Expression of TNFAIP3 in intestinal epithelial cells protects from DSS- but not TNBS-induced colitis

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INTESTINAL EPITHELIAL CELLS (IEC) absorb nutrients, generate a dynamic barrier, and participate in innate immune defenses of the gut. The barrier generated by IEC is functional and physical, in that the epithelium produces mucus and antimicrobial peptides (9). In addition, IEC maintain dynamic control of tight junctions, which serve to decrease microbial contact with the mucosa (7). One way that the barrier function of the gut is lost is through ulceration, wherein IEC death exposes surfaces of the mucosa to direct contact with microbes of the gut lumen. Prevention of IEC death may be one way to reduce inflammation due to ulceration in the gut.

TNFα-induced protein 3 (TNFAIP3, also known as A20) is a cytosolic protein that inhibits signaling induced by the TNF receptor and Toll-like receptors (TLRs) (5, 15). TNF signaling includes the activation of NF-κB, JNK, and death-inducing caspases, and all these signals are inhibited by TNFAIP3 to prevent inflammation and apoptosis in most cells (13). In addition, TNFAIP3 inhibits NF-κB- and JNK-mediated signals from all the known TLRs (5). TNFAIP3 expression is rapidly induced by TLR ligands in IEC, and expression of TNFAIP3 promotes tolerance and inhibition of TLR signals in these cells (21, 29). TNFAIP3 expression is induced by NF-κB, indicating that TNFAIP3 acts in a negative-feedback loop to inhibit signals that induce its own expression (13). Mice lacking TNFAIP3 develop widespread inflammation and die prematurely (15). This is triggered by TLR-dependent activation of innate immune cells (26). TNFAIP31/– mice exhibit prolonged and excessive activation of NF-κB but are nonetheless sensitive to TNF-induced apoptosis, suggesting that TNFAIP3 mediates some of the antiapoptotic effects of NF-κB activation (15). Lineage-specific deletion of TNFAIP3 in IEC results in increased susceptibility of these cells to TNF-induced death and renders mice more sensitive to dextran sodium sulfate (DSS)-induced colitis (27). Transgenic expression of TNFAIP3 in IEC promotes barrier function by increasing tight junction stability (14). As TNFAIP3 expression in IEC is required for protection from apoptosis and promotes tight junction integrity, we tested whether transgenic expression of TNFAIP3 in IEC might protect from colitis.

METHODS

Animal studies. All animals were housed in specific pathogen-free conditions, and studies were approved by the University of Chicago Institutional Animal Care and Use Committee (Protocols 71661 and 72089). Villin-TNFAIP3 transgenic (Tg) mice and wild-type (WT) littermates, age 6–7 wk, were generated as previously described (14). For acute DSS-induced colitis, groups of four to six mice were fed DSS (2% wt/vol in water; 36,000–50,000 mol wt; MP Biosciences) ad libitum for 5 days and then returned to regular drinking water for recovery and euthanized. For 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, groups of mice were administered, by enema and under anesthesia, one dose of 100 μl of a 1:1 solution of 5% (wt/vol) TNBS in water mixed with absolute alcohol. Mouse weight and disease activity index were recorded blindly for the duration of the DSS cycle, and occurrences of euthanasia resulting from mice exceeding Institutional Animal Care and Use Committee-approved end points (weight loss >20% of maximum body weight, persistent positive fecal blood test or rectal bleeding, hunched posture, or rectal prolapse) were recorded as the percentage of survival for these experiments. The disease activity index was calculated by averaging

