TLR8-mediated NF-κB and JNK Activation Are TAK1-independent and MEKK3-dependent

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TLR8-mediated NF-κB and IRF7 activation are abolished in human IRAK-deficient 293 cells and IRAK4-deficient fibroblast cells. Both wild-type and kinaseinactive mutants of IRAK and IRAK4, respectively, restored TLR8-mediated NF-κB and IRF7 activation in the IRAK- and IRAK4-deficient cells, indicating that the kinase activity of IRAK and IRAK4 is probably redundant for TLR8-mediated signaling. We recently found that TLR8 mediates a unique NF-κB activation pathway in human 293 cells and mouse embryonic fibroblasts, accompanied only by IkBα phosphorylation and not IkBβ degradation, whereas interleukin (IL)-1 stimulation causes both IkBα phosphorylation and degradation. The intermediate signaling events mediated by IL-1 (including IRAK modifications and degradation and TAK1 activation) were not detected in cells stimulated by TLR8 ligands. TLR8 ligands trigger similar levels of IkBα phosphorylation and NF-κB and JNK activation in TAK1−/− mouse embryo fibroblasts (MEFs) as compared with wild-type MEFs, whereas lack of TAK1 results in reduced IL-1-mediated NF-κB activation and abolished IL-1-induced JNK activation. The above results indicate that although TLR8-mediated NF-κB and JNK activation are IRAK-dependent, they do not require IRAK modification and are TAK1-independent. On the other hand, TLR8-mediated IkBα phosphorylation, NF-κB, and JNK activation are completely abolished in MEKK3−/− MEFs, whereas IL-1-mediated signaling was only moderately reduced in these deficient MEFs as compared with wild-type cells. The differences between IL-1R- and TLR8-mediated NF-κB activation are also reflected at the level of IkB kinase (IKK) complex. TLR8 ligands induced IKKγ phosphorylation, whereas IKKα/β phosphorylation and IKKγ ubiquitination that can be induced by IL-1 were not detected in cells treated with TLR8 ligands. We postulate that TLR8-mediated MEKK3-dependent IKKγ phosphorylation might play an important role in the activation of IKK complex, leading to IkBα phosphorylation.

The IL-1β/Toll receptors play essential roles in inflammation and innate immunity. The defining feature of members of the superfamily is a Toll/IL-1 receptor (TIR) domain on the cytoplasmic side of the receptors. The members of the IL-1 receptor subfamily contain three Ig domains in their extracellular regions (1). The other group in the superfamily is the recently identified pathogen-associated pattern recognition receptors, the Toll-like receptors (TLRs), 11 members of which contain two major domains characterized by extracellular leucine-rich repeats and an intracellular TIR domain (2–7).

Much progress has been made in understanding the IL-1R-mediated signaling. Upon IL-1 stimulation, the TIR domain-containing adaptor molecule MyD88 (8) is recruited to the TIR domain of the receptor complex, which then recruits serine-threonine kinases IRAK4 (IL-1 receptor associated kinase 4) (9, 10) and IRAK (11, 12). Whereas IRAK4 is the kinase that functions upstream of and phosphorylates IRAK, the phosphorylated IRAK mediates the recruitment of TRAF6 to the receptor complex (13). IRAK-TRAF6 then leaves the receptor complex to interact with TAK1, a member of the mitogen-activated protein kinase kinase family, and the proteins that bind to it, TAB1, TAB2, and TAB3 on the membrane (14, 15). TAK1 and TAB2 are phosphorylated on the membrane, followed by the formation and translocation of TRAF6-TAK1-TAB1-TAB2, from the membrane to the cytosol (15), where TAK1 is activated. Whereas genetic studies show that IRAK is required for the IL-1-induced activation of TAK1, in vitro biochemical analyses reveal that TRAF6-mediated ubiquitination may also play an important role in TAK1 activation (16). The activation of TAK1 eventually leads to the activation of IkB kinase (IKK) by an unknown mechanism. Activated IKK phosphorylates IkB proteins, which are degraded, releasing NF-κB to activate transcription in the nucleus (17–20). Activated TAK1 has also been implicated in the IL-1-induced activation of MKK6 and JNK (14). The definitive evidence for an essential role of TAK1 in IL-1 signaling is from studies with TAK1-deficient cells. Two groups (21, 22) independently reported that TAK1 deficiency leads to a defect in IL-1 signaling. MEKK3 has also been implicated in IL-1-mediated IKK and JNK activation, possibly through its interaction with TRAF6 (23–25).

