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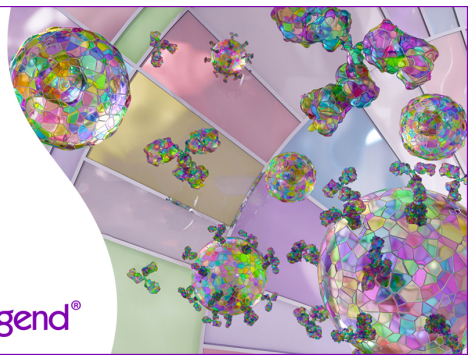

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Raf Kinase Inhibitor Protein Preferentially Promotes TLR3-Triggered Signaling and Inflammation

Rongrong Lai,*[,1] Meidi Gu,*[,1] Wei Jiang,[†,1] Wenlong Lin,* Penglei Xu,* Zhiyong Liu,* He Huang,[‡] Huazhang An,[†] and Xiaojian Wang*

Raf kinase inhibitor protein (RKIP) protects against host immunological responses in nematodes and *Drosophila*. Whether RKIP functions in innate immune responses in mammals remains unknown. In this article, we report that RKIP preferentially regulates the TLR3-mediated immune response in macrophages. RKIP deficiency or silencing significantly decreases polyinosinic:polycytidylic acid [Poly(I:C)]-induced IFN-β, IL-6, and TNF-α production without affecting the counterpart induced by LPS or CpG. Compared with their wild-type counterparts, RKIP-deficient mice produce less IFN-β, IL-6, and TNF-α in serum and display decreased lethality upon peritoneal Poly(I:C) plus D-galactosamine injection. Mechanistically, RKIP interacts with TBK1 and promotes the Poly(I:C)-induced TANK-binding kinase 1/IRF3 activation. Simultaneously, RKIP enhances the Poly(I:C)-induced interaction between TGF-β–activated kinase 1 and MAPK kinase 3 (MKK3), thus promoting MKK3/6 and p38 activation. We further demonstrated that Poly(I:C) treatment, but not LPS treatment, induces RKIP phosphorylation at S109. This action is required for RKIP to promote TANK-binding kinase 1 activation, as well as the interaction between TGF-β–activated kinase 1 and MKK3, which lead to activation of the downstream IRF3 and p38, respectively. Therefore, RKIP acts as a positive-feedback regulator of the TLR3-induced inflammatory response and may be a potential therapeutic target for inflammatory disease. *The Journal of Immunology*, 2017, 198: 4086–4095.

Toll-like receptors are essential components of the immune system and play an important role in infectious and noninfectious disease processes. For example, TLR3 plays a protective role in enteric bacterial infection (1). Bipolar disorder patients exhibit high expression of TLR2 and TLR5 (2). Thus, TLRs and their signaling pathways are appealing targets for therapeutics. As pattern recognition receptors, the TLR family recognizes different conserved pathogen-associated molecular patterns (PAMPs) to initiate the innate immune response and regulate the subsequent adaptive immunity (3). For example, TLR2 or TLR5 recognizes bacterial components, such as lipopeptides or flagellin. Endosomal TLRs, such as TLR3, TLR7, TLR8, and TLR9, typically sense pathogen-derived nucleic acids. TLR4, which can signal from both plasma and endosomal membranes, recognizes bacterial LPS (4).

TLRs have an extracellular ligand-binding domain, a single membrane-spanning segment, and a cytosolic Toll/IL-1R (TIR) domain (5). After the recognition of PAMPs, the TIR domain recruits the adapter MyD88 and/or the TIR domain–containing adaptor inducing IFN-β (TRIF) (6), leading to the activation of the transcription factors NF-κB, AP-1, IRF3, and/or IRF7; these transcription factors then induce the production of proinflammatory cytokines and type I IFN. The MyD88-dependent pathway is used by all TLRs except TLR3 (6). After activation, MyD88 recruits protein kinases of the IRAK family, such as IRAK1 and/or IRAK2; this recruitment leads to the polyubiquitination of TRAF6 and the phosphorylation of the downstream TGF-β–activated kinase 1 (TAK1) and MAPKs. Then, phosphorylated TAK1 activates the IKK complex, which induces the activation of the transcription factor NF-κB. Activated MAPKs trigger proinflammatory cytokine production via the transcription factor AP-1 (7). For the activation of TLR3, the TIR domain recruits TRIF, which leads to the activation of the TRAF6, TRAF3, and RIP1 pathways. TRAF6 activates MAPKs and TAK1 in a manner similar to the MyD88 pathway. Furthermore, TRAF3 activates the TANK-binding kinase 1 (TBK1)–IKKi complex and induces the oligomerization and phosphorylation of IRF3 and IRF7 (8), which consequently induce the production of type I IFN. Excessive activation of TLRs induces inflammatory responses, such as endotoxin shock (9) and tissue injury (10). Numerous inflammatory diseases, such as systemic lupus erythematosus (11), rheumatoid arthritis (12), and multiple sclerosis (13), are associated with excessive activation of TLR signaling. Therefore, it is essential to investigate the exact regulatory mechanism of TLR signaling.

Raf kinase inhibitor protein (RKIP), a member of the evolutionary conserved phosphatidylethanolamine-binding protein superfamily,

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Abbreviations used in this article: HA, human influenza hemagglutinin; MKK3, MAPK kinase 3; MOI, multiplicity of infection; PAMP, pathogen-associated molecular pattern; Poly(I:C), polyinosinic:polycytidylic acid; RKIP, Raf kinase inhibitor protein; RSV, respiratory syncytial virus; siRKIP, RKIP-specific siRNA; siRNA, small interfering RNA; TAK1, TGF-β–activated kinase 1; TBK1, TANK-binding kinase 1; TIR, Toll/IL-1R; TRIF, TIR domain–containing adaptor inducing IFN-β; WT, wild-type.

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was originally identified as a RAF-1 binding protein that could inhibit MEK phosphorylation and activation (14). Further research demonstrated that RKIP is engaged in many signaling cascades, including G protein–coupled receptor (15), NF-κB (16), and GSK3β signaling pathways (17). RKIP plays an important role in the pathophysiological process by regulating cell growth and differentiation, accelerating the apoptosis of tumor cells, and limiting tumorigenesis and metastasis (18). Recent research has revealed that RKIP participates in the development of inflammation. RKIP deficiency apparently inhibits the IFN-γ secreted by $CD8^+$ T cells in the systemic inflammatory reaction syndrome induced by staphylococcal enterotoxin A (19). We recently demonstrated that RKIP contributes to colitis in mice and humans via mediating intestinal epithelial cell apoptosis (16). Simultaneously, RKIP positively regulates the antiviral innate immune response via promoting TBK1 activation (20). However, whether RKIP affects the TLR-mediated innate immune response remains unknown.

In this study, we demonstrate that RKIP preferentially positively regulates TLR3-induced inflammation. RKIP knockout or knockdown remarkably impaired polyinosinic:polycytidylic acid [Poly(I:C)]-induced TBK1/IRF3 and MAPK kinase 3 (MKK3)/p38 activation, and significantly inhibited Poly(I:C)-induced proinflammatory cytokine production in macrophages. Compared with control mice, RKIP-deficient mice produced fewer proinflammatory cytokines and type I IFNs and were more resistant to Poly(I:C)-induced death. We further demonstrate that phosphorylation of RKIP serine 109 is required for RKIP to promote TLR3-mediated signaling and inflammation.

Materials and Methods

Mice and animal experiments

$RKIP^{-/-}$ mice on a C57BL/6J background were kindly provided by Prof. John Sedivy from Brown University. Mice were genotyped by PCR analysis of DNA isolated from tails using the following primers: RKIP sense (5′-GAGCCCTGGCCGGTCTCCCTTGTCCCAAACTTT-3′), RKIP–wild-type (WT) antisense (5′-CACAAAACCAATCTTAAAGAGCCA-3′), and RKIP-knockout antisense (5′-CCAAAAGGGTCTTTGAGCACCAGAGGACATCCG-3′). The mice were bred in specific pathogen-free conditions and intercrossed to obtain homozygous knockout mice and WT control littermates. For in vivo experiments, 7- to 8-wk-old and sex-matched mice received i.p. injections of Poly(I:C) (8 μg/g; Sigma) plus D-galactosamine (0.5 mg/g; Merck) or LPS (*E. coli* O111:B4; 10 mg/kg; Sigma) (21) alone. The survival of the injected mice was monitored every 4 h for 3 d or every 12 h for 5 d. All mice were housed in the University Laboratory Animal Center. The animal experiment protocols were approved by the Review Committee of Zhejiang University School of Medicine (Hangzhou, China) and were in compliance with institutional guidelines.

