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NF-κB Activation Pathway is Essential for the Chemokine Expression in Intestinal Epithelial Cells Stimulated with *Clostridium difficile* Toxin A

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Abstract

Intestinal epithelial cells are known to upregulate the expression of several chemokines in response to stimulation with bacterial toxin. However, the cellular mechanisms of *Clostridium difficile* toxin A-induced mucosal inflammation have not yet been fully elucidated. In this study, we investigated whether nuclear factor-kappa B (NF-κB) could regulate chemokine expression in intestinal epithelial cells. Toxin A increased the levels of NF-κB complexes containing p65/p50 heterodimers and p65/p65 homodimers. Concurrently, toxin A decreased the levels of IκBα. Toxin A stimulation also increased the signals of phosphorylated IκB kinase (IKK)α/β and NF-κB-inducing kinase (NIK). In the toxin A-stimulated HT-29 cells, the suppression of IKK or NIK inhibited the upregulation of downstream target genes of NF-κB such as IL-8 and monocyte-chemotactic protein (MCP)-1 and similarly, inhibition of NF-κB also downregulated the expression of IL-8, growth-related oncogene-α, and MCP-1. These results suggest that NF-κB signalling events may be involved in the inflammatory responses to toxin A produced by toxigenic *C. difficile*.

Introduction

Clostridium difficile causes antibiotic-associated diarrhoea and pseudomembranous colitis in humans [1]. Pathogenic strains of *C. difficile* release two types of exotoxins: toxin A (308 kDa) and toxin B (269 kDa). In animal models, toxin A causes fluid secretion, mucosal oedema and villous disruption by inducing massive acute inflammation with neutrophil infiltration [1, 2]. Theoretically, the first host cells that *C. difficile* toxins interact with are epithelial cells in the colon. Thus, mucosal inflammatory signals may be initiated from intestinal epithelial cells in response to *C. difficile* toxins. Working from this hypothesis, several reports have demonstrated that intestinal epithelial cells can produce the chemokine interleukin (IL)-8, growth-related oncogene (GRO)-α, and monocyte-chemotactic protein (MCP)-1 in response to *C. difficile* toxin A [1, 3–5]. However, the exact pathogenic mechanism of toxin A-induced inflammation has not yet been fully investigated.

Many of the genes that are activated in intestinal epithelial cells after bacterial infection or enterotoxin stimulation are target genes of the transcription nuclear factor-kappa B (NF-κB). NF-κB is a dimeric transcription factor composed of homodimers or heterodimers of Rel protein, of which there are five family members in mammalian cells [RelA (p65), c-Rel, Rel B, NF-κB1 (p50) and NF-κB2 (p52)] [6]. Heterodimers of p65 and p50 are the predominant NF-κB complexes that translocate to the nucleus after enterotoxin stimulation or bacterial infection of intestinal epithelial cells [7, 8]. NF-κB dimers are held in the cytoplasm in an inactive state by inhibitory proteins called IκB [9]. IκB kinase (IKK) is known to directly phosphorylate IκB, which then undergoes ubiquitin-mediated proteolysis, thereby releasing NF-κB dimers to translocate to the nucleus [10]. Although toxin A can activate NF-κB in monocytes [11] and intestinal epithelial cells [3], the role of NF-κB in the toxin A-induced signal transduction pathway in intestinal epithelial cells has not been clarified. Thus, the present study tested whether intestinal epithelial cells could use a NF-κB signal transduction pathway to activate the host's chemokine response to *C. difficile* toxin A stimulation.

Materials and methods

Clostridium difficile toxin A and cell culture. C. difficile toxin A was purchased from Sigma Chemical Co. (St Louis, MO, USA). To confirm that toxin A did not

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contain contaminating toxin B, we performed ELISA for toxin B using the TechLab C. difficile TOX A/B ELISA test kit (Blacksburg, VA, USA). In this experiment, the toxin A preparation from Sigma Chemical Co. did not have detectible toxin B activity. In addition, the cytotoxic activity against the HT-29 cells was completely neutralized with specific antitoxin A antibody and that the addition of antitoxin B antibody had no effect on the activity.

HT-29 human colon epithelial cells (ATCC HTB 38) were grown in DMEM (pH 7.4, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA), 2 mM L-glutamine, 25 mM HEPES, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin), as described previously [12]. Primary human colon epithelial cells were obtained from normal-appearing mucosa of surgically resected colons from individuals with colon cancer, as described previously [7, 13]. Freshly isolated colon epithelial cells were cultured at 2×10^6/ml in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). Epithelial cell preparations had less than 3% contamination with B cells and monocytes/macrophages, as assessed by flow cytometry using CD19/20 and CD14 as markers. T-cell contamination was less than 3% in the epithelial cell preparation, as assessed by flow cytometry using CD3 as a marker.

In some experiments, intestinal epithelial cells were treated with an NF-κB essential modifier (NEMO)-binding domain (NBD) peptide (200 μM, Peptron, Daejeon, Korea) or an inhibitor of NF-κB, calpain-1 inhibitor (25 μM, Calbiochem, La Jolla, CA, USA) for 1 h before addition of toxin A. An NBD peptide can block association of NEMO with the IKK complex and inhibit NF-κB activation [14, 15]. Sequences of the wild-type and mutant peptides are drqikiwfqnrrmkwkkTALDWSWLQTE (wild) and drqikiwfqnrrmkwkkTALDASALQTE (mutant). Positions of the W→)A mutations are underlined [14].

