EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

TNF- α Modulation of Intestinal Tight Junction Permeability Is Mediated by NIK/IKK- α Axis Activation of the Canonical NF- κ B Pathway

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Tumor necrosis factor (TNF)- α , a key mediator of intestinal inflammation, causes an increase in intestinal epithelial tight junction (TJ) permeability by activating myosin light chain kinase (MLCK; official name MYLK3) gene. However, the precise signaling cascades that mediate the TNF- α -induced activation of MLCK gene and increase in TJ permeability remain unclear. Our aims were to delineate the upstream signaling mechanisms that regulate the TNF- α modulation of intestinal TJ barrier function with the use of *in vitro* and *in vivo* intestinal epithelial model systems. TNF- α caused a rapid activation of both canonical and noncanonical NF- κ B pathway. NF- κ B-inducing kinase (NIK) and mitogen-activated protein kinase kinase-1 (MEKK-1) were activated in response to TNF- α . NIK mediated the TNF- α activation of inhibitory κ B kinase (IKK)- α , and MEKK1 mediated the activation of IKK complex, including IKK- β . NIK/IKK- α axis regulated the activation of both NF- κ B p50/p65 and RelB/p52 pathways. Surprisingly, the siRNA induced knockdown of NIK, but not MEKK-1, prevented the TNF- α activation of both NF- κ B p50/p65 and RelB/p52 and the increase in intestinal TJ permeability. Moreover, NIK/IKK- α /NF- κ B p50/p65 axis mediated the TNF- α -induced MLCK gene activation and the subsequent MLCK increase in intestinal TJ permeability. In conclusion, our data show that NIK/IKK- α regulates the activation of NF- κ B p50/p65 and plays an integral role in the TNF- α -induced activation of MLCK gene and increase in intestinal TJ permeability. (*Am J Pathol* 2016, 186: 1151–1165; <http://dx.doi.org/10.1016/j.ajpath.2015.12.016>)

It is well established that in intestinal permeability disorders, the defective intestinal tight junction (TJ) barrier allows paracellular permeation of luminal antigens, which can induce or propagate inflammatory response.^{1,2} Previous studies from our laboratory and others have shown that proinflammatory cytokines, including tumor necrosis factor (TNF)- α , IL-1 β , IL-6, and interferon- γ , cause an increase in intestinal TJ permeability and contribute to the inflammatory process by allowing luminal antigenic penetration.^{3–6} Conversely, anti-inflammatory cytokine IL-10 was shown to promote intestinal TJ barrier function.^{7,8} In IL-10^{−/−} mice, the development of intestinal inflammation was preceded by an increase in intestinal permeability,^{7,9} and enhancement of intestinal TJ barrier with a TJ barrier enhancing agent (AT-1001) prevented the development of intestinal inflammation.^{10,11}

TNF- α is a multifunctional proinflammatory cytokine that was shown to play a central role in intestinal inflammation

of Crohn disease (CD).^{2,12,13} Patients with CD have marked increase in TNF- α levels in their intestinal tissues, sera, and stool. Treatment with anti-TNF- α antibodies was shown to be an effective therapeutic strategy in CD.^{14,15} Previous studies have shown that TNF- α causes an increase in intestinal TJ permeability *in vitro* and *in vivo* and that the TNF- α -induced increase in intestinal permeability contributes to the development of intestinal inflammation by allowing increased antigenic penetration.^{16–19} Clinical studies have also shown that anti-TNF- α therapy leads to a rapid re-tightening of the intestinal barrier and early resolution of active CD.^{20–22}

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Previous studies have shown that NF- κ B signaling plays an important role in TNF- α modulation of intestinal epithelial TJ barrier by targeting myosin light chain kinase (MLCK; official name *MYLK3*) gene activation.^{5,23} These studies indicated that TNF- α causes a rapid activation of NF- κ B p50/p65 dimer. The inhibition of NF- κ B p50/p65 activation by pharmacologic inhibitors or siRNA silencing of p65 subunit completely inhibited the TNF- α -induced activation of MLCK gene and increase in intestinal TJ permeability.²³ The upstream signaling pathways that mediate the TNF- α modulation in NF- κ B p50/p65 activation, MLCK gene activity, or increase in intestinal epithelial TJ permeability remain unknown. Mitogen-activated protein kinase kinases (MAP3 kinases) are recruited by TNF- α receptor complex after TNF- α binding and play a crucial regulatory role in a variety of biological activities in intestinal epithelial cells.^{24–26} The MAP3 kinases mitogen-activated protein kinase kinase kinase-1 (MEKK-1) and NF- κ B-inducing kinase (NIK), are important regulators of NF- κ B pathways.^{24,25,27} Two distinct pathways were described that lead to activation of NF- κ B dimers: the canonical (or classic) and the noncanonical (or alternative) pathways.^{28–32} In the canonical pathway, TNF- α binding to the cell surface receptor leads to the TNF- α receptor complex recruitment of membrane shuttle kinases that ultimately lead to the phosphorylation and degradation of inhibitory κ B (IkB)- α and activation of NF- κ B dimer p50/p65.^{33,34} The TNF- α -induced activation of the noncanonical pathway results in the phosphorylation of p100 subunit, leading to the generation and activation of RelB/p52 dimer.^{30,35}

The role of MAP3 kinases in TNF- α modulation of intestinal TJ barrier remains unknown. Our aim was to determine the regulatory role of MAP3 kinases MEKK-1 and NIK in TNF- α -induced increase in intestinal epithelial TJ permeability, using filter-grown Caco-2 intestinal epithelial monolayers and recycling mouse intestinal perfusion as *in vitro* and *in vivo* model systems, respectively. Herein, we show for the first time that NIK mediates the TNF- α -induced activation of MLCK gene and increase in intestinal TJ permeability by regulating the activation of the canonical (NF- κ B p50/p65) pathway. Our results also show for the first time that NIK, a MAP kinase known to regulate the noncanonical pathway (NF- κ B p50/p52), regulates the activation of the canonical pathway via IkB kinase (IKK)- α -induced activation of NF- κ B p50/p65. This is in direct contrast to what we previously described with IL-1 β where the canonical pathway of NF- κ B and MLCK gene activity were regulated by MEKK1 activation of IKK- β (a part of the IKK complex).³⁶

Materials and Methods

Chemicals

Cell culture media (Dulbecco's modified Eagle's medium), trypsin, fetal bovine serum, glutamine, penicillin, streptomycin,

and phosphate-buffered saline (PBS) were purchased from GIBCO-BRL (Grand Island, NY). Anti-MEKK-1, NIK, IKK- α , IKK- β , IkB- α , MLCK, and anti- β -actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Anti-phospho-MEKK1, phospho-NIK, phospho-IKK- α / β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NF- κ B p65 and p100/p52 antibodies were purchased from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies for Western blot analysis were purchased from Invitrogen (San Francisco, CA). siRNA of MEKK-1, NIK, IKK- α , IKK- β , p65, and p100 and transfection reagents were obtained from Dharmacon (Lafayette, CO). All other chemicals were purchased from Sigma-Aldrich, VWR (West Chester, PA), or Fisher Scientific (Pittsburgh, PA).

