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RESEARCH ARTICLE

Innate Immunity

TRAF5 Deficiency Ameliorates the Severity of Dextran Sulfate Sodium Colitis by Decreasing TRAF2 Expression in Nonhematopoietic Cells

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ABSTRACT

TNFR-associated factor 5 (TRAF5) is a cytosolic adaptor protein and functions as an inflammatory regulator. However, the in vivo function of TRAF5 remains unclear, and how TRAF5 controls inflammatory responses in the intestine is not well understood. In this study, we found that intestinal epithelial cells from *Traf5*$^{-/-}$ mice expressed a significantly lower level of NF-κB–regulated proinflammatory genes, such as *Tnf*, *Il6*, and *Cxcl1*, as early as day 3 after dextran sulfate sodium (DSS) exposure when compared with wild-type mice. The intestinal barrier integrity of DSS-treated *Traf5*$^{-/-}$ mice remained intact at this early time point, and *Traf5*$^{-/-}$ mice showed decreased body weight loss and longer colon length at later time points. Surprisingly, the protein level of TRAF2, but not TRAF3, was reduced in colon tissues of *Traf5*$^{-/-}$ mice after DSS, indicating the requirement of TRAF5 for TRAF2 protein stability in the inflamed colon. Experiments with bone marrow chimeras confirmed that TRAF5 deficiency in nonhematopoietic cells caused the attenuated colitis. Our in vitro experiments demonstrated that proinflammatory cytokines significantly promoted the degradation of TRAF2 protein in *Traf5*$^{-/-}$ nonhematopoietic cells in a proteasome-dependent manner. Collectively, our data suggest a novel regulatory function of TRAF5 in supporting the proinflammatory function of TRAF2 in nonhematopoietic cells, which may be important for acute inflammatory responses in the intestine. *ImmunoHorizons*, 2020, 4: 129–139.

INTRODUCTION

TNFR-associated factor 5 (TRAF5), a member of the TRAF family of cytoplasmic adaptor proteins, plays a critical role in signal transduction mediated by the TNFR superfamily and cytokine receptors (1–9). TRAF5 is highly expressed in hematopoietic cells, such as B and T lymphocytes, and controls important functions in lymphocytes (10–16). Another member of the TRAF family that

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H.T.P. designed, performed, and interpreted most experiments and wrote the manuscript. H.N., S.K., T.M., T. Sakurai, S.T., A.A., N.A., A.I., T.K., and Y.O. provided reagents, supported experiments, and gave critical advice. N.I. and T. So designed and supervised the project, provided funding, and wrote the manuscript.

Abbreviations used in this article: DSS, dextran sulfate sodium; FITC-dextran, FITC-conjugated dextran; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; KO-LT, knockout littermate; MEF, mouse embryonic fibroblast; Primed, prestimulated with both TNF-α and IFN-γ; TRAF5, TNFR-associated factor 5; WT-LT, wild-type littermate.

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is functionally similar to TRAF5 is TRAF2 (8). Although TRAF2 and TRAF5 play a redundant role in activating the TNFR1–NF-κB signaling in mouse embryonic fibroblasts (MEFs) (17) and in limiting IL-6R signaling in $CD4^+$ T cells (10, 16), the in vivo function of TRAF5 seems to be different from that of TRAF2. *Traf2*$^{-/-}$ mice die prematurely because of spontaneous colitis, and the phenotype could be rescued by knocking out *Tnfr1* (18–20). However, *Traf5*$^{-/-}$ mice are viable and exhibit no obvious abnormality (11, 14). These studies suggest TRAF5 plays a less-important homeostatic role. Nonetheless, only a limited number of studies have attributed the effect of TRAF5 to specific cell types, such as nonlymphocytes. Thus, we thought that dextran sulfate sodium (DSS) colitis, which is not dependent on T or B lymphocytes (21), might be a good model system to evaluate the significance of TRAF5 in nonlymphocytes in living animals. We aim to discover novel functions of TRAF5 in the context of inflammation.

In this study, we found that *Traf5*$^{-/-}$ intestinal epithelial cells (IECs) expressed significantly lower levels of NF-κB–dependent genes as early as day 3 after DSS administration. In line with this result, at this early time point, the intestinal barrier integrity of *Traf5*$^{-/-}$ mice remained intact, whereas wild-type mice showed compromised barrier functions and increased infiltration of neutrophils. Accordingly, *Traf5*$^{-/-}$ mice displayed a more attenuated DSS colitis phenotype at later time points. Furthermore, by generating bone marrow chimeras, we confirmed that TRAF5 expressed by recipient mice exacerbated colitis. Importantly, the proinflammatory cytokines TNF-α and IFN-γ promoted the degradation of TRAF2 in TRAF5-deficient nonhematopoietic cells in a proteasome-dependent manner, suggesting that this event could dampen the TNFR–TRAF2/5–NF-κB–dependent acute inflammatory response in the inflamed colon. Our data suggest, to our knowledge, a novel regulatory function of TRAF5 in supporting the proinflammatory function of TRAF2 in nonhematopoietic cells, which may be important for barrier functions and tissue inflammation in the intestine.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 (B6) mice were purchased from Japan SLC. *Traf5*$^{+/-}$ mice with a C57BL/6 (B6) background were intercrossed to generate *Traf5*$^{+/+}$ and *Traf5*$^{-/-}$ mice (10). All mice were bred and maintained under specific pathogen-free conditions, and all experiments were performed with protocols approved by the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

Mice experiments

DSS-induced colitis. DSS salt (MP Biomedicals) was used to induce colitis in mice. Wild-type and *Traf5*$^{-/-}$ mice (8–12 wk old) that matched for gender and age were cohoused for at least 1 wk before treatment with DSS dissolved in water. Colitis was also induced in *Traf5*$^{+/+}$ and *Traf5*$^{-/-}$ mice. Mice were exposed to 3% DSS for 7 d. The mice's body weight loss and health status were monitored daily. When body weight loss exceeded 25%, the mice were immediately euthanized. To analyze the recovery phase of acute colitis, mice were exposed to 3% DSS for 4 d, followed by 6 d with normal drinking water.

Bone marrow chimeras. Six-week-old wild-type and *Traf5*$^{-/-}$ mice were irradiated sublethally with one dose of 9.5 Gy, and bone marrow hematopoietic cells from wild-type or *Traf5*$^{-/-}$ mice were transferred into the irradiated mice (5×10^6 cells per mouse via i.v. injection) the following day. Eight weeks after cell transfer, recipient mice were exposed to 2% DSS for 7 d and monitored as described above.

Preparation of colonic cells

Colons were dissected longitudinally, washed with PBS (Dulbecco's PBS), and cut into 0.5-cm pieces. The fragments were agitated in RPMI 1640 medium containing 2 mM EDTA and 2% FCS at 37°C for 30 min, and the mixture was passed through a 100-μm cell strainer. The filtrated supernatants were used in the preparation of IECs, and the colonic pieces were used in the preparation of lamina propria cells. Centrifuged pellets containing IECs were suspended in RPMI 1640 containing 2% FCS and layered over 37% Percoll (17-0891-01; GE Healthcare), and IECs were enriched by centrifugation through Percoll gradient. Isolated IECs were washed once with PBS containing 2% FCS prior to further analysis. To prepare colonic lamina propria cells, the colonic pieces were further minced and incubated in RPMI 1640 containing 2% FCS, collagenase D (1 mg/ml; Roche), and DNase I (200μg/ml, DN25; Sigma-Aldrich) at 37°C for 45 min, and the mixture was passed through a 70-μm cell strainer. Centrifuged pellets containing lamina propria cells were suspended in RPMI 1640 containing 2% FCS and layered over 37% Percoll. Isolated lamina propria cells were treated with an erythrocyte lysis ACK buffer before FACS analysis.