Electrophoreticmobility shift assays. Cells were harvested, and nuclear extracts were prepared as previously described [7, 8]. The concentrations of proteins in the extracts were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Electrophoretic mobility shift assays (EMSA) were performed according to the protocol of the manufacturer (Promega, Madison, WI, USA). In brief, 5 μg of nuclear extracts were incubated for 30 min at room temperature with γ^{32}P-labelled oligonucleotide probe corresponding to a consensus NF-κB binding site. After incubation, bound and free DNA were resolved on 5% native polyacrylamide gels as described previously [7]. Supershift assays were used to identify the specific members of the NF-κB family activated by stimulation with toxin A. EMSA was performed as described above, except that rabbit antibodies (1 μg/reaction) against NF-κB proteins p50, p52, p65, c-Rel and Rel B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added during the binding reaction period [7].

Immunoblots. Confluent monolayers in six-well plates were washed with ice-cold PBS and lysed in a 0.5 ml/well lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0.1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotonin) as described previously [7, 8]. Protein concentrations in the lysates were determined by the Bradford method (Bio-Rad). Fifteen to fifty microgram protein/lane was size-fractionated on 6% polyacrylamide minigel (Mini-PROTEIN II; Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (0.1 μm pore size). Specific proteins were detected using mouse anti-human IκBα (Santa Cruz Biotechnology), IKKα, IKKβ, phospho-IKKα/IKKβ and NF-κB-inducing kinase (NIK) (Cell Signaling Technology, Beverly, MA, USA) as primary antibodies, and peroxidase-conjugated anti-mouse IgG (Transduction Laboratories, Lexington, KY, USA) as a secondary antibody. Specifically bound peroxidase was detected by enhanced chemiluminescence (ECL system; Amersham Life Science, Buckinghamshire, England) and exposure to X-ray film (XAR5; Eastman Kodak Company, Rochester, NY, USA).

Plasmids and transfection. A mammalian expression vector encoding a haemagglutinin (HA) epitope-tagged mutant IκBα (IκBα-AA) with substitutions of serine with alanine at positions 32 and 36 (a gift of Joseph A. DiDonato, Cleveland Clinic Foundation, Cleveland, OH) [16] and an expression vector encoding a FLAG-tagged IKKβ in which lysine was replaced by alanine at position 44 (IKKβ-AA) (a gift of Dr Mercurio, Signal Pharmaceuticals, San Diego, CA, USA) [17] were used to block NF-κB activation as described previously [7, 8]. An expression vector for an NIK catalytic mutant that has a double replacement of lysine with alanine residues at positions 429 and 430 and lacks kinase activity (NIK-AA) was a gift of Dr Karin in the University of California, San Diego [8]. pIL8-luciferase, p2x NF-κB-luciferase and pβ-actin-luciferase transcriptional reporters were provided by Dr Kagnoff in the University of California, San Diego [8]. A pMCP-1-luciferase transcriptional reporter was provided by Dr Ik-Sang Kim in the Seoul National University College of Medicine, Seoul, Korea [18]. Cells in six-well dishes were transfected with 1.5 μg of plasmid DNA, using Lipofectamine Plus (Gibco BRL), according to the manufacturer's instructions [7]. The transfected cells were incubated for 48 h at 37 °C in a 5% CO_2 incubator. Cells were incubated with C. difficile toxin A or recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) as a control for 6 h, after which cells were harvested and whole-cell lysates were prepared as described previously [7]. Briefly, cells were lysed at 4 °C for 25 min in a whole-cell lysis buffer (0.1 M KPO_4, 0.1 M DTT, 0.5% Triton X-100, pH 7.8).

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Luciferase activity was determined in accordance with the manufacturer's instruction (Tropix Inc., Bedford, MA, USA) and light release was quantitated for 10 s using a luminometer (MicroLumat Plus, Berthold GmbH & Co. KG, Bad Wildbad, Germany) [7].

Recombinant retrovirus and small interfering RNA transfection. Dominant-negative IκBα (S32A, S36A) [16] was amplified with sense (5′-aaccATGGCATACCC ATACGACGTCCCAGACTACGCTttccaggcggccgagcgc-ccccaggag-3′) and antisense (5′-aaaaGGATCC tcataacgtca-gacgctggcct-3′) primers by using high-fidelity Taq polymerase (Life Technology), in which nucleotides in uppercase indicate an HA tag. The PCR products were digested with NcoI and BamHI restriction enzymes and cloned into the corresponding sites in MFG retroviral vector to replace the GFP sequence of MFG.GFP.IRES.puro. The retroviral plasmids obtained were introduced into 293 gpg retrovirus packaging cell line by transient transfection with Lipofectamine (Life Technology) [19]. After 72 h, the supernatants were harvested and used for retroviral infection. The virus titres, measured in NIH3T3 cell line by puromycin-resistant colony formation, were between 10^5 and 5×10^5/ml (retrovirus-IκBα-AA). The infection and selection of target cells with puromycin was performed as described previously [19].

Small interfering RNA (siRNA) against NIK was constructed as described [20]. A NIK-specific siRNA and a negative (nonsilencing) control siRNA were purchased from Dharmacon Research Inc. (Lafayette, CO, USA). For transfection, HT-29 cells were cultured in 24-well plates to 50–80% confluence. The cells were then transfected with NIK-specific siRNA and nonsilencing siRNA using RNAiFect (QIAGEN, Valencia, CA, USA) as transfection reagent, following the manufacturer's instructions. Briefly, 100 nM of NIK siRNA or nonsilencing siRNA was diluted in serum-free medium to give a final volume of 100 μl and incubated with 5 μl of RNAiFect for 15 min at room temperature. The transfection mixture was added to the respective wells, each containing 300 μl of medium (10% FBS content). Transfections were incubated for 72 h prior to assay.