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the scores of weight loss, stool consistency, and rectal bleeding (0 = healthy, 4 = maximum colitis). For weight, no weight loss was scored as 0, weight loss of 1–5% from baseline as 1, 5–10% as 2, 10–15% as 3, and >15% as 4. Fecal blood was determined with hemoccult cards (Beckman Coulter). For bleeding, no blood was scored as 0, hemoccult-positive stool as 0, and rectal stool or diarrhea as 4. Colons were excised and measured for length analysis. For histological analysis, colons were fixed in 10% formalin and embedded in paraffin as longitudinal sections. Sections (5 μm) were stained with hematoxylin-eosin and scored by a pathologist blinded to the genotype using the following criteria: 0 = histologically normal; 1 = crypt architectural distortion or increase in lamina propria lymphocytes, but no increase in granulocytes; 2 = increase in lamina propria granulocytes, but absence of intraepithelial granulocytes; 3 = presence of intraepithelial granulocytes, but absence of crypt abscesses; 4 = crypt abscesses in <50% of crypts; 5 = crypt abscesses in >50% of crypts or presence of erosions.

ELISAs. On day 15, after DSS treatment and recovery, colons from villin-TNFAIP3 Tg mice and WT littermates were dissected out of the mesentery. Colons were cut longitudinally and washed twice in ice-cold PBS. The lumen was scraped with a glass slide for collection of mucosal protein by lysis and sonication in 500 μl of buffer [10 mM Tris, 5 mM MgSO4, 250 μg DNase 2, and 1× protease inhibitor cocktail (Roche)]. Protein lysates were quantified using a bicinchoninic acid protein assay (Pierce). Cytokine levels were assayed by ELISA using equivalent amounts of protein (100 μg/well) according to the manufacturer’s protocol for the indicated cytokines with OptEIA kits (BD Biosciences).

IEC death assays. Villin-TNFAIP3 Tg mice and WT littermates, age 6 wk, were treated with 2% DSS in drinking water for 5 days, and colons were then fixed in 10% formalin, embedded in paraffin as longitudinal sections, and sectioned at 5 μm. Apoptosis in the epithelium was analyzed by fluorescence microscopy after terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of the colonic tissue with the appropriate treatment using the In Situ Cell Death Detection Kit (POD, Roche), and TUNEL positivity was quantified on random tissue sections (5–10 sections per mouse) as a percentage of IEC per field of view.

Intestinal permeability assay. To assess intestinal permeability, we utilized a mouse ex vivo intestinal loop model previously described (4, 8). Villin-TNFAIP3 Tg mice and WT littermates were treated with 2% DSS in drinking water for 2 days and then euthanized. Colonic segments were filled with dialyzed FITC-dextran (1 mg/ml, 4,000 mol wt in PBS, Sigma), transferred into wells containing 2 ml of PBS, incubated at 37°C, and sampled for FITC-dextran flux and fluorescence by Bio Tek Synergy2 plate reader. FITC-dextran flux was measured as change in fluorescence per centimeter of intestine over time.

Statistical analysis. Statistical analyses were performed by Prism GraphPad 4.0 using two-way ANOVA with post hoc tests. Student’s t-test was used for comparison of two samples in a single condition. Values are means ± SE and represent a minimum of three experi-

![Fig. 1. Epithelial-specific TNFα-induced protein 3 (TNFAIP3) protects against dextran sodium sulfate (DSS)-induced colitis and inflammation.](http://c143.ajpgi.org/)

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 Lineage deletion of TNFAIP3 in IEC renders mice more susceptible to DSS-induced colitis, indicating that TNFAIP3 is required for protection of the intestine in that model (27). To determine whether IEC expression of TNFAIP3 is sufficient to protect mice from colitis, we generated villin-TNFAIP3 Tg mice and tested their response to DSS-induced colitis. Villin-TNFAIP3 Tg mice displayed reduced weight loss and more rapid recovery than WT littermates exposed to 2% DSS in drinking water for 5 days (Fig. 1A). Clinical scores indicated that WT littermates and villin-TNFAIP3 Tg mice initially became sick from the exposure to DSS but that villin-TNFAIP3 Tg mice recovered more quickly than WT littermates (Fig. 1B). Morbidity and mortality were decreased in the villin-TNFAIP3 Tg DSS-treated group, consistent with less mucosal damage than in WT littermates (Fig. 1C). One feature of mucosal injury in this model is a pronounced shortening of the DSS-treated mouse colon. This was also abrogated in villin-TNFAIP3 Tg mice (Fig. 1, D and E). Thus expression of TNFAIP3 in IEC promotes better clinical outcomes in mice exposed to DSS.

