Rip1 Mediates the Trif-dependent Toll-like Receptor 3- and 4-induced NF-κB Activation but Does Not Contribute to Interferon Regulatory Factor 3 Activation*

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Nicole Cusson-Hermance, Smriti Khurana, Thomas H. Lee, Katherine A. Fitzgerald, and Michelle A. Kelliher

From the Departments of Cancer Biology and Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Rip1 is required for IκB kinase activation in response to tumor necrosis factor α (TNF-α) and has been implicated in the Toll-like receptor 3 (TLR3) response to double-stranded RNA. Cytokine production is impaired when rip1−/− cells are treated with TNF-α, poly(I-C), or lipopolysaccharide, implicating Rip1 in the Trif-dependent TLR3 and TLR4 pathways. To examine the role of Rip1 in the Trif-dependent TLR4 pathway, we generated rip1−/− MyD88−/− cells. Lipopolysaccharide failed to stimulate NF-κB activation in rip1−/−/MyD88−/− cells, revealing that Rip1 is also required for the Trif-dependent TLR4-induced NF-κB pathway. In addition to activating NF-κB, TLR3/4 pathways also stimulate interferon regulatory factor 3 activation. However, we find that Rip1 expression stimulates NF-κB but not interferon regulatory factor 3 activity. In the TNF-α pathway, Rip1 interacts with the E3 ubiquitin ligase Traf2 and is modified by polyubiquitin chains. Upon TLR3 activation, Rip1 is also modified by polyubiquitin chains and is recruited to TLR3 along with Traf6 and the ubiquitin-activated kinase Tak1. These studies suggest that Rip1 uses a similar, ubiquitin-dependent mechanism to activate IκB kinase-β in response to TNF-α and TLR3 ligands.

The death domain kinase Rip1 (receptor-interacting protein) mediates TNF-α-induced NF-κB and p38 mitogen-activated protein kinase activation (1–4). Although Rip1 is required for IKK activation (5), its kinase activity is dispensable (3, 6), suggesting that Rip1 may mediate IKK activation by recruiting other MAP kinases such as Mekk3 or by employing other mechanisms to achieve IKK activation. Upon recruitment to the TNF receptor (TNFR1), Rip1 is initially modified by K63-linked polyubiquitin chains. These K63-linked polyubiquitin chains are recognized by the ubiquitin receptor Tab2 (7), resulting in the recruitment of the ubiquitin-activated kinase Tak1 (TGF-β-activating kinase 1) enzyme to the TNFR1.

Recently, Rip1 has also been implicated in the Toll-like receptor 3 (TLR3)-mediated NF-κB response to dsRNA (8). Rip1 interacts with the TLR3- and TLR4-specific adapter Trif (Toll-interleukin-1 receptor domain-containing adapter inducing IFN-β). Trif is a TIR domain-containing adapter protein that is essential for all signaling by TLR3 and some signaling by TLR4. Rip1 binds the C terminus of the Trif protein via a Rip homotypic interaction motif (8). Trif also binds Traf6 (tumor necrosis factor receptor-associated factor-6) and TBK-1 via its N terminus, and these interactions result in interferon regulatory factor 3 (IRF-3) activation. The TLR4 ligand LPS induces NF-κB activation by engaging the MyD88-dependent and Trif-dependent pathways. Thus, we reasoned that Rip1 may also participate in late phase NF-κB activation induced by TLR4 Trif-dependent pathway.

We find NF-κB responses and cytokine production ablated when rip1−/− murine embryonic fibroblasts (MEF) or splenocytes are stimulated with dsRNA or TNF-α. Although the NF-κB responses to TLR4 appear unaffected due to activation of the MyD88-dependent pathway, LPS-induced cytokine production was impaired in the absence of Rip1. Importantly, LPS-induced NF-κB activation was completely ablated in rip1−/−/MyD88−/− MEF, providing genetic evidence that Rip1 contributes to the Trif-dependent, TLR4-induced NF-κB pathway. Moreover, we find Rip1 phosphorylated and polyubiquitinated in TLR3-stimulated cells and demonstrate that Rip1, Traf6, and the ubiquitin-activated kinase Tak1 are recruited to TLR3 in response to poly(I-C) treatment. Taken together, these studies suggest that Rip1 may use similar ubiquitin-dependent mechanisms to activate IKK-β in TLR3-stimulated cells.