RT-PCR analysis and chemokine ELISA. Confluent monolayers of HT-29 cells in 24-well plates were stimulated with toxin A for the indicated times, after which total cellular RNA was extracted from the cells using an acid guanidinium thiocyanate–phenol–chloroform method [5]. Oligonucleotide primers used for PCR amplification and the sizes of the PCR products obtained from target cellular RNA and synthetic standard RNA are described in the previous report [21]. Quantitative RT-PCR using an internal standard was used to quantify chemokine mRNA levels, as assessed previously [5]. Synthetic standard RNA was kindly provided by Dr Kagnoff in the University of California, San Diego. PCR amplification consisted of 35 cycles of 1 min denaturation at 95 °C, 2.5 min annealing and extension at either 60 °C (IL-8 and GRO-α), 65 °C (MCP-1) or 72 °C (β-actin).

Chemokines in culture supernatants were assayed by ELISA. Before chemokine proteins were measured, the supernatants were filtered through 0.22-μm filters to remove any contaminants. The levels of human IL-8, GRO-α and MCP-1 were determined by Quantikine immunoassay kits (R&D Systems). Chemokine proteins were tested in triplicate.

Statistical analyses. Data are presented as mean ± standard deviation (SD) for quantitative RT-PCR and mean ± standard error of the means (SEM) for ELISA and luciferase assay. Wilcoxon's rank sum test was used for statistical analysis. $P<0.05$ was considered statistically significant.

Results

Clostridium difficile toxin A induces NF-κB activation and IκB degradation in human colon epithelial cells

To determine whether toxin A activates NF-κB in intestinal epithelial cells, DNA-binding studies were performed using HT-29 cell extracts after stimulation with toxin A. Stimulation of these cells with toxin A increased NF-κB DNA binding, as shown by EMSA (Fig. 1A). In contrast, degradation of IκBα was observed in toxin A-stimulated cells. Similar results were also obtained from the primary human colon epithelial cells (Fig. 1B). In this experiment, NF-κB activation in primary epithelial cells was not increased after 2 h in the absence of toxin A. At 10 ng/ml of toxin A, approximately 88% of HT-29 cells and approximately 82% of primary colon epithelial cells were viable 24 h after incubation. At the unstimulated condition, the viability was approximately 98% of HT-29 cells and approximately 93% of primary epithelial cells 24 h after incubation, as assessed by Trypan Blue exclusion. The uptake of dye Trypan Blue indicates the loss of membrane integrity. The magnitude of the NF-κB response was dependent on the concentration of stimulating toxin A per epithelial cell. Stimulating HT-29 cells with increasing concentrations of toxin A led to increased luciferase activity in cells transfected with 2× NF-κB promoter plasmid. At concentrations of 1, 10, 100, and 1000 ng/ml of toxin A, reporter gene activity of 2× NF-κB increased 1.1-, 2.9-, 5.6- and 5.2-fold at 6 h after stimulation, respectively, relative to those of unstimulated controls (mean value, $n = 3$). However, heat-inactivated toxin A, which was heated for 30 min at 60C°, did not activate the NF-κB- or IL-8-reporter genes in HT-29 intestinal epithelial cells (Table 1).

NF-κB exists as either homo- or heterodimeric complexes [22]. To identify the specific NF-κB subunits that comprise the NF-κB signal detected by EMSA in toxin A-stimulated HT-29 cells, supershift assays were performed. Specific antibodies to p50, p52, p65, c-Rel and Rel B were used for these experiments. Supershift studies demonstrated that antibodies to p65 and p50 shifted the NF-κB signals

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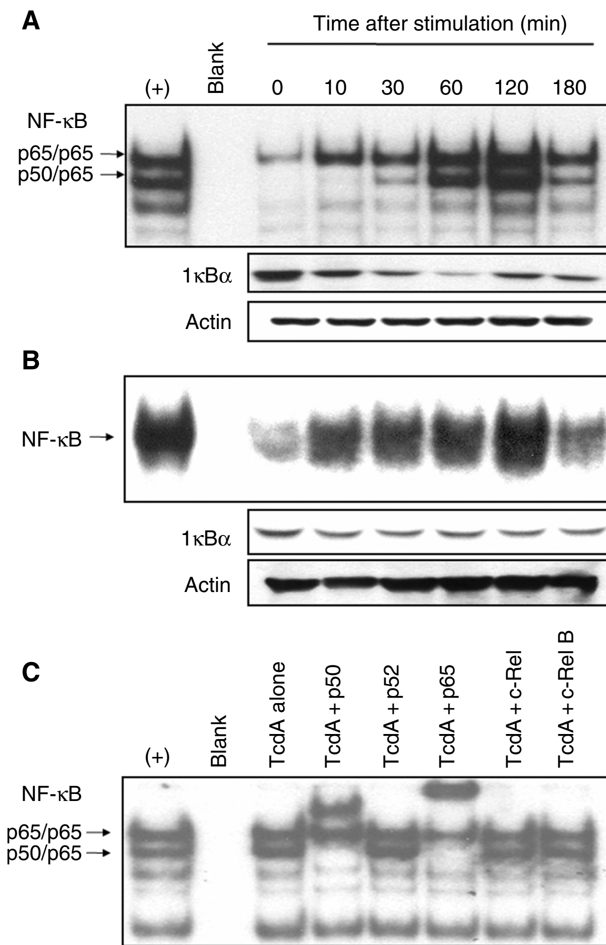

Figure 1 NF-κB activation and IκB degradation in human intestinal epithelial cells stimulated with *Clostridium difficile* toxin A. HT-29 cell lines (A) and primary colon epithelial cells (B) were stimulated with toxin A (20 ng/ml). NF-κB DNA-binding activity was assessed by electrophoretic mobility shift assays (EMSA) at the indicated times. Immunoblots for concurrent IκBα levels in cells under the same conditions are provided beneath each EMSA time point. (C) Supershift assays using nuclear extracts from HT-29 cells stimulated with *C. difficile* toxin A (TcdA) for 1 h were performed using antibodies to p50, p52, p65, c-Rel and Rel B. The results are representative of more than five repeated experiments. (+) represents positive control in which HT-29 cells were treated with TNF-α (20 ng/ml) for 1 h (–) represents negative control.

