Mucosal T Cells Bearing TCR$\gamma\delta$ Play a Protective Role in Intestinal Inflammation[1]

Kyoko Inagaki-Ohara,[2*†] Takatoshi Chinen,[†] Goro Matsuzaki,[‡] Atsuo Sasaki,[†] Yukiko Sakamoto,* Kenji Hiromatsu,* Fukumi Nakamura-Uchiyama,* Yukifumi Nawa,* and Akihiko Yoshimura[2†]

Intestinal intraepithelial lymphocytes (IEL) bearing TCR$\gamma\delta$ represent a major T cell population in the murine intestine. However, the role of $\gamma\delta$ IEL in inflammatory bowel diseases (IBD) remains controversial. In this study, we show that $\gamma\delta$ IEL is an important protective T cell population against IBD. $\gamma\delta$ T cell-deficient (C$\delta^{-/-}$) mice developed spontaneous colitis with age and showed high susceptibility to Th1-type 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis at a young age. Transfer of $\gamma\delta$ IEL to C$\delta^{-/-}$ mice ameliorated TNBS-induced colitis, which correlated with decrease of IFN-γ and TNF-α production and an increase of TGF-β production by IEL. Furthermore, a high level of IL-15, which inhibits activation-induced cell death to terminate inflammation, was expressed more in intestinal epithelial cells (EC) from TNBS-treated C$\delta^{-/-}$ mice than in those from wild-type mice. EC from wild-type mice significantly suppressed the IFN-γ production of IEL from TNBS-treated C$\delta^{-/-}$ mice, whereas EC from TNBS-treated C$\delta^{-/-}$ mice did not. These data indicate that $\gamma\delta$ IEL play important roles in controlling IBD by regulating mucosal T cell activation cooperated with EC function. Our study suggests that enhancement of regulatory $\gamma\delta$ T cell activity is a possible new cell therapy for colitis. *The Journal of Immunology*, **2004, 173: 1390–1398.**

Intestinal mucosal lymphocytes, including both intestinal intraepithelial lymphocytes (IEL)[3] and lamina propria lymphocytes (LPL), may serve a critical role in the mucosal immune system by providing immune surveillance of the epithelium (1–3). Murine IEL comprise an approximately equal frequency of population bearing TCR$\alpha\beta$ and TCR$\gamma\delta$ (4). $\gamma\delta$ IEL are considered to be a T cell population playing for the first line of host defense against a variety of Ags and pathogens (5, 6). Important roles of intestinal $\gamma\delta$ T cells in maintenance of homeostasis in intestine have been reported, such as production of keratinocyte growth factor, which stimulates epithelial cell (EC) proliferation (7) and the regulation of the generation and differentiation of EC (8). $\gamma\delta$ IEL also participate in immune regulation at epithelial site by contributing to the help for Ig class switching (9) and requirement for oral tolerance (10).

Inflammatory bowel diseases (IBD) are immune-mediated diseases that are induced by a complex chronic inflammatory process. The pathogenesis of IBD is deteriorated by proinflammatory cytokines such as IFN-γ and TNF-α, and a dysregulation of T cell functions or cytokine balance has been suggested as a cause of colitis. $CD4^+CD45RB^{high}$ cells have been shown to develop spontaneous colitis (11). In contrast, transfer of $CD4^+CD45RB^{low}$ T cells with $CD4^+CD45RB^{high}$ cells prevents development of colitis mediated by involvement in TGF-β and IL-10, regulatory cytokines (12, 13). However, little is known concerning the role of $\gamma\delta$ T cells in inflammatory disease, such as IBD. Some reports have indicated an increase in susceptibility to 2,4,6-trinitrobenzene sulfonic acid (TNBS), which is an inducer of Th1-type colitis, in $\gamma\delta$ T cell-depleted rats (14), and to dextran sodium sulfate, which is the inducer of T cell-independent colitis, in the C$\delta^{-/-}$ mice (15). Therefore, $\gamma\delta$ T cells seem to be involved in the regulation of colitis by T cell-dependent and/or -independent mechanisms. However, these protective roles against IBD remain to be investigated.

In this study, we evaluated whether intestinal $\gamma\delta$ T cells play a protective role and which mechanisms are involved in it. Our data suggest that $\gamma\delta$ IEL is a significant protective T cell population against colitis, because colitis induced in C$\delta^{-/-}$ mice is suppressed by $\gamma\delta$ IEL transfer mediated by TGF-β production. Furthermore, aberrant cell communication between EC and IEL following TNBS administration is discussed.

Materials and Methods

Mice and induction of colitis

C57BL/6 mice were purchased at the age of 8 wk from the Japan SLC (Hamamatsu, Japan). TCR$\alpha^{-/-}$ mice were purchased from the The Jackson Laboratory (Bar Harbor, ME). C$\delta^{-/-}$ mice were a kind gift from S. Itohara (Kyoto University, Kyoto, Japan). All mice were used between 8 and 12 wk of age and were approved by the institutional review board for animal experiments of Kyushu University and University of Miyazaki.

*Department of Infectious Diseases, Division of Parasitic Disease, University of Miyazaki, Miyazaki Medical College, Kiyotake, Miyazaki, Japan; †Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Maidashi, Fukuoka, Japan; and ‡Division of Molecular Microbiology, Center of Molecular Biosciences, University of the Ryukyus, Senbaru, Nishihara, Okinawa, Japan

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[2] Address correspondence and reprint requests to Dr. Kyoko Inagaki-Ohara, Department of Infectious Diseases, Division of Parasitic Disease, University of Miyazaki, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan (E-mail address: INAGAKI@med.miyazaki-u.ac.jp); or Dr. Akihiko Yoshimura, Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan (E-mail address: yakihiko@bioreg.kyushu-u.ac.jp).

[3] Abbreviations used in this paper: IEL, intestinal intraepithelial lymphocytes; AICD, activation-induced cell death; AP, allophycocyanin; DP, double positive; EC, epithelial cell; IBD, inflammatory bowel disease; LI, large intestine; LPL, lamina propria lymphocyte; PI, propidium iodide; SI, small intestine; TNBS, 2,4,6-trinitrobenzene sulfonic acid; WT, wild type.

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Colitis was induced according to a modification of published methods (16). On days 0 and 7, the mice were administered a TNBS enema, and 3 days after the last administration, they were sacrificed to assess tissues and cells.

Detection of anaerobes

After the mice were scarified, the contents of their small intestines (SI) and large intestines (LI) were collected and suspended in saline. An aliquot of suspension was diluted, and 100 μl of the diluted suspension was plated on brain heart agar (BD Microbiology Systems, Sparks, MD). These plates were incubated in anaerobe chamber at 37°C overnight.

Histological analysis

Intestines were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. The paraffin sections were stained with H&E.