Histological analysis

Livers from control or Poly(I:C) plus D-galactosamine–infected mice were dissected, fixed in 4% paraformaldehyde, embedded in paraffin, cut into sections, stained with H&E solution, and examined by light microscopy for histology changes. Immunohistochemical staining was performed using standard procedures.

Plasmids

Mouse RKIP, TAK1, TRAF6, MKK3, and MKK6 cDNAs were reverse transcribed from mRNA prepared from RAW264.7 cells and cloned into the pcDNA3.1 enhanced GFP vector. Sequences were confirmed by Sanger sequencing.

RNA interference

The small interfering RNA (siRNA) duplexes were transfected into mouse peritoneal macrophages or 293T cells using INTERFERin reagent (Polyplus) according to a standard protocol.

The siRNA sequences for RKIP or TBK1 used in this study were as follows: control siRNA: 5′-TTCTCCGAACGTGTCACGT-3′, si-mouse-RKIP: 5′-UGGUCAACAUGAAGGGUAATT-3′, and si-mouse-TBK1: 5′-CUGUGAAAGUGUAUGAGAATT-3′.

Cell culture and stimulation

Peritoneal macrophages were collected by peritoneal washing 3 d after injection of thioglycollate. In brief, C57BL/6 mice (6–8 wk old) were i.p. injected with 2 ml 3% sodium thioglycollate (Sigma) solution. Three days later, cells in the abdominal cavity were collected by lavage with 10–15 ml RPMI 1640 medium (Invitrogen) without FBS and then centrifuged. Cells were seeded and maintained in RPMI 1640 medium containing 10% heat-inactivated FBS (Biology Industries). After the floating cells were removed 4 h later, the adhered peritoneal macrophages were subjected to further experiments. The RAW264.7 cell line and 293T cell line were maintained in DMEM (Life Technologies) containing 10% heat-inactivated FBS. For immunoblot analysis, quantitative RT-PCR, and immunoprecipitation, 1.6×10^6, 2.4×10^6, and 20×10^6 macrophages, respectively, were used per 12-well plate or 100-mm dish, respectively. Before samples were collected, cells were treated with Poly(I:C) (20 μg/ml; Sigma), LPS (100 ng/ml; Sigma), CpG (0.3 μM; Sangon Biotech), or respiratory syncytial virus (RSV) (multiplicity of infection [MOI] 0.1) or pretreated with the p38 inhibitor SB203580 (Sigma) for 1 h.

Quantitative real-time PCR

Real-time PCR was performed using SYBR Green fluorescence as previously described (22). Primers were synthesized by the Sangon Company: mouse β-Actin sense 5′-AACAGTCCGCCTAGAAGCAC-3′; mouse β-Actin antisense 5′-CGTTGACATCCGTAAAGACC-3′; mouse IFN-β sense 5′-CCCTATGGAGATGACGGAGA-3′; mouse IFN-β antisense 5′-CTGTCTGCTGGTGGAGTTCA-3′; mouse IL-6 sense 5′-AGTTGCCTTCTTGGGACTGA-3′; mouse IL-6 antisense 5′-TCCACGATTTCCCAGAGAAC-3′; mouse TNF-α sense 5′-CTGGGACAGTGACCTGGACT-3; mouse TNF-α antisense 5′-GCACCTCAGGGAAGAGTCTG-3′.

Measurement of cytokines

Serum from mice injected with Poly(I:C) plus D-galactosamine or LPS was collected at the indicated times. The concentrations of TNF-α (eBioscience), IL-6 (eBioscience), and IFN-β (BioLegend) were measured using ELISA kits according to the manufacturers' instructions. Peritoneal macrophages or RAW264.7 cells were stimulated with various TLR ligands for the indicated times, and the culture medium was collected for measurement of the TNF-α, IL-6, and IFN-β levels by ELISA.

Immunoblot analysis and immunoprecipitation

Total cells were washed twice with ice-cold PBS and lysed with cell lysis buffer (Cell Signaling Technology). The protein concentration was determined using the BCA protein assay kit (Thermo). Cell lysates (20–50 μg) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and probed with a primary Ab against the target protein. Mouse mAbs against the Flag and human influenza hemagglutinin (HA) epitopes and β-actin (Abmart) and rabbit polyclonal Abs against p-TBK1, TBK1, p-IRF3, p-p38, p38, p-MKK3/6, MKK3, MKK6, p-JNK1/2 p-ERK1/2, p-TAK1(T187), MKK4, MKK7 (Cell Signaling Technology), TLR4, TLR3, TAK1 (Abcam), RKIP, and IKKα/β (Santa Cruz Biotechnology) were purchased from the indicated manufacturers. The polyclonal Ab against mouse p-RKIP-S109 was generated by immunizing a rabbit (Abmart) with the following peptide: C-SDYVG(pS)GPPSG. For immunoprecipitation, cell extracts were prepared using lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, and 0.5% [v/v] Nonidet P-40, 1 mM EDTA) supplemented with a protease inhibitor mixture (Roche). Lysates were coimmunoprecipitated with anti-Flag (M2)-agarose (Sigma), anti-HA agarose (Abmart), or Ab-coupled beads (Bio-Rad) for 3 h at 4°C. The immunoprecipitates were washed three times with the same buffer and subjected to immunoblot analysis.

Statistical analysis

Statistical analysis was performed with Prism v5.0 (GraphPad Software). For in vivo experiments, values are expressed as the mean + SEM of n animals, and the presented data are representative of at least three independent experiments. Mouse survival curves and statistics were analyzed with the Mantel–Cox test. The level for statistically significant differences was defined as $p < 0.05$.

Results

RKIP specifically promotes TLR3-activated IFN-β and proinflammatory cytokine production in macrophages

To explore the role of RKIP in the TLR signaling pathway, we first used primary peritoneal macrophages from WT ($RKIP^{+/+}$) and

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RKIP-deficient ($RKIP^{-/-}$) mice to investigate the effect of RKIP on the TLR3-, TLR4-, and TLR9-mediated inflammatory responses. We measured the IFN-β, IL-6, and TNF-α production and mRNA levels in cells stimulated with Poly(I:C), LPS, or CpG. As shown in Fig. 1A, the TLR3 ligand Poly(I:C)-induced secretion and mRNA expression of IFN-β, IL-6, and TNF-α were significantly lower in the RKIP-deficient group than in the WT group. Simultaneously, we treated $RKIP^{+/+}$ or $RKIP^{-/-}$ peritoneal macrophages with the TLR4 ligand LPS or the TLR9 ligand CpG and observed no significant difference in IFN-β, IL-6, and TNF-α protein and mRNA expression (Supplemental Fig. 1A, 1B) between WT and RKIP-deficient macrophages. The purity of macrophage was up to 94.9% (Supplemental Fig. 1C). RKIP-specific siRNA (siRKIP) was synthesized and transfected into peritoneal macrophage cells to inhibit endogenous RKIP expression (Supplemental Fig. 1D). RNA interference–based silencing of RKIP led to substantial decreases in the IFN-β, IL-6, and TNF-α mRNA expression in macrophages treated with Poly (I:C) (Fig. 1B). To further investigate the preferential role of RKIP in the TLR3-mediated inflammatory response, we overexpressed RKIP in RAW264.7 cells, screened stable cell lines, and then challenged the transfectants with Poly(I:C), LPS, or CpG. As shown in Fig. 1C, RKIP overexpression significantly increased the Poly(I:C)-induced IFN-β, IL-6, and TNF-α mRNA