Table 1 NF-κB activation and IL-8 expression in HT-29 cells stimulated with *Clostridium difficile* toxin A*

Stimuli	Activation of luciferase reporter gene†		
	NF-κB	IL-8	β-actin
Unstimulated control	1	1	1
Toxin A	3.1 ± 0.3	6.9 ± 1.1	0.9 ± 0.2
Heat-inactivated toxin A	0.9 ± 0.2	1.1 ± 0.2	1.1 ± 0.3

*HT-29 cell lines were transfected with p2x NF-κB-, pIL-8- or pβ-actin-luciferase transcriptional reporter. After 48 h, the transfected cells were stimulated with toxin A (20 ng/ml) or heat-inactivated toxin A for 2 h (NF-κB) or 6 h (IL-8 and β-actin).

†Data are expressed as mean fold induction ± SEM of luciferase activity relative to unstimulated controls ($n = 5$).

significantly. In contrast, antip52, anti-c-Rel or anti-Rel B antibodies did not shift the NF-κB signal (Fig. 1C). Similar findings were found with primary colon epithelial cells (data not shown). These results suggest that NF-κB activation by toxin A stimulation may be mediated predominantly by heterodimers of p65/p50 and homodimers of p65/p65.

The inhibition of NF-κB suppresses chemokine expression in intestinal epithelial cells stimulated with *Clostriium difficile* toxin A

To confirm that the activated NF-κB was directly related to toxin A-induced chemokine production, we determined if the inhibition of NF-κB could influence the chemokine expression in HT-29 cells. As shown in Fig. 2A, transfection with retrovirus-IκBα-AA completely blocked NF-κB activity in toxin A-stimulated cells, whereas the control retrovirus containing *GFP* plasmid did not change the NF-κB activity. In this system, blocking the activation of NF-κB in HT-29 cells transfected with retrovirus-IκBα-AA significantly inhibited the expression of chemokines upregulated by toxin A (Fig. 2B and Table 2). Similar results were obtained in the primary colon epithelial cells

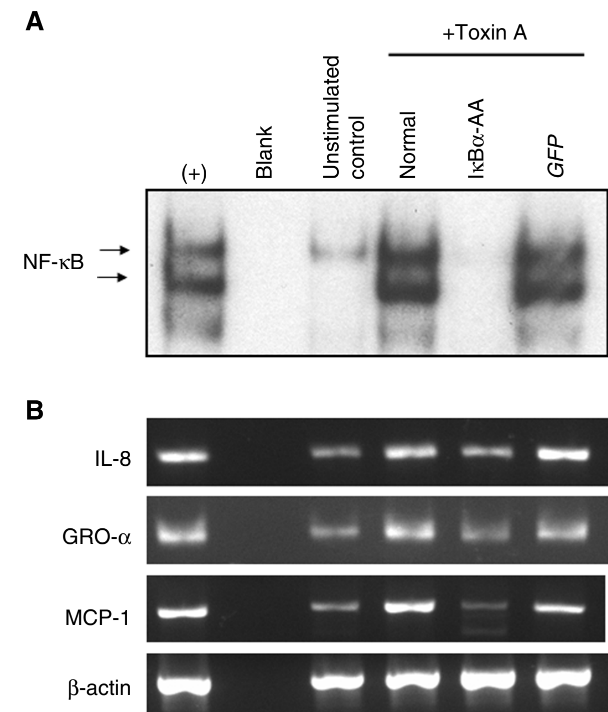

Figure 2 Expression of chemokine genes in *Clostridium difficile* toxin A-stimulated HT-29 cells transfected with retrovirus containing IκBα super-repressor. (A) HT-29 cells were transfected with either retrovirus containing IκBα-superrepressor (IκBα-AA) or control virus (*GFP*). At 48 h after transfection, the cells were stimulated with toxin A (20 ng/ml) for 1 h. NF-κB-binding activity was assayed by electrophoretic mobility shift assay. (B) Transfected HT-29 cells were then stimulated with toxin A (20 ng/ml) for 6 h. Total RNA was reverse-transcribed using an oligo(dT) primer and amplified by PCR using chemokine gene-specific primers.

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Table 2 Transfection with retrovirus containing IκBα superrepressor reduced the levels of chemokine mRNA transcripts in HT-29 cells stimulated with *Clostridium difficile* toxin A*

	Control	Toxin A	Toxin A + IκBα-AA	Toxin A + *GFP*
IL-8 ($\times 10^5$)	5.9 ± 2.3 (1)	346 ± 282 (59)	50 ± 62† (8)	334 ± 278 (57)
GRO-α ($\times 10^5$)	2.2 ± 1.1 (1)	99 ± 26 (45)	22 ± 11† (10)	102 ± 59 (46)
MCP-1 ($\times 10^4$)	6.7 ± 3.9 (1)	117 ± 38 (17)	28 ± 21† (4)	119 ± 86 (18)
β-actin ($\times 10^6$)	5.3 ± 1.9 (1)	4.6 ± 4.1 (0.9)	6.1 ± 3.2 (1.2)	5.4 ± 3.2 (1.0)

GRO, growth-related oncogene; MCP, monocyte-chemotactic protein.
*HT-29 cells were transfected with either retrovirus containing IκBα- superrepressor (IκBα-AA) or control virus (*GFP*). At 48 h after transfection, the cells were stimulated with toxin A (20 ng/ml) for 6 h, at which point total cellular RNA was extracted. The values represent mean numbers of mRNA transcripts ± SD per microgram of total RNA ($n = 5$). Values in parentheses are mean fold induction compared with the unstimulated control.
†Values are significantly different from those for samples treated with toxin A alone ($P < 0.05$).

stimulated with toxin A in the presence of an inhibitor of NF-κB, calpain-1 inhibitor [control, 102.7 ± 16.5; toxin A, 1085.3 ± 182.0; toxin A + calpain-1 inhibitor, 208.7 ± 24.8; mean ± SEM (pg/ml of IL-8) of three separate experiments]. These results demonstrate a direct association between toxin A-induced NF-κB activation and chemokine production.

