Tumor Necrosis Factor Receptor-associated Factor (TRAF) 2 Controls Homeostasis of the Colon to Prevent Spontaneous Development of Murine Inflammatory Bowel Disease

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Fine-tuning of host cell responses to commensal bacteria plays a crucial role in maintaining homeostasis of the gut. Here, we show that tumor necrosis factor receptor-associated factor (TRAF)2−/− mice spontaneously developed severe colitis and succumbed within 3 weeks after birth. Histological analysis revealed that apoptosis of colonic epithelial cells was enhanced, and B cells diffusely infiltrated into the submucosal layer of the colon of TRAF2−/− mice. Expression of proinflammatory cytokines, including Tnfα, Il17a, and Il23a, was up-regulated, whereas expression of antimicrobial peptides was down-regulated in the colon of TRAF2−/− mice. Moreover, a number of IL-17-producing helper T cells were increased in the colonic lamina propria of TRAF2−/− mice. These cellular alterations resulted in drastic changes in the colonic microbiota of TRAF2−/− mice compared with TRAF2+/+ mice. Treatment of TRAF2−/− mice with antibiotics ameliorated colitis along with down-regulation of proinflammatory cytokines and prolonged survival, suggesting that the altered colonic microbiota might contribute to exacerbation of colitis. Finally, deletion of Tnfr1, but not Il17a, dramatically ameliorated colitis in TRAF2−/− mice by preventing apoptosis of colonic epithelial cells, down-regulation of proinflammatory cytokines, and restoration of wild-type commensal bacteria. Together, TRAF2 plays a crucial role in controlling homeostasis of the colon.

The microenvironment of intestinal lumen is a unique site of the body that is constantly exposed to a large amount of bacteria (1, 2). Although it is crucial for epithelial and immune cells to prevent invasion of bacteria into the subepithelial tissues, these cells need to suppress exaggerated immune responses to the colonic microbiota and food antigens. Therefore, fine-tuning of these opposing immune mechanisms is crucial to maintain homeostasis of the gut. Dysregulation of the balance elicits human inflammatory bowel disease (IBD)2 such as ulcerative colitis and Crohn disease (2, 3). In murine models, deletion of the Il10, Il12, and Foxp3 genes induces colitis because of a defect in the development of regulatory T cells (1–3). In addition, impaired barrier function induced by an increase in apoptosis of intestinal epithelial cells, as in mice with an intestinal epithelial cell-specific deletion of the Nemo or Tak1 gene, also promotes the development of colitis due to invasion of commensal bacteria (4, 5).

Tumor necrosis factor (TNF) receptor-associated factors are composed of six members of the family and act as adaptors that transmit signals through the TNF receptor superfamily, IL-1, and Toll-like receptors (6, 7). Among them, TRAF2, -5, and -6 activate NF-κB. The pathways leading to activation of NF-κB are currently divided into two pathways, the canonical and non-canonical pathways. Although degradation of inhibitors of κB (IkB), followed by nuclear translocation of RelA/p50, is a hallmark of the activation of the canonical pathway, processing of p100/NF-κB2 to p52 and the subsequent nuclear translocation of RelB/p52 are the hallmarks of activation of the noncanonical pathway (6, 7). Consistent with that, NF-κB prevents apoptosis (8), and TRAF2−/− mice are more susceptible to TNFα-induced apoptosis and succumb soon after birth (9–11). However, the cause of premature death is not fully understood. We previously reported that TRAF2−/− mice show a defect in Peyer patch but not mesenteric lymph node development (12). In addition, recent studies have shown that TRAF2 and TRAF3 negatively

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2 The abbreviations used are: IBD, inflammatory bowel disease; BM, bone marrow; LP, lamina propria; IPC, lamina propria cell; MLN, mesenteric lymph node; TNFR, TNF receptor; Tnfr1, 17 cell, IL-17-producing helper T cell; qPCR, quantitative PCR; DGGE, denaturing gradient gel electrophoresis.
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regulate the noncanonical pathway, and deletion of the Traf2 or Traf3 gene constitutively activates the noncanonical pathway (12–14).