Cell Cultures

Caco-2 cells (passage 20) were purchased from the ATCC (Rockville, MD) and maintained at 37°C in a culture medium composed of Dulbecco's modified Eagle's medium with 4.5 mg/mL glucose, 50 U/mL penicillin, 50 U/mL streptomycin, 4 mmol/L glutamine, 25 mmol/L HEPES, and 10% fetal bovine serum. The cells were kept at 37°C in a 5% CO₂ environment. Culture medium was changed every 2 days. Caco-2 cells were subcultured after partial digestion with 0.25% trypsin and 0.9 mmol/L EDTA in Ca²⁺- and Mg²⁺-free PBS.^{4,37}

Determination of Epithelial Monolayer Resistance and Paracellular Permeability

An epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) was used for measurements of the trans-epithelial electrical resistance (TER) of the filter-grown Caco-2 intestinal monolayers as previously reported.^{17,37} The effect of TNF- α on Caco-2 paracellular permeability was determined with an established paracellular marker inulin (mol. wt. = 5000 g/mol).³⁸ For determination of mucosal-to-serosal flux rates of inulin, Caco-2-plated filters with epithelial resistance of 400 to 500 $\Omega \cdot \text{cm}^2$ were used. Known concentrations of inulin (2 $\mu\text{mol/L}$) and its radio-active tracer were added to the apical solution.

Assessment of Protein Expression by Western Blot Analysis

Caco-2 monolayers were treated with 10 ng/mL TNF- α for various time periods. At the end of the experimental period, Caco-2 monolayers were immediately rinsed with ice-cold PBS, and cells were lysed with lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 500 $\mu\text{mol/L}$ NaF, 2 mmol/L EDTA, 100 $\mu\text{mol/L}$ vanadate, 100 $\mu\text{mol/L}$ phenylmethylsulfonyl fluoride, 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin A, 40 mmol/L paranitrophenyl phosphate, 1 $\mu\text{g/mL}$ aprotinin, and 1% Triton X-100) and scraped, and the cell lysates were placed in Microfuge tubes. Cell lysates were

centrifuged to yield a clear lysate. Supernatant fluid was collected, and protein measurement was performed with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Laemmli gel loading buffer was added to the lysate that contained 10 to 20 μg of protein and boiled for 7 minutes, after which time proteins were separated on SDS-PAGE gel. Proteins from the gel were transferred to the membrane (Trans-Blot Transfer Medium, Nitrocellulose Membrane; Bio-Rad Laboratories) overnight. The membrane was incubated for 2 hours in blocking solution (5% dry milk in tris-buffered saline—Tween 20 buffer). The membrane was incubated with appropriate primary antibodies in blocking solution. After being washed in tris-buffered saline—1% Tween buffer, the membrane was incubated in appropriate secondary antibodies and developed with the Santa Cruz Western Blotting Luminol Reagents (Santa Cruz Biotechnology) on the Kodak BioMax MS film (Fisher Scientific, Pittsburgh, PA).

siRNA of MEKK-1, NIK, IKK- α , IKK- β , p65, and p52

Targeted siRNAs were obtained from Dharmacon, Inc. (Chicago, IL). Caco-2 monolayers were transiently transfected with DharmaFect transfection reagent (Dharmacon).⁴ Briefly, 5×10^5 cells per filter were seeded into a 12-well transwell plate and grown to confluency. Caco-2 monolayers were then washed with PBS twice, and 1.0 mL Opti-MEM medium was added to the apical compartment of each filter, and 1.5 mL was added to the basolateral compartment of each filter. Five nanograms of the siRNA of interest and 2 μL of DharmaFect reagent were preincubated in Opti-MEM. After 5 minutes of incubation, two solutions were mixed and incubated for another 20 minutes, and the mixture was added to the apical compartment of each filter. The TNF- α experiments were performed 96 hours after transfection. The efficiency of silencing was confirmed by Western blot analysis.

Nuclear Extracts and ELISA for Transcription Factor Activation

Filter-grown Caco-2 monolayers were treated with 10 ng/mL TNF- α for 30 minutes. Caco-2 monolayers were washed with ice-cold PBS, scraped, collected, and centrifuged at $20,800 \times g$ for 30 seconds. The cell pellets were resuspended in 200 μL of buffer A (in millimoles: 10 HEPES-KOH, 1.5 MgCl_2 , 10 KCl, 0.5 dithiothreitol, and 0.2 phenyl-methylsulfonyl fluoride; pH 7.9), and incubated on ice for 15 minutes. After centrifugation at $20,800 \times g$ for 30 seconds, pelleted nuclei were resuspended in 30 μL of buffer C [in millimoles: 20 HEPES-KOH (25% glycerol), 420 NaCl, 1.5 MgCl_2 , 0.2 EDTA, 0.5 dithiothreitol, and 0.2 phenyl-methylsulfonyl fluoride; pH 7.9]. After incubation on ice for 20 minutes, the lysates were centrifuged at $20,800 \times g$ for 20 minutes. Protein concentrations were determined with the Bradford method. The NF- κB p65 and p52 DNA-binding assay was performed with Trans-AM enzyme-linked

immunosorbent assay (ELISA)-based kits from Active Motif (Carlsbad, CA) according to the manufacturer's protocol. In brief, the binding reactions contained 1 pmol/L biotinylated probe (Integrated DNA Technologies, Coralville, IA) and 5 μg of nuclear extract in complete binding buffer with a total volume of 50 μL . After 30 minutes of incubation, the solution was transferred to an individual well on a 96-well plate and incubated for 1 hour. Appropriate antibody (2 $\mu\text{g}/\text{mL}$) was added to the well to bind the target protein in nuclear extract. After incubation for 1 hour, the antibody was removed, and 100 μL of horseradish peroxidase-conjugated secondary antibody was added to the well and incubated for 1 hour. Subsequently, 100 μL of developing solution was added for 2 to 10 minutes, and 100 μL of stop solution were added. The absorbance at 450 nm was determined with the SpectraMax 190 (Molecular Devices, Sunnyvale, CA).

RNA Isolation and Reverse Transcription

Caco-2 cells (5×10^5 per filter) were seeded into -well transwell permeable inserts and grown to confluence. Filter-grown Caco-2 cells were then treated with appropriate experimental reagents for desired time periods. At the end of the experimental period, cells were washed twice with ice-cold PBS. Total RNA was isolated with Qiagen RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA concentration was determined by absorbance at 260/280 nm with the use of SpectraMax 190 (Molecular Devices). The reverse transcription was outperformed with the GeneAmp Gold RNA PCR core kit (Applied Biosystems, Foster city, CA). Two micrograms of total RNA from each sample were reverse transcribed into cDNA in a 40- μL reaction that contained $1 \times$ RT-PCR buffer, 2.5 mmol/L MgCl_2 , 250 $\mu\text{mol}/\text{L}$ of each dNTP, 20 U RNase inhibitor, 10 mmol/L dithiothreitol, 1.25 $\mu\text{mol}/\text{L}$ random hexamer, and 30 U multiscribe reverse transcriptase. The reverse transcription reactions were performed in a thermocycler (MyCycler; Bio-Rad Laboratories) at 25°C for 10 minutes, 42°C for 30 minutes, and 95°C for 5 minutes.

