ T cell IL-10 synthesis was significantly low in TCR$\delta^{-/-}$ mice given small oral doses of OVA, although IL-10 production by CD4$^+$ T cells from control TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice was comparable. These results were confirmed at the RNA level by quantitative RT-PCR analysis of IL-10 transcripts (data not shown). When production of other cytokines was examined, significant differences were not seen between the levels produced by CD4$^+$ T cells from TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice. These findings suggest that IL-10 production may play an important role in low-dose oral tolerance induction and that γδ T cells may regulate this response by CD4$^+$ T cells.

In order to examine a possible role for TGF-β in the induction and maintenance of low-dose oral tolerance, we measured levels of TGF-β in CD4$^+$ T cell cultures from orally treated TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice. The levels of TGF-β did not increased in culture supernatants of splenic CD4$^+$ T cells after OVA stimulation, suggesting that TGF-β may not have an effect on the induction of systemic unresponsiveness by low doses of oral OVA.

Table 3. Splenic CD4$^+$ T_h1 and T_h2 cytokine synthesis in TCRδ$^{+/+}$ and TCRδ$^{-/-}$ mice given low oral doses of OVA[a]

Mouse strain	Orally immunized with	T_h1 type[b]		T_h2 type[b]			
		IFN-γ (ng/ml)	IL-2 (U/ml)	IL-4 (pg/ml)	IL-5 (U/ml)	IL-6 (ng/ml)	IL-10 (pg/ml)
TCRδ$^{+/+}$							
	PBS	5.1 ± 0.5[c]	3.1 ± 0.6	250.2 ± 78.0	12.5 ± 2.3	11.1 ± 2.5	883.2 ± 56.2
	OVA	5.1 ± 0.5	ND[d]	ND	9.5 ± 3.0	12.2 ± 3.0	1446.3 ± 50.2
TCRδ$^{-/-}$							
	PBS	4.0 ± 0.4	4.3 ± 0.7	187.5 ± 2.0	9.9 ± 2.3	4.1 ± 1.1	912.5 ± 126.3
	OVA	5.2 ± 0.5	ND	ND	7.0 ± 0.5	7.6 ± 1.5	73.4 ± 10.4

[a]Splenic CD4$^+$ T cells (1×10^6/ml) from each group of mice were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and irradiated splenic feeder cells (2×10^6/well).
[b]Culture supernatants were harvested following 4 days (2 days for IL-2) of incubation and analyzed by the respective cytokine-specific ELISA.
[c]Results represent the values (mean ± SEM) from three separated experiments.
[d]Not detected.

Table 4. γδ T cells enhance *In vitro* IL-10 synthesis by CD4$^+$ T cells from TCRδ$^{-/-}$ mice given low oral doses of OVA[a]

	Levels of IL-10 (pg/ml) in CD4$^+$ T cell cultures[b]					
			Splenic γδ T cells[c]		IEL γδ T cells[c]	
Days	None	Naive splenic γδ T cells	Direct	Indirect	Direct	Indirect
7	172.7 ± 8.0[d]	184.8 ± 36.2	344.7 ± 0.4	248.0 ± 38.0 *	412.0 ± 44.5	308.1 ± 18.3
14	190.8 ± 56.7	ND[e]	462.5 ± 6.3	347.2 ± 36.4 *	516.5 ± 84.1	403.3 ± 18.5

[a]Splenic CD4$^+$ T cells (2×10^6/ml) from TCRδ$^{-/-}$ mice fed low doses of OVA were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and irradiated splenic feeder cells (4×10^6/well).
[b]Culture supernatants were harvested following 7 and 14 days of incubation and analyzed by IL-10 specific ELISA. The control wells without antigen stimulation and those with γδ T cells alone contained 13.1 ± 4.1 and 4.4 ± 0.2 pg/ml of IL-10 respectively.
[c]γδ T cells (2.5×10^5 – 1×10^6/well) from spleen or IEL of TCRδ$^{+/+}$ mice fed low doses of OVA were added into CD4$^+$ T cell cultures (Direct). In some cases, γδ T cells were added to the upper well of double chamber plate (Indirect). The levels of IL-10 were statistically increased ($P < 0.03$) in comparison to the cultures without γδ T cells.
[d]Results represent the value (mean ± SEM) from three separate experiments.
[e]Not determined.
*$P = 0.02$.

