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Epithelial Cell IκB-Kinase β Has an Important Protective Role in Clostridium difficile Toxin A-Induced Mucosal Injury

Sungwon Chae,* Lars Eckmann,* Yukiko Miyamoto,* Charalabos Pothoulakis,† Michael Karin,‡ and Martin F. Kagnoff2*§

Toxin A released by Clostridium difficile interacts with the single layer of intestinal epithelial cells that lines the host’s intestinal tract and leads to mucosal damage and inflammation that manifests clinically as antibiotic-associated diarrhea and pseudomembranous colitis. Activation of the transcription factor NF-κB in intestinal epithelial cells is important for regulating the expression of epithelial cell proinflammatory genes and cell survival. However, the role of NF-κB activation in the pathogenesis of C. difficile toxin A–induced colitis is unknown. To determine the functional importance in vivo of NF-κB activation in intestinal epithelium in the pathogenesis of C. difficile–induced colitis, we used mutant mice that do not activate the classical NF-κB signaling pathway in intestinal epithelial cells due to a conditional deficiency in those cells of the IκB-kinase β (IKKβ) subunit of IKK. C. difficile toxin A challenge of intestinal loops in intestinal epithelial cell IKKβ-deficient mice induced a rapid and significant increase in intestinal epithelial apoptosis compared with littermate controls. This was accompanied by a significant increase in acute mucosal inflammation, mucosal injury, luminal fluid secretion, and bacterial translocation. We conclude that activation of intestinal epithelial cell NF-κB by toxin A plays an important host mucosal protective role after C. difficile toxin A exposure that is mediated, at least in part, through promoting epithelial cell survival by abrogating epithelial cell apoptosis. The Journal of Immunology, 2006, 177: 1214–1220.

Clostridium difficile is a Gram-positive anaerobic enteric bacterium that is the major cause of colitis associated with antibiotic usage (1–3). C. difficile colitis is characterized by diarrhea, acute mucosal inflammation with an infiltration of neutrophils, epithelial cell destruction, microabscesses, pseudomembrane formation, and increased production of proinflammatory mediators. C. difficile colonizes the colon, but do not cause disease by invading the intestinal mucosa. Rather, C. difficile colitis is caused by exotoxins produced by the bacteria (3).

C. difficile produces two exotoxins, toxin A and toxin B. Studies of C. difficile colitis have focused mainly on the role of toxin A in disease pathogenesis. Intestinal epithelium is the initial site of host contact with C. difficile toxin A after its release at sites of bacterial colonization. Toxin A (308 kDa) binds to the apical surface of intestinal epithelial cells, is internalized, and has glucosyltransferase activity that results in glucosylation of small GTPases of the β family (RhoA, Rac, Cdc42) (4, 5). This was shown to result in disorganization of F-actin, with increased paracellular permeability and compromised epithelial tight junction function in monolayers of cultured intestinal epithelial cell lines (3, 6). The induction of mucosal inflammation by toxin A is thought to be initiated by cytokines and other mediators, which are released initially by intestinal epithelial cells (3, 7, 8). Whereas toxin A induces a necroinflammatory response in the intestinal mucosa of humans, rodents, and rabbits (3, 7, 9, 10), toxin B (270 kDa) has been reported also to cause colitis in humans, but not in several laboratory animal models (3, 11–13).

Inflammatory cells and enteric neurons have a role in toxin A–induced mucosal inflammation and tissue destruction. The mucosal inflammatory response is characterized by the recruitment of neutrophils and the release of proinflammatory and immune mediators, including IFN-γ, TNF-α, platelet-activating factor, PGE2, leukotrienes, and the chemokines CXCL8, MIP-2, CCL3, and CCL5 (7, 14–19). The importance of some of those mediators has been demonstrated in mutant mice. For example, enteritis and fluid secretion in response to C. difficile toxin A was attenuated in mice that did not produce IFN-γ (19), and in mice that lack the chemokine receptor CCR1 (i.e., receptor for CCL3 and CCL5) (18). In vitro, exposure of cultured intestinal epithelial cells to C. difficile toxin A was shown to cause mitochondrial damage, cytochrome c release, oxidative stress, and ultimately apoptosis and necrosis of those cells (3, 20, 21).

