MEK Is a Key Modulator for TLR5-induced Interleukin-8 and MIP3α Gene Expression in Non-transformed Human Colonic Epithelial Cells

Sang Hoon Rhee, Andrew C. Keates, Mary P. Moyer, and Charalabos Pothoulakis

From The Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215 and the INCELL Corporation, San Antonio, Texas 78249

Flagellin, a specific ligand for Toll-like receptor 5 (TLR5), is a molecular pattern associated with several bacterial species. Recently, TLR signaling has been intensively studied. However, TLR5-associated signaling in non-transformed colonocytes has not been investigated. Here we studied the expression of cytokines induced by flagellin in non-transformed human colonic NCM460 cells and the signaling mechanisms mediating these responses. Cytokine expression array experiments showed that exposure of the cells to flagellin (100 ng/ml) for 12 h increased the expression of interleukin (IL)-8 and macrophage-inflammatory protein 3α (MIP3α) in a TLR5-specific manner. Flagellin also activated MAP kinases (ERK1/2, JNK, and p38) and degraded IκBα. Dominant negative MEK1 (a kinase that activates ERK1/2) blocked flagellin-stimulated IL-8 and MIP3α transcriptional activity, while the MEK-specific inhibitors PD98059 and U0126 reduced protein production of these cytokines. Conversely, transfection with a constitutively active MEK1 increased IL-8 and MIP3α transcriptional activity in a NFκB-independent manner. Furthermore, overexpression of the constitutively active MEK1 induced IL-8 and MIP3α protein production. We also demonstrated that C-terminal coiled-coil and TRAF-C domains of TRAF6, unable to mediate NFκB activation, are involved in MEK-mediated IL-8 and MIP3α expression. Thus, in non-transformed human colonocytes, MEK activation following flagellin/TLR5 engagement is a key modulator for NFκB-independent, IL-8 and MIP3α expression.

Mammalian toll-like receptors (TLRs) are a family of type 1 receptors composed of an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1R (TIR) domain. TLRs recognize several pathogen-associated molecular patterns such as Gram-negative bacterial lipopolysaccharide, lipoprotein from Gram-positive bacteria, double-stranded RNA, bacterial hypomethylated DNA, or flagellin and then triggers innate and adaptive immune responses against these pathogens (2). Studies on TLR signaling provided information on several adaptor molecules of TLR such as MyD88 (myeloid differentiation factor 88), TIRAP/Mal (TIR domain-containing adapter protein/MyD88-adapter-like), TRIF (TIR domain-containing adapter inducing interferon β), and TRAM (TRIF-related adaptor molecule) (3, 4). These adaptor molecules mediate NFκB and MAP kinase activation, leading to proinflammatory cytokine gene expression and immune cell maturation. Among the various pathogen-associated molecular patterns, flagellin, the major component of the flagellar filament, is produced from Gram-negative and Gram-positive bacterial species and stimulates TLR5 leading to cytokine gene expression (5, 6). So far MyD88 is the only known immediate downstream adaptor molecule of TLR5, mediating TLR5-associated signaling.

The human gut harbors a large collection of commensal microbes (~500–1000 bacterial species) at the concentration of ~10^{11} organisms/ml of proximal colonic contents (7, 8). These commensal microbiota are compartmentalized in the intestinal lumen by the intestinal epithelium that serves as an interface between the host and the bacterial micelle. Several clinical observations and animal experiments suggest that intestinal bacteria play a major role in the pathogenesis of chronic bowel inflammation (8–11). In addition, enteroinvasive pathogens such as Salmonella, Shigella, Yersinia, and Listeria can invade the epithelium and provoke inflammatory responses characterized by secretion of proinflammatory cytokines (12–14). TLR5 is highly expressed in intestinal epithelial cells lining the gastrointestinal tract (15). Thus TLR5 engagement by flagellin may activate various signal transduction pathways leading to proinflammatory responses.

