Brief Definitive Report

γ/δ T Cell–deficient Mice Have Impaired Mucosal Immunoglobulin A Responses

By Kohtaro Fujihashi,* Jerry R. McGhee,*‡ Mi-Na Kweon,* Max D. Cooper,‡§‖¶ Susumu Tonegawa,** Ichiro Takahashi,‡‡ Takachika Hiroi,§§ Jiri Mestecky,‡§ and Hiroshi Kiyono*‡§§

*From The Immunobiology Vaccine Center, Mucosal Immunization Research Group, Departments of *Oral Biology, ‡Microbiology, §Medicine, and ‖Pediatrics; ¶Howard Hughes Medical Institute, the University of Alabama at Birmingham, Medical Center, Birmingham, Alabama 35294; **Center for Cancer Research, Department of Biology, Howard Hughes Medical Institute and Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; ‡‡Department of Oral Microbiology, Faculty of Dentistry; and §§Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan*

Summary

Mucosal tissues of mice are enriched in T cells that express the γ/δ T cell receptor. Since the function of these cells remains unclear, we have compared mucosal immune responses in γ/δ T cell receptor–deficient (TCRδ$^{-/-}$) mice versus control mice of the same genetic background. The frequency of intestinal immunoglobulin (Ig) A plasma cells as well as IgA levels in serum, bile, saliva, and fecal samples were markedly reduced in TCRδ$^{-/-}$ mice. The TCRδ$^{-/-}$ mice produced much lower levels of IgA antibodies when immunized orally with a vaccine of tetanus toxoid plus cholera toxin as adjuvant. Conversely, the antigen-specific IgM and IgG antibody responses were comparable to orally immunized control mice. Direct assessment of the cells forming antibodies against the tetanus toxoid and cholera toxin antigens indicated that significantly lower numbers of IgA antibody–producing cells were present in the intestinal lamina propria and Peyer's patches of TCRδ$^{-/-}$ mice compared with the orally immunized control mice. The selective reduction of IgA responses to ingested antigens in the absence of γ/δ T cells suggests a specialized role for γ/δ T cells in mucosal immunity.

The mucosal immune system is considered as a separate functional entity quite independent of the systemic immune compartment, since it possesses unique anatomical features and is composed of specialized subsets of lymphoid cells. For example, the γ/δ T cells are prevalent among the intestinal intraepithelial lymphocyte (IEL) population (1–5). The underlying lamina propria of the small intestine also contains a higher frequency of γ/δ T cells than the systemic lymphoid tissues (6). Most of the resident γ/δ T cells in intestinal epithelium express cell surface CD8 molecules composed of αα homodimeric chains (7, 8), and their Vγ gene repertoire differs from that of the systemic γ/δ T cells. Thus the intestinal, uterine, and skin γ/δ T cells express the Vγ7, Vγ6, and Vγ5 genes, respectively, whereas systemic γ/δ T cells predominantly express the Vγ4 gene (9, 10).

Studies of TCR-deficient mice suggest an important role for γ/δ T cells in immune responses to intracellular bacteria and parasites (11–13). The γ/δ T cells appear to be required for control of mycobacterial infection (11) and contribute to immunity after *Plasmodium yoelii* vaccination, since γ/δ TCR-deficient (TCRδ$^{-/-}$) mice do not respond normally to these intracellular microorganisms (12). The γ/δ T cells also play an accessory role in the late stages of protective immune responses to *Mycobacterium bovis* Bacillus Calmette-Guerin (13). These observations clearly implicate γ/δ T cells in microbial immunity, but the precise function of γ/δ T cells in specific immune responses remains unclear. Our previous studies suggested that γ/δ T cells are important for the maintenance of mucosal IgA responses in the presence of systemic unresponsiveness or tolerance induced by oral immunization (14, 15).