The transcription factor NF-κB regulates the expression of genes whose products have a central role in regulating acute inflammation, innate immunity, and cell survival (22–28). NF-κB can be activated by diverse stimuli (e.g., proinflammatory cytokines, microbes and microbial products, ionizing radiation, and oxidative stress) that signal its activation through the catalytic IκB-kinase β (IKKβ)3 unit of the IKK complex (26–28). IKKβ phosphorylates NF-κB-bound IκBs in the cytoplasm and targets their degradation, thereby freeing NF-κB dimers to translocate from the cytoplasm to the nucleus, where they regulate the transcription of NF-κB-dependent target genes (29).

‡Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093.

§Address correspondence and reprint requests to Dr. Martin F. Kagnoff, Professor of Medicine and Pediatrics, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0623. E-mail address: mkagnoff@ucsd.edu

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3 Abbreviations used in this paper: IKK, IκB-kinase; COX, cyclooxygenase; MLN, mesenteric lymph node; MPO, myeloperoxidase.
Understanding intestinal epithelial cell responses to *C. difficile* toxin A in vivo is essential for defining key steps in the early pathogenesis of *C. difficile* colitis, and may lead to the identification of targets useful for preventing disease. *C. difficile* toxin A was reported recently to activate NF-κB and up-regulate the expression of NF-κB proinflammatory target genes in intestinal epithelial cell lines (30). Given the importance of NF-κB activation in regulating epithelial cell proinflammatory genes and intestinal epithelial cell survival, we sought to determine whether NF-κB activation in intestinal epithelial cells in vivo has an important functional role in the pathogenesis of *C. difficile* toxin A-induced colitis. For these studies, we used as a model mice that cannot activate the classical NF-κB signaling pathway in intestinal epithelial cells due to a conditional deficiency of IKKβ.

**Materials and Methods**

**Mice**

Adult villin-Cre/Ikkβ−/− mice deficient in IKKβ in small intestinal and colonic epithelial cells were generated on a mixed C57BL/6j/129 background, as described (25). Mice were healthy and fertile, and did not manifest abnormalities in gastrointestinal tract development or function (25, 25). Littermate Ikkβ+/− mice, whose intestinal epithelial cells express IKKβ normally, were used as controls (25). Genotype was confirmed by PCR analysis of tail DNA (25).

**Toxins**

Toxin A was purified to homogeneity from culture supernatants of *C. difficile* strain VPI 10463 (American Type Culture Collection) (31). Protein concentrations were determined as described before (32). The toxin A used in these studies was confirmed to activate NF-κB and CXCL8 production by the HT-29 colon epithelial cell line (30). *Vibrio* cholera toxin was from EMD Biosciences.

**Ileal loops and toxin challenge**

To generate ileal loops, mice fasted overnight were anesthetized with ketamine/xylazine, a laparotomy was performed, and distal ileal loops, 4 cm in length, were constructed with the mesenteric vessels intact. Ileal loops were injected with toxin in PBS or PBS alone in 200 μl after which loops were returned to the abdominal cavity and mice were maintained under a heat lamp for the duration of the experiment. After sacrifice, ileal loops were returned to the abdominal cavity and mice were maintained under a heat lamp for the duration of the experiment. After sacrifice, ileal loops and toxin challenge were injected with toxin in PBS or PBS alone in 200 μl, after which loops were returned to the abdominal cavity and mice were maintained under a heat lamp for the duration of the experiment. After sacrifice, ileal loops were removed and processed for further studies. Fluid accumulation in ileal loops was determined by weighing loops before and after draining intraluminal fluid. All studies were approved by the University of California Institutional Animal Care and Use Committee.