A few studies indicate that bacterial flagellin can induce proinflammatory responses in the intestine. For example, in colonic adenocarcinoma HT-29, T84, or Caco-2 cells flagellin stimulates basolaterally expressed TLR5 to induce IL-8 gene expression (14, 15). Exposure of HT-29 cells to flagellin also induces expression of MIP-3α (macrophage-inflammatory protein 3α) (12, 13). Since overexpression of IκBα suppressed flagellin-induced IL-8 and MIP3α gene expression, the transcription factor NFκB was suggested to be a central regulator for IL-8 and MIP3α gene expression in response to flagellin (12, 13, 16). However, studies with pharmacologic inhibitors suggested that the MAP kinases ERK and p38 are involved in flagellin-induced IL-8 expression in colonic adenocarcinoma cells (14, 17, 18). Thus, the signaling pathways involved in...
**FIG. 1.** Flagellin stimulation specifically up-regulates the expression of IL-8 and MIP3α in the non-transformed human colonocytes. The human cytokine array map is shown in A. Pos, positive; Neg, negative; ENA, epithelial cell-derived neutrophil-activating peptide; GCSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; GRO, growth-regulated concogene; IL, interleukin; IFN, interferon; MCP, monocyte chemotactic protein; MCSF, macrophage colony stimulating factor; MDC, macrophage-derived chemokine; MIF, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; RANTES, regulated upon activation in normal T cells, expressed, and secreted; SCF, stem cell factor; SDF, stromal cell-derived factor; TARC, thymus and activation regulated chemokine; TGF, transforming growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; Ang, angiotensin; OSM, oncostatin; Tpo, thrombopoietin; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; BDNF, brain-derived neurotrophic factor; BLC, B-lymphocyte chemotactic factor; Ck8-1, β-chemokine; FGF, fibroblast growth factor; GCP, glutamate carboxypeptidase; GDNF, glial cell line derived neurotrophic factor; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor binding protein; IP-10, interferon-inducible protein 10; LIF, leukemia inhibitory factor; LIGHT, lymphotoxins, inducible expression, competes with HSV glycoprotein D for HVEM, a receptor expressed on T-lymphocytes; MIF, macrophage migration inhibitory factor; NAP, neutrophil activating peptide; NT, neurotrophin; PARC, pulmonary and activation-regulated chemokine; PIGF, placenta growth factor; TIMP, tissue inhibitors of metalloproteinases. Cytokine microarray assay revealed the expression of IL-8 and MIP3α by flagellin in the non-transformed human colonocytes NCM460 (B). 1.5 × 10⁶ cells were plated in 60-mm dishes 1 day prior to the experiment. The cells were then stimulated without (B, left panel) or with (B, right panel) purified flagellin (100 ng/ml) for 12 h.
proinflammatory cytokine expression by TLR5 engagement in intestinal epithelial cells remain to be elucidated.

Here, we used non-transformed human colonic NCM460 cells (19, 20) to investigate which cytokines are induced by TLR5 and studied the signaling pathways involved in cytokine expression. We found that exposure of NCM460 cells to flagellin specifically increased IL-8 and MIP3α expression, which was blocked by a TLR5-dominant negative mutant. We also present novel evidence indicating that MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase), a kinase involved in ERK1/2 activation, mediates IL-8 and MIP3α expression via NFκB-independent pathway(s) in flagellin/TLR5-induced signal transduction pathways.

MATERIALS AND METHODS

Reagents—Purified flagellin was purchased from Apotech Corporation (Lausen, Switzerland). The transfection reagent TransIT-LT1 was obtained from Mirus (Madison, WI). The plasmid constructs encoding a dominant negative MEK1 (MEKI (S1218E/S1222E), MEKI-CA) and a constitutively active MEK1 (MEKI (S218E/S222E), MEKI-CA) were gifts from Dr. Kun-Liang Guan (University of Michigan, Ann Arbor, MI) (21, 22). The expression constructs encoding wild type TRAF6 and TRAF6-CC (N-terminal Ring finger and zinc finger domains were truncated) were kindly provided by Dr. Jun-Ichi Inoue (University of Tokyo, Tokyo) (23). The luciferase reporter constructs for human IL-8 and human MIP3α were previously described (19, 24). The endothelial leukocyte-adhesion molecule NFκB-luciferase reporter construct was also described elsewhere (25). For a dominant negative human TLR5 (TLR5(ΔTLR)), the cytoplasmic TIR domain of TLR5 was truncated, as described elsewhere (15). All plasmids were prepared using the EndoFree plasmid kit as recommended by the manufacturer (Qiagen, Valencia, CA). The polyclonal antibody for IL-8 was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-p38, p38, phospho-JNK, phospho-ERK1/2, ERK1/2, phospho-MEK1/2, MEK1/2, Akt, and the MEK1/2-specific inhibitors, PD98059 and U0126, were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against TRAF6 and JNK1 were from Santa Cruz Biotechnology, Inc. The interleukin-1 receptor-associated kinase (IRAK) antibody was purchased from Upstate (Charlottesville, VA). The human non-transformed colonocytes NCM460 and the culture medium M3D were obtained from INCELL Corporation (San Antonio, TX) (19, 26). NCM460 cells were cultivated in M3D media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% L-glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in air supplemented with 5% CO₂.

Human Cytokine Expression Array Assay—The human cytokine array 5.1 was purchased from Ray Biotech (Norcross, GA) and used following the manufacturer's instructions (27). Briefly, NCM460 cells were plated in 6-well plates (0.4 × 10⁶ cells/well) and transfected with the appropriate plasmid DNA, including a β-galactosidase expression plasmid (HS70-β-gal) as an internal control, using TransIT-LT1 from Mirus (Madison, WI) according to the manufacturer's instructions. One day after transfection, cells were stimulated with flagellin (100 ng/ml) for 6 h, and the relative luciferase activity was determined by normalization with β-galactosidase activity as described previously (25, 28). The total amount of plasmid DNA was kept consistent by adding the empty vector for each transfection. All assays were performed in triplicate, and a single representative experiment is shown. Data are expressed as mean values ± S.E.