Preparation of MEFs and cell cultures

Preparation of MEFs. To prepare MEFs, embryos (embryonal days 13–15) were harvested from crosses of *Traf5*$^{+/-}$ parents. After removing the placenta and yolk sac, each embryo was placed in a 100-mm dish containing PBS. The tail and visible red organs were removed with forceps, and the remaining body was washed with PBS and placed in one well of a six-well plate containing a trypsin–EDTA (0.25% trypsin and 1 mM EDTA) solution. Each embryo was cut with scissors into pieces, and the fragments were incubated at 37°C for 15 min. After adding DMEM containing 10% FCS, the mixture was passed through a 100-μm cell strainer. The isolated cells were cultured in DMEM containing 10% FCS, penicillin, streptomycin, and 2-ME, and the following day, the culture medium was removed and replaced with fresh medium. The attached MEFs were further cultured for 2–3 d, and the cells were stored at −80°C.

Cell culture. After two to six passages of MEFs, wild-type and *Traf5*$^{-/-}$ MEFs were cultured in DMEM containing 10% FCS and

used for in vitro experiments. MEFs were seeded in culture dishes and cultured for 12 h. MEFs were then starved in DMEM containing 1% FCS for 1–2 h, followed by stimulation with cytokines indicated in figure legends. For experiments using cycloheximide and an anti-TNFR2 blocking Ab (10 μg/ml), MEFs were incubated with these reagents for 30 min before adding cytokines.

Intestinal permeability assay

Translocation of a macromolecule from the intestinal lumen to the blood was evaluated according to a modified protocol as previously described (22). Briefly, wild-type and *Traf5*$^{-/-}$ mice were treated with DSS for 3 d and then gavaged with 44 mg/100 g body weight/mouse FITC-conjugated dextran (FITC-dextran; FD4, BCBT0198; Sigma-Aldrich) in PBS. After 4 h, mice were anesthetized by isoflurane inhalation, and the blood was collected via cardiac puncture. After 30 min at room temperature, the blood was centrifuged at 4°C and 3000 × *g* for 10 min. Serum FITC-dextran concentrations were measured by spectrophotofluorometry (FlexStation 3; Molecular Devices) at excitation and emission wavelengths of 485 and 528 nm, respectively.

Histology and immunohistochemistry

Colons were dissected longitudinally and washed with ice-cold PBS. The distal colon (2 cm long) was placed in a histology cassette, fixed overnight in 10% formalin solution (060-01667; Wako Chemicals), and embedded in paraffin. The sections (5 μm in thickness) were used for H&E staining or immunohistochemistry for ZO-1 (bs-1329R; Bioss Antibodies) and Cleaved Caspase-3 (Asp^{175}; CST-9664; Cell Signaling Technology).

The presence of colitis was determined based on the histological analysis of H&E staining and using a scoring system for DSS colitis (23). Briefly, the scoring system for colon damage includes two subscores: a subscore for tissue damage (0, none; 1, isolated focal epithelial damage; 2, mucosal erosions and ulcerations; and 3, extensive damage deep into the bowel wall) and a subscore for lamina propria cell infiltration (0, infrequent; 1, increased, some neutrophils; 2, submucosal presence of inflammatory cell clusters; and 3, *trans*-mural cell infiltration). Total scores are the sum of the two subscores, ranging from 0 to 6. Immunohistochemistry samples were analyzed using ImageJ software.

Chemicals, Abs, and cytokines

Chemicals and media. DSS (160110, m.w. = 36,000–50,000) was purchased from MP Biomedicals. Proteasome inhibitor MG132 (139-18451), DMSO (043-29355), cycloheximide (CHX; 037-20991), isoflurane (099-06571), DMEM (with L-glutamine, 044-29765), and RPMI 1640 (with L-glutamine, 189-02025) were obtained from Wako Chemicals.

Abs. Anti-TRAF2 (M112-3, 1 μg/ml) and anti–α-tubulin (M175-3, 2 μg/ml) were acquired from Medical & Biological Laboratories Anti-TRAF5 (SC-6195, 0.4 μg/ml), anti-TRAF3 (SC-947, 0.2 μg/ml), and anti-p65 (SC-372-G, 0.4 μg/ml) were procured from Santa Cruz Biotechnology. Anti-phospho–p-65 (CST-3031, 1:1000 dilution) was purchased from Cell Signaling Technology. PE–anti-mouse CD3ε (100308), PE–anti-CD45 (103106), PE–anti-CD45.1 (110708), allophycocyanin–anti-CD45.2 (109813), allophycocyanin–anti-CD11b (101212), FITC–anti-Gr1 (108406), BV421–anti-F4/80 (123137), allophycocyanin–anti-EPCAM1 (118214), anti-CD120b (113302), and control IgG (400916) were obtained from BioLegend (1:100–200 dilution).

Cytokines. Mouse TNF-α (315-01A) and human IFN-γ (300-02) were acquired from PeproTech.

Plasmids and transfection

A retrovirus was produced by transfecting Plat-E retrovirus packaging cells with a pMX-IRES-EGFP-TRAF5 retroviral plasmid vector (16). On days 2 and 3, pooled virus-containing supernatants were filtered by 0.45-μm membranes, and the filtrates were centrifuged at 8000 × *g* and at 4°C for 16 h. The pellets were diluted in DMEM containing 5 μg/ml polybrene, and the mixture was added to MEF cell cultures. The cells were cultured for 12 h, and virus-containing culture medium was replaced by fresh medium.

Flow cytometry

Cells were incubated with anti-CD16/CD32 Ab (2.4G2, produced in-house) prior to staining with Abs for FACS analysis and cell sorting. FACS data were acquired via an FACSCanto II (BD Biosciences). Cells were sorted by FACSAria II (BD Biosciences). The FACS data were analyzed using FlowJo software (Tree Star).

Real-time PCR

Total RNA was extracted from cells or tissues using RNAiso Plus (9109; Takara Bio), and cDNA was synthesized with a SuperScript III Reverse Transcriptase kit (4374966; Applied Biosystems). Diluted cDNA was used for quantitative RT-PCR with SYBR premix Ex Taq (Takara Bio) and 7500 Real-Time PCR System (Life Technologies). Primers were as follows: mouse *Tnf* (forward: 5′-CCCTCACACTCAGATCATCTTCT-3′; reverse: 5′-GCTACGAC GTGG GCTACAG-3′), mouse *Il6* (forward: 5′-TCTCTGCAAGA GACTTCCATCCA-3′; reverse: 5′-ACAGGTCTGTTGGGAGTGGT-3′), mouse *Cxcl1* (forward: 5′-CAATGAGCTGCGCTGTCAGTG-3′; reverse: 5′-CTTGGGGACACCTTTTAGCATC-3′), mouse *Traf2* (forward: 5′-CTGTCTGTCCCAATGATG GA-3′; reverse: 5′-CATTC CTGCTCAGTGTGGTG-3′), mouse *Traf5* (forward: 5′-CCGACACCG AGTACCAGTTTG-3′; reverse: 5′-CGGCACCGAGTTCAATTCTC-3′), mouse *Tnfrsf1b* (*Tnfr2*) (forward: 5′-CGAGTGCCAGATCTCA CAGG-3′; reverse: 5′-TCCGAGGTCTTGTTGCAGAA-3′), mouse *Ptprc* (*Cd45*) (forward: 5′-GGGTTGTTCTGTGCCTTGTT-3′; reverse: 5′-GGATAGATGCTGGCGATGAT-3′), and mouse *Gapdh* (forward: 5′-AGGTCGGTGTGAACGGATTTG-3′; reverse: 5′-TGTAGAC CATGTAGTTGAGGTCA-3′).