Quantification of Gene Expression Using Real-Time PCR

The real-time PCRs were performed with ABI prism 7900 sequence detection system and TaqMan universal PCR master mix kit (Applied Biosystems, Branchburg, NJ) as previously described.²³ Each real-time PCR reaction contained 10 μL reverse transcription reaction mix, 25 μL $2 \times$ TaqMan universal PCR master mix, 0.2 $\mu\text{mol}/\text{L}$ probe, and 0.6 $\mu\text{mol}/\text{L}$ primers. Primer and probe design for the real-time PCR was made with Primer Express version 2 from Applied Biosystems. [The primers used in this study are as follows: MLCK-specific primer pairs consisted of 5'-AGG-AAGGCAGCATTGAGGTTT-3' (forward), 5'-GCTTTTCAGCAGGCAGAGGTAA-3' (reverse); probe specific for MLCK consisted of FAM 5'-TGAAGATGCTGGCTCC-3' TAMRA; the internal control glyceraldehyde-3-phosphate

dehydrogenase (GAPDH)-specific primer pairs consisted of 5' CCACCCATGGCAAATTCC-3' (forward), 5'-TGGG-ATTTCATTGATGACCAG-3' (reverse); probe specific for GAPDH consisted of JOE 5'-TGGCACCGTCAAG-GCTGAGAACG-3' TAMRA.] All runs were performed according to the default PCR protocol (50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute). For each sample, real-time PCR reactions were performed in triplicate, and the average threshold cycle (Ct) was calculated. A standard curve was generated to convert the Ct to copy numbers. Expression of MLCK mRNA was normalized with GAPDH mRNA expression. The average copy number of MLCK mRNA expression in control samples was set to 1.0. The relative expression of MLCK mRNA in treated samples was determined as a fold increase compared with control samples.

Transfection of MLCK DNA and Measurement of Promoter Activity

The MLCK promoter region was cloned with Genome-Walker system (Clontech Laboratories, Inc., Mountain View, CA). A 2091-bp DNA fragment (−2109 to −18) was amplified by PCR.²³ The amplification condition was 1 cycle at 94°C for 2 minutes, followed by 43 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes and 1 cycle at 72°C for 5 minutes. The resultant PCR product was digested with HindIII and KpnI and inserted into pGL3-basic luciferase reporter vector (Promega, Madison, WI). The sequence was confirmed by DNA services at the University of New Mexico. MLCK promoter was transiently transfected into Caco-2 cells with the use of transfection reagent lipofectamine 2000 (Life Technologies, Carlsbad, CA). Renilla luciferase vector (pRL-TK; Promega) was cotransfected with each plasmid construct as an internal control. Cells (5×10^5 per filter) were seeded into a 6-well transwell plate and grown to confluence. Caco-2 monolayers were then washed with PBS twice, 1.0 mL Opti-MEM medium was added to the apical compartment of each filter, and 1.5 mL was added to the basolateral compartment of each filter. One microgram of each plasmid construct and 0.25 µg pRL-TK or 2 µL lipofectamine 2000 was preincubated in 250 µL Opti-MEM, respectively. After 5 minutes of incubation, two solutions were mixed and incubated for another 20 minutes, and the mixture was added to the apical compartment of each filter. After incubation for 3 hours at 37°C, 500 µL Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum was added to both sides of the filter to reach a 2.5% final concentration of fetal bovine serum. Subsequently, media were replaced with normal Caco-2 growth media 16 hours after transfection. Specific experiments were performed 48 hours after transfection. At the completion of specific experimental treatments, Caco-2 cells were washed twice with 1 mL ice-cold PBS, followed by the addition of 400 µL $1 \times$ passive lysis buffer, incubated at room temperature for 15 minutes, scraped, transferred into an Eppendorf

tube, and centrifuged for 15 seconds at $19,000 \times g$ in a microcentrifuge. Luciferase activity was determined with the dual luciferase assay kit (Promega). Twenty microliters of the supernatant fluid was used for each assay. Luciferase values were determined by Lumat LB 9507 (EG&G Berthold, Oak Ridge, TN). The value of reporter luciferase activities were then divided by that of renilla luciferase activities to normalize for differences in transfection efficiencies. The average activity value of the control samples was set to 1.0. The luciferase activity of MLCK promoter in treated samples was determined relative to the control samples.

In Vivo Mouse Intestinal Permeability Measurements

The Laboratory Animal Care and Use Committee at the University of New Mexico approved all experimental protocols. The mouse intestinal permeability was measured by recycling small intestinal perfusion as previously described.^{39–41} After the experimental period, mice were anesthetized with isoflurane. After midline incision of the abdomen, 5 cm of intestine segment was isolated and 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 was first perfused through the intestine at 1 mL per minute for 20 minutes, followed by air flush to remove residual contents with the use of an external pump (Bio-Rad Laboratories). This was followed by perfusion of 5 mL perfusate solution (85 mmol/L NaCl, 10 mmol/L HEPES, 20 mmol/L sodium ferrocyanide, 5 mmol/L KCl, 5 mmol/L CaCl_2 , pH 7.4.) that contained Texas Red-labeled dextran (10 kDa) in a recirculating manner at 0.75 mL per minute for 2 hours. The abdominal cavity was covered with moistened gauze, body temperature was measured via rectal thermometer, and temperature was maintained at $37.5^\circ\text{C} \pm 0.5^\circ\text{C}$ with the use of a heating lamp. One-milliliter aliquots of test solution were removed at the beginning and end of the perfusion. After perfusion, the animal was sacrificed, and the perfused intestine segment was excised and the length was measured. The excised intestinal tissue was then snap-frozen in optimal cutting temperature compound or used for protein and RNA analyses. Ferrocyanide concentration in the perfusate was measured with the colorimetric assay. Texas Red-labeled dextran 10 kDa concentration was measured with an excitation wavelength of 595 nm and an emission wavelength of 615 nm in a microplate reader. Probe clearance was calculated as

$$C_{\text{probe}} = (C_i V_i - C_f V_f) / (C_{\text{avg}} TL) \quad (1)$$

In the equation, C_i represents the measured initial probe concentration; C_f represents the measured final probe concentration; V_i represents the measured initial perfusate volume; V_f was calculated as $V_i[(\text{ferrocyanide})_i/(\text{ferrocyanide})_f]$; C_{avg} was calculated as

$$(C_i - C_f) / \ln(C_i/C_f) \quad (2)$$

T represents hours of perfusion, and L represents the length of the perfused intestine section in centimeters.

Figure 1 Time course effect of TNF- α on Caco-2 NIK and MEKK-1 activation. **A:** TNF- α (10 ng/mL) causes a time-dependent increase in Caco-2 NIK phosphorylation (total NIK was used for equal protein loading). **B:** TNF- α causes a time-dependent increase in MEKK-1 phosphorylation (total MEKK-1 was used for equal protein loading). MEKK-1, mitogen-activated protein kinase kinase kinase-1; NIK, NF- κ B-inducing kinase; TNF, tumor necrosis factor.

