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TGF-β–Activated Kinase 1 Signaling Maintains Intestinal Integrity by Preventing Accumulation of Reactive Oxygen Species in the Intestinal Epithelium

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The intestinal epithelium is constantly exposed to inducers of reactive oxygen species (ROS), such as commensal microorganisms. Levels of ROS are normally maintained at nontoxic levels, but dysregulation of ROS is involved in intestinal inflammatory diseases. In this article, we report that TGF-β–activated kinase 1 (TAK1) is a key regulator of ROS in the intestinal epithelium. tak1 gene deletion in the mouse intestinal epithelium caused tissue damage involving enterocyte apoptosis, disruption of tight junctions, and inflammation. Disruption of TNF signaling, which is a major intestinal damage inducer, rescued the inflammatory conditions but not apoptosis or disruption of tight junctions in the TAK1-deficient intestinal epithelium. We found that TAK1 deficiency resulted in reduced expression of several antioxidant-responsive genes and reduced the protein level of a key antioxidant transcription factor NF-E2–related factor 2, which resulted in accumulation of ROS. Exogenous antioxidant treatment reduced apoptosis and disruption of tight junctions in the TAK1-deficient intestinal epithelium. Thus, TAK1 signaling regulates ROS through transcription factor NF-E2–related factor 2, which is important for intestinal epithelial integrity. The Journal of Immunology, 2010, 185: 4729–4737.
deletion of TNFR1 greatly reduces intestinal damage in neonatal intestinal epithelial-specific TAK1-deletion mice, the mice spontaneously develop ileitis and colitis at approximately postnatal day 15 (10). Thus, ablation in enterocyte-derived TAK1 signaling results in TNF-dependent and -independent intestinal damage. In the current study, we investigated the mechanism by which TAK1 prevents TNF-dependent and -independent intestinal epithelial damage.

Materials and Methods

Mice

Mice carrying a floxed Map3k7 allele (TAK1^{FLo/FLo}) (7) were backcrossed to C57BL/6 mice for at least five generations. TNFR1-deficient C57BL/6 mice (TNFR1^{−/−}) (21) and villin-Cre transgenic mice (22) with a C57BL/6 background were from The Jackson Laboratory (Bar Harbor, ME). villin-CreER^{T2} transgenic mice with a C57BL/6 background were described previously (23). The backcrossed TAK1^{FLo/FLo} mice were used to generate villin-CreTAK1^{FLo/FLo}, villin-CreER^{T2}TAK1^{FLo/FLo}, and villin-CreERT2TAK1^{FLo/FLo}NFRI^{−/−} mice. In all experiments, littersmates were used as controls. To induce TAK1 gene deletion, 4-wk-old mice were given i.p. injections of tamoxifen (1 mg/20 g body weight) for two to five consecutive days. Some mice were fed food containing 0.7% butylated hydroxyanisole (BHA) beginning 1 wk prior to the tamoxifen treatment. The following primers were used for genotyping: floxed TAK1: 5′-CAGACGCTGCTGATTCTTTTGTAGCC-3′; villin-CreER^{T2}: 5′-CAAGCTGTCGACGGCC-3′ and 5′-CGGGAACCTCCATGTTGAGT-3′; and TNFR1: 5′-GTGAAGGCCCCACCTTTACGGCC-3′ and 5′-GGGTGACGTGACCCAGGG-3′. Mice were bred and maintained under specific pathogen-free conditions. All animal experiments were done with the approval of the North Carolina State University Institutional Animal Care and Use Committee.

Short hairpin RNA and cell culture

Caco-2 cells (ATCC HTB-37) were cultured in DMEM with 10% bovine growth serum (HyClone, Logan, UT) and penicillin-streptomycin at 33°C in 8% CO2. C57BL/6J (B6) mice (Harbor, ME) were described previously (22) with a C57BL/6 background were from The Jackson Laboratory (Bar Harbor, ME). Nuclei were counterstained with DAPI. Images were visualized using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI). Sections were stained with H&E for histological analysis. Sections were analyzed using the comparative threshold cycle method. Values were normalized to the level of β-actin mRNA in SYBR Green and to the level of GAPDH mRNA in TaqMan gene-expression assays.