To circumvent the embryonic lethal phenotype of Traf2−/− mice on a C57BL/6 background (10), we crossed Traf2−/− mice with BALB/c mice. We found that Traf2−/− mice on a BALB/c background spontaneously developed severe colitis and succumbed within 3 weeks after birth. In this study, we investigate the mechanisms underlying colitis in Traf2−/− mice and reveal that TNFα-induced apoptosis of colonic epithelial cells is primarily responsible for the development of colitis through an altered colonic microbiome and subsequent alterations of the colonic microbiota in Traf2−/− mice.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Lipopolysaccharide (R595), phorbol 12-myristate 13-acetate, and ionomycin were purchased from List Biological Laboratories, Inc., and Sigma, respectively. The following antibodies were used in this study and were obtained from the indicated sources: anti-CD3 (2C11.1), anti-CD4 (L3T4), anti-CD8 (M1/70), anti-CD11c (HL3), anti-Gr1 (RB6-8C5), and anti-B220 (RA3-6B2) (from BD Biosciences); anti-F4/80 (BM8), anti-IgM (II/41), anti-PDCA-1 (CD317 and BST2), and anti-IL-17 (17B7) (from eBiosciences); and anti-cleaved caspase 3 (9661) (from Cell Signaling).

**Mice and Treatments**—Traf2−/− and Tnfr1−/− mice (provided by W.-C. Yeh and T.-W. Mak) were described previously (9, 15). Traf2−/− and Tnfr1−/− were backcrossed with BALB/c mice at least 10 and 5 generations, respectively. Il17a−/− mice on a BALB/c background were described previously (16). Wild-type BALB/c mice were purchased from Japan Clea.

To administer antibiotics to newborn mice, we treated Traf2−/− mice with drinking water containing 1 mg/ml ampicillin and 1 mg/ml neomycin sulfate during pregnancy and the lactation period. Under these conditions, antibiotics were centrifuged at 1,800 rpm at 4 °C for 5 min to remove supernatants. The pellets were further incubated in 10 ml of 5 mM EDTA/Hanks’ balanced salt solution at 37 °C for 20 min. After incubation, epithelial cells were removed by gently scraping with forceps. The colon was then incubated in 10 ml of 4% FCS/RPMI 1640 medium containing 0.5 mg/ml liberase CI (Roche Applied Science) and 40 μg/ml DNase I (Sigma) at 37 °C with gentle shaking. After a 35-min incubation, cell suspensions were centrifuged at 1,800 rpm at 4 °C for 5 min to remove supernatants. The pellets were further incubated in 10 ml of 5 mM EDTA/Hanks’ balanced salt solution at 37 °C for 5 min with gentle shaking. After washing with Hanks’ balanced salt solution, cell suspensions were passed through nylon mesh and used for FACS analysis and culture. Plasma cytolytic dendritic cells were subsequently isolated by auto-MACS (Miltenyi Biotec) using anti-PDCA-1 microBeads (Miltenyi Biotec) according to the manufacturer’s instructions. Isolated cells were unstimulated or stimulated with 100 ng/ml LPS for 18 h.

For intracellular cytokine staining, immediately after isolation, cells were incubated with 50 ng/ml phorbol 12-myristate 13-acetate, 500 ng/ml ionomycin, and 10 μg/ml GolgiPlug (BD Biosciences) for 4 h. Surface and intracellular cytokine staining was performed as described elsewhere (17).

**Adoptive Bone Marrow Transplantation**—BM cells were prepared from 2-week-old Traf2−/+ and Traf2−/− mice, and 4–5 × 10⁶ cells were injected into wild-type BALB/c mice that had been exposed to lethal irradiation (9 gray). Four weeks after transplantation, the body weight of wild-type mice reconstituted with Traf2−/+ or Traf2−/− BM cells was measured once a week. Because donor and recipient mice were on a BALB/c background, we determined chimerism of recipient versus donor cells by examining relative expression levels of Traf2 mRNA by qPCR using BM cells from reconstituted mice.

**ELISA**—TNFα (eBiosciences), IL-22 (R&D Systems), and IgA (Bethyl Laboratories) were determined by ELISA kits from the indicated sources according to the manufacturer’s instructions. Serum IgM levels were determined by ELISA as described previously (18).

**qPCR**—Colon sections were homogenized with Polytron in TRIzol (Tel-Test) according to the manufacturer’s instructions; total RNAs were extracted, and cDNAs were synthesized using SuperScript II (Invitrogen). qPCR was performed using the 7500 Real Time PCR detection system using TaqMan Universal PCR master mix and Assays-on-Demand gene expression products of the mouse target genes along with an endogenous control (Gapdh; Mm99999915_g1) (Applied Biosystems). The following primers were used in this study: Tnfα (Mm00443258_m1); Il17a (Mm00439619_m1); Il17f (Mm00521423_m1); Ifng (Mm00801778_m1); Il22 (Mm01226722_g1); Il23p19 (Mm00518984_m1); Il6 (Mm00446190_m1); Muc1 (Mm00449604_m1); Defa4 (Mm00651736_g1); and Defa1 (Mm0254428_g1). The expression levels of these
genes were expressed relative to those of Gapdh using a 7500 SDS software (Applied Biosystems).

