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Homeostatic regulation of intestinal epithelia by intraepithelial γδ T cells

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ABSTRACT **Although T cells bearing γδ T-cell receptors have long been known to be present in the epithelial lining of many organs, their specificity and function remain elusive. In the present study, we examined the intestinal epithelia of T-cell-receptor mutant mice, which were deficient in either γδ T cells or αβ T cells, and of normal littermates. The absence of γδ T cells was associated with a reduction in epithelial cell turnover and a downregulation of the expression of major histocompatibility complex class II molecules. No such effects were observed in αβ T-cell-deficient mice. These findings indicate that intraepithelial γδ T cells regulate the generation and differentiation of intestinal epithelial cells.**

Two classes of T cells, namely, T cells expressing αβ T-cell receptors (TCRs) and T cells expressing γδ TCRs, have been identified in all vertebrates examined to date. It is now clear that most αβ T cells recognize peptide fragments of antigens presented by major histocompatibility complex (MHC) molecules and are engaged in most characterized cell-mediated antigen-specific immune responses. In contrast, the general rules for γδ T-cell recognition remain undefined and the biologic role of γδ T cells in immune responses is not well understood (1, 2). It has been demonstrated, however, that this distinct class of T cells is abundant in various epithelia (3) such as the epidermis of the mouse (4, 5) and intestinal epithelium of many species (6–10). Recent findings (11–14) indicated a fundamental difference in antigen recognition between γδ T cells and αβ T cells, suggesting that γδ T cells may contribute to the immune system differently than αβ T cells do (1, 2).

In this study, we analyzed the physiological significance of one major γδ T-cell subset that is localized in the mouse intestinal epithelium. The pool of intestinal intraepithelial T lymphocytes (IELs) of adult mice is comparable in size to the T-cell pool in the spleen (15). It consists of both αβ T cells (40–70%) and γδ T cells (30–60%) (15, 16). The striking fact that IELs interdigitate between the basolateral faces of intestinal epithelial cells (IECs) (17) suggests a close functional relationship between IELs and adjacent IECs (1, 18–20). To investigate this issue, we examined tissue sections of the small intestines prepared from normal wild-type (WT) mice and TCR mutant mice that lack either αβ-TCR-expressing cells ($\beta^{-/-}$ mice) (21) or γδ-TCR-expressing cells ($\delta^{-/-}$ mice) (22) along with the IECs and IELs isolated from these mice by immunofluorescence analysis. Our results show that the generation of crypt cells, their migration toward the top of the villi, and the expression of MHC class II molecules by villus IECs are all downregulated significantly in $\delta^{-/-}$ mice compared with those activities observed in WT and $\beta^{-/-}$ mice.

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MATERIALS AND METHODS

Mice. TCRβ mutant ($\beta^{-/-}$) mice (21) and TCRδ mutant ($\delta^{-/-}$) mice (22) have been described. In brief, embryonic stem cell lines E14 (129/Ola mouse origin) and D3 (129/Sv mouse origin) were used for the targeted disruptions of TCR genes (21, 22). Unless otherwise stated, the starting $\beta^{-/-}$ and $\delta^{-/-}$ mice used in the present study had a genetic background of (129/Ola × C57BL/6)F_1 mice that had been backcrossed three times to C57BL/6 parent. In some experiments, we also used $\delta^{-/-}$ mice either in a genetic background of (129/Sv × BALB/c)F_1 mice that had been backcrossed two times to BALB/c parent ($\delta^{-/-}$-B/c1) or in a (129/Ola × BALB/c)F_1 × BALB/c genetic background ($\delta^{-/-}$-B/c2). $\delta^{+/-}$ (WT) and $\delta^{-/-}$ (mutant) mice were littermates from an intercross between $\delta^{+/-}$ and $\delta^{-/-}$ mice. Mice were typed by PCR analysis of tail DNA with a set of primers to the neomycin-resistance gene (5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′, 280-bp PCR fragment) (23) and a set of primers to the WT TCRδ allele (5′-AAAAGCCAGCCTCCGGCCAAA-3′ and 5′-AACTGAACATGTCACTGAATT-3′, 222-bp PCR fragment) (24). Likewise, $\beta^{+/-}$ (WT) and $\beta^{-/-}$ (mutant) mice were littermates from an intercross between $\beta^{+/-}$ and $\beta^{-/-}$ mice. Mice were typed by FACScan analysis of peripheral blood T cells expressing αβ TCRs. We also obtained WT, $\delta^{-/-}$, and $\beta^{-/-}$ littermates from the F_2 generation of an intercross between $\beta^{-/-}$ and $\delta^{-/-}$ mice. C57BL/6 and BALB/c mice were from colonies in our laboratory. Mice of both sexes, 8–24 weeks of age, were used in the experiments.