**Isolation of intestinal epithelial cells**

Ileal loops were opened longitudinally, cut into 2- to 3-mm pieces, placed in HBSS containing 30 mM EDTA, and gently rocked for 10 min at 37°C, as described before (33). Supernatants were removed and centrifuged, and the resulting cell pellet, which contained few, if any, lamina propria cells, was washed in ice-cold PBS. Cell pellets and remaining tissue fragments were snap frozen in liquid nitrogen.

**Epithelial cell lysates**

Cell extracts were obtained by lysing epithelial cell pellets at 4°C in lysis buffer (150 mM NaCl, 20 mM HEPES (pH 7.6), 1.5 mM MgCl2, 0.2 mM EDTA, and 1% Triton X-100), supplemented with 1 mM DTT, 1:200 protease inhibitor mixture III (Calbiochem), 100 mM β-glycerophosphate, 1 mM NaF, and 1 mM sodium orthovanadate.

**Kinase assay, EMSA, and Western blotting**

IKK immune complex kinase assays and EMSAs were done, as described before (25, 33). For Western blotting, protein lysates were separated by SDS-gel electrophoresis and transferred to polyvinylidene difluoride membranes (33). Anti-IKKα Ab was from Imgenex.

**Myeloperoxidase (MPO) assay**

MPO activity in ileal loop segments was determined by enzymatic assay (34), using MPO purified from human leukocytes as a standard (Sigma-Aldrich).

**Real-time RT-PCR**

RNA was extracted with TRIzol reagent (Invitrogen Life Technologies), cDNA was generated using Superscript II kits (Invitrogen Life Technologies), as described before (35). Primers were designed using Primer Express 2.0 software (Applied Biosystems). Amplification of the expected single PCR product for each set of primers was confirmed by electrophoresis of the product on 1% agarose gels stained with ethidium bromide. The relative abundance of mRNAs was determined by real-time PCR, as described before (35, 36). Amplification conditions were 95°C, 15 s; 60°C, 1 min; 40 cycles. Data analysis was performed using PE Biosystems 7700 sequence detection system software (36).

The following primer pairs were used: MIP-2, sense, 5′-ACTCTCAAAAGACATCCAGAGCTTGA-3′ and antisense, 5′-CTAGATGCTCTGTTGGCGCC-3′; TNF-α, sense, 5′-ATGAGCACAGAACAGACTGATC-3′ and antisense, 5′-TACAGGCTTGTACCTGAAAT-3′; IL-6, sense, 5′-ACAACCCAGGGGCTTCCATAC-3′ and antisense, 5′-ACAATCAAGGATGACATTGAC-3′; cyclooxygenase (COX)-2, sense, 5′-CAGAACCGCATTTGCTCTG-3′ and antisense, 5′-TTGGACTTATGCGACAGAG-3′; X-linked inhibitor of apoptosis protein, sense, 5′-AATGGGACAGCGCTGTCTGGCAGG-3′ and antisense, 5′-TTACTGGACCCCTTGGTGGACC-3′ and antisense, 5′-TATTTGGACCCCTTGATGC-3′; c-FLIP, sense, 5′-GGTGAAGACATTCCAGGGA-3′ and antisense, 5′-CGGATGTCGGGGATAGGAAA-3′; survivin, sense, 5′-ATCCACTGCTCACCGAGAG-3′ and antisense, 5′-AAAAAAAACACTGGGGGA-3′; Bel-2, sense, 5′-GGTTGCAGGCTGGGCTTCT-3′; antisense, 5′-TGACCTCCAAATCCACGCT-3′; Bel-2, sense, 5′-CGCCGGAGAACAGGCTATGAAA-3′ and antisense, 5′-CCCCACTCTGATGCCCCCTTG-3′; cellular inhibitor of apoptosis proteins-2, sense, 5′-TTCAAGAAGCCGCTTTCTTACC-3′ and antisense, 5′-GCGTGAATACGTGGGAATCG-3′; p53, sense, 5′-AGATCCGGGCGGCTAAC-3′ and antisense, 5′-TCTGGTAGCATGGCATCCTTTT-3′; and GAPDH, sense, 5′-ATCAAGACCCCTTGTTGACC-3′ and antisense, 5′-CCAGTACTGCTCACGACATC-3′ and antisense, 5′-CCAGTACTGCTCACGAGATC-3′.