Histology and immunohistochemistry

Sections were stained with H&E for histological analysis. Sections were scored in a blinded fashion on a scale from 0 to 4, based on the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion, as previously described (25). To detect apoptotic cells, the TUNEL assay was performed on paraffin sections using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI), according to the manufacturer’s instructions. Immunofluorescent staining was performed on paraffin-embedded sections or cryosections using a polyclonal Abs against claudin-3 (1:500; Zymed, San Francisco, CA), occludin (1:50; Zymed),ZO-1 (1:50; Zymed),Gr-1 (1:100; eBioScience, San Diego, CA), and cleaved-caspase 3 (1:100; Cell Signalling Technology, Beverly, MA). Bound Abs were visualized by Cy3- or Cy2-conjugated secondary Abs against rabbit (1:500; GE Healthcare, Piscataway, NJ). Nuclei were counterstained with DAPI. Images were visualized using a microscope (BX41; Olympus, Melville, NY) controlled by the iPLab imaging software (Scanalytics, Fairfax, VA).

Isolation of enterocytes

The small intestine was harvested and flushed with PBS to remove fecal contents. One end of the intestine was tied off, filled with HBSS (Sigma-Aldrich) containing 10 mM EDTA, and incubated in a PBS bath at 37°C for 5 min. After removing the contents, the intestine was filled with 10 nM EDTA in HBSS and incubated in PBS again for 10 min. The contents were collected into tubes and centrifuged at 1200 rpm for 5 min. The resulting pellets containing predominantly epithelial cells were washed twice in cold PBS.

Immunoblot analyses

Nuclear and cytoplasmic extracts from enterocytes and keratinocytes were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Proteins from cell lysates were electrophoresed on SDS-PAGE and transferred to Hybond-P (GE Healthcare). The membranes were immunoblotted with polyclonal Abs against NF-κB-related factor 2 (Nrf2; Santa Cruz Bio-technology, Santa Cruz, CA), TAK1 (8), TAK1-binding protein (TAB)2 (26), and mAbs against Lamin B1 (Zymed). Bound Abs were visualized with HRP-conjugated Abs against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Quantitative real-time PCR

Total RNA from the small intestine was isolated using RNAeasy Mini (Qiagen, Valencia, CA). cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). mRNA levels of NQO1 and β-actin were analyzed by real-time PCR with SYBR Green (Applied Biosystems). NQO1 primers 5′-CATCTCGAAGGGCTGTTGTA-G 3′ and 5′-CTAGTTTGTACGTCGTTGTCG-T 3′; and β-actin primers 5′-GCCAGGCAGCAAGATGTC-3′ and 5′-AGAGCATAGCCCTCAGGTG-3′. Mice were bred and maintained under specific pathogen-free conditions. All animal experiments were done with the approval of the North Carolina State University Institutional Animal Care and Use Committee.