EXPERIMENTAL PROCEDURES

Generation of rip1−/− and rip1−/−/MyD88−/− Murine Embryonic Fibroblasts—Rip1+/− mice were interbred, females were sacrificed between embryonic days 15.5 and 17, and MEF were prepared as described in Ref. 1. For other studies, rip1+/−/tnfr1−/− mice were intercrossed, and splenocytes were isolated from day 2 rip1+/−/tnfr1−/− or rip1−/−/tnfr1−/− mice. MyD88−/− mice were gifts from S. Akira (Osaka, Japan). The MyD88−/− mice used for this study were backcrossed into the C57BL/6 background for 11 generations. To generate rip1−/−/MyD88−/− MEF, rip1+/− mice were mated with MyD88−/− mice, and then rip1+/−/MyD88−/− mice were intercrossed, and females were sacrificed to generate rip1+/− or rip1+/−/MyD88−/− MEF. Traf6−/− MEF were provided by Dr. J.-I. Inoue (Tokyo, Japan).

Cell Lines and Reagents—HEK293 cells were stably transfected with FLAG-tagged human TLR3 as described in Ref. 9. LPS derived from Escherichia coli strain 0111:B4 was purchased from Sigma, dissolved in deoxycholate, and re-extracted by phenol/chloroform as described in Ref. 10. Poly(I-C) was purchased from Amersham Biosciences, and MALP2 and peptidoglycan were purchased from EMC Microcollec-
Rip1 Mediates TLR3 and TLR4 NF-κB Pathways

Coimmunoprecipitation and Western Blotting—HEK293 cells stably transfected with FLAG-tagged human TLR3 left unstimulated or treated with poly(I-C) (100 μg/ml) were lysed in endogenous lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) and immunoprecipitated with anti-FLAG antibody, and associated proteins were detected by immunoblotting with anti-Rip1, -Traf6, or -Tak1 antibodies. To measure NF-κB activity, wild type or rip1−/− MEFs were left untreated or stimulated with TLR2 ligand peptidoglycan (PGN at 10 μg/ml) or IL-1β (10 ng/ml) for the time periods indicated. Activation of NF-κB was measured by probing the cell lysates with a phosphospecific IκBα antibody. To ensure that an equivalent amount of total protein was achieved, cell lysates were probed with an IKK-α antibody. Three independent rip1−/− MEF lines were examined, and a representative experiment is shown.

RESULTS

Rip1 Mediates the Trif-dependent NF-κB Pathway—Recently, the Rip1 protein has been implicated in the innate immune response to double-stranded RNA viruses (8, 12). Mouse embryonic fibroblasts that lack Rip1 fail to activate NF-κB when stimulated with poly(I-C) (8); however, the response to other Toll-like ligands has not been examined. To test whether Rip1, like Rip2, mediates multiple Toll-like receptor pathways, we treated wild type and rip1−/− MEFs with IL-1α (10 ng/ml), TNF-α (10 ng/ml), or TLR ligands poly(I-C) (100 μg/ml), purified LPS (100 μg/ml), peptidoglycan, or MALP2 or infected with Sendai virus, and total cell lysates were examined for evidence of phosphorylated IκBα using a phosphospecific IκBα antibody (Cell Signaling). To ensure that an equivalent amount of total protein was examined, lysates were probed with either an IKK-α antibody (Santa Cruz) or anti- extra-cellular signal-regulated kinase antibody. To measure TNF- and LPS-induced NF-κB activation in the rip1−/− MyD88−/− MEF, IκBα degradation was measured by probing equivalent amounts of total protein with an anti-IκBα antibody (Santa Cruz Biotechnology). Polyubiquitinated Rip1 was detected by stimulating the RAW264.7 macrophage or murine embryonic fibroblasts with TLR2 ligand peptidoglycan. To measure NF-κB activity, we assayed cell lysates for phosphorylated IκBα by immunoblotting with an anti-ubiquitin antibody.
Rip1 Mediates TLR3 and TLR4 NF-κB Pathways