Immunoprecipitation—NCM460 cells (2.5 × 10⁶) were plated in 60-mm dishes and stabilized overnight. Cells were stimulated with flagellin (100 ng/ml) for various time points, and cell lysates were prepared in lysis buffer as described above. Equal amounts of protein were mixed with the IRAK antibody, incubated at 4 °C for 3–4 h, and then incubated with protein A beads (Pierce) for 6 h followed by washing. The precipitated mixture was washed three times with lysis buffer, proteins were fractionated on 10% SDS-polyacrylamide gel, followed by immunoblotting as described above.

RESULTS

Cytokine Microarray Assay Revealed the Selective Expression of IL-8 and MIP3α by TLR5 Engagement in Non-transformed Human Colonocytes—Understanding the expression array of proinflammatory mediators by bacterial flagellin is important to study the pathophysiology of inflammatory responses in the human gut. Therefore, using a human cytokine microarray assay, we first screened the expression profile of cytokines from non-transformed human NCM460 colonocytes stimulated with flagellin. The microarray membrane was immobilized with capture antibodies against 79 different cytokines as listed in Fig. 1A. The membranes were hybridized with 300 μg of total protein from flagellin-stimulated (100 ng/ml, for 12 h) or control NCM640 cells. The corresponding proteins were detected by a mixture of detection antibodies and visualized by an ECL system. Among various cytokines, the expression of IL-8 and MIP3α was substantially up-regulated (Fig. 1B). Subsequently, the supernatant from flagellin-stimulated cells was used for ELISA to confirm the increased expression of IL8 and MIP3α observed with the cytokine microarray assay. As shown in Fig. 1, C and D, IL-8 and MIP3α production detected by ELISA was substantially increased in response to the TLR5 agonist. These
MEK Mediates TLR5-induced IL-8 and MIP3α Expression

Fig. 2. TLR5 engagement leads to NFκB and MAP kinase activation. NCM460 cells were plated in 1.5 × 10^6 cells in 60-mm dishes 1 day prior to the experiment and stimulated with purified flagellin (100 ng/ml) for the indicated times. Cell lysates were fractionated by SDS-PAGE and then analyzed by Western blotting using antibodies directed against phospho-ERK1/2 (A), phospho-JNK (B), phospho-p38 (C), and IκBα (D), respectively. Membranes were then stripped and re-probed using an antibody against total ERK1/2 (A), JNK1 (B), p38 (C), and Akt (A and D), respectively, as control for equal loading. A single representative experiment is shown from three different experiments.

Results strongly suggest that, after 12 h incubation, flagellin stimulation selectively up-regulates IL-8 and MIP3α in human colonocytes.

Additionally, we observed that mRNA for TLR5 is also expressed in human colonic epithelial NCM460 cells.2 Although flagellin has been suggested as a specific ligand for TLR5, it is necessary to verify if IL-8 and MIP3α expression induced by flagellin is specifically mediated by TLR5 engagement and not by putative contaminants present in the purified flagellin preparation. To test this, we used a dominant negative TLR5 mutant (cytoplasmic TIR domain deleted TLR5(DTIR)). Cells were transiently co-transfected with IL-8 or MIP3α luciferase reporter constructs with or without dominant negative TLR5 mutant. As shown in Fig. 1, E and F, DTIR overexpression substantially inhibited flagellin-induced IL-8 and MIP3α transcriptional activity. In contrast, we confirmed that overexpressing dominant negative TLR5 mutant did not alter TNFα-mediated responses (data not shown), indicating the TLR5 specificity of this response. Together, these findings indicate that, in human colonocytes, flagellin stimulation selectively up-regulates IL-8 and MIP3α expression in a TLR5-specific manner.

Engagement of TLR5 Results in the Activation of MAP Kinases and NFκB Signaling—Previous results in macrophages demonstrate that TLR signaling leads to the activation of MAP kinases and the transcription factor NFκB (2). Subsequent studies suggested that bacterial infection with Salmonella or Escherichia coli results in NFκB and p38 activation in colon adenocarcinoma HT-29 and Caco-2 cells (16, 17, 29). Therefore, we tested whether TLR5 engagement can give rise to a typical TLR-related signaling response. NCM460 cells were transfected with flagellin as indicated in Fig. 2 and whole cell lysates were then prepared and analyzed by immunoblot analysis. Activation of MAP kinases was determined using specific antibodies against phospho-ERK1/2, phospho-JNK, and phospho-p38. As shown in Fig. 2, flagellin induced activation of ERK1/2 (A), JNK (B), and p38 (C) at early time points. Moreover, flagellin exposure triggered degradation of IκBα (D). Thus, engagement of TLR5 leads to rapid activation of MAP kinases and NFκB in non-transformed human colonocytes, indicating the presence of intact TLR5 signaling in the cells.