Preparation of IEL, EC, and LPL

Mucosal lymphocytes were isolated and prepared according to a modification of previously published methods (17, 18). Dissected small segments of the intestines were incubated at 37°C for 30 min in an RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FCS and 1 mM DTT with vigorous shaking. The tissue suspension was passed through a nylon mesh to remove debris and centrifuged through a 25/40/75% discontinuous Percoll (Sigma-Aldrich) gradient at 600 $\times$ *g* at 20°C for 20 min. The cells collected from the interface of 40/75% and 25/40% were IEL and EC, respectively. To isolate LPL, after removal of EC and IEL, tissues were incubated for 30 min at 37°C in RPMI 1640 containing collagenase type VIII (Sigma-Aldrich). The cell suspension was centrifuged through a 40/75% discontinuous Percoll gradient, and the cells at the interface were LPL. For the cell culture, $CD4^+$, $CD4^+CD8^+$ double-positive (DP), and $CD8^+$ IEL were separated by using anti-FITC MultiSort Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instruments. For the isolation of $CD4^+$ and DP IEL, IEL were stained with anti-FITC-conjugated CD4 mAb (RM4-5), following MultiSort anti-FITC MicroBeads. All $CD4^+$ IEL were positively selected using a MS^+ column in MACS (Miltenyi Biotec). The MicroBeads were then released and stained with anti-PE CD8α mAb (53-6.7), following anti-PE microbeads. DP IEL were positively sorted, and $CD4^+CD8^-$ IEL were negatively sorted. For the isolation of $CD4^-CD8^+$ IEL, IEL stained with anti-FITC-conjugated CD8α mAb were positively sorted, and then cells were restained with anti-PE-conjugated CD4 and were negatively sorted. For culture, EC were isolated, as described previously (19). Briefly, minced intestine was digested at 20°C for 30 min in a DMEM medium (Sigma-Aldrich) containing collagenase (type XI; Sigma-Aldrich) and dispase (neutral protease type I; Roche Diagnostic Systems, Indianapolis, IN). The tissue suspension was pipetted vigorously, transferred through a nylon mesh to remove debris, and washed at 200 $\times$ *g* for 2 min until the supernatant was completely clear.

Transfer of IEL

Donor $\alpha\beta$ and $\gamma\delta$ IEL from the SI and $\gamma\delta$ IEL from the LI were negatively sorted by MACS and labeled with or without 5 μM CFSE (Sigma-Aldrich). After incubating IEL for 15 min at 37°C, cells were washed and resuspended in 200 μl of sterile PBS. These IEL (3×10^6) were injected i.p. into the recipient $C\delta^{-/-}$ mice, which were irradiated with 5 Gy from a cesium source γ irradiator.

FACS analysis

Cells were stained, as described previously (18). The mAbs used in this study were as follows: FITC-conjugated CD4 (RM4-5), TCR$\gamma\delta$ (GL3) mAb; PE-conjugated CD8α (53-6.7) and TCRβ (H57-597) mAb; biotin-conjugated TCRβ (H57-597) mAb, followed by streptavidin-allophycocyanin (AP); AP-conjugated CD3ϵ (145-2C11) mAb. Intracellular FACS of TGF-β in the small intestinal IEL was performed using Cytofix/Cytoperm Plus kit (BD Pharmingen, San Jose, CA), according to the manufacturer's instructions. After permeabilization, IEL were stained with biotin-conjugated anti-TGF-β mAb (A75-3.1), followed by streptavidin-PE. For the detection of apoptotic cells, IEL were stained with FITC-conjugated annexin V and propidium iodide (PI). All mAbs were purchased from BD Pharmingen. Flow cytometry analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Cell culture and cytokine analysis

Whole and sorted IEL (1×10^6/ml) were added to a 96-well plate precoated with 2.5 μg of anti-CD3 mAb (145-2311; BD Pharmingen) and were cultured for 48 h in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. Isolated EC by collagenase/dispase digestion were plated on collagen (Cellmatrix type I-P; Nitta Gelatin, Osaka, Japan)-coated dish in DMEM containing 2.5% FCS. For the coculture of EC with IEL, EC were trypsinized and replated on 96-well plates with a 1:10 ratio of IEL for 48 h. The supernatants were collected to estimate the cytokine contents. The cytokine contents in the culture supernatant were assayed by an ELISA using mouse IFN-γ, IL-4, and TNF-α (*e*Bioscience, San Diego, CA) and TGF-β (BioSource International, Camarillo, CA). ELISA systems were according to the manufacturer's instructions. Intracellular staining of TGF-β in the IEL was performed by using Cytofix/Cytoperm Plus kit (BD Pharmingen), according to the manufacturer's instructions. After permeabilization, IEL were stained with biotin-conjugated anti-TGF-β mAb (A75-3.1), followed by streptavidin-PE.

Table I. *Survival rate of C57BL/6 mice after TNBS administration*[a]

Dose (μg/g weight)	No. of Survivors per No. of Treated Mice (%)	Body Weight (g) (% decrease)
0[b]	8/8 (100)	23.1 $\pm$ 1.1 (100)
20	8/8 (100)	21.4 $\pm$ 0.9 (93)
40	10/10 (100)	20.0 $\pm$ 0.7 (87)[c]
60	4/7 (57)	18.2 $\pm$ 1.0 (79)[c]
80	0/10 (0)	ND[d]

[a] Survival was counted on day 14 after administration of TNBS.
[b] Mice were treated with 50% ethanol alone.
[c] Value of $p < 0.05$ compared with mice without TNBS treatment.
[d] Not determined due to death of TNBS-treated mice.

Semiquantitative RT-PCR

Total RNA was extracted from isolated cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) and primed with 20 pmol of a random primer in mixtures for reverse transcription. The synthesized cDNA was amplified by PCR using primers specific for the murine IL-15 and β-actin cDNA sequence. The primer sets were as follows: IL-15 sense, 5′-GGA AGG CTG AGT TCC ACA TC-3′; IL-15 antisense, AGG GAG ACC TAC ACT GAC AC-3′; β-actin sense, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′; β-actin antisense, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′.

Western blotting analysis

Lysates of EC from both small and large intestinal tissues of wild-type (WT) and $C\delta^{-/-}$ mice were obtained, according to a modification of previously published methods (20). Goat anti-mouse IL-15 Ab was purchased from R&D Systems (Minneapolis, MN), and mouse anti-β-actin Ab from Sigma-Aldrich.

Statistics analysis

Student's *t* test was used to determine the significant difference. A *p* value of <0.05 was taken as significant. Kaplan-Meier analysis (log-rank test) was used to determine the significant difference of survival following TNBS administration.

