TNFR2 Activates MLCK-Dependent Tight Junction Dysregulation to Cause Apoptosis-Mediated Barrier Loss and Experimental Colitis

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BACKGROUND & AIMS: Tight junction dysregulation and epithelial damage contribute to barrier loss in patients with inflammatory bowel disease. However, the mechanisms that regulate these processes and their relative contributions to disease pathogenesis are not completely understood. We investigated these processes using colitis models in mice. **METHODS:** We induced colitis by adoptive transfer of $CD4^{+}CD45RB^{hi}$ cells or administration of dextran sulfate sodium to mice, including those deficient in tumor necrosis factor receptor (TNFR) 1, TNFR2, or the long isoform of myosin light chain kinase (MLCK). Intestinal tissues and isolated epithelial cells were analyzed by immunoblot, immunofluorescence, enzyme-linked immunosorbent assay, and real-time polymerase chain reaction assays. **RESULTS:** Induction of immune-mediated colitis by $CD4^{+}CD45RB^{hi}$ adoptive transfer increased intestinal permeability, epithelial expression of claudin-2, the long isoform of MLCK, and TNFR2 (but not TNFR1) and phosphorylation of the myosin II light chain. Long MLCK upregulation, myosin II light chain phosphorylation, barrier loss, and weight loss were attenuated in $TNFR2^{-/-}$, but not $TNFR1^{-/-}$, recipients of wild-type $CD4^{+}CD45RB^{hi}$ cells. Similarly, long $MLCK^{-/-}$ mice had limited increases in myosin II light chain phosphorylation, claudin-2 expression, and intestinal permeability and delayed onset of adoptive transfer–induced colitis. However, coincident with onset of epithelial apoptosis, long $MLCK^{-/-}$ mice ultimately developed colitis. This indicates that disease progresses via apoptosis in the absence of MLCK-dependent tight junction regulation. In support of this conclusion, long $MLCK^{-/-}$ mice were not protected from epithelial apoptosis-mediated, damage-dependent dextran sulfate sodium colitis. **CONCLUSIONS: In immune-mediated inflammatory bowel disease models, TNFR2 signaling increases long MLCK expression, resulting in tight junction dysregulation, barrier loss, and induction of colitis. At advanced stages, colitis progresses by apoptosis and mucosal damage that result in tight junction- and MLCK-independent barrier loss. Therefore, barrier loss in immune-mediated colitis occurs via two temporally and morphologically distinct mechanisms. Differential targeting of these mechanisms can lead to improved inflammatory bowel disease therapies.**

Keywords: Inflammatory Bowel Disease; Epithelial Barrier; Intestinal Permeability; Disease Progression.

Intestinal homeostasis requires an epithelial barrier that regulates interactions between luminal material and the interstitium, which, respectively, include the gut microbiome and mucosal immune cells. It is not surprising that mild or severe intestinal barrier disruption can enhance or directly trigger experimental inflammatory bowel disease (IBD).[1–3] When the epithelium is intact, intestinal barrier function is largely defined by permeability characteristics of the epithelial tight junction. However, epithelial cell death also causes barrier loss, regardless of tight junction function.[4] It is challenging to distinguish between these two types of barrier loss in vivo, and many investigators have grouped these under the single heading of barrier function without greater mechanistic analysis. However, without detailed understanding of the relative roles of tight junction– and apoptosis-dependent barrier loss in disease progression, it is impossible to identify appropriate targets for therapeutic intervention. This has complicated the interpretation of studies linking increased intestinal epithelial tight junction permeability to progression of experimental colitis,[1–3] as most of these models also demonstrate both immune activation and ongoing damage, as demonstrated by increased epithelial turnover. The relative contributions of tight junction dysregulation– and epithelial damage–induced intestinal barrier loss to immune-mediated colitis have not been defined.

Tumor necrosis factor (TNF) is central to Crohn's disease pathogenesis. Although TNF signaling in immune cells is clearly of great importance, and one mechanism by which anti-TNF agents exert their therapeutic effect is TNFR2-dependent induction of lamina propria T-cell apoptosis,[5] other processes are also targeted. Epithelial TNFR1 and TNFR2 are less well studied, but have been implicated in epithelial apoptosis, proliferation, migration, and tight junction regulation.[6–8] The

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Abbreviations used in this paper: CA-MLCK, constitutively active myosin light chain kinase; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; MLC, myosin II regulatory light chain; MLCK, myosin light chain kinase; *Rag1*, recombinase activating gene 1; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

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relationship of these effects to IBD pathogenesis is poorly understood.

Here we show that TNFR2-mediated signaling on intestinal epithelial cells results in increased long myosin light chain kinase (MLCK) expression and that the resulting MLCK-dependent tight junction regulation is critical to initiation and progression of immune-mediated colitis. In contrast, epithelial apoptosis drives barrier loss in more advanced disease. These data might, in part, explain the utility of anti-TNF treatment as maintenance therapy in IBD[9,10] and further suggest that other approaches targeting this early, MLCK-mediated tight junction regulation can also be useful in preventing reactivation and limiting disease progression.

Materials and Methods

Mice and Colitis Models

All experiments used C57BL/6 mice bred and maintained at the University of Chicago in accordance with Institutional Animal Care and Use Committee regulations. $CD4^+CD45RB^{hi}$ and dextran sulfate sodium (DSS) colitis was induced in 6- to 8-week-old mice as described previously.[1] For adoptive transfer colitis, $CD4^+$ splenocytes were isolated using magnetic-activated cell sorting beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and enriched for $CD45RB^{hi}$ lymphocytes using a MoFlo Cell Sorter (Beckman Coulter, Brea, CA). Five hundred thousand $CD4^+CD45RB^{hi}$ T cells were injected intravenously into the specified mice, all on a recombinase-activating gene 1 $(Rag1)^{-/-}$ background. Acute colitis experiments used 3% DSS in drinking water of $Rag1^{+/+}$ mice. Numbers of mice per experimental group for the data shown, as well as the total number of experiments represented, are indicated in each legend. Results of all experiments were similar in male and female mice.

Intestinal Permeability Assay

Mice were denied access to food but allowed water for 3 hours. One hundred and fifty microliters of 80 mg/mL fluorescein isothiocyanate–4 kD dextran (Sigma, St Louis, MO) was gavaged and serum was harvested 1 and 3 hours later. Serum recovery was measured in a Synergy HT plate reader (BioTek, Winooski, VT).