FIGURE 2. Decreased IFN-β expression and cytokine production in poly(I-C)-stimulated rip1−/− MEF. A, IFN-β expression is reduced in poly(I-C)-stimulated rip1−/− cells. Wild type (wt) and rip1−/− MEF were left untreated or treated with poly(I-C) (100 ng/ml) for the time periods indicated, and real-time PCR analysis was performed using primers specific for IFN-β and β-actin. B, poly(I-C) or TNF-α treatment of rip1−/− cells results in impaired cytokine production. Wild type and rip1−/− MEF were left untreated or stimulated with poly(I-C) (100, 10, or 1 μg/ml), Sendai virus (200, 20, or 2 HAU), or murine TNF-α (100, 10, or 1 ng/ml). Twenty-four-hour later, culture supernatants were examined for RANTES production by ELISA. Three independent rip1−/− MEF lines were examined in triplicate, and one representative experiment is shown.

FIGURE 3. Poly(I-C) and LPS-induced cytokine production is severely impaired in Rip1-deficient cells. A, Rip1-deficient MEF are impaired in cytokine response to poly(I-C) and LPS. Wild type and rip1−/− MEF were left unstimulated or stimulated with poly(I-C) (100 or 10 ng/ml), LPS (100 or 10 ng/ml), or mTNF-α (10 ng/ml). Culture supernatants were examined for RANTES production by ELISA. B, poly(I-C)- and LPS-induced cytokine production is impaired in rip1−/−/tnfr1−/− splenocytes. rip1+/+tnfr1−/− or rip1−/−/tnfr1−/− splenocytes were left untreated or stimulated with poly(I-C) (100 or 10 μg/ml) or purified LPS (100 or 10 ng/ml). Twenty-four-hour later, culture supernatants were examined for RANTES production by ELISA. A minimum of three rip1−/− MEF and splenocytes from three rip1+/+tnfr1−/− and two rip1−/−/tnfr1−/− mice were examined in triplicate. A representative experiment for each is shown. wt, wild type.

ligands (Fig. 1, B and C). Thus, the NF-κB responses to Toll-like receptor 2 and 4 ligands appear unaffected by a Rip1 deficiency (Fig. 1, B and C), indicating that, unlike Rip2, Rip1 may only mediate the Trif-dependent pathways.

The lack of TLR3 responsiveness in rip1−/− MEF could reflect decreased TLR3 expression in Rip1-deficient cells. Thus, we examined wild type and rip1−/− MEF for expression of TLR3 and TLR4 using reverse transcription-PCR. We found similar TLR3 and TLR4 expression levels in wild type and rip1−/− cells (not shown), suggesting that the TLR3 signaling defect is not due to decreased TLR3 receptor levels in Rip1-deficient cells.

Decreased IFN-β and RANTES Production in Poly(I-C)-stimulated rip1−/− Cells—To foster antiviral responses, Rip3 and TLR3 and TLR4 activate the transcription factors NF-κB and IRF-3 and induce IFN-β expression. The transcriptional enhancer of the IFN-β promoter contains four positive regulatory domains, which function cooperatively to induce IFN-β expression in response to viral infection. The transcription factors that regulate IFN-β expression include NF-κB, IRF-3, and the ATF-2-c-Jun heterodimer (9, 13). Thus, the initial induction of IFN-β expression requires the activation of NF-κB, yet in our previous published studies, we observed induction of IFN-β in poly(I-C)-treated rip1−/− cells (8). Since these studies were performed using reverse transcription-PCR (13), we reexamined IFN-β expression in poly(I-C)-stimulated rip1−/− MEF using quantitative real-time PCR. At early time points, induction of IFN-β expression was observed in poly(I-C)-stimulated, wild type MEF but not in rip1−/− MEF stimulated with poly(I-C) (Fig. 2A). In contrast, late phase (24-h) IFN-β expression appears less affected, although expression remained significantly reduced in poly(I-C)-treated rip1−/− MEF (Fig. 2A). Similarly, RANTES production was reduced when rip1−/− cells were stimulated with either poly(I-C) or TNF-α (Fig. 2B). However, cytokine production was unaffected when rip1−/− MEF were infected with Sendai virus, a single-stranded RNA virus, that induces IFN-β expression via a Trif- and TLR3-independent pathway (14). These data suggest that Rip1 contributes to anti-viral responses mediated by TLR3 but is not required for NF-κB activation by the intracellular retinoic acid-inducible gene I pathway (15).