Results

Susceptibility of $C\delta^{-/-}$ mice to colitis induction

We found that ~5% of $C\delta^{-/-}$ mice over 8 mo of age developed colitis with soft stool, diarrhea, and rectal prolapse when housed in a conventional environment (data not shown). When healthy young $C\delta^{-/-}$ mice were housed with the aged mice with rectal prolapse, the young mice showed no signs or symptoms. Thus, these observations suggested that the colitis of $C\delta^{-/-}$ mice was not induced by infection in the colony, and $C\delta^{-/-}$ mice spontaneously develop colitis with age.

Development of spontaneous colitis in aged $C\delta^{-/-}$ mice led us to examine whether young $C\delta^{-/-}$ mice are susceptible to colitis induction. The ability of $\gamma\delta$ T cells to protect the intestinal mucosa from TNBS has been reported in Lewis rats treated with anti-TCR$\gamma\delta$ Ab (14). Thus, we used $C\delta^{-/-}$ mice to investigate significance of $\gamma\delta$ T cells for suppression of TNBS-induced colitis. In advance to this experiment, we optimized the dose of TNBS required to induce colitis in C57BL/6 mice because this strain is

considered to be resistant to TNBS-induced colitis, and Cδ$^{-/-}$ mice are C57BL/6 background. Intrarectal inoculation of TNBS at 20 μg/g body weight induced no colitis, and 80 μg/g body weight was a lethal dose. The TNBS inoculation at 40 μg/g body weight induced mild loss of body weight without lethality (Table I). Thus, we used the dose of 40 μg/g body weight of TNBS in the following experiments. As shown in Fig. 1*A*, ~50% of young Cδ$^{-/-}$ mice died within 14 days after TNBS administration, whereas WT mice survived. The TNBS-treated Cδ$^{-/-}$ mice showed a more severe thickening and shortening of the colon than the TNBS-treated WT mice. In addition, the SI was also impaired by TNBS administration (Fig. 1*B*), because the tissue wall became thin and fragile. As it was found that the TNBS-treated Cδ$^{-/-}$ mice showed diarrhea and it has been reported that anaerobe number was increased in IBD patient (21), we examined changes of colony number of anaerobe. Anaerobe number was greater in Cδ$^{-/-}$ mice than WT mice following TNBS administration (Fig. 1*C*). These results suggest a strong correlation between the high sensitivity to TNBS-induced colitis and the lack of γδ T cells in the intestine; they also indicate that the γδ T cells have a beneficial role for protection against TNBS-induced colitis.

Preventive role of γδ IEL against TNBS-induced colitis

To investigate the regulatory role of γδ T cells against colitis, we examined whether transfer of intestinal γδ IEL protects Cδ$^{-/-}$ mice from TNBS-induced colitis. Transferred small intestinal γδ IEL prepared from the WT mice were repopulated in the SI of Cδ$^{-/-}$ mice at the level comparable to WT mice (Fig. 2*A*). The small intestinal γδ IEL also repopulated in the LI of the Cδ$^{-/-}$ mice, although the number is lower than that of WT mice. However, the transferred γδ T cells were not detected in other tissues such as the liver and spleen. The large intestinal γδ IEL transferred to Cδ$^{-/-}$ mice repopulated preferentially in LI, but not in SI (Fig. 2*A*). When small intestinal γδ IEL-transferred mice were administered with TNBS, they exhibited significant increase of survival rate (Fig. 2*B*). In contrast, small intestinal αβ IEL-transferred Cδ$^{-/-}$ mice showed exacerbated colitis. Interestingly, γδ IEL prepared from LI did not improve the odds of survival against TNBS-induced colitis. On gross examination, nontransferred Cδ$^{-/-}$ mice showed severe shortening and thickening of colonic wall after TNBS treatment. In contrast, small intestinal γδ IEL-transferred Cδ$^{-/-}$ mice showed milder thickening of the colon after TNBS treatment compared with the nontransferred Cδ$^{-/-}$ mice (Fig. 2*C*, *left*). Small intestinal αβ IEL- and large intestinal γδ IEL-transferred Cδ$^{-/-}$ mice exaggerated thickening. In histological analysis, we observed the thickening of intestinal wall and the ulcers reaching the muscle layer, diffuse cell infiltration, and distortion of crypts in the TNBS-treated Cδ$^{-/-}$ mice that had not received any IEL and TNBS-treated small intestinal αβ IEL- or large intestinal γδ IEL-transferred Cδ$^{-/-}$ mice (Fig. 2*C*, *right*). However, small intestinal γδ IEL-transferred Cδ$^{-/-}$ mice showed only mild thickening and cell infiltration, although distortion of crypts was observed. These results suggest that small intestinal γδ IEL suppress colitis when transferred to colitis-prone mice with no γδ IEL.

We next investigated the mechanism of γδ IEL-mediated suppression of colitis. IEL prepared from Cδ$^{-/-}$ mice produced a higher level of IFN-γ and TNF-α, but a lower level of TGF-β and IL-4 than the WT mice (Fig. 3*A*). IEL from Cδ$^{-/-}$ mice treated with TNBS enhanced the production of Th1-type cytokines, such as IFN-γ and TNF-α. By contrast, the γδ IEL-transferred Cδ$^{-/-}$ mice showed a greater decrease in IFN-γ and TNF-α and a greater increase in TGF-β production than the Cδ$^{-/-}$ mice that had not received the IEL (Fig. 3*B*, *left*). These results suggest that Cδ$^{-/-}$ mice lack the suppressive ability against proinflammatory cytokine production by IEL, which enhance colitis. To further confirm the production of TGF-β by transferred γδ IEL, CFSE-labeled γδ IEL

