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Epithelial NF-κB Enhances Transmucosal Fluid Movement by Altering Tight Junction Protein Composition after T Cell Activation

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In inflammatory bowel disease (IBD), aberrant activation of innate and adaptive immune responses enhances mucosal permeability through mechanisms not completely understood. To examine the role of epithelial nuclear factor (NF-κB) in IBD-induced enhanced permeability, epithelial-specific IκBα mutant (NF-κB super repressor) transgenic (TG) mice were generated. NF-kB activation was inhibited in TG mice, relative to wild-type mice, following T cell-mediated immune cell activation using an anti-CD3 monoclonal antibody. Furthermore, epithelial NF-κB super repressor protein inhibited diarrhea and blocked changes in transepithelial resistance and transmucosal flux of alexa350 (0.35 kDa) and dextran3000 (3 kDa). *In vivo* perfusion loop studies in TG mice revealed reversed net water secretion and reduced lumenal flux of different molecular probes (bovine serum albumin, alexa350, and dextran3000). Cell-imaging and immunoblotting of low-density, detergent-insoluble membrane fractions confirmed that tight junction proteins (occludin, claudin-1 and zona occludens-1) are internalized through an NF-κB-dependent pathway. Taken together, these data suggest that IBD-associated diarrhea results from NF-κB-mediated tight junction protein internalization and increased paracellular permeability. Thus, reduction of epithelial NF-κB activation in IBD may repair defects in epithelial barrier function, reduce diarrhea, and limit protein (eg, serum albumin) losses. Epithelial NF-κB activation induced by mucosal T cells, therefore, actively plays a role in opening paracellular spaces to promote transmucosal fluid effux into the intestinal lumen. *(Am J Pathol 2010, 176:158–167; DOI: 10.2353/ajpath.2010.090548)*

Aberrant innate and adaptive immune responses in inflammatory bowel disease (IBD) compromise mucosal permeability.[1–3] Intact intestinal barrier function is essential for nutrient and water absorption, while preventing microbial penetration.[3–7] Barrier function is maintained through transepithelial and paracellular processes. Enterocyte apical plasma membranes and intercellular tight junctions (TJs) provide the first barrier.[8] Remodeling and internalization of TJs play an important role in the regulation of intestinal barrier permeability.[9–13] Patients with IBD suffer from the consequences of barrier dysfunction including dehydration, as well as protein-losing enteropathy. In IBD, the use of lumenal probes suggests that mucosal permeability is increased.[14] In addition, enteric loss of water, albumin, and α_1-antitrysin indicate that mucosal inflammation increases efferent movement of macromolecules into the lumen.[15–17]

The pro-inflammatory transcription factor nuclear factor (NF-κB) is a central regulator of host defense responses in epithelial cells.[1] Epithelial NF-κB activation occurs in response to enteric pathogens (enteropathogenic *Escherichia coli*, *Salmonella*, etc), toll-like receptor signaling, and cytokines such as interleukin-6 and tumor necrosis factor (TNF).[18] Additionally, TNF and interferon-γ induction of NF-κB compromise barrier integrity of

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cultured cells including increased TJ permeability and altered occludin expression.[8,19,20] In IBD patients, NF-κB activation localizes to crypts as opposed to villus or plateau regions.[21] Previous *in vitro* data suggested that epithelial NF-κB activation increased proliferation while inhibiting apoptosis.[22,23] Furthermore, another *in vitro* study using the NF-κB inhibitor curcumin indicated that barrier function and subcellular localization of zona occludens (ZO)-1 is dependent on NF-κB following TNF-α administration.[24] Interestingly, results in epithelial-specific IKK knockout mice showed that colitis-induced cell proliferation was unaffected while crypt cell apoptosis increased in the absence of NF-κB signaling.[25] These studies highlight the importance of using site-specific models of NF-κB-deficient signaling to understand NF-κB biology in the intestine.

To examine *in vivo* NF-κB function in epithelial permeability, a NF-κB super repressor was expressed under the control of the enterocyte-specific fatty acid binding protein (FABP)-promoter. $FABP_{4x}$-rtTA mice were generated by Gordon and colleagues for restricting doxycycline-inducible transgenes to small bowel and proximal colonic epithelia.[26] The NF-κB super repressor construct was used previously, but not under the control of a "Tet-On" system.[27] Here, we generated TetOIκBα mutant (TetO- $I\kappa B\alpha_m$) mice crossed to $FABP_{4x}$-rtTA mice to examine epithelial NF-κB signaling in T cell-induced diarrhea. T cell activation was induced via i.p. injection of a T cell receptor cross-linking monoclonal antibody (mAb) specific for the CD3ε subunit. Anti-CD3 has been used in multiple *in vivo* studies to examine T cell activation on epithelial apoptosis, intestinal permeability, and fluid loss.[3,14,28–30]

Data from anti-CD3-treated wild-type and TNF receptor-1 knockout mice indicate that T cell-induced TNF reduces sodium and water absorption by epithelial cells, in part, by inhibiting epithelial Na/K ATPase.[14] Further studies reveal that epithelial myosin light chain kinase (MLCK) activation contributes to T cell-induced diarrhea by reducing barrier function.[28] Analyses of human tissue confirms that epithelial Na/K ATPase activity, reduced sodium and water absorption, and elevated myosin light chain phosphorylation, are consistent features of IBD compared with normal tissue.[3,14] Data from the current study suggest that T cell-induced NF-κB activation opens paracellular spaces and enhances transmucosal fluid movement by altering TJ protein composition. Our findings support the view that diarrhea resulting from NF-κB-mediated changes of epithelial TJs is important for mucosal host defense responses.

Materials and Methods

Generation of Super Repressor Mutant IκBα Transgenic Mice

To prevent signal-induced degradation, point mutations at sites of ubiquitination were introduced into the human IκBα cDNA clone MAD3 to generate the NF-κB super repressor as described.[27] The super repressor was cloned into the pTRE2 Vector (Clontech)[31] and a 2.33 kb Sap I-Put I fragment was transfected into Hela "turn-on" cells (Clontech). mIκBα protein was induced by doxycycline in a dose-dependent manner (data not shown). The mIκBα fragment was microinjected into C57/B6 fertilized eggs to generate transgenic mice.[26] Screening was performed by PCR from tail DNA, (5′-CGCCTGGAGACGCC-3′ and 5′-CATTCTAAACAACACCCTG-3′). Double transgenic mice were created by crossing pTRE2-mIκBα founders to $FABP_{4x}$-rtTA mice generated by Gordon and colleagues.[26] Transgenic protein expression was induced with 14 days of doxycycline (2 mg/ml) in the drinking water[26] as detected by Western Blotting. Mice were injected with TNF and analyzed after 1 hour by electromobility shift analysis. The pattern of protein expression was determined by crossing $FABP_{4x}$-rtTA mice with Tet-O-lacZ mice (Jackson Laboratory). Immunohistochemistry staining of β-gal was performed as described.[32]

All animal experiments were performed in accordance with National Institutes of Health guidelines under protocols approved by the Northwestern University Institutional Animal Care and Use Committee.