Decreased RANTES Production When rip1−/− Splenocytes Are Stimulated with Purified LPS—Although Rip1 has been shown to interact with the TLR3 and TLR4 adapter Trif, cytokine responses to
Rip1 Mediates TLR3 and TLR4 NF-κB Pathways

poly(I-C) or purified LPS were not examined in our previous study (8). Thus, Rip1 may contribute to NF-κB activation when MEF are treated with poly(I-C) but may not contribute significantly to cytokine production in vivo, particularly when the TLR4 pathway is stimulated, since NF-κB can be activated by the MyD88-dependent pathway. Due to the lethality associated with a Rip1 deficiency (1), we were unable to examine TLR3/4 responses in Rip1-deficient macrophages or dendritic cells. To evaluate the contribution of Rip1 to TLR3- and TLR4-induced innate immune responses, we stimulated neonatal day 2 splenocytes from rip1−/− mice and control littermates with poly(I-C), purified LPS, or TNF-α. We found RANTES production significantly reduced when neonatal rip1−/− splenocytes are stimulated with poly(I-C) and LPS (Fig. 3). These findings suggest that Rip1 contributes to both the Trif-dependent TLR3 and TLR4 pathways and demonstrate that activation of both the Trif-Rip1 and MyD88-dependent TLR4 pathways is required for robust cytokine production.

Rip1 Mediates the Trif-dependent TLR4 Pathway—The decreased cytokine responses to the TLR4 ligand LPS observed in rip1−/− MEF and splenocytes suggested that Rip1 may also mediate the Trif-dependent, TLR4-induced NF-κB pathway. To test this possibility, we mated our rip1+/− mice with MyD88−/− mice (generously provided by S. Akira) and then stimulated wild type, rip1+/+ MyD88−/−, or rip1−/− MyD88−/− and rip1+/+ MyD88−/− MEF with TNF-α or LPS and examined cytokine-induced IkBα degradation. TNF-α-induced IkBα degradation was observed in wild type cells and in rip1+/+ MyD88−/− 10 min following cytokine treatment (Fig. 4, A and B). As expected, no TNF-α-induced IkBα degradation is observed in the double knockout rip1−/− MyD88−/− cells due to the absence of Rip1 (Fig. 4C). Wild type MEF exhibit IkBα degradation 15 min following LPS treatment, whereas IkBα degradation is delayed in rip1+/+ MyD88−/− cells with evidence of IkBα degradation detected between 45 min and 1 h poststimulation (Fig. 4, A and B). In contrast, no LPS-induced IkBα degradation was observed in cells deficient for both Rip1 and MyD88 even at 120 min following LPS treatment (Fig. 4C). The double knock out cells failed to respond to TNF-α, LPS, and poly(I-C) but remained capable of activating NF-κB in response to treatment with phorbol 12-myristate 13-acetate/ ionomycin (not shown). These studies provide genetic evidence that Rip1 contributes to the Trif-dependent TLR4-induced NF-κB pathway.

Rip1 Expression Stimulates NF-κB but Not IRF-3 Activation—The presence of a Rip homotypic interaction domain in Trif and Rip1 suggests that Rip1 and Trif may interact to mediate both NF-κB and IRF-3 activation. Deletion of the Rip homotypic interaction motif in Trif ablates Trif-mediated NF-κB activation but has no effect on IRF-3 activation (8). To test whether Rip1 can stimulate both NF-κB and IRF-3 activation, we transfected HEK293 cells with a NF-κB or IRF reporter constructs (ISG54 and ISRE) with expression plasmids containing p65 (RelA), Rip1, or Trif or with a constitutively active IRF-3 expression construct, the phosphomimetic IRF3-5D. Twenty-four h after transfection, luciferase gene activity was measured. The luciferase assays were done in triplicate and repeated three times.

FIGURE 4. Rip1 mediates the Trif-dependent TLR4-induced NF-κB pathway. Wild (wt) type MEF (A), rip1+/+ MyD88−/− (B) and double knockout rip1−/− MyD88−/− (C) were left untreated or stimulated with LPS (10 μg/ml) or TNF-α (10 ng/ml) for the time periods indicated, and NF-κB activity was measured by immunoblotting with an anti-IκBα antibody. To ensure that equal amounts of total protein were loaded, cell lysates were probed with an anti-extracellular signal-regulated kinase (α-ERK) antibody. One wild type, one rip1+/+ MyD88−/−, and three rip1−/− MyD88−/− MEF were examined. One representative experiment is shown.

