A Novel IRAK1–IKKε Signaling Axis Limits the Activation of TAK1–IKKβ Downstream of TLR3

Daniela Bruni, Jordi Sebastia, Susan Dunne, Martina Schröder, and Marion P. Butler

IRAK1 is involved in the regulation of type I IFN production downstream of TLR3. Previous work indicated that IRAK1 negatively regulates TRIF-mediated activation of IRF3 and IRF7. We report that IRAK1 limits the activation of the TLR3–NF-κB pathway. Following TLR3 stimulation, IRAK1-deficient macrophages produced increased levels of IL-6 and IFN-β compared with wild type macrophages. Pharmacological inhibition of TAK1 reduced this increase in IFN-β, together with the heightened activation of IRF3 and p65 found in TLR3-ligand stimulated IRAK1-deficient macrophages. Recently, IKKε and TANK-binding kinase 1 (TBK1) were reported to limit activation of the NF-κB pathway downstream of IL-1R, TNFR1, and TLRs. We show that TBK1 has a positive role in the TLR3–NF-κB pathway, because we detected reduced levels of IL-6 and reduced activation of p65 in TBK1-deficient macrophages. In contrast, we show that IKKε limits the activation of the TLR3–NF-κB pathway. Furthermore, we show that IRAK1 is required for the activation of IKKε downstream of TLR3. We report impaired activation of ERK1/2 in IRAK1– and IKKε-deficient macrophages, a novel finding for both kinases. Importantly, this work provides novel mechanistic insight into the regulation of the TLR3-signaling pathway, providing strong evidence that an IRAK1-IKKε–signaling axis acts to limit the production of both type I IFNs and proinflammatory cytokines by regulating TAK1 activity. ***The Journal of Immunology*****, 2013, 190: 2844–2856.**

Interleukin-1 receptor associated kinase (IRAK)-1 is a serine/threonine kinase that was previously shown to be essential for IFN-α production downstream of TLR-7 and TLR-9 signaling in plasmacytoid dendritic cells (1). In contrast, in TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent signaling, IRAK1 has been proposed to act as a negative regulator of type I IFN production, negatively regulating the activation of IRF3 and IRF7 (2).

TLR-3–mediated activation of NF-κB and IRF3 is thought to bifurcate at the level of TRIF (3). The N terminus of TRIF interacts with TNFR-associated factor (TRAF)6 and TRAF family member–associated NF-κB activator (TANK)-binding kinase (TBK)1 (4), whereas the C terminus of TRIF binds receptor-interacting protein (RIP)1 (5). TRAF6 and RIP1 have both been implicated in NF-κB activation downstream of TLR3, and recruitment of RIP1, TRAF6, and transforming growth factor-β-activated kinase 1 (TAK1) to the receptor following ligand stimulation has been demonstrated (6). In TLR3 signaling, TAK1 has an essential role in NF-κB activation, because TAK1-deficient cells fail to activate NF-κB (7). TRAF6-mediated K63-linked polyubiquitination was proposed to be required for TAK1 activation (8). Phosphorylation at key residues located in the activation loop of IκB kinase (IKK)α (S176/180) and IKKβ (S177/181) is required for their activation, and TAK1 was shown to phosphorylate IKKβ in the activation loop (9). IKKα and IKKβ were found to phosphorylate the NF-κB inhibitor IκB (10, 11), which targets IκB for degradative polyubiquitylation, allowing for NF-κB nuclear translocation. Phosphorylation of RelA/p65 at several serine residues, including those in the transactivation domain, is required for optimal NF-κB activity, and several kinases have been implicated, including IKKα/β, TBK1, and IKKε (12–14). In TLR signaling, TAK1 is involved in the activation of the MAPKs JNK, p38, and ERK1/2 (9, 15).

Both TBK1 and IKKε can directly phosphorylate IRF3 at key serine residues in the C terminus of IRF3, leading to its homodimerization and nuclear translocation. Studies of IKKε- and TBK1-knockout mice confirmed a critical role for TBK1 in the induction of type I IFNs in response to viral infection, whereas IKKε deficiency had no significant impact on type I IFN production (16, 17). The coordinate assembly of a number of transcription factors on the IFN-β enhancer is required for the efficient transcriptional regulation of the IFN-β gene. The IFN-β enhancer contains four overlapping positive regulatory domains (PRDs) designated PRDII, PRDIII-I, and PRIV, which bind NF-κB, IRF3/7 and ATF-2/c-Jun heterodimer, respectively. IRF3 and/or IRF7 have essential roles in the production of IFN-β and other type I IFNs following viral infection. NF-κB and AP-1 have critical roles in maintaining constitutive IFN-β expression, which primes cells to a viral-ready state, and a less important role in IFN-β production following viral infection (18, 19).