***In Vivo* Labeling and *in Situ* Immunocytochemical Staining Procedures of Proliferating IECs.** DNA replicating cells were determined as described by Gratzner (25). Mice were injected i.p. with bromodeoxyuridine (BrdU; 20 mg/kg of body weight) five times at 6-hr intervals. One hour after the last injection, the small intestines were removed and fixed with 70% ethanol for 12 hr, and paraffin-embedded tissue sections (3 μm thick) were deparaffinized by xylene, rinsed sequentially with ethanol and water, treated with 0.1 M boric acid (pH 10), and finally washed with 2 M HCl. Endogenous peroxidase activity was blocked by a 30-min incubation in 0.3% hydrogen peroxide in methanol. The tissue sections were further rinsed three times with PBS and then incubated with anti-BrdU monoclonal antibody (mAb; Becton Dickinson), followed by incubation with biotinylated horse anti-mouse immunoglobulin antibodies (Vector Laboratories) and avidin-biotin-peroxidase complex (Vector Laboratories). Finally, the tissue sections were stained

Abbreviations: IEC, intestinal epithelial cell; IEL, intestinal intraepithelial T lymphocyte; TCR, T-cell receptor; MHC, major histocompatibility complex; WT, wild type; mAb, monoclonal antibody; DETC, dendritic epidermal T cell.

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with diaminobenzidine. Five arbitrary fields of the small intestine from each mouse were photographed (original magnification, ×80), and BrdU-incorporated cells were enumerated.

Immunohistochemical Procedure. Immunohistochemical staining was as described (9). In brief, tissue segments were sectioned with a cryostat at 5 μm and sections were preincubated with 3% bovine serum albumin in PBS to block nonspecific binding of the mAb. The sections were reacted with biotinylated anti-IA^b mAb (clone 25-9-3; PharMingen), washed three times with PBS, incubated with peroxidase-conjugated avidin (Organon Teknika), and finally stained with 3-amino-9-ethylcarbazol. Endogenous peroxidase was blocked with 1% sodium periodate in distilled water.

Isolation of IECs and IELs. We isolated IECs and IELs as described (9, 16). In brief, an inverted intestine was cut into four segments and the segments were transferred to a 50-ml conical tube containing 45 ml of RPMI 1640 medium/5% fetal calf serum/25 mM Hepes/penicillin (100 units/ml)/streptomycin (100 μg/ml). The tube was shaken at 37°C for 45 min (horizontal position on an orbital shaker at 150 rpm). Cell suspensions were collected and passed through a glass-wool column to deplete cell debris and sticky cells (crude cell preparation). Subsequently, the cells were suspended in 30% (wt/vol) Percoll and centrifuged for 20 min at 400 × *g*. After centrifugation, cells at the top of the 30% Percoll were enriched with IECs devoid of CD3-positive cells. Cells at the bottom of the solution was then subjected to Percoll discontinuous-gradient centrifugation (16) and IELs were recovered at the interphase of 44 and 70% Percoll (>95% were CD3-positive).

Flow Cytometry. For immunofluorescence analysis of MHC molecules on IECs, IECs were incubated first with a mixture of anti-IA^b mAbs of different specificities, i.e., clone 7-16.17 (PharMingen) and clone AF6 (provided by N. Shinohara, Mitsubishi Kasei Institute of Life Science, Tokyo) or with anti-K^b mAb (clone AF6-88.5; PharMingen) and then with biotinylated $F(ab')_2$ fragments of sheep anti-mouse IgG (Cappel), followed by an incubation with streptavidin-phycoerythrin (Becton Dickinson). For immunofluorescence analysis of MHC molecules on IELs, IELs were incubated first with fluorescein isothiocyanate-conjugated anti-IA^b mAb (clone K25-8.7; provided by T. Shirai and Y. Hirose, Juntendo University, Tokyo) and phycoerythrin-conjugated anti-mouse κ-light chain antibody (Beckton Dickinson) or fluorescein isothiocyanate-conjugated anti-K^b mAb (clone AF6-88.5; PharMingen). To eliminate the dead cells from the data, we used propidium iodide (0.5 μg/ml) and the data were analyzed by FACScan LYSIS II software. Three-color analysis of IEL subsets was performed as described (9).