16 S rRNA Gene Analysis and Denaturing Gradient Gel Electrophoresis (DGGE) of Bacteria—Bacterial 16 S rRNA gene analysis and DGGE were performed as described previously (19). Briefly, the 16 S rRNA gene was amplified by 35 cycles of PCR at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, using the broad range bacterial primers 5'-CCAAACTCCTAGGGAGGAGC-3' and 5'-CATGGAATACAGGGTTATCTAATC-3' from the bacterial DNA pool (1:50 total DNA) of the colon. Amplified products were verified by agarose gel electrophoresis and purified by QIAEX II (Qiagen). The PCR products were then ligated into pGEM-T (Promega) for sequencing analysis or used as templates for DGGE analysis. The cloned 16 S rRNA gene fragments were then sequenced, and the resulted sequences were subjected to an on-line BLASTN analysis (National Center for Biotechnology Information, National Institutes of Health) for bacterial identification.

For DGGE analysis, we conducted nested PCR as follows. In the first PCR, samples were initially PCR-amplified by using the above-mentioned broad range bacterial primers. PCR amplification was carried out using the following program: 2 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C; and 5 min at 72 °C. The first PCR products were diluted 1:1,000 and used as a template for a second PCR with 518r and GC-clumped 341f primers as described previously (20). The second PCR was performed with 10 cycles of touchdown PCR (denaturation at 94 °C for 1 min, annealing for 1 min with 1 °C/ cycle decrements from 65 to 56 °C, and elongation at 72 °C for 1 min), followed by 15 cycles of regular PCR (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C) at 94 °C for 5 min, and a final elongation for 30 min at 72 °C. The PCR products were purified with a commercial kit (Promega). DGGE analysis was performed with a D-code system (Bio-Rad) as described previously (20). Individual DNA fragments extracted from the gel were amplified by PCR with the primers 341f and 518r, subcloned into the pGEM-T vector, and sequenced as described above. The homogeneity of the DNA fragments was confirmed by sequencing multiple clones.

Whole-intestine Culture—Two hundred mg of the small intestine or colon were washed in cold PBS supplemented with penicillin and streptomycin. Segments were cut into small pieces and cultured in 12-well plates in serum-free RPMI 1640 medium. A high concentration of penicillin and streptomycin was supplemented to prevent bacterial growth. After incubation at 37 °C for 24 h, culture supernatants were collected. Production of IgA in culture supernatants was determined by ELISA and represented as per g of the small intestine or colon.

Colitis Score—The colitis score of individual mice represents the sum of the following clinical and histological findings of the colon as described elsewhere with slight modifications (21). When moribund, mice were killed and assessed for the score of three parameters as follows: body weight loss, 0 or 1; stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool); grade of cell infiltration based on H&E-stained sections of the colon, 0–3 (0, no infiltration; 1, mild; 2, moderate; 3, severe). Maximum score was 7.

![Figure 1](image)

**Figure 1.** *Traf2*−/− mice spontaneously develop colitis. A, H&E-stained sections of the duodenum, jejunum, ileum, and colon of 2-week-old *Traf2*−/− and *Traf2*+/+ mice. Scale bars, 100 μm. B, Immunohistochemical analysis of the colon of 2-week-old *Traf2*−/− and *Traf2*+/+ mice. Frozen colon sections were stained with the indicated antibodies. Scale bars, 100 μm.

**Statistical Analysis**—Statistical analysis was performed by two-way unpaired t test. p < 0.05 was considered to be significant.

**RESULTS**

Spontaneous Development of Severe Colitis in *Traf2*−/− Mice—Because we could not obtain viable pups of *Traf2*−/− mice on a C57BL/6 background, we backcrossed *Traf2*−/− mice with BALB/c mice. BALB/c-*Traf2*−/− mice were born at half-expected Mendelian ratios (+/+; +/+; −/+; −/− = 1:2:0:5) (supplemental Table 1), but all mice were runted and succumbed within 2 weeks after birth (supplemental Fig. 1A). Thus, we referred to *Traf2*−/− mice on a BALB/c background as *Traf2*−/− mice hereafter. We found that *Traf2*−/− mice began to suffer from diarrhea and bloody stool 10 days after birth, suggesting that *Traf2*−/− mice spontaneously developed colitis. Macroscopic analysis revealed that the colon of *Traf2*−/− mice contained bloody stool (supplemental Fig. 1B). Histological analysis showed that a large number of mononuclear cells diffusely infiltrated in the submucosal layers of the colon and that goblet cells completely disappeared in the colon of *Traf2*−/− mice (Fig. 1A). No histological differences were detected in the duodenum and jejunum between *Traf2*+/+ and *Traf2*−/− mice, but mild atrophic changes were observed in the ileum of *Traf2*−/− but not *Traf2*+/+ mice (Fig. 1A). Notably, *Traf2*+/+ mice were indistinguishable from *Traf2*−/− mice and did not show any sign of colitis based on macroscopic and histological analysis (supplemental Fig. 1C and data not shown). Immunohistochemical analysis of colon sections revealed that the majority of infiltrating cells were B220+ B cells in *Traf2*−/−.
mice, whereas CD11c+ cells were modestly increased in the colon of Traf2+/− mice compared with Traf2+/+ mice (Fig. 1B). Although B cells accumulated in isolated lymphoid follicles in the colon of Traf2+/+ mice, no lymphoid follicles were found in the colon of Traf2−/− mice (supplemental Fig. 1D). The contribution of accumulated B cells to the development of colitis in Traf2−/− mice is currently unknown.