Immunoblot analysis

Cells and colon tissues were lysed in an ice-cold 1% Nonidet P-40 lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, and 10 mM

N-ethylmaleimide) with a protease inhibitor mixture (P8340; Sigma-Aldrich). Protein concentrations of cell lysates were determined by bicinchoninic acid assay (Thermo Fisher Scientific). Cell lysates were reduced for 10 min at 70°C in a 4× lithium dodecyl sulfate sample buffer (NP0007; Life Technologies) containing 2-ME, separated by SDS-PAGE ($NaDodSO_4$-PAGE), transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore-Sigma), and analyzed by immunoblot with the appropriate Abs. The reaction was visualized with a chemiluminescence detection system using Immobilon Western HRP Substrate (Millipore-Sigma) and VersaDoc MP 5000 (Bio-Rad Laboratories).

Statistics

A Student *t* test with two-sided distributions was performed to assess the statistical significance of results.

RESULTS

Traf5$^{-/-}$ mice show reduced inflammation in DSS-induced colitis

To identify novel in vivo functions of TRAF5, we administered DSS to wild-type and *Traf5*$^{-/-}$ mice to induce acute colitis, which reflects the compromised epithelial barrier function and activation of cells of innate immunity via the translocation of bacterial components into lamina propria (24, 25). After administering drinking water containing 3% DSS for 7 d, *Traf5*$^{-/-}$ mice displayed a milder inflammatory phenotype in terms of body weight loss (Fig. 1A), colon length (Fig. 1B), and colonic histology scores (Fig. 1C) than wild-type mice. These results indicate that TRAF5 plays a proinflammatory role in DSS-induced colitis. To understand the role of TRAF5 in the restitution phase of DSS colitis, wild-type and *Traf5*$^{-/-}$ mice were administered 3% DSS for the first 4 d and normal drinking water for the following 6 d. In this setting, both mice showed comparable body weight changes and colonic histological scores (Supplemental Fig. 1A, 1B), suggesting that TRAF5 plays an important role in the early/acute phase of DSS colitis.

Traf5$^{-/-}$ mice colons display milder damage after 3 d of DSS administration

On days 3–4 after 3% DSS, a significant difference between wild-type and *Traf5*$^{-/-}$ mice was observed in terms of diarrhea and rectal bleeding. It was previously reported that increasing epithelial barrier leakage occurred after 1–3 d of DSS (24, 26), implying that TRAF5 expressed by the colons plays a pathogenic role. To understand the attenuated DSS colitis phenotype in *Traf5*$^{-/-}$ mice, we evaluated the translocation of FITC-dextran (~4000-Da macromolecules) from the lumen to the blood. On day 3, after 3% DSS, *Traf5*$^{-/-}$ mice displayed no significant increases in FITC-dextran in the blood when compared with wild-type mice (Fig. 2A). This result suggests that the integrity of the colonic epithelium of *Traf5*$^{-/-}$ mice remains intact at this stage. On day 3, the colons of *Traf5*$^{-/-}$ mice expressed lower levels of *Tnf* and *Il6* (Fig. 2B) and contained fewer neutrophils than wild-type mice (Fig. 2C), although macrophages remained similar in both groups on day 3 (Fig. 2C). However, on day 7, the number of neutrophils was comparable between groups (Supplemental Fig. 1C), and more macrophages had accumulated in the colons

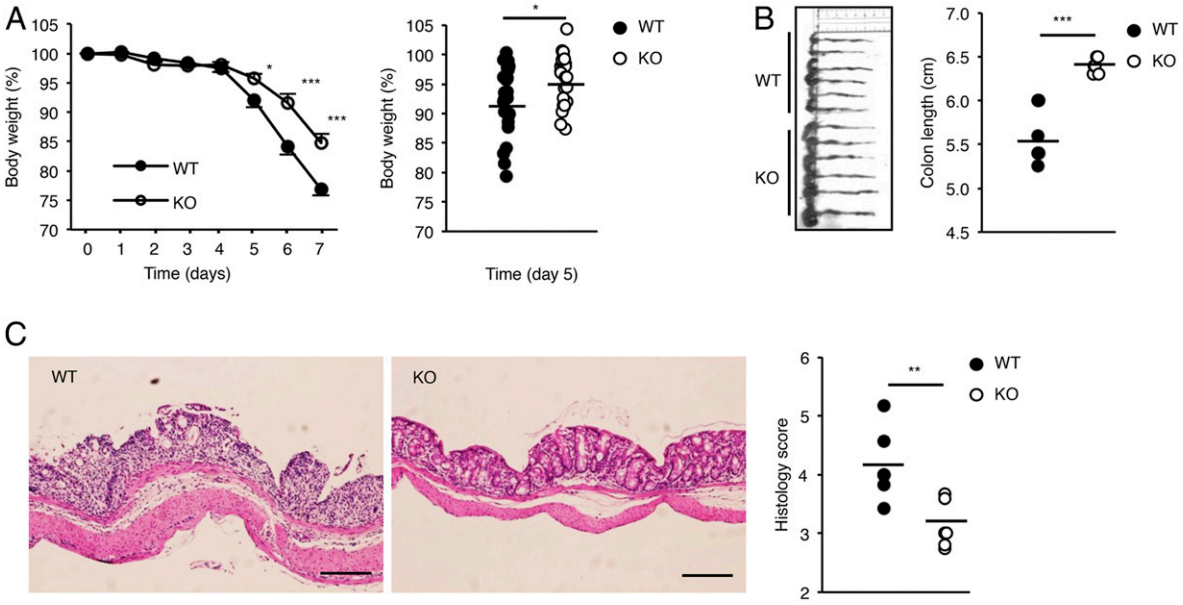

FIGURE 1. *Traf5$^{-/-}$* mice are less susceptible to DSS-induced colitis.

(**A**) Body weight loss of wild-type (WT) (n = 23) and *Traf5*$^{-/-}$ (KO) (n = 24) mice treated with 3% DSS for 7 d. The right panel shows percentage of body weight on day 5. Data are pooled from four independent experiments. (**B**) Colon length of WT and KO mice on day 7 as in (A) (WT, n = 6; KO, n = 6). (**C**) H&E staining of distal colons on day 7 as in (A). The right panel shows histology scores for colitis (WT, n = 6; KO, n = 6). Data are mean ± SEM. Scale bar, 200 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student *t* test).