Immunoblot, Immunofluorescence, Enzyme-Linked Immunosorbent Assay, and Real-Time Polymerase Chain Reaction

Immunoblots of isolated intestinal epithelia and immunofluorescence of frozen sections were performed as described previously.[11] Mucosal cytokines were quantified using Ready-SET-Go! kits (eBioscience, San Diego, CA). Quantitative real-time polymerase chain reaction was performed as described previously using GCGTGATCAGCCTGTTCTTTCTAA and GCCCCATCTGCCCTTCTTTGACC as long MLCK primers.[1]

Data Analysis

All data presented are representative of 3 or more experiments, each with similar results. Quantitative data are shown as mean ± SEM. Statistical significance was determined using Student's *t* test ($^*P < .05$ and $^{**}P < .01$).

Results

$CD4^+CD45RB^{hi}$ Adoptive Transfer Colitis Induces Changes in Tight Junction Protein Expression and Organization That Are Similar to Human IBD

Recent studies have shown that ileal and colonic epithelial long MLCK expression and activity as well as claudin-2 expression are increased in human ulcerative colitis and Crohn's disease.[12,13] Immunofluorescence analysis confirmed increased long MLCK expression and activity, measured as increased myosin light chain (MLC) phosphorylation, increased claudin-2 expression, and occludin endocytosis in ileal enterocytes during $CD4^+CD45RB^{hi}$-induced disease (Figure 1*A*). As expected, similar changes were noted in the colon after $CD4^+CD45RB^{hi}$ adoptive transfer, both by immunofluorescence (Figure 1*B*) and sodium dodecyl sulfate polyacrylamide gel electrophoresis immunoblot (Figure 1*C*). In addition, analyses of fluorescein isothiocyanate-4 kD dextran recovery in serum at 1 and 3 hours after gavage indicate increased small intestinal and colonic permeability, respectively, during $CD4^+CD45RB^{hi}$-induced disease (Figure 1*D*). These data show that induced by $CD4^+CD45RB^{hi}$ adoptive transfer in the small intestine and colon are similar. Our subsequent analyses focus on the colon on the basis of these observations and the fact that most studies have concentrated on the more severe colitis, relative to ileitis, induced in this model.

Epithelial TNFR2 Expression and Apoptosis Are Increased in $CD4^+CD45RB^{hi}$ Colitis

TNF is pivotal to the pathogenesis of Crohn's disease, and expression of this cytokine is markedly increased in $CD4^+CD45RB^{hi}$ adoptive transfer colitis.[14] To better understand the potential roles of epithelial TNFR1 and TNFR2 in colitis, their expression in colonocytes was assessed after adoptive transfer. Remarkably, although TNFR1 expression was unaffected, TNFR2 was markedly upregulated (Figure 2*A*). To further define the roles of TNFRs in colitis, wild-type $CD4^+CD45RB^{hi}$ lymphocytes were transferred to TNFR knockout recipients. This is imperfect, as recipient innate immune cells also lack TNFRs, but tissue-specific TNFR knockout mice are not available. Cleaved caspase-3 staining was used to identify apoptotic epithelial cells. $CD4^+CD45RB^{hi}$ adoptive transfer induced a marked increase in epithelial apoptosis (Figure 2*B* and C; $P < .01$). In contrast, adoptive transfer of wild-type $CD4^+CD45RB^{hi}$ T cells into $TNFR1^{-/-}/Rag1^{-/-}$ or $TNFR2^{-/-}/Rag1^{-/-}$ recipients resulted in far fewer cleaved caspase-3–positive cells (Figure 2*B* and C; both $P < .02$ vs $Rag1^{-/-}$). These data show that both TNFR1 and TNFR2 signaling on recipient cells, including but not limited to, epithelial cells, contribute to pathways that trigger epithelial apoptosis during adoptive transfer colitis.

TNFR2 Expression Contributes to Barrier Loss and Colitis Progression

The data above implicate apoptosis as a potential mechanism of the intestinal barrier loss observed in

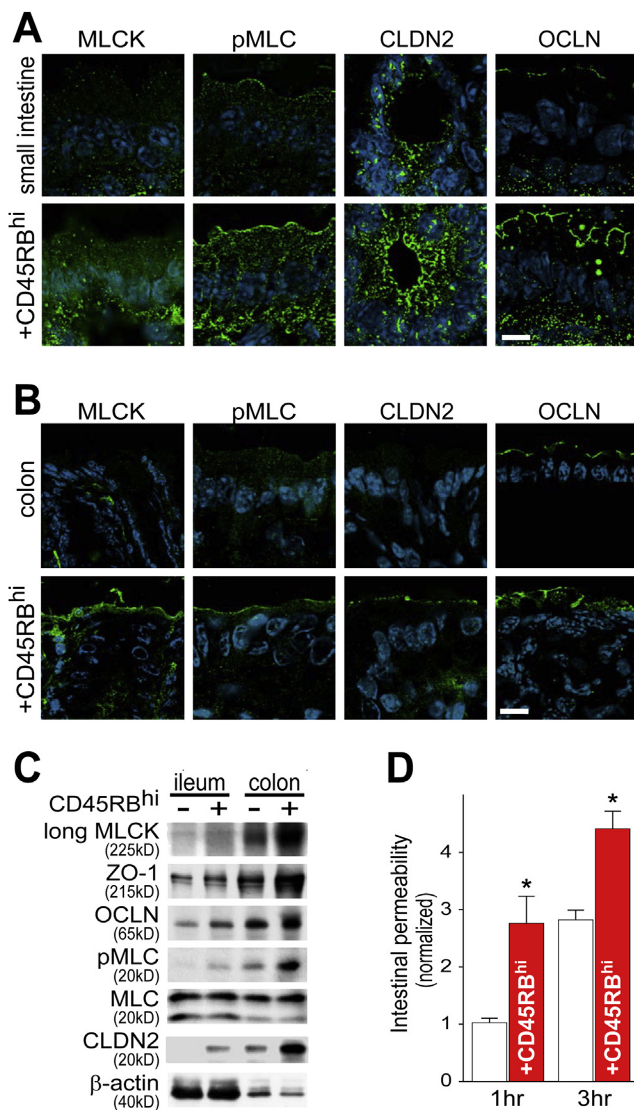

Figure 1. Adoptive transfer colitis is associated with altered tight junction structure and composition as well as reduced intestinal barrier function. (*A, B*) Immunofluorescence (*green*) of MLCK, phosphorylated MLC (pMLC), claudin-2 (CLDN2), and occludin (OCLN) in small intestine (*A*) and colon (*B*) before and after adoptive transfer of $CD4^{+}CD45RB^{hi}$ T cells into $Rag1^{-/-}$ mice. Nuclei are *blue*. Bar = 10 μm. (*C*) Immunoblots of isolated small intestinal (ileal) and colonic epithelia showing long MLCK, ZO-1, occludin (OCLN), pMLC, total MLC (MLC), claudin-2 (CLDN2), and β-actin expression before and after adoptive transfer. (*D*) Intestinal permeability to fluorescein isothiocyanate-4 kD dextran at 1 and 3 hours after gavage (n = 4). Data in this figure are representative of at least 6 independent experiments.