Electromobility Shift Assay Analysis

Electromobility shift assay analyses were performed as previously described.[33] Briefly, 50 to 100 mg of mucosa tissue was added 4 ml of buffer A (150 mmol/L NaCl, 10 mmol/L HEPES [pH 7.9], 0.6% [v/v] NP-40, 0.2 mol/L EDTA, 0.1 mol/L phenylmethylsulfonyl fluoride) and then mechanically homogenized. The homogenate was transferred to a 15-ml Falcon tube and centrifuged at 850 × *g* in a tabletop centrifuge for 30 seconds to remove cellular debris. The supernatant was then transferred to a 50-ml Falcon tube and incubated on ice for 5 minutes before being centrifuged for 10 minutes at 3500 × *g*. Supernatant was collected as a cytoplasmic extract. The pellet was resuspended in 300 μl of buffer B (sterile water, 25% [v/v] glycerol, 20 mmol/L HEPES [pH 7.9], 5 mol/L NaCl, 1 mol/L $MgCl_2$, 0.2 mol/L EDTA,0.1 mol/L phenylmethylsulfonyl fluoride, 1 mol/L dithiothreitol, 10 mg of benzamidine per ml, 1 mg of pepstatin per ml, 1 mg of leupeptin per ml, 1 mg of aprotinin per ml) and incubated on ice for 30 minutes. Following centrifugation at 14,000 rpm in an Eppendorf microcentrifuge for 2 minutes, the supernatant was collected as the nuclear extract and frozen at −70°C. Protein concentrations in nuclear and cytoplasmic extracts were determined by using the Bradford assay.

An oligonucleotide (5′-AGTTGA**GGGGACTTTCC**CAGGC-3′, 3.5 pmol) containing NF-κB binding site (bold) was incubated for 10 minutes at 37°C in 10 μl containing 10 μCi of [γ-^{32}P]ATP, 5 U of T4 polynucleotide kinase and 1X kinase buffer (supplied with the kinase). The labeling reaction was terminated by the addition of 100 mmol/L EDTA, after which the reaction mixture was centrifuged through a Sephadex G-25 column to remove unincorporated ^{32}P. For electromobility shift assay, nuclear protein extract (10 μg) was incubated for 30 minutes at room temperature in a final volume of 10 μl containing 0.03 pmol/L of ^{32}P-end-labeled oligonucleotide, 40 mmol/L HEPES–KOH (pH 7.8), 10%

glycerol, 1 mmol/L $MgCl_2$, 0.1 mmol/L DTT and 1 μg of poly(dI-dC). The binding reaction was terminated by the addition of electrophoresis sample buffer, and the samples were fractionated on 5% nondenaturing polyacrylamide gels in 0.5× Tris-boric acid–EDTA (TBE) buffer. The gels were then subjected to autoradiography. No NF-κB binding activity was found in samples without NF-κB probe and all NF-κB binding activity was inhibited with excess unlabeled probe (data not shown).

Laser Capture Microdissection

One cm segments of small intestine were prepared for laser capture microdissection as described.[34] Intestinal epithelial cells and lamina propria were harvested using a PixCell II LCM system (Arcturus). Around 1000 intestinal epithelial cells or lamina propria cells were harvested from each mouse for RNA isolation. Total cellular RNA was extracted from these 1000 cells (PicoPure RNA isolation kit, Arcturus) and the RNA from each sample was reconstituted in 5 μl and was reverse transcribed to cDNA without amplification. The reverse transcription (RT) was performed using High Capacity RNA-to-cDNA Kit (Applied BioSystems) following the manufacturer's protocol. The final reaction volume for RT was 20 μl and 5 μl of RT product was used to real-time PCR analysis. The expression of the TNF-α, keratin 20, and vimentin was determined by real-time quantitative PCR that used the ABI 7700 sequence detector system (PerkinElmer, Foster City, CA) and a TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA). Primers and probes were selected on the basis of nucleotide sequences downloaded from the National Center for Biotechnology Information data bank and designed by Primer Express 1.0 software (Applied Biosystems). The TaqMan probe consists of an oligonucleotide with a 5′-reporter dye (carboxyfluorescein) and a 3′-quencher dye (carboxytetramethylrhodamine). The sequences for the primers and probes were listed in Table 1.

The reaction mix was prepared according to the manufacturer's protocol, and it contains 1× PCR buffer, 200 μmol/L deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and 400 μmol/L deoxyuridine triphosphate, 5.5 mmol/L $MgCl_2$, 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems), 0.5 U of Amp-Erase (Applied Biosystems) uracil-*N*-glycocylase, 200 nmol/L of each primer, and 100 nmol/L probe. The thermal cycling conditions included 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute for denaturing and annealing/extension, respectively. Relative quantitative gene expression was calculated as follows. First, the threshold cycle (Ct) value for each target gene was determined and corrected by subtracting the Ct for glyceraldehyde-3-phosphate dehydrogenase (ΔCt) of each sample assayed (the Ct value represents the cycle when the sequence detection application begins to detect the increase in signal associated with an exponential growth of PCR product). Second, untreated controls were chosen as the reference samples, and the ΔCt for all treated experimental samples were subtracted from the ΔCt for the control samples ($\Delta\Delta$Ct). Finally, treated target gene mRNA abundance relative to control was calculated by the formula $2^{-(\Delta\Delta Ct)}$. All assays were performed in duplicate.[35]

Enteropooling

Intestinal fluid accumulation was determined by sacrificing mice after i.p. injection of 0.2 mg anti-CD3 (2C11) mAb or control hamster mAb (UC8-1B9). Diarrheogenic activity was quantified with a technique adapted from the "enteropooling" method used to assess accumulation of fluid into the small intestine.[14] Mice were fasted overnight, but allowed to drink water *ad libitum*. Mice were then sacrificed at the indicated time.