Short hairpin RNA and cell culture

Caco-2 cells (ATCC HTB-37) were cultured in DMEM with 10% bovine growth serum (HyClone, Logan, UT) and penicillin-streptomycin at 37°C in 5% CO2. TAK1 small interfering RNA target sequence, corresponding to nucleotides 88–106 of the TAK1-coding region, was used to generate a retrovirus vector expressing short hairpin RNA (shRNA) against TAK1, pSUPERRetro-puro-shTAK1 (24). Caco-2 cells were transfected with pSUPERRetro-puro vector or pSUPERRetro-puro-shTAK1 and selected with puromycin for 2 wk. Pools of puromycin-resistant Caco-2 cells were used. TAK1^{−/−} and TAK1Δα keratinocytes were isolated from TAK1^{−/−} and K5-Cre TAK1^{−/−} mice described previously (11). Spontaneously immortalized keratinocytes derived from the skin of postnatal day 0–2 mice were cultured in Ca2+-free MEM (Lonza, Walkersville, MD) supplemented with 4% Chelex-treated bovine growth serum (HyClone, 10 mg/ml insulin growth factor (Invitrogen, Carlsbad, CA), 0.05 mM calcium chloride, and penicillin-streptomycin at 33°C in 8% CO2. Reagents were used. BSA (Sigma-Aldrich, St. Louis, MO), and 4% fetal bovine serum maintained at 37°C. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuiting through Ag–AgCl electrodes using a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance (TER; Ω cm2) was calculated from the spontaneous PD and short-circuit current. The Ussing-chamber experiments were run for up to 3 h after an initial equilibration period of 15 min. Duplicate tissues were studied from each animal in each Ussing-chamber experiment.

Analysis of ROS production

Harvested small intestines were embedded and frozen in OCT compound, and frozen sections were prepared. Sections were stained with 5 μM 5-(and -6)-chloromethyl-2-dichlorodihydrofluorescein diacetate (CM-H2DCFDA (Invitrogen) for 40 min at 37°C. Images were taken using a fluorescent microscope (BX41; Olympus) controlled by the iPLab (Scanalytics). Three to five randomly selected areas were photographed with the same exposure time. The images were processed using the same fixed threshold in all samples by Photoshop software, and representative images are shown.

Statistical analyses

Statistical comparisons were made using independent, Student t tests on data with normal variance.

Results

TAK1 prevents TNF-dependent and -independent intestinal damage

Mice with intestinal epithelial-specific deletion of TAK1 (TAK1^{HKO}) have a lethal defect within 24–48 h after birth due to severe damage in the intestine (10). We previously demonstrated that TNFR1 deletion rescues this early lethality (10), indicating that the severity of damage is mainly caused by TNF. However, mice having
intestinal epithelial-specific TAK1 deletion, even on a TNFR1−/− background, develop ileitis and colitis at postnatal day 15–17 (10). This suggests that TAK1 prevents TNF-dependent damage and that TAK1 is also important for blockade of TNF-independent epithelial dysregulation. In this study, we aimed to determine the mechanism by which TAK1 prevents TNF-dependent and -independent epithelial dysregulation. Enterocyte survival and tightly connected cell–cell junctions are essential for maintenance of intestinal epithelial integrity. Therefore, we first examined apoptosis and tight junctions in control and TAK1-deficient intestinal epithelium on a wild type or TNFR1−/− background. To compare the level of damage at the same age in the mouse model, we generated intestinal epithelial-specific inducible TAK1-deletion mice on a wild type background (TAK1IE-IKO) and TNFR1−/− background (TAK1IE-IKO-TNFR1−/−). In this system, we induce tak1 gene deletion by tamoxifen injection. We used 4-wk-old mice for all experiments and initially analyzed the small intestine at day 3 of tamoxifen injection. TAK1 deletion caused epithelial damage, including extensive evidence of apoptosis in the crypts (Fig. 1A,1B). The TAK1-deficient epithelium also exhibited extensive separation of villus epithelium from the lamina propria throughout the upper two thirds of the affected villi. This damage was observed, regardless of TNFR1 status in the ileum (Fig. 1A,1B). The numbers of apoptotic enterocytes were similar in the TAK1IE-IKO and TAK1IE-IKO-TNFR1−/− ileum epithelium. These results indicate that apoptosis was induced mainly through a TNF-independent mechanism in the TAK1-deficient ileum. We noted that TAK1IE-IKO mice exhibited damage in the small intestine and colon, whereas damage in TAK1IE-IKO-TNFR1−/− mice was observed primarily in the ileum (Supplemental Fig. 1). As reported previously, TAK1IE-IKO mice develop severe damage, which becomes lethal at 4–5 d after the initiation of tamoxifen injection (10). In contrast, the level of damage in TAK1IE-IKO TNFR1−/− intestinal epithelium was not changed following 3 d of tamoxifen injection. We injected TAK1IE-IKO-TNFR1−/− mice with tamoxifen for five consecutive days and maintained them without