To examine the role of γ/δ T cells in the induction and regulation of mucosal immunity, we compared the immune responses in TCRδ$^{-/-}$ and control mice to oral immunization with tetanus toxoid (TT) and cholera toxin (CT) as mucosal adjuvants. This immunization regimen induces intestinal secretory IgA (S-IgA) responses as well as circulating IgG and IgA antibodies to both antigens in normal mice (16, 17), thus allowing examination of the role of γ/δ T cells in both mucosal and systemic responses. In this communication, we report that in contrast to their normal counterparts, TCRδ$^{-/-}$ mice display significant alterations in IgA responses induced by oral immunization.

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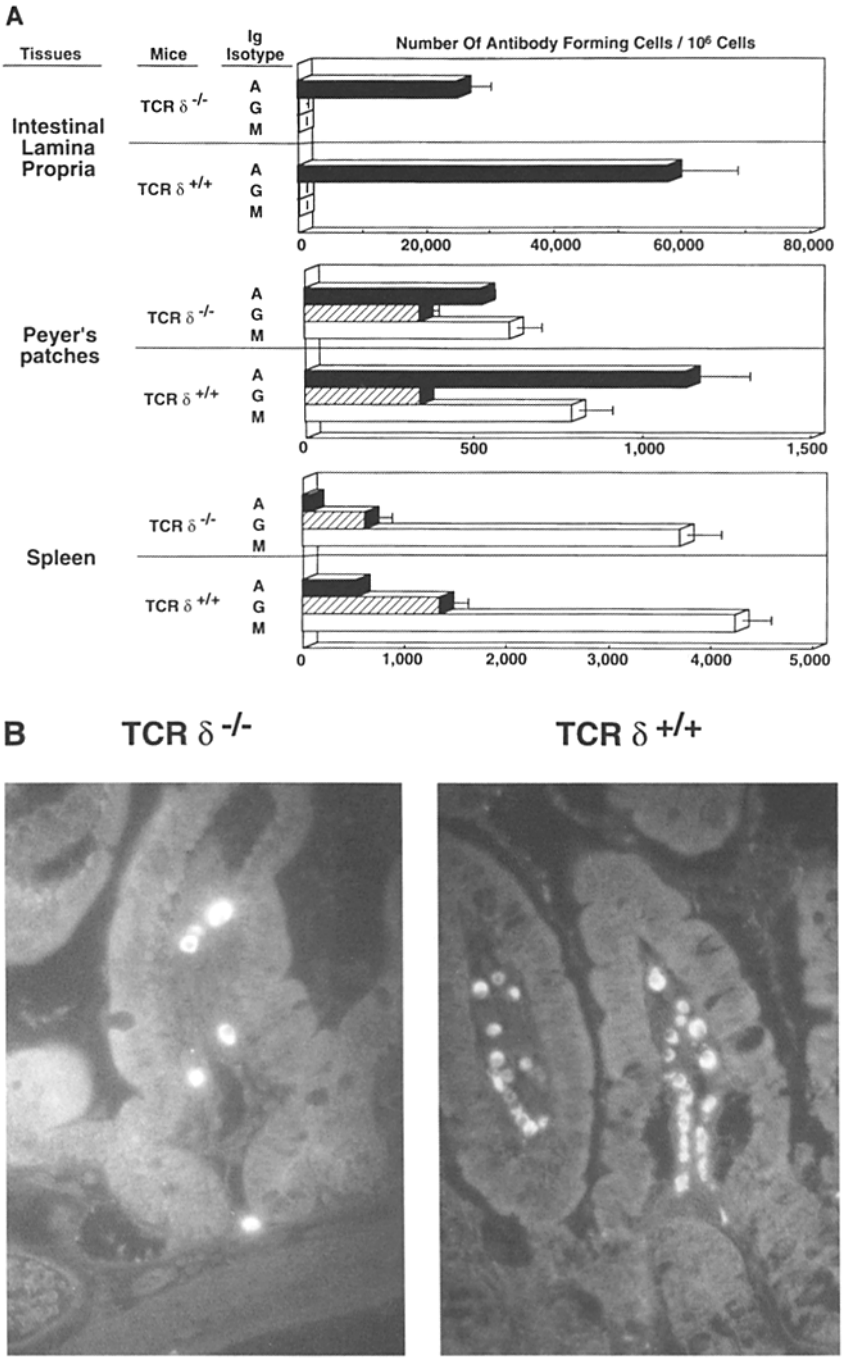