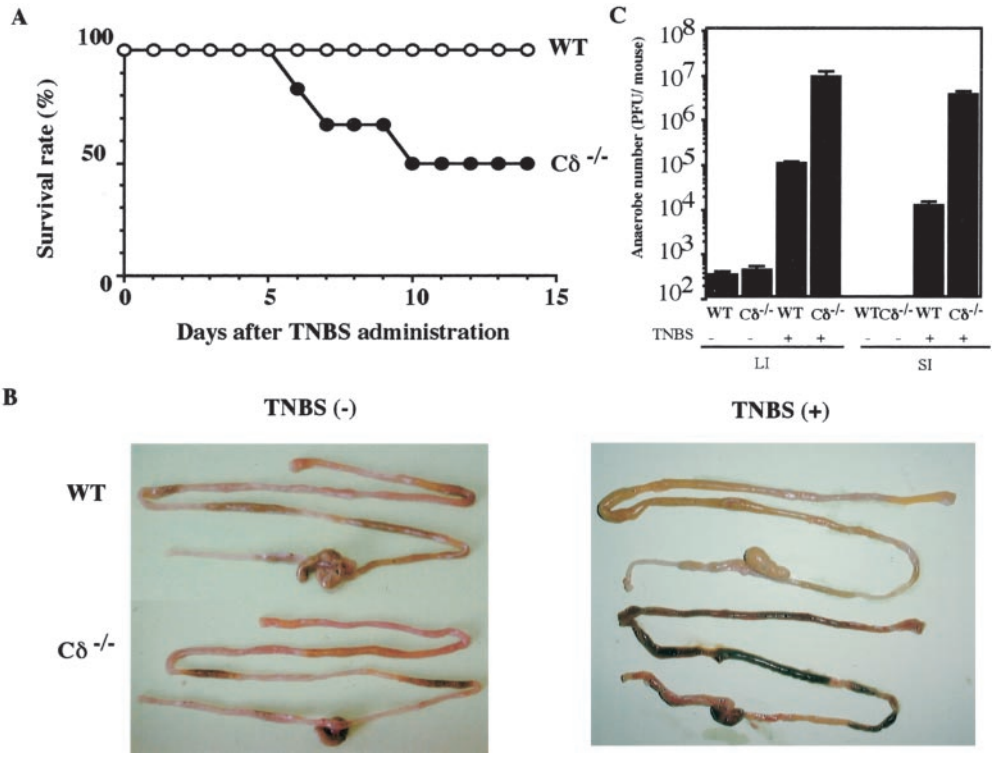

FIGURE 1. Susceptibility of Cδ$^{-/-}$ mice to TNBS. *A*, Cδ$^{-/-}$ mice and WT mice ($n = 10$) were administered 40 μg of TNBS per gram of body weight. Survival rates were obtained daily after the first TNBS administration. *B*, Gross photographs of dissected SI and LI of WT and Cδ$^{-/-}$ mice on day 10 after TNBS administration. *C*, Anaerobe number on day 10 after TNBS administration. Values represent the mean ± SD of three individual experiments.

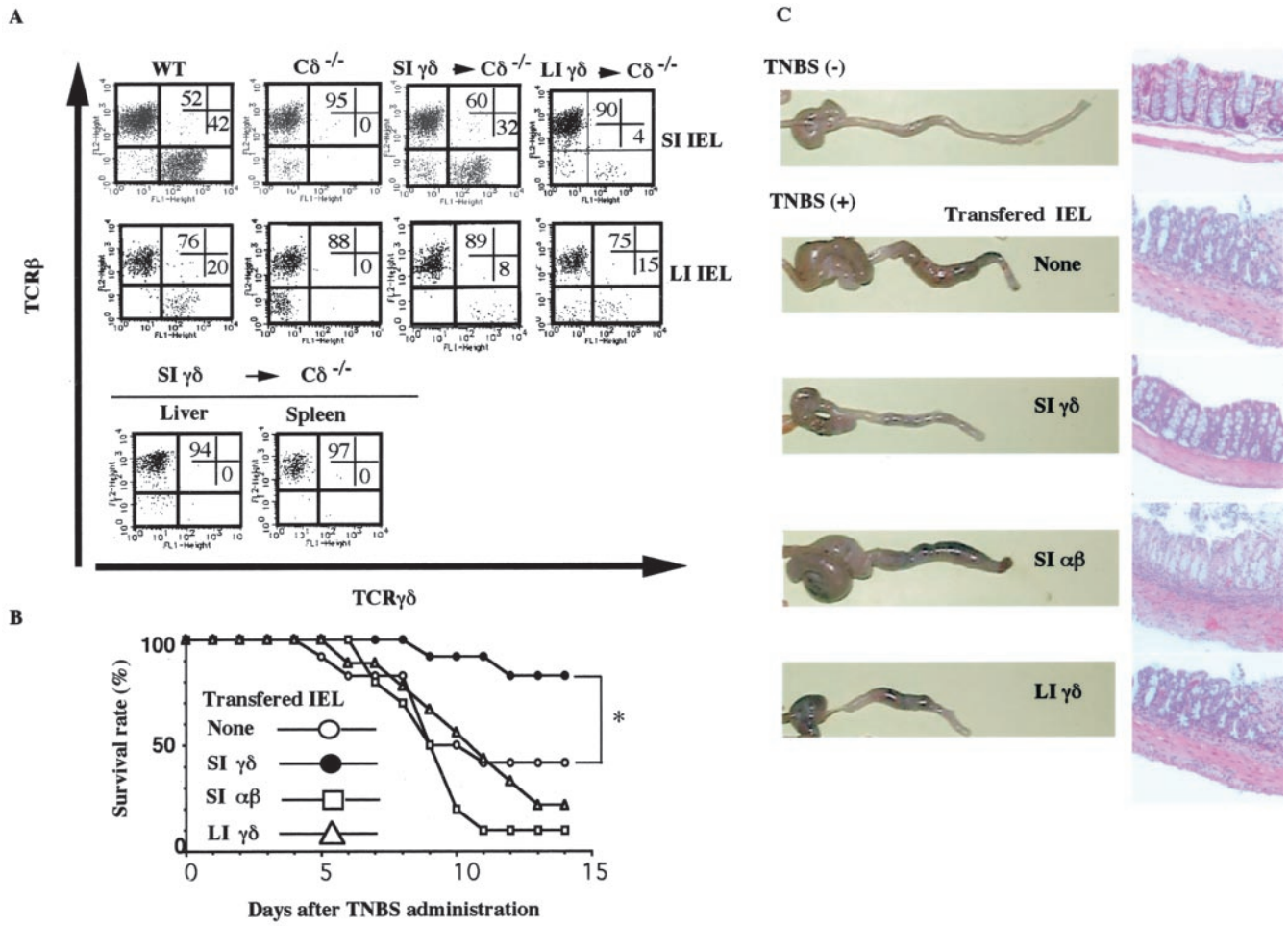

FIGURE 2. Suppressive role of γδ IEL in the development of colitis. *A*, Reconstitution of transferred IEL in the recipient Cδ$^{-/-}$ mice. γδ or αβ IEL in the SI and γδ IEL in the LI of WT mice were transferred into irradiated Cδ$^{-/-}$ mice, as described in *Materials and Methods*. Three weeks later, IEL in both the SI and LI were prepared in these IEL-transferred recipient mice for FACS analysis. Representative data show IEL staining in mice, which are γδ IEL transferred. The numbers indicate the percentages of fluorescence-positive cells in the corresponding squares. *B*, Survival rate of Cδ$^{-/-}$ mice. None of IEL or SI γδ IEL were transferred to Cδ$^{-/-}$ mice ($n = 12$). SI αβ ($n = 10$) or LI γδ IEL ($n = 9$) were transferred to Cδ$^{-/-}$ mice. After the transfer of cells, the mice were administered 40 μg of TNBS per gram of body weight. The survival rates were observed daily after the first TNBS administration. Data shown represent the survival in terms of percentage obtained by Kaplan-Meier analysis. ∗, $p < 0.05$ was determined by the log-rank test (Mantel-Cox). *C*, The gross appearance (*left*) and H&E staining of transverse sections of the colon in each group on day 14 (*right*, magnification, ×100).

prepared from WT mice were transferred to irradiated Cδ$^{-/-}$ mice. Intracellular FACS analysis revealed that most of CFSE^{+} γδ IEL transferred into Cδ$^{-/-}$ mice produced TGF-β (Fig. 3*B*, *right*). These results demonstrated that Cδ$^{-/-}$ mice develop Th1-type colitis, and we speculate from the results that γδ IEL down-regulate colitis through TGF-β-mediated suppression of the Th1-type immune response.