Paracellular Permeability, Ussing Chambers

Mucosa to serosal unidirectional permeability and ion flux was measured in modified Ussing chambers (Physiological Instruments, Inc.) from jejunum and ileum as described.[14] To assess paracellular permeability, selective permeability markers of 350, 3000, and 10,000 molecular weight (mw) fluorophores were used. Equimolar amounts (20 μmol/L) of Alexa (350) and dextran-fluorescein (3000) were added to mucosal reservoir and samples were taken every 20 minutes from serosal chambers. Markers were validated with radioactively labeled inulin (5000) and mannitol, (182) as published.[4,14,36,37] To correct for differences in probe concentration in the source bath we used non-labeled probes on the serosal side during equilibration. The chambers were connected to dual channel voltage/current clamps (VCC MC2, Physiological Instruments) with a computer interface allowing for real time data acquisition and analysis (Acquire & Analyze software, Physiological Instruments).

In Vivo *Measurement of Plasma to Lumen Permeability*

Similar to described methodology,[28] mice were injected intravenously with 250 μl of different molecular weight macromolecular probes (Alexa 0.35 kDa, Dextran 3 kDa, and bovine serum albumin 66 kDa; Invitrogen). The ab-

Table 1. Primers and Probes

Genes	Sense	Antisense	TaqMan probe (FAM)
TNF	5′-GGCTGCCCCGACTACG-3′	5′-CTGAAAGAGGACCATACTCTATCGTT-3′	5′-CCTCACCCACACCGTCAGCCG-3′
Keratin 20	5′-TGATGTCGTGTCCAAGTGAATG-3′	5′-CTCTGCCATACAGCTGCAGG-3′	5′-CACTGAAGTCCTTGCCAGCCTGAGC-3′
Vimentin	5′-GCATGTCCAGATCGATGTGG-3′	5′-ATACTGCTGGCGCACATCAC-3′	5′-CAAGCCTGACCTCACTGCTGCCCT-3′

domen was opened by a midline incision, and a 5-cm loop of jejunum was cannulated at the proximal and distal ends with 0.76-mm internal diameter polyethylene tubing. Flushing solution (140 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4), warmed to 37°C, and perfused through the jejunal loop at 1 ml/min for 10 minutes using a peristaltic pump (Bio-Rad Laboratories). Next, 5 ml of test solution (50 mmol/L NaCl, 5 mmol/L HEPES, 2 mmol/L sodium ferrocyanide, 2.5 mmol/L KCl, 20 mmol/L glucose, pH 7.4) was perfused in a recirculating manner at 1 ml/min for 3 hours, beginning 90 minutes after anti-CD3 or vehicle treatment. The abdominal cavity was covered with moistened gauze and body temperature maintained at 37°C. One-milliliter aliquots of test solution were removed at the beginning and end of the perfusion. The perfused jejunal segment length was then measured. Ferrocyanide concentration in the perfusate was measured using a colorimetric assay.[28] The concentration of Alexa-conjugated macromolecular probes was measured with a microplate reader (Synergy HT; Bio-Tek). Probe clearance was calculated as $C\text{probe} = (CiVi - CfV_f)/(C\text{avg}TL)$; water flux was calculated as $(Vi - Vf)/(\text{TL})$. C_i is the initial probe concentration; C_f is the final probe concentration; V_i is the initial perfusate volume; V_f was calculated as $V_i([\text{ferrocyanide}]_i/[\text{ferrocyanide}]_f)$; C_{avg} was calculated as $(C_i - C_f)/\ln(C_i/C_f)$; T represents hours of perfusion; and L represents the length of the perfused jejunum (cm).

Intestinal Epithelial Cell Isolation

Intestinal epithelial cells were purified similar to described methodology.[38] Lengthwise sections of intestine were washed in 4°C Ca^{2+}- and Mg^{+}-free Hanks Balanced Salt Solution. The tissue was transferred to CMF-HBSS containing 10 mmol/L dithiothreitol and 50 nmol/L calyculin A (Calbiochem) for 30 minutes, 4°C. Tubes were shaken and then tissue was transferred to fresh tubes containing Ca^{2+}- and Mg^{+}-free Hanks Balanced Salt Solution with 1 mmol/L EDTA and 50 nmol/L calyculin A, 4°C for 1 hour. Epithelial cells were then dislodged by vigorous shaking. Large pieces of tissue were discarded. Epithelial cells were harvested by centrifugation at 500 × *g* for 10 minutes. The purity of cells isolated using this protocol, was determined by immunohistochemistry. More than 97% of the isolated cells were intestinal epithelial cells that were positive for cytokeratin.[38] Studies in our lab using flow cytometry confirmed that less than 3% of contaminating cells stained for the pan-leukocyte marker, CD45 (data not shown).

Subcellular Fractionation

Membrane and cytosol proteins from isolated epithelium were extracted as described.[39] Tissue samples were homogenized in 5 ml of 20 mmol/L Tris-HCl, ph7.4, 2 mmol/L EDTA, 10 mmol/L EGTA, 0.25 mol/L sucrose, and protease inhibitor cocktail (Sigma), and centrifuged at 200 × *g* at 4°C for 10 minutes. The supernatant was centrifuged at 10^3 × g at 4°C for 10 minutes followed by ultracentrifugation at 10^5 × *g* at 4°C for 1 hour. The resulting supernatant was enriched for cytosolic protein. The pellet containing membrane proteins was homogenized with 0.1% Triton X-100, sonicated, mixed vigorously, and incubated for 1 hour on ice followed by ultracentrifugation at 10^5 × *g* at 4°C for 1 hour. Centricon filtration (Millipore) was used to concentrate samples.

Sucrose Gradient Fractionation

Isolated intestinal epithelial cells were lysed in 2.5 ml of Tris-buffered saline containing 1% Triton X-100. Two milliliters of lysate was combined with 2 ml of 80% sucrose (w/v) in Tris-buffered saline and layered over a 1-ml cushion of 50% sucrose. Sucrose layers were added over the lysate as follows: 2 ml of 35%, 2 ml of 30%, 3 ml of 15%, and 1 ml of 5% sucrose. Sucrose gradients were centrifuged at 280,000 × *g* for 18 hours, at 4°C. 0.5 ml fractions were collected. Protein concentration was measured by DC or BCA Protein Assay kits (Bio-Rad Laboratories).