Animal Surgery and *in Vivo* Transfection of NF- κ B p65, MEKK-1, and NIK siRNA

Mice were deprived of food for 24 hours before the surgery. Mice were anesthetized with isoflurane (4% for surgical induction, 1% for maintenance) with the use of oxygen as carrier during surgical procedures. Surgical procedures were performed with sterile technique. The abdomen was opened by a midline incision, and 6 cm of intestine segment was isolated at the proximal and distal ends and tied with sutures. siRNA transfection solution (0.5 mL; containing Accell medium; 2.5 nmol p38 or ATF-2 siRNA and 50 μ L transfecting agent lipofectamine) was introduced into the isolated intestine segment (surface area 6 cm²) for 1-hour transfection period. Control animals underwent sham-operation, where the siRNA transfection solution contained Accell medium; 2.5 nmol nontarget siRNA, and 50 μ L transfecting agent lipofectamine. The abdominal cavity was covered with moistened gauze. Body temperature was monitored continuously with a rectal probe and maintained at 37.5°C $\pm$ 0.5°C with the use of a heating pad. After 1-hour transfection period, each end of the intestinal segment was untied, the intestine was placed back in the abdominal cavity, and the abdomen was closed. Three days after transfection, functional studies of intestinal epithelial barrier were performed. The surgery and the *in vivo* transfection procedures had no effect on the food intake and the body weight of the animals during the experimental period. The average animal weight averaged between 23 and 25 g during the experimental period.

Statistical Analysis

Statistical significance of differences between mean values was assessed with Student's *t*-tests for unpaired data and analysis of variance analysis whenever required. All

reported significance levels represent two-tailed *P* values. *P* < 0.05 was used to indicate statistical significance. All *in vitro* experiments that used Caco-2 monolayers, including assessment of TJ barrier, biochemical and molecular studies, and kinase activity measurements, were performed in triplicates or quadruplicates and were repeated at a minimum of three times for reproducibility. The immunoblot analysis and cell imaging studies were repeated three to four times. The animal studies were performed individually, and each experimental group consisted of three to six animals.

Results

Role of MAP3 Kinase Pathways in TNF- α -Induced Increase in Caco-2 TJ Permeability

The role of MAP3 kinases NIK and MEKK-1 in mediating the TNF- α -induced increase in Caco-2 TJ permeability was investigated. TNF- α caused a time-dependent increase in NIK phosphorylation (Thr 559) in Caco-2 cells, starting at about 10 minutes and continuing up to 60 minutes as determined by phospho-NIK immunoblotting (Figure 1A). TNF- α treatment (10 ng/mL) also caused a rapid activation of MEKK-1 as assessed by phospho-MEKK-1 (Thr 1381) immunoblotting (Figure 1B). These results suggested that TNF- α causes rapid activation of both NIK and MEKK-1 in filter-grown Caco-2 monolayers.

The requirement of NIK and/or MEKK-1 in TNF- α -induced increase in Caco-2 TJ permeability was examined by targeted knockdown of NIK or MEKK-1 by siRNA transfection of filter-grown Caco-2 monolayers. Two different controls were used in these experiments; a control group exposed to normal media and a control group transfected with nontargeted siRNA. The NIK siRNA transfection resulted in a near-complete depletion of NIK expression in Caco-2 cells (Figure 2A); NIK silencing by

Figure 2 Effect of siRNA-induced NIK and MEKK-1 knockdown on TNF- α -induced increase in Caco-2 TJ permeability. **A:** NIK siRNA transfection results in a near complete depletion in NIK protein expression. **B:** NIK silencing prevents the TNF- α -induced drop in Caco-2 TER. **C:** NIK silencing by siRNA transfection prevents the TNF- α -induced increase in inulin flux. **D:** MEKK-1 siRNA transfection results in a near complete depletion in MEKK-1 protein expression. **E:** siRNA-induced knockdown of MEKK-1 does not prevent the TNF- α -induced drop in Caco-2 TER. **F:** siRNA-induced knockdown of MEKK-1 does not prevent the TNF- α -induced increase in inulin flux. **G:** NIK inhibitor (4H-isoquinoline-1,3-dione; 50 μ M) prevents the TNF-phosphorylation of NIK. **H:** NIK inhibitor prevents the TNF- α -induced drop in Caco-2 TER. Data are expressed as means $\pm$ SEM. $n = 4$. ** $P < 0.005$ versus control; †† $P < 0.005$ versus TNF- α treatment. C, no siRNA controls; inh, inhibitor; MEKK-1, mitogen-activated protein kinase kinase kinase-1; NIK, NF- κ B-inducing kinase; NT, non-targeted siRNA; TER, trans-epithelial electrical resistance; TJ, tight junction; TNF, tumor necrosis factor.

siRNA transfection prevented the TNF- α -induced drop in Caco-2 TER and increase in apical-to-serosal flux of paracellular marker inulin (Figure 2, B and C). In contrast, the siRNA-induced knockdown of MEKK-1 (Figure 2D) did not affect the TNF- α -induced drop in Caco-2 TER (Figure 2E) or the increase in inulin flux (Figure 2F). The treatment of Caco-2 monolayers with pharmacologic NIK inhibitor 4H-isoquinoline-1,3-dione (50 μ M) also inhibited the TNF- α -induced drop in Caco-2 TER (Figure 2, G and H). These data suggested that NIK activation, but not MEKK-1, was required for the TNF- α -induced increase in Caco-2 TJ permeability.

Involvement of Canonical versus NonCanonical NF- κ B Pathways

The role of noncanonical pathway (NF- κ B p52/Rel B) or canonical pathway (NF- κ B p50/p65) in mediating the TNF- α modulation of Caco-2 TJ permeability was

examined. In the following studies, the TNF- α effect on canonical pathway was determined by assessing the degradation of I κ B- α and activation of NF- κ B p50/p65 dimer, and the effect on noncanonical pathway was determined by the activation of p100/p52 subunit with the use of ELISA-based assay.^{5,37} TNF- α caused a rapid degradation of I κ B- α in Caco-2 cells (Figure 3A). Interestingly, TNF- α caused activation of both p50/p65 and p100/p52 dimers as evidenced by the binding of the active dimers to the respective κ B binding site on the oligonucleotide probe as determined by ELISA binding assay (Figure 3, B and C), indicating that both canonical and noncanonical NF- κ B pathways were activated in Caco-2 cells. The siRNA-induced silencing of p65 resulted in a complete inhibition of TNF- α -induced drop in Caco-2 TER and increase in inulin flux (Figure 3, D and E). In contrast, siRNA-induced knockdown of p52 did not affect the TNF- α drop in Caco-2 TER or increase in inulin flux (Figure 3, F and G). Collectively, these data suggested that TNF- α activation of the canonical pathway,