Blocking MEK1 Inhibited Flagellin/TLR5-induced IL-8 and MIP3α Expression—Based on ample evidence suggesting involvement of several cytokines in the pathogenesis of bowel inflammation (30, 31), it is important to study the mechanism by which the inflammatory mediators are up-regulated in response to inflammatory stimuli, such as flagellin. Previous studies (12, 13, 16) showed that the expression of IL-8 and MIP3α by pathogenic bacteria was attenuated by the overexpression of IκBα, indicating that expression of these cytokines by enteric pathogens is NFκB-dependent.

However, as shown in Fig. 2, apart from NFκB activation, TLR5 signaling also leads to activation of MAP kinases. Therefore, we next determined the role of MAP kinase activation in TLR5-derived IL-8 and MIP3α gene expression. Since TLR5 engagement induces ERK1/2 activation, which is phosphorylated on its threonine and tyrosine residues by the MEK1/2 class of MAP kinase kinases, we stimulated NCM460 cells with flagellin in the presence or absence of the MEK-specific inhibitors U0126 or PD98059 and then measured IL-8 and MIP3α protein in culture supernatants by ELISA. As shown in Fig. 3, A–D, pharmacologic antagonism of MEK activity inhibited IL-8 and MIP3α protein production in response to flagellin in dose-dependent manner.

To directly examine whether flagellin stimulation induces MEK1/2 activation in NCM460 cells, we performed immunoblot analysis in cell lysates from flagellin-stimulated cells using an antibody directed against phospho-MEK1/2. As shown in Fig. 3E, flagellin stimulation induced MEK1/2 activation at early time points. Since MEK1/2 is the upstream enzyme to directly activate ERK1/2, this result is compatible with flagellin-induced ERK1/2 activation (Fig. 2A).

To obtain further evidence for the involvement of MEK in TLR5-induced IL-8 and MIP3α expression, we next determined whether a dominant-negative MEK1 mutant could influence IL-8 or MIP3α transcriptional activity in flagellin-stimulated human colonocytes. NCM460 cells were transiently co-transfected with the IL-8- or MIP3α-luciferase reporter plasmid with or without an expression plasmid encoding for a dominant negative MEK1 (MEK1-DN) mutant. As shown in Fig. 3, F and G, overexpression of a dominant negative MEK1 mutant in these cells resulted in the complete inhibition of IL-8 transcriptional activities, whereas MEK1-DN expression partially inhibited flagellin-induced MIP3α promoter activity. Together, these results suggest that flagellin-induced IL-8 and MIP3α expression is, at least in part, mediated by activation of MEK.

Constitutive MEK1 Activation Induces IL-8 and MIP3α Gene Expression—The finding that MEK activation is required for the induction of IL-8 and MIP3α gene expression by flagellin in NCM460 cells raises the possibility that activated MEK1 can up-regulate IL-8 and MIP3α gene expression in the absence of any upstream stimulation. To address this issue, a plasmid encoding for constitutively active MEK1 (MEK1-CA) was co-transfected with IL-8 or MIP3α-luciferase reporter plasmid constructs into NCM460 cells and luciferase activity was measured. As shown in Fig. 4A, IL-8 promoter activity was substantially increased by the specific activation of MEK1 without flagellin stimulation. Surprisingly, the level of increase in IL-8 promoter activity in cells overexpressing constitutively active MEK1 alone is higher compared the level obtained following flagellin

---

2 S. H. Rhee, A. C. Keates, M. P. Moyer, and C. Pothoulakis, unpublished observation.
MEK Mediates TLR5-induced IL-8 and MIP3α Expression

**Fig. 3.** MEK1 activation is required for TLR5-induced IL-8 and MIP3α expression. NCM460 cells were pretreated 60 min with the MEK specific inhibitors, U0126 (A and C) or PD98059 (B and D), and then stimulated for 12 h with purified flagellin (100 ng/ml). The supernatants were then harvested, and ELISA was performed to measure the amount of IL-8, as described under "Materials and Methods." Immunoblot analysis was performed in total cell lysates from cells stimulated with flagellin (100 ng/ml) for the indicated times. Activation of MEK1/2 was determined with an antibody against phospho-MEK1/2. Membranes were then stripped and incubated with total MEK1/2 antibody, as equal loading control (E). NCM460 cells were transiently co-transfected with an IL-8 (F)- or MIP3α (G)-luciferase reporter plasmid (1 μg), and a dominant negative MEK1 (MEK1-DN, 2 μg) in the indicated combinations. The total amount of plasmid DNA was kept consistent by adding the empty vector for each transfection. Transfections were performed in triplicate, and a single representative experiment is shown. Data are reported as mean values ± S.E. (n = 3). U, U0126; PD, PD98059.
The transcription factor NFκB is known to be involved in proinflammatory cytokine gene expression in various cell types (28). Previous results indicate that overexpression of IκBα to inhibit NFκB activation inhibited IL-8 gene expression in colon carcinoma cells stimulated with flagellin-expressing enteropathogenic bacteria (12, 13, 16). Thus, it is necessary to determine whether MEK1-mediated IL-8 expression in response to flagellin involves NFκB activation. To test this possibility, an IκBα expression construct was transiently co-transfected with an IL-8 reporter construct in NCM460 cells, in the presence/absence of MEK1-CA. As shown in Fig. 5A, overexpression of IκBα did not alter MEK1-derived IL-8 transcriptional activity. In contrast, flagellin-induced IL-8 transcriptional activity was abolished by IκBα overexpression (Fig. 5B). Thus, in NCM460 colonocytes, MEK1 activation appears to induce IL-8 transcriptional activity in a NFκB-independent manner. Moreover, as shown in Fig. 5C, transfection with the MEK1-CA construct does not result in NFκB activation. Together, these results demonstrate that, in non-transformed human colonocytes, MEK signaling is a parallel to the NFκB signaling molecular event in response to flagellin/TLR5 engagement and mediates IL-8 expression in a NFκB-independent manner.