**FIGURE 1.** TAK1 deletion causes TNF-dependent and -independent intestinal damage. A, H&E staining of ileal sections from littermate control (TAK1F/F, CT) and villin-CreERT2TAK1FL/FL (TAK1IE-IKO) mice and littermate control TNFR1−/− (TNFR1−/−) and villin-CreERT2TAK1FL/FL-TNFR1−/− (TAK1IE-IKO TNFR1−/−) mice. Four-week-old mice were treated with tamoxifen for two consecutive days, and samples were prepared 1 d after the second injection (day 3). Scale bars, 50 μm. Arrows indicate examples of apoptotic enterocytes. B, TUNEL staining of sections in A. Scale bars, 20 μm. TUNEL+ cells were counted in ≥180 crypts of each ileum. Bar graph shows mean (± SEM) TUNEL+ cells per crypt (n = 3). C, Immunofluorescence staining of claudin-3 using the sections prepared as described in A. Scale bars, 20 μm. D, Real-time PCR analysis of TAK1FL/FL (CT, open bars) and villin-CreERT2TAK1FL/FL-TNFR1−/− (TAK1IE-IKO TNFR1−/−) mice. Four-week-old mice were treated with tamoxifen for two consecutive days, and mRNA samples were prepared 1 d after the second injection (day 3). mRNA levels relative to GAPDH mRNA are shown. The data represent means ± SEM (n = 7). *p < 0.05.
additional tamoxifen injection. We confirmed that the tak1 gene was deleted at 3 d and at 8 wk after the termination of tamoxifen injection. TAK1IE-IKO/+,TNFR1+/− mice were viable for ≥6 mo without showing any clinical signs, but they exhibited tissue damage in the ileum at similar levels to those at 3 d after initiation of tamoxifen injection. These results indicate that TAK1 prevents TNF-independent apoptosis in the ileum and that TNF is not a primary mediator of intestinal damage, but it amplifies the damage.

We examined whether the tight junctions were also affected by ablation of TAK1. We analyzed the localization of three major tight junction-associated proteins in enterocytes: claudin-3, occludin, and tight junction plaque protein ZO-1. All three proteins were diffusely localized in the ileum of TAK1IE-IKO/− and TAK1IE-IKO/− mice (Supplemental Fig. 2 and data not shown). Although localization of occludin and ZO-1 was marginally altered, the mislocalization of claudin-3 was striking in TAK1-deficient intestinal epithelium (Fig. 1C); there was very little evidence of claudin-3 precisely at the region of the tight junction apical lateral membrane, rather it appeared to be within the cytoplasm. The level of claudin-3 mislocalization was very noticeably different between TAK1IE-IKO/− and TAK1IE-IKO/−TNFR1+/− mice.

To assess the levels of inflammation, we measured the mRNA levels of inflammatory cytokines that were expressed in the small intestine in TAK1IE-IKO/− and TAK1IE-IKO/−TNFR1+/− mice at day 3 of tamoxifen injection (Fig. 1D). TAK1IE-IKO/− mice had markedly increased levels of inflammatory cytokines, whereas TAK1IE-IKO/− TNFR1+/− mice did not exhibit a significant increase in those cytokines. Collectively, TAK1 seems to be essential for enterocyte survival and integrity of tight junctions, primarily in the ileum. This effect is independent of TNF signaling. When TNF signaling is intact, TAK1 deletion causes more severe tissue damage in the ileum as well as in other regions of the intestine. These results suggest that TAK1 is primarily important for maintenance of enterocyte survival and tight junction integrity and that TNF signaling amplifies TAK1 deficiency-induced damage by promoting inflammation.