Transfer of Traf2−/− BM Cells into Lethally Irradiated Wild-type Mice Does Not Induce Colitis—To test whether Traf2 deficiency in hematopoietic lymphoid precursors is responsible for the development of colitis, we first examined the population of lymphocytes of the BM, spleen, and MLN from Traf2−/− and Traf2+/− mice. In sharp contrast to an increase in B cells in the colon of Traf2−/− mice, the frequency of B220+ cells was decreased in Traf2−/− BM and spleen but not MLN (Fig. 2A). We next performed adoptive bone marrow transfer experiments. BM cells from Traf2+/+ or Traf2−/− mice were transplanted into lethally irradiated wild-type BALB/c mice. Because allotype-specific antibodies, such as anti-CD45.1 or anti-CD45.2, used to determine the origin of hematopoietic cells are not available in BALB/c recipient mice, we examined the expression levels of Traf2 genes by quantitative PCR using BM cells from recipient wild-type BALB/c mice. Traf2 expression was high in BM cells of recipient mice transplanted with Traf2+/+ BM cells but not Traf2−/− BM cells (Fig. 2B). Although a lower percentage of B cells was found in BM cells of recipient mice reconstituted with Traf2−/− BM cells compared with mice reconstituted with Traf2+/+ BM cells, the percentages of B cells in the spleen and MLN from Traf2−/− mice were comparable with those from Traf2+/+ mice at 8 weeks after transfer (Fig. 2C). Under these experimental conditions, Traf2−/− BM-transplanted wild-type mice did not show body weight loss, diarrhea, or bloody stool (Fig. 2D). Histological analysis of the colon reconstituted with Traf2−/− BM cells did not show any abnormality (Fig. 2E). Together, Traf2 deficiency in lymphoid precursors/stem cells was not sufficient to induce colitis.

Alterations in the Colonic Microenvironment of Traf2−/− Mice—To further investigate the mechanisms underlying colitis in Traf2−/− mice, we next examined mRNA expression of proinflammatory cytokines by qPCR. As shown in Fig. 3A, mRNA expression levels of proinflammatory cytokines, including Ifnα, Il17a, Il17f, Il6, Ilng, and Il22 but not Il23p19, were significantly up-regulated in the colon of Traf2−/− mice compared with those of Traf2+/+ mice. In addition, the colonic LPCs spontaneously produced TNFα and IL-22, and production of these cytokines by LPCs from the colon of Traf2−/− mice was further enhanced upon stimulation with LPS compared with those from the colon of Traf2+/+ mice (Fig. 3B). Moreover, isolated plasmacytoid dendritic cells in the colonic LP of Traf2−/− mice spontaneously produced TNFα. Intriguingly, the numbers of IL-17-producing helper T (T\text{h}17) cells
were significantly increased in the colonic LP of Traf2<sup>−/−</sup> mice compared with Traf2<sup>+/+</sup> mice (supplemental Fig. 2A). Accumulating studies have shown that TH17 cells play a critical role in tissue damage or prevention of infection of pathogenic bacteria (22, 23); however, deletion of the Il17a gene did not rescue embryonic lethality or prevent the development of colitis in Traf2<sup>−/−</sup> mice (supplemental Table 2 and Fig. 2B). These results suggest that IL-17A was not primarily involved in the development of colitis in Traf2<sup>−/−</sup> mice.