FIGURE 5. Rip1 expression stimulates NF-κB but not IRF-3 activation. HEK 293 cells were transfected with an NF-κB reporter plasmid (A) or with IRF3 reporter plasmids (B) or with a Viperin-dependent reporter construct (C), together with expression constructs encoding p65, Rip1, Trif, or IRF-3. After 24 h, luciferase gene activity was measured. The luciferase assays were done in triplicate and repeated three times.
Rip1 Mediates TLR3 and TLR4 NF-κB Pathways

A.

B.

C.

FIGURE 6. Poly(I-C) and LPS-induced cytokine production is impaired in traf6−/− MEF. A, poly(I-C) or LPS treatment does not stimulate NF-κB activation in traf6−/− MEF. Wild type (wt) and traf6−/− MEF were left untreated or stimulated with poly(I-C) (100 μg/ml), LPS (100 ng/ml), or TNF-α (10 ng/ml) for the indicated time periods. Cell lysates were separated on a 12% SDS-polyacrylamide gel, and NF-κB activity was assayed by immunoblotting with an anti-phospho-IκBα antibody. B, poly(I-C) or LPS treatment fails to stimulate cytokine production in traf6−/− MEF. Wild type and traf6−/− MEF were left untreated or stimulated with various concentrations of poly(I-C) (100, 10, or 1 μg/ml), LPS (100, 10, or 1 ng/ml), 10 nm MALP2, or 10 ng/ml murine TNF-α. Twenty-four hours later, culture supernatants were examined in triplicate for RANTES production. Each experiment was repeated a minimum of three times.

FIGURE 7. Poly(I-C) treatment stimulates the recruitment of Rip1, Traf6, and Tak1 to the TLR3. HEK293 cells expressing a FLAG-tagged version of human TLR3 were left untreated or stimulated with 100 μg/ml poly(I-C) for the time periods indicated. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, and TLR3-associated proteins were isolated. The presence of Rip1, Traf6, and Tak1 was detected by immunoblotting cell lysates with an anti-Rip1, Traf6, or Tak1 antibody. Total cell lysates were then probed with an anti-Rip1 antibody to assure equal loading. The coimmunoprecipitation experiments were repeated twice.

Rip1, Traf6, and Tak1 Are Recruited to TLR3 upon Poly(I-C) Stimulation—Our data suggest that Rip1 and Traf6 proteins contribute to the Trif-dependent NF-κB pathway. To test whether the endogenous proteins are recruited to TLR3 upon receptor activation, we stimulated HEK 293 that express a FLAG-tagged TLR3 with poly(I-C) for 30, 60, and 120 min. We immunoprecipitated TLR3 using the anti-FLAG antibody and tested whether Rip1 or Traf6 associated with TLR3. We detected Rip1 and Traf6 recruitment to TLR3 30 min following poly(I-C) treatment (Fig. 7, A and B). We also examined the TLR3-associated proteins for evidence of Tak1 recruitment. Tak1 recruitment to TLR3 was observed at 60 min when maximal phosphorylated IκBα protein is also detected (Fig. 7C). Interestingly, Rip1 and Traf6 recruitment appears to precede Tak1 recruitment, suggesting that Traf6 may modify Rip1 and thereby signal Tak1 recruitment.

Rip1 Is Phosphorylated and Polyubiquitinated in TLR3-stimulated Cells—We have shown that the kinase activity of Rip1 is responsive to TNFR1 activation (6), suggesting that the kinase activity of Rip1 is responsive to specific ligands or stresses. Studies in cells expressing kinase-inactive Rip1 have revealed that Rip1 autophosphorylation is induced in TNF-α-stimulated cells (6) (Fig. 8, A and B). Similarly, the related Rip2 kinase and IRAK kinases are responsive to TLR and IL-1 receptor activation (16). To provide additional evidence that Rip1 responds to TLR3/4 activation, we stimulated wild type cells with TNF-α or with the TLR ligand LPS or poly(I-C) and examined cell lysates for phosphorylated Rip1. As expected, phosphorylated Rip1 was detected as early as 5 min following TNF-α treatment. We find Rip1 autophosphorylation also rapidly induced upon poly(I-C) treatment or LPS treatment (Fig. 8, A and B). These studies reveal that the Rip1 kinase activity responds to TNFR1, TLR3, and TLR4 activation and further implicates Rip1 in a TLR3 and TLR4 Trif-dependent pathway.