**TAK1 deficiency reduces TER**

To verify whether the increased apoptosis and disruption of tight junctions impair intestinal barrier function, we measured TER in TAK1-deficient intestinal epithelium. We chose TNFR1+/− background (TAK1IE-IKO/+,TNFR1+/−) mice to rule out the possibility that inflammatory conditions could indirectly affect the barrier function. TER was significantly lower in TAK1IE-IKO/− mice compared with control TNFR1+/− mice (Fig. 2). Thus, TAK1 is important for maintenance of the intestinal barrier function through modulating enterocyte survival and tight junctions.

![Figure 2](http://www.jimmunol.org/) **FIGURE 2.** TAK1 deficiency impairs intestinal barrier. TNFR1−/− (○) and villin-CreERT2TAK1FL/FL,TNFRI−/− (●) mice were treated with tamoxifen for five consecutive days, and mRNA samples were prepared 1 d after the third tamoxifen injection (day 4). Relative mRNA levels were calculated using b-actin mRNA. The data represent means ± SEM (n = 4). *p < 0.05; **p < 0.01. B, Nrf2 mRNA levels were determined by real-time PCR analysis in the small intestine in TAK1IE-IKO/− and TAK1IE-IKO/−TNFR1+/− animals (left panel). The mice were treated with tamoxifen for two consecutive days, and mRNA samples were prepared 1 d after the third tamoxifen injection (day 3). Relative mRNA levels were calculated using GAPDH mRNA. The data represent means ± SEM (n = 7). Immunoblot analysis of Nrf2 in the nuclear extracts (Nuc) from TAK1IE-IKO/− (right panel). The enterocytes were prepared at day 3 of tamoxifen injection. Lamin B1 was used as a loading control. C, Nrf2 mRNA and protein levels of TNFR1−/− and villin-CreERT2-TAK1FL/FL,TNFRI−/− (TAK1IE-IKO/− TNFR1+/−) small intestine were analyzed as described in B. The mRNA data are means ± SEM (n = 7).
TAK1 deficiency downregulates the levels of antioxidant-responsive genes

To determine the mechanism by which TAK1 mediates enterocyte survival, we measured the mRNA levels of genes associated with cell survival in control and TAK1-deficient intestine. We initially analyzed samples from the intestine having control genotype and intestinal epithelial-specific constitutive deletion of TAK1 (TAK1\textsuperscript{IE-KO}). The expression levels of antiapoptotic genes, including \textit{bcl2} and \textit{bclxL}, were not altered (Supplemental Fig. 3).

We found that several antioxidant-responsive genes (i.e., \textit{nqo1}, \textit{gstm1}, and \textit{gstm4}), were downregulated in the constitutive and inducible TAK1-deficient small intestine and the colon (Fig. 3A). These antioxidant-responsive genes are known to be regulated by a key antioxidant transcription factor, Nrf2. Therefore, we examined the levels of Nrf2 in inducible TAK1-deficient intestinal epithelium on wild type and TNFR1\textsuperscript{−/−}/2 background (TAK1\textsuperscript{IE-IKO} and TAK1\textsuperscript{IE-IKO}TNFR1\textsuperscript{−/−})(Fig. 3B,3C). Although the mRNA levels of Nrf2 were not significantly altered by ablation of TAK1, the protein level of nuclear Nrf2 was greatly reduced in the TAK1-deficient intestine. Nrf2 was not detectable in the cytoplasmic fraction in intestinal epithelium (data not shown). Downregulation of Nrf2 was independent of TNFR1 status. Nrf2 regulation of antioxidant-responsive genes plays an integral role in ROS metabolism (28, 29), which is critically involved in cell viability and tight junction integrity. We postulated that TAK1 might regulate the protein level of Nrf2, thereby modulating cell survival and tight junctions.