**TRAF6 Is an Upstream Mediator of MEK for IL-8 and MIP3α Expression**—TNF receptor-associated factors are identified as downstream signal transducers of the TNF receptor family. TRAF6 has been determined as a downstream signaling molecule in TLRs and IL-1R engagement, which is associated with transforming growth factor β-activated kinase 1 (TAK1) via the TAK1-binding protein to activate NFκB-inducing kinase leading to NFκB activation (32). Kopp et al. (33) identified a novel TRAF6-binding protein, ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), which interacts with the MEKK1 and thereby links TRAF6 to MEKK1, leading to NFκB and AP-1 activation in TLR4 response. More recently, Huang et al. (34) demonstrated that MEKK3 associates with TRAF6 to link MAP kinase (JNK and p38, but not ERK1/2) activation in response to lipopolysaccharide or IL-1. Furthermore, several studies already showed that TRAF6 associates with IRAK following the engagement of TLRs and IL-1 receptor (35–38).

Based on this evidence, we hypothesized that TRAF6 is involved in the flagellin/TLR5 signal transduction pathway, functions as a bridge to MEK1 activation, and mediates IL-8 and MIP3α gene expression following TLR5 engagement. To test this, IRAK was co-immunoprecipitated in flagellin-stimulated cell lysates, and co-precipitation of TRAF6 was determined by immunoblot analysis using a TRAF6 antibody. As shown in Fig. 6A, flagellin stimulation induced the association of IRAK and TRAF6. Additionally, we transfected the cells with a TRAF6 expression plasmid together with an IL-8 or MIP3α reporter construct. In some cases, MEK1-DN was included in the indicated combination. As shown in Fig. 6, B and C, overexpression of TRAF6 induced IL-8 and MIP3α transcriptional activity without flagellin/TLR5 stimulation. However transfection with the MEK1-DN construct completely inhibited IL-8 transcriptional activity induced by TRAF6, whereas TRAF6-derived MIP3α promoter activity was partially inhibited by
Transfections were performed in triplicate, and a single representative experiment is shown. Data are reported as mean values ± S.E. (n = 3).

**Materials and Methods.**

- **Overexpression of MEK1-CA induces IL-8 transcriptional activity in a NFκB-independent manner.** NCM cells were co-transfected with IL-8 (A)- or MIP3α (B)-luciferase reporter constructs (1 µg) with or without a MEK1-CA expression construct (2 µg). The expression construct encoding IκBα (2 µg) was also included in some experiments as indicated. Various amounts of plasmid encoding MEK1-CA (2, 3, and 4 µg) were co-transfected with the NFκB-luciferase reporter construct (1 µg) (C). One day after transfection, cell lysates were harvested for luciferase reporter assay. For a positive control (C, lane 5), the NFκB-luciferase construct (1 µg) was transfected, and after 1 day, cells were stimulated with flagellin (100 ng/ml) for 6 h before luciferase assays were performed. Detailed procedures for transfection and luciferase assay were described under “Materials and Methods.” The total amount of plasmid DNA was kept consistent by adding the empty vector for each transfection. Transfections were performed in triplicate, and a single representative experiment is shown. Data are reported as mean values ± S.E. (n = 3).