The abbreviations used are: IL, interleukin; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; IKK, IkB kinase; PBS, phosphate-buffered saline; IL-1R, IL-1 receptor; MEF, mouse embryonic fibroblast; JNK, c-Jun N-terminal kinase; IFNα, interferon α.
TLR8-mediated Signaling

Recent studies have begun to unravel how a subset of TLRs, TLR7, TLR8, and TLR9, employ a novel MyD88-dependent pathway to mediate the activation of transcription factors NF-κB, IRF5, and IRF7 and induction of interferon-α. Whereas TLR9 has been shown to recognize bacterial DNA, a synthetic compound (imidazoquinoline compound R848) with antiviral activity and single-stranded RNA have been described as ligands for TLR7 and TLR8. It has been recently reported that the transcription factor IRF7 interacts with MyD88 to form a complex in the cytoplasm, and this interaction resulted in activation of IFNα-dependent promoters (26, 27). IRAK4 and TRAF6 have also been implicated in this pathway, and ubiquitin ligase activity of TRAF6 has been shown to mediate IRF7 activation. The detailed molecular mechanisms for this novel TLR7-, TLR8-, and TLR9-mediated MyD88-dependent pathway are still unclear.

In this paper, we report that TLR8 mediate a unique NF-κB activation pathway. The intermediate signaling events mediated by IL-1 (including IRAK modifications and degradation and TAK1 activation) were not detected in cells stimulated by TLR8 ligands. Using IRAK- and TAK1-deficient cells, we found that although TLR8-mediated NF-κB and JNK activation are IRAK-dependent, they do not require IRAK modification and are TAK1-independent. On the other hand, TLR8-mediated IκB phosphorylation and NF-κB and JNK activation are completely abolished in MEKK3−/− MEFs, whereas IL-1-mediated signaling was only moderately reduced in these deficient MEFs as compared with wild-type cells. The differences between IL-1R- and TLR8-mediated NF-κB activation are also reflected at the level of the IKK complex. TLR8 ligands induced IKKγ phosphorylation, whereas IKKα/β phosphorylation and IKKγ ubiquitination that can be induced by IL-1 were not detected in cells treated with TLR8 ligands. We postulate that TLR8-mediated MEKK3-dependent IKKγ phosphorylation might play an important role in the activation of IKK complex, leading to IκBα phosphorylation and NF-κB activation.

MATERIALS AND METHODS

Biological Reagents and Cell Culture—Recombinant human IL-1β was provided by the National Cancer Institute. Antibodies against phosphorylated IκBα (Ser32), ERK, p38, JNK, IKKα/β (Ser176/Ser180), and IKKγ (Ser376) and total IκBα, JNK, IKKα, IKKβ, IKKγ, and ubiquitin were purchased from Cell Signaling. Antibody to FLAG (anti-FLAG) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 3M-002 and 3M-003 were provided by the National Cancer Institute. Interferon-α2 (IFNa2) or interferon-α4 (IFNa4)-dependent-luciferase reporter constructs were generated by PCR and inserted into pBasicLuc. The human TLR7/8 and IKKγ expression constructs were created by inserting cDNA fragments, amplified by PCR, into the pcDNA3.1 expression vector. Wild-type, kinase-inactive (KK213AA), two lysine residues in the ATP binding pocket were mutated to alanine, abbreviated as IRAK4mt) IRAK-4 cDNA was cloned into the retrovirus vector, pBabe-puro. Mammalian expression vectors encoding wild-type and kinase-inactive (K293A) IRAK (driven by the thymidine kinase promoter) were described elsewhere (12). Transfection of the indicated plasmids by FuGENE 6 transfection reagents was done as recommended by the manufacturer (Roche Applied Science). Transfection solution was prepared by mixing 1 μg of plasmid DNA and 3 μl of FuGENE 6 transfection reagent in 100 μl of serum-free medium. After incubation at room temperature for 15 min, the mixture was added to tissue culture wells containing 1 × 105 cells in 2 ml of complete culture medium. MEK3 siRNA sequence 5′-GATTCCCCGCTTTAGATATTGCTGTTTTCAAGAGAAACA-GCAAATATCCATAAGGCTTTTGGAAA-3′ was cloned into vector pSUPER, which was obtained from Dr. Reuven Agami’s group (Center for Biomedical Genetics, The Netherlands) (31).