Reconstitution of γδ IELs. Small intestines were prepared from $\delta^{-/-}$ mice that had been i.v. injected 4 weeks in advance with 1×10^6 WT IELs. Setting aside a quarter of jejunum, we isolated IELs from each intestine and determined the number of γδ IELs and αβ IELs by flow cytometry. Histological examination of the saved jejunum sections was then carried out on ≈50% of the reconstituted $\delta^{-/-}$ mice in which the numbers of γδ IELs had reached those of γδ IELs in WT mice.

RESULTS

Crypt Cell Numbers Are Reduced in $\delta^{-/-}$ Mice. We examined hematoxylin/eosin-stained tissue sections of the small intestines prepared from $\delta^{+/-}$, $\delta^{-/-}$, $\beta^{+/-}$, and $\beta^{-/-}$ mice, and noticed that the cellularity of crypts was reduced in $\delta^{-/-}$ mice. This first impression was confirmed by counting the number of crypt cells in $\delta^{-/-}$, $\beta^{-/-}$, and normal mice (Fig. 1*B*). Similar differences in the cellularity of crypts were observed among aged animals, i.e., >12-month-old $\delta^{-/-}$, $\beta^{-/-}$, and normal mice (data not shown). We also counted crypt cells in two $\delta^{-/-}$ mouse lines carrying a BALB/c genetic background, and the cellularity of crypts was reduced in $\delta^{-/-}$ mice as follows: $\delta^{+/-}$-B/c1, 40.0 ± 1.16 cells (n = 5); in $\delta^{-/-}$-B/c1, 24.8 ± 1.64 cells (n = 5); in $\delta^{+/-}$-B/c2, 34.0 ± 0.50 cells (n = 3); in $\delta^{-/-}$-B/c2, 28.2 ± 1.50 cells (n = 3). Furthermore, the depletion of crypt cells in $\delta^{-/-}$ mice was reversed by adoptive transfer of γδ IELs from normal mice (Fig. 1*B*).

Generation of IECs Is Downregulated in $\delta^{-/-}$ Mice. Epithelial stem cells proliferate at the base of the crypt. The newly formed cells move upward and differentiate into mature columnar IECs with striated borders (26, 27). In $\delta^{-/-}$ mice, the generation of crypt cells appeared to be downregulated. To examine this possibility, we injected BrdU into mice, to visualize the IECs that had passed through S phase of the cell cycle (25). Indeed, the number of cells that had incorporated BrdU and their migration toward the top of the villi were significantly reduced in $\delta^{-/-}$ mice (Fig. 1*A*). Since γδ IELs are located between IECs at the stem of the villi but not in the crypt nor in the lamina propria (28, 29), they might secrete a growth-promoting factor or, alternatively, might act on neighboring IECs, which in turn could influence the proliferation of epithelial stem cells.

MHC Class II Molecules on IECs Is Decreased in $\delta^{-/-}$ Mice. Maturation of the IEC membrane occurs as the cell migrates from the crypt to the villus (26, 27) and corresponds to the expression of various brush border enzymes and MHC class II antigens (30, 31). In an attempt to investigate whether γδ IELs had any effect on upward-moving IECs, we examined the expression of MHC class II molecules by flow cytometry of IECs isolated from TCR mutant and WT mice. As shown in Fig. 2*B*, the expression of IA^b (MHC class II) but not of K^b (MHC class I) molecules by IECs was drastically reduced in the $\delta^{-/-}$ mice compared with $\beta^{-/-}$ and WT mice. The reduction of IA^b expression was also noted in double mutant $\beta \times \delta^{-/-}$ mice (data not shown). On the other hand, IELs lacked MHC class II molecules and expressed MHC class I molecules at the same level in all mice (Fig. 2*C*). To examine the MHC class II expression by IECs *in situ*, we used anti-IA^b mAbs to stain frozen sections of the small intestines. Again, the expression of IA^b was much lower on IECs from $\delta^{-/-}$ mice than on IECs from $\beta^{-/-}$ and WT mice (Fig. 3).