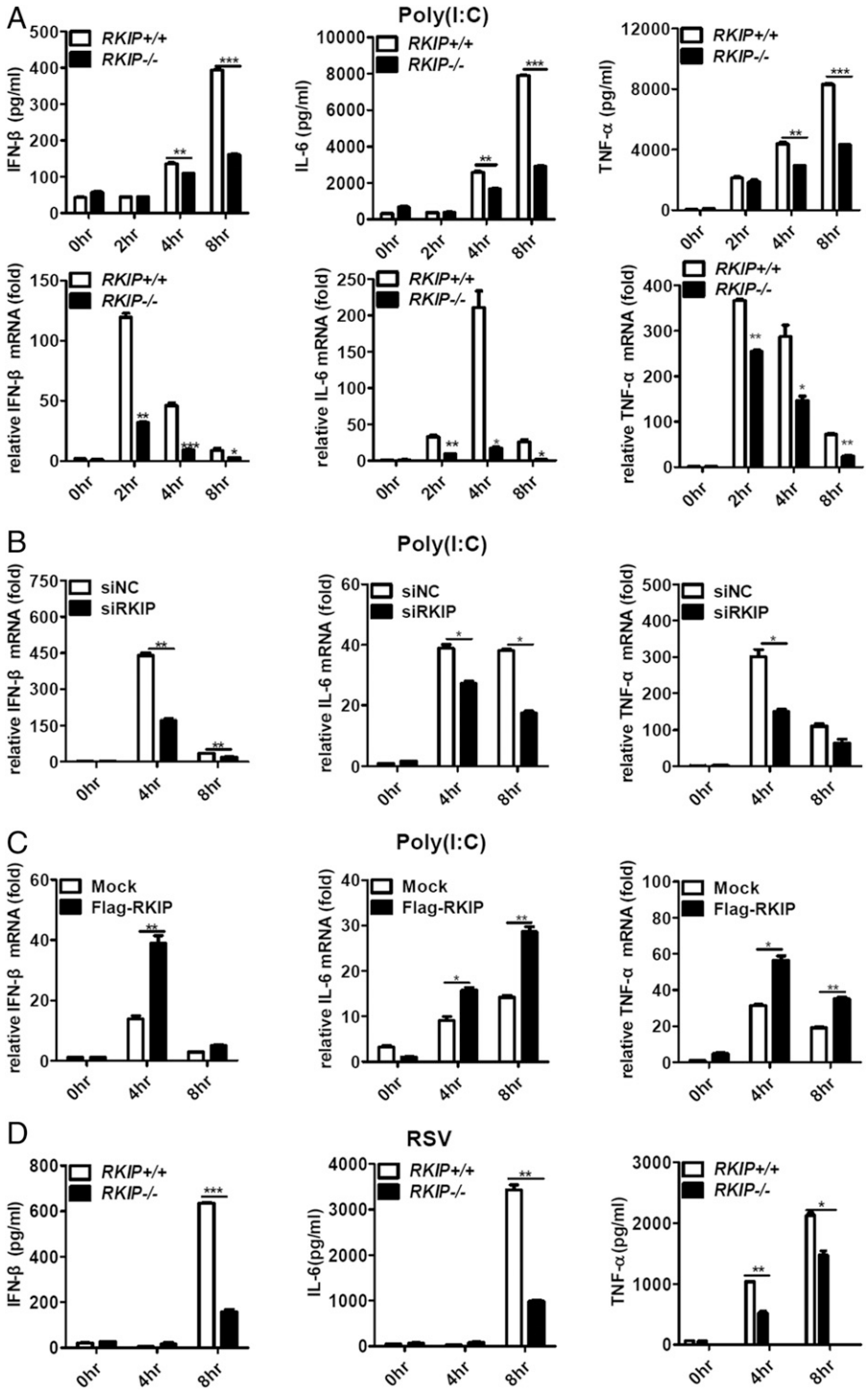

FIGURE 1. RKIP specifically promotes the TLR3-induced IFN-β and proinflammatory cytokine production in macrophages. (**A**) ELISA or real-time PCR analysis of IFN-β, IL-6, and TNF-α in peritoneal macrophages isolated from RKIP-deficient mice and control littermates (WT) treated with Poly(I:C) (20 μg/ml) for the indicated times. (**B**) Real-time PCR analysis of IFN-β, IL-6, and TNF-α mRNA in peritoneal macrophages treated with siRKIP or scrambled siRNA and then stimulated with Poly(I:C) (20 μg/ml) for the indicated times. (**C**) Real-time PCR analysis of IFN-β, IL-6, and TNF-α mRNA in RAW264.7 cells stably transfected with RKIP and then stimulated with Poly(I:C) (20 μg/ml) for the indicated times. (**D**) ELISA analysis of IFN-β, IL-6, and TNF-α in peritoneal macrophages isolated from RKIP-deficient mice and control littermates (WT) infected with RSV (MOI 0.1) for the indicated times. Data are the means ± SEM. Similar results were obtained for at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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expression but had no effect on the LPS- or CpG-induced IFN-β, IL-6, and TNF-α mRNA expression (Supplemental Fig. 2A, 2B). We further infected *RKIP*$^{+/+}$ or *RKIP*$^{-/-}$ peritoneal macrophages with RSV, which is sensed by TLR3 (23, 24). As shown in Fig. 1D, RKIP deficiency significantly inhibited RSV-induced IFN-β, IL-6, and TNF-α protein production. The viral loads in the *RKIP*$^{+/+}$ and *RKIP*$^{-/-}$ macrophages were determined by 50% tissue culture infective dose assay. There was no difference in the viral loads between *RKIP*$^{+/+}$ and *RKIP*$^{-/-}$ macrophage at 2 h post RSV infection (Supplemental Fig. 2C), indicating the same infectivity of WT and RKIP knockout cells with RSV. However, the increased viral loads were shown in the *RKIP*$^{-/-}$ macrophage at 8 h post RSV infection when compared with that in *RKIP*$^{+/+}$ macrophage, which suggest knockout of RKIP renders the cells susceptible to RSV infection. These results indicate that RKIP preferentially promotes TLR3-activated type I IFN and proinflammatory cytokine production in macrophages.

RKIP-deficient mice are more resistant to Poly(I:C)-induced inflammation

To further investigate whether RKIP regulates the TLR-mediated innate immune response in vivo, we i.p. injected RKIP knockout (*RKIP*$^{-/-}$) mice and control littermates (*RKIP*$^{+/+}$) with Poly(I:C) plus D-galactosamine or LPS alone. Serum cytokines were measured 2 h after Poly(I:C) injection or 1.5 h after LPS injection. As shown in Fig. 2A, the Poly(I:C)-induced production of IFN-β, IL-6, and TNF-α was significantly reduced in sera from *RKIP*$^{-/-}$ mice compared with WT counterparts. In contrast, WT and *RKIP*$^{-/-}$ mice challenged with LPS exhibited no significant difference of IFN-β, IL-6, and TNF-α production (Supplemental Fig. 2D). The survival was evaluated every 4 h for 3 d. Consis-