During DSS-induced colitis, mucosal inflammation results in production of cytokines that can induce tissue damage but can also promote healing of the gut. We therefore examined the tissue levels of cytokines in villin-TNFAIP3 Tg mice treated with DSS. WT littermates treated with DSS exhibited high levels of monocyte chemotactic protein 1 (MCP-1) and IL-6 in the intestinal mucosa that were not observed in villin-TNFAIP3 Tg mice (Fig. 2). We did not observe similarly high levels of IL-4 or IFNγ in WT or villin-TNFAIP3 Tg mice. Comparable levels of IL-10 were found in the mucosa of WT and villin-TNFAIP3 Tg mice (Fig. 2). Thus expression of TNFAIP3 in the epithelium reduces the expression of inflammation-associated cytokines in the mucosa of DSS-treated mice.

To determine why villin-TNFAIP3 Tg mice display improved clinical outcomes following exposure to DSS, we examined histological specimens from mice after 5 days of DSS treatment followed by 10 days of recovery. Intestines from WT mice remained noticeably inflamed, with extensive loss of epithelial cells, large areas of active inflammation, erosions, thickened muscularis propria, polymorphonucleocyte invasion, and disrupted crypt architecture (Fig. 3A). However, in DSS-treated villin-TNFAIP3 Tg mice, intestinal inflammation and injury were significantly diminished compared with WT littermates (Fig. 3B). The lamina propria had increased granulocytes, but without prominent crypt abscesses or erosions. This was confirmed by scoring of the histopathology of these mice, wherein scores were reduced in DSS-treated villin-TNFAIP3 Tg mice compared with WT littermates (Fig. 3C).

One early effect of DSS treatment is increased intestinal permeability, and we previously showed that TNFAIP3 expression in IEC promotes barrier function and tight junction integrity (14). We therefore examined the acute effects of DSS exposure on intestinal permeability in villin-TNFAIP3 Tg mice. After 2 days of DSS exposure, WT mice, but not villin-TNFAIP3 Tg mice, exhibited increased intestinal permeability compared with untreated controls (Fig. 4). Thus one effect of TNFAIP3 during DSS treatment is prevention of the early loss of barrier function that occurs in this model.

The persistence of epithelial cells and the protection from early loss of barrier function in the mucosa of DSS-treated villin-TNFAIP3 Tg mice led us to assess whether expression of TNFAIP3 promotes epithelial survival in this model. After 5 days of DSS treatment, histological evaluation of cell death indicated that the IEC of villin-TNFAIP3 Tg mice were significantly protected from cell death compared with WT littermates (Fig. 5, C and D). This reduced cell death was also seen in IEC after 2 days of DSS treatment (Fig. 5, A and B). Thus TNFAIP3 expression in IEC protects against DSS-induced cell death, and this may explain the preservation of IEC and promotion of intestinal barrier function in these mice.

The TNBS model of inflammatory bowel disease (IBD) involves the mechanical disruption of the epithelial barrier fol-
followed by haptenization of mucosal or luminal antigens and a T cell-mediated response leading to Th1-type inflammation. As TNFAIP3 expression in IEC promotes barrier function, we tested whether villin-TNFAIP3 Tg mice are protected from TNBS-induced colitis. WT and villin-TNFAIP3 Tg mice lost weight and developed intestinal inflammation following administration of TNBS, and there was no significant difference in the extent or severity of IBD, as measured by body weight or histological score, between these groups (Fig. 6). In fact, there was a trend toward worse colitis in villin-TNFAIP3 Tg mice, with a significant decrease in colon length. This indicates that, unlike the protection observed in the DSS model of IBD, IEC expression of TNFAIP3 does not protect from TNBS-induced colitis. Thus TNFAIP3 expression in IEC protects against IBD driven by IEC cell death, but not against IBD driven by T cell-mediated inflammation.