Development of the Remaining γδ and αβ IELs in TCR Mutant Mice. The above described findings indicate that the generation and differentiation of intestinal epithelial cells are influenced by γδ IELs but not by αβ IELs. To further rule out the possibility of indirect effects resulting from interactions between αβ and γδ IELs, we analyzed the number and surface marker expression of IELs from both $\delta^{-/-}$ and $\beta^{-/-}$ mice. As shown in Fig. 4*A*, there was a compensatory increase in the number of αβ IELs and of γδ IELs in $\delta^{-/-}$ mice and $\beta^{-/-}$ mice, respectively. The compensatory increase in cell numbers was not associated with any gross alteration of IEL subpopulations as defined by the expression of CD4, CD8α, and CD8β chains (Fig. 4*B*). Thus, while there appears to be a mechanism that controls the total number of IELs, no evidence was obtained for any interactions between αβ and γδ IELs.

DISCUSSION

IELs are thought to provide a first line of defense against microbial pathogens (1, 2). Support for this idea has been obtained for αβ IELs but not for γδ IELs. Thus, the comparison of IELs in normal and germ-free mice has shown that microorganisms in the intestine have a strong influence on the number and functional properties of αβ IELs but have no significant effect on γδ IELs (32–34). More recently, Findly *et al.* (35) found that infection of mice with the coccidian parasite *Eimeria* led to an increase in the number of γδ T cells in the intestinal epithelia. However, the analysis of γδ TCR expression in the infected epithelia suggested that the additional cells