Increase in CD4^{+}CD8^{+} DP IEL in Cδ$^{-/-}$ mice treated with TNBS

We also noticed that CD4^{+}CD8^{+} (DP) IEL in the SI increased more in Cδ$^{-/-}$ mice than it did in WT mice at 20 and 40 wk of age (Fig. 4*A*). Similar changes were observed in LPL in the SI (Fig. 4*A*) and IEL in the LI (data not shown). As the increase of DP T cells was most marked in the IEL of the SI, we focused on the IEL in the SI in the following experiments. We examined whether the increased number of DP T cells in Cδ$^{-/-}$ mice contributes to increased susceptibility to TNBS-induced colitis in the mice. When Cδ$^{-/-}$ mice were treated with TNBS, an increase in DP IEL was observed even in young mice, but TNBS administration did not induce DP IEL in WT mice (Fig. 4*B*). The transfer of γδ IEL into Cδ$^{-/-}$ mice reduced increase of DP IEL after TNBS administration. No such increase of DP IEL was observed in TCRα$^{-/-}$ mice, which develop spontaneous Th2-type colitis with age. Even though TCRα$^{-/-}$ mice showed symptoms of colitis, such as prolapse at 30 wk, they did not show the alterations of the DP population (Fig. 4*C*). These results suggest that the γδ T cell is involved in the regulation of DP cells, and increase of DP cells may be associated with Th1-type, but not Th2-type, colitis.

Regulatory cytokines produced by CD4^{+}CD8^{+} DP IEL and inflammatory cytokines by CD4^{-}CD8^{+} IEL

Next, we tried to identify the cytokine produced by the DP IEL, and the functional differences between the DP IEL in aged WT mice and those induced in TNBS in Cδ$^{-/-}$ mice. As shown in Fig. 5, the DP IEL from aged WT mice and TNBS-treated Cδ$^{-/-}$ mice produced a high level of TGF-β as well as CD8^{+} IEL from the mice. IFN-γ and TNF-α were predominantly produced by CD8^{+} IEL rather than DP IEL, and TNF-α production in Cδ$^{-/-}$ mice was much more than that in aged WT mice. In contrast, CD4^{+} IEL

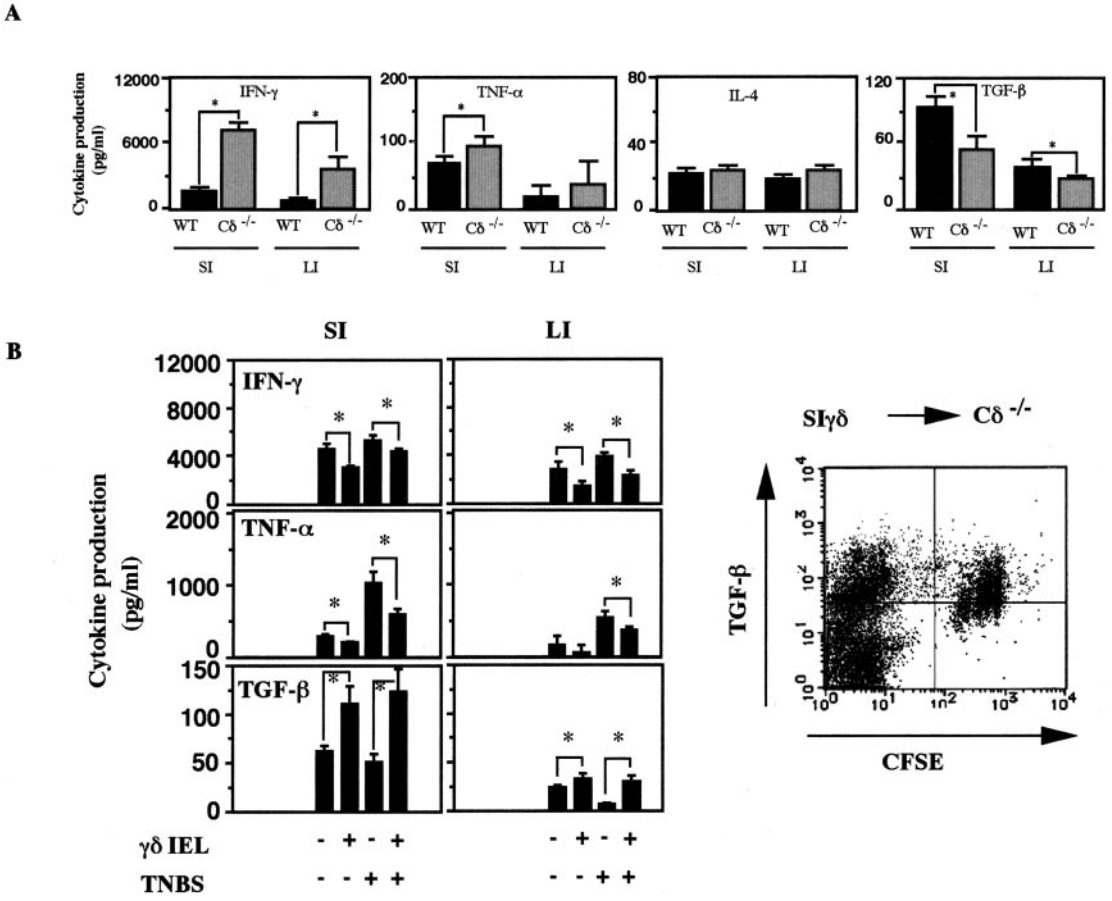

FIGURE 3. Changes of cytokine production following TNBS administration. *A*, Cytokine profiles in IEL in both SI and LI before TNBS administration. IEL were prepared before TNBS administration and on day 10 after the first TNBS enema. Freshly isolated IEL were cultured with plate-bound anti-CD3 mAb for 48 h, and the supernatants were collected to determine the concentration of each cytokine by ELISA for IFN-γ, IL-4, TNF-α, and TGF-β. *B*, Cytokine production of the SI and LI IEL in $C\delta^{-/-}$ mice following TNBS administration. Intracellular FACS of TGF-β was performed in the SI IEL of the recipient $C\delta^{-/-}$ mice, which received CFSE-labeled SI γδ IEL (*right*). Values represent the mean ± SD of three individual experiments. ∗, Represents a statistically significant difference ($p < 0.05$).

were not involved in the production of these cytokines. These results suggest that the increase of DP IEL in $C\delta^{-/-}$ mice after TNBS administration may play a protective role against colitis because these cells produced TGF-β. We speculate that the TGF-β-producing DP IEL increased in $C\delta^{-/-}$ mice to compensate for the lack of a regulatory function of γδ IEL.