Clostridium difficile toxin A increases phosphorylated IKK and NKK signals in instestinal epithelial cells

Major pathways for NF-κB activation involve the activation of IKK, which is followed by IκB degradation. In addition, activation of IKK can be mediated through NIK [10]. In the present study, toxin A increased the signals of phosphorylated IKKα/β and phosphorylated NIK in HT-29 cell lines (Fig. 3). In this system, addition of an IKK inhibitor, NBD peptide, into HT-29 cells significantly attenuated the increased IL-8, GRO-α, and MCP-1 secretion induced by toxin A stimulation (Fig. 4). There was no significant difference in cell number between toxin A-treated group and toxin A + NBD peptide-treated group in this experiment (data not shown). Therefore, the reduction of chemokine expression resulting from NBD peptide treatment was not because of decreased cell density.

Activation of NF-κb, IL-8 and MCP-1 reporter genes induced by *Clostridium difficile* toxin A is inhibited by IκBα, IKKβ or NIK superrepressors

We next determined whether the activation of IκB, IKK and/or NIK was involved in signalling pathway mediating the response to toxin A stimulation in HT-29 cells. As shown in Fig. 5, the activation of the IL-8, MCP-1, and NF-κB transcriptional reporter was inhibited in HT-29 cells when cotransfected with IκBα, IKKβ or NIK superrepressor plasmid, but not when cotransfected with control plasmid. These superrepressor plasmids significantly suppressed the phosphoylated protein signals of IKKα/β and NIK in HT-29 cells stimulated with toxin A (Fig. 6). However, the inhibition of IL-8, MCP-1 and NF-κB transcriptional reporter by a NIK superrepressor was less than that by IκBα or IKKβ superrepressor. To confirm this, an experiment using NIK-specific siRNA was performed. NIK siRNA or control non-silencing siRNA was transfected into HT-29 cells for 72 h; the cells were then treated with toxin A (20 ng/ml) for 6 h. As shown in Fig. 7B, transfection with NIK siRNA partially

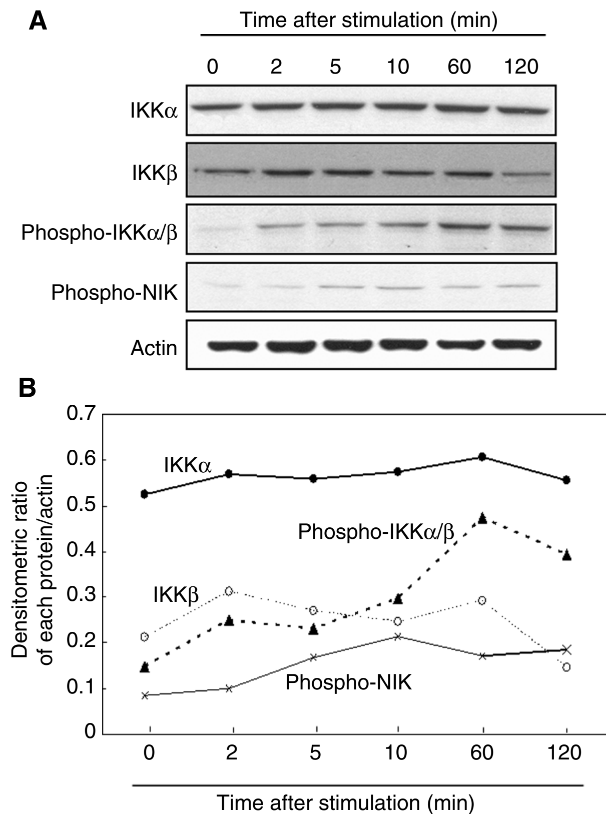

Figure 3 Phosphorylation of IκB kinase (IKK) and NF-κB-inducing kinase (NIK) in *Clostridium difficile* toxin A-stimulated HT-29 cells. Phosphorylation of IKK and NIK in *C. difficile* toxin A-stimulated HT-29 cells. (A) HT-29 cells were incubated with toxin A (20 ng/ml) for the indicated times. Phosphorylation and protein expression of IKKα/β, NIK and actin were assessed by immunoblot. The results are representative of at least five repeated experiments. (B) Densitometric analysis for the expressed proteins. The values represent the relative densities of each protein compared with actin using the TINA program. ●, IKKα; ○, IKKβ; ▲, phospho-IKKα/β; ×, phospho-NIK.