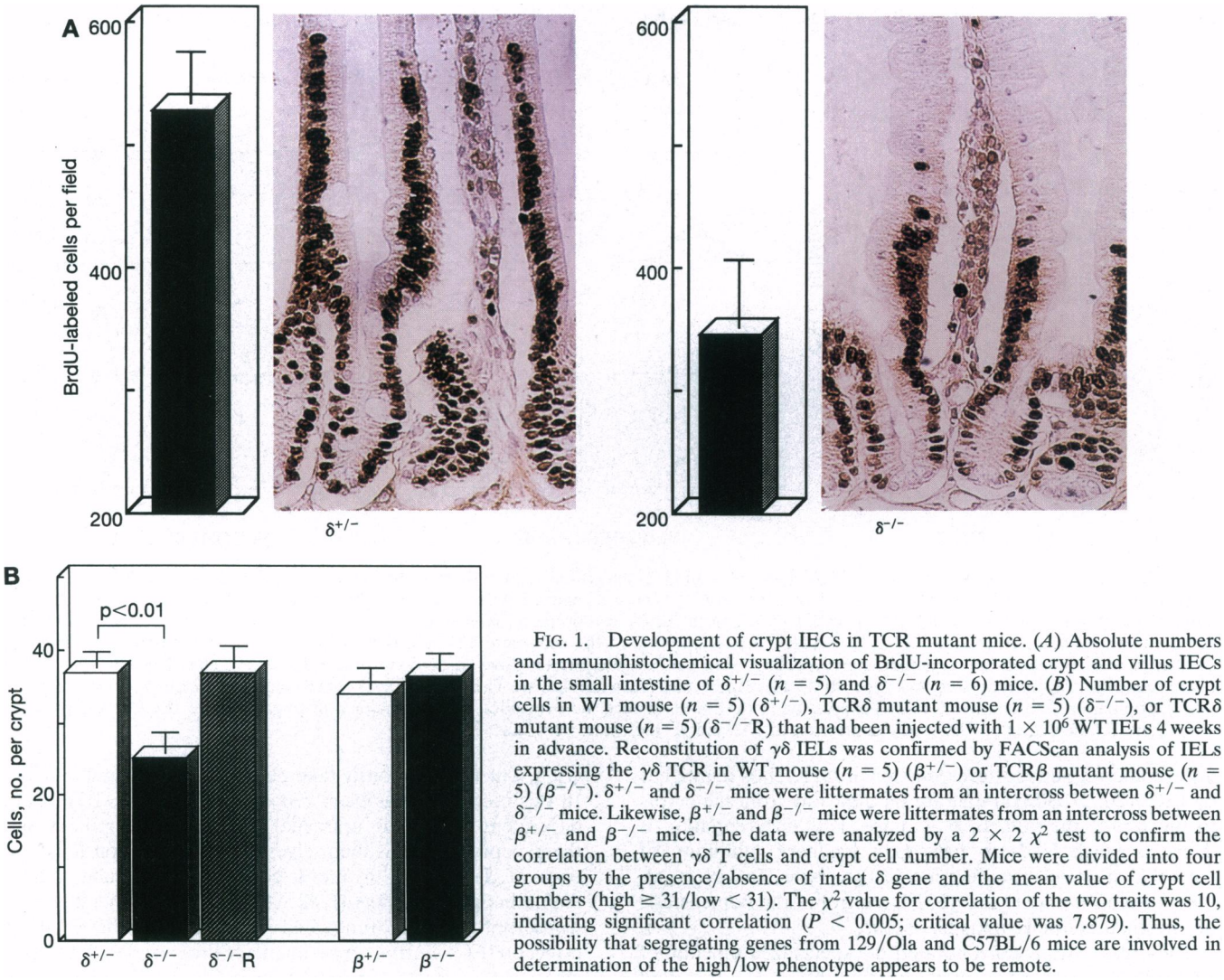

FIG. 1. Development of crypt IECs in TCR mutant mice. (*A*) Absolute numbers and immunohistochemical visualization of BrdU-incorporated crypt and villus IECs in the small intestine of $\delta^{+/-}$ ($n = 5$) and $\delta^{-/-}$ ($n = 6$) mice. (*B*) Number of crypt cells in WT mouse ($n = 5$) ($\delta^{+/-}$), TCRδ mutant mouse ($n = 5$) ($\delta^{-/-}$), or TCRδ mutant mouse ($n = 5$) ($\delta^{-/-}$R) that had been injected with 1×10^6 WT IELs 4 weeks in advance. Reconstitution of $\gamma\delta$ IELs was confirmed by FACScan analysis of IELs expressing the $\gamma\delta$ TCR in WT mouse ($n = 5$) ($\beta^{+/-}$) or TCRβ mutant mouse ($n = 5$) ($\beta^{-/-}$). $\delta^{+/-}$ and $\delta^{-/-}$ mice were littermates from an intercross between $\delta^{+/-}$ and $\delta^{-/-}$ mice. Likewise, $\beta^{+/-}$ and $\beta^{-/-}$ mice were littermates from an intercross between $\beta^{+/-}$ and $\beta^{-/-}$ mice. The data were analyzed by a 2×2 χ^2 test to confirm the correlation between $\gamma\delta$ T cells and crypt cell number. Mice were divided into four groups by the presence/absence of intact δ gene and the mean value of crypt cell numbers (high $\geq$ 31/low $<$ 31). The χ^2 value for correlation of the two traits was 10, indicating significant correlation ($P < 0.005$; critical value was 7.879). Thus, the possibility that segregating genes from 129/Ola and C57BL/6 mice are involved in determination of the high/low phenotype appears to be remote.

were not derived from resident $\gamma\delta$ IELs but were recruited from $\gamma\delta$ T-cell pools at other sites (35). To our knowledge, there is no convincing evidence for a role of resident $\gamma\delta$ IELs in the defense against infections. A recent study of $\gamma\delta$ T cells in the uterine epithelium suggests that these cells may also have functions that are unrelated to infections (36).