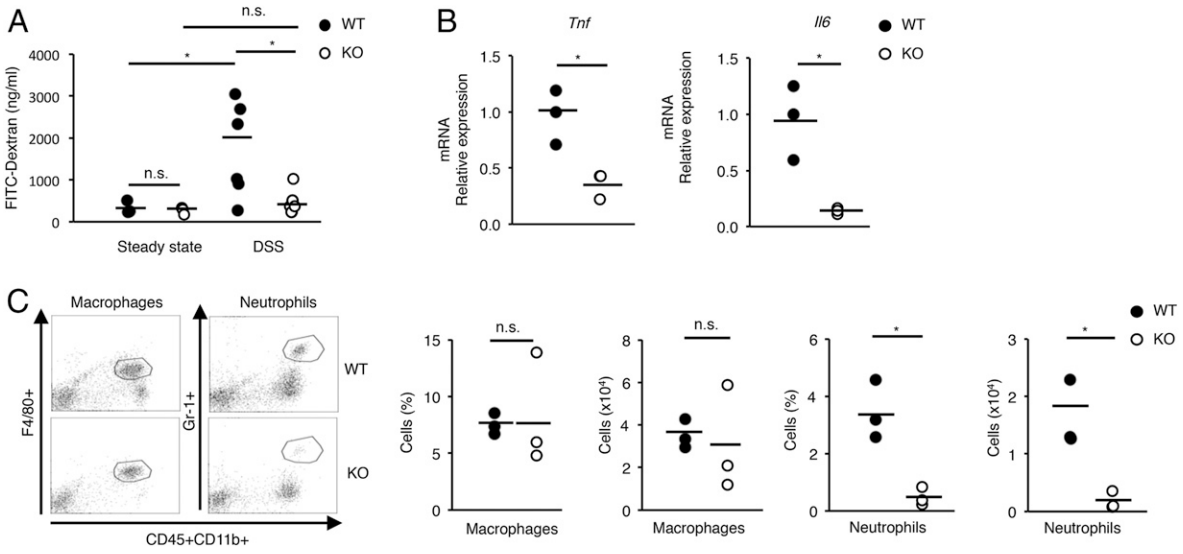

FIGURE 2. Colon damage and inflammatory responses after DSS are attenuated in *Traf5*$^{-/-}$ mice.

(**A**) FITC-dextran (4 kDa) translocation across gastrointestinal barrier into blood serum in wild-type (WT) and *Traf5*$^{-/-}$ (KO) mice at steady-state (WT, n = 3; KO, n = 3) and on day 3 after 3% DSS (WT, n = 6; KO, n = 7). Data are from pooled two independent experiments. (**B**) Quantitative RT-PCR analysis of the expression of proinflammatory cytokine genes *Tnf* and *Il6* in WT (n = 3) and KO (n = 3) colons on day 3 after 3% DSS, presented relative to the expression of *Gapdh*. (**C**) The percentages and numbers of neutrophils (CD45$^+$CD11b$^+$Gr1$^+$) and macrophages (CD45$^+$CD11b$^+$F4/80$^+$) in the colon tissues of 3% DSS-treated WT (n = 3) and KO (n = 3) mice on day 3. Data in (B) and (C) are from one experiment representative of at least two independent experiments with similar results. Lines indicate the mean values of mouse samples. *$p < 0.05$ (Student t test). n.s., not significant.

of wild-type mice (data not shown). In addition, although both groups of mice displayed comparable cleaved caspase-3$^+$ apoptotic cells in their colons (Supplemental Fig. 1D), the expression level of tight junction protein ZO-1 in the colons of *Traf5*$^{-/-}$ mice was significantly higher than that of wild-type mice (Supplemental Fig. 1E), indicating a more resilient colonic epithelial barrier in *Traf5*$^{-/-}$ mice. Thus, these results suggest that TRAF5 contributes to the activation of inflammatory signaling pathways in the colon, which disrupts tight junction structures or functioning.

IECs from Traf5$^{-/-}$ *mice express decreased NF-κB–regulated genes on day 3 after DSS*

To evaluate the proinflammatory role of TRAF5 expressed by IECs, we isolated colonic IECs from both mice on day 3 after 3% DSS and performed immunoblot analysis of phosphorylated NF-κB p65 in the cell lysates. As expected, *Traf5*$^{-/-}$ IECs displayed significantly decreased levels of phosphorylated p65 when compared with wild-type IECs (Fig. 3A, Supplemental Fig. 3D). In support of this finding, purified CD45$^-$EpCAM1$^+$ colonic IECs from wild-type mice expressed significantly higher levels of NF-κB pathway–regulated genes, such as *Tnf*, *Il6*, and *Cxcl1*, than *Traf5*$^{-/-}$ IECs (Fig. 3B, Supplemental Fig. 1F). These results are consistent with the results shown in Fig. 2B and suggest that TRAF5 expressed by IECs promotes the activation of inflammatory signaling mediated by NF-κB.

TRAF5 deficiency in nonhematopoietic cells attenuates DSS colitis

To investigate the possibility that reduced DSS colitis in *Traf5*$^{-/-}$ mice is caused by TRAF5 deficiency in nonhematopoietic cells in

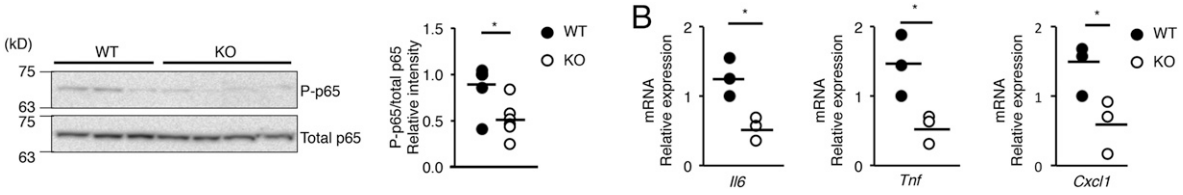

FIGURE 3. IECs from *Traf5*$^{-/-}$ mice express decreased levels of NF-κB–regulated proinflammatory genes after DSS.

(**A**) Immunoblot analysis of NF-κB p65 phosphorylated at Ser536 (P-p65) and total p65 in colonic IECs isolated from wild-type (WT) and *Traf5*$^{-/-}$ (KO) mice on day 3 after 3% DSS. Each lane indicates an individual mouse sample. The right panel shows the densitometric ratio of P-p65 to total p65. Data are pooled from two independent experiments (WT, n = 5; KO, n = 6). (**B**) Quantitative RT-PCR analysis of the expressions of proinflammatory cytokine genes *Tnf*, *Il6*, and *Cxcl1* in CD45$^-$EpCAM1$^+$ IECs purified from WT (n = 3) and KO (n = 3) mice on day 3 after 3% DSS. Data are from one experiment representative of two experiments with similar results. Lines indicate the mean values of mouse samples. *$p < 0.05$ (Student t test).

the colon, we adoptively transferred bone marrow cells from wild-type mice to wild-type or *Traf5*$^{-/-}$ recipient mice that had been sublethally irradiated; then, these recipient mice were treated with 2% DSS for 7 d. We found that *Traf5*$^{-/-}$ recipient mice exhibited milder inflammatory responses in terms of body weight change, colon length, histology scores, and barrier function than wild-type recipient mice (Fig. 4, Supplemental Fig. 1G, 1H). Next, to reveal further insights into the cellular source of TRAF5 during DSS colitis, we performed additional transplantation experiments in which bone marrow from wild-type or *Traf5*$^{-/-}$ mice was transferred to wild-type or *Traf5*$^{-/-}$ recipient mice. Body weight loss by DSS-treated mice was significantly reduced when TRAF5 was deficient in recipient mice (Supplemental Fig. 1I). Collectively, these results demonstrate that TRAF5 expressed by nonhematopoietic cells is responsible for the pathogenesis of DSS colitis.

Decreased TRAF2 protein in the colons of DSS-treated Traf5$^{-/-}$ *mice*

We initially thought that the cellular level of TRAF5 would be correlated with inflammatory responses in the colon and found that the level of TRAF5 protein marginally increased in the colons of wild-type mice on day 3 after DSS (Fig. 5A, 5B, Supplemental Fig. 3A, 3B). However, the levels of TRAF5 protein and mRNA in wild-type IECs were relatively low and rather unchanged after DSS (Fig. 5C, Supplemental Figs. 1J, 3C).