Figure 3 Effect of 10 ng/mL TNF- α on Caco-2 NF- κ B pathway (p65 and p52) activation. **A:** TNF- α causes a time-dependent degradation in I κ B- α expression. **B:** TNF- α treatment causes a significant increase in Caco-2 NF- κ B p65 binding to the DNA probe as assayed by ELISA-based DNA binding assay of NF- κ B p65. **C:** TNF- α treatment causes a significant increase in Caco-2 NF- κ B p52 binding to the DNA probe as assayed by ELISA-based DNA binding assay of NF- κ B p52. **D:** NF- κ B p65 siRNA transfection prevents the TNF- α -induced drop in Caco-2 TER. **E:** NF- κ B p65 siRNA transfection prevents the TNF- α -induced increase in inulin flux. **F:** NF- κ B p52 siRNA transfection does not prevent the TNF- α -induced drop in Caco-2 TER. **G:** NF- κ B p52 induced silencing by siRNA transfection does not prevent the TNF- α -induced increase in inulin flux. Data are expressed as means $\pm$ SEM. $n = 4$. $^{**}P < 0.005$, $^{****}P < 0.0001$ versus control; $^{\dagger\dagger}P < 0.005$ versus TNF- α treatment. C, no siRNA controls; ELISA, enzyme-linked immunosorbent assay; I κ B, inhibitory κ B; NT, non-targeted siRNA; TER, transepithelial electrical resistance; TNF, tumor necrosis factor.

but not the noncanonical pathway, was required for the TNF- α -induced increase in intestinal TJ permeability.

Next, the regulatory role of NIK or MEKK-1 on TNF- α -induced activation of p50/p65 dimer was examined. The siRNA-induced knockdown of NIK prevented the TNF- α -induced degradation of I κ B- α and activation of p50/p65 in Caco-2 cells (Figure 4, A and B). However, the siRNA knockdown of MEKK-1 did not affect the degradation of I κ B- α or the activation of p50/p65 (Figure 4, C and D). These data suggested that NIK (but not MEKK1) mediated the TNF- α activation of the canonical pathway.

Role of IKK Catalytic Subunits in TNF- α -induced Increase in Caco-2 TJ Permeability

The role of IKKs in mediating the TNF- α -induced increase in intestinal TJ permeability was examined. In these studies, the involvement of IKK catalytic subunits, IKK- α and IKK- β , was examined. TNF- α (10 ng/mL) caused a rapid activation of both IKK- α and IKK- β subunits as assessed by subunit phosphorylation (Ser176/177) (Figure 5A). To identify which IKK subunit mediated the TNF- α effect on Caco-2 TJ permeability, the expression of IKK- α or IKK- β was selectively silenced via siRNA transfection. The IKK- α knockdown, but not IKK- β knockdown (Figure 5, D and E), prevented both the TNF- α -induced drop in Caco-2 TER and increase in paracellular permeability (Figure 5, B and C), suggesting that IKK- α but not IKK- β was required for the increase in Caco-2 TJ permeability. Next, the involvement of IKK subunits in the TNF- α activation of NF- κ B p65 was also examined. IKK- α , but not IKK- β , silencing

inhibited the TNF- α -induced degradation of I κ B- α and activation of p65 subunit (Figure 6). These were unexpected findings and suggested that IKK- α , and not IKK- β (as part of IKK complex), was the catalytic subunit responsible for

Figure 4 Effect of siRNA-induced MAP3 kinase knockdown on TNF- α activation of NF- κ B p65. **A:** NIK siRNA transfection prevents the TNF- α -induced degradation of I κ B- α as assessed by Western blot analysis. **B:** NIK silencing inhibits the TNF- α -induced binding of p65 to its binding site on DNA probe as measured by DNA ELISA-binding assay. **C:** siRNA-induced knockdown of MEKK-1 does not prevent the TNF- α -induced degradation of I κ B- α . **D:** siRNA-induced knockdown of MEKK-1 does not prevent the TNF- α -induced binding of p65 to its binding site on DNA probe as measured by DNA ELISA-binding assay. Data are expressed as means $\pm$ SEM. $^{****}P < 0.0001$ versus control; $^{\dagger\dagger\dagger}P < 0.005$, $^{\dagger\dagger\dagger\dagger}P < 0.0001$ versus TNF- α treatment. C, no siRNA controls; ELISA, enzyme-linked immunosorbent assay; I κ B, inhibitory κ B; MAP3 kinase, mitogen-activated protein kinase, kinase; MEKK-1, mitogen-activated protein kinase kinase kinase-1; NIK, NF- κ B-inducing kinase; NT, non-targeted siRNA; TNF, tumor necrosis factor.

Figure 5 Time course effect of TNF- α on Caco-2 IKK catalytic subunit activation. **A:** TNF- α (10 ng/mL) causes a time-dependent increase in Caco-2 IKK- α and IKK- β phosphorylation. **B:** IKK- α siRNA transfection completely prevents the TNF- α -induced drop in Caco-2 TER. **C:** siRNA-induced knockdown of IKK- α prevented the TNF- α -induced increase in inulin flux. **D:** IKK- β siRNA transfection does not prevent the TNF- α -induced drop in Caco-2 TER. **E:** siRNA-induced knockdown of IKK- β does not inhibit the TNF- α -induced increase in inulin flux. Data are expressed as means $\pm$ SEM. $n = 4$. ** $P < 0.005$ versus control; †† $P < 0.005$ versus TNF- α treatment. C, no siRNA controls; IKK, inhibitory κ B kinase; NT, non-targeted siRNA; TER, transepithelial electrical resistance; TNF, tumor necrosis factor.

mediating the TNF- α -induced degradation of I κ B- α and activation of NF- κ B p65 and the subsequent increase in TJ permeability.

Next, the MAP3 kinase responsible for the TNF- α activation of IKK- α was determined. The siRNA knockdown of NIK inhibited the TNF- α -induced phosphorylation of IKK- α ; however, MEKK-1 knockdown did not have any effect (Figure 7, A and B). Interestingly, the siRNA depletion of MEKK-1, but not NIK, inhibited the phosphorylation of IKK- β (data not shown). These results suggested that NIK was responsible for the TNF- α -induced activation of IKK- α and MEKK-1 mediated the phosphorylation of IKK- β .

Role of NIK in TNF- α Regulation of MLCK Gene Activity

Previous studies have shown that the TNF- α -induced increase in Caco-2 TJ permeability was regulated by the activation of MLCK gene.⁴² In the following studies, we examined the involvement of NIK in MLCK gene regulation by assessing MLCK promoter activity and MLCK mRNA transcription. To assess the MLCK promoter activity, Caco-2 cells were transfected with plasmid vector encoding the MLCK promoter region and the luciferase

reporter gene. The TNF- α treatment resulted in an increase in MLCK promoter activity, MLCK mRNA expression, and MLCK protein expression (Figure 7, C–E).^{23,42} The siRNA knockdown of NIK prevented the TNF- α -induced increase in MLCK promoter activity, MLCK mRNA expression, and MLCK protein expression (Figure 7, C–E). The siRNA silencing of MEKK-1 did not have any effect (Figure 7, F–H). These data indicated that the TNF- α -induced increase in Caco-2 TJ permeability was regulated by NIK pathway activation of MLCK gene.