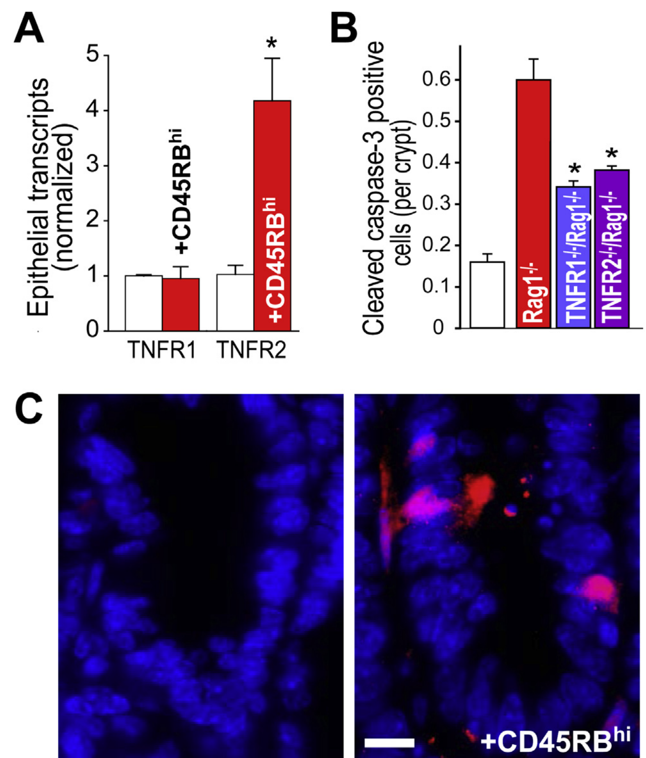

Figure 2. Epithelial TNFR2 expression is selectively increased in colitis. (*A*) Colonic epithelial TNFR1 and TNFR2 messenger RNA transcript expression in $Rag1^{-/-}$ mice that did (*red bars*) or did not (*white bars*) receive $CD4^{+}CD45RB^{hi}$ lymphocytes (n = 4–7). (*B*) Both $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice were partially protected from epithelial apoptosis after adoptive transfer (n = 3–5). (*C*) Representative cleaved caspase-3 (*red*) immunostains with nuclear counterstain (*blue*) before or after adoptive transfer. Bar = 10 μm. Data in this figure are representative of at least 3 independent experiments.

$CD4^{+}CD45RB^{hi}$ colitis. We therefore assessed the impact of TNFR deficiency on intestinal permeability increases induced by adoptive transfer colitis (Figure 3*A*). Only *$TNFR2^{-/-}/Rag1^{-/-}$* recipients showed significant protection from colitis-associated barrier loss (Figure 3*A*; $P < .05$). Weight loss paralleled barrier loss, with partial protection of *$TNFR2^{-/-}/Rag1^{-/-}$* recipients (Figure 3*B*; $P < .05$). Similarly, mucosal TNF production was reduced in *$TNFR2^{-/-}/Rag1^{-/-}$* recipients relative to *$TNFR1^{-/-}/Rag1^{-/-}$* and *$Rag1^{-/-}$* recipients (Figure 3*C*; $P < .05$). *$TNFR2^{-/-}/Rag1^{-/-}$* recipients were also protected in terms of histopathology (Figure 3*D*; $P < .05$). Consistent with this, a direct correlation between TNF induction and histological damage has been previously reported in $CD4^{+}CD45RB^{hi}$ adoptive transfer colitis.[15]

To better understand the protection afforded by TNFR2 knockout, we considered the possibility that tight junction barrier function was preserved in these mice during colitis initiation. Expression of long MLCK transcripts was therefore assessed in colonocytes of *$Rag1^{-/-}$*, *$TNFR1^{-/-}/Rag1^{-/-}$*, and *$TNFR2^{-/-}/Rag1^{-/-}$* mice 15 days after adoptive transfer. Long MLCK transcription was increased to 2.7 ± 0.3 and 2.3 ± 0.5 in *$Rag1^{-/-}$* and *$TNFR1^{-/-}/Rag1^{-/-}$* recipients, relative to mice that did not receive $CD4^{+}CD45RB^{hi}$ T cells (Figure 3*E*; both $P < .05$ vs controls without adoptive transfer). In contrast, long MLCK transcription was not increased in *$TNFR2^{-/-}/Rag1^{-/-}$* recipients (Figure 3*E*; 1.3 ± 0.2; $P < .05$ vs of *$Rag1^{-/-}$* and *$TNFR1^{-/-}/Rag1^{-/-}$* recipients, NS vs controls without adoptive transfer). Consistent with this, colonic epithelial MLC phosphorylation in *$TNFR2^{-/-}/Rag1^{-/-}$* recipients was only 51% ± 10% of that observed in *$Rag1^{-/-}$* recipients (Figure 3*F*; $P < .05$). MLC phosphorylation was variably reduced in *$TNFR1^{-/-}/Rag1^{-/-}$* recipients, but this did not reach statistical significance. These data therefore suggest that TNFR2-dependent MLCK upregulation is an important contributor to intestinal barrier loss and colitis