Interaction between EC and IEL regulates the cytokine production of IEL

EC play an essential role in the onset and development of colitis, and IEL and EC regulate each other (8, 22). Therefore, we next analyzed EC-mediated regulation of IEL in $C\delta^{-/-}$ mice. As an EC-derived immunoregulator, we first analyzed the expression of IL-15 by EC because: 1) IL-15 is a cytokine produced by various cells including EC (18, 23); 2) IL-15 affects the survival of T cells by inhibiting activation-induced cell death (AICD) (24, 25); and 3) IL-15 mRNA increased in the inflamed intestinal tissue of IBD (26, 27). Although the $C\delta^{-/-}$ and WT mice showed similar expression levels before TNBS administration in the SI, the $C\delta^{-/-}$ mice showed an enhanced IL-15 mRNA level after TNBS administration, whereas WT mice showed only a slight increase (Fig. 6*A*). The $C\delta^{-/-}$ mice also showed a slight increase of the IL-15 message in the LI following TNBS administration. We next examined expression of IL-15 at the protein level by Western blotting analysis. In accordance with the result of RT-PCR, larger amount of IL-15 was produced by the EC of the $C\delta^{-/-}$ mice than those of WT mice following TNBS administration (Fig. 6*B*). We further analyzed AICD of intestinal T cells in the $C\delta^{-/-}$ mice. αβIEL of the $C\delta^{-/-}$ mice contained ~30% of apoptotic annexin V-positive PI-negative cells, but those of WT mice contained 60% of apoptotic cells (Fig. 6*C*), indicating that less IEL of $C\delta^{-/-}$ mice undergo AICD than those of WT mice. These results suggest that EC of $C\delta^{-/-}$ mice protect proinflammatory intestinal T cells from AICD through expression of IL-15 at a high level. These results also suggest that the sustained activation of IEL could be caused by a failure to terminate the inflammation.

Cell-to-cell interactions between EC and IEL are assumed to be an important mechanism to control aberrant activation of IEL, which may be necessary for the prevention of inflammatory responses (22). Thus, we examined whether EC affect the activation of IEL in $C\delta^{-/-}$ mice treated with TNBS. As freshly isolated EC prepared by using Percoll gradient rapidly died in vitro, we used EC grown in vitro after preparation with collagenase and dispase (19). Isolated EC were once grown on a collagen-coated plate, and the EC then were replated on the well without a collagen coat. The EC were alive within 3 days without growth, and plate-bound anti-CD3 mAb did not interrupt the EC attachment to the plate (data not shown). As shown in Fig. 7, EC from WT mice reduced production

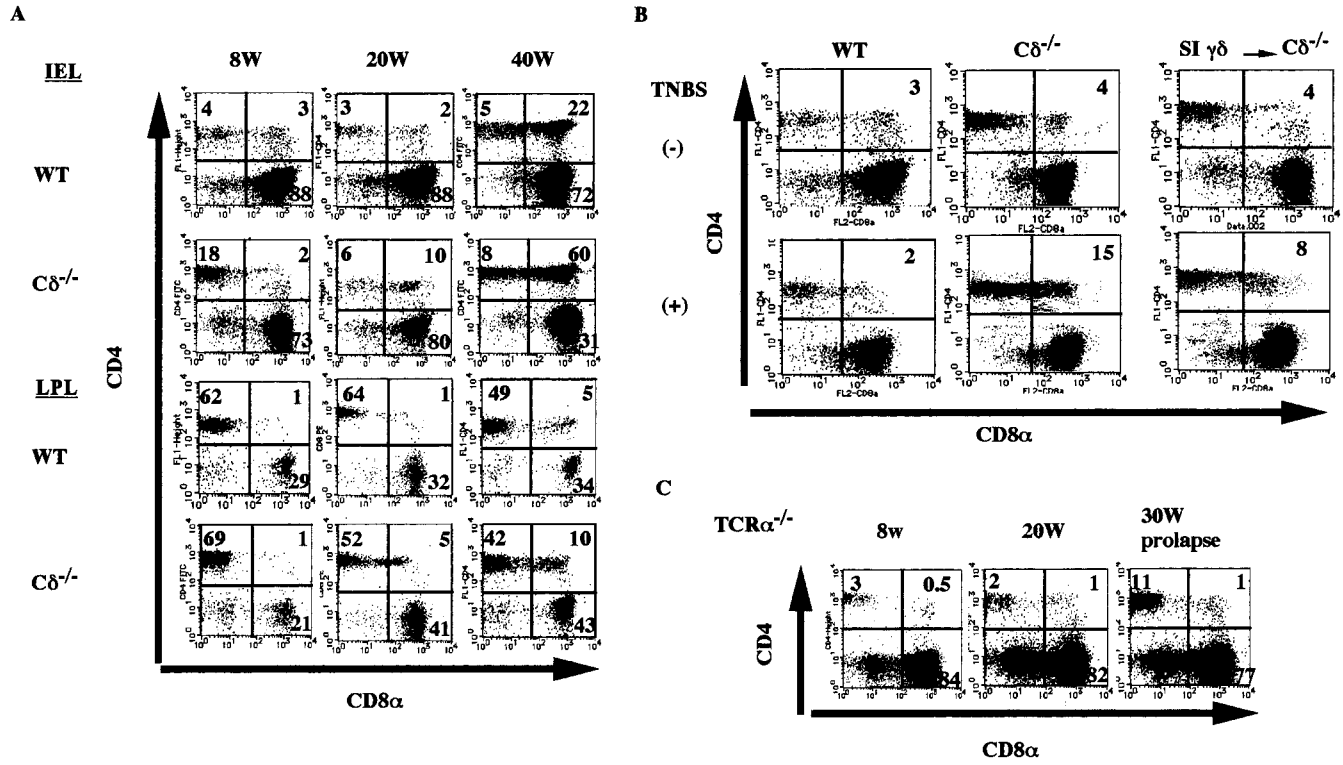

FIGURE 4. Age- or TNBS-dependent changes of intestinal T cells in C$\delta^{-/-}$ mice. *A*, CD4 and CD8α expression of SI IEL and LPL cells in WT and C$\delta^{-/-}$ mice at 8, 20, and 40 wk of age. These CD4 and CD8α expressions are gated on CD3$^+$ cells. *B*, Expression of CD4 and CD8α on SI IEL. SI IEL obtained from WT, C$\delta^{-/-}$, and C$\delta^{-/-}$ mice transferred with SI $\gamma\delta$ IEL in WT mice, which were treated with or without TNBS. *C*, The expression of CD4 and CD8α of SI IEL in TCR$\alpha^{-/-}$ mice.

of IFN-γ from IEL in both WT and TNBS-treated C$\delta^{-/-}$ mice. However, EC from TNBS-treated C$\delta^{-/-}$ mice did not affect IFN-γ production from whole IEL in WT mice, and rather tended to enhance IFN-γ production from IEL isolated from TNBS-treated CD8$^+$ IEL. TGF-β production was not significantly affected by coculture with EC. These data suggest that EC from TNBS-treated C$\delta^{-/-}$ mice could not suppress the activation of IEL, which may contribute to the development of colitis.