Ablation of TAK1 downregulates Nrf2 and sensitizes cells to oxidative stress

To further investigate the mechanism by which TAK1 regulates Nrf2 and cell survival, we used two lines of cultured epithelial cells exhibiting TAK1 ablation: Caco-2 cells stably expressing an shRNA targeted against TAK1 and TAK1-deficient skin epithelial cells (keratinocytes) that were isolated from the epidermal-specific TAK1-deletion mice (11). In both cell types, Nrf2 was not de-
tectable in the cytoplasmic fractions (data not shown), and the protein level of Nrf2 was lower in TAK1-deficient cells compared with control cells (Fig. 4A, 4B). The protein level of Nrf2 is known to be primarily regulated by protein degradation through the proteasome pathway (30). Blockade of the proteasome pathway by MG-132 treatment greatly increased the levels of Nrf2 in control and TAK1-deficient cells (Fig. 4A, 4B). This suggests that Nrf2 is always highly degraded through the proteasome pathway and that TAK1 might be involved, in part, in Nrf2 stability. We asked whether activation of TAK1 could alter Nrf2 stability. Coexpression of TAK1 with TAB1 highly activates TAK1 (31). We determined the Nrf2 stability with and without coexpression of TAK1 and TAB1 in 293 cells (Fig. 4C). The protein level of Nrf2 was almost completely diminished within 5 h after blockade of protein synthesis when Nrf2 alone was expressed, whereas the level of Nrf2 decreased much more slowly in cells with coexpression of TAK1 and TAB1. These results suggest that TAK1 may participate in Nrf2 stability. Therefore, ablation of TAK1 might cause increased degradation of Nrf2.

Nrf2 is important for preventing oxidative stress (29, 32). We next examined whether TAK1 deficiency could cause increased apoptosis and disruption of tight junctions in response to oxidative stress. We treated Caco-2 and keratinocytes with TBHP, a prototypical organic oxidant, and the tight junctions and apoptotic cells were observed by immunostaining with anti-cleaved caspase 3 and anti–ZO-1, respectively (Fig. 4D, 4E). We noted that claudin-3 was less clearly detected in Caco-2 cells compared with ZO-1, and we used ZO-1 to visualize the tight junctions in those cultured cells. Compared with control cells, the tight junctions were relatively more damaged, and apoptotic cells were significantly increased in TAK1-deficient cells. These results indicate that TAK1 deficiency caused hypersensitivity to oxidative stress in cultured epithelial cells. Collectively, we postulated that ablation of TAK1 causes dysregulation of Nrf2 stability and ROS, which results in impaired tight junctions and increased apoptosis in the intestinal epithelium.

ROS is the cause of TNF-dependent and -independent intestinal damage in TAK1-deficient intestinal epithelium

We examined the hypothesis that TAK1 regulates ROS levels in the intestinal epithelium. ROS were measured in the TAK1IE-IEKO and TAK1IE-IEKO/TNFFR1IE-IEKO intestinal epithelium at day 3 of tamoxifen injection. The unfixed fresh cryosections of the ileum were used to detect ROS by CM-H2-DCFDA staining (Fig. 5A). The levels of ROS were greatly increased in TAK1-deficient intestinal epithelium. The increased ROS might be generated from infiltrated myeloid cells, because TAK1IE-IEKO intestinal epithelium was highly inflamed (Fig. 1). However, the levels of ROS were not different between highly inflamed TAK1IE-IEKO mice and TAK1IE-IEKO TNFR1IE-IEKO mice, which did not exhibit significant inflammatory conditions. We detected some Gr-1+ cells in control TNFR1IE-IEKO and TAK1IE-IEKO/TNFFR1IE-IEKO intestinal epithelium; however, the number of Gr-1+ cells was much smaller than that of ROS positive cells in TAK1IE-IEKO/TNFFR1IE-IEKO intestinal epithelium (Supplemental Fig. 4). We found that the ROS positive cells were highly overlapped with the cells having cleaved caspase 3 (Fig. 5B), suggesting that ROS are produced in apoptotic intestinal epithelial cells. These results indicate that TAK1 signaling is essential for preventing ROS accumulation in the intestinal epithelial cells. We next attempted to reduce ROS and tested whether reduction of ROS could rescue the apoptosis and tight-junction disruption in the TAK1-deficient epithelium. We fed the mice a chow diet containing the antioxidant BHA for 1 wk prior to tak1 gene deletion. We found that the levels of ROS were greatly reduced by BHA feeding in TAK1IE-IEKO and TAK1IE-IEKO/TNFFR1IE-IEKO mice (Fig. 5A). Histological evaluation and TUNEL assays revealed that intestinal damage and apoptotic enterocytes were significantly reduced with BHA feeding (Fig. 6). BHA feeding was equally effective in TAK1-deficient intestinal epithelium on a TNFR1+/- background. Furthermore, BHA feeding greatly improved tight-junction integrity (Fig. 7A). The mRNA levels of inflammatory cytokines were not upregulated in BHA-treated TAK1IE-IEKO mice (Fig. 7B). These results indicate that ablation of TAK1 causes enterocyte apoptosis and impairs barrier function, most likely as the result of increased ROS.