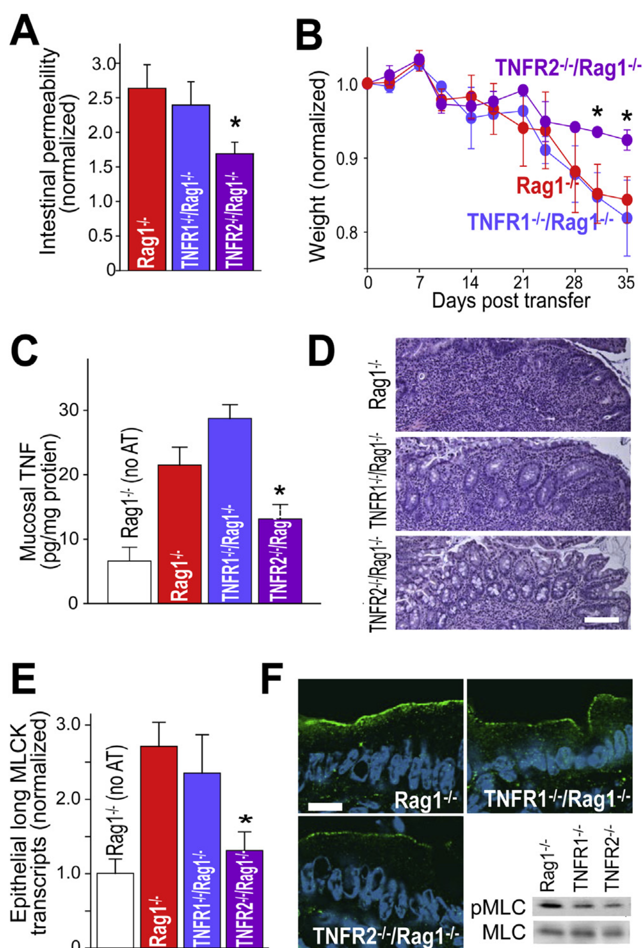

Figure 3. *TNFR2*$^{-/-}$ recipients of wild-type CD4$^+$CD45RB^{hi} T cells are partially protected from colitis. (*A*) Intestinal permeability to fluorescein isothiocyanate-4 kD dextran at early times, eg, 15 days, after adoptive transfer in *Rag1*$^{-/-}$, *TNFR1*$^{-/-}$*/Rag1*$^{-/-}$, and *TNFR2*$^{-/-}$*/Rag1*$^{-/-}$ mice. Data are normalized to mice that did not receive adoptive transfer (n = 3–7). (*B*) Weight loss after adoptive transfer in *Rag1*$^{-/-}$, *TNFR1*$^{-/-}$*/Rag1*$^{-/-}$, and *TNFR2*$^{-/-}$*/Rag1*$^{-/-}$ mice (n = 4–5). (*C*) Mucosal TNF expression at early times after adoptive transfer (n = 3–5). (*D*) Colonic histopathology at late times, eg, 35 days, after adoptive transfer. Bar = 100 μm. (*E*) Colonic epithelial long MLCK messenger RNA expression at early times after adoptive transfer (n = 3). (*F*) Immunofluorescence for phosphorylated MLC (green) at early times after adoptive transfer. Nuclei are *blue*. Bar = 10 μm. *Inset*: colonic epithelial immunoblots for phosphorylated MLC (pMLC) and total MLC (MLC) at early times days after adoptive transfer (representative of n = 3 in this experiment). Data in this figure are representative of at least 3 independent experiments.

initiation. However, they do not discriminate between effects of TNFR2 on nonepithelial and epithelial recipient cells. In addition, these data do not resolve differences between barrier loss due to tight junction regulation vs apoptosis.

Long MLCK Deletion Delays Barrier Defects and Reduces Histologic Severity of Immune-Mediated Colitis

The data presented here suggest that failure to upregulate intestinal epithelial long MLCK expression might contribute to the protection observed in TNFR2-deficient CD4$^+$CD45RB^{hi} T-cell recipients. This is consistent with the critical role of intestinal epithelial MLCK in tight junction barrier dysregulation in rodent models of acute TNF-induced diarrhea.[11] However, no studies have examined the utility of MLCK inhibition in chronic colitis. This, in part, reflects the nonspecific nature of widely used MLCK inhibitors, such as ML-7 and ML-9,[16] which inhibit long (epithelial) and short (smooth muscle) MLCK[17] as well as many other kinases.[18] To selectively inhibit the long MLCK expressed in human and murine intestinal epithelia[19] without affecting short MLCK-dependent intestinal motility,[20] *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$ mice were used. These mice have previously been shown to be phenotypically normal in the absence of stressors but are protected from tight junction barrier loss and net fluid secretion in models of acute, apoptosis-independent, TNF-mediated diarrhea.[15]

After wild-type CD4$^+$CD45RB^{hi} T-cell transfer, intestinal epithelial MLC phosphorylation in *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$ mice was only 24% ± 4% of that in *long MLCK*$^{+/+}$*/Rag1*$^{-/-}$ mice (Figure 4*A*; $P < .05$). Claudin-2 upregulation was also reduced by 46% ± 8% in *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$, relative to *long MLCK*$^{+/+}$*/Rag1*$^{-/-}$, mice (Figure 4*A*; $P < .05$). This suggests that MLCK-dependent tight junction barrier loss drives events that lead to increased claudin-2 expression during disease progression.

Consistent with the absence of marked increases in epithelial MLC phosphorylation, intestinal permeability of *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$ was not increased at early times (eg, 15 days) after adoptive transfer (Figure 4*B*; $P < .05$). However, this protection was lost at later times (eg, 35 days) as disease progressed. *Rag1*$^{-/-}$ recipients displayed progressive increases in epithelial apoptosis from early to late times after adoptive transfer (Figure 4*C*). In contrast, frequency of apoptotic cells in *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$ at early times after adoptive transfer (Figure 4*C*) was similar to mice that had not received adoptive transfer (Figure 2*B*) and significantly less than that observed in *Rag1*$^{-/-}$ recipients (Figure 4*C*; $P < .05$). The protection from apoptosis afforded by long MLCK knockout was lost at later times (Figure 4*C*), similar to loss of barrier protection (Figure 4*B*). Similarly, long MLCK knockout also prevented early, but not late, increases in mucosal TNF production (Figure 4*D*; $P < .05$). These data suggest that inhibition of MLCK-mediated tight junction barrier loss limits early disease progression, but that epithelial apoptosis at later times causes MLCK-independent barrier loss that facilitate disease progression.