Luciferase Reporter Assays—Cells (2 × 105) were transiently transfected using FuGENE 6 (Roche Applied Science), following the manufacturer’s protocol. Cells were transfected with the indicated expression vectors plus 100 ng of the luciferase reporter plasmids and 10 ng of β-galactosidase plasmid for normalization, with a 1:3 ratio of DNA/FuGENE 6. Transfection of empty vector was used to ensure that all samples received equal amounts of DNA. At 36 h after transfection, cells were stimulated with ligands for 6 h. Cells were lysed, and luciferase activity was assessed using reporter lysis buffer and luciferase assay reagent (Promega). All results reported represent duplicate experiments with at least three independent transfections.

Western Blot Analysis—Cells that were not treated or treated with IL-1 (10 ng/ml), 3M-02 (5 μg/ml), or 3M-03 (5 μg/ml) were harvested, washed in cold PBS buffer, pelleted, and lysed in ice-cold lysis buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) for 30 min. Cell debris was pelleted by centrifugation for 10 min at 13,000 × g. Supernatants were separated on 10% SDS-PAGE, transferred to supported nitrocellulose membrane (Millipore), and blocked in a 5% solution of nonfat dry milk prepared in 1× PBS and 0.05% Tween 20. Blots were incubated with primary antibody diluted in PBS overnight at 4 °C, washed three or four times for 10 min each with PBS, and then detected with horse-radish peroxidase-conjugated secondary antibody diluted 1:5000 in PBS plus 5% nonfat milk and developed using the enhanced chemiluminescence method (ECL Plus; Amershams Biosciences) following the manufacturer’s protocol.

Coimmunoprecipitation and Immunoblotting—Cell were harvested and lysed in a Triton-containing lysis buffer (0.5% Triton X-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 10 mM NaF, 2 mM dithio-
threitol, 1 mM sodium orthovanadate, 2 mM EGTA, 20 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell extracts were incubated with 1 μg of antibody or preimmune serum (negative control) for 2 h followed by a 2-h incubation with 20 μl of protein A-Sepharose beads (prewashed and resuspended in phosphate-buffered saline at a 1:1 ratio). After incubation, the beads were washed four times with lysis buffer, separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting.

**IkB Kinase Assay**—For IkB kinase assays, cell lysates were immunoprecipitated with anti-IKKα/β and collected on protein A-Sepharose beads. Kinase reactions were performed in 50 μl of buffer containing 20 mM Hepes (pH 7.0), 20 mM MgCl₂, 1 mM ATP, 10 μCi of [γ-32P]ATP at 30 °C for 30 min. The substrate was 2 μg of glutathione S-transferase-IkB (residues 1–54) (Joseph DiDonato, Cleveland Clinic Foundation, Cleveland, OH). Samples were analyzed by 10% SDS-PAGE, followed by autoradiography.

**Nuclear Extracts**—Nuclear extracts were prepared from cells plated in 100-mm dishes. Cells were washed twice with ice-cold PBS, harvested with a cell scraper, and centrifuged at 4 °C for 5 min at 1500 × g. Cell pellets were resuspended in 10 volumes of ice-cold lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.3 mM Na₂VO₄, 5 mM NaF, 1× protease inhibitor mixture (Roche Applied Science), and 0.05% Nonidet P-40), incubated on ice for 10 min, and centrifuged for 10 min at 2000 × g. Nuclear pellets were resuspended in 2 volumes of ice-cold storage buffer (20 mM HEPES (pH 7.9), 500 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.3 mM Na₂VO₄, 5 mM NaF, 1× mixture of protease inhibitor (Roche Applied Science), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, and 25% glycerol) and incubated on ice for 30 min. After centrifugation for 10 min at 14,000 × g, the supernatant fraction was frozen in aliquots and stored at −80 °C.