The majority of mouse Thy-1$^+$ dendritic epidermal T cells (DETCs) express virtually homogeneous TCRs encoded by Vγ5 and Vδ1 genes (4) and exist in intimate contact with keratinocytes. Recent *in vitro* studies have indicated a functional link between DETCs ($\gamma\delta$ T cells) and keratinocytes (20, 37). Thus, it has been demonstrated that DETCs respond to self-antigen presented by keratinocytes and produce interleukin 2 (37). The activated DETCs also express the epithelial-cell mitogen keratinocyte growth factor and promote the growth of cultured epithelial cells (20). Furthermore, the authors (20) presented evidence that keratinocyte growth factor is inducibly expressed by $\gamma\delta$ IELs but not $\alpha\beta$ IELs. In view of these findings, we regard the following four mechanisms as more likely for the effect of $\gamma\delta$ IELs on IECs *in situ*. (*i*) Since $\gamma\delta$ IELs are located between villus IECs but not in proliferating cells of the intestinal crypt (28, 29), they produce a certain growth-promoting factor such as keratinocyte growth factor and the factor secreted would reach crypt cells in the mucosal circulation. (*ii*) Since $\alpha\beta$ and $\gamma\delta$ IELs constitutively display cytolytic activity (16, 32, 34), perhaps a substantial number of IECs moving upward from the crypts to the top of the villi are continuously eliminated by cytolytic $\gamma\delta$ IELs but not cytolytic $\alpha\beta$ IELs, which leads in turn to a feedback acceleration of crypt-cell proliferation. Thus, in the absence of cytolytic $\gamma\delta$ IELs, proliferation of crypt cells is shifted into low gear in $\delta^{-/-}$ mice. (*iii*) After autonomous self-renewal in the crypt, a significant number of IECs in $\delta^{-/-}$ mice would die within the crypts and/or along the crypt-to-villus axis, and $\gamma\delta$ IELs and/or their product(s) would rescue the dying IECs. In this context, it should be pointed out that the number of crypt cells in S phase of the cell cycle enumerated 1 hr after the single i.p. infusion of BrdU was not significantly different between $\delta^{+/-}$ and $\delta^{-/-}$ mice ($\approx$60 cells per field containing six to seven crypts), although the net accumulation of BrdU-incorporated IECs determined after the multiple i.p. infusions of BrdU was significantly reduced in $\delta^{-/-}$ mice (Fig. 1*A*). E-cadherin and/or other intestinal cadherins are produced in IECs (38), play a central role in maintaining the integrity of cell–cell and cell–matrix interactions and in regulating the differentiation program of enterocytes during the first 48-hr residence within the villus, and thus, rescue the IECs from apoptosis (38). We found by immunohistochemistry that the expression of E-cadherin on villus IECs was reduced to some extent in $\delta^{-/-}$ mice (H.K., H. I., and S. I., unpublished work). These observations suggest that the rate of generation of daughter cells at the intestinal crypt of $\delta^{-/-}$ mice is comparable with that of $\delta^{+/-}$ mice but a certain fraction of the newly formed cells are dying in the absence of $\gamma\delta$ IELs, supporting the third rather than the second proposition described above. Lastly, our results suggest that $\gamma\delta$ IELs enhance the migration-associated