Interestingly, the level of TRAF2 protein, but not TRAF3 protein, was greatly diminished in the colons and IECs of *Traf5*$^{-/-}$ mice after DSS (Fig. 5, Supplemental Fig. 3A–C), although colon tissues from both groups of mice expressed comparable levels of TRAF2 mRNA after DSS (data not shown). Thus, these results indicate that TRAF5 supports the expression of TRAF2 protein after DSS. Because both TRAF2 and TRAF5 are critical in activating the NF-κB signaling pathway (8, 17), it is reasonable that NF-κB–regulated genes were significantly reduced in colons and IECs of *Traf5*$^{-/-}$ mice after DSS (Figs. 2B, 3B).

Proinflammatory cytokines downregulate TRAF2 protein in Traf5$^{-/-}$ *nonhematopoietic cells in vitro*

The above-reported results suggest that, in an inflammatory cytokine milieu induced by DSS, TRAF5 supports the expression of TRAF2 protein in the colon and IECs. To test this possibility, nonhematopoietic MEFs from wild-type and *Traf5*$^{-/-}$ mice were stimulated with proinflammatory cytokines in vitro. Because TNF-α and IFN-γ are induced in whole colons as early as day 1 after DSS (26), we decided to activate MEFs with these cytokines. Upon stimulation with both cytokines, but not with either cytokine alone, *Traf5*$^{-/-}$ MEFs exhibited significantly decreased TRAF2 protein expression when compared with wild-type MEFs (Fig. 6A, 6B, Supplemental Fig. 4A, 4B). Furthermore, when MEFs that had been prestimulated with both TNF-α and IFN-γ (Primed) were restimulated with TNF-α alone, NF-κB–regulated signaling events, such as the phosphorylation of p65 and the mRNA expression of *Tnf*, were significantly reduced in *Traf5*$^{-/-}$ MEFs when compared with wild-type MEFs (Fig. 6C, 6D,

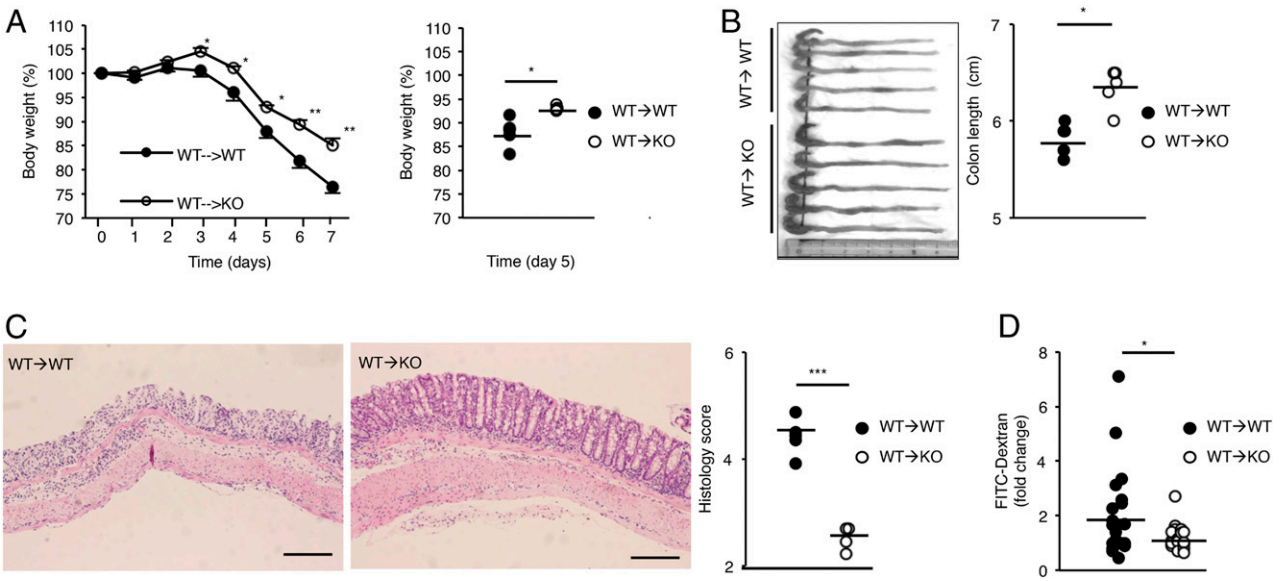

FIGURE 4. TRAF5 expressed by recipient mice promotes DSS colitis in bone marrow chimeras.

(**A**) Body weight loss of wild-type (WT) (n = 5) and *Traf5*$^{-/-}$ (KO) (n = 5) recipient mice adoptively transferred WT bone marrow cells, treated with 2% DSS for 7 d. The right panel shows body weight loss on day 5. Data are from one experiment representative of three experiments with similar results. (**B**) Colon length on day 7, as in (A) (WT→WT, n = 5; WT→KO, n = 5). (**C**) H&E staining of distal colons on day 7 as in (A) (WT→WT, n = 5; WT→KO, n = 5). The right panel shows histological score of colitis (WT→WT, n = 5; WT→KO, n = 5). Scale bar, 200 μm. (**D**) FITC-dextran (4 kDa) translocation across the gastrointestinal barrier into blood serum in WT and KO recipient mice on day 3 after 2% DSS. Data are pooled from four experiments (WT→WT, n = 23; WT→KO, n = 19). Lines indicate the mean values of mouse samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student t test).

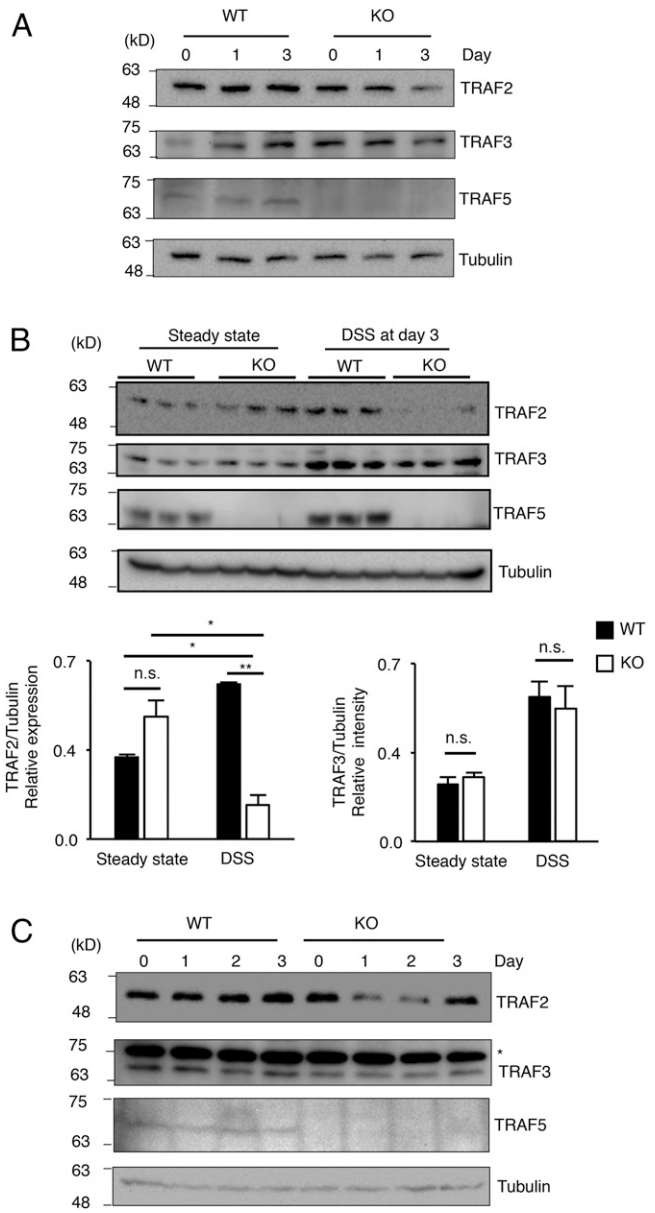

FIGURE 5. TRAF5 deficiency in colons and IECs renders TRAF2 protein unstable after DSS.