**Histological analysis**

Ileal loops were opened longitudinally, fixed as Swiss rolls in 10% Formalin overnight, and paraffin embedded. H&E-stained sections (5 μm) were scored for tissue injury using the following scale: no mucosal damage = 0; epithelial damage at villous tips with otherwise normal appearing villi = 1; epithelial damage extending beyond the villous tip without villous shortening = 2; subtotal villous destruction = 3; total villous destruction with mild to moderate crypt damage = 4; and total villous destruction and severe crypt damage = 5 (see Fig. 1A). Tissue injury was scored by two independent observers blinded to the experimental conditions. Each observer scored the three most severely affected areas of each section, after which scores were averaged to obtain the histologic damage score. The correlation coefficient on the scores of the two observers was r = 0.98, indicating that the scoring procedure was reproducible and observer independent.

**Apoptosis assays**

Apoptosis was assessed by TUNEL assay and by immunostaining for active caspase 3. TUNEL assay were done using the In Situ Cell Death Detection Kit (Roche Diagnostic Systems), according to the manufacturer’s instructions. For immunostaining active caspase 3, frozen sections were fixed in acetone and blocked with 1% BSA in TBS-Tween 20, and then incubated overnight at 4°C with affinity-purified rabbit anti-active caspase 3 (1/100 dilution) (BD Pharmingen), or rabbit IgG as a control, followed by incubation with Cy3-conjugated goat anti-rabbit IgG (1/1000 dilution) (Jackson ImmunoResearch Laboratories) for 1 h at room temperature.

**Bacterial translocation**

Mesenteric lymph nodes (MLN) from mice with ileal loops were homogenized in Bacto tryptic soy broth (BD Biosciences). For aerobic cultures, homogenates were plated on tryptic soy blood agar containing 10% sheep blood (REML) and then incubated at 37°C overnight. For anaerobic cultures, serial dilutions of homogenates were plated on Columbia blood agar with 10% sheep blood and then incubated at 37°C for 48 h in an anaerobic culture system (Oxide Atmosphere Generation System for Anaerobic Bacteriology and Anaero-Gen), according to the manufacturer’s instructions (Oxide), after which CFU were determined.
Statistical analysis
Differences between means were compared by t tests and by ANOVA with post hoc Tukey tests. Values of p < 0.05 were considered significant.

Results
Mucosal injury and fluid secretion in response to C. difficile toxin A
Ileal loops of control Ikkβ+/− mice were challenged by intraluminal injection of C. difficile toxin A (0.25–4 μg), after which histologic damage scores were determined. Representative examples of mucosal damage and accompanying assigned histologic damage scores are shown in Fig. 1A. Increasing doses of toxin A were accompanied by greater mucosal damage (Fig. 1B). Significant histologic damage was noted by 3 h after toxin A (4 μg) injection, with marked mucosal destruction in all loops by 4–5.5 h (Fig. 1C), whereas significant fluid accumulation was delayed and first seen 4 h after toxin A (4 μg) challenge (Fig. 1D). In contrast to C. difficile toxin A, there was no histologic damage, but significantly greater luminal fluid accumulation after injection of ileal loops with cholera toxin (4 μg) (fluid accumulation over 4 h: cholera toxin, 83.7 ± 8.2 mg/cm vs C. difficile toxin A, 18.5 ± 1.7; n = 3; p < 0.01).