Potential Crosstalk between NIK and MAP Kinases ERK1/2 and p38 Kinase

Previous studies from our laboratory showed that the TNF- α -induced increase in intestinal TJ permeability was mediated in part by extracellular signal-regulated kinase (ERK)1/2 signaling pathway¹⁶ and that IL-1 β increase in intestinal TJ permeability involved p38 kinase pathway.⁴⁰ Because, TNF- α is known to activate MAP kinases, the possible crosstalk between MAP3 kinase NIK and MAP kinases ERK1/2 and p38 kinase in TNF- α -induced activation of the canonical pathway was also examined. TNF- α caused an activation of both ERK1/2 and p38 kinase, as

Figure 6 Effect of siRNA IKK subunit knockdown on TNF- α -induced activation of NF- κ B p65. **A:** siRNA-induced knockdown of IKK- α completely abolishes the TNF- α -induced degradation of I κ B- α . **B:** IKK- α siRNA transfection inhibits the TNF- α -induced binding of p65 to its binding site on DNA probe as measured by DNA ELISA-binding assay. **C:** IKK- β siRNA transfection does not prevent the TNF- α -induced degradation of I κ B- α . **D:** siRNA-induced knockdown of IKK- β does not inhibit the TNF- α -induced binding of p65 to its binding site on DNA probe as measured by DNA ELISA-binding assay. **** $P < 0.0001$ versus control; †††† $P < 0.0001$ versus TNF- α treatment. C, no siRNA controls; ELISA, enzyme-linked immunosorbent assay; I κ B, inhibitory κ B; IKK, inhibitory κ B kinase; NT, non-targeted siRNA; TNF, tumor necrosis factor.

evidenced by the phosphorylation of ERK1/2 and p38 kinase (Figure 7, I and J). To determine the possible regulatory role of NIK in the MAP kinase activation, the effect of siRNA knockdown of NIK on TNF- α -induced activation of ERK1/2 and p38 kinase was examined. The siRNA knockdown of NIK did not affect the TNF- α -induced phosphorylation of ERK1/2 but inhibited the p38 kinase phosphorylation (Figure 7, I and J), indicating that p38 kinase activation depended on NIK. Note that MEKK1 knockdown completely prevented the TNF- α -induced phosphorylation of ERK1/2 (data not shown). Interestingly, the siRNA-induced knockdown of p38 kinase did not affect the TNF- α -induced degradation of I κ B- α (Figure 7K). In combination, our results suggested that the TNF- α -induced activation of p38 kinase is regulated by NIK, but p38 kinase is not involved in TNF- α -induced degradation of I κ B- α . Thus, it appears that the NIK pathway activation of NF- κ B p50/p65 is independent of MAP kinases ERK1/2 and p38 kinase.

Role of NIK in TNF- α -Induced Increase in Intestinal Permeability *in Vivo*

The above-mentioned studies indicated that NIK/IKK- α /p50/p65 axis mediated the TNF- α -induced activation of MLCK gene and Caco-2 permeability. However, whether similar kinase axis also plays a role in *in vivo* animal system remains unclear. In the following studies, we examined the

role of NIK signaling cascade in the TNF- α -induced increase in mouse intestinal permeability by *in vivo* recycling intestinal perfusion of mouse small intestine.³⁹ The intraperitoneal injection of TNF- α (5 μ g) caused a threefold increase in mouse intestinal permeability to paracellular marker dextran 10 kDa (Figure 8A). TNF- α treatment also resulted in a time-dependent degradation of I κ B- α in mouse intestinal tissue (Figure 8B), consistent with the activation of NF- κ B p50/p65. The TNF- α administration also caused a time-dependent phosphorylation of NIK (Thr 559) and MEKK-1 (Thr 1381) in mouse intestinal tissue (Figure 8C), confirming the activation of the upstream MAP3 kinases in response to the TNF- α treatment. TNF- α also caused an increase in intestinal tissue MLCK mRNA and protein expression in mouse small intestine (Figure 9, A and B). To determine the requirement of NIK or MEKK-1 in TNF- α regulation of MLCK expression and intestinal permeability *in vivo*, intestinal tissue NIK or MEKK-1 expression was selectively silenced with the *in vivo* siRNA transfection method.^{39–41} Briefly, 6 cm of mouse small intestine was isolated with sutures, and the mucosal surface was exposed to siRNA transfection solution that contained NIK or MEKK-1 siRNA for 1 hour. The sutures were removed, the intestinal segment was reinserted into the original location in the abdomen, and the abdominal cavity was closed with sutures. After 2 days, mice were treated with TNF- α , and intestinal permeability studies were performed on day 3. The *in vivo* siRNA knockdown of intestinal tissue NIK, but not MEKK-1 (Figure 9, C and F), prevented the TNF- α -induced increase in intestinal MLCK expression (Figure 9, D and G) and intestinal permeability (Figure 9, E and H), demonstrating the requirement of NIK in TNF- α -induced increase in MLCK expression and intestinal permeability *in vivo*. Next, the effect of siRNA-induced silencing of NIK or MEKK-1 on I κ B- α degradation was examined. The siRNA-induced knockdown of NIK but not MEKK-1 prevented the TNF- α -induced degradation of I κ B- α in mouse intestine (Figure 10, A and B), suggesting that NIK mediated the TNF- α -induced degradation of I κ B- α and activation of NF- κ B p50/p65 in mouse intestine. The *in vivo* siRNA knockdown of intestinal NF- κ B p65 prevented the TNF- α -induced increase in intestinal tissue expression of MLCK and increase in mouse intestinal permeability (Figure 10, C and D). Together, these data suggested that the TNF- α -induced increase in mouse intestinal permeability was also regulated by NIK/NF- κ B p50/p65 axis activation of MLCK gene and MLCK-dependent increase in mouse intestinal permeability.

Discussion

Previous studies have shown that TNF- α causes an increase in intestinal TJ permeability.^{5,17} The TNF- α -induced increase in intestinal TJ permeability is an important factor that contributes to the observed increase in intestinal