Antimicrobial peptides play a crucial role in controlling the numbers of bacteria in the colon (24, 25). A previous study has shown that expression of β-Defensin 3 is significantly down-regulated in the colon of intestinal epithelial cell-specific Nemo-deficient mice (4). Because the activation of the canonical pathway induced by members of the TNF receptor superfamily is impaired in Traf2<sup>−/−</sup> mice (10), we hypothesized that expression of Defensin family genes might be reduced in the colon of Traf2<sup>−/−</sup> mice compared with Traf2<sup>+/+</sup> mice. Our qPCR analysis showed that expression of Muc1, Defensin a1, and Defensin a4 was significantly down-regulated in the colon of Traf2<sup>−/−</sup> mice compared with Traf2<sup>+/+</sup> mice (Fig. 3C). Production of IgA plays a crucial role in local defense and prevention of exacerbated immune responses against bacteria in the gut (26). We therefore tested whether production of IgA by the small intestine and colon of Traf2<sup>−/−</sup> mice was impaired using a whole-intestine culture system. IgA production by the small intestine but not the colon of Traf2<sup>−/−</sup> mice is significantly reduced compared with that of Traf2<sup>+/+</sup> mice.
TRAF2 Controls Homeostasis of the Colon

**FIGURE 4.** *Traf2*⁺/− mice develop colitis soon after birth and administration of antibiotics partially ameliorates colitis in *Traf2*⁺/− mice. A, H&E-stained colon sections of *Traf2*⁺/+ and *Traf2*⁺/− mice at the indicated days after birth. Green arrows indicate mononuclear cell infiltration. Scale bars, 100 μm. B, survival curves of *Traf2*⁺/− mice untreated or treated with antibiotics. *p* value was calculated with a log rank test. C, H&E-stained colon sections of 2-week-old *Traf2*⁺/+ and *Traf2*⁺/− mice untreated or treated with antibiotics. **Scale bars**, 100 μm. D, preservation of goblet cells in the colon of *Traf2*⁺/− mice treated with antibiotics. Colon sections of 2-week-old *Traf2*⁺/+ and *Traf2*⁺/− mice untreated or treated with antibiotics were stained with periodic acid-Schiff to visualize goblet cells. **Scale bars**, 100 μm. E, down-regulation of expression of inflammatory cytokine genes in the colon of *Traf2*⁺/− mice treated with antibiotics. RNAs were extracted from the colon of 15−16-day-old *Traf2*⁺/+ or *Traf2*⁺/− mice treated with antibiotics, and the expression of each gene was determined by qPCR. Results are means ± S.E. (*n* = 6 mice per genotype). ns, not significant. F, DGGE analysis of the colonic microbiota of the indicated genotyped mice untreated or treated with antibiotics. *Traf2*⁺/+ and *Traf2*⁺/− mice untreated (n = 2) and *Traf2*⁺/+ and *Traf2*⁺/− mice treated with antibiotics (n = 4) were analyzed using DGGE. The DNA fragment corresponding to Pasteurellales was observed. DNA size markers are indicated on the right.

The intestine of *Traf2*⁻/⁻ mice was significantly decreased compared with *Traf2*⁺/+ mice possibly due to a defect in Peyer patch development of *Traf2*⁻/⁻ mice (Fig. 3D) (12). In contrast, IgA production by the colon was rather increased in *Traf2*⁻/⁻ mice compared with *Traf2*⁺/+ mice, which may be caused by an increase in the number of B cells in the colon of *Traf2*⁻/⁻ mice (Fig. 3D). In sharp contrast, serum IgM levels were slightly increased in *Traf2*⁻/⁻ mice compared with *Traf2*⁺/+ mice (Fig. 3D). Together, these results suggest that the colonic microenvironment in *Traf2*⁻/⁻ mice was significantly altered from that of *Traf2*⁺/+ mice.