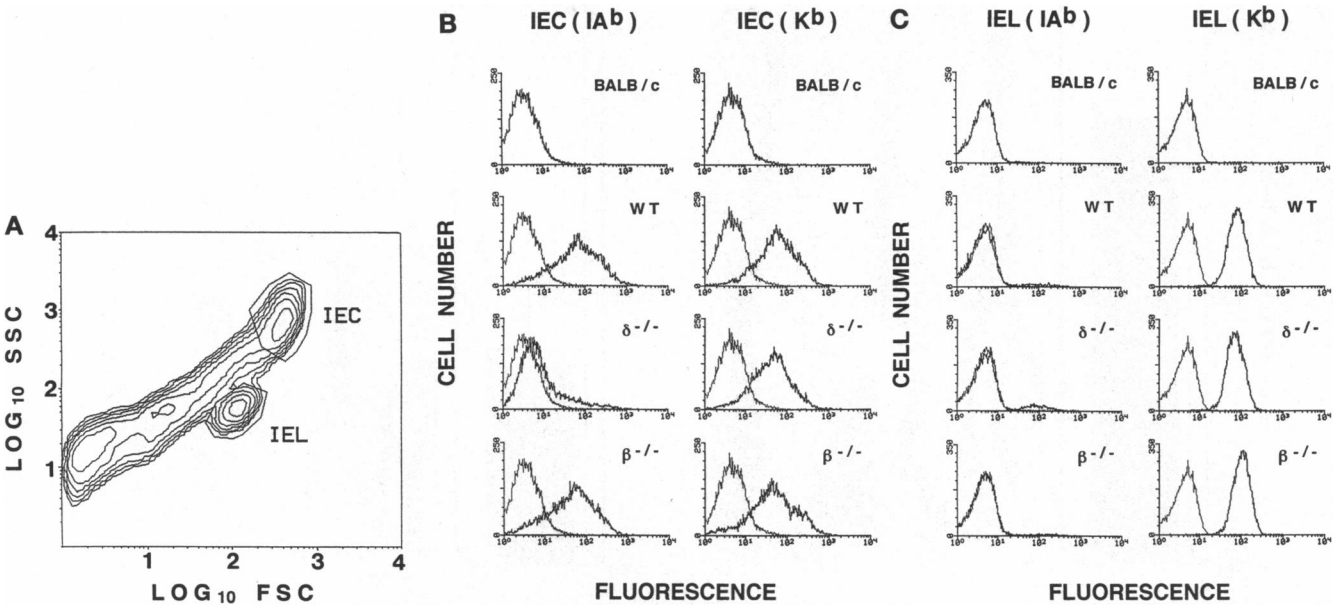

FIG. 2. Immunofluorescence analysis of MHC molecules on IECs and IELs isolated from TCR mutant mice. WT, $\delta^{-/-}$, and $\beta^{-/-}$ mice were littermates of the F_2 generation from an intercross between $\beta^{-/-}$ and $\delta^{-/-}$ mice. BALB/c mice (H-2^d) were used as a negative control strain of mouse. (*A*) Forward (FSC) and side (SSC) scatter profile of the crude cell preparation isolated from epithelia of the small intestine. Two major cell populations indicated as IECs (gate 1) and IELs (gate 2; >95% of the cells were CD3-positive) were identified. Four months after lethal irradiation and reconstitution with bone-marrow cells, >90% cells in gate 2 were shown to be donor derived by MHC class I typing, whereas all of the cells in gate 1 still expressed host-type MHC class I molecules (data not shown). Thus, on the basis of cell volume (FSC), cell surface property (SSC), population size, and MHC class I expression in the chimeric mice, we concluded that the major cells in gate I were IECs. (*B*) Expression of IA^b and K^b molecules on IECs. (*C*) Expression of IA^b and K^b molecules on IELs.

differentiation, such as the expression of MHC class II antigens by IECs (26, 27, 30, 31) that are on their way from the crypts to the top of the villi. In $\delta^{-/-}$ mice, IEC differentiation is delayed, which leads in turn to a feedback inhibition of epithelial-stem-cell proliferation in the crypts. In any event, the mechanism underlying γδ IEL control of the homeostasis of intestinal epithelia remains obscure.

The present study corroborated the specialized function of γδ T cells in the mouse intestinal epithelium. γδ IELs are found in the intestinal epithelia of all vertebrates examined (2) and may have evolved prior to the thymus in invertebrates (39). Murine γδ IELs develop extrathymically in the intestinal mucosa (7, 8, 10, 15, 40). IECs are most likely required for the development of γδ IELs. The present findings indicate that γδ IELs in turn influence the generation and differentiation of IECs, which continuously take place in the intestinal mucosa. In this context, it has been reported that thymic T cells are dependent on thymic epithelial cells for development and thymic epithelial cells themselves are dependent on the presence of developing thymic T cells to differentiate and to maintain their integrity (41, 42). At the present time, however, we do not know the functional significance of the γδ IEL's effect on IECs. Further experiments are required to determine whether the effects of γδ IELs on IECs described here have functional implications related to defense against neoplasia or to the regulation of immune responses to antigens passing through the intestinal tract.