Toxin A increases intestinal epithelial cell I KK activity and activates NF-κB in control, but not epithelial cell I KKβ-deficient mice
To determine whether toxin A activates NF-κB in normal intestinal epithelium in vivo, as was the case for intestinal epithelial cell lines (30), ileal loops of control Ikkβ+/− and intestinal epithelial cell I KKβ-deficient villin-Cre/Ikkβ−/− mice were challenged with toxin A (4 μg), after which IKK activity and NF-κB activation were determined in epithelial cells isolated from the loops. As shown in Fig. 2, toxin A increased IKK activity and activated NF-κB in epithelial cells isolated from ileal loops of control, but not epithelial cell I KKβ-deficient mice.

C. difficile toxin A causes greater mucosal damage and fluid secretion in epithelial cell I KKβ-deficient mice
To determine the importance of epithelial cell I KKβ in the host response to C. difficile toxin A, ileal loops in I KKβ-deficient mice were injected with toxin A (4 μg) and histologic damage was assessed over the ensuing 4 h. Ileal loops in villin-Cre/Ikkβ−/− mice manifested a more rapid onset of mucosal damage and significantly greater mucosal damage than those in control Ikkβ+/− mice (Fig. 3, A and B). Consistent with this, fluid secretion was significantly greater in the ileal loops of villin-Cre/Ikkβ−/− than control Ikkβ+/− mice (Fig. 3C).

Increased epithelial cell apoptosis in C. difficile toxin A-challenged I KKβ-deficient mice
Toxin A causes apoptosis of intestinal epithelial cell lines (3, 20, 21). To determine whether NF-κB activation in intestinal epithelial cells in vivo has an antiapoptotic role and promotes cell survival, we assessed apoptosis in toxin A-challenged ileal loops. Toxin A (4 μg) resulted in a significant increase in epithelial cell apoptosis in the ileal loops of epithelial cell I KKβ-deficient compared with control mice, as assessed by TUNEL assay (Fig. 4A), immunostaining for active caspase 3 (Fig. 4B), and H&E staining (Fig. 5). Moreover, increased epithelial cell apoptosis in loops from epithelial cell I KKβ-deficient mice was detected by 1–2 h after toxin A challenge. These data indicate that NF-κB-regulated genes in intestinal epithelial cells can have an important role in promoting

![FIGURE 1. Mucosal damage and fluid accumulation in C. difficile toxin-challenged ileal loops. Ileal loops were stained with H&E after intraloop instillation of C. difficile toxin A or PBS. A, H&E staining of loops that are representative of each scoring category. B, Histologic damage scores in loops of Ikkβ+/− mice 4 h after administration of different toxin doses. Values are mean ± SE; n = 4–14 loops per group; ANOVA, p < 0.0001; *, post hoc test; p < 0.01 compared with PBS-injected control. C, Histologic damage scores in loops of Ikkβ−/− mice at various times after intraloop injection of 4 μg of toxin A. Values are means ± SE; n = 4–14 loops per group; ANOVA, p < 0.0001; *, post hoc test; p < 0.01 compared with 1 h. D, Fluid accumulation in loops of Ikkβ−/− mice at various times after intraloop injection of 4 μg of toxin. Values are mean ± SE; n = 4–12 loops per group; ANOVA, p < 0.0001; *, post hoc test; p < 0.01 compared with 1, 2, and 3 h.](image1)

![FIGURE 2. Epithelial cell NF-κB and IKK activity in C. difficile toxin challenged in ileal loops. Nuclear and cytoplasmic extracts were prepared from isolated epithelial cells obtained from ileal loop mucosa of Ikkβ+/− and villin-Cre/Ikkβ−/− mice before and 30 min after intraloop challenge with C. difficile toxin A (4 μg). Cytoplasmic extracts were analyzed for IKK kinase (KA) activity by an in vitro kinase assay (KA) using GST-IκBα as substrate. IKK recovery was determined by Western blotting (WB) using anti-IKKα Ab. NF-κB DNA-binding activity was determined by EMSA. NF-Y-binding activity was used as a loading control.](image2)
epithelial cell survival after epithelial encounter with *C. difficile* toxin A.