Consistent with the observed barrier preservation and reductions in apoptosis and TNF production, both weights and clinical scores of *long MLCK*$^{-/-}$*/Rag1*$^{-/}$, recipients were improved, relative to *long MLCK*$^{+/+}$*/Rag1*$^{-/-}$ mice, at early times after adoptive transfer (Figure 4E; $P < .05$). This protective effect persisted for 4 weeks, but like the other parameters, clinical scores and weight loss of *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$ recipients ultimately progressed and, at late times (eg, 35 days) after adoptive transfer were comparable with those in *long MLCK*$^{+/+}$*/Rag1*$^{-/-}$ recipients. In contrast, colonic histopathology was significantly reduced in

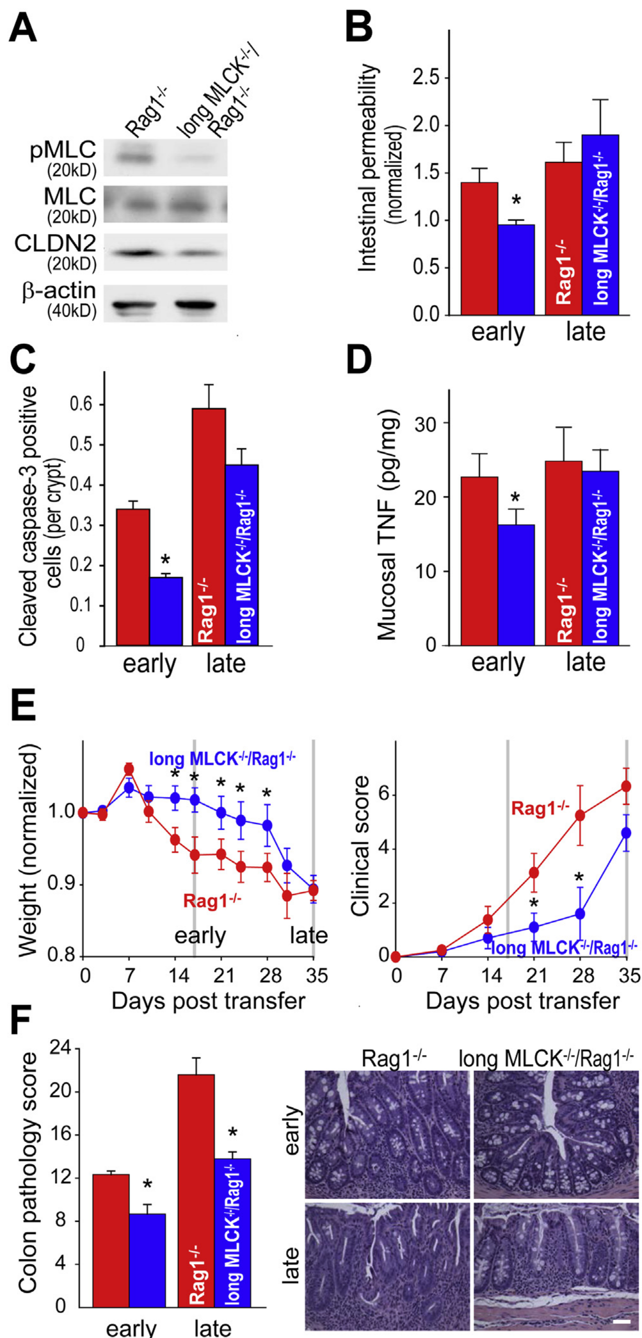

Figure 4. *Long MLCK*$^{-/-}$ mice are protected from CD4^{+}CD45RB^{hi} colitis induced by wild-type CD4^{+}CD45RB^{hi} T cells. (*A*) Colonic epithelial immunoblots at early times, eg, 15 days, after adoptive transfer. (*B*) Intestinal permeability to fluorescein isothiocyanate-4 kD dextran at early and late, eg, 35 days, after adoptive transfer. Data are normalized to mice that did not receive adoptive transfer (n = 6–7). (*C*) Frequency of cleaved caspase-3–positive cells at early and late, eg, 35 days, after adoptive transfer (n = 3–5). (*D*) Mucosal TNF expression at early and late times after adoptive transfer (n = 5–7). (*E*) Weight and clinical scores during adoptive transfer colitis (n = 8–10). (*F*) Colon histopathology at early and late times after adoptive transfer (n = 3–5). Bar = 100 μm. Data in this figure are representative of at least 5 independent experiments.

long MLCK$^{-/-}$/*Rag1*$^{-/-}$ recipients at both early and late times after adoptive transfer (Figure 4*F*). These data show that, despite epithelial damage, intestinal barrier loss, and increased TNF production at later times, the histological protection afforded by long MLCK deficiency persists and represents a durable reduction in disease severity.

Long MLCK Deletion Does Not Inhibit Acute Cytokine-Induced or Chemically Induced Epithelial Damage

As a whole, these data suggest that long MLCK inhibition delays progression of immune-mediated colitis by preventing tight junction barrier loss. However, an alternative explanation, that long MLCK inhibition prevents epithelial apoptosis, could also be considered. The latter hypothesis is consistent with the observation that apoptotic frequency is reduced in *long MLCK*$^{-/-}$/*Rag1*$^{-/-}$ mice, relative to *long MLCK*$^{+/+}$/*Rag1*$^{+/+}$ mice, at early times after adoptive transfer. To test this hypothesis, the sensitivity of *long MLCK*$^{-/-}$ and *long MLCK*$^{+/+}$mice to acute, TNF-induced apoptosis was assessed. Acute in vivo TNF administration causes massive intestinal epithelial apoptosis within 4 hours (Figure 5*A*). However, there was no difference between *long MLCK*$^{-/-}$ and *long MLCK*$^{+/+}$ mice upon TNF administration (Figure 5*A*). Therefore, long MLCK inhibition does not prevent acute TNF-induced intestinal epithelial apoptosis.

To more fully examine the potential of long MLCK as an intermediate in direct epithelial damage, the sensitivity of *long MLCK*$^{-/-}$ and *long MLCK*$^{+/+}$ mice to chemical damage-induced, (ie, DSS), colitis was assessed. Barrier loss (Figure 5*B*), gross and histologic pathology (Figure 5*C*), weight loss, and clinical score increases (Figure 5*D*) induced by DSS were similar or of greater severity in *long MLCK*$^{-/-}$ mice relative to *long MLCK*$^{+/+}$ mice. This lack of protection from chemically induced cell injury and colitis emphasizes the inability of MLCK inhibition to prevent barrier loss and disease triggered by epithelial damage. In addition, these data support the conclusion that *long MLCK*$^{-/-}$ mice are protected early in the course of immune-mediated colitis due to their resistance to cytokine-induced, tight junction dysregulation rather than inhibition of epithelial apoptosis.

Intestinal Epithelial MLCK Drives Progression and Increases Severity of CD4^{+} CD45RB^{hi} Colitis

Tissue-specific long MLCK knockout mice are not available. To determine if loss of long MLCK in non-epithelial cell types, including endothelium and neutrophils,[21] contributed to the observed protection from adoptive transfer colitis, *long MLCK*$^{-/-}$/*Rag1*$^{-/-}$ mice were crossed to transgenic mice that express constitutively active MLCK (CA-MLCK) specifically within the intestinal epithelium.[1] After adoptive transfer, *long MLCK*$^{-/-}$/*Rag1*$^{-/-}$ mice failed to increase intestinal epithelial MLC phosphorylation above that seen in mice that did not receive adoptive transfer (Figure 6*A*). Intestinal epithelial-specific CA-MLCK expression corrected this defect, as colitis-associated MLC phosphorylation in *CA-MLCK/long MLCK*$^{-/-}$/*Rag1*$^{-/-}$ mice was similar to that observed in *long MLCK*$^{+/+}$/*Rag1*$^{-/-}$ mice (Figure 6*A*).