γδ T cells can up-regulate IL-10 synthesis in CD4$^+$ T cells from TCRδ$^{-/-}$ mice fed low doses of OVA

In order to address the potential role of γδ T cells in the induction of IL-10-mediated low-dose tolerance, γδ T cells and CD4$^+$ T cells respectively were isolated from TCRδ$^{+/+}$ and TCRδ$^{-/-}$ mice previously fed low doses of OVA. These T cell subsets were cultured separately, mixed together or separated by filters in the presence of antigen and feeder cells for 7 or 14 days. The culture supernatants were harvested and IL-10 levels were examined by ELISA. The control wells without antigen stimulation and those with γδ T cells alone contained 13.1 ± 4.1 and 4.4 ± 0.2 pg/ml of IL-10 respectively. The levels of IL-10 production by CD4$^+$ T cells were increased in the presence of γδ T cells from spleen or intestinal epithelium of OVA fed and immunized (Table 4, $P < 0.03$), but not by γδ T cells taken from naive mice or from high-dose tolerant TCRδ$^{-/-}$ mice. Increased levels of IL-10-specific mRNA expression in CD4$^+$ T cells but not in γδ T cells after 7 days of culture further confirmed our findings (data not shown). Interestingly, when direct cell–cell interactions between γδ T cells and CD4$^+$ T cells were prevented by using a double-chamber well system, reduced IL-10 production was noted (Table 4). Splenic γδ T cells in the trans-well system showed marked decreases in IL-10 production when compared with direct cell–cell contact cultures (Table 4, $P < 0.02$). These results provide direct evidence that γδ T cells serve an immunoregulatory role for CD4$^+$ T cells of IL-10 production under conditions of low-dose oral tolerance. The present findings further suggest that both soluble factors produced by γδ T cells and cell–cell interactions between γδ T cells and CD4$^+$ T cells are essential for maximum IL-10 synthesis which leads to an inhibition of systemic immune responses.

Discussion

The results of the present study indicate that the role of γδ T cells in oral tolerance depends on the antigen dose. The γδ T cells are not required for the systemic B or T cell unresponsiveness by relatively high doses of orally administered antigen. To the contrary, this T cell subset regulates low-dose oral tolerance via modulation of IL-10 production by $CD4^+$ T cells. This antigen dose-dependent regulatory dichotomy provides an interesting paradox with regard to the local function of intestinal γδ T cells in oral antigen responses. The sentinel location and increased numbers of γδ T cells in the intestinal epithelium intuitively suggest a significant role in the responses to orally administered antigens. Their absence has been associated with the loss of certain epithelial cell functions (29) and to the diminishment of mucosal IgA responses to oral antigens (21), a finding confirmed in the present studies. The absence of the γδ T cell subset would therefore be expected to influence the immunologic homeostasis pathway involved in mucosally induced hyporesponsiveness and our present findings indeed indicate compromised oral tolerance in $TCR\delta^{-/-}$ mice given relatively low doses of OVA. We further demonstrated that γδ T cells are able to up-regulate IL-10 synthesis in $TCR\delta^{+/+}$ mice tolerized with low oral antigen doses. This finding provides direct evidence that γδ T cells regulate IL-10 synthesis by $CD4^+$ T cells in the induction of low-dose oral tolerance. Our results also confirm studies in which γδ T cells were shown to regulate the induction and maintenance of systemic tolerance to antigens administered via oral (22,23), respiratory mucosa (4) or portal vein routes (30,31).