**Drastic Alterations in the Gut Microbiota of Traf2⁻/⁻ Mice—**

We found that infiltration of mononuclear cells was not detected in the colon of *Traf2*⁻/⁻ mice 1 day after birth, and the earliest it was detected was day 5 after birth (Fig. 4A), suggesting that colonization with the microbiota derived from breastfeeding may be involved in the development of colitis. To test this possibility, we investigated whether administration of antibiotics could ameliorate colitis in *Traf2*⁻/⁻ mice. Because *Traf2*⁻/⁻ mice died soon after birth (supplemental Fig. 1A), we treated pregnant *Traf2*⁻/⁻ and *Traf2*⁺/− mice with drinking water containing ampicillin and neomycin that kills both Gram-positive and Gram-negative bacteria. Under these conditions, antibiotics were transferred to the pups through breastfeeding. Interestingly, administration of antibiotics substantially prolonged the survival of *Traf2*⁻/⁻ mice (Fig. 4B) and ameliorated colitis based on preservation of goblet cells and a decrease in infiltration of mononuclear cells (Fig. 4, C and D). Moreover, expression of proinflammatory cytokines, including *Tnfa*, *Il17a*, *Il17f*, *Il6*, *Ifng*, and *II22* in the colon of antibiotic-treated *Traf2*⁻/⁻ mice, returned to levels comparable with those of *Traf2*⁺/+ mice (Fig. 4E). Likewise, the colitis score of *Traf2*⁻/⁻ mice treated with antibiotics was significantly decreased compared with untreated *Traf2*⁻/⁻ mice (Fig. 5E). Although the numbers of mononuclear cells still infiltrated in the colon of antibiotic-treated *Traf2*⁻/⁻ mice were comparable with *Traf2*⁺/+ mice, immunohistochemical analysis revealed that infiltrated cells were almost B cells but not dendritic cells or CD4⁺ T cells (supplemental Fig. 3). Therefore, expression of proinflammatory cytokines returned to levels comparable with those of *Traf2*⁺/+ mice despite the presence of mononuclear cell infiltration. Together, these results suggest that the colonic microbiota of *Traf2*⁻/⁻ mice might contribute to the development and/or exacerbation of colitis through up-regulating proinflammatory cytokines.

We next investigated whether the microbiota composition is altered in the colon of *Traf2*⁻/⁻ mice compared with *Traf2*⁺/+ mice. We determined the sequences of 16 S rRNA genes of bacteria in the colon of *Traf2*⁺/+ and *Traf2*⁻/⁻ mice. Consistent with our previous study (19), obligate anaerobic bacteria,
including Clostridiales and Bacteroidales, predominantly colonized the colon of 12–16-day-old Traf2+/− and Traf2+/+ mice (Table 1). In sharp contrast, Pasteurellales and Enterobacteriales were the predominant orders in the colon of 16-day-old Traf2−/− mice (Table 1). Together, expansion of Pasteurellales and Enterobacteriales that belong to the γ-Proteobacteria class is correlated with the development of colitis in Traf2−/− mice.

Because the administration of antibiotics significantly attenuated colonic inflammation in Traf2−/− mice, it would be intriguing to test whether treatment with antibiotics may affect the gut microbiota composition of Traf2−/− mice. DGGE and direct sequencing of the PCR products of bacterial 16 S rRNA genes revealed that the DNA fragment corresponding to Pasteurellales was a major band in untreated Traf2−/− mice (Fig. 4F). However, the DNA fragment corresponding to Pasteurellales completely disappeared in antibiotic-treated Traf2−/−, Traf2+/−, and Traf2+/+ mice (Fig. 4F). Collectively, antibiotics ameliorated colonic inflammation along with restoring commensal bacterial flora, including Clostridiales and Bacteroidales, in Traf2−/− mice.

**Development of Spontaneous Colitis in Traf2−/− Mice Is Largely Dependent on Signaling through TNR1—**Production of TNFa is enhanced in the colon of Traf2−/− mice (Fig. 3, A and B), and susceptibility to TNFα-induced apoptosis is increased in Traf2−/− mice (9), prompting us to test whether apoptosis of colonic epithelial cells is increased in Traf2−/− mice. Taken that activation of caspase 3 is a hallmark of apoptosis, we stained colon sections with anti-active caspase 3 or anti-IgM antibodies (red). Nuclei were stained with Hoechst 33258 (blue). Scale bars, 100 μm.

**FIGURE 5. Development of spontaneous colitis in Traf2−/− mice is completely dependent on signaling through TNR1.** A, active caspase 3-positive cells of the colon of Traf2−/− mice. Frozen colon sections were stained with anti-active caspase 3 antibody (red). Nuclei were stained with Hoechst 33258 (blue). The numbers of active caspase 3-positive cells were counted using randomly picked high power fields. Results are means ± S.E. (n = 5 mice per genotype). Scale bars, 100 μm. B, survival curves of Traf2+/− Tnfr1−/− and Traf2−/− Tnfr1−/− mice. p value was calculated with a log rank test. C, microscopy of the colon of 8-week-old genotyped mice. D, H&E-stained sections of the colon of 3-week-old genotyped mice. Scale bars, 100 μm. E, colitis score of 2–3-week-old genotyped mice. The colitis score of individual mice represents the sum of the clinical and histological findings as described under “Experimental Procedures.” Maximal score is 7. Dots indicate individual mice. Traf2−/− (n = 8), Traf2+/− with antibiotics (n = 5), Traf2−/− Tnfr1−/− (n = 5), and Traf2−/− IL17α−/− mice (n = 5) are shown. **, p < 0.01; ***, p < 0.001; ns, not significant. F, development of spontaneous colitis in Traf2−/− mice is completely dependent on signaling through TNR1. Immunohistochemical analysis of the colon of 3-week-old genotyped mice. Frozen colon sections were stained with anti-active caspase 3 or anti-IgM antibodies (red). Nuclei were stained with Hoechst 33258 (blue). Scale bars, 100 μm.