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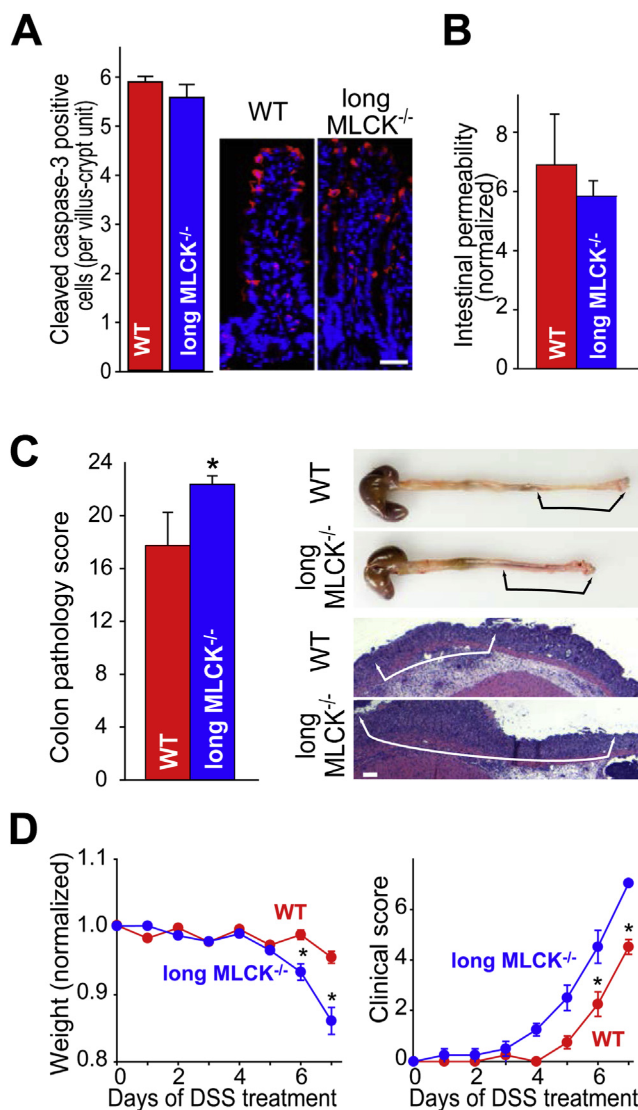

Figure 5. *Long MLCK*$^{-/-}$ mice are not protected from TNF-induced apoptosis or DSS colitis. (*A*) Morphometric analysis (n = 3) and representative cleaved caspase-3 (*red*) immunostains with nuclear counterstain (*blue*) 4 hours after intraperitoneal administration of 10 μg TNF to immunocompetent wild-type and *long MLCK*$^{-/-}$ mice. Bar = 50 μm. (*B*) Intestinal permeability to fluorescein isothiocyanate-4 kD dextran at day 6 of DSS treatment (n = 3). (*C*) Colon gross and histologic pathology after 6 days of DSS treatment. For scores, n = 3–5. *Bracketed arrows* in gross photo show extended area of erythema in shortened colon of *long MLCK*$^{-/-}$ mouse. *Bracketed arrows* in micrographs show extended area of colonic ulceration in *long MLCK*$^{-/-}$ mouse. The *left arrows* indicate the anorectal junction, where ulceration begins, and the *right arrows* show the proximal extent contiguous ulceration. Bar = 150 μm. (*D*) Weight and clinical scores during DSS-induced colitis (n = 3). Data in this figure are representative of at least 3 independent experiments.

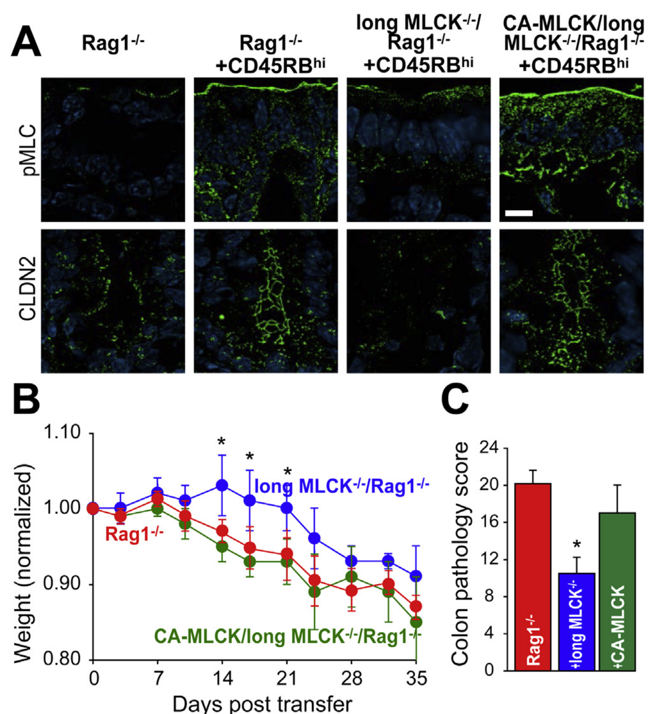

Figure 6. Transgenic, intestinal epithelial-restricted expression of constitutively active-MLCK restores sensitivity of *long MLCK*$^{-/-}$ mice to CD4^{+}CD45RB^{hi} colitis. (*A*) Immunofluorescence (green) of phosphorylated MLC (pMLC) and claudin-2 (CLDN2) before or after adoptive transfer, as indicated. Nuclei are *blue*. Bar = 10 μm. (*B*) Weight of indicated genotypes after adoptive transfer (n = 5–7). (*C*) Colon pathology scores at 35 days (n = 4). Data in this figure are representative of 5 independent experiments.

Similar to MLC phosphorylation, intestinal epithelial-specific CA-MLCK expression also restored colitis-associated upregulation of intestinal epithelial claudin-2 expression (Figure 6*A*). In addition, CA-MLCK expression restored both disease sensitivity and histopathology of *long MLCK*$^{-/-}$/*Rag1*$^{-/-}$ mice to that of *long MLCK*$^{+/+}$/*Rag1*$^{-/-}$ mice (Figure 6*B* and *C*). These data show that intestinal epithelial-restricted MLCK expression reversed the protective effect of long MLCK knockout on immune-mediated colitis. This demonstrates that the protection conferred by long MLCK knockout is due to loss of intestinal epithelial long MLCK rather than long MLCK expressed in other cell types.