Figure 1. Effect of TCR-δ gene disruption on immunoglobulin production (*A*) Antibody-producing cells of IgM (□), IgG (▨), and IgA (■) isotypes were examined in gut-associated tissues and the spleen by an ELISPOT assay (*B*) Immunofluorescence staining of small intestine tissue sections from TCRδ$^{-/-}$ and control TCRδ$^{+/+}$ mice. IgA-containing cells were visualized by incubation with biotinylated goat anti-mouse α F(ab')$_2$ followed by avidin-AMCA. Results represent the values (mean ± SEM) for 12 mice in each experimental group.

Materials and Methods

γ/δ T Cell–deficient Mice. The TCRδ$^{-/-}$ mice were constructed by introducing germline mutations in the TCR-δ chain gene on a (129/Ola × C57Bl/6) (H-2^b) background (18). The δ chain–deficient and (129/O1a × C57Bl/6)F$_2$ normal mice (TCRδ$^{+/+}$) were barrier maintained in Trexler isolators and have remained pathogen antibody 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 lib. The mice were between 7 and 10 wk of age at the beginning of the experiment.

Immunization. Mice were immunized orally on days 0, 7, and 14 with 0.25 ml PBS containing a mixture of vaccine-grade TT (250 μg/mouse; kindly provided by Dr. Patricia J. Freda Pietrobon [Connaught Laboratories Inc., Swiftwater, PA]) and CT (10 μg/mouse; List Biologic Laboratories, Inc., Campbell, CA) (16, 17).

Antibody Assays. Antibody titers in serum and fecal extracts were determined by ELISA (16, 17). Assay plates (Falcon Microtest; Becton Dickinson & Co., Oxnard, CA) were coated with an optimal concentration of TT (100 μl of 5 μg/ml TT, equivalent to 0.8 floculation units/ml) or recombinant CT-B (100 μl of 5 μg/ml of CT-B; List Biological Laboratories Inc.) in PBS. End

point titers were expressed as the last dilution yielding an optical density at 414 nm (OD414) of >0.1 U above negative control values after a 15-min incubation. In some experiments, the plates were coated with goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) at 2 μg/ml (100 μl/well). Total IgM, IgG, and IgA levels in serum saliva, bile, and fecal extracts were estimated by comparison with serial dilutions of mouse IgM, IgG, and IgA standards (Southern Biotechnology Associates).

Enumeration of Antibody-producing Cells. The spleen was removed aseptically, and single-cell suspensions were prepared as described (14, 15). Peyer's patches carefully excised from the intestinal wall were dissociated using the neutral protease enzyme Dispase® (Boehringer Mannheim Corp., Indianapolis, IN) in Joklik-modified medium (Life Technologies, Inc., Gaithersburg, MD) to obtain single-cell preparations (16, 17). Mononuclear cells in the lamina propria were isolated after removal of PP from the small intestine using a combination of enzymatic dissociation and discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden) (6, 19). Mononuclear cells in the interface between the 40 and 75% layers were removed, washed, and resuspended in RPMI 1640 containing 10% FCS. An enzyme-linked immuno spot assay was used to detect cells producing IgM, IgG, and IgA antibodies (14, 16). 96-well nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, MA) were coated with goat anti-mouse Ig at 2 μg/ml (100 μl/well) to detect total IgM, IgG, and IgA antibody-forming cells (AFC), 5 μg/ml of CT-B (100 μl/well) for anti-CT-B-specific AFCs, or 5 μg/ml of TT (100 μl/well) for anti-TT-specific AFC (16, 19).