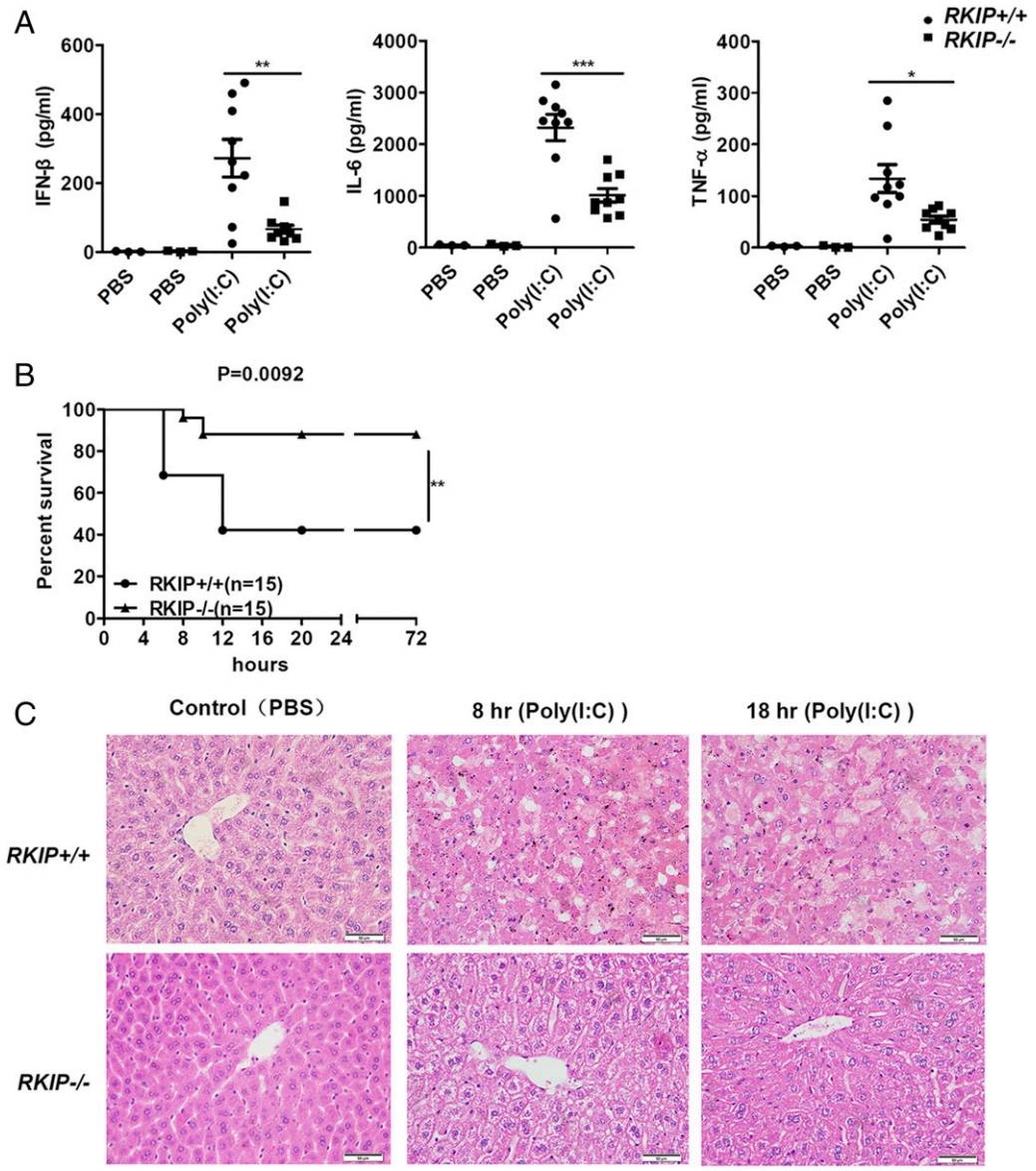

FIGURE 2. RKIP-deficient mice are more resistant to Poly(I:C)-induced inflammation. (**A**) Sex- and age-matched *RKIP*$^{+/+}$ and *RKIP*$^{-/-}$ mice were i.p. injected with Poly(I:C) (8 μg/g) plus D-galactosamine (0.5 mg/g). The ELISA analysis of IFN-β and proinflammatory cytokines in serum was performed 2 h later ($n = 9$ per group). (**B**) Survival curve for sex- and age-matched *RKIP*$^{+/+}$ and *RKIP*$^{-/-}$ mice i.p. injected with Poly(I:C) (8 μg/g) plus D-galactosamine (0.5 mg/g). The survival was monitored every 4 h for 3 d, $n = 15$. (**C**) The liver pathology was assayed after Poly(I:C) treatment as in (A). The tissue was stained with H&E. Scale bars, 50 μm. Similar results were obtained for three independent experiments. ns, no significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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tent with the results of cytokine production, $RKIP^{-/-}$ mice exhibited delayed death and a decreased percentage of lethality compared with that in the $RKIP^{+/+}$ counterparts after the injection of Poly(I:C) plus D-galactosamine (Fig. 2B). Notably, WT and $RKIP^{-/-}$ mice exhibited similar survival curves after LPS treatment (Supplemental Fig. 2E). Furthermore, liver parenchyma analysis revealed less inflammatory infiltrate and fewer eosinophil and monocyte cells in $RKIP^{-/-}$ mice than in WT mice. Ferric ion effusion from hepatocytes was observed in the liver of Poly(I:C)-treated $RKIP^{+/+}$ mice, but not Poly(I:C)-treated $RKIP^{-/-}$ mice (Fig. 2C). These results suggest that RKIP preferentially positively regulates TLR3-mediated inflammatory cytokine production, which leads to more serious injury in mice.

RKIP promotes Poly(I:C)-induced TBK1, IRF3, MKK3/6, and p38 phosphorylation

TLR3 signaling mediates type I IFN and proinflammatory cytokine production by triggering TRIF-related adaptor molecule and the downstream kinases TBK1 and TAK1, which lead to the activation of the IRF3, MAPK, and NF-κB pathways (25). To investigate the mechanism underlying the RKIP regulation of the TLR3-mediated IFN-β, IL-6, and TNF-α production, we first explored whether RKIP influences the TBK1, IRF3, MAPK, or NF-κB signaling pathways triggered by Poly(I:C) stimulation or RSV infection in peritoneal macrophages. As shown in Fig. 3A, RKIP deficiency significantly inhibited the Poly(I:C)-induced TBK1, IRF3, MKK3/6, and p38 phosphorylation but did not affect the TAK1, ERK1/2, JNK, IKKα/β, and NF-κB phosphorylation. Simultaneously, RKIP-deficient macrophage showed impaired activation of TBK1, IRF3, MKK3/6, and p38 triggered by RSV infection (Fig. 3B). Moreover, RKIP deficiency had no effect on the LPS- or CpG-induced TBK1, IRF3, MKK3/6, and p38 phosphorylation (Supplemental Fig. 3A, 3B). These results demonstrate that RKIP preferentially promotes the Poly(I:C)-induced TBK1-IRF3 and MKK3/6-p38 pathway activation in macrophages. Consistently, knockdown of RKIP with siRNA remarkably inhibited the Poly(I:C)-induced TBK1, IRF3, MKK3/6, and p38 phosphorylation (Fig. 3C). To further investigate the role of RKIP in regulating TLR3-mediated signaling, we next evaluated the TLR3-mediated signaling in RAW264.7 cells stably overexpressing RKIP. As shown in Fig. 3D, overexpression of RKIP remarkably increased the Poly(I:C)-induced TBK1, IRF3, MKK3/6, and p38 phosphorylation in RAW264.7 macrophages. To further examine whether RKIP-mediated inflammatory cytokine production depends on facilitating the TAK1-MKK3-p38 pathway, we pretreated RAW264.7 cells stably overexpressing RKIP with a p38 MAPK-specific inhibitor (SB203580). As shown in Fig. 3E, the IL-6 and TNF-α mRNA expression were considerably increased in stably RKIP-overexpressing transfectants treated with Poly(I:C) compared with control transfectants treated with Poly(I:C). Pretreatment with SB203580 not only inhibited the Poly(I:C)-induced IL-6 and TNF-α mRNA expression but also reversed the potentiating effect of RKIP overexpression on the Poly(I:C)-induced IL-6 and TNF-α mRNA expression. Taken together, these results demonstrate that RKIP increases type I IFN and inflammatory cytokine production by positively regulating TBK1-IRF3 and MKK3/6-p38 activation in the TLR3-triggered innate immune response.