Discussion

Increased intestinal permeability has been linked to a variety of autoimmune and inflammatory disorders. The case is strongest in Crohn's disease, where reduced barrier function is a marker of impending disease reactivation.[22] However, increased intestinal permeability is also increased in ulcerative colitis.[23] Despite this association, intestinal barrier loss alone is insufficient to cause disease in either animal models or human subjects.[1–3,24] Nevertheless, intestinal barrier defects do accelerate onset and enhance severity of experimental colitis when coupled with disease-inducing stimuli.[1–3] These data suggest that preservation of the tight junction barrier can be beneficial in colitis.

We and others have described two critical mechanisms of immune-mediated tight junction regulation. The first, which can be driven by TNF, LIGHT, or IL-1β, depends on transcriptional and enzymatic activation of epithelial long MLCK that leads to phosphorylation of peri-junctional MLC.[15,25–27] The presence of increased epithelial long MLCK expression and activity in both ulcerative colitis and Crohn's disease indicates that this form of tight junction dysregulation is active in human IBD.[12] A second mechanism of barrier loss is associated

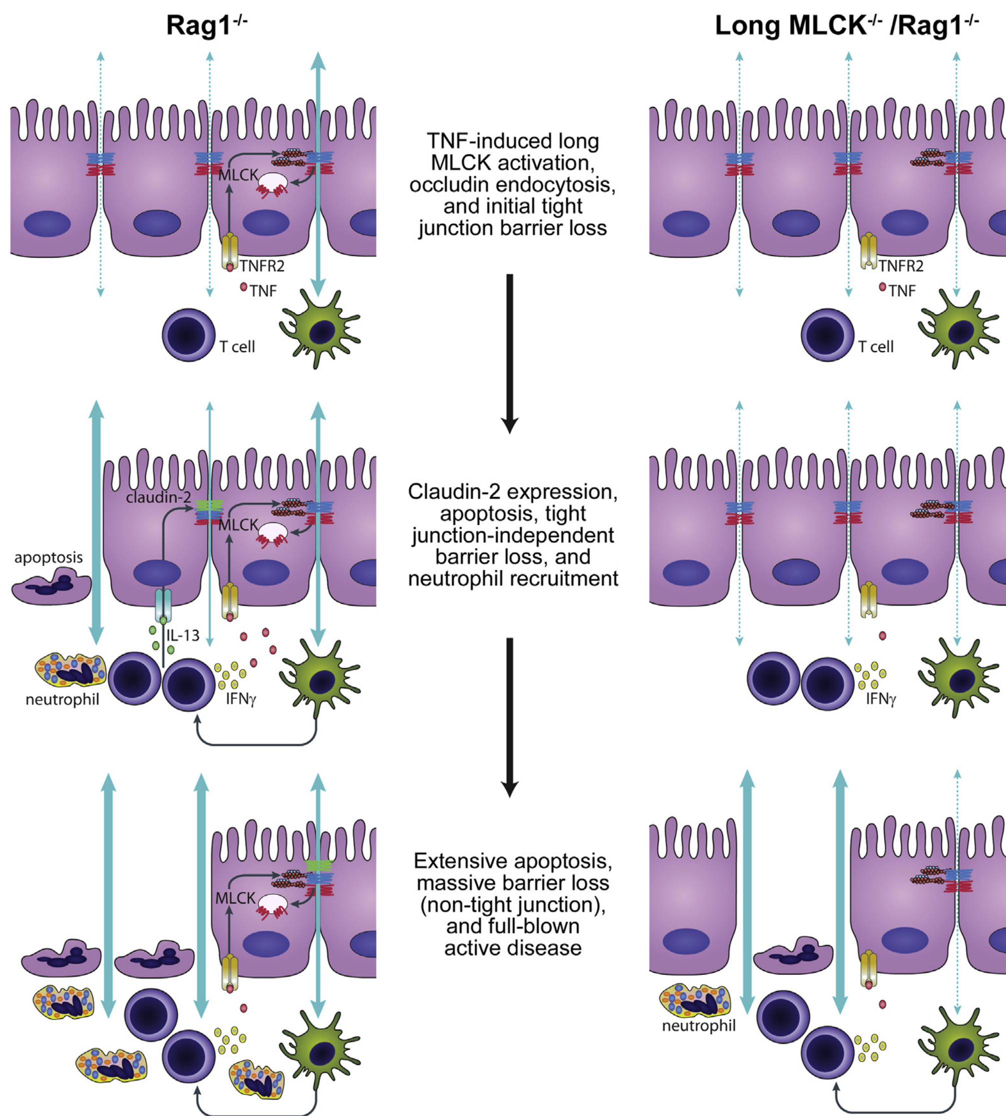

Figure 7. Proposed roles of TNFR2-dependent, MLCK-mediated tight junction barrier loss and apoptosis-driven barrier loss during colitis progression. Adoptive transfer of $CD4^{+}CD45RB^{hi}$ T cells results in TNF production that activates epithelial TNFR2 in $Rag1^{-/-}$ (*left*) and long $MLCK^{-/-}/Rag1^{-/-}$ (*right*) mice. This leads to transcriptional upregulation and enzymatic activation of epithelial long MLCK, MLCK-mediated occludin internalization, and increased permeability of large macromolecules via the paracellular leak pathway.[40] These events do not occur in the absence of long MLCK (depicted) or TNFR2-dependent long MLCK upregulation Increased tight junction permeability results in enhanced paracellular flux of luminal materials, mucosal activation, and release of cytokines, eg, IL-13. This induces epithelial claudin-2 expression that enhances flux of small cations and water via the paracellular pore pathway.[40] Additional amplifying cycles of immune activation and tight junction regulation lead to inflammation that causes scattered epithelial apoptosis and tight junction-independent barrier loss. As numbers of apoptotic epithelial cells increase, erosions and ulcers develop and cause massive permeability increases due to epithelial loss that, in turn, accelerate disease progression. Although tight junction dysregulation and associated barrier loss do not occur in *long* $MLCK^{-/-}$ mice, $CD4^{+}CD45RB^{hi}$ T cells are eventually activated sufficiently to cause significant epithelial apoptosis. As in *long* $MLCK^{+/+}$ mice, the resulting massive, tight junction-independent barrier loss facilitates disease progression, culminating in full-blown active disease.

with increased epithelial claudin-2 expression, and this is also present in ulcerative colitis and Crohn's disease.[11,28] The data reported here implicate both forms of tight junction dysregulation in immune-mediated chronic colitis. In addition, the results show that epithelial MLCK inhibition prevents claudin-2 upregulation, suggesting that long MLCK activation triggers both forms of IBD-associated tight junction dysregulation. Intestinal epithelial MLCK might, therefore, be a convergence point at which inhibition of a single mediator, ie, long MLCK, can correct or prevent both forms of IBD-associated barrier loss.