Immunohistology of the Small Intestine. Samples of mouse jejunum and ileum were obtained from TCRδ$^{-/-}$ and control mice for conventional histology and staining of antibody-containing cells (19). 1-cm portions of intestine were opened longitudinally, mounted on thin cards, and washed in three changes of PBS at 4°C over a period of 2 h to remove tissue-associated Ig. The tissue was then fixed in 5% glacial acetic acid in 95% ethanol at −20°C for 24 h before paraffin embedding. 4-μm-thick serial tissue sections were mounted on glass slides, and Ig-containing cells were visualized by three-color staining with FITC-labeled goat $(Fab')_2$ anti-mouse μ (Southern Biotechnology Associates), RITC-labeled goat $F(ab')_2$ anti-mouse γ (Southern Biotechnology Associates), and biotinylated $F(ab')_2$ anti-mouse α (Southern Biotechnology Associates) followed by avidin-7-amino-4-methylocoumarin-3-acetic acid (AMCA; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA [19]).

Statistics. Mean numbers of AFC in the different tissues were compared among experimental and control groups by Wilcoxon signed-rank test. Mean percentages of Ig-containing cells per villi were calculated from multiple determinations of experimental and control groups and compared by Wilcoxon signed-rank test.

Results

Deficiency of IgA-producing Cells in TCRδ$^{-/-}$ Mice. The vast majority of γ/δ T cells are located in the epithelium of the small intestine of normal mice (1–3), suggesting that absence of this T cell subset in this mucosal effector site could influence immunological homeostasis. Since previous studies have not assessed γ/δ T cells for their participation in regulation of humoral and mucosal immune responses, we initially examined the effects of TCR-δ gene disruption on the numbers of IgM-, IgG-, and IgA-producing cells in systemic and mucosal tissues, and levels of IgM, IgG, and IgA present in serum, saliva, bile, and fecal extracts. When the frequency of Ig-producing cells was compared between spleens of nonimmunized TCRδ$^{-/-}$ mice and control mice of the same (129 × B6)F_2 background (TCRδ$^{+/+}$), comparable numbers of IgM- or IgG-producing cells were seen. In constrast, the numbers of IgA-secreting cells in the intestinal lamina propria and Peyer's patches of TCRδ$^{-/-}$ mice were significantly lower than in control TCRδ$^{+/+}$ mice ($P < 0.02$, Fig. 1 *A*).

The frequency of IgA-containing cells was also evaluated in tissue sections of jejunum and ileum by immunohistological analysis. Enumeration of the IgA-producing cells in the lamina propria of the small intestine indicated a reduction of IgA plasma cells in TCRδ$^{-/-}$ mice. The numbers of intestinal IgA plasma cells in normal and mutant mice were 460 ± 63 and 116 ± 22/10 fields, respectively ($P < 0.002$, Fig. 1 *B*). The reduction in IgA-producing cells in TCRδ$^{-/-}$ mice was confirmed by an assessment of antibody levels in serum, saliva, bile, and fecal extracts using an isotype-specific ELISA. The IgA levels were reduced by ~80% in fecal extracts obtained from TCRδ$^{-/-}$ mice compared with fecal IgA levels in control TCRδ$^{+/+}$ mice ($P < 0.001$, Table 1). Serum IgA levels were also reduced in TCRδ$^{-/-}$ mice ($P < 0.003$), whereas IgM and IgG levels were normal (Fig. 2, Table 1). Further, IgA levels in saliva and bile of TCRδ$^{-/-}$ mice were significantly lower than controls ($P < 0.005$, Table 1).