In striking contrast, the present results indicate that systemic tolerance following oral antigen administration can be induced normally by relatively high antigen doses in the complete absence of γδ T cells. Equivalent reductions in systemic IgG and IgE antibody responses, DTH, T cell proliferation, and both T_h1 and T_h2 cytokine responses to OVA were all seen in both $TCR\delta^{-/-}$ and $TCR\delta^{+/+}$ mice fed with this antigen prior to parenteral immunization. The same results were observed in experiments in which $TCR\delta^{+/+}$ mice were given anti-γδ mAb treatment. Moreover, TNBS and oxazolone feedings induced tolerance to contact sensitization in $TCR\delta^{-/-}$ mice (data not shown). Although these results differ from those of similar studies in a previous report (22), others have shown that γδ T cells are not essential for the induction of aerosol-induced IgE unresponsiveness to OVA (32). We conclude that, whereas the γδ T cells are required for normal mucosal epithelial function and IgA responsiveness (21,31), they do not play an obligatory role in the establishment of high-dose oral or mucosally induced tolerance.

A major difference between earlier studies of γδ T cell involvement in oral tolerance and the present ones involved the manner in which the γδ T cells were manipulated. Some studies have relied primarily on anti-γδ mAb treatment of mice (22,23), while others involved the transfer of antigen-stimulated γδ T cells to naive recipients (4,23,30,31). Engagement of the γδ T cells via their TCR was a common feature in most of these experiments and one proposed mechanism for the tolerization was the production of inhibitory cytokines by the γδ T cells (31). This possibility gains support from the demonstration that treatment of mice with anti-γδ TCR antibodies does not eliminate γδ T cells, but instead leads indirectly to the activation of recipient αβ T cells (33,34). It is therefore possible that while γδ T cells are non-essential for the induction of tolerance to antigens administered by mucosal routes, they may still play an active role in this process under certain natural and experimental conditions. Our results in $TCR\delta^{-/-}$ mice and in an anti-γδ mAb treatment system have provided evidence that γδ T cells may indeed play distinct roles in the induction of orally induced tolerance depending on the dose of antigen. Specifically, our data indicates that low-dose oral tolerance requires γδ T cells for development of the systemic unresponsiveness and high-dose oral tolerance does not.

The difference in outcomes between the present studies and those of Ke *et al*. (23), are not easily reconciled. These investigators found that $TCR\delta^{-/-}$ mice were refractory to tolerance induction by various doses of oral antigen as assessed by reduction in IgG antibody responses, and T cell proliferative and cytotoxic T lymphocyte responses to subsequent systemic immunization. The resolution of this discrepancy must await additional studies. However, a second potentially important difference in the present study and those of other investigations (23) is the manner in which oral tolerance was induced by oral feeding. This is a significant consideration since the mechanisms involved in mucosally induced tolerance may differ according to the antigen dose (1). Indeed, we found systemic tolerance was not induced in $TCR\delta^{-/-}$ mice pre-administered orally with multiple small doses of OVA, but was induced in identically treated $TCR\delta^{+/+}$ mice. Prolonged oral administration or feeding with doses of antigen can selectively activate $CD4^+$ or $CD8^+$ T cells which secrete suppressive cytokines, e.g. TGF-β, IL-4 and IL-10, that may down-regulate antigen-specific immune responses (1). In tolerance induced by small doses of oral antigen, γδ T cells may contribute to unresponsiveness by the production of TGF-β, considered to be a key cytokine for the induction of active suppression (35). However, we could not demonstrate an inhibitory effect for TGF-β for the induction of low-dose oral tolerance. Instead, in the present study, splenic $CD4^+$ T cells in $TCR\delta^{+/+}$ mice given low doses of OVA produced higher levels of IL-10 than did those of non-tolerized $TCR\delta^{+/+}$ mice, while those from $TCR\delta^{-/-}$ mice fed low doses of OVA failed to produce IL-10. A recent study showed that regulatory $CD4^+$ T cell clones which selectively produce IL-10, termed T regulatory cell 1 (T_r1), can dramatically reduce T cell proliferative responses (36). This T cell subset was found to be responsible for preventing the development of inflammatory bowel disease in a mouse model (36). These results support the importance of IL-10 production for induction of low-dose oral tolerance. The present results indicate that γδ T cells are able to up-regulate the synthesis of this important cytokine by $CD4^+$ T cells. The γδ T cells may thus induce IL-10 producing regulatory $CD4^+$ T cells that can limit systemic immune responses in mice primed with low oral antigen doses.