(**A**) Immunoblot analysis of TRAF2, TRAF3, and TRAF5 in whole-colon lysates from wild-type (WT) and *Traf5*$^{-/-}$ (KO) mice on day 0 and on days 1 and 3 after 3% DSS. Each lane indicates an individual mouse sample. (**B**) Immunoblot analysis of TRAF2, TRAF3, and TRAF5 in whole-colon lysates from WT and KO mice on day 0 (steady-state) (WT, $n = 3$; KO, $n = 3$) and on day 3 after 3% DSS (WT, $n = 3$; KO, $n = 3$). Each lane indicates an individual mouse sample. The lower panels show the densitometric ratio of TRAF2 (left) or TRAF3 (right) to α-tubulin. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ (Student t test). n.s., not significant. (**C**) Immunoblot analysis of TRAF2, TRAF3, and TRAF5 in IECs from WT and KO mice on day 0 and on days 1, 2, and 3 after 3% DSS. Each lane indicates an individual mouse sample. Data are from one experiment representative of two experiments with similar results. *, nonspecific band.

Supplemental Fig. 4C), indicating attenuated TNFR–TRAF2–NF-κB signaling in Primed *Traf5*$^{-/-}$ MEFs. Although TRAF2 plays a protective role in the TNFR1-mediated apoptosis (8, 17, 27), these cytokines showed no significant cytotoxic effect on both MEFs (Supplemental Fig. 2A, 2B). Finally, retroviral transduction of *Traf5* restored the expression of TRAF2 protein in Primed *Traf5*$^{-/-}$ MEFs (Fig. 6E, Supplemental Fig. 4D). These results show that TRAF5 inhibits TRAF2 protein degradation mediated by TNF-α and IFN-γ, suggesting that TRAF5 is required for TRAF2-mediated NF-κB activation in nonhematopoietic cells in inflamed colons.

TRAF5 limits proteasome-dependent TRAF2 degradation by TNFR2 in nonhematopoietic cells

Signals from TNFR2 promote the degradation of TRAF2 in a proteasome-dependent manner (28, 29). Indeed, the reduction of TRAF2 protein in Primed *Traf5*$^{-/-}$ MEFs could be rescued by treatment with the proteasome inhibitor MG132 or anti-TNFR2 blocking Ab (Fig. 7A, 7B, Supplemental Fig. 4E, 4F). Stimulation with TNF-α and IFN-γ had no significant effect on *Tnfr2* (*Tnfrsf1b*) and *Traf2* mRNA levels in both groups of MEFs (Supplemental Fig. 2C), indicating that TRAF5 protects TRAF2 from TNFR2- and proteasome-dependent degradation mediated by TNF-α and IFN-γ in nonhematopoietic cells.

Taken together, the results obtained in this study support the hypothesis that TRAF5 expressed by nonhematopoietic cells in the colon inhibits TRAF2 protein degradation mediated by TNFR2 and proteasome pathways, which promotes TNF-α–TNFR1–TRAF2/5–NF-κB–dependent inflammatory responses in the colon (Fig. 8).

DISCUSSION

The effects of TRAF5 deficiency on nonhematopoietic cells and acute DSS colitis documented in this study indicate that TRAF5 is required for TRAF2 activity to induce the TNF-α–NF-κB–dependent acute inflammation process in vivo. TRAF2 protein was downregulated in *Traf5*$^{-/-}$ colons and IECs after a few days of DSS exposure, and simultaneously, the expression of NF-κB–dependent proinflammatory genes was significantly decreased in *Traf5*$^{-/-}$ colons and IECs. In the absence of TRAF5, inflammatory stimuli triggered proteasome-dependent TRAF2 degradation, and the decreased expression of TRAF2 protein rendered *Traf5*$^{-/-}$ cells less responsive to TNF-α. Therefore, we have identified a novel function of TRAF5 that is important for TRAF2 protein stability in nonhematopoietic cells, which may be critical for the development of acute inflammation in the colon.

Piao et al. (18) demonstrated an antiapoptotic function of TRAF2 by preventing TNFR1-mediated IEC death and spontaneous colitis using *Traf2*$^{-/-}$ mice. In this study, TRAF2 protein was downregulated, but did not disappear completely, in *Traf5*$^{-/-}$ colons and IECs after a few days of DSS exposure. In this context, the induction of NF-κB–dependent proinflammatory genes was significantly attenuated in *Traf5*$^{-/-}$ colons and IECs (Figs. 2, 3), whereas the level of apoptosis of IECs was equivalent between

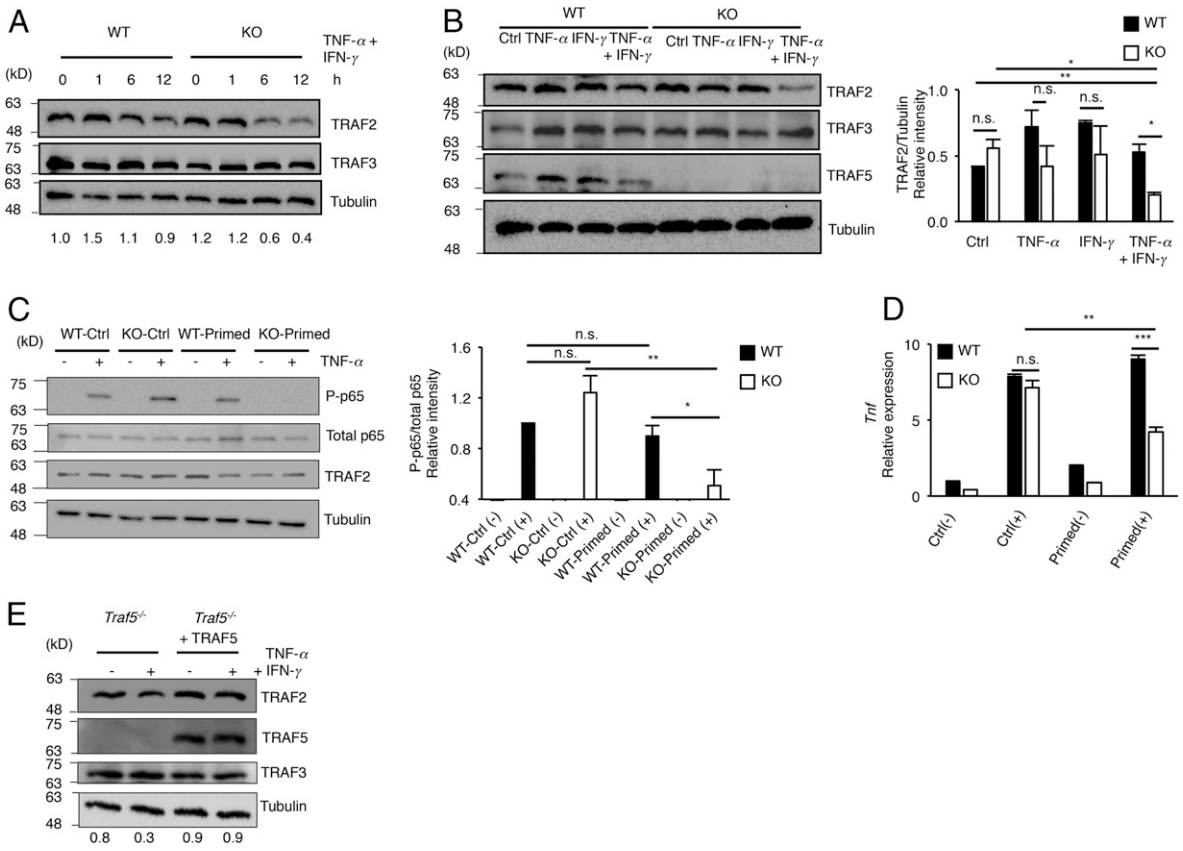

FIGURE 6. TRAF5 deficiency in MEFs renders TRAF2 protein unstable after stimulation with TNF-α and IFN-γ, which causes reduced NF-κB activity mediated by TNF-α.