Increased mucosal production of proinflammatory mediators in toxin A-challenged ileal loops of epithelial IKKB-deficient mice

NF-κB is a central regulator of proinflammatory gene transcription by intestinal epithelial cell lines (37). Therefore, one might predict that a deficiency in epithelial IKKβ and NF-κB activation would result in decreased intestinal mucosal inflammation. However, the opposite was the case, and we found increased mucosal inflammation in ileal loops of epithelial IKKβ-deficient mice. This was associated with an increased influx of neutrophils in IKKβ-deficient mice compared with controls, as assessed by mucosal MPO activity. Thus, MPO activity 4 h after toxin A (4 μg) challenge in epithelial cell IKKβ ileal loops was 3.6 ± 0.5 U/g vs 0.9 ± 0.2 U/g in loops of control mice (n = 4; p < 0.01). Consistent with this, we found increased expression of several NF-κB target genes whose products regulate leukocyte migration and inflammation, and mediators that are putatively relevant for mucosal cytoprotection and repair, in epithelial cell IKKβ-deficient ileal loops. As shown in Fig. 6, relative mRNA levels of the neutrophil chemoattractant MIP-2 were significantly greater in the intestinal mucosa of epithelial cell IKKβ-deficient than control mice at each time after toxin A challenge. Moreover, MIP-2 in the loops of epithelial cell IKKβ-deficient mice derived from cells in the lamina propria (data not shown). The most marked increase in MIP-2 expression, as well as the expression of TNF-α, another NF-κB target gene, occurred 2 h after toxin A injection (Fig. 6). IL-6 mRNA levels were initially similar in the mucosa of toxin A-challenged control and epithelial cell IKKβ-deficient loops, but increased in the epithelial IKKβ-deficient loops by 4 h after challenge. This was also the case for inducible COX-2. The latter finding is consistent with the increased toxin A-induced luminal secretory response in intestinal loops of IKKβ-deficient compared with control mice.

Expression of NF-κB-regulated antiapoptotic genes

Notably, little, if any, increase in apoptosis was observed in cells in the lamina propria of epithelial cell IKKβ-deficient cell compared with control mice after toxin A challenge (Figs. 4 and 5). Whereas the intestinal epithelium of epithelial cell IKKβ-deficient mice is deficient in IKKβ and NF-κB activation, this is not the case for cells in the lamina propria, as we showed before (25). Given the increase in tissue damage in epithelial IKKβ-deficient mice with little apoptosis of cells in the lamina propria, we posited that cells in the lamina propria of epithelial IKKβ-deficient mice might manifest increased expression of NF-κB-regulated antiapoptotic target genes as part of the host’s mucosal protective response. This was the case, as shown in Fig. 7. In the absence of epithelial cell IKKβ and NF-κB activation, which is associated with a significant increase in epithelial apoptosis after *C. difficile* toxin challenge, cells in the lamina propria more markedly up-regulated a group of NF-κB-regulated genes that are important for cell survival.

Increased bacterial translocation in epithelial cell IKKβ-deficient mice

Bacteria and bacterial products that cross the epithelial barrier can activate and increase mucosal inflammation. To determine whether the greater acute mucosal inflammation and tissue damage in epithelial IKKβ-deficient mice after toxin A challenge were accompanied by increased bacterial translocation, MLN from epithelial
cell IKKβ-deficient and control mice were cultured for aerobic and anaerobic bacteria 4 h after C. difficile toxin A (4 μg) challenge of ileal loops. MLN of epithelial IKKβ-deficient compared with control mice contained significantly greater numbers of aerobic and anaerobic bacteria (9.7 × 10^3 vs 2.5 × 10^3 aerobes and 3.0 × 10^4 vs 1.8 × 10^4 anaerobes, respectively; values are CFU/g tissue; n = 3; p = 0.01). MLN from PBS-injected mice were sterile in both the epithelial cell IKKβ-deficient and control mice.