**Electrophoretic Mobility Shift Assays**—An NF-κB oligonucleotide containing the NF-κB consensus sequence 5’-AGTTGAGGGGACTTTGCCAGGC-3’ was labeled with [γ-32P]ATP using T4 polynucleotide kinase. The NF-κB promoter was incubated with TPA at 20 μg/ml at room temperature in the presence of 10 mmol/liter Tris-HCl buffer, pH 7.5, containing 50 mmol/liter NaCl, 0.5 mmol/liter EDTA, 0.5 mmol/liter dithiothreitol, 4% (w/v) glycerol, and 1 mmol/liter MgCl₂. Nuclear extracts (5 or 10 μg of protein) were incubated for 15 min at room temperature in the presence of 2 μg of poly(dI-dC) in a reaction mixture containing 25 mM HEPES (pH 7.9), 60 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.75 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and the end-labeled oligonucleotide probe (50,000 cpm). The samples were loaded onto a prerun, 16-cm-long, 1.5-mm-thick 4% acrylamide-bisacrylamide (29:1) gel prepared in 1× TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 8.0)). After electrophoresis for 2 h at 200 V (at 4 °C), the gel was dried and exposed to Eastman Kodak Co. films at −80 °C for 3–24 h with intensifying screens. The radiolabeled bands were detected by autoradiography. Each gel mobility shift assay was repeated with at least two independently prepared nuclear extracts.

**RESULTS**

**TLR8-mediated NF-κB Activation Is Accompanied by IkBα Phosphorylation but Not IkBα Degradation**—Although the same upstream signaling components (MyD88, IRAK1, IRAK4, and TRAF6) are utilized by TLR7/8 and IL-1R, TLR7/8 mediate the activation of IRF7 in addition to the common signaling events (NF-κB and JNK activation) shared with IL-1R, implicating differential usage of the upstream signaling components by these receptors. To compare TLR7- and TLR8-mediated signaling with IL-1R, TLR7 or TLR8 was co-transfected with an NF-κB-dependent E-selectin-luc construct into human embryonic kidney 293 cells transfected with IL-1R. As shown in Fig. 1a, both TLR7 and TLR8 induced NF-κB-dependent luciferase activity in 293 cells in response to stimulation with single-stranded poly(U), single-stranded RNA40, and imidazoquinoline compounds (3M-002 and 3M-003). Since TLR8-mediated NF-κB activation is much stronger than TLR7, we stably transfected TLR8 into 293 cells transfected with IL-1R (293-IL-1R-TLR8). Imidazoquinoline compounds (3M-002 and 3M-003) induced similar levels of NF-κB DNA binding activity as compared with that induced by IL-1 stimulation in 293-IL-1R-TLR8 cells (Fig. 1b). Furthermore, TLR8 ligands also induced p38 and JNK activation, albeit at reduced levels compared with IL-1 stimulation (Fig. 1c). However, whereas IL-1 stimulation led to both IkBα phosphorylation and rapid IkBα degradation, the imidazoquinoline compounds only induced IkBα phosphorylation and not IkBα degradation (Fig. 1c).

To determine whether such differential signaling outcomes are only restricted to 293 cells, we isolated primary mouse kidney epithelial cells. As shown in Fig. 1d, both IL-1 and imidazoquinoline compounds induced the activation of NF-κB, JNK, ERK, and p38 in the primary kidney epithelial cells. However, whereas IL-1 stimulation induced both IkBα phosphorylation and degradation, IkBα was phosphorylated but not degraded upon imidazoquinoline compounds (3M-002 and 3M-003) in these primary cells. Since the murine TLR8 gene is not functional, the imidazoquinoline-induced signaling in mouse kidney cells is probably through TLR7. Taken together, these results suggest that TLR7/8 and IL-1R might mediate differential signaling pathways to lead to NF-κB activation.