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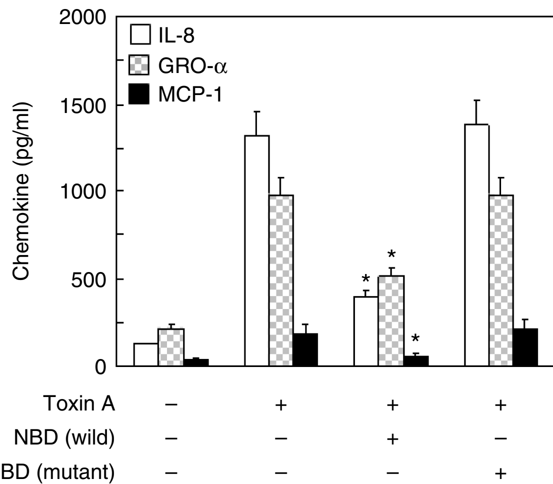

Figure 4 HT-29 cells were incubated with an NF-κB essential modifier (NEMO)-binding domain (NBD) peptide (200 μM). After 1 h, toxin A (20 ng/ml) was added, and the culture supernatants were harvested 24 h later. The protein levels of each chemokine in the culture supernatant were determined by ELISA. Data are expressed as mean ± SEM ($n = 5$). Asterisks indicate values of toxin A + NBD peptide that are significantly different from those of toxin A alone ($P < 0.05$).

downregulated the increased IL-8 mRNA expression induced by toxin A. In this experiment, NIK siRNA completely suppressed NIK signals, whereas the control nonsilencing siRNA did not change phospho-NIK signals or IL-8 mRNA expression (Fig. 7A). These results suggest that NF-κB activation and the upregulation of IL-8/MCP-1 expression in intestinal epithelial cells by toxin A stimulation may be caused by other kinases, although NIK is partially involved.

Discussion

Human intestinal epithelial cells stimulated with *C. difficile* toxin A were shown to express and secrete several chemokines that can chemoattract and activate neutrophils [1, 4, 5]. In this study, NF-κB was activated in HT-29 cells stimulated with toxin A. NF-κB DNA-binding activities reached a maximum 120 min after stimulation, and then decreased. This kinetic pattern in intestinal epithelial cells was observed in other experiments, including stimulation with *Bacteroides fragilis* enterotoxin [7] and infection with *Salmonella dublin* [8]. Our supershift studies showed that toxin A stimulation induced NF-κB complexes of p65/p50 heterodimers and p65/p65 homodimers. In addition, we noted IκBα degradation in toxin A-stimulated intestinal epithelial cells. Moreover, the inhibition of toxin A-induced NF-κB signals suppressed the expression of CXC chemokines such as IL-8 and GRO-α as well as CC chemokine MCP-1. These findings indicate that NF-κB is an essential transcription factor for integrating the host epithelial cell chemokine response to stimulation with toxin A.

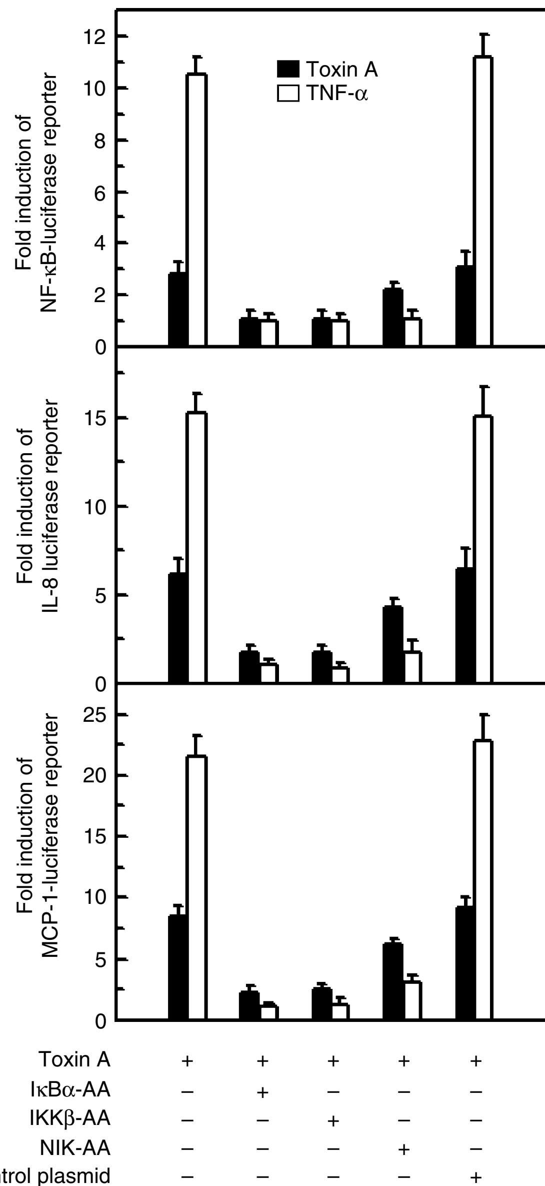

Figure 5 The effects of IκBα, IκB kinase (IKK)β and NF-κB-inducing kinase (NIK) superrepressors on reporter gene activation in *Clostridium difficile* toxin A-stimulated HT-29 cells. HT-29 cell lines were transfected with pIL8-, pMCP-1 or p2x NF-κB-luciferase transcriptional reporter together with IκBα-AA, IKKβ-AA or NIK-AA expression vector, as indicated. After 48 h, the transfected cells were stimulated with toxin A (20 ng/ml, black bar) or TNF-α (20 ng/ml, open bar) for 2 h (NF-κB) or 6 h (IL-8 and MCP-1). Data are expressed as mean fold induction ± SEM of luciferase activity relative to unstimulated controls ($n = 7$). The mean fold induction of β-actin reporter gene activity relative to unstimulated controls remained relatively constant throughout each experiment.