**Discussion**

We show that C. difficile toxin A activates NF-κB in intestinal epithelium in vivo. Because toxin A produced by C. difficile initially interacts with intestinal epithelium, we posited that the activation of epithelial cell NF-κB by C. difficile toxin A in vivo might have a significant role in the pathogenesis of C. difficile-induced mucosal injury and inflammation. Using a genetic model in which IKKβ was conditionally depleted in intestinal epithelial cells in vivo, we found that NF-κB activation by C. difficile toxin A in intestinal epithelial cells has important functional consequences for host mucosal protection. In the absence of toxin A-induced activation of NF-κB in intestinal epithelial cells, in vivo challenge with C. difficile toxin A caused significantly greater mucosal damage that was characterized by a more rapid onset, increased epithelial cell apoptosis, increased mucosal inflammation, increased intestinal secretion, and increased bacterial translocation.

NF-κB regulates the transcription of genes that govern proinflammatory responses and apoptosis of intestinal epithelial cells (23, 25, 33, 37). The finding that toxin A challenge caused greater apoptosis of epithelial cells in ileal loops of IKKβ-deficient than control mice is consistent with the importance of NF-κB target genes in promoting epithelial cell survival by inhibiting apoptosis. Increased epithelial apoptosis occurred within 1–2 h of in vivo

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**FIGURE 5.** Increased apoptosis in ileal loops of toxin A-challenged villin-Cre/Ikkβ^+/− mice. A, Ileal loops were injected with PBS (a) or C. difficile toxin A (4 μg) (b–d) and stained with H&E. a, Loop from villin-Cre/Ikkβ^+/− mouse 2 h after PBS injection. b, Loop from control Ikkβ^+/− mouse 2 h after toxin injection. c and d, Loop from villin-Cre/Ikkβ^+/− mouse 1 h (c) or 2 h (d) after toxin injection. Original magnification, ×40. B, Mean ± SE of numbers of apoptotic cells in crypt sections 2 h after injection of ileal loops with toxin or PBS. ANOVA, p < 0.005; *, post hoc test; p < 0.01 villin-Cre/Ikkβ^+/− vs PBS and p < 0.05 villin-Cre/Ikkβ^+/− vs Ikkβ^+/−.

**FIGURE 6.** Expression of proinflammatory genes. Relative mRNA expression levels were examined in ileal loops of villin-Cre/Ikkβ^+/− (●) and control Ikkβ^+/− (○) mice 1, 2, and 4 h after C. difficile toxin A challenge. Levels of the indicated mRNAs were quantitated by real-time PCR and normalized to the level of GAPDH mRNA in the same loop. Values are from a representative experiment. Similar results were obtained in two or more repeated experiments.

**FIGURE 7.** Expression of antiapoptotic genes. Relative mRNA expression levels were examined in ileal loops of villin-Cre/Ikkβ^+/− (●) and Ikkβ^+/− (○) mice 1, 2, and 4 h after C. difficile toxin A challenge. Levels of the indicated mRNAs were quantitated by real-time PCR and normalized to the level of GAPDH mRNA in the same loop. Values are from a representative experiment. Similar results were obtained in two or more repeated experiments.
toxin A challenge in epithelial cell IKKβ-deficient loops, suggesting decreased epithelial cell survival as a mechanism underlying later changes in mucosal inflammation and damage, increased fluid secretion, and bacterial translocation. In cultured human colon cancer epithelial cell lines, C. difficile toxin A was shown to be internalized and localized to mitochondria, where it caused mitochondrial damage, cytochrome c release, and the generation of reactive oxygen intermediates (3, 38). Notably, those events preceded toxin A-induced p-glucoysylation and the disorganization of F actin by 15–30 min, were accompanied by NF-κB activation, and culminated in epithelial cell apoptosis or necrosis (3, 38). In other studies, inhibition of monoglucoysylation of p by toxin A was shown to have little effect on the induction of apoptosis of an intestinal epithelial cell line by toxin A (C. Pothoulakis, unpublished observations).