**TLR8 Do Not Lead to IRAK Modification and Degradation**—To further compare intermediate signaling events mediated by TLR8 and IL-1R, we employed MyD88-deficient cells (I3A), IRAK-deficient cells (I1A), and IRAK4-deficient cells. Similar to IL-1 signaling (12, 15, 32, 33), MyD88, IRAK, and IRAK4 are also required for TLR8-mediated activation of NF-κB-dependent (E-selectin; Fig. 2, a, c, and d) and IRF5/7-dependent (IFNα/β; Fig. 2, b, d) promoters (data not shown). We previously showed that IRAK and IRAK4 kinase-inactive mutants had the same ability as the wild-type IRAK and IRAK4 in restoring IL-1-mediated signaling in IRAK- and IRAK4-deficient cells, respectively, indicating that the kinase activity of IRAK4 is not necessary for the IL-1 pathway (12, 33). The fact that only the impairment of the kinase activity of both IRAK and IRAK4 efficiently abolished the IL-1 pathway suggests that the kinase activity of IRAK and IRAK4 is probably redundant for IL-1-mediated signal-
As shown in Fig. 2, c and d, both wild-type and kinase-inactive IRAK and IRAK4 can also restore TLR7/8-mediated activation of NF-κB-dependent (E-selectin) and IRF5/7-dependent (IFN-α/β) promoters in IRAK- and IRAK4-deficient cells, respectively. These results indicate that whereas IRAK and IRAK4 are necessary for TLR8 signaling, the individual kinase activity of IRAK and IRAK4 is not required, implicating potential redundancy of the kinase activity of these two kinases in TLR8-mediated signaling (Fig. 2 and data not shown).

Hallmarks of IRAK activation upon IL-1 stimulation include its phosphorylation and ubiquitination followed by its degradation (12, 33–35, 35–37). IRAK was clearly modified upon IL-1 stimulation in 293-IL-1R-TLR8 cells (Fig. 2e). Treatment with calf intestinal phosphatase leads to loss of several IL-1-induced shifted IRAK bands, confirming that they are phosphorylated forms (12) (Fig. 2f). IL-1-induced IRAK ubiquitination was confirmed by immunoprecipitation with anti-IRAK antibody followed by Western analysis with anti-ubiquitin antibody (36) (data not shown). However, IRAK modification was not detected in response to the imidazoquinoline compounds in 293-IL-1R-TLR8 cells (Fig. 2e). These results indicate that although IRAK is required for both IL-1R and TLR8-mediated signaling, it might have different signaling roles in these two pathways.

**TLR8 Does Not Lead to TAK1/TAB1 Phosphorylation**—We and others have previously shown that TAK1 is activated upon IL-1 stimulation and is important for IL-1-induced NF-κB and JNK activation (14, 15, 21, 22, 37–39). TAK1 and TAB1 (a TAK1-binding protein and substrate) were clearly modified upon IL-1 stimulation in 293-IL-1R-TLR8 cells (Fig. 3a). Treatment with calf intestinal phosphatase eliminated the IL-1-induced slowly migrating TAK1 and TAB1 bands, confirming that they are phosphorylated forms (38) (Fig. 2f). Furthermore, it has been shown that IL-1-induced phosphorylation of TAK1 and TAB1 correlates with TAK1 activity (15, 38) (data not shown). However, TAK1 and TAB1 were not modified upon imidazoquinoline stimulation in 293-IL-1R-TLR8 cells (Fig. 3a), suggesting that TAK1 may not be activated by TLR8-mediated signaling.

Recently, TAK1-deficient mice were generated by gene targeting using the Cre-loxP system (21). TAK1-deficient (Map3k7−/−) mouse embryonic fibroblasts (MEFs) were generated by in vitro transduction of Cre in MEFs homologous for loxP.
flanked (floxed) Map3k7fl/o/flox, and confirmed by genomic Southern, reverse transcription-PCR, and Western analysis (21).