RKIP promotes the interaction between TAK1 and MKK3

Stimulation of TLR3 triggers a TRIF-dependent signaling cascade, recruiting the downstream TRAF6 and leading to activation of TAK1. Subsequently, TAK1 activates the NF-κB, AP-1, or CREB transcription factors through the IKK complex, MKK4/7/JNK pathway, or MKK3/6/p38 pathway, respectively (4). As shown in Fig. 3A–D, RKIP specifically promotes MKK3/6 and p38 MPAK activation. TRAF6, TAK1, MKK3, and MKK6 (26, 27) are involved in the TLR3-triggered p38 activation. To further investigate the mechanism by which RKIP promotes the TLR3-triggered p38 activation, we examined the effect of RKIP on the p38 activation mediated by these molecules. As shown in Fig. 4A, RKIP overexpression enhanced the p38 phosphorylation induced by TAK1 and TRAF6 overexpression, but not MKK3 or MKK6 overexpression. In contrast, RKIP silencing led to a reduction in the p38 phosphorylation induced by TAK1 and TRAF6 overexpression, but not that induced by MKK3 or MKK6 overexpression (Fig. 4B). Given that RKIP deficiency or knockdown inhibited TLR3-activated MKK3/6 phosphorylation, but not TAK1 phosphorylation (Fig. 3A, 3C), RKIP may function as an adaptor to facilitate the interaction between TAK1 and MKK3/6 and promote MKK3/6 phosphorylation. To test this hypothesis, we first performed a coimmunoprecipitation assay in 293T cells to examine the interaction between RKIP and MKK3, MKK6, or TAK1. As shown in Fig. 4C, RKIP interacted with MKK3, MKK6, and TAK1, but not GFP. We immunoprecipitated RKIP from lysates of Poly(I:C)-treated RAW264.7 macrophages stably overexpressing Flag-RKIP. RKIP physically associated with TAK1, IKKα/β, MKK3, and MKK6, but not MKK4, MKK7, or TLR3 (Fig. 4D). We noticed that the interaction between TAK1 and RKIP and between MKK3 and RKIP increased after Poly(I:C) stimulation, peaked at 30 min, and then declined to the basal levels of resting cells (Fig. 4D), indicating that RKIP may be involved in the interaction between TAK1 and MKK3. We next performed coimmunoprecipitation assays in primary peritoneal macrophages using an anti-TAK1 Ab. As shown in Fig. 4E, Poly(I:C) induced the interaction between TAK1 and MKK3, but this interaction was remarkably reduced in RKIP-deficient macrophages (Fig. 4E). To confirm these results, we performed coimmunoprecipitation in RAW264.7 macrophages stably overexpressing Flag-RKIP. Compared with the control cell line, RKIP-overexpressing macrophages exhibited increased interaction between TAK1 and MKK3, but not MKK6 (Fig. 4F). Notably, RKIP had no effect on the interaction between TAK1 and IKKα/β or TAK1 and MKK4/7 (Fig. 4E, 4F), consistent with the result that RKIP did not affect the Poly(I:C)-induced NF-κB or JNK pathway. These results suggest that RKIP strengthened the interaction between TAK1 and MKK3 and promoted the phosphorylation of MKK3 and p38, subsequently increasing the production of proinflammatory cytokines during the TLR3-mediated inflammatory immune response.

RKIP promotes TLR3-activated signaling pathways in a serine 109 phosphorylation-dependent manner

We previously reported that upon viral infection, TBK1 mediates RKIP S109 phosphorylation, and the phosphorylated RKIP subsequently interacts with TBK1 and promotes TBK1 activation, ultimately leading to IRF3 phosphorylation and the production of type I IFN (20, 28). We therefore tested whether Poly(I:C), LPS, or CpG induces RKIP S109 phosphorylation in primary peritoneal macrophages. As shown in Fig. 5A, Poly(I:C), but not LPS or CpG, induced phosphorylation of RKIP serine 109. Silencing of TBK1 expression significantly inhibited the Poly(I:C)-induced RKIP phosphorylation in macrophages, indicating that TBK1 was required for the Poly(I:C)-induced RKIP phosphorylation (Fig. 5B). Because the S109 phosphorylation in RKIP was regulated by Poly(I:C) treatment, we evaluated whether this phosphorylation is essential for the RKIP promotion of the Poly(I:C)-induced signaling pathway and cytokine production. We constructed RKIP mutant vectors by mutating the RKIP S109 residue to alanine to mimic an unphosphorylated state (S109A) and screened RAW264.7 cells stably overexpressing the S109A mutant. As shown in Fig. 5C, the overexpression of WT RKIP in RAW264.7 cells enhanced the phosphorylation of TBK1, IRF3, MKK3/6, and p38 triggered by Poly(I:C), whereas the RKIP

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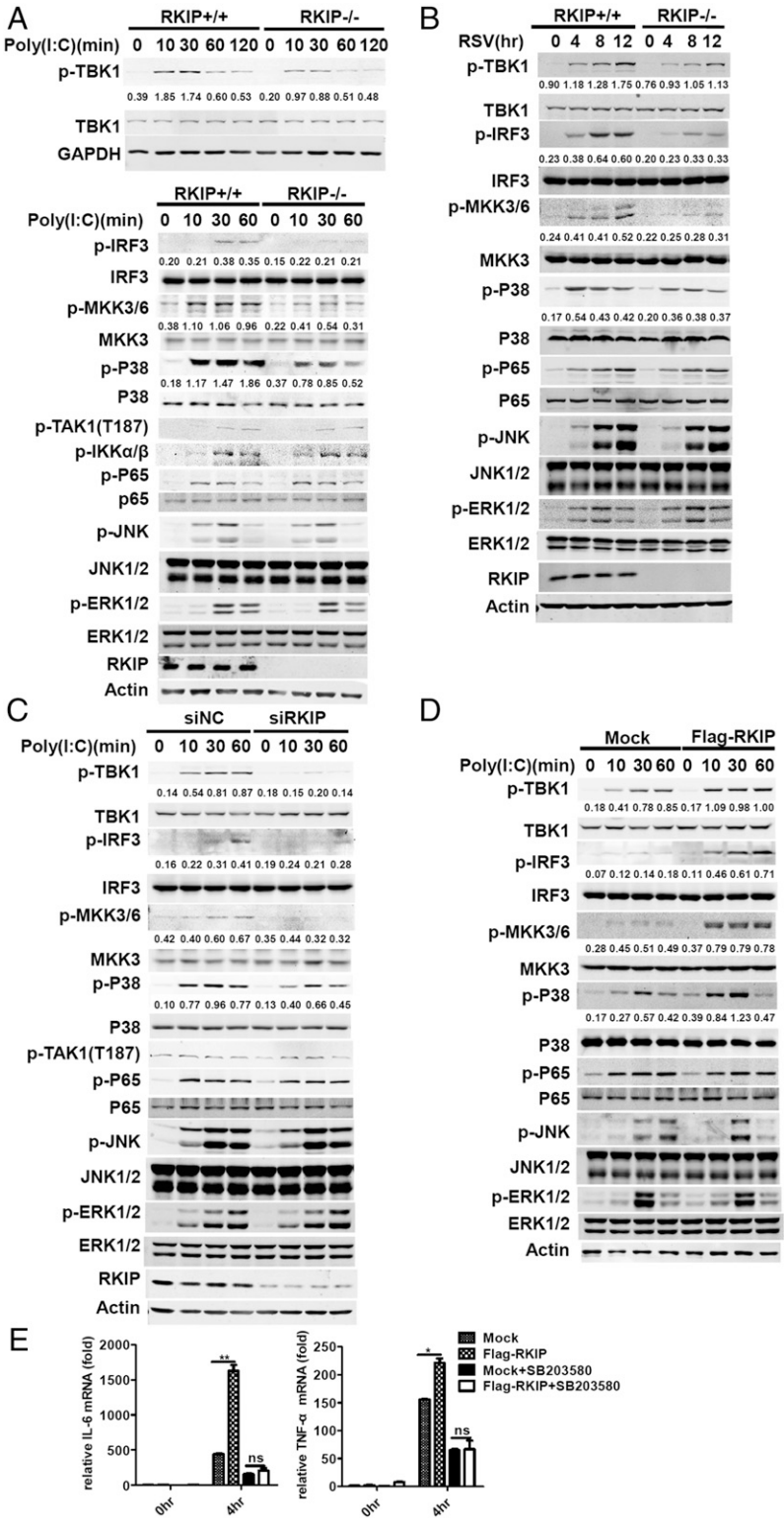