We found a significantly greater increase in the activation of NF-κB-regulated genes that are important in mediating antiapoptotic responses in cells in the subepithelial region (i.e., lamina propria) of epithelial IKKβ-deficient compared with control mice. In that IKKβ deficiency is restricted to the epithelial cells of intestinal epithelial cell IKKβ-deficient mice, and does not affect cells in the lamina propria, the marked increase in expression of NF-κB-regulated antiapoptotic genes by lamina propria cells may explain, at least in part, the relative paucity of apoptotic cells in the lamina propria. We also found little apoptosis of lamina propria cells in vivo in toxin A-challenged control ileal loops, indicating that apoptosis of cells in the subepithelial region was not a major functional consequence of luminal exposure to toxin A in vivo in normal intestine in this model. Nonetheless, it has been reported that isolated lamina propria cells (e.g., macrophages, T cells, eosinophils, and mast cells) incubated with toxin A in vitro undergo apoptosis (39, 40).

Increased mucosal inflammation in C. difficile toxin A-challenged ileal loops from intestinal epithelial cell IKKβ-deficient relative to control mice was characterized by significantly greater mucosal mRNA expression of the neutrophil chemotactic protein MIP-2, and an increased mucosal influx of neutrophils. MIP-2 was previously reported to be important in the toxin A-induced neutrophil predominant inflammatory response in rat intestinal loops (7). Although MIP-2, a prototypic NF-κB target gene, can be produced in normal mice by intestinal epithelial cells and cells in the lamina propria, the marked increase in MIP-2 expression in intestinal epithelial cell IKKβ-deficient mice was most due to MIP-2 expression by cells in the lamina propria. This could result from the direct stimulation of lamina propria cells by toxin A that gains access across a damaged epithelial barrier (41) or reflect increased activation of lamina propria cells by enteric bacteria or their products that crossed the damaged epithelial barrier. Although expression of IL-6 transcript levels in the mucosa of epithelial cell IKKβ-deficient loops at later times after toxin A challenge may mediate cytoprotective responses in the intestinal mucosa (42), toxin A-induced up-regulation of COX-2, through its role in regulating PG production, may play a role in the increased intestinal fluid secretion (43, 44) noted in IKKβ-deficient ileal loops. These latter events are consistent with the increased activation of proinflammatory mediators in a chemical model of colon epithelial injury in epithelial IKKβ-deficient mice (33) and could reflect the host’s attempt to control the severe tissue damaging inflammatory response and/or to promote mucosal healing in the more markedly injured intestinal mucosa.

In summary, we show that activation of epithelial cell NF-κB can have significant functional consequences in C. difficile toxin A-induced mucosal inflammation and injury in vivo. Moreover, the mechanism of its host-protective role appears to be mediated, in part, through abrogating epithelial cell apoptosis and promoting epithelial cell survival, which is important for ameliorating damage to the epithelial barrier. In the absence of activation of the classical NF-κB pathway in intestinal epithelial cells, increased epithelial cell injury was accompanied by increased mucosal inflammation, mucosal damage, and fluid secretion into the intestinal lumen. These findings have potential therapeutic implications, because concurrent use of pharmacologic agents that inhibit NF-κB activation in individuals with C. difficile colitis might be predicted to increase, rather than ameliorate, mucosal inflammation and damage.

Disclosures
The authors have no financial conflict of interest.

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