Discussion

The role of $\gamma\delta$ T cells in the colitis model is yet to be determined. An important finding in this study is that the lack of mucosal $\gamma\delta$ T cells results in the enhancement of colitis induction. We showed that the transfer of $\gamma\delta$ IEL suppressed mortality and the severity of TNBS-induced colitis (Fig. 2). This is the first evidence that $\gamma\delta$ IEL play a direct role in protection against the development of colitis. It has been reported that $\gamma\delta$ T cells play an important role in the maintenance of mucosal homeostasis. C$\delta^{-/-}$ mice showed a reduction in the turnover of EC and the down-regulation of the expression of the MHC class II molecule (8). $\gamma\delta$ IEL can produce keratinocyte growth factor (7) and TGF-β (28), resulting in the possible suppression of inflammation. Consistently with this result, IEL in the SI play a protective role against colonic inflammation (29). In fact, we demonstrated that C$\delta^{-/-}$ mice show reduced production of TGF-β (Fig. 3), but that the TGF-β level was restored after the transfer of small intestinal $\gamma\delta$ IEL. Furthermore, the $\gamma\delta$ IEL-transferred C$\delta^{-/-}$ mice showed reduction of mortality. It was also reported that autoimmune, inflammatory lupus-like disease of MRL/*lpr* mice was markedly exacerbated by backcross to the C$\delta^{-/-}$ mice (30), suggesting that an exaggerated $\alpha\beta$ T cell response is down-regulated by $\gamma\delta$ T cells. Classified as immunoregulatory cells, it might be predicted that animals would show pathological immune dysregulation due to its absence, and reconstitution of the subset restores normal immune response. Collectively, it is likely that $\gamma\delta$ T cells play an important role as immunoregulatory cells.

Germfree mice rarely develop IBD, which implies that the presence of microbes influences the development of IBD. Our results showed that the intestine of C$\delta^{-/-}$ mice with TNBS-induced colitis contained an increased number of anaerobes (Fig. 1*C*). These results could be supported by the fact that the pathology of colitis is associated with a high level of anaerobe (21) and a reduction of $\gamma\delta$ T cell (31). It has been also reported that a decrease in the humoral immune response is associated with an increase of several species of enteric bacteria (32, 33). It was also demonstrated that mice with a deficiency in Ig class switch and IgA production showed an increase of anaerobes in the intestine (34). Because colitis-prone C$\delta^{-/-}$ mice showed a decrease of TGF-β production and TGF-β is an important cytokine in the IgA class switch, it is possible that the reduction of IgA secretion in C$\delta^{-/-}$ mice causes an increase of anaerobes and the resultant colitis. The hypothesis is now under investigation.

As IL-15 is produced by the EC and is a growth factor of IEL, the possible roles of IL-15 in the mucosal intranet between EC and IEL have been investigated (18, 35). In contrast, negative regulatory systems controlling IL-15 production may be required because IL-15 is also an inflammatory cytokine. The expression of IL-15 mRNA was found to be increased in the inflamed intestinal tissue of IBD (26, 27). IL-15 has been reported to be overexpressed in rheumatoid arthritis, and IL-15-recruited and -activated autoreactive T cells in the synoviral membrane have led to TNF-α secretion in synoviral fluid (36, 37). Localized overexpression of intestinal IL-15 led to the development of a unique subset of

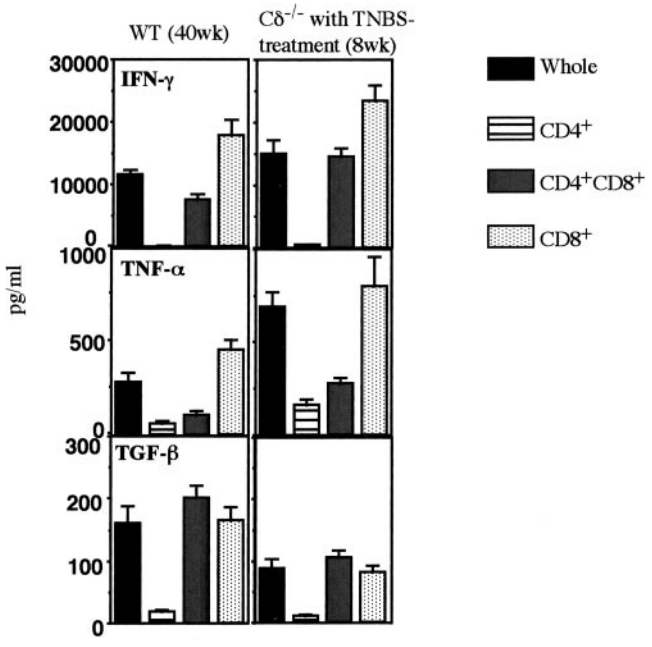

FIGURE 5. Comparison of cytokine production between aged WT mice and $C\delta^{-/-}$ mice treated with TNBS. Concentrations of each cytokine in whole, $CD4^+$, $CD4^+CD8^+$, and $CD8^+$ subsets are shown. Values represent the mean ± SD of three individual experiments.

AICD-resistant Th1-type $CD8\alpha\beta^+NK1.1^+$ T cells and the induction of inflammation in the SI (38). The TNBS-treated mice showed a high level of inflammatory cytokines from IEL (Figs. 3 and 5) and a high level of IL-15 mRNA expression and protein (Fig. 6, *A* and *B*). IL-15 sustains the Bcl-2 level of IEL (18), causing IEL to survive and become resistant against apoptosis in IBD. Collectively, the activation observed in $C\delta^{-/-}$ mice might be due to effete or aberrant cells failing to undergo apoptosis.