To further investigate the role of TAK1 in TLR7/8-mediated signaling, we transfected human TLR8 into TAK1/H11001/H11001 (Map3k7fl/o/flox) and TAK1/H11002/H11002 (Map3k7fl/o/flox) MEFs (21). As shown in Fig. 3b, IL-1-induced NF-κB and JNK activation are greatly reduced in TAK1−/− MEFs as compared with that in TAK1+/− MEFs. However, NF-κB and JNK activation were
MEKK3 Is Required for TLR8-mediated NF-κB and JNK Activation—Since MEKK3 has been implicated in TLR-IL-1R signaling, we examined the role of MEKK3 in TLR8-mediated signal-
ing in MEKK3-deficient MEFs (24, 40). We transfected human TLR8 into MEKK3+/+ and MEKK3−/− MEFs. Imidazoquinoline-induced 1xβ phosphorylation (Fig. 4b), NF-
κB and JNK activation (Fig. 3b) were completely abolished in MEKK3-deficient MEFs as compared with that in wild-type control cells. The lack of MEKK3 in MEKK3-deficient MEFs was confirmed by Western analysis (data not shown). These results clearly indicate that MEKK3 is required for TLR8-mediated signal-
ing. The TLR8-mediated signaling was diminished in 293-IL-1R-TLR8 cells where MEKK3 was knocked down by siRNA, confirming the role of MEKK3 in TLR8 signaling (Fig. 3c).

We then examined the impact of imidazoquinoline on IKK. Interestingly, we found that whereas IL-1 stimulation led to the phosphorylation of IKKα/β detected with anti-
Ser(P)177/181IKKβ antibody in 293-IL-1R-TLR8 cells (Fig. 4a) and MEFs (Fig. 4b), imidazoquinoline compounds failed to induce such phosphorylation of IKKα/β in these cells (Fig. 4, a and b). Furthermore, whereas IL-1 stimulation induced IKKγ phosphorylation and ubiquiti-
ation in 293-IL-1R-TLR8 cells (Fig. 4a) and MEFs (Fig. 4b), imidazoquinoline treatment only induced IKKγ phosphorylation but not ubiquitination (Fig. 4, a and c). Imidazoquinoline-induced IKKγ phosphorylation was completely abolished in MEKK3-deficient MEFs as compared with that in wild-type control cells (Fig. 4d). These results clearly indicate that MEKK3 is required for TLR8-mediated IKKγ phosphorylation.

The next question is whether the kinase activity of IKK is activated at all upon activation of TLR8. As shown in Fig. 4, e and f, imidazoquino-
lne compounds can activate 1xβ kinase in 293-IL-1R-TLR8 cells at similar levels as IL-1 stimulation.

consistent with this finding, imidazoquinoline-induced 1xβ phosphorylation was abolished in IKKα/β−/− MEFs (29), whereas JNK activation was intact in these cells. The lack of IKKα and IKKβ in these IKKα/β-deficient MEFs was con-
ferred by Western analysis (data not shown). Taken together, these results suggest that imidazoquinoline-induced NF-κB activation is IKK-dependent, although the mechanism of

induced to similar levels by imidazoquinoline compounds in TAK1+/+ and TAK1−/− MEFs (Fig. 3b). These results clearly indicate that TAK1 is not required for TLR8-mediated NF-κB and JNK activation.

**MEKK3 Is Required for TLR8-mediated NF-κB and JNK Activation**—Since MEKK3 has been implicated in TLR-IL-1R signaling, we examined the role of MEKK3 in TLR8-mediated signal-

**TLR8-mediated Signaling**

![Figure 3. TLR8-mediated signaling is TAK1-independent and MEKK3-dependent. a, TLR8 does not lead to TAK1 and TAB1 phosphorylation. 293 cells transfected with TLR8 were either left untreated or treated (times shown above blot) with IL-1 (10 ng/ml), 3M-002 (5 μg/ml), or 3M-003 (5 μg/ml). The cells were then lysed and analyzed by immunoblot with antibodies against TAK1, TAB1, or actin. b and c, TLR8-mediated signaling is TAK1-independent and MEKK3-dependent. b, wild-type (+/+), TAK1-deficient (−/−), and MEKK3-deficient (−/−) MEFs transiently transfected with human TLR8 were stimulated with IL-1 (10 ng/ml) or 3M-002 (5 μg/ml) for the indicated durations. Nuclear extracts were prepared, and NF-κB DNA binding activity was determined by an electrophoretic mobility shift assay using a probe specific for NF-κB. Whole-cell lysates were prepared and subjected to Western blot analysis using anti-

![Figure 3](image-url)
IKK activation by TLR8 might be different from that by IL-1R (Fig. 5).