**TABLE 1**

| Mouse No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-----------|---|---|---|---|---|---|---|---|
| Total     | 5 | 6 | 6 | 13| 8 | 15| 13| 19| 85|

**Analysis of the colonic microbiota of Traf2+/+*, Traf2+/−, and Traf2−/− mice**

Bacterial 16 S rRNA genes were amplified by PCR using DNAs from the colon of 12–16-day-old Traf2+/+, Traf2+/−, and Traf2−/− mice. The sequences of bacterial 16 S rRNA genes were determined as described under “Experimental Procedures.” The numbers of the sequences corresponding to each bacterial rRNA gene were counted and are shown per individual mouse (n = 8 per group).

**Analysis of the colonic microbiota of Traf2+/+*, Traf2+/−, and Traf2−/− mice**

Mouse No. 1 2 3 4 5 6 7 8
Total 5 6 6 13 8 15 13 19 85

**Largely Dependent on Signaling through TNR1—**Production of TNFa is enhanced in the colon of Traf2−/− mice (Fig. 3, A and B), and susceptibility to TNFα-induced apoptosis is increased in Traf2−/− mice (9), prompting us to test whether apoptosis of colonic epithelial cells is increased in Traf2−/− mice. Taken that activation of caspase 3 is a hallmark of apoptosis, we stained colon sections with anti-active caspase 3 antibody. The numbers of active caspase 3-positive epithelial cells were increased in the colon of Traf2−/− mice compared with Traf2+/− mice (Fig. 5A). These results suggest that TNFα-dependent apoptosis may be involved in the development of colitis in Traf2−/− mice. We then crossed Traf2−/− mice with Tnfr1−/− mice that had been backcrossed into BALB/c mice by at least five generations. Notably, in contrast to Traf2−/− Tnfr1−/− mice on a C56BL/6 and 129 mixed genetic background (10), Traf2−/− Tnfr1−/− mice were born at Mendelian ratios, indicating that deletion of the Tnfr1 gene on a BALB/c background completely rescued Traf2−/− mice from embryonic lethality (supplemental Table 3). Moreover, 50% of Traf2−/− Tnfr1−/− mice grew normally and appeared to be healthy even at 6 months after birth (Fig. 5B). Consistently, microscopy of the colon of 8-week-old Traf2−/− Tnfr1−/−
mice was comparable with that of Traf2+/+ Tnfr1+/+ mice (Fig. 5C). Histological analysis showed that the architecture of colonic tissues and the numbers of goblet cells were preserved (Fig. 5D). However, mild inflammation was still observed in the colon of Traf2−/− Tnfr1−/− mice. The colitis score of Traf2−/− Tnfr1−/− mice was significantly improved compared with that
of Traf2−/− mice (Fig. 5E). Furthermore, active caspase 3-positive epithelial cells were not increased in the colon of Traf2−/− Tnfr1−/− mice compared with Traf2+/+ Tnfr1−/− mice (Fig. 5F), suggesting that enhanced apoptosis of colonic epithelial cells is tightly associated with the development of colitis. In addition, IgM+B cells were only slightly increased in the colon of Traf2−/− Tnfr1−/− mice compared with Traf2+/+ Tnfr1−/− mice (Fig. 5F). Collectively, the development of colitis in Traf2−/− mice was largely dependent on TNFα-induced apoptosis of colonic epithelial cells.

Deletion of Tnfr1 Gene Almost Completely Restores the Colonic Microenvironment of Traf2−/− Tnfr1−/− Mice—We next investigated whether ablation of the TNFR1-dependent pathway may restore the colonic microenvironment of Traf2−/− mice to that of Traf2+/+ mice. Consistent with amelioration of colitis in Traf2−/− Tnfr1−/− mice, expression of proinflammatory cytokines, including Il22, Il17a, Il6, and Ifng but not Il22, in the colon of Traf2−/− Tnfr1−/− mice returned to levels comparable with those of Traf2+/+ Tnfr1+/− mice (Fig. 6A). However, expression of Muc1 and Defensin a1 (Defa1) was still decreased in the colon of Traf2−/− Tnfr1−/− mice compared with those of Traf2+/+ Tnfr1+/− mice (Fig. 6B). Unexpectedly, the numbers of T17 cells were still increased in the colonic LP from Traf2−/− Tnfr1−/− mice compared with those of Traf2+/+ Tnfr1+/− or Traf2+/+ Tnfr1−/− mice (Fig. 6C). This increase might contribute to mild and persistent inflammation and be associated with up-regulation of Il22 mRNA in the colon of Traf2−/− Tnfr1−/− mice (Fig. 6A).