The only barrier between the Ag-rich luminal environment and the mucosal immune system is the single layer of EC. EC would not only form a purely physical barrier, but would also be capable of producing a series of cytokines, chemokines, and defensins (39, 40). Cell-to-cell interactions between IEL and EC would be mediated by cell surface molecules, suggesting that these interactions prevent potential inflammatory responses at the intestinal mucosal surface (22). We demonstrated the significance of EC in the regulation of IEL function. The activation of IEL in TNBS-treated mice was suppressed by normal EC (Fig. 7). However, EC from TNBS-treated $C\delta^{-/-}$ mice failed to suppress IFN-γ production of IEL. Therefore, γδ T cell-mediated acquisition of EC-suppressive function can be an important mechanism of γδ T cell-mediated suppression of IEL. In this study, we showed the suppressive effect of γδ T cells on colitis. However, γδ T cells are not only anti-inflammatory cells in the intestine. Our data also demonstrated that DP IEL produce TGF-β. We speculate that the DP IEL may also participate in suppression of colitis, which is in accordance with previous reports showing regulatory function of DP IEL against a colitis model (41). In addition, intestinal EC express CD1d. CD1d-restricted NKT cells have been reported to suppress dextran sodium sulfate-induced colitis (42). It is assumed that multiple regulatory T cell subsets are necessary for protecting against colitis. To elucidate the different reactivity of these regulatory T cells is valuable in therapeutics for colitis.

We demonstrated in the present study that DP IEL increase in $C\delta^{-/-}$ mice from early stage of ontogeny, although such increase of DP IEL was observed at later stage of ontogeny in WT mice. Although the mechanism of the early increase of DP IEL in $C\delta^{-/-}$ mice is not clear at this moment, there are two possible mechanisms for the increase. The increase of anaerobic bacteria in the intestinal lumen of $C\delta^{-/-}$ mice may induce the increase of DP IEL because it has been reported that the increase of DP IEL depends on the recognition of bacterial Ags (43, 44). Alternatively, inflammation in the intestine recruits $CD4^+$ T cells to the intestine and converts its phenotype to DP. The second possibility is supported by the observation that splenic $CD4^+CD8^-$ T cells change their phenotype to DP when transferred to RAG-$1^{-/-}$ mice (41). We need further information to prove the hypotheses.

In summary, our study demonstrates that γδ T cells have protective ability against developing colitis, and suggests the possibility of a novel therapeutic strategy using regulatory γδ T cells for the suppression of colitis, including transfer of the γδ T cells or activation of the cells in vivo. In this study, we proposed that the protective role of γδ T cells against colitis is mediated by TGF-β

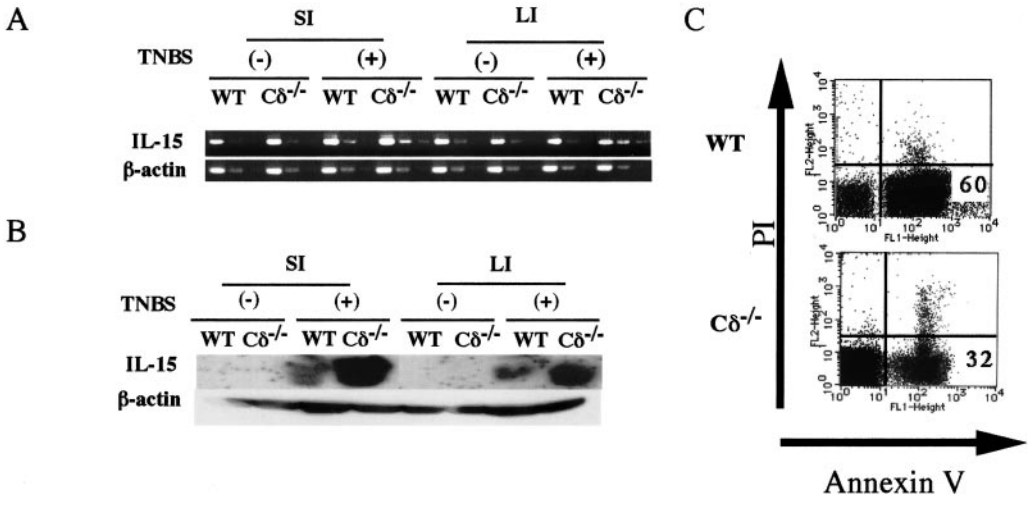

FIGURE 6. Increased IL-15 level in EC and following TNBS administration. *A*, Expression of IL-15 mRNA in both EC of SI and LI in WT and $C\delta^{-/-}$ mice with or without TNBS administration. Semiquantitative RT-PCR was performed by using cDNA diluted with 4-fold serially as templates. *B*, Lysates from EC in WT and $C\delta^{-/-}$ mice were prepared and were subsequently analyzed by Western blot for IL-15. *C*, IEL were incubated with FITC-conjugated annexin V, PI, and AP conjugated with TCRβ mAb.

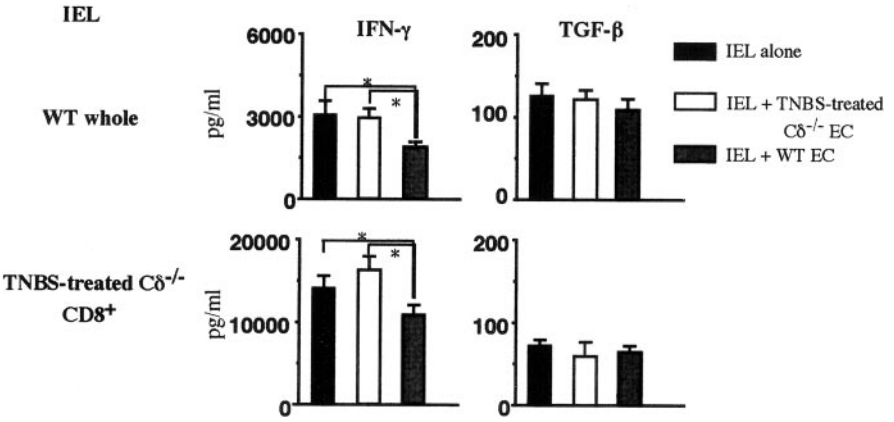

FIGURE 7. Inhibition of the activation of IEL stimulated with anti-CD3 mAb by EC. IEL were cultured with EC in the presence of immobilized anti-CD3 mAb at EC to IEL ratios of 1:10. These cells were then incubated for 48 h at 37°C to collect supernatants for the determination of cytokine production by ELISA for IFN-γ and TGF-β. Values represent the mean $\pm$ SD of three individual experiments. $*$, Represents a statistically significant difference ($p < 0.05$).

production. Furthermore, $\gamma\delta$ T cell-mediated regulation of the immunoregulatory function of EC is also suggested to be involved in the regulation of colitis. However, it remains to be determined when and how $\gamma\delta$ T cells develop and are induced as regulatory cells during the development of colitis, and whether there is communication or a relationship between $\gamma\delta$ T cells and other regulatory cells such as DP cell. Therefore, it will be important to characterize $\gamma\delta$ T cells as regulatory T cells.

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