(**A**) Immunoblot analysis of TRAF2 and TRAF3 in MEFs from wild-type (WT) and *Traf5*$^{-/-}$ (KO) mice after stimulation for the indicated times with both TNF-α (10 ng/ml) and IFN-γ (20 ng/ml). The values indicated below are the densitometric ratio of TRAF2 to α-tubulin. (**B**) Immunoblot analysis of TRAF2, TRAF3, and TRAF5 in MEFs from WT and KO mice. Cells were left unstimulated (Ctrl) or stimulated for 4 h with TNF-α (10 ng/ml) or IFN-γ (20 ng/ml) or both and then rested for 2 h before harvesting. The right panel shows the densitometric ratio of TRAF2 to α-tubulin. (**C**) Immunoblot analysis of NF-κB p65 phosphorylated at Ser536 (P-p65), total p65, and TRAF2 in MEFs from WT and KO mice. Cells were left Ctrl or stimulated for 4 h with both TNF-α (10 ng/ml) and IFN-γ (20 ng/ml) (Primed), rested for 2 h, and then left unstimulated (−) or restimulated with TNF-α (10 ng/ml) (+) for 10 min. The right panel shows the densitometric ratio of P-p65 to total p65. (**D**) Quantitative RT-PCR analysis of the expression of *Tnf* mRNA in MEFs from WT and KO mice. Ctrl or Primed cells were left unstimulated (−) or restimulated with TNF-α (10 ng/ml) (+) for 2 h. (**E**) Immunoblot analysis of TRAF2, TRAF3, and TRAF5 in KO MEFs stably transfected with control or TRAF5 retroviral vector. Cells were left unstimulated (−) or stimulated for 6 h with both TNF-α (10 ng/ml) and IFN-γ (20 ng/ml) (+) before harvesting. The values indicated below are the densitometric ratio of TRAF2 to α-tubulin. Data are mean ± SEM and from one experiment representative of two experiments (A, D, and E) or three experiments (B and C) with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student *t* test). n.s., not significant.

Traf5$^{-/-}$ and wild-type mice (Supplemental Fig. 1D). In support of this, we did not observe any differences in cell survival/death between *Traf5*$^{+/+}$ and *Traf5*$^{-/-}$ MEFs (Supplemental Fig. 2A, 2B). Thus, the transient loss of TRAF2 does not lead to severe colitis but causes a decrease in NF-κB–driven intestinal inflammation without affecting apoptosis.

There are several limitations of this study in terms of microbiota differences, littermate controls, and the small sample size in some experiments. Our in vivo results contradict previous observations reported by Shang et al. (30). By using littermate mice from *Traf5*$^{+/-}$ parental mice, Shang et al. (30) showed that *Traf5*$^{-/-}$ mice were more susceptible to DSS-induced colitis with elevated *Ifng*, *Il4*, and *Il17a* mRNA in the colon when compared with wild-type littermate (WT-LT) mice. We prepared *Traf5*$^{+/+}$ and *Traf5*$^{-/-}$ littermate mice (WT-LT and knockout littermate [KO-LT], respectively) and performed DSS colitis experiments. However, KO-LT mice were less susceptible to DSS colitis than WT-LT mice (Supplemental Fig. 2D). We also analyzed the mRNA levels of Th cytokines, such as *Ifng*, *Il4*, and *Il17a*, in colonic tissues from WT-LT and KO-LT mice on day 6.5 after 3% DSS but found no differences (Supplemental Fig. 2E). On day 6.5 after 3% DSS, the percentage and number of CD4^{+} T cells in the colonic tissues of both littermate mice groups were comparable, whereas a significant reduction in neutrophil numbers

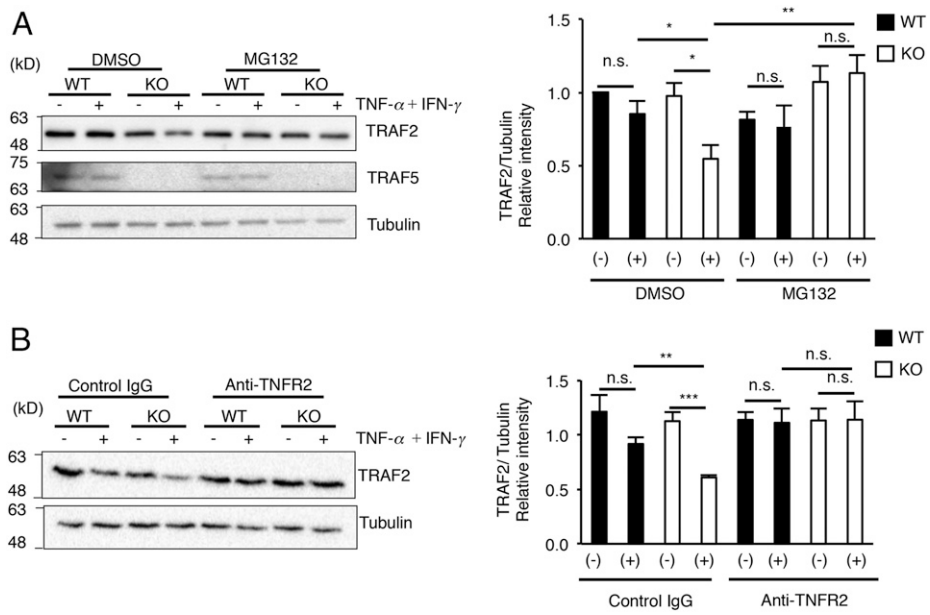

FIGURE 7. Proteasome inhibitor or TNFR2 blockade abrogates the degradation of TRAF2 mediated by TNF-α and IFN-γ in *Traf5*$^{-/-}$ MEFs. (**A**) Immunoblot analysis of TRAF2 and TRAF5 in wild-type (WT) and *Traf5*$^{-/-}$ (KO) MEFs. Cells were left unstimulated (−) or stimulated for 12 h with both TNF-α (10 ng/ml) and IFN-γ (20 ng/ml) (+). DMSO or MG132 (5 μM) was added the last 6 h before harvesting. (**B**) Immunoblot analysis of TRAF2 in WT and KO MEFs. Control IgG or anti-TNFR2 (10 μg/ml) was added from the beginning of the culture. The right panels show the densitometric ratio of TRAF2 to α-tubulin. Data are mean ± SEM and from one experiment representative of four experiments (A) or three experiments (B) with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student *t* test). n.s., not significant.

was observed in KO-LT mice (data not shown). Therefore, our results using littermate mice demonstrate that TRAF5 deficiency ameliorates DSS colitis. The discrepancy may be explained by differences in the intestinal microbiota of mice maintained in two separate facilities. In addition, the weight loss difference in DSS colitis observed in our study seems less pronounced when using littermate controls as compared with cohoused controls (Fig. 1A, Supplemental Fig. 2D). Thus, the discrepancy may also be due to differences in the gender and age of mice derived from many different heterozygous parents or the different numbers of mice used in each experiment.