The findings obtained in a trans-well response system provide evidence indicating that both soluble factors produced by γδ T cells and direct cell–cell interactions are essential for maximum IL-10 production. Monocyte chemotactic protein (MCP)-1, a CC chemokine, is a candidate

soluble factor for the induction of IL-10 synthesis. In support of this idea, MCP-1 enhanced T_h2-type cytokine responses when transgenic T cells were stimulated via the TCR–CD3 complex (37). MCP-1 may also play an immunoregulatory role in the induction of oral tolerance by inhibition of T_h1-type responses (38). The γδ T cells in spleen and in intestinal epithelium of low-dose tolerized mice could produce MCP-1 since γδ T cells from spleen, intestinal epithelium or epidermis have been reported to exhibit distinct mRNA levels for chemokines such as macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES and lymphotactin (39). Indeed, our separate study has shown that intestinal intraepithelial γδ T cells stimulated with IL-7 and stem cell factor expressed mRNA specific for MCP-1 (manuscript in preparation). Our current findings provide direct evidence that γδ T cells can regulate $CD4^+$ T cell synthesis of IL-10, a cytokine that is important in the induction of low-dose oral tolerance, and the mechanism by which γδ T cells regulate IL-10 synthesis by $CD4^+$ T cells is currently under investigation. Interestingly, levels of IL-6, an important cytokine for terminal B cell differentiation, was higher in $TCR\delta^{-/-}$ mice given low oral doses of OVA than in $TCR\delta^{-/-}$ mice fed PBS. The findings that γδ T cells can play a regulatory role in the induction of T_h2-type cytokine production by $CD4^+$ T cells complements those in previous studies, indicating that γδ T cells may modulate T_h2-type cytokine-mediated B cell responses (e.g. for IgE and IgA) (4,21).

A large single oral dose of protein can lead to T cell anergy or clonal deletion (1,40). In accordance with this outcome, our studies indicate that both T_h1- and T_h2-type OVA responses of splenic T cells were reduced when a large oral dose of OVA was given prior to parenteral challenge. A recent study involving adoptive transfer of OVA-specific transgenic T cells also indicated that a large oral dose of OVA elicited T cell anergy (41). In addition, mice rendered deficient in IL-4 production and thus T_h2 defective could be tolerized with a large oral dose of OVA, and IL-4-regulated IgG1 and IFN-γ-induced IgG2a antibody responses were both down-regulated (9). On the other hand, our previous study had shown that elevated levels of IFN-γ production occur in orally tolerized BALB/c mice (25). The difference between the previous study (25) and our present investigation can be explained by the use of different experimental systems and strains of mice. The previous study was performed by using BALB/c mice which are known to be a T_h2 prone strain. Further, these mice were immunized twice with OVA and CFA after OVA feeding. Thus, it is possible and even likely that high IFN-γ synthesis was required in orally tolerized BALB/c mice in order to down-regulate preferential T_h2-type responses. In addition, although purified $CD4^+$ T cells were used in the *in vitro* system for T_h1 and T_h2 cytokine analysis in the present study, our previous work was performed with whole T cell populations. This could be an additional explanation for the high level of IFN-γ synthesis in the previous report since both $CD4^+$ and $CD8^+$ T cells are sources of this cytokine.

In summary, the present studies also indicate that γδ T cells regulate IL-10-producing $CD4^+$ T cells for subsequent inhibition of systemic IgG responses in mice orally tolerized with low doses of antigen. Thus, γδ T cells regulate the induction of low-dose oral tolerance, but are not essential participants in the induction of systemic unresponsiveness to high doses of orally ingested antigen.

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Abbreviations

AFC	antibody-forming cells
APC	antigen-presenting cell
CFA	complete Freund's adjuvant
DTH	delayed-type hypersensitivity
HEL	hen egg lysozyme
MCP	monocyte chemotactic protein
MIP	macrophage inflammatory protein
OVA	ovalbumin
TGF	transforming growth factor

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