Western Blot Analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incubated with primary antibodies specific for IκBα, ZO-1, occludin, claudin5, junctional adhesion molecule (JAM)-A (Santa Cruz), and claudin-1 (Cell Signaling). As per manufacturer's specifications, the anti-claudin-1 antibody may cross-react with claudin-2. Molecular weight (Kaleidoscope Prestained Standards, Bio-Rad, Cat#161-0324) markers were used to compare the position of the tested bands to known standards. Thus, the bands detected were confirmed to be the correct size according to the manufacturer's protocol. Bands appearing on films were quantified by densitometry using Eagle Eye (Strategene). Fold-induction was determined based on values from control mice.

The Student's *t*-test was used to evaluate differences between the control and experimental groups. When more than two groups were compared, a one-way analysis of variance followed by a post hoc Tukey's *t*-test was used to evaluate differences. Differences were considered significant with P values of less than 0.05.

Immunohistochemistry

Immunostaining was performed as described.[40] Five-micron frozen sections were collected on coated slides, fixed in 1% paraformaldehyde, washed with PBS, and blocked with 1% normal goat serum in PBS. After incubation with affinity-purified rabbit anti-occludin (5 μg/ml; Zymed Laboratories), sections were washed and incubated with Alexa 594-conjugated goat anti-rabbit antisera, Alexa 488-conjugated phalloidin, and Hoechst 33342 (Invitrogen). Sections were imaged using a Leica DMLB epifluorescence microscope equipped with an 88000 filter set (Chroma Technology Corp.) and a Coolsnap HQ camera (Roper Scientific Inc.) controlled by MetaMorph 6 (Universal Imaging Corp.).

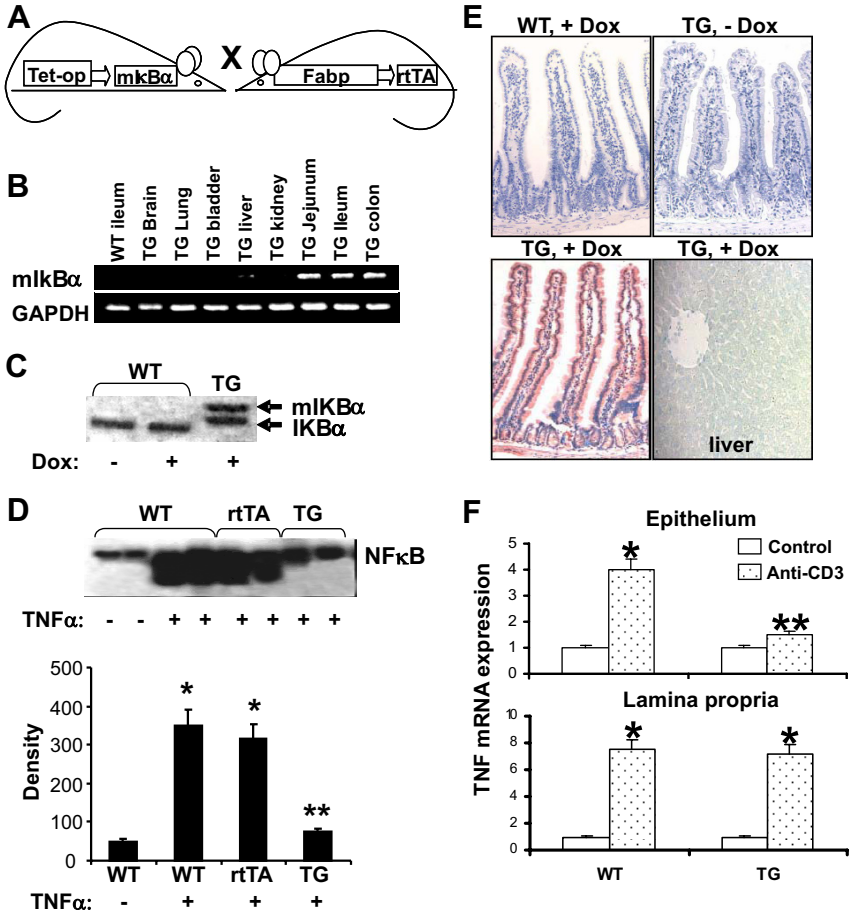

Figure 1. Generation of inducible intestinal epithelial cell-specific mutant IκBα transgenic mice (mIκBα). **A:** Double transgenic (TG) mice with inducible intestinal epithelial cell-specific mIκBα were created as described in the *Materials and Methods*. Transgenic expression was induced with a 14-day doxycycline (Dox) feeding (2 mg/ml in drinking water). **B:** Expression levels of mIκBα mRNA in different tissues of transgenic mice after doxycycline-induction were tested by RT-PCR. Results show mIκBα expression in jejunum, ileum, and colon tissue in TG mice. **C:** Crypt epithelial cells were isolated and protein probed with polyclonal anti-IκBα. TG mice expressed a higher molecular weight mIκBα in small intestine crypt epithelial cells. **D:** After activation of NF-κB by TNF injection (i.p. 5 μg/mice), nuclear extracts from intestine epithelial cells of nontransgenic wild-type (WT), single transgenic (rtTA), or double transgenic (TG) mice, were analyzed by electromobility shift assay. No NF-κB binding activity was found in samples without NF-κB probe and all NF-κB binding activity was inhibited with excess unlabeled probe. Densitometry evaluation of the blots indicated that TNF-induced NF-κB nuclear DNA binding activity in small intestine of transgenic mice was significantly reduced. $^{*}P < 0.05$ compared with wild-type group; $^{**}P < 0.05$ compared with TNF treatment group. $N = 4$. **E:** Immunohistochemical evidence for limited microchimerism in TG mice. $FABP_{4x}$-rtTA expression was examined by crossing TG mice with Tet-O-lacZ transgenic mice. Small intestine (ileum) and liver from mice were examined without (−Dox) and with Dox feeding × 2 weeks (+Dox). Tissues were stained for β-galactosidase (red) indicating positive staining throughout crypt and villus structures of nearly all crypts with rare hepatic expression detected. **F:** Specificity of NF-κB blockade was tested by examination of T cell-induced TNF mRNA levels in epithelial and lamina propria (LP) cells. mRNA from epithelial and LP cells were captured by using laser capture microdissection 3 hours after injection with control or anti-CD3 mAb. TNF mRNA induction was selectively inhibited in epithelial but not LP cells captured from Dox-fed TG but not wild-type mice. $^{*}P < 0.05$, anti-CD treated wild-type versus wild-type; $^{**}P < 0.05$, TG versus wild-type injected with anti-CD3.