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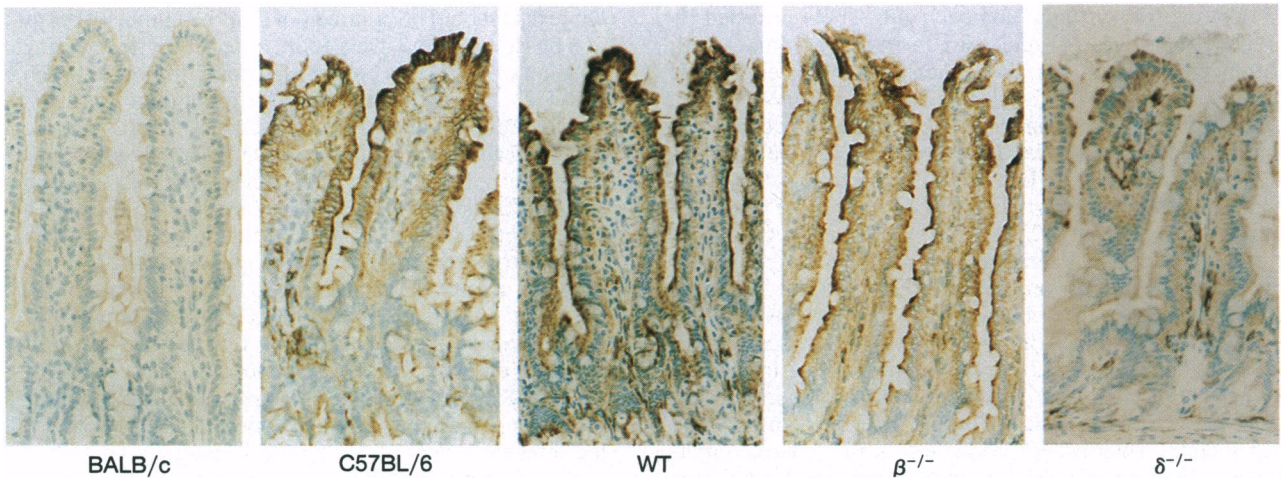

FIG. 3. Immunohistochemical analysis of IA^b molecules on the small intestinal epithelia in TCR mutant mice. WT, $\beta^{-/-}$, and $\delta^{-/-}$ mice employed for this study were exactly the same as those described in Fig. 2. BALB/c (IA^d) and C57BL/6 (IA^b) mice were also used as negative and positive control strains of mice, respectively. Staining was observed on columnar IECs in C57BL/6, WT, and $\beta^{-/-}$ mice.

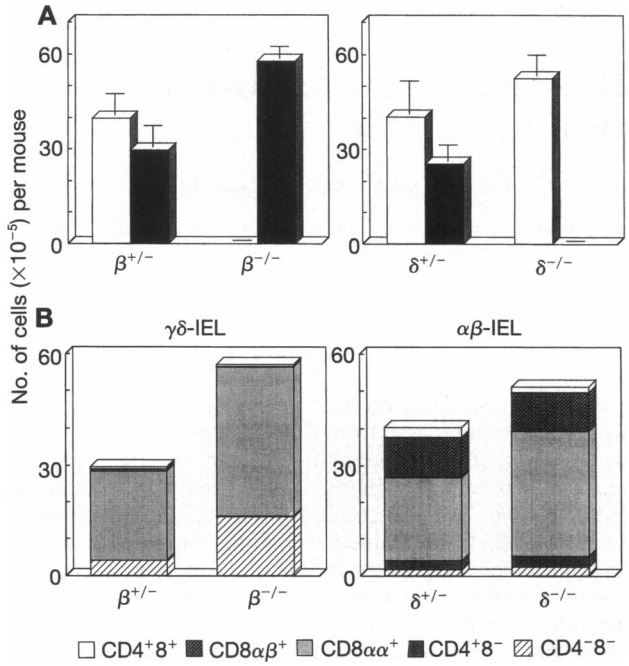

FIG. 4. Immunofluorescence analysis of IELs isolated from TCR mutant mice. WT and mutant mice were the same as those described in Fig. 1. (*A*) Numbers of αβ and γδ IELs recovered from WT ($\beta^{+/-}$ or $\delta^{+/-}$), $\beta^{-/-}$, and $\delta^{-/-}$ mice. Absolute numbers of αβ and γδ IELs were calculated on the basis of total numbers of CD3/TCR-positive IELs. The results (open bars, αβ IELs; solid bars, γδ IELs) are the mean ± SD of data obtained from five experiments. (*B*) Three-color analysis of IEL subsets isolated from WT ($\beta^{+/-}$ or $\delta^{+/-}$), $\beta^{-/-}$, and $\delta^{-/-}$ mice. Absolute numbers of double-positive ($CD4^+CD8^+$ or $CD4^+CD8\alpha\alpha^+$) or single-positive ($CD8\alpha\beta^+$, $CD8\alpha\alpha^+$, or $CD4^+CD8^-$) subsets were calculated on the basis of total numbers of αβ and γδ IELs.

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