In addition to phosphorylation, TNF-α stimulates the conjugation of K63-linked polyubiquitin chains on Rip1 (6, 17–19). K63-linked polyubiquitin chains on Rip1, Traf6, or IKK-γ (NEMO) are recognized by

experiment suggests that Rip1 may not participate in the Trif-dependent IRF-3 pathway and is recruited by Trif to mediate IKK-β activation.

LPS- and Poly(I-C)-induced Cytokine Production Is Diminished in traf6−/− MEF—The MyD88-dependent NF-κB pathway is mediated by the recruitment of IRAK kinases and Traf6 and the Tak1-Tab1-Tab2 complex. Thus, we reasoned that Traf6 may also contribute to the Trif-dependent NF-κB pathway. To test this possibility, we stimulated wild type and traf6−/− MEF with TLR ligand poly(I-C) or LPS or with TNF-α and examined IKK activity using a phospho-IκBα antibody. As poly(I-C) or LPS treatment fails to stimulate cytokine production in traf6−/− MEF. Wild type and traf6−/− MEF were left untreated or stimulated with various concentrations of poly(I-C) (100, 10, or 1 μg/ml), LPS (10, 10, or 1 ng/ml), 10 nm MALP2, or 10 ng/ml murine TNF-α. Twenty-four hours later, culture supernatants were examined in triplicate for RANTES production. Each experiment was repeated a minimum of three times.
Rip1 Mediates TLR3 and TLR4 NF-κB Pathways

The TLR3 and TLR4 pathways employ the adapter protein Trif that signals both NF-κB and IRF-3 activation. Trif recruits Traf6 and TBK-1 via its N terminus, and TBK-1 is responsible for IRF-3 phosphorylation and activation (11). However, it remains unclear how Trif activates the IKK-β and JNK2 pathways. Data base screening for proteins containing regions homologous to Trif revealed that Traf-induced NF-κB activation may be mediated by Rip1 (8). Studies in Rip1-deficient MEF then confirmed defects in poly(I-C)-induced NF-κB activation, implicating Rip1, a mediator of TNF-α-induced IKK activation, in the Trif-dependent NF-κB pathway (8).

In this study, we address the biologic contribution of Rip1 to innate immune responses by examining poly(I-C)- and LPS-induced cytokine production in rip1−/− MEF and splenocytes. Consistent with the published studies on Trif-deficient and MyD88-deficient cells (20, 21), we find poly(I-C)- and LPS-induced cytokine production impaired in Rip1-deficient MEF and splenocytes. Taken together, these studies support the model that TLR4-induced cytokine responses to bacterial pathogens require activation of both the Trif-dependent and MyD88-dependent pathways.

The involvement of Rip1 in the Trif-dependent TLR3 NF-κB pathway suggested that Rip1 may also contribute to the Trif-dependent TLR4 NF-κB activation. Consistent with this idea, we find that LPS, like TNF-α and poly(I-C), stimulates the kinase activity of Rip1, and we find LPS-induced cytokine levels diminished in rip1−/− cells. To test the contribution of Rip1 to the Trif-dependent TLR4 NF-κB pathway, we generated rip1−/− MyD88−/− cells and stimulated them with either TNF-α or LPS. As expected, NF-κB activation is delayed in LPS-treated rip1−/− MyD88−/− cells; however, in the absence of both MyD88 and Rip1, LPS-induced NF-κB activation is not observed. Taken together, these studies demonstrate that Rip1 is responsive to TLR4 activation and demonstrates that Rip1 mediates both the Trif-dependent TLR3 and TLR4 NF-κB pathways. Interestingly, the dsRNA-activated protein kinase PKR has also been implicated in poly(I-C)-induced NF-κB activation and in apoptotic responses to poly(I-C), TNF-α, and LPS (22, 23), raising the possibility that dsRNA-induced NF-κB activation may involve PKR and Rip1. Consistent with this model, Li and co-workers (24) find PKR recruited to Trif upon poly(I-C) stimulation. However, unlike PKR-deficient cells that are resistant to poly(I-C) and TNF-α-induced apoptosis, Rip1-deficient cells are sensitive to TNF-α and poly(I-C)-induced cell death, suggesting that Rip1 and PKR have opposing functions in the TNF-α and dsRNA-activated pathways.3