In terms of clinical signs of disease, we observed significant differences between wild-type and *Traf5*$^{-/-}$ mice around day 3 after DSS. For this reason, we thought that TRAF5 played an important role in the early stage of acute inflammation in the colon. On day 3 after DSS, the level of TRAF2 protein was significantly decreased in the colons and IECs of *Traf5*$^{-/-}$ mice, and inflammation-related events, such as intestinal barrier leakage and the NF-κB–driven induction of proinflammatory mediators in the colon, were significantly attenuated in *Traf5*$^{-/-}$ mice. Although the recruitment of neutrophils in the colon was significantly decreased in *Traf5*$^{-/-}$ mice, colons of both groups of mice had comparable CD4$^+$ T cells and expressed equivalent levels of genes related to CD4$^+$ T cells, such as *Ifng*, *Il17a*, *Rorc*, *Gata3*, *Foxp3*, and *Tbx21* (see above and data not shown). Thus, we concluded that the observed inflammatory phenotype in *Traf5*$^{-/-}$ mice might not be related to the regulatory mechanism driven by CD4$^+$ T cells.

We also initially thought that TRAF5 expressed by the colon might affect DSS-driven inflammatory responses via the modulation of the IL-6/gp130 signaling pathway, because we had previously identified a negative regulatory function of TRAF5 in IL-6R signaling in CD4$^+$ T cells (10). After 3 d of 3% DSS, the level of phosphorylated STAT3 protein in the colon was greatly increased, but we found no differences between wild-type and *Traf5*$^{-/-}$ colons (Supplemental Fig. 2F). Additionally, both wild-type and *Traf5*$^{-/-}$ MEFs displayed comparable levels of phosphorylated STAT3 after stimulation with a complex of IL-6R and IL-6 (Supplemental Fig. 2G). Our previous studies indicate that TRAF5 can exhibit a negative regulatory function in the IL-6R signaling pathway if a cell type, including lymphocytes, expresses substantial amounts of endogenous TRAF5 and gp130 (10, 16). Our data suggest that fibroblasts or epithelial cells may not have enough TRAF5 molecules to inhibit gp130 signaling (Supplemental Fig. 1J). Thus, we concluded that the regulatory function of TRAF5 in the IL-6/gp130 signaling pathway might not be associated with the DSS colitis phenotype in *Traf5*$^{-/-}$ mice.

TRAF2 is degraded by the proteasome pathway regulated by the TNFR family members, such as CD30, CD40, and TNFR2 (28, 31–34). NF-κB activation mediated by TNF-α/TNFR1 or LPS/TLR4 signaling increases the surface expression of TNFR2, which negatively regulates the level of TRAF2 protein and TNFR1–TRAF2/5–NF-κB signaling (28, 31, 32, 35). In this study, blocking TNFR2 restored the expression of TRAF2 in *Traf5*$^{-/-}$ MEFs, indicating the TNFR2-mediated degradation of TRAF2 in *Traf5*$^{-/-}$ MEFs. Although we tested different combinations of cytokines

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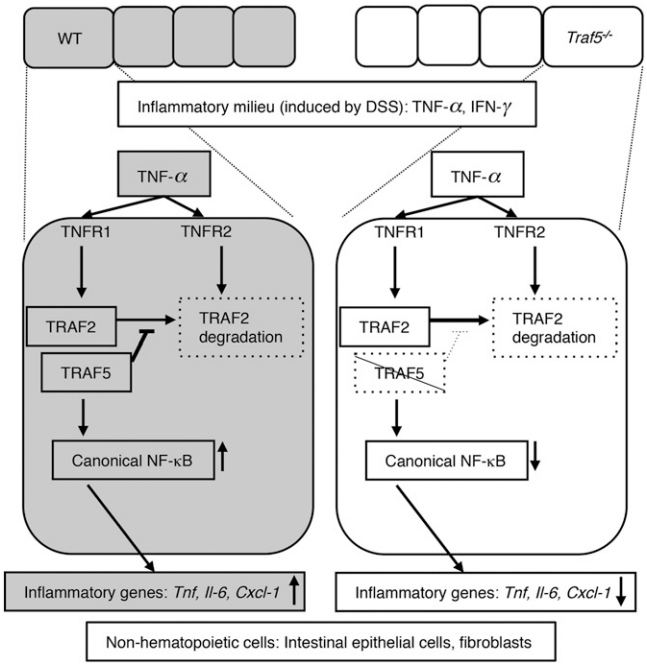

FIGURE 8. A proposed model of TRAF5-dependent regulation of TNFR1 and TNFR2 signaling in nonhematopoietic cells, which promotes NF-κB–driven acute colonic inflammation.

In the context of an inflammatory cytokine milieu, TRAF5 and TRAF2 expressed by nonhematopoietic cells promote the activation of the TNF-α/TNFR1/canonical NF-κB pathway, which leads to the expression of inflammatory genes, such as *Tnf*, *Il6*, and *Cxcl1*, and augments colonic inflammation. However, in the absence of TRAF5, TRAF2 protein exhibits a higher susceptibility to degradation mediated by TNFR2, which renders *Traf5*$^{-/-}$ nonhematopoietic cells less responsive to TNF-α because of the insufficiency of both TRAF2 and TRAF5 proteins in the cells. Therefore, the expression of TRAF5 in nonhematopoietic cells augments inflammatory responses that are dependent on the canonical NF-κB pathway induced by TNF-α.

other than TNF-α and IFN-γ, it is currently unclear why a combination of TNF-α and IFN-γ induced a significant TRAF2 protein reduction in *Traf5*$^{-/-}$ MEFs. One possible explanation may be that IFN-γ induces proteasome activators. RANK-induced TRAF6 degradation in bone marrow–derived monocyte/macrophage osteoclast precursor cells became more pronounced when RANK synergistically worked with IFN-γR signaling to induce the proteasome activators PA28α and PA28β (36). The mechanism that TRAF5 regulates TNFR2- and the proteasome-mediated degradation of TRAF2 will need to be clarified in future research.

Our findings may be relevant to previous human and mouse studies regarding inflammatory bowel diseases and cancer. Liu et al. (37) recently demonstrated that the histone methyltransferase EZH2 expressed by IECs suppressed the expression of epithelial TRAF2 and TRAF5. This study also showed that EZH2 might dampen proinflammatory TNF-α–TRAF2/TRAF5–NF-κB signaling during DSS-induced murine colitis. Furthermore, in human inflammatory bowel disease (IBD) specimens, the mRNA level of EZH2 was negatively correlated with that of TRAF2 or TRAF5, suggesting that the decreased EZH2 expression in IBD patients causes augmented TNF-α–induced NF-κB signaling mediated by TRAF2 and TRAF5. In support of this, the expression of TRAF2 and TRAF5 was upregulated in the inflamed colonic tissues of IBD patients (38, 39). In addition, the overexpression of TRAF2 or TRAF5 might be involved in colon cancer development (40, 41). Thus, identifying the role of TRAF2 and TRAF5 in the pathogenesis of inflammatory bowel diseases and cancer would be an interesting topic to explore in the future.

Collectively, our data demonstrate, to our knowledge, for the first time, that TRAF5 promotes the induction of acute intestinal inflammation by supporting TRAF2 protein stability in nonhematopoietic cells. Although it is currently unknown whether the proposed regulatory function of TRAF5 exists in all cell types that express TRAF5, we would propose a novel mechanism by which the induction of proinflammatory activity of TRAF2 is dependent on the cellular expression of TRAF5 in an inflammatory milieu.

DISCLOSURE

The authors have no financial conflicts of interest.

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The seventh author's first name was incorrect as originally published. The correct name is Shunichi Tayama. The author's name has been corrected in the author line and in the author contribution footnote in the online article.

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