Figure 7 Effect of siRNA-induced MEKK-1 and NIK knockdown on TNF- α activation of IKK- α . **A:** siRNA-induced knockdown of NIK prevents the TNF- α -induced phosphorylation of IKK- α as assessed by Western blot analysis. **B:** siRNA-induced knockdown of MEKK-1 does not prevent the TNF- α -induced phosphorylation of IKK- α . Effect of siRNA induced knockdown of NIK and MEKK-1 on TNF- α -induced increase in MLCK gene activity and protein expression. **C:** siRNA-induced knockdown of NIK results in a complete inhibition of TNF- α -induced increase in MLCK promoter activity. **D:** siRNA-induced knockdown of NIK prevents the TNF- α -induced increase in MLCK mRNA levels. **E:** NIK silencing by siRNA transfection prevents the TNF- α -induced increase in MLCK protein expression. **F:** siRNA-induced knockdown of MEKK-1 does not prevent the TNF- α -induced increase in MLCK promoter activity. **G:** Knocking-down MEKK-1 by siRNA does not prevent the TNF- α -induced increase in MLCK mRNA levels. **H:** Knocking-down MEKK-1 by siRNA does not affect the TNF- α -induced increase in MLCK protein expression. **I:** Knocking-down NIK by siRNA prevents the TNF- α -induced phosphorylation of p38 kinase (siNIK: siRNA NIK transfection). **J:** Knocking-down NIK by siRNA does not inhibit the TNF- α -induced phosphorylation of ERK1/2. **K:** Knocking-down p38 kinase by siRNA does not inhibit the TNF- α -induced degradation of I κ B- α . * P < 0.05, ** P < 0.005 versus control; †† P < 0.005, ††† P < 0.001 versus TNF- α treatment. C, no siRNA controls; ERK, extracellular signal-related kinase; IKK, inhibitory κ B kinase; MEKK-1, mitogen-activated protein kinase kinase kinase-1; MLCK, myosin light chain kinase; NIK, NF- κ B-inducing kinase; NT, nontargeted siRNA; siNIK: siRNA NIK transfection; sip38: siRNA p38 kinase transfection; T, TNF- α for 30 minutes; TNF, tumor necrosis factor.

permeability in inflammatory bowel disease patients.^{13,43} In this regard, treatment of inflammatory bowel disease patients with anti-TNF- α antibody therapy resulted in rapid retightening of the intestinal barrier and improvement of the disease.^{20,22,44,45} Previous studies from our laboratory have shown that NF- κ B p50/p65 plays a central role in the TNF- α -induced increase in intestinal TJ permeability by targeting MLCK gene activation.^{17,23,42} However, the signaling pathways and the intracellular mechanisms that mediate TNF- α -induced activation of NF- κ B p50/p65 and MLCK gene in intestinal epithelial cells remain unknown. Herein,

we investigated the involvement of canonical and noncanonical NF- κ B pathways and the specific protein kinases that mediate the TNF- α -induced activation of NF- κ B and MLCK gene and increase in intestinal TJ permeability. Our results demonstrate a new signaling pathway activation of NF- κ B p50/p65 or canonical pathway in which NIK, a MAP3 kinase shown to be involved primarily in the activation of the noncanonical pathway, activates the canonical pathway via IKK- α degradation of I κ B- α .

The MAP3 kinases, including NIK and MEKK-1, are important upstream regulators of NF- κ B activation.^{26,46} The

Figure 8 Effect of TNF- α activation of NF- κ B pathway in mouse intestinal permeability. **A:** TNF- α (5 μ g) causes an increase in mouse intestinal mucosal-to-serosal flux of dextran 10 kDa. **B:** TNF- α causes a time-dependent increase in I κ B- α degradation in mouse intestinal tissue, starting at 2 hours and continuing up to 24 hours as assessed by Western blot analysis. **C:** TNF- α caused a time-dependent increase in phosphorylation of NIK and MEKK-1 in mouse intestinal tissue as assessed by Western blot analysis. $^{**}P < 0.01$. I κ B, inhibitory κ B; MEKK-1, mitogen-activated protein kinase kinase kinase-1; NIK, NF- κ B-inducing kinase; TNF, tumor necrosis factor.

NF- κ B dimers are formed by the individual Rel subunits, including p65 (often referred to as RelA), RelB, c-Rel, p50, and p52.^{47,48} Two distinct NF- κ B signaling pathways have been identified that regulate the activation of the

canonical (or classic) and the noncanonical (or alternative) pathways.^{30,49} In the canonical pathway, the ligand binding to the membrane receptor leads to the phosphorylation and degradation of I κ B protein (most common subtype being

Figure 9 Effect of TNF- α on MLCK expression *in vivo*. **A:** TNF- α causes an increase in mouse intestinal tissue MLCK mRNA transcript as assessed by real-time PCR; TNF- α 24 hours of treatment. **B:** TNF- α causes a time-dependent increase in mouse intestinal tissue MLCK protein expression as assessed by Western blot analysis. **C:** NIK siRNA transfection *in vivo* results in a near-complete knockdown of NIK expression in mouse intestinal tissue. **D:** NIK siRNA transfection *in vivo* prevents the TNF- α -induced increase in mouse intestinal MLCK protein expression as assessed by Western blot analysis. **E:** NIK siRNA transfection *in vivo* prevents the TNF- α -induced increase in mouse intestinal mucosal-to-serosal flux of dextran 10 kDa. **F:** MEKK-1 siRNA transfection *in vivo* results in a near-complete knockdown of MEKK-1 expression in mouse intestinal tissue. **G:** siRNA-induced knockdown of MEKK-1 *in vivo* does not prevent the TNF- α -induced increase in mouse intestinal MLCK protein expression as assessed by Western blot analysis. **H:** MEKK-1 siRNA transfection *in vivo* does not prevent the TNF- α -induced increase in mouse intestinal mucosal-to-serosal flux of dextran 10 kDa. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\dagger\dagger}P < 0.005$ versus TNF- α treatment. MEKK-1, mitogen-activated protein kinase kinase kinase-1; MLCK, myosin light chain kinase; NIK, NF- κ B-inducing kinase; TNF, tumor necrosis factor.

Figure 10 Effect of siRNA-induced silencing of NIK and MEKK-1 on NF- κ B signaling pathway and mouse intestinal permeability. **A:** NIK siRNA transfection *in vivo* prevents the TNF- α -induced degradation of I κ B- α in mouse intestinal tissues. **B:** MEKK-1 siRNA transfection *in vivo* does not inhibit the TNF- α -induced degradation of I κ B- α in mouse intestinal tissues. **C:** NF- κ B p65 siRNA transfection *in vivo* prevents the TNF- α -induced increase in mouse intestinal MLCK protein expression as assessed by Western blot analysis. **D:** NF- κ B p65 siRNA transfection *in vivo* prevents the TNF- α -induced increase in mouse intestinal mucosal-to-serosal flux of dextran 10 kDa. *** P < 0.001 versus control; †† P < 0.005 versus TNF- α treatment. I κ B, inhibitory κ B; MEKK-1, mitogen-activated protein kinase kinase kinase-1; MLCK, myosin light chain kinase; NIK, NF- κ B-inducing kinase; TNF, tumor necrosis factor.

the I κ B- α) and activation of NF- κ B dimer p50/p65.^{49–52} NF- κ B p50/p65 is the dominant NF- κ B dimer and makes up >90% of the NF- κ B dimers present in the cells. In the noncanonical pathway, ligand binding leads to the activation of NIK, which, in turn, leads to the phosphorylation and processing of p100 subunit into p52 and generation of activated RelB/p52 dimer.^{29,53} To date, NIK is the only MAP3 kinase that has been shown to signal RelB/p52 activation or the noncanonical pathway.^{54,55}