FIGURE 3. RKIP promotes TLR3-induced TBK1, IRF3, MKK3/6, and p38 phosphorylation in macrophages. (**A** and **B**) Immunoblot analysis of p-TBK1, p-P65, p-TAK1, p-IRF3, p-p38, p-MKK3/6, p-JNK, and p-ERK1/2 in lysates of $RKIP^{+/+}$ and $RKIP^{-/-}$ peritoneal macrophages stimulated by Poly(I:C) (20 μg/ml) (A) or RSV (MOI 0.1) (B) for the indicated times. (**C** and **D**) Immunoblot analysis of p-TBK1, p-P65, p-TAK1, p-IRF3, p-p38, p-MKK3/6, p-JNK, and p-ERK1/2 in lysates of peritoneal macrophages transfected with siRKIP or scrambled siRNA (B) or RAW264.7 cells stably overexpressing RKIP and (C) stimulated with Poly(I:C) (20 μg/ml). (**E**) Real-time PCR analysis of IFN-β, IL-6, and TNF-α mRNA in RAW264.7 cells stably transfected with RKIP, followed by treatment with SB203580 (p38 inhibitor, 20 μM) for 1 h and then stimulation with Poly(I:C) (20 μg/ml) for the indicated times. Numbers between two blots indicate densitometry of phosphorylated proteins relative to that of total proteins, respectively. Similar results were obtained for at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. ns, no significance.

S109A mutant failed to promote the TLR3-activated phosphorylation of TBK1, IRF3, MKK3/6, and p38. Consistently, the Poly(I:C)-induced expression of IFN-β, IL-6, and TNF-α mRNA was increased in WT RKIP stable transfectants, but not in RKIP S109A stable transfectants, compared with control transfectants (Fig. 5D).

We reported that virus infection induced RKIP S109 phosphorylation, which is crucial for RKIP to interact with TBK1 and promote TBK1 activation. Poly(I:C) stimulation increased the interaction between TBK1 and RKIP in RAW264.7 stable transfectants (Figs. 4D, 5E), and this interaction correlated with RKIP S109 phosphorylation. In addition, no interaction between TBK1 and the RKIP S109A mutant was detected in RKIP S109A-overexpressing macrophages. Coimmunoprecipitation experiments in primary peritoneal macrophages using an anti-TBK1 Ab further demonstrated that the interaction between TBK1 and RKIP was increased with Poly(I:C) stimulation and was correlated with RKIP S109 phosphorylation (Fig. 5F). Taken together, the results indicate that in addition to virus infection, TLR3 signaling triggers RKIP S109

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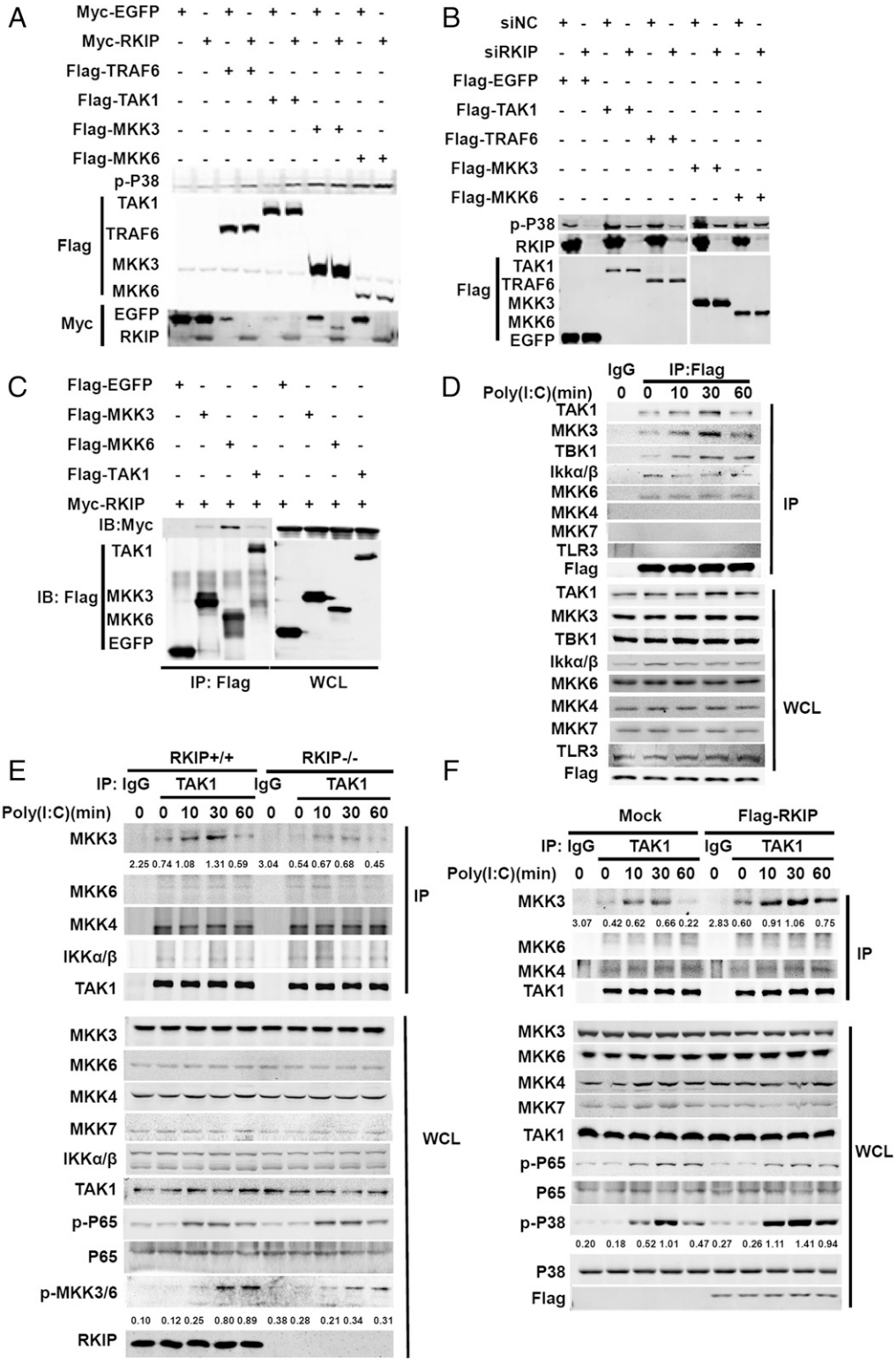

FIGURE 4. RKIP promotes the interaction between TAK1 and MKK3, but not MKK6. (**A**) Immunoblot analysis of the lysate of 293T cells transfected with an empty vector or myc-RKIP vector together with the plasmid for flag-TRAF6, flag-TAK1, flag-MKK3, or flag-MKK6. (**B**) Immunoblot of extracts of 293T cells transfected with plasmids for flag-TRAF6, flag-TAK1, flag-MKK3, or flag-MKK6 together with siRKIP or scrambled siRNA. (**C**) Immunoblot analysis of 293T cells transfected with the indicated combinations of vectors for myc-RKIP, flag-MKK6, flag-MKK3, or flag-TAK1 or an empty vector, followed by immunoprecipitation by anti-flag beads. (**D**) Immunoblot of the lysate of RAW264.7 cells stably expressing RKIP and treated with Poly(I:C) (20 μg/ml) for the indicated times, followed by immunoprecipitation with anti-flag beads probed with the indicated Ab. (**E**) Immunoblot of extracts of peritoneal macrophages isolated from *RKIP*$^{+/+}$ and *RKIP*$^{-/-}$ mice treated with Poly(I:C) (20 μg/ml) followed by immunoprecipitation with an anti-TAK1 Ab. (**F**) Immunoblot of the lysate of RAW264.7 cells stably expressing RKIP and treated with Poly(I:C) (20 μg/ml) for the indicated time, followed by immunoprecipitation with anti-TAK1. Numbers below the blots indicate densitometry of phosphorylated proteins relative to that of total proteins, respectively. Similar results were obtained from three independent experiments. WCL, whole-cell lysates.