Discussion

In this study, we demonstrated that enterocyte-derived TAK1 signaling plays a critical role in ROS metabolism, possibly through transcription factor Nrf2 and its target genes. Ablation of this TAK1 pathway caused accumulation of ROS, resulting in enterocyte apoptosis and disruption of tight junctions. We previously reported that deletion of TNFR1 can rescue TAK1 deficiency-induced apoptosis and inflammatory conditions in the intestinal epithelium in neonatal mice (10) and in the epidermis of the skin (11). In cultured cells, we demonstrated that TNF greatly increases ROS in TAK1-deficient keratinocytes, which causes TNF-induced apoptosis (11, 20). Thus, we concluded that TAK1 signaling principally reduces TNF-induced ROS and prevents TNF-induced apoptosis in the intestine of neonatal mice and epidermis of the skin.
However, in the adult intestinal epithelium, we found that the TAK1 deficiency-induced ROS was not altered by TNFR1 deletion (Fig. 5A). This indicates that TNF is not the major inducer of ROS in the adult intestinal epithelium. Mice are sterile in utero and are inoculated with bacteria at birth, and populations of intestinal commensal bacteria are known to be dramatically altered during postnatal development (33). We speculate that commensal bacteria in the adult intestines may be the major trigger of ROS. TAK1 signaling reduces those non-TNF–induced ROS, which is essential for enterocyte survival and integrity of tight junctions. How does TAK1 regulate ROS? In this study, we showed that the level of Nrf2 was downregulated in TAK1-deficient intestinal epithelium. Although Nrf2 knockout increases susceptibility to intestinal injury, it alone does not increase enterocyte apoptosis or

FIGURE 6. Antioxidant prevents TNF-dependent and -independent intestinal damage in the TAK1-deficient intestinal epithelium. A, H&E staining of the ileum sections from control (TAK1F/F; CT), villin-CreER2TAK1FL/FL (TAK1IE-IKO), TNFR1−/−, and villin-CreER2TAK1FL/FLTNFR1−/− (TAK1IE-IKO TNFR1−/−) mice fed a normal or BHA chow diet for 1 wk and subsequently treated with tamoxifen for two consecutive days. Sections were prepared from the ileum 1 d after the second injection (day 3). Scale bars, 50 μm. Arrows indicate examples of apoptotic enterocytes. CT, TAK1IE-IKO, and TAK1IE-IKO +BHA, n = 3; TNFR1−/−, TAK1IE-IKO TNFR1−/−, and TAK1IE-IKO TNFR1−/− +BHA, n = 4. B, Histological scores for samples shown in A. Data are the means ± SEM. C, TUNEL staining of the sections shown in A. D, TUNEL+ cells were counted in 180 crypts of each small intestinal segment. The numbers shown are means (± SEM) of TUNEL positive cells per crypt (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001.