**Coiled-coil and TRAF-C Regions of TRAF6 Mediate TLR5-dependent Signaling—**TRAF6 is characterized by a N-terminal Ring finger and five zinc finger domains together with a C-terminal coiled-coil and TRAF-C domains (23). TRAF6 lacking the N-terminal Ring finger and zinc finger domains acts as a dominant negative mutant and blocks NFκB activation in response to lipopolysaccharide or IL-1 (23, 39). We found that transfection with TRAF6-CC (TRAF6 lacking N-terminal Ring finger and zinc finger domains but harboring C-terminal coiled-coil and TRAF-C domains) inhibited flagellin-induced NFκB activation (Fig. 7A), indicating that TRAF6 participates in this TLR5 signaling response. Based on this evidence, we next studied whether TRAF6 transduces the upcoming TLR5 response to MEK-mediated cytokine expression without activating the NFκB pathway. To test this, cells were co-transfected with IL-8 or MIP3α reporter constructs, together with TRAF6-CC, and/or MEK1-DN constructs in combinations indicated in Fig. 7. Our results show that overexpression TRAF6-CC, which is unable to mediate NFκB activation, did not inhibit flagellin-induced IL-8 and MIP3α promoter activity. IL-8 and MIP3α promoter activities, however, were substantially inhibited in the presence of MEK1-DN. These data demonstrate that the C-terminal coiled-coil and TRAF-C regions of TRAF6 mediate IL-8 and MIP3α expression via MEK1. Taken together, our results strongly suggest that MEK mediates flagellin/TLR5-induced IL-8 and MIP3α expression in an NFκB-independent manner.

**Discussion**

An important finding of our study is the novel demonstration of specific flagellin/TLR5-induced proinflammatory responses in non-transformed human colonic epithelial cells. Among various cytokines, TLR5 engagement in human colonocytes stimulates the specific expression of the potent chemokine IL-8 and MIP3α. Flagellin-stimulated TLR5 is also linked to activation of several MAP kinases, including ERK1/2, p38, and JNK, and of the transcription factor NFκB. Activation of MEK, an upstream kinase of ERK1/2, is an important step for flagellin/TLR5-induced IL-8 and MIP3α gene expression in these cells. In contrast, flagellin/TLR5-derived IL-8 and MIP3α expression does not involve activation of JNK.

Our results may be pertinent to the pathophysiology of colonic inflammation. Increased MIP3α and IL-8 expression has been observed in colonic tissues obtained from patients with inflammatory bowel disease (30, 31). Several reports underline the importance of MIP3α and IL-8 in inflammatory cell recruitment during colonic inflammation. For example, MIP3α attracts CCR6-expressing memory T cells and immature DCs, and IL-8 is a chemoattractant for neutrophils in the subepithelial compartment (12, 13, 24). Thus, IL-8 and MIP3α participate in infiltration of immune cells to the subepithelial compartment seen during intestinal inflammatory responses.

Previous studies in colon carcinoma HT-29 and Caco-2 cells showed that overexpression of IκBα significantly reduced IL-8 and MIP3α expression in response to flagellin-containing bacteria (12, 13, 16). Using non-transformed human colonocytes, we also found that the NFκB/IκB system is involved in flagel-
lin-stimulated IL-8 and MIP3α promoter activity (see “Results”). Consistent with these findings, previous studies demonstrated the importance of NFκB binding sites on the promoter regions of IL-8 and MIP3α for transcriptional activation of these genes (24, 40, 41). Thus, NFκB appears to play an important role in IL-8 and MIP3α expression following exposure to flagellin-containing bacteria.

Using molecular as well as pharmacologic approaches we provide strong evidence that NFκB-independent MEK-mediated signaling pathways are also involved in flagellin/TLR5-induced IL-8 and MIP3α expression. MEK-associated signaling has been linked to activation of several, other than NFκB, transcription factors regulating IL-8 and MIP3α gene expression. For example, DNA binding sites for the transcription factors AP-1 and C/EBP (NF-IL6) have been identified within the IL-8 promoter (40). Moreover, in gastric cancer cells, MEK signaling induces the transactivation of SRE and AP-1 in response to *Helicobacter pylori* and inhibition of MEK activation reduces *H. pylori*-induced IL-8 secretion, indicating that MEK participates in IL-8 expression in response to this pathogen (41). MEK signaling is also related to activation of transcription factors such as Elk-1 and C/EBP in human monocytes and colon adenocarcinoma T84 cells, respectively (18, 42). In addition, in transformed endothelial cells, IL-8 gene expression induced by TNFα is regulated by AP-1 and C/EBP (43). Moreover, bindings sites for Sp1, the Ets nuclear factor ESE-1, and p50/p65 NFκB heterodimers have been identified within the 5′-flanking region of MIP3α and substitutions in these elements alter MIP3α promoter activity induced by IL-1 in colon adenocarcinoma Caco-2 cells (24).

We found that TLR5-induced expression of IL-8 and MIP3α was not altered by exposure of cells to the specific JNK inhibitor SP600125 and by overexpressing a dominant negative JNK, implying that JNK does not mediate the up-regulation of IL-8 and MIP3α gene expression by TLR5 engagement. Our results also indicate increased p38 activation following exposure of NCM460 cells to flagellin (Fig. 2), consistent with prior observations in colon adenocarcinoma HT-29 cells (17). Although we did not directly examine involvement of p38 in flagellin-induced IL-8 and MIP3α expression in NCM460 cells, Yu *et al.* (17) presented evidence indicating that p38 activation is functionally linked to IL-8 production in response to flagellin activation in HT-29 cells.