Reduction of TT-specific IgA Responses in Orally Immunized TCRδ$^{-/-}$ Mice. The antibody response to TT was examined in TCRδ$^{-/-}$ mice that were immunized orally with a vaccine containing TT and CT to examine the potential involvement of γ/δ T cells in antigen-specific IgA responses. When TT-specific serum antibody responses were compared after three oral doses of the combined vaccine, the TCRδ$^{-/-}$ mice and their normal littermates (TCRδ$^{+/+}$) produced almost identical levels of IgG antibodies to both proteins, whereas lower serum IgA responses were seen in TCRδ$^{-/-}$ mice ($P < 0.003$, Table 2). When TT-specific antibodies were assessed in fecal samples, lower IgA responses were noted in the TCRδ$^{-/-}$ mice ($P < 0.005$, Table 2). To evaluate these findings at a cellular level, we examined the frequency of antigen-specific AFC in different tissues of mice immunized orally with the combined vaccine by using the ELISPOT assay. A reduction in the numbers of TT-specific IgA AFC was noted in both Peyer's patches and intestinal lamina propria in the TCRδ$^{-/-}$ mice when compared with control mice ($P < 0.01$, Fig. 3 *A*).

Characterization of Antibody Response to Cholera Toxin B Subunit in TCRδ$^{-/-}$ Mice. It is possible that hyporesponsiveness of TCRδ$^{-/-}$ mice to combined TT was due to unresponsiveness to the adjuvant effect of CT. Since the CT molecule possesses strong immunogenicity in addition to mucosal adjuvant activity for IgA responses, we could also measure IgA responses to the CT antigen. Serum and fecal extracts as well as mononuclear cells from various tissues of both TCRδ$^{-/-}$ mice and controls were obtained 1 wk after the last oral dose of the combined vaccine for analysis of CT-B-specific responses by ELISA and ELISPOT assays.

Table 1. *Low Levels of Total Serum and Secretory IgA Antibodies in TCRδ$^{-/-}$ Mice*

Mouse strains	Fecal	Saliva	Bile	Serum
	μg/ml	*ng/ml*	*μg/ml*	*μg/ml*
TCRδ$^{-/-}$	2 ± 1	244 ± 19	270 ± 30	25 ± 3
TCRδ$^{+/+}$	11 ± 2	753 ± 100	849 ± 155	125 ± 10

Values represent the mean endpoint titer ± SEM for nine mice in each experimental group.

When CT-B–specific serum responses were compared between TCRδ$^{-/-}$ and normal mice, lower CT-B–specific IgA titers were detected in the former ($P < 0.008$, Table 2). When CT-B–specific antibody titers were examined in fecal extracts, a significant decrease in the antigen-specific IgA levels was observed in TCRδ$^{-/-}$ mice ($P < 0.01$, Table 2). Reductions in CT-B–specific IgA-producing cells in both intestinal lamina propria and Peyer's patches were also observed in orally immunized TCRδ$^{-/-}$ mice ($P < 0.03$, Fig. 3 *B*).

Discussion

These studies suggest that γ/δ T cells exert an unexpected role in mucosal immunity. Decreased levels of total serum and secretory IgA as well as the reduction of IgA responses to both tetanus toxoid and cholera toxin antigens in TCRδ$^{-/-}$ mice suggests that γ/δ T cells can regulate the IgA response. In contrast, we found that IgM and IgG antibody responses were unimpaired in TCRδ$^{-/-}$ mice when compared with normal controls.

CD4$^+$ α/β T cells have been shown to be essential for IgA B cell responses (for review see reference 20), whereas the results of this study suggest that γ/δ T cells also may regulate the mucosal IgA immune response. The helper T cells that preferentially produce IL-4, IL-5, IL-6, and IL-10, the Th2 subset of CD4$^+$ cells, promote IgA responses (16, 21–23). Our earlier studies indicated that oral immunization of normal mice with the combined TT and CT vaccine induced Th2-type cells in the IgA mucosal response (16), and TCR-β$^{-/-}$ mice are essentially devoid of mucosal IgA-producing cells (Fujihashi, K., M.-N. Kweon, M. Marinaro, K. Imaoka, R.J. Jackson, J.R. McGhee, and H. Kiyono, manuscript in preparation). Function-loss mutations in the CD40L gene in humans and mice result in the failure to generate T cell–dependent secondary humoral immune responses, including antibodies of IgG and IgA isotype (24, 25). A severe impairment of IgA responses is also observed in anti-CD4–treated and athymic mice, which also have reduced germinal center development and few IgA-producing cells in the intestinal lamina propria (19, 26). The interaction between CD4$^+$ Th2 cells and B cells that are induced to undergo isotype switching normally occurs in germinal centers (20), where the interaction between CD40L on the activated CD4$^+$ α/β T cells and the CD40 molecule on B cells also initiates a signal needed for the isotype switch process (for review see reference 27). A special subset of the CD4$^+$ α/β T cells is thus essential for the induction of systemic IgA responses.