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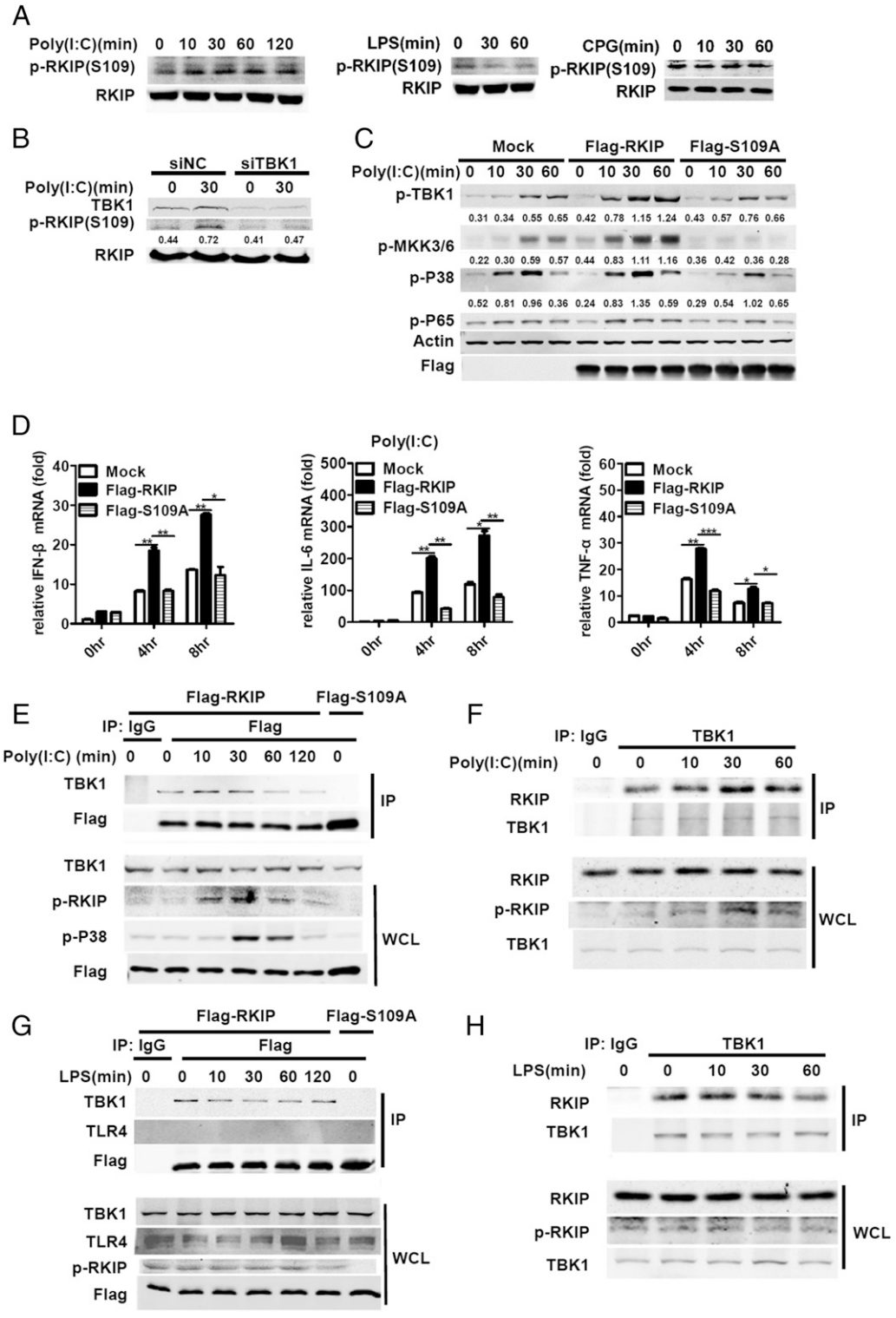

FIGURE 5. RKIP promotes TLR3-triggered signaling in a S109 phosphorylation-dependent manner. (**A**) Immunoblot analysis of the p-RKIP of peritoneal macrophages stimulated with Poly(I:C) (20 μg/ml), LPS (100 ng/ml), and CpG (0.3 μM) for the indicated times. (**B**) Immunoblot analysis of the p-RKIP of peritoneal macrophages after transfection with TBK1-specific or scrambled siRNA and then stimulated with Poly(I:C) (20 μg/ml). Numbers between two blots indicate densitometry of phosphorylated proteins relative to that of total proteins. (**C**) Immunoblot analysis of p-TBK1, p-MKK3/6, p-p38, and p-P65 in the lysate of RAW264.7 cells stably transfected with RKIP or RKIP-S109A and stimulated with Poly(I:C) (20 μg/ml). Numbers below the blots indicate densitometry of phosphorylated proteins relative to that of Actin protein, respectively. (**D**) Real-time PCR analysis of the IFN-β, IL-6, and TNF-α mRNA in RAW264.7 cells stably overexpressing WT RKIP or RKIP-S109A and treated with Poly(I:C) (20 μg/ml). (**E**) Immunoblot of the lysate of RAW264.7 cells stably overexpressing flag-tagged RKIP and stimulated with Poly(I:C) (20 μg/ml) for the indicated times, followed by immunoprecipitation with anti-flag beads. (**F**) Immunoblot of extracts of peritoneal macrophages isolated from $RKIP^{+/+}$ and $RKIP^{-/-}$ mice treated with Poly(I:C) for the indicated times, followed by immunoprecipitation with the anti-TBK1 Ab. (**G**) Immunoblot of the lysate of RAW264.7 cells stably overexpressing flag-tagged RKIP and stimulated with LPS for the indicated times, followed by immunoprecipitation with anti-flag beads. (**H**) Immunoblot of extracts of peritoneal macrophages isolated from $RKIP^{+/+}$ and $RKIP^{-/-}$ mice treated with LPS (100 ng/ml) for the indicated times, followed by immunoblotting with anti-TBK1 Ab. Similar results were obtained for at least three independent experiments.

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phosphorylation via TBK1, which is critical for RKIP to bind to TBK1 and promote TBK1-IRF3 activation and the production of IFN-β. We also investigated the interaction between RKIP and TBK1 upon LPS stimulation. As shown in Fig. 5G and 5H, the interaction between RKIP and TBK1 was not enhanced but was slightly reduced after LPS treatment in both RKIP-overexpressing cells and primary peritoneal macrophages. There was no interaction of RKIP with TLR4 in the macrophage with or without LPS stimulation (Fig. 5G). Consistently, LPS stimulation did not induce RKIP phosphorylation at S109 (Fig. 5A, 5G), which explains why RKIP has no effect on LPS-induced signaling and inflammation given that Ser^{109} phosphorylation is essential for RKIP to promote TLR3-activated signaling. Simultaneously, we constructed RKIP mutant vectors by mutating the RKIP S109 residue to aspartic acid to mimic constitutive phosphorylation (S109D) and screened RAW264.7 cells stably overexpressing the S109D mutant. As shown in Supplemental Fig. 2C, constitutive phosphorylation of the RKIP mutant led to increased mRNA expression of IFN-β, IL-6, and TNF-α after LPS administration, whereas WT RKIP had no effect on the LPS-induced cytokine expression. Taken together, these results demonstrate that RKIP promotes TLR3 signaling and type I IFN and inflammatory cytokine production in a Ser^{109} phosphorylation-dependent manner.

RKIP promotes the interaction between TAK1 and MKK3 in a S109 phosphorylation-dependent manner

Given that RKIP S109 phosphorylation is required for Poly(I:C)-induced MKK3 phosphorylation, we next explored whether RKIP positively regulates the Poly(I:C)-induced interaction between TAK1 and MKK3 in a manner that is dependent on S109 phosphorylation. Poly(I:C)-treated WT or stable RKIP S109A mutant RAW264.7 transfectants were used to perform coimmunoprecipitation assays. As shown in Fig. 6A, overexpression of RKIP increased the Poly(I:C)-induced interaction between TAK1 and MKK3 compared with that in control transfectants. However, RKIP S109A mutants did not promote the Poly(I:C)-induced interaction between TAK1 and MKK3 (Fig. 6A). Consistently, 293T cells overexpressing WT RKIP, but not S109A mutant, exhibited increased interaction between TAK1 and MKK3 compared with that in control transfected cells (Fig. 6B). Taken together, these data suggest that RKIP S109 phosphorylation is essential for the interaction between TAK1 and MKK3, the downstream phosphorylation of p38, and the production of proinflammatory cytokines.