Results

To address the role of epithelial NF-κB in immune-mediated diarrhea, double transgenic (TG) mice were generated. TetO-mIκBα mice were generated by cloning the NF-κB super repressor[27] into a *Tet*-On expression vector. Founders were bred to $FABP_{4x}$-rtTA mice to restrict expression of mIκBα to the distal small intestine and colon (Figure 1, A and B). mRNA expression of the transgene was confirmed by RT-PCR from a variety of tissues and found increased after 14 days of doxycycline feeding. Transgene mRNA expression was largely restricted to jejunum, ileum, and colon with faint expression detected in the liver.

To examine intestinal tissue for epithelial cell transgene protein expression, epithelial cells were isolated and protein extracts were probed for IκBα, as originally described.[27] The mIκBα protein was detected as a slower migrating band compared with endogenous IκBα in wild-type mice (Figure 1C). Electromobility shift assay was performed on nuclear extracts from small bowel epithelial cells isolated from wild-type, $FABP_{4x}$-rtTA and double transgenic mice after TNF injection. As expected, NF-κB activation was evident in wild-type and FABP-rtTA control mice. However, levels of NF-κB activation in epithelial cells from TG mice treated with TNF were equivalent to levels detected in unstimulated wild-type mice (Figure 1D).

To examine TG mice for microchimerism, double transgenic mice were crossed to TetO-lacZ "reporter" mice. Immunostaining for β-galactosidase revealed lacZ expression throughout crypt and villus structures in doxycycline-fed mice, but not in control TG mice. Rare (<0.1%) crypts in the jejunum and ileum failed to exhibit *lacZ* expression. Low levels of *lacZ* staining in the liver were consistent with mRNA data (Figure 1E).

Cellular populations within the intestine were examined by laser capture microdissection for site-specific functional effects by NF-κB. T cell induced expression of NF-κB-dependent TNF mRNA was evaluated in epithelial and lamina propria (LP) cells. Mucosal T cell activation was induced with systemic anti-CD3 mAb.[14] The purity for each fraction was validated by assessing mRNA levels of cytokeratin for epithelial cells, and vimentin for mesenchymal cells or macrophages in the LP (See Supplemental Figure S1 at *http://ajp.amjpathol.org*).[34] Figure 1F shows that T cell activation causes robust TNF mRNA

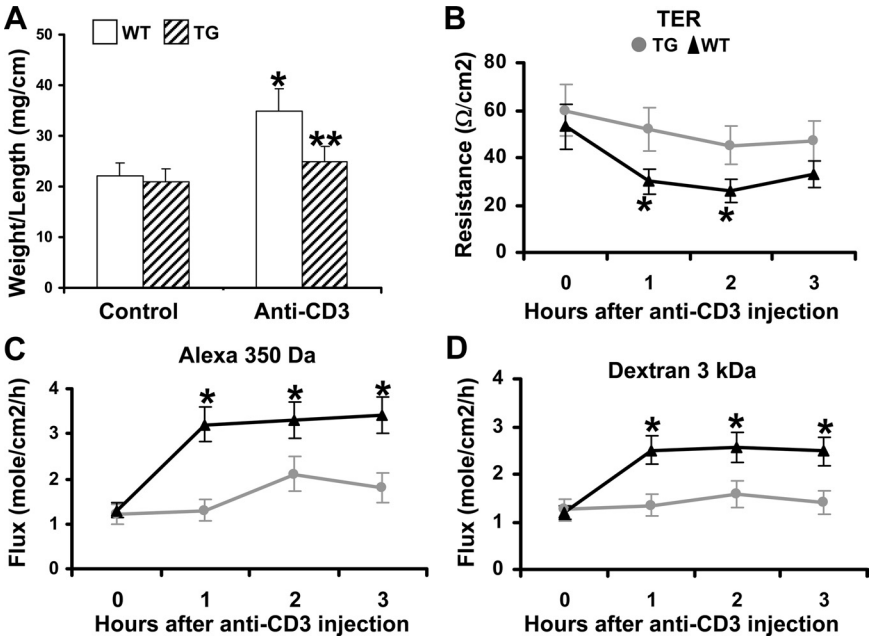

Figure 2. Epithelial NF-κB mediates T cell-induced diarrhea and increased intestinal paracellular permeability. Control and transgenic (TG) mice were injected with 0.2 mg anti-CD3 mAb. **A:** Loop wt/L ratios were measured 3 hours after anti-CD3 injection. Whereas T cell stimulation increased the wt/L ratios of wild-type (WT) SB segments by 55%, diarrhea was abrogated in TG mice. $^{*}P < 0.05$, anti-CD treated wild-type versus wild-type; $^{**}P < 0.05$, TG versus wild-type injected with anti-CD3. **B:** Deficient NF-κB signaling (TG mice) reduced T cell-induced transepithelial resistance (TER) changes measured in SB tissue from wild-type mice (see *Materials and Methods*) ($^{*}P < 0.05$ TER in Tg versus wild-type mice). **C, D:** Deficient NF-κB signaling (TG mice) reduced T cell-induced transmucosal fluxes of Alexa (350 Da) (**C**) and Dextran (3 kDa) (**D**) measured in modified Ussing chambers (see *Materials and Methods*). Values are means ± SE for four mice (repeated three times). $^{*}P < 0.05$ TG (filled circles) as compared with wild-type (filled triangles) injected with anti-CD3.

expression in both epithelial and LP compartments of wild-type mice. However, TNF mRNA expression was dramatically inhibited in epithelial cells isolated from doxycycline-fed TG mice. Therefore, TG mice express a functionally competent NF-κB super repressor in SB epithelial cells.

The Role of Epithelial NF-κB in T Cell-Induced Diarrhea

To examine the role of NF-κB *in vivo* in T cell-induced diarrhea, wild-type and TG mice were injected with anti-CD3. Within one to three hours after anti-CD3 injection, mice developed watery stools and rectal prolapse. Additional examination of the small intestine revealed fluid-filled loops of duodenum, jejunum, and ileum after 3 hours. To quantify the volume of fluid produced (enteropooling), random segments of SB were isolated to determine the wt/L ratio. The T cell stimulated wt/L ratios of SB segments increased 55% in wild-type mice after 3 hours (Figure 2A), but only 13% in TG mice ($P < 0.05$), suggesting that epithelial expression of an NF-κB super repressor attenuates T cell-induced diarrhea.