We show here that long MLCK knockout, which has no apparent toxicity, is beneficial in immune-mediated colitis. However, although MLCK deficiency resulted in sustained histologic benefit, all other measures of disease, such as barrier loss, cytokine production, weight loss, and clinical scores of *long* $MLCK^{-/-}/Rag1^{-/-}$ and *long* $MLCK^{+/+}/Rag1^{-/-}$ recipients ultimately equalized. This long-term failure could simply be due to the well-recognized inexorable nature of $CD4^{+}CD45RB^{hi}$ T-cell–induced colitis. For example, despite the clinical efficacy of anti-$\alpha 4$ integrin antibodies, which inhibit $\alpha 4\beta 7$ integrin-mediated leukocyte adhesion,[29] and anti-TNF antibodies,[30,31] neither $\beta 7$

integrin deficiency[32] nor anti-TNF therapy[13] completely prevented CD4$^+$CD45RB^{hi} colitis. Similarly, anti-TNF therapy has failed to provide sustained benefit in chemically induced colitis models.[33,34] The inability of long MLCK knockout alone to provide sustained benefit in experimental immune-mediated colitis can thus paint an excessively pessimistic picture of the potential for therapeutic long MLCK inhibition. It is also important to recognize that long MLCK inhibition may be most useful as part of multi-agent maintenance therapy, along with immunomodulatory or immunosuppressive agents.

The weight and clinical score curves suggest that intestinal epithelial MLCK inhibition delays initiation but does not ultimately prevent the development of colitis. It is, therefore, important to understand the mechanisms by which CD4$^+$CD45RB^{hi} colitis overcomes this delay to cause colitis in long *MLCK*$^{-/-}$ mice. Together, the observations that long MLCK knockout does not impact acute TNF-induced apoptosis or apoptosis-driven DSS colitis; the number of apoptotic epithelial cells increases over time during CD4$^+$CD45RB^{hi} T cell-induced colitis; and differences between *long MLCK*$^{-/-}$*/Rag1*$^{-/-}$ and *long MLCK*$^{+/+}$*/Rag1*$^{-/-}$ in epithelial apoptotic frequency, barrier function, cytokine production, and clinical features of disease are lost in parallel during progression of CD4$^+$CD45RB^{hi} colitis suggest that apoptosis-induced barrier loss is this mechanism. Based on these data, a model emerges where immune activation, in this case driven initially by CD4$^+$CD45RB^{hi} T-cell transfer, causes MLCK-mediated tight junction-dependent barrier loss that promotes additional immune activation, culminating in full-blown active disease (Figure 7). In the absence of epithelial long MLCK, tight junction-dependent barrier loss is prevented and the signaling loop is short circuited. However, although immune activation progresses at a slower rate without MLCK-mediated increases in tight junction permeability, immune activation is not entirely inhibited. This is because this model depends on transfer of naïve T-effector cells to immunodeficient recipients that lack regulatory T cells. Over time, a critical level of T-cell activation is reached that, in turn, triggers apoptotic barrier loss. This overcomes the inhibition of tight junction–dependent barrier loss and allows disease progression. Whether the delay imposed by epithelial long MLCK inhibition would be more durable in other immune-mediated IBD models or in human disease remains to be determined.

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Although both TNFR1 and TNFR2 knockout in T-cell recipients reduced the numbers of apoptotic epithelial cells at early times after adoptive transfer, only TNFR2 knockout improved clinical outcomes. Like long MLCK knockout, this was associated with preservation of intestinal barrier function and reduced TNF expression relative to TNFR sufficient and TNFR1 knockout mice. In addition, colitis-associated increases in long MLCK transcription and epithelial MLC phosphorylation were largely blocked in TNFR2 knockout mice. This suggests that the protective effect of TNFR2 inhibition reflects a critical role for TNFR2 in TNF-induced long MLCK upregulation and subsequent colitis progression. This conclusion, which is consistent with in vitro data showing that TNFR2 signaling mediates TNF-induced long MLCK expression and tight junction regulation,[6] might explain the association of TNFR2 polymorphisms with Crohn's disease.[35] Although a protective effect of TNFR2 knockout has recently been reported in DSS colitis,[36] this result is controversial, as other studies have demonstrated no significant change or increased DSS sensitivity in TNFR2 knockout mice.[37,38] One possible explanation for this disparity between studies is that differences in mouse strain used, local microbiome, or other factors that affect DSS colitis development highlighted or suppressed the well known role of TNFR2 in promoting epithelial migration and wound healing.[8] Consistent with this, knockout of epithelial long MLCK, which is also involved in wound healing,[39] slightly enhanced severity of DSS-induced disease. However, it is important to note that all of these studies used universal TNFR knockout mice, making it difficult to distinguish TNF signaling in immune cells from that in other cell types. This likely explains the difference between previous reports demonstrating increased DSS colitis severity in TNFR1 knockout mice[36–38] and our studies, which did not detect any clinically evident impact of TNFR1 knockout, including contributions of TNFR1 signaling on myeloid lineage cells, to promote mucosal recovery.[37] As a whole, our data combined with this literature demonstrate that the contributions of TNFR1 and TNFR2 to colitis vary by with disease model and cell type, and, possibly, by gut microbiome composition and mouse strain.[36–38] Additional analysis of TNFR expression by different cell types in human IBD might be useful in defining functions. Our data do demonstrate that one important role of TNFR2 is to stimulate increased epithelial long MLCK expression that, in turn, enhances severity of immune-mediated colitis.

Overall, our data demonstrate that barrier loss in immune-mediated colitis occurs by two temporally and morphologically distinct mechanisms (Figure 7). MCLK-dependent tight junction permeability increases occur early and promote disease initiation. Later, as disease progresses, apoptosis and gross ulceration of the epithelium causes tight junction-independent barrier loss. We conclude that targeted inhibition of intestinal epithelial long MLCK may be therapeutically effective, particularly in preventing reactivation of quiescent IBD.

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The authors disclose no conflicts.

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