Infection of intestinal epithelial cells with enteroinvasive bacteria or stimulation of the cells with proinflammatory cytokines, such as IL-1 and TNF-α, activates a signalling cascade that culminates in the phosphorylation of IκBα [7, 8, 23]. Two IκB kinases, IKKα and IKKβ, directly phosphorylate IκBs on serine residues and are components of a

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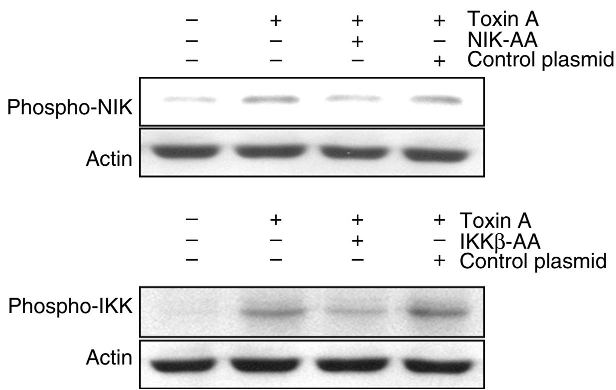

Figure 6 The effects of IκB kinase (IKK)β and NF-κB-inducing kinase (NIK) superrepressors on protein expression in *Clostridium difficile* toxin A-stimulated HT-29 cells. HT-29 cells were transfected with IKKβ or NIK supperrepressor for 24 h, at which point toxin A (20 ng/ml) was added to NIK-AA-transfected cells for 10 min or IKKβ-AA-transfected cells for 60 min. Cell lysates were prepared, size-fractionated and blotted onto a nitrocellulose membrane. Phospho-NIK, phosphor-IKKα/β and actin were detected with specific antibodies and enhanced chemiluminescence. The results are representatives of three independent experiments.

high-molecular-weight cytoplasmic IKK complex [17, 24]. Activation of IKK can be mediated through the mitogen-activated protein-3 (MAP-3) kinase, NIK, and MEK kinase 1 (MEKK-1). Phosphorylation of IκBs on conserved serine residues targets the IκBs for subsequent ubiquitination and degradation [22]. After IκB degradation, dimers of NF-κB (e.g. p65/p50) are free to translocate to the nucleus where they transactivate NF-κB target genes. Our observations that an IKKβ or NIK superrepressor alone was sufficient to inhibit the activation of an IL-8 transcriptional reporter indicate that both IKK and NIK could be involved in the epithelial cell signalling transduction pathway that is responsible for NF-κB activation following toxin A stimulation. These findings are consistent with previous reports showing that NIK and IKK play physiologic roles in IκB phosphorylation and NF-κB activation [17, 25, 26]. Considering that toxin A induced mitochondrial damage and eventual activation of NF-κB in intestinal epithelial cells [3], our data delineate a pathway for NF-κB activation in response to toxin A.

In the present study, the inhibition of IL-8 and NF-κB transcriptional reporters by NIK superrepressor was less than that by IκBα or IKKβ superrepressors, although toxin A could induce phosphorylated NIK signals. This finding suggests that NF-κB activation and IL-8 expression in intestinal epithelial cells stimulated with toxin A may also be initiated by other pathways such as MAPK.

C. difficile toxin A induces expression of the stress-induced early gene product RhoB [27]. In addition, mitochondria-derived reactive oxygen intermediate generation induced by toxin A is independent of Rho inactivation and involves nuclear translocation of NF-κB before IL-8 release [27]. Until now, there have been no reports of

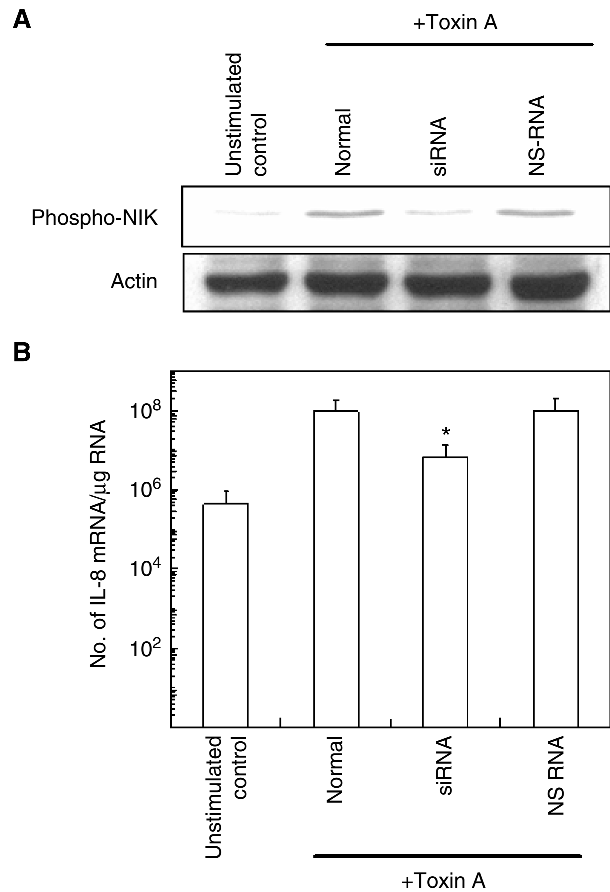

Figure 7 Effects of NF-κB-inducing kinase (NIK)Small interfering RNA (siRNA) on IL-8 mRNA expression in *Clostridium difficile* toxin A-stimulated HT-29 cells. (A) Cells were transfected with NIK-specific silencing siRNA or nonsilencing siRNA (NS-RNA) for 24 h, at which point the transfected cells were stimulated with toxin A (20 ng/ml) for 10 min. Cell lysates were prepared, size-fractionated and blotted onto a nitrocellulose membrane. Phospho-NIK and actin were detected with specific antibodies and enhanced chemiluminescence. The results are representatives of three independent experiments. (B) After treatment with toxin A (20 ng/ml) for 6 h, total RNA was reverse-transcribed using an oligo(dT) primer and a synthetic internal IL-8 RNA standard and amplified by PCR. Data represent mean ± SD of the results from five different cultures. An asterisk indicates values significantly different from toxin A-stimulated cells without siRNA ($P < 0.05$). The β-actin mRNA levels in each group were not significantly different (approximately 5×10^6 transcripts/μg RNA).

glucosylation of Rho proteins induced by toxin A leading to kinase activation that leads to NF-κB activation. However, considering that the p38 MAPK inhibitor SB202190 reduces the expression of RhoB [27], it is possible that the molecular action of toxin A (glucosylation of Rho proteins) might lead to the kinase activation leading to NF-κB activation. Therefore, further study is needed to clarify this hypothesis.