FIGURE 7. BHA feeding blocks disruption of tight junctions and inflammation. A, Immunofluorescence analysis of claudin-3 of the ileum sections from control (CT; TAK1F/F), villin-CreER2TAK1FL/FL (TAK1IE-IKO), TNFR1−/−, and villin-CreER2TAK1FL/FLTNFR1−/− (TAK1IE-IKO TNFR1−/−) mice fed a normal or BHA chow diet. Scale bars, 20 μm. B, Real-time PCR analysis was performed in the small intestine from control (CT; TAK1F/F) and villin-CreER2TAK1FL/FL (TAK1IE-IKO) mice, with a normal or BHA chow diet. The mice were treated with the procedure described in A. mRNA levels relative to GAPDH mRNA are shown. The data are means ± SEM. CT, TAK1IE-IKO, and TAK1IE-IKO + BHA, n = 7; CT + BHA, n = 4.
cause inflammatory conditions (34). Therefore, we would not expect that ablation of Nrf2 alone would be sufficient to induce all of the noted disruptions caused by intestinal epithelial-specific deletion of TAK1. In our previous study, we found that an AP-1 family transcription factor c-Jun is downregulated in TAK1-deficient keratinocytes (20). Similar to Nrf2, AP-1 family transcription factors are critical to the transcriptional regulation of antioxidant-responsive genes (35–38). Overexpression of c-Jun partially blocks accumulation of TNF-induced ROS (20). In addition, TAK1 is an integral upstream kinase of IκB kinases, leading to activation of transcription factor NF-κB (11, 39), which is also a major transcription factor for several cellular antioxidant genes (40, 41). Taken together, we believe that TAK1 signaling regulates multiple antioxidant transcription factors, including Nrf2, c-Jun, and possibly other unidentified factors that modulate the level of ROS.

We showed that TAK1 deficiency downregulates the protein levels of Nrf2 in the nucleus. Nrf2 is normally localized in the cytoplasm by its binding partner Keap1 and is constantly degraded through the proteasome pathway (28). In the intestinal epithelium and cultured Caco-2 and keratinocytes, Nrf2 was not detectable in the cytoplasmic fraction. Antioxidants and oxidative stress oxidize Keap1, which results in the release of Nrf2. Dissociation from Keap1 stabilizes and translocates Nrf2 into the nucleus (28). We showed that TAK1 regulates Nrf2 stability; therefore, ablation of TAK1 downregulates the level of Nrf2. TAK1 signaling is likely to modulate Nrf2 or Keap1 and blocks Keap1-dependent Nrf2 degradation. Further studies are needed to define the mechanism by which TAK1 modulates the Nrf2-Keap1 complex.

Intestinal epithelial-specific deletion of TAK1 causes increased apoptosis and disruption of cell–cell tight junctions, primarily in the ileum. Those pathological conditions are very similar to the pathology noted in inflammatory bowel disease (IBD). Anti-TNF therapy has recently been extensively used for the effective treatment of IBD (13). In the TAK1-deficient intestinal epithelium, although the deletion of TAK1 causes sustained severe intestinal damage, additional gene deletion of TNFRI greatly reduces the inflammatory conditions, enabling the mice to survive. Thus, the effects of TNF downregulation have some similarities between IBD and the mouse model with intestinal epithelial-specific deletion of TAK1. In the intestinal epithelial-specific TAK1-deletion mouse model, TNFRI deletion did not block increased ROS, apoptosis, or disruption of tight junctions. Although anti-TNF therapy is effective in the treatment of select IBD patients, it does not block all of the associated pathologic conditions. Our results raise the possibility that upregulation of the TAK1-Nrf2 pathway could reduce the level of ROS and enhance enterocyte survival and integrity of tight junctions. The TAK1 pathway may be a novel target involved in regulating intestinal barrier function.

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References