Previous studies have shown MEKK-1 to be an important regulator of the canonical pathway; MEKK-1 induces the activation of the trimeric IKK complex, consisting of the catalytic IKK subunits, IKK- α and IKK- β , and the regulatory subunit IKK- γ /NEMO.^{36,56–58} IKK- γ does not have catalytic activity but plays a critical role in the IKK complex formation.^{59,60} The activation of MEKK-1 leads to the activation of IKK complex with phosphorylation of IKK- α and IKK- β .⁵⁶ The final outcome of the canonical pathway signaling is the phosphorylation and degradation of I κ B- α and activation of p50/p65 dimer, and the noncanonical pathway is the phosphorylation and processing of p100 and the generation of RelB/p52 dimer.^{26,35,51,56,61}

Herein, we show that TNF- α causes activation of both canonical and noncanonical pathways in Caco-2 monolayers as evidenced by the activation of p50/p65 and RelB/p52 dimers. Our data suggested that NIK was responsible for activating both the canonical and the noncanonical pathways, because siRNA-induced silencing of NIK inhibited the TNF- α activation of p50/p65 and RelB/p52 dimers. Surprisingly, MEKK-1 depletion did not affect the TNF- α activation of p50/p65. These data suggested that NIK but not MEKK-1 mediated the TNF- α -induced activation of both noncanonical (RelB/p52) and canonical (p50/p65) pathways. It had been previously shown that in the noncanonical pathway, NIK phosphorylates IKK- α at Ser-176 and Ser-180 without affecting the trimeric IKK complex.⁴⁶ However in the canonical pathway, MAP3 kinases, including MEKK-1, activate the IKK complex, leading to the phosphorylation of serine residues within the activation loop of the catalytic subunits IKK- α (Ser-176 and Ser-180) and IKK- β (Ser-177 and Ser-181).^{46,56} The activated IKK catalytic

subunits then phosphorylate I κ B- α protein at Ser-32 and Ser-36, leading to the ubiquitination and proteasome-induced degradation and nuclear translocation of p50/p65 dimer.^{62–65} Although both IKK- β and IKK- α can catalyze I κ B- α phosphorylation, IKK- β is the dominant catalytic subunit that catalyzes the I κ B- α phosphorylation.^{65–67} NIK is known to phosphorylate and activate IKK- α , but it can also directly phosphorylate p100.^{68,69} Our results indicate that TNF- α caused the activation of both canonical and noncanonical pathways, but only the siRNA depletion of p65 but not p52 inhibited the TNF- α -induced activation of MLCK gene and increase in Caco-2 TJ permeability, confirming that p50/p65 but not RelB/p52 dimer mediated the TNF- α modulation of MLCK gene activity and increases in Caco-2 TJ permeability. As described previously, the increase in MLCK expression leads to MLCK-dependent opening of the TJ barrier.^{70,71} It has been shown that MLCK catalyzes the phosphorylation of MLC; which in turn activates Mg²⁺-myosin ATPase, leading to the contraction of perijunctional actomyosin filaments and mechanical tension-induced opening of the TJ barrier.^{72,73} MLCK expression has been shown to be markedly increased in intestinal tissue of patients with inflammatory bowel disease.⁷⁴ MLCK is also essential to the permeability of intestinal epithelial barrier function *in vitro* and *in vivo*; MLCK gene is activated in response to proinflammatory cytokine, including TNF and IL-1 β in the inflamed intestinal tissues.^{5,36,75} In addition, several recent studies have found the pathogenic role of MLCK in both intestinal barrier dysfunction and intestinal inflammation in animal models of inflammatory bowel disease.^{39,76} In addition, other studies found that interferon- γ induces endocytosis of TJ transmembrane proteins and increase in intestinal epithelial TJ permeability in T-84 monolayers by triggering selective vacuolarization of the apical plasma membrane that was mediated by myosin II-driven contraction.⁷⁷

An important novel finding of our study is the demonstration that the TNF- α -induced activation of NF- κ B p50/p65 in Caco-2 monolayers is mediated by what is traditionally considered to be a noncanonical pathway NIK/IKK- α axis degradation of I κ B- α . Although both IKK- β and IKK- α were activated in response to TNF- α treatment, only

IKK- α depletion inhibited the TNF- α degradation of I κ B- α and subsequent MLCK gene activation. Neither MEKK-1 (which catalyzed the IKK- β phosphorylation and activation) nor IKK- β depletion affected the I κ B- α degradation, p50/p65 activation or MLCK gene activation. This is in contrast to previous published reports that show IKK- β (as part of the IKK complex) to be the kinase mainly responsible for I κ B- α phosphorylation in various cell types.^{78–80} Although there are a number of reports showing MEKK-1/IKK- β to be the upstream kinases that regulate NF- κ B p50/p65 activation,^{56,57,81} there are also a few reports showing that NIK can also mediate p50/p65 activation.^{82,83} In this regard, previous studies with peptidoglycan and IL-1 α showed that IKK- α activation was associated with the canonical NF- κ B pathway activation in HeLa and fibroblast cells.^{84–86} Herein, we show for the first time that NIK/IKK- α axis, independent of MEKK-1 and IKK- β , regulates the TNF- α -induced activation of NF- κ Bp50/p65 in intestinal epithelial cells and, thereby, also mediates MLCK gene activation and increase in intestinal epithelial TJ permeability. These present findings with TNF- α are quite distinct from our previous studies showing that IL-1 β -induced activation of MLCK gene was mediated by MEKK1/IKK- β -induced degradation of I κ B- α and NF- κ B p50/p65 activation.³⁶ Thus, our results demonstrate that the TNF- α and IL-1 β activation of NF- κ B p50/p65 is mediated by distinct MAP3 kinases and IKK catalytic subunits.

The *in vivo* mouse intestinal perfusion studies were also performed to determine the involvement of the NIK in TNF- α -induced increase in mouse intestinal permeability. Similar to the cell culture studies with Caco-2 monolayers, the *in vivo* mouse studies also showed that TNF- α causes activation of both NIK and MEKK-1 in mouse small intestine. The siRNA depletion of NIK prevented the TNF- α -induced increase in mouse intestinal expression of MLCK and increase in intestinal permeability, whereas MEKK-1 knockdown did not have any effect. These data suggested that intestinal tissue activation of NIK was required for the TNF- α -induced increase in mouse intestinal MLCK expression and intestinal permeability. Our *in vivo* studies also showed that NIK was a key regulator of TNF- α -induced I κ B- α degradation and p50/p65 activation. Thus, *in vivo* data indicated that NIK also plays an integral role in mediating the TNF- α -induced activation of the canonical pathway, increase in MLCK gene expression and increase in mouse intestinal permeability.

Conclusion

In conclusion, the results of this study provide important novel insight into the signaling processes that mediate TNF- α modulation of intestinal epithelial TJ permeability *in vitro* and *in vivo*. Our data show that TNF- α -induced increase in intestinal TJ permeability is mediated by NIK/IKK- α /NF- κ B p50/p65 axis activation of MLCK gene. Although

TNF- α also caused the activation of MEKK-1/IKK- β axis, the traditional pathway known to regulate the canonical pathway, this axis was not involved in the TNF- α -induced activation of NF- κ B p50/p65 or the increase in intestinal TJ permeability. Thus, our results identify an important new pathway activation of NF- κ B p50/p65 in the intestinal epithelial cells and provide novel insight into the mechanism of intestinal TJ barrier regulation.

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