Several studies indicate participation of TRAF6 in other than TLR5, TLR-related signaling. Here we present direct evidence that in human colonocytes TRAF6 mediates flagellin-induced TLR5 proinflammatory signaling (Figs. 6 and 7) and that the C-terminal coiled-coil and TRAF-C domains of TRAF6 relay the upcoming signal from TLR5 to MEK activation, leading to IL-8 and MIP3α gene expression (Fig. 7). Thus, in non-transformed human colonocytes, the C-terminal coiled-coil and TRAF-C domains of TRAF6 are responsible for linking TLR5 signaling to MEK1 activation and MEK-mediated IL-8 and MIP3α expression in a NFκB-independent manner. However, the molecule(s) involved in TRAF6-MEK signaling pathway in flagellin-stimulated human colonocytes have not been examined in our study. Previous results indicate that the TRAF6-associating adaptor molecule ECSIT, which links MEKK1 to TRAF6, leads to JNK and NFκB activation in response to TLR4 engagement (33). More recently, MEKK3 has been suggested to interact with TRAF6, resulting in activation...
MEK Mediates TLR5-induced IL-8 and MIP3α Expression

Fig. 7. The Coiled-coil and TRAF-C regions of TRAF6 mediate TLR5 signaling to IL-8 and MIP3α promoter activation via MEK1 but not NFκB. NCM460 cells were transiently co-transfected with NFκB (A), IL-8 (B), or MIP3α (C)-luciferase reporter plasmids (1 μg) together with either an expression construct encoding the C-terminal coiled-coil and TRAF-C region of TRAF6 (TRAF6-CC, 2 μg) or an empty vector (2 μg). A dominant negative MEK1 (MEK1-DN, 2 μg) expression construct was also included in the indicated combinations. The HSP70-β-gal reporter construct (0.5 μg) was included as the internal control. Transfected cells were cultivated for a day and then stimulated with flagellin (100 ng/ml) for 6 h, as indicated. Transfections were performed in triplicate, and a single representative experiment is shown. Data are reported as mean ± S.E. (n = 3).

of p38 and JNK, but not ERK1/2 in response to TLR4 activation (34). However, both ECSIT and MEKK3 are known to directly interact with TRAF6, but not with the N-terminally truncated form of TRAF6 (33, 34). Thus, it is quite likely that another molecule(s) mediates TRAF6-induced MEK activation in response to TLR5 engagement.

Our findings demonstrate that a TRAF6-mediated MEK-dependent pathway, that does not involve NFκB activation, as well as TRAF6-NFκB-dependent pathways are important modulators of IL-8 and MIP3α gene expression following TLR5 engagement in non-transformed human colonocytes. Thus, TRAF6-mediated activation of MEK and NFκB-dependent pathways synergize to regulate expression of IL-8 and MIP3α genes in response to flagellin/TLR5 engagement in human colonocytes. We hypothesize that similar pathways may be important in the pathogenesis of several forms of colonic inflammation, including inflammatory bowel disease.

Acknowledgements—We thank Dr. Kun-Liang Guan and Dr. Jun-Ichio Inoue for kindly providing some of plasmids used in this study.