As studies indicate that T cell activation reduces SB mucosal resistance, we measured transepithelial resistance (TER) in wild-type and TG mice using modified (murine) Ussing chambers. Figure 2B shows that TER was decreased 56% in wild-type mice, whereas there was 80% less change 2 hours following T cell activation in TG mice. To address whether reductions in TER are related to enhanced paracellular flux, we measured the paracellular flux of Alexa (0.35 kDa) and dextran (3 kDa; Figure 2, C and D). T cell activation increased the flux of Alexa threefold and dextran 2.5-fold, whereas Alexa and Detran flux decreased by 50% and 80% respectively, in TG mice indicating that epithelial NF-κB activation increases intestinal paracellular permeability.

Role of Epithelial NF-κB in T Cell-Induced Water Movement and Macromolecular Flux

In wild-type mice, T cell activation changes net water absorption into net water secretion. In contrast, T cell-induced water movement was reversed in TG mice (Figure 3A). The increased flux of macromolecular probes into lumenal perfusates indicates that intestinal paracellular permeability is increased. The movement of differently sized probes was analyzed to accurately reflect the movement of biologically active macromolecules.[14,36,41] T cell activation increased movement of Alexa (0.35 kDa), and Dextran (3 kDa) from blood vessels into the lumenal perfusate (Figure 3, B and C). In all cases, enhanced probe flux was blocked by epithelial expression of mIκBα protein. Transmucosal movement of BSA (66 kDa) into the lumen increased three-fold compared with control (Figure 3D). Finally, *in vivo* inhibition of NF-κB reduced BSA movement by 60%. Thus, NF-κB mediated T cell-induced paracellular permeability of both relatively small (0.35 kDa) and large (66 kDa) molecules.

Effect of Epithelial NF-κB Activation on the Redistribution of TJ Protein

In epithelial cells, TJ proteins are partitioned into membrane-associated and cytosolic compartments. Previous results suggested that exposure to inflammatory cytokines such as TNF, increases paracellular permeability and induces TJ protein redistribution.[6,9,42,43] Changes in TJ protein localization were assessed by immunofluorescent staining of SB tissue from wild-type and TG mice before and after T cell activation. Occludin was dramatically rearranged from the extracellular membrane to the cytoplasm in wild-type, but not in TG epithelial cells after T cell stimulation (Figure 4A). Biochemically occludin

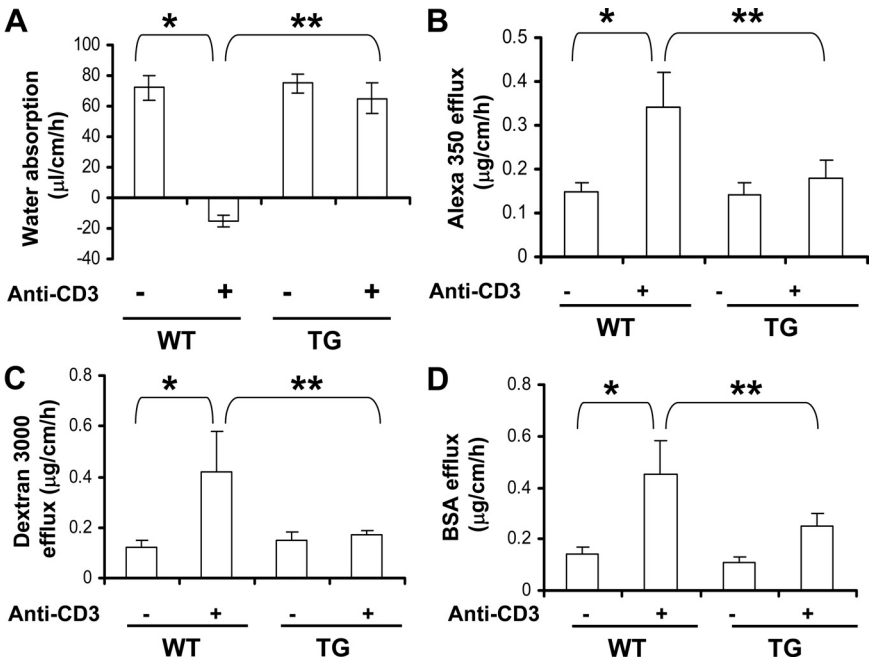

Figure 3. NF-κB regulates T cell-induced mucosal permeability changes *in vivo*. An *in vivo* perfusion system was developed to measure movement of water and paracellular flux of macomolecules in a segment of distal small bowel with intact neurovascular supply.[28] Water movement was detected by changes in ferrocyanide in the perfusate while paracellular fluxes were measured as changes in perfusate concentration of intravenously-injected fluorescently-tagged probes 3 hours after anti-CD3 injection. **A:** T cell activation induced net water movement into loops of wild-type (WT) (net secretion) but not TG mice (net absorption). **B–D:** Anti-CD3-induced the flux of molecular probes Alexa 0.35 kDa (**B**), and dextran 3 kDa (**C**), and BSA 66 kDa (**D**) from the bloodstream to intestinal perfusate lumen in wild-type but not TG mice. Values are means ± SE for four mice (repeated three times). $^{*}P < 0.05$, anti-CD treated wild-type versus wild-type; $^{**}P < 0.05$, TG versus wild-type injected with anti-CD3.

redistribution was analyzed in low-density, detergent-insoluble glycolipid- and cholesterol-rich membrane fractions from wild-type and TG mice following anti-CD3 treatment (Figure 4, B and C). Following ultracentrifugation, fractions 7 through 10 represent cholesterol-rich membrane fractions. T cell activation caused a significant loss of membrane-associated occludin in wild-type, but not in TG mice. Total cellular protein levels of occludin were unchanged in all mice, as indicated by immunoblotting of the soluble fractions (fractions 24 and 25, Figure 4C). These data show that occludin redistribution biochemically correlate with morphological observations seen by immunofluorescent analysis of stained tissue. Claudins are essential components of TJs for establishing the paracellular barrier.[44] T cell activation also induced claudin-1 redistribution in wild-type but not in TG mice (Figure 4D and see Supplemental Figure S2 at *http://ajp.amjpathol.org*). It is possible that lower bands identified represent claudin-2 due to sequence homology with claudin-1. Taken together, epithelial NF-κB mediates T cell-induced occludin and claudin-1 internalization.