DISCUSSION

We here report that TLR8 mediates a novel MyD88-dependent NF-κB activation pathway that is significantly different from the classical MyD88-dependent NF-κB pathway mediated by IL-1R. One striking difference between these two pathways is IRAK modification. IL-1 stimulation leads to IRAK phosphorylation, ubiquitination, and modification. IRAK modifications and degradation were not detected in cells stimulated by TLR8 ligands, although TLR8-mediated NF-κB activation is IRAK-dependent. It is possible that additional adaptor molecules are associated with IL-1R and/or TLR8, influencing IRAK modification upon ligand stimulation.

Following IRAK modification, TAK1 is activated upon IL-1 stimulation, which is critical for IL-1-induced NF-κB activation. Our unpublished data showed that IL-1-induced IRAK modification is required for TAK1 activation.3 Consistent with

3 J. Yao and X. Li, unpublished data.
the fact that TLR8 does not lead to IRAK modification, imidazoquinoline treatment failed to activate TAK1. Using TAK1-deficient MEFs, we found that TLR8-mediated NF-κB and JNK activation are TAK1-independent. Taken together, our results indicate that the unmodified IRAK in TLR8 pathway probably does not utilize TAK1 to mediate downstream signaling. On the other hand, TLR8-mediated IkBα phosphorylation and NF-κB and JNK activation are completely abolished in MEKK3−/− MEFs, whereas IL-1-mediated signaling was only moderately reduced in these deficient MEFs as compared with wild-type cells. Therefore, instead of TAK1, the unmodified IRAK in the TLR8 pathway probably utilizes MEKK3 to mediate NF-κB and JNK activation.

The differences between IL-1R- and TLR8-mediated pathways are also reflected at the level of the IKK complex. TLR8 ligands induced IKKγ phosphorylation, whereas IKKα/β phosphorylation and IKKγ ubiquitination that can be induced by IL-1 were not detected in cells treated with TLR8 ligands. Previous studies suggest that ligand-induced phosphorylation of IKKα/β plays a critical role in IKK activation (41). However, although imidazoquinoline treatment does not lead to IKKα/β phosphorylation, TLR8 ligands still activate the kinase activity of IKK, indicating that IKK is activated through a differential mechanism in TLR8 signaling. Using MEKK3-deficient MEFs, MEKK3 is shown to be required for TLR8-induced IkBα phosphorylation. We postulate that TLR8-mediated MEKK3-dependent IKKγ phosphorylation might play an important role in the activation of IKK complex, leading to IkB phosphorylation and NF-κB activation.

The dogma for NF-κB activation is that signal-induced phosphorylation of IkBα targets this inhibitor of NF-κB for ubiquitination and subsequent degradation, thus allowing NF-κB to enter the nucleus to turn on the target gene (42). One intriguing finding is that whereas IL-1 stimulation leads to IkBα phosphorylation and degradation, imidazoquinoline treatment only causes IkBα phosphorylation but not degradation. By NF-κB DNA binding assay and NF-κB-dependent luciferase reporter assay, it is clear that imidazoquinoline treatment can lead to NF-κB activation. It is possible that the TLR8-mediated IkBα phosphorylation might lead to dissociation of IkBα from NF-κB without IkBα degradation. The dissociated NF-κB migrates to the nucleus to activate gene transcription. Alternately, the phosphorylation of IkBα by imidazoquinoline treatment changes the confirmation of the NF-κB-IkBα complex, which exposes the nuclear localization signal on NF-κB, leading to nuclear localization of NF-κB. Previous studies showed that phosphorylation of IkBα in the C-terminal PEST region is critical for IkBα degradation (42, 43). It is possible that imidazoquinoline treatment might miss such specific phosphorylation on IkBα, which leads to a lack of IkBα degradation. Future studies are required to investigate the detailed molecular mechanism of TLR8-mediated NF-κB activation.

The physiological significance of this novel TLR8-mediated MyD88-dependent pathway still needs to be further investigated. Our unpublished studies showed that this novel MyD88-dependent pathway is predominantly present in primary kidney and intestine/colon epithelial cells. Therefore, it is possible that this unique pathway plays a critical role in maintaining the homeostasis of epithelium. Recent studies showed that the TLR8-MyD88-IRAK4 pathway is required to reverse the suppressive function of Treg cells (44). It should be very interesting to investigate whether this novel MyD88-dependent pathway plays a role in reversing the suppressive function of Treg cells.

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