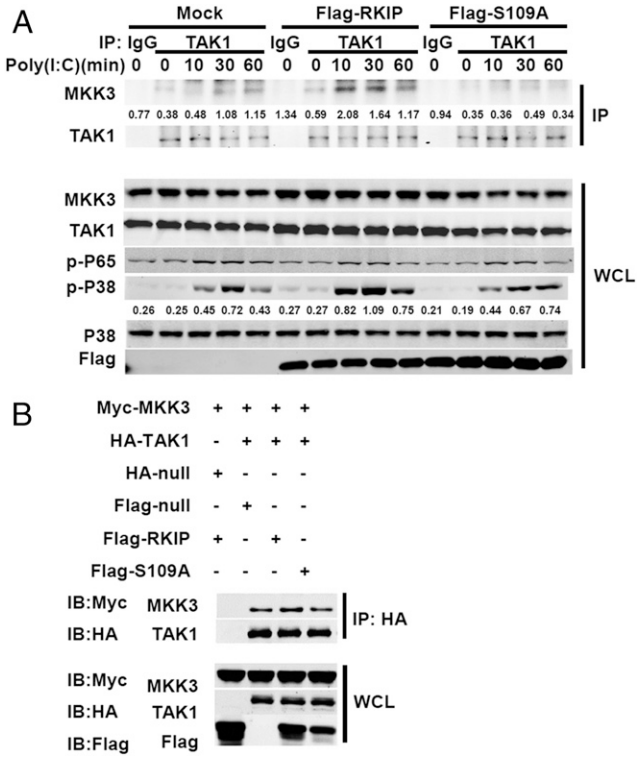

FIGURE 6. RKIP promotes the interaction between TAK1 and MKK3 in a Ser^{109} phosphorylation-dependent manner. (**A**) Immunoblot analysis of RAW264.7 cells stably overexpressing WT RKIP or RKIP-S109A and treated with Poly(I:C) for the indicated times, followed by immunoprecipitation with anti-TAK1 Ab. Numbers between the blots indicate densitometry of phosphorylated proteins relative to that of total proteins, respectively. (**B**) Immunoblot analysis of the lysate of 293T cells transfected with the indicated combinations of vectors for Myc-MKK3, HA-TAK1, flag-WT-RKIP, or flag-RKIP-S109A or an empty vector, followed by immunoprecipitation with an anti-HA Ab. Similar results were obtained for at least three independent experiments.

Discussion

Inflammation is implicated in various diseases, including aging, autoimmune diseases, cancer, cardiovascular diseases, infectious diseases, metabolic diseases, obesity, and respiratory diseases (29). As pattern recognition receptors, TLRs sense PAMPs and damage-associated molecular patterns. Activation of TLRs leads to increased production of various inflammatory cytokines and is involved in numerous inflammatory disease processes (11, 12). Accordingly, the TLR inflammatory response must be tightly regulated. In this report, RKIP preferentially positively regulated TLR3-triggered signaling and inflammation in an RKIP S109 phosphorylation-dependent manner. RKIP-deficient mice were more resistant to Poly(I:C)-induced death.

RKIP is a well-known metastasis suppressor, and reduced RKIP expression is observed in a number of human cancers (30). For example, the downregulation of RKIP in hepatocellular cancer associated with hepatitis B infection indicates aggressive tumor behavior and predicts a worse clinical outcome (31). RKIP also performs a protective role against host immunological responses in nematodes and *Drosophila* (32). Our laboratory recently demonstrated that RKIP protects mice from vesicular stomatitis virus infection (20). In this study, we first identified RKIP as a positive regulator of TLR3-induced inflammation in vitro and in vivo. Compared with WT macrophages, RKIP-deficient or RKIP-silenced macrophages exhibited reduced Poly(I:C)- or RSV-induced IFN-β and proinflammatory cytokine production, whereas RKIP overexpression robustly increased the Poly(I:C)-induced expression of proinflammatory cytokines and type I IFN. Compared with WT mice, RKIP-knockout mice produced less IFN-β and proinflammatory cytokines following Poly(I:C) plus D-galactosamine injection and exhibited decreased mortality, suggesting that RKIP plays an important role in accelerating TLR3 activation–induced inflammatory cytokine production and inflammation in vivo and in vitro. TLR3-mediated IFN-β production promoted HBV clearance in a mouse model (33). Given that RKIP facilitates Poly(I:C)-induced IFN-β production, it is important to investigate whether RKIP participates in the anti-HBV process and inhibits the development of hepatic carcinoma.

Upon activation, TLR3 recruits the downstream adaptor molecules TRIF or TRIF-related adaptor molecule, and these adaptor molecules trigger various downstream signaling cascades, including the TAK1/MKK3/6/p38 and TAK1/MKK4/7/JNK signals, the TRAF6/TAK1/NF-κB pathway, and the TBK1/IRF3 pathway (34). Ultimately, these processes induce the transcription of type I IFN and proinflammatory cytokines. In this article, we showed that RKIP significantly increased the Poly(I:C)-induced TBK1/IRF3 and

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MKK3/6/p38 activation but had no effect on NF-KB activation and ERK1/2 and JNK activation. RKIP promotes TRAF6- and TAK1-induced p38 activation, but not MKK3- and MKK6-induced p38 activation. Coimmunoprecipitation experiments showed that RKIP interacts with MKK3, MKK6, and IKKα/β in macrophages but specifically promotes the Poly(I:C)-induced interaction between TAK1 and MKK3, which accelerates MKK3/p38 activation and the production of proinflammatory cytokines. Upon viral infection, TBK1 mediates RKIP S109 phosphorylation. Phosphorylated RKIP subsequently interacts with TBK1 and directly promotes TBK1 activation, ultimately leading to the production of type I IFN to clear the invading virus (20). In this study, we also found that Poly(I:C) induced RKIP S109 phosphorylation via TBK1, and that S109 phosphorylation is critical for RKIP to promote TBK1/IRF3 and MKK3/p38 activation and the production of proinflammatory factors and type I IFN. Consistently, the RKIP S109A mutant failed to promote the Poly(I:C)-induced interaction between TAK1 and MKK3. Although both activated TLR4 and TLR3 recruit TRIF and induce subsequent TBK1 activation, only Poly(I:C) induces RKIP serine 109 phosphorylation. This finding may explain why RKIP has no effect on the TLR4-mediated signaling pathway and the production of proinflammatory factors and type I IFN. The interaction between RKIP and TBK1 was increased upon Poly(I:C) stimulation but slightly reduced with LPS stimulation. We examined the location of RKIP and TBK1 in LPS- or Poly(I:C)-treated macrophages and observed no difference (data not shown). It might be possible that LPS stimulation induces other modifications of RKIP that prevent RKIP phosphorylation at S109, which is essential for RKIP to promote TLR3-triggered inflammation.

In summary, our study demonstrates that RKIP is required for TLR3-mediated TBK1-IRF3 and MKK3-p38 activation and the production of the downstream type I IFN and proinflammatory cytokines in macrophages. TLR3 activation induces RKIP S109 phosphorylation via TBK1. Phosphorylated RKIP promotes TBK1 activation and the interaction between TAK1 and MKK3, thus activating the downstream IRF3 and p38, respectively. RKIP-deficient mice produce reduced levels of proinflammatory cytokines and type I IFNs and are more resistant to Poly(I:C)-induced death. As a positive regulator of TLR3-mediated inflammation, RKIP may be a potential therapeutic target for inflammatory disease.

Disclosures

The authors have no financial conflicts of interest.

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