REFERENCES
1. Medzhitov, R., Preston-Hurlbut, P., and Janeway, C. A., Jr. (1997) Nature 386, 394–397.
2. Underhill, D. M., and Ozinsky, A. (2002) Curr. Opin. Immunol. 14, 103–110.
3. Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2002) Nat. Immunol. 3, 1144–1150.
4. O'Neill, L. A., Fitzgerald, K. A., and Bowie, A. G. (2003) Trends Immunol. 24, 286–290.
5. Smith, K. D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M. A., Barrett, S. L., Cookson, B. T., and Aderem, A. (2003) Nat. Immunol. 4, 1247–1253.
6. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, R. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001) Nature 410, 1099–1103.
7. Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001) Science 291, 881–884.
8. Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M., and Lohs, H. (2002) Gastroenterology 122, 44–54.
9. Stagg, A. J., Hart, A. L., Knight, S. C., and Kamm, M. A. (2003) Gut 52, 1522–1529.
10. Hart, A. L., Stagg, A. J., and Kamm, M. A. (2003) J. Clin. Gastroenterol. 36, 111–119.
11. Cummings, J. H., Macfarlane, G. T., and Macfarlane, S. (2003) Curr. Issues Intest. Microbiol. 4, 9–20.
12. Izadpanah, A., Dwinnell, M. B., Eckmann, L., Varki, N. M., and Kagnoff, M. F. (2001) J. Immunol. 166, 875–881.
13. Sierrro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J. P., and Sirard, J. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13722–13727.
14. Zhou, X., Giron, J. A., Torres, A. G., Crawford, J. A., Negrete, E., Vogel, S. N., and Kaper, J. B. (2003) Infect. Immun. 71, 2129–2139.
15. Gewertz, A. T., Navas, T. A., Lyons, S., Godowski, P. J., and Maradja, J. L. (2001) J. Immunol. 167, 1882–1885.
16. Elewaut, D., DiDionato, J. A., Kim, J. M., Truong, F., Eckmann, L., and Kagnoff, M. F. (1999) J. Immunol. 163, 1457–1466.
17. Yu, Y., Zeng, H., Lyons, S., Carlson, A., Merlin, D., Neish, A. S., and Gewirtz, A. T. (2003) Am. J. Physiol. 285, G282–G290.
18. Salmenpera, P., Hamalainen, S., Hakkanen, M., and Kankuri, E. (2003) Am. J. Physiol. 284, C1133–C1139.
19. Zhao, D., Keates, A. C., Kuhnt-Moore, S., Moyer, M. P., Kelly, C. P., and Pothoulakis, C. (2001) J. Biol. Chem. 276, 44464–44471.
20. Moyer, M. P., Manzano, L. A., Merriman, R. L., Stauffer, J. S., and Tanzer, L. R. (1996) In Vitro Cell Dev. Biol. Anim. 32, 315–317.
21. Sugimoto, T., Stewart, S., Han, M., and Guan, K. L. (1998) EMBO J. 17, 1717–1727.
22. Li, W., Chong, H., and Guan, K. L. (2001) J. Biol. Chem. 276, 34728–34737.
23. Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S., and Inoue, J. (2001) EMBO J. 20, 1271–1280.
24. Kwon, J. H., Keates, S., Simeonidou, S., Grall, F., Libermann, T. A., and Keates, A. C. (2003) J. Biol. Chem. 278, 875–884.
25. Bhee, S. H., Jones, B. W., Toshchakov, V., Vogel, S. N., and Fenton, M. J. (2003) J. Biol. Chem. 278, 21296–21302.
26. Saihi, J., Nataraja, S. G., Layden, T. J., Goldestein, J. L., Moyer, M. P., and Rao, M. C. (1998) Am. J. Physiol. 275, C1048–C1102.
27. Huang, R., Lin, Y., Wang, C. C., Gano, J., Lin, B., Shi, Q., Boynton, A., Burke, J., and Huang, R. P. (2002) Cancer Res. 62, 2806–2912.
28. Bhee, S. H., and Hwang, D. (2000) J. Biol. Chem. 275, 34035–34040.
29. Madrazo, D. R., Tranguch, S. L., and Marriott, I. (2003) Infect. Immun. 71, 5418–5421.
MEK Mediates TLR5-induced IL-8 and MIP3α Expression

30. Kwon, J. H., Keates, S., Bassani, L., Mayer, L. F., and Keates, A. C. (2002) Gut 51, 818–826
31. Banks, C., Biteman, A., Payne, R., Johnson, P., and Sheron, N. (2003) J. Pathol. 199, 28–35
32. O’Neill, L. A. (2002) Curr. Top. Microbial. Immunol. 270, 47–61
33. Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C. A., and Ghosh, S. (1999) Genes Dev. 13, 2059–2071
34. Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z. G., and Su, B. (2004) Nat. Immunol. 5, 98–103
35. Takasuzu, G., Ninomiya-Tsuji, J., Kishida, S., Li, X., Stark, G. R., and Matsumoto, K. (2001) Mol. Cell. Biol. 21, 2475–2484
36. Ye, H., Arron, J. R., Lamorte, B., Cirilli, M., Kobayashi, T., Sherde, N. K., Segal, D., Drivenu, O. K., Vologodskaja, M., Yin, M., Du, K., Singh, S., Pike, J. W., Darnay, B. G., Choi, Y., and Wu, H. (2002) Nature 418, 443–447
37. Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002) Mol. Cell. Biol. 22, 7158–7167
38. Xu, H., An, H., Yu, Y., Zhang, M., Qi, R., and Cao, X. (2003) J. Biol. Chem. 278, 36334–36340
39. Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T., and Inoue, J. (1999) J. Biol. Chem. 274, 28745–28748
40. Roebuck, K. A. (1999) J. Interferon Cytokine Res. 19, 429–438
41. Mitsuno, Y., Yoshida, H., Maeda, S., Ogura, K., Hirata, Y., Kawabe, T., Shiratori, Y., and Omata, M. (2001) Gut 49, 18–22
42. Gaha, M., O’Connell, M. A., Pawliński, B., Hollis, A., McGovern, P., Yan, S. F., Stern, D., and Mackman, N. (2001) Blood 98, 1429–1439
43. Natarajan, R., Gupta, S., Fisher, B. J., Ghosh, S., and Fowler, A. A., III (2001) Exp. Cell Res. 266, 203–212
MEK Is a Key Modulator for TLR5-induced Interleukin-8 and MIP3α Gene Expression in Non-transformed Human Colonic Epithelial Cells
Sang Hoon Rhee, Andrew C. Keates, Mary P. Moyer and Charalabos Pothoulakis

J. Biol. Chem. 2004, 279:25179-25188.
doi: 10.1074/jbc.M400967200 originally published online April 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400967200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 23 of which can be accessed free at http://www.jbc.org/content/279/24/25179.full.html#ref-list-1