Our present results suggest that γ/δ T cells may serve an important regulatory role for mucosal IgA responses in that TCRδ$^{-/-}$ mice possess normal numbers of functional α/β T cells but display markedly decreased IgA responses to oral immunization. The previous studies also suggested that γ/δ T cells may play an important role in the maintenance of mucosal IgA responses in the presence of oral tolerance (14, 15). One possible explanation for this unexpected finding is that γ/δ T cells positively influence α/β Th2 cells that regulate IgA immune responses in mucosal tissues. In this scenario, a triad interaction between mucosal γ/δ T cells, α/β Th2-type cells, and IgA B cell precursors is involved in the induction of maximal IgA responses to oral antigens. Experiments in mice (11, 13, 28) and in chickens (29, 30) in-

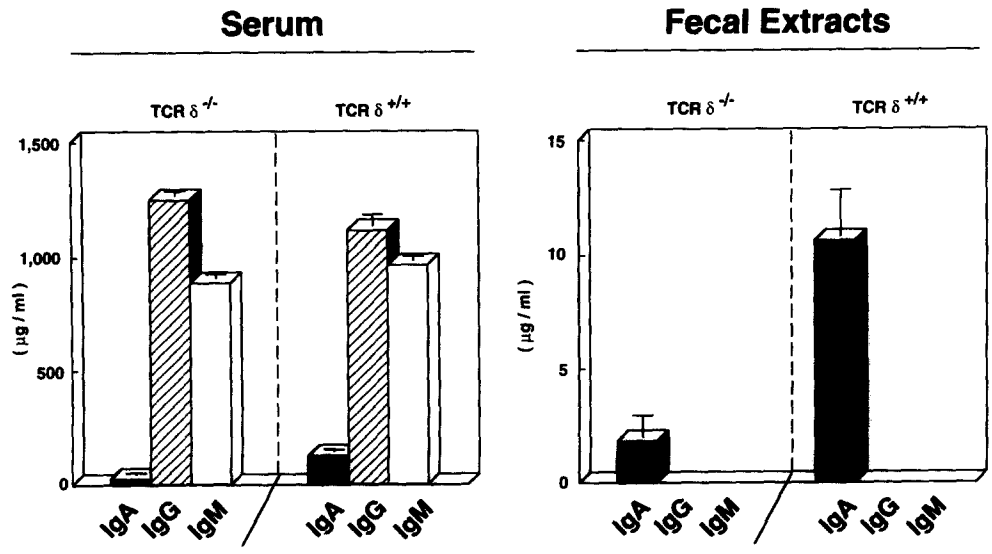

Figure 2. IgM, IgG, and IgA levels in serum and fecal extracts of TCRδ$^{-/-}$ and TCRδ$^{+/+}$ mice. These levels were determined by an ELISA using mouse IgM, IgG, and IgA standards. Values are mean ± SEM for nine mice in each experimental group.