To further examine epithelial NF-κB induced changes in TJ protein redistribution *in vivo*, levels of ZO-1 were measured in membrane and cytosolic fractions of epithelial cells isolated from SB of wild-type and TG mice.[45] T cell activation induced ZO-1 redistribution from membrane to cytosol fractions (See Supplemental Figure S3 at *http://ajp.amjpathol.org*).[39,45] Although the change in membrane TJ protein was diluted by the larger membrane pool size, effects of T cell activation on cytosolic levels were more apparent as amounts were about 60% greater than control levels. Data obtained from TG mice suggest that epithelial NF-κB inhibition prevents T cell activation-induced "internalization" of ZO-1. These findings confirm that changes in T cell-induced TJ protein redistribution are dependent on epithelial NF-κB signaling.

Discussion

IBD is associated with mucosal T cell activation and diarrhea. Our previous studies show that T cell activation induces lumenal fluid accumulation by increasing mucosal permeability and reducing epithelial Na/K-ATPase activity leading to decreased intestinal sodium and water absorption.[14] However, the initial step causing intestinal fluid accumulation was not delineated. To explore this question further, we focused on the role of epithelial NF-κB in T cell-induced the diarrhea. Our results show that NF-κB suppression inhibited T cell-induced intestinal permeability measured by blunted changes in wt/L ratios, as well as blocked reduction of TER and abrogated flux of macromolecules measured in Ussing chambers. Perfusion loop studies revealed that NF-κB suppression blocked T cell-induced changes in paracellular permeability *in vivo*. Importantly, changes in permeability were associated with a net movement of water and solute into the bowel lumen. These data not only correlated with clinical signs of diarrhea but also help explain the initial fluid accumulation observed in previous studies.[14,39] Thus, taken together with prior data, the findings presented here suggest that NF-κB activation opens paracellular spaces and promotes movement of fluid into bowel lumen.

The most characterized and studied NF-κB inhibitor is IκBα. This protein binds avidly to the p65 (RelA) subunit of NF-κB. NF-κB activation requires the removal of IκBα from NF-κB. Phosphorylation of IκBα by an activated IKK complex is a necessary step for inducible IκBα degradation and NF-κB activation.[18] Mutation of IκBα at key sites impairs phosphorylation and prevents activation of NF-κB.[18] Mutation of IκBα at serine-32 and serine-36 effectively blocks gene expression of interleukin-1, interleukin-8, inducible nitric oxide synthase, and cyclooxygenase-2.[46,47]