DISCUSSION

The results from this study show that epithelial-specific expression of TNFAIP3 protects against DSS-induced colitis. Mice with an IEC-specific deletion of TNFAIP3 are more susceptible to DSS-induced colitis and TNF-induced IEC death (27). Together, these complementary studies demonstrate that TNFAIP3 expression in IEC is critical for intestinal homeostasis subsequent to damage of the epithelium. Genetic deletion of IkB kinase (IKKβ), a key component of the NF-κB signaling pathway, also renders mice more susceptible to DSS-induced colitis (33). Complete ablation of the IKK complex in
IEC results in spontaneous colitis due to TNF-induced IEC death (19). Thus it is clear that NF-κB activation is protective in IEC. TNFAIP3 is an inhibitor of NF-κB activation; therefore, villin-TNFAIP3 Tg mice might be expected to phenocopy mice with inhibition of NF-κB in IEC. However, our results suggest that the ability of TNFAIP3 to prevent cell death can compensate for the inhibition of NF-κB activation by TNFAIP3. Indeed, TNFAIP3 is a potently NF-κB-induced gene and may be one of the essential antiapoptotic genes that accounts for the prosurvival function of NF-κB in the epithelium. Cells that lack NF-κB activation do not produce TNFAIP3; therefore, it is possible that some of the phenotype seen in mice with an IEC-specific deletion of NF-κB may be the result of a failure to induce TNFAIP3 in the IEC of those mice (19, 33). Thus, although NF-κB inhibition in IEC renders mice susceptible to colitis, the use of TNFAIP3 to inhibit NF-κB activation may be beneficial, because TNFAIP3 inhibits NF-κB activation and apoptosis and protects from colitis.

TNFAIP3 also negatively regulates MAPK signaling, and this may also account for the protective effects of IEC expression of TNFAIP3 in our model (29, 34). Decreases in p38 or JNK signaling protect from DSS-induced colitis, as these factors control TNF production by myeloid cells (2, 12). However, expression of p38 in IEC and expression of JNK are protective against DSS-induced colitis, suggesting that inhibition of MAPK by TNFAIP3 does not account for the protective effects of IEC expression of TNFAIP3 (6, 22). Conversely, prolonged NF-κB activation in IEC leads to profound inflammation when there is concurrent activation of the p38 and ERK MAPK pathways, as these signals are necessary for full production of TNF in vivo (10). Thus the combined activation of NF-κB and MAPK pathways may lead to less inflammation than either signal alone (10). Therefore, in the context of concurrent NF-κB activation, inhibition of MAPK by TNFAIP3 might provide additional protection from IBD.

The protection of villin-TNFAIP3 Tg mice from DSS-induced colitis was manifest as reduced ulceration and general preservation of IEC. Early in the course of DSS treatment, expression of TNFAIP3 in IEC prevented DSS-induced increases in intestinal permeability and death of IEC. We previously found that TNFAIP3 promotes intestinal barrier function by inhibiting TNF- or LPS-induced loss of tight junction integrity (14). The protection of barrier function in that context

Fig. 4. TNFAIP3 expression in epithelial cells preserves intestinal barrier function when challenged with DSS. Flux of FITC-dextran over time across explanted loops in WT (Δ) and villin-TNFAIP3 Tg (■) mice after a 2-day DSS challenge and in untreated WT (○) and villin-TNFAIP3 Tg (□) mice was measured in relative fluorescent units (RFU) per centimeter of colon (n = 3 separate experiments). *P < 0.05. **P < 0.01. ***P < 0.001.