Table 2. *Selective Impairment of IgA Responses to Oral Immunization with TT and CT Antigens in TCRδ$^{-/-}$ Mice*

Antigen	Mouse strains	Antibody titer (Log_2) Fecal IgA	Serum IgA	Serum IgG	Serum IgM
TT	TCRδ$^{-/-}$	5.0 ± 0.3	8.0 ± 0.3	16.6 ± 0.3	11.6 ± 0.3
	TCRδ$^{+/+}$	7.5 ± 0.5	10.8 ± 0.4	16.8 ± 0.7	11.4 ± 0.4
CT-B	TCRδ$^{-/-}$	5.7 ± 0.5	10.6 ± 0.4	16.2 ± 0.2	11.0 ± 0.2
	TCRδ$^{+/+}$	8.3 ± 0.3	12.5 ± 0.3	17.0 ± 0.5	10.7 ± 0.4

Values represent the mean end point titer ± SEM for nine mice in each experimental group. The antibody end point titration was performed by ELISA as described in Materials and Methods.

deed suggest that bidirectional interactions between α/β and γ/δ T cells may have functionally important consequences. Alternatively, the γ/δ T cells could have a direct effect either on the differentiation of sIgA$^+$ B cells or in the induction of the mucosal IgA switch process. A recent study indicates that activated γ/δ T cells can express CD40L and thereby induce an IgE B cell response (31). It thus seems possible that mucosal CD40L$^+$ γ/δ T cells are capable of direct interaction with CD40$^+$ B cells to induce IgA responses. The observation of significant IgA production in CD40$^{-/-}$ and CD40L$^{-/-}$ mice (25, 32, 33) also raises the possibility of an alternative set of interaction molecules that could allow γ/δ (or α/β) T cells in the mucosal compartment to induce IgA responses to ingested antigens.

Our previous studies and those of others indicate that γ/δ IEL can produce an array of Th1- and Th2-type cytokines, as well as TNF-α and TGF-β (34, 35). Specifically, the γ/δ IELs can secrete IL-5 and IL-6, which are key cytokines for inducing sIgA$^+$ B cells to differentiate into IgA plasma cells (34, 35). Moreover, γ/δ T cells in other mucosal effector tissues, such as the salivary glands, are committed to produce IL-5 and IL-6 (36), and the frequency of IgA-producing cells is reduced in the salivary glands of TCRδ$^{-/-}$ mice (data not shown). IgA-producing cells are also greatly reduced in intestinal tissues of IL-6$^{-/-}$ mice (37). The impaired IgA responses in TCRδ$^{-/-}$ mice could therefore reflect the absence of mucosal γ/δ T cells that produce IL-5 and IL-6.

Yet another possible explanation for our findings is that the lack of γ/δ T cells in intestinal epithelium negatively influences epithelial cell production of TGF-β and IL-6,