In conclusion, this study demonstrates that the exposure of intestinal epithelial cells to toxin A results in the rapid activation of NF-κB signalling pathways and the activated

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NF-κB then mediates the expression of IL-8, GRO-α and MCP-1. Based on these findings, we suggest that NF-κB signalling events contribute to the biological effects of toxin A produced by toxigenic *C. difficile*.

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References

1 Kelly CP, LaMont JT. *Clostridium difficile* infection. Annu Rev Med 1998;49:375–90.
2 Burakoff R, Zhao L, Celifarco AJ *et al.* Effects of purified *Clostridium difficile* toxin A on rabbit distal colon. Gastroenterology 1995;109:348–54.
3 He D, Sougioultzis S, Hagen S *et al. Clostridium difficile* toxin A triggers human colonocyte IL-8 release via mitochondrial oxygen radical generation. Gastroenterology 2002;122:1048–57.
4 Mahida YR, Makh S, Hyde S, Gray T, Borriello SP. Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: induction of interleukin 8 production and apoptosis after cell detachment. Gut 1996;38:337–47.
5 Kim JM, Kim JS, Jun HC, Oh YK, Song IS, Kim CY. Differential expression and polarized secretion of CXC and CC chemokines by human intestinal epithelial cancer cell lines in response to *Clostridium difficile* toxin A. Microbiol Immunol 2002;46:333–42.
6 Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–79.
7 Kim JM, Cho SJ, Oh YK, Jung HY, Kim YJ, Kim N. Nuclear factor-kappa B activation pathway in intestinal epithelial cells is a major regulator of chemokine gene expression and neutrophil migration induced by *Bacteroides fragilis* enterotoxin. Clin Exp Immunol 2002;130:59–66.
8 Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, Kagnoff MF. NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. J Immunol 1999;163:1457–66.
9 Thanos D, Maniatis T. NF-κB: a lesson in family values. Cell 1995;80:529–32.
10 May MJ, Ghosh S. Signal transduction through NF-κB. Immunol Today 1998;19:80–8.
11 Jefferson KK, Smith MF Jr, Bobak DA. Roles of intracellular calcium and NF-kappa B in the *Clostridium difficile* toxin A-induced up-regulation and secretion of IL-8 from human monocytes. J Immunol 1999;163:5183–91.
12 Kim JM, Eckmann L, Savidge TC, Lowe DC, Witthoft T, Kagnoff MF. Apoptosis of human intestinal epithelial cells after bacterial invasion. J Clin Invest 1998;102:1815–23.
13 Kim JM, Jung HY, Lee JY, Youn J, Lee CH, Kim KH. Mitogen-activated protein kinase and activator protein-1 dependent signals are essential for *Bacteroides fragilis* enterotoxin-induced enteritis. Eur J Immunol 2005;35:2648–57.
14 May MJ, D'Acquisto F, Madge LA, Glockner J, Pober JS, Ghosh S. Selective inhibition of NF-kappaB activation by a peptide that blocks the interaction of NEMO with the IkappaB kinase complex. Science 2000;289:1550–4.
15 Kim JM, Oh YK, Kim YJ, Youn J, Ahn MJ. *Escherichia coli* up-regulates proinflammatory cytokine expression in granulocyte/macrophage lineages of CD34+ stem cells via p50 homodimeric NF-κB. Clin Exp Immunol 2004;137:341–50.
16 DiDonato JA, Mercurio F, Rosette C *et al.* Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Mol Cell Biol 1996;16:1295–04.
17 Mercurio F, Zhu H, Murray BW *et al.* IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-kappa B activation. Science 1997;278:860–6.
18 Cho NH, Seong SY, Huh MS, Kim NH, Choi MS, Kim IS. Induction of the gene encoding macrophage chemoattractant protein 1 by *Orientia tsutsugamushi* in human endothelial cells involves activation of transcription factor activator protein 1. Infect Immun 2002;70:4841–50.
19 Kim JM, Kim JS, Jung HC *et al. Helicobacter pylori* infection activates the NF-κB signaling pathway to induce iNOS and protect human gastric epithelial cells from apoptosis. Am J Physiol Gastrointest Liver 2003;285:G1171–80.
20 Choudhary S, Boldogh S, Garofalo R, Jamaluddin M, Brasier AR. Respiratory syncytial virus influences NF-kappaB-dependent gene expression through a novel pathway involving MAP3K14/NIK expression and nuclear complex formation with NF-kappaB2. J Virol 2005;79:8948–59.
21 Jung HC, Eckmann L, Yang SK *et al.* A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest 1995;95:55–65.
22 Baldwin AS. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–83.
23 DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine- responsive IκB kinase that activates the transcription factor NF-κB. Nature 1997;388:548–54.
24 Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-κB/IκB family. intimate tales of association and dissociation. Genes Dev 1995;9:2723–35.
25 Mercurio F, Murray BW, Shevchenko A *et al.* IκB kinase (IKK) -associated protein 1, a common component of the heterogeneous IKK complex. Mol Cell Biol 1999;19:1526–38.
26 Zandi E, Chen Y, Karin M. Direct phosphorylation of IκB by IKKα and IKKβ discrimination between free and NF-κB-bound substrate. Science 1998;281:1360–3.
27 Gerhard R, Tatge H, Genth H *et al. Clostridium difficile* toxin A induces expression of the stress-induced early gene product RhoB. J Biol Chem 2005;280:1499–05.

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