We finally investigated whether suppression of apoptosis of colonic epithelial cells may affect the gut microbiota composition of Traf2−/− mice. DGGE analysis and direct sequencing of PCR products of bacterial 16 S rRNA genes revealed that Pasteurellales disappeared, and Clostridiales predominantly colonized the colon of Traf2−/− Tnfr1−/− mice (Fig. 6D). Together, these results suggest that deletion of the Tnfr1 gene largely restored the colonic microenvironment of Traf2−/− mice to those of Traf2+/+ mice by preventing apoptosis of colonic epithelial cells, down-regulation of expression of proinflammatory cytokines, and normalization of the gut microbiota.

DISCUSSION

In this study, we have shown that Traf2−/− mice on a BALB/c background spontaneously developed severe colitis and succumbed within 3 weeks after birth. Production of TNFα by the colonic LPCs in response to commensal bacteria appears to be a critical event that triggers the development of colitis in Traf2−/− mice. TNFα induced apoptosis of colonic epithelial cells that subsequently disrupted barrier function of the epithelium and also altered the colonic microenvironment of Traf2−/− mice. The alterations in the microenvironment subsequently induced drastic changes in the colonic microbiota of Traf2−/− mice, resulting in further enhancement of TNFα production by LPCs. Thus, blockade of the TNFα/TNFRI-dependent signaling pathway by crossing Traf2−/− mice with Tnfr1−/− mice suppressed the development of colitis in Traf2−/− mice.

Drastic alterations in the gut microbiota observed in Traf2−/− mice appear to be a common feature of IBD patients as well as murine IBD models (27). The gut microbiota in Traf2−/− mice were mainly composed of obligate anaerobic bacteria, including Clostridiales and Bacteroidales. In sharp contrast, the gut microbiota in Traf2+/− mice consisted of facultative anaerobic and Gram-negative bacteria such as Pasteurellales and Enterobacteriales that grow well under aerobic conditions. The changes in the gut microbiota in Traf2−/− mice might be a consequence of an altered colonic microenvironment of Traf2−/− mice but also a contributing factor to exacerbate colonic inflammation. Indeed, expression of proinflammatory cytokines was up-regulated due to apoptosis of colonic epithelial cells along with massive infiltration of B cells in the colon of Traf2−/− mice. Moreover, mucosal immunity-associated IgA production by the small intestine and expression of Defensin genes were reduced in the colon of Traf2−/− mice. Accumulating studies have shown that reduced expression of Defensin genes might increase susceptibility to bacterial infection and subsequent development of IBD (24). These changes might result in an expansion of the Proteobacteria that potentially enhance inflammation. Therefore, administration of antibiotics partially, but not completely, ameliorated colitis in Traf2−/− mice through down-regulation of expression of proinflammatory cytokines.

The reason why antibiotic-treated Traf2−/− mice died prematurely is not fully elucidated in this study. Notably, colitis was substantially ameliorated in antibiotic-treated Traf2−/− mice, suggesting that these mice might die due to another cause. In accordance with a previous study (9), we confirmed that the numbers of myeloid progenitors in Traf2−/− BM cells were significantly reduced compared with Traf2+/+ BM cells (supplemental Fig. 4). It is therefore conceivable that hemopoietic stress due to inflammation (e.g. mild colitis) might provoke bone marrow failure, resulting in the premature death of Traf2−/− mice. Further studies will be required to address this possibility.

The most critical event to induce colitis in Traf2−/− mice might be enhanced apoptosis of colonic epithelial cells, which is dependent on the TNFα/TNFRI signaling pathway. Consistent with intestinal and epithelial cell-specific Nemo- or Tak1-deficient mice (4, 28), deletion of the Tnfr1 gene drastically ameliorated colitis in Traf2−/− mice through suppression of apoptosis of colonic epithelial cells. Although these studies have shown that TNFR1-induced apoptosis plays a crucial role in the development of colitis, it is not clear which pathways leading to NF-κB activation are required to prevent apoptosis of colonic epithelial cells and the subsequent development of colitis. Indeed, deletion of Nemo or Tak1 completely abolishes canonical but not noncanonical NF-κB activation induced by all members of the TNFR superfamily and TLR family (6, 7). In this respect, this study has revealed for the first time that Traf2-dependent survival signals are crucial for maintaining homeostasis of the colon by preventing colonic epithelial cells from apoptosis.

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