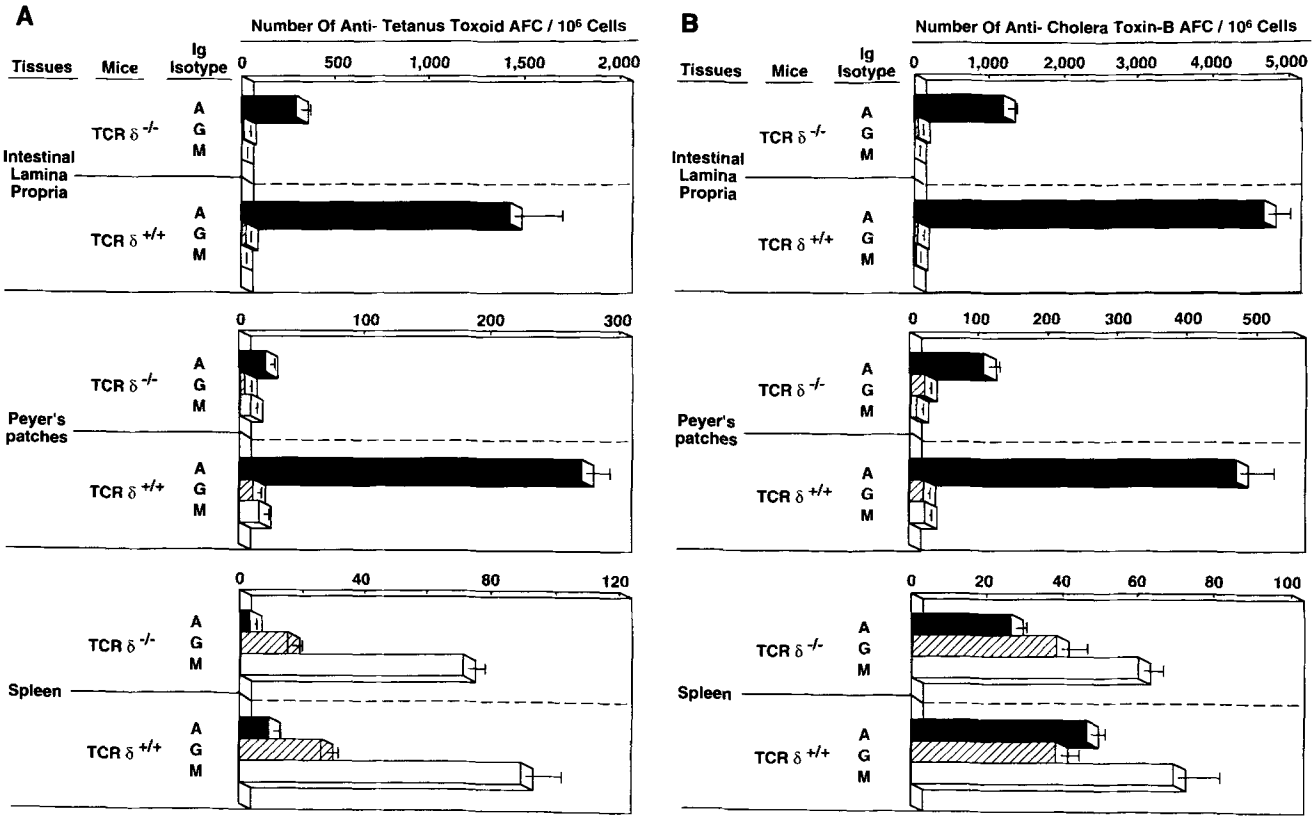

Figure 3. Comparison of (*A*) TT- and (*B*) CT-B–specific antibody responses in TCRδ$^{-/-}$ and control mice immunized orally with a combined vaccine containing TT and CT. Mononuclear cells isolated from spleen, Peyer's patch, and lamina propria of both TCRδ$^{-/-}$ and control mice were subjected to the TT- and CT-B–specific ELISPOT assay. Antigen-specific IgM (□), IgG (▨) and IgA (■) AFC were enumerated. Results represent the values (mean ± SEM) for nine mice in each experimental group.

which serve as IgA isotype switching and differentiation factors, respectively (38, 39). T cell–derived cytokines, including IFN-γ, TNF-α, and IL-4, can influence epithelial cell functions (40, 41). All of these cytokines can be produced by γ/δ IEL (34, 35), and γ/δ T cells may influence epithelial cell growth and function. In fact, reduction in both the numbers of intestinal epithelial cells and their level of MHC class II expression have been observed in TCR-δ gene-disrupted mice (42). Further, intraepithelial γ/δ T cells have been shown to modulate growth of epithelial cells via the production of keratinocyte growth factor (43). The absence of γ/δ T cells in the intestinal epithelium could therefore compromise TGF-β and IL-6 production by epithelial cells, which, in turn, may result in diminished IgA responses.

In conclusion, γ/δ T cells may influence IgA B cell responses to ingested antigens via their interactions with other T cells and mucosal epithelial cells. Mucosal γ/δ T cells may thus regulate production of key cytokines for IgA B cell development by $CD4^+$ α/β T cells (e.g., IL-5, IL-6, and IL-10) and epithelial cells (e.g., TGF-β and IL-6). The interactions between γ/δ T cells, α/β T cells, and epithelial cells in the induction and regulation of mucosal immune responses could thus represent a fertile field for future investigation.

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Address correspondence to Hiroshi Kiyono, Departments of Oral Biology and Microbiology, BBRB Room 761, Immunobiology Vaccine Center, University of Alabama at Birmingham, Medical Center, Birmingham, AL 35294-2170.

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