We demonstrate that, like TNFR1 activation, TLR3 activation stimulates Rip1 recruitment and Rip1 polyubiquitination. In TNF-α-treated cells, ubiquitin chain conjugation is mediated by the Ubc13-Uev1a complex and facilitated by Traf2, the E3 ubiquitin ligase recruited to the TNFR1 that also interacts with Rip1 (6, 19). We find TLR3 and TLR4 responses diminished in traf6−/− cells and observe ligand-dependent recruitment of Rip1, Traf6, and Tak1 to TLR3 (Fig. 7). Our studies suggest that Traf6 also contributes to TLR3/4-induced antimicrobial responses. Traf6 has been shown by others to be recruited to TLR3 and bind Trf (24, 25) and has been implicated in TLR signaling with decreased cytokine responses observed when traf6−/− macrophages are stimulated with TLR2, -4, -7, and -9 ligands (26).

Rip1 polyubiquitination is induced upon TNF-α or poly(I-C)-treatment of cells, suggesting that Rip1 may use similar mechanisms to activate NF-κB in the TNFR1- and Trif-dependent TLR pathways. Thus, polyubiquitinated Rip1 may be recognized by the Tab2 protein (7), a

the ubiquitin receptor protein Tab2, a component of the ubiquitin-activated Tak1 complex (7). Thus, Rip1 may use similar mechanisms to mediate IKK-β activation in TNF-α- and TLR3- or TLR4-stimulated cells. To test this possibility, we stimulated the macrophage cell line RAW264.7 and the U373 astrocytoma line with TNF-α for 10 min or treated the cells with poly(I-C) for 30, 60, or 120 min. We immunoprecipitated Rip1 from the untreated and treated cells and examined the lysates for polyubiquitinated Rip1 protein by immunoblotting with an anti-ubiquitin antibody. We observed evidence of Rip1 polyubiquitination in both the poly(I-C) and TNF-α-treated cell lysates (Fig. 8C), suggesting that polyubiquitinated Rip1 may contribute to IKK-β activation in both the TNFR1 and TLR3 pathway. It remains unclear whether Rip1 is modified by K63-linked and/or K48-linked polyubiquitin chains in the TLR3-stimulated cells; however, Rip1 degradation does not appear induced in the poly(I-C)-treated cells, suggesting that Rip1 may be modified by K63-linked polyubiquitin chains. Moreover, the recruitment of Traf6 and Tak1 would support the idea that K63-linked polyubiquitinated Rip1 stimulates IKK-β activation in TLR3/4-stimulated cells. These studies provide genetic and biochemical evidence that Rip1 is an essential mediator of the TLR3 and TLR4 NF-κB responses and suggest that the Trif-dependent NF-κB pathway may be mediated by polyubiquitinated Rip1 and the ubiquitin-activated Tak1 complex.

3 G. Wen and M. A. Kelliher, unpublished data.
Rip1 Mediates TLR3 and TLR4 NF-κB Pathways

component of the Tak1 complex, and thereby, the ubiquitin-activated kinase Tak1 may be recruited to the TLR3. Consistently, we find Traf6 and Tak1 recruited to TLR3, and we find TLR3/4-induced cytokine production ablated in traf6−/− cells. Additionally, TLR3 and TLR4 NF-κB and IRF-3 responses have been shown to be negatively regulated by the ubiquitin-modifying enzyme A20 (27–29).

Parallels can also be made to Drosophila melanogaster, where the Imd protein, the mammalian counterpart to Rip1, mediates the antimicrobial immune response to Gram-negative bacteria. Imd is required for the antimicrobial response and mechanism of IKK activation in flies and mammals appear highly conserved and may be dependent on the ubiquitin-modifying enzyme A20 (27–29).

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