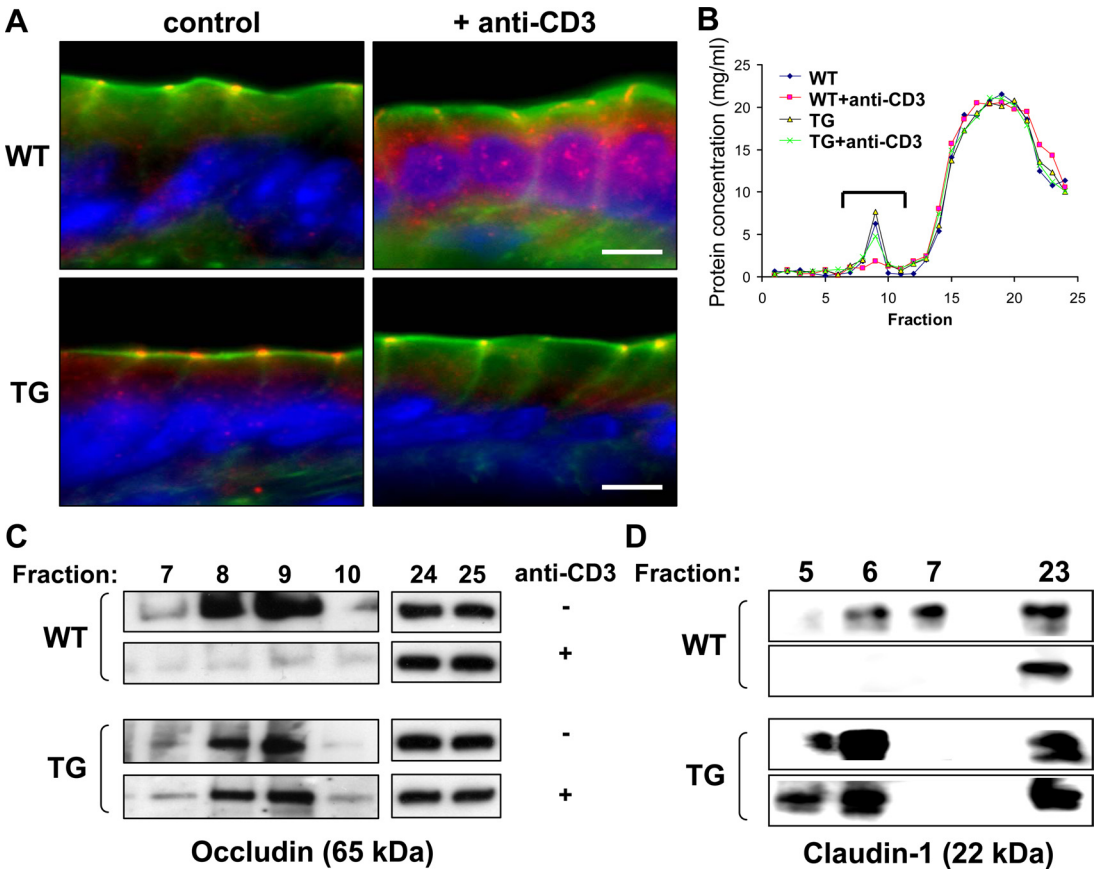

Figure 4. Effect of epithelial NF-κB deficiency on T cell-induced changes of tight junction protein localization. **A:** Immunofluorescent localization of occludin. The nuclei were stained with Hoechst 33342 in blue. Occludin (Red) was localized to TJs (F-actin, Green) in control tissue, but after anti-CD3 treatment, occludin was internalized into intracellular vesicles. In contrast, anti-CD3 treatment in TG mice failed to alter occludin internalization as occludin remained confined to tight junction without evidence of internalization. Scale bar = 5 μm. **B:** Detergent-insoluble subcellular fractions of small bowel epithelial cells were isolated on isopycnic gradients. Protein concentrations were determined for each gradient fraction prepared from control and anti-CD3-treated wild-type (WT) and TG mice. A protein peak associated with the low-density, detergent-insoluble membrane fraction was present (bracket). These fractions were assessed by immunoblot. **C:** The immunoblot for occludin in low-density, detergent-insoluble membrane fractions (fraction 8–9) showed loss of occludin from the detergent-insoluble membrane fraction after anti-CD3 treatment in wild-type mice. In contrast, mutation of IkBα in TG mice inhibited anti-CD3-induced occludin internalization. The immunoblot for occludin in high-density load regions (fraction 24–25) showed no significant difference between different groups. **D:** The immunoblot for claudin-1 in low-density, detergent-insoluble membrane fractions (fraction 5–7) showed loss of claudin-1 from the detergent-insoluble membrane fraction after anti-CD3 treatment in wild-type mice. In contrast, mutation of IkBα in TG mice inhibited anti-CD3-induced claudin-1 internalization. The immunoblot for claudin-1 in high-density load regions (fraction 23) showed no significant difference between different groups.

In the model used here, several additional point mutations were introduced into the human IκBα cDNA.[27] Along with mutation of Ser32 and Ser36 of IκBα, sites of ubiquitination, tyrosines for T-cell receptor signaling, and the C-terminal PEST-region of IκBα were mutated. These mutations function to enhance the stability of the mutant IκBα as evidenced by reductions in NF-κB nuclear binding and expression of NF-κB target genes (Figure 1, D and F). Interestingly expression of mutant IκBα does not completely abrogate DNA binding by active nuclear NF-κB (Figure 1D), this may be due to microchimerism and expression of wild-type IκBα in induced TG mice as suggested by data in Figure 1C.

Our study extends knowledge about the role of NF-κB in inflammation-induced transmucosal fluid movement. Evidence presented here supports the hypothesis that T cell-induced epithelial NF-κB activation alters epithelial permeability by restructuring TJ organization. Opening epithelial paracellular spaces allows fluid movement into the intestinal lumen (Figure 3). We also found that T cell activation increases lumenal movement of relatively small (Alexa 350, .35 kDa) and large (BSA, 66 kDa; Dextran 3000, 3 kDa) macromolecules. In studies submitted elsewhere, we detected enhanced lumenal movement of antibody (eg, sIgA, IgG1, IgG2) also induced by T cell activation (data not shown). Although not directly addressed here, it is possible that inducing rapid fluid entry into the lumen dilutes invading bacteria and hydrostatically opposes movement of organisms into intestinal crypts. Thus, we speculate that NF-κB-mediated effects on epithelial TJ proteins may contribute to host defenses by allowing the movement of fluid and biologically active macromolecules through open paracellular gates into the lumen.

Our previous data suggested that T cell activation induced diarrhea through a cytokine-mediated pathway dominated by TNF.[14,28] TNF levels are significantly increased in IBD patients.[48–50] Clinical studies show that anti-TNF is an effective agent in the treatment of IBD.[50,51] TNF also increases epithelial TJ permeability in different cell lines with conflicting results.[52,53] Pharmacological NF-κB inhibitors prevented TNF-induced increases in

paracellular permeability.[8] Another report found that some NF-κB inhibition failed to prevent barrier defects induced by TNF and interferon-γ.[43] The discrepancy is likely explained by the fact that Ma and colleagues[8] used a relatively-undifferentiated Caco-2 cell line, while Wang et al[43] used the well-differentiated Caco-2BBe subclone.[54] Given that both groups demonstrated TNF-induced transcriptional and enzymatic activation of MLCK, the conflicting data are likely explained by the presence of both NF-κB and AP-1 sites in the human MLCK promoter and distinct patterns of transcription factor activation by TNF during epithelial differentiation.[55] Cell culture models require 6 to 24 hours of TNF exposure to cause barrier loss, while anti-CD3 treatment induces barrier loss within 2 hours *in vivo*.[28,42,43] In contrast, MLCK transcripts are not increased until 4 hours after *in vivo* TNF exposure.[55] Thus, while MLCK plays critical roles during *in vitro* and *in vivo* TNF-mediated barrier loss, increased MLCK transcription is likely not essential for the process observed in this study. It is likely that NF-κB regulates MLCK via non-genomic activation of MLCK or other targets.

NF-κB activation plays an integral role in multiple aspects of epithelial responses to pathogen invasion. Gene array studies examining the effects of *Listeria monocytogenes* infection of epithelial cells found several NF-κB-dependent genes induced.[56] For example, genes encoding chemokines (eg, Gro), cell cycle proteins (cMyc), and inhibitors of apoptosis (IAP-2) were induced, as well as genes involved in actin-cytoskeleton dynamics.[57] These studies highlight the complexity of studying NF-κB in intestinal epithelium. Studies by Karin and colleagues[25] reinforce this contention in their colitis-induced cancer model. Results of epithelial and myeloid-specific models of deficient NF-κB signaling revealed that epithelial NF-κB did not affect tissue inflammation or epithelial proliferation, but rather was associated with increased tumor incidence. By comparison, Karin and colleagues[25] found myeloid NF-κB had dramatic effects on tissue inflammation and tumor size suggesting a key role in tumor growth. The failure of epithelial NF-κB inhibition to suppress proliferation, despite *in vitro* studies to the contrary, indicates that to determine the role of molecular regulators, such as NF-κB, models that allow for *in vivo* factors to exert their effects are required.

Furthermore, colitis is detected in mice deficient in epithelial NEMO[58] and IKKβ.[59] In both models, deficient NF-κB signaling attenuated host defense responses associated with bacterial invasion and mucosal inflammation. In our model, however, the time points considered, immediately after T cell stimulation, showed NF-κB was required for the development of diarrhea. If this is part of a host defense response to pathogen invasion, then NF-κB deficiency diminishes host defense at the mucosal barrier. In mice with NEMO or IKKβ deficiencies, loss of epithelial NF-κB function reduces the production of NF-κB-dependent host defense molecules (chemokines, defensins, prostaglandins, etc) and promotes bacterial translocation and inflammation. Thus, our *in vivo* analysis suggests that NF-κB-induced effects on TJ reorganization mediate epithelial permeability changes seen in mucosal inflammation. Our data thus highlights a novel paradigm shift where an NF-κB induced compromise to epithelial barrier integrity is not entirely detrimental to the host.

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