Fig. 5. Epithelial TNFAIP3 expression protects against DSS-induced cell death. A and C: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; green) of longitudinal colon sections after a 2- or 5-day DSS challenge. (Hoechst stain was used to show nuclei.) B and D: scoring of TUNEL-positive cells per high-power field (HPF; n = 2–3 per group) *P < 0.02.
was independent of IEC death. In the present work, however, the loss of intestinal barrier function was coincident with IEC death induced by DSS. It is difficult to determine the temporal relationship between IEC death and loss of barrier function in vivo. Decreased barrier function can result in microbial activation of mucosal innate immune cells, leading to increased production of TNF, which can cause IEC death. Alternatively, IEC death can lead to loss of intestinal barrier function. In the context of DSS-induced colitis, where the epithelium is damaged, cytokines such as TNF and IL-6 promote healing and are required for protection against inflammation. We observed increased levels of IL-6 in WT mice compared with villin-TNFAIP3 Tg mice following DSS treatment and interpret this as a heightened response of WT mice to IEC damage. We did not measure TNF expression in our studies, but prevention of TNF-induced barrier disruption and IEC death might also be a mechanism of protection from DSS-induced colitis in the villin-TNFAIP3 Tg colon (20). It is likely that IEC death and decreased barrier function occur simultaneously in the DSS model, and our data suggest that TNFAIP3 prevents colitis by preventing IEC death and supporting intestinal barrier function.

Using the TNBS model of IBD, we did not consistently observe protection of villin-TNFAIP3 Tg mice from T cell-mediated colitis. The TNBS model involves disruption of the mucosal barrier with ethanol followed by haptenization of intestinal antigens and a Th1 immune response and inflammation (31). This model largely protects the intestinal epithelium from death, although cell death within the first 24 h of TNBS treatment might be caused by the ethanol used as a carrier. The cause of this IEC death was once considered to be FasL, but recent data do not support this mechanism (23, 25). Instead, IEC death may proceed through induction of peroxynitrites or other radicals generated early in the treatment (32). Results from the studies presented here, showing that villin-TNFAIP3 Tg mice are not protected from TNBS-induced colitis, suggest that, regardless of the form of IEC death that occurs in this model, it is not prevented by IEC expression of TNFAIP3. Indeed, we observed a trend toward worse colitis in TNBS-treated villin-TNFAIP3 Tg mice, which may indicate that the intestinal epithelium may restrain the colitis in this model in a way that is inhibited by IEC expression of TNFAIP3. The effects of IEC expression of TNFAIP3 in other T cell-mediated models of IBD, such as the IL-10−/− mouse, may help unveil...
new roles for the intestinal epithelium in the regulation of intestinal homeostasis.

TNFAIP3 is essential for IEC homeostasis and tight junction integrity and also for the normal regulation of innate and adaptive immune responses (5, 11, 14, 15, 26, 27). Thus genetic variants that alter TNFAIP3 expression or activity might be expected to be associated with IBD. Genetic variants in or near the TNFAIP3 gene have been implicated in a number of human immune disorders, including celiac disease, diabetes, rheumatoid arthritis, psoriasis, and systemic lupus erythematosus (13, 16, 17, 24, 28). Although there have been modest IBD associations with TNFAIP3 in genome-wide association studies, results have not consistently identified strong genetic associations in this locus with IBD. Other studies of rare or coding variants in TNFAIP3 have found associations with IBD susceptibility (16, 18). Decreased TNFAIP3 expression is one component of a five-gene signature that marks the severity of Crohn’s disease and responses to therapy (1). Since TNFAIP3 controls TLR and TNF activation of innate and adaptive immune cells, genetic variations that reduce TNFAIP3 expression are likely to lead to increased inflammation. In addition, our data suggest that alterations in TNFAIP3 expression will affect IEC survival and intestinal barrier function. Reduced barrier function in the gut can lead to increased microbial exposure and contribute to systemic immune responses and autoimmune disorders (3, 30). Thus autoimmunity associated with genetic variants in TNFAIP3 may arise in part because of the loss of TNFAIP3 control of IEC homeostasis. Conversely, protection from IBD and autoimmunity might occur when TNFAIP3 expression is increased in the intestinal epithelium.

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