Recently, IKKα and IKKβ were reported to phosphorylate and activate the noncanonical kinases, TBK1 and IKKε, in TNF and TLR signaling (20). A second pathway coexists in IL-1R, TLR3, and TLR4 signaling that culminates in the autocatalytic activity of TBK1 and IKKε (20). TBK1 and IKKε were reported to activate a negative-feedback loop that limits the activation of NF-κB, and IKKα, IKKβ, and NEMO were identified as potential targets (20).

Institute of Immunology, National University of Ireland Maynooth, Maynooth, County Kildare, Ireland

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Address correspondence and reprint requests to Dr. Marion Butler, Institute of Immunology, National University of Ireland Maynooth, Maynooth, County Kildare, Ireland. E-mail address: marion.butler@nuim.ie

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; EV, empty vector; IKK, IκB kinase; IRAK1 KD, IRAK1 kinase-inactive expression construct; poly(I:C), polyinosinic-polycytidylic acid; PRD, positive regulatory domain; RIP, receptor-interacting protein; siRNA, small interfering RNA; TANK, TRAF family member–associated NF-κB activator; TBK, TRAF family member–associated NF-κB activator-binding kinase; TRAF, tumor necrosis factor receptor-associated factor, TNFR-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β; WT, wild type.

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We present novel findings implicating IRAK1 and IKKε as key signaling molecules that regulate the intensity of the TLR3-activated NF-κB–signaling pathway.

Materials and Methods

Cell lines

HEK293 cells stably expressing TLR3 (TLR3 HEK293) were purchased from InvivoGen. Parental HEK293 (wild type [WT]) and IRAK1-deficient HEK293I1A cells were provided by Dr. Xiaoxia Li (Cleveland Clinic Foundation, Cleveland, OH). WT, IRAK1$^{-/-}$, IKKε$^{-/-}$, TBK1$^{+/+}$ TNFR1$^{-/-}$, and TBK1$^{-/-}$ TNFR1$^{-/-}$ bone marrow–derived macrophages (BMDMs) were gifts from Dr. Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA). The human microglial cell line CHME3 was provided by Dr. M. Naghavi (Columbia University, New York, NY).

Plasmids

Constructs encoding WT and kinase-dead IRAK1 (IRAK1 D340N) were from Tularik (San Francisco, CA). The pRL-TK plasmid encoding for *Renilla* luciferase was purchased from Promega, and pcDNA3.1 was from Invitrogen. Constructs encoding TBK1-flag, IKKε-flag, IFN-β, PRDII, and PRDIII-I luciferase reporters were provided by Dr. Kate Fitzgerald. IRF3 activation was assessed using pFR-Luc and the *trans*-activator plasmid pFA-IRF3 (IRF3 fused downstream of the yeast Gal4 DNA binding domain; provided by Dr. Kate Fitzgerald). The Gal4-p65$^{(1-551)}$ construct encoding for full-length p65 fused to the Gal4 DNA-binding domain was a gift from Dr. Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany).

Abs and reagents

Anti–phospho-IKKα/β clone 16A6, anti–phospho–NF-κB p65 (Ser^{536}) clone 93H1, anti–NF-κB p65 clone 93H1, anti–phospho-TBK1/NAK (Ser^{172}) clone D52C2, anti–phospho-IRF3 (Ser^{396}) clone 4D4G, anti-IRF3 clone D83B9, anti-IKKε clone D61F9, anti–phospho-p44/42 MAPK (Erk1/2; Thr^{202}/Tyr^{204}) clone D13.14.4E, anti-p44/42 MAPK (Erk1/2) clone 137F5, anti–phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) clone 81E11, anti-SAPK/JNK clone 56G8, anti–phospho-p38 MAPK (Thr^{180}/Tyr^{182}) clone D3F9, anti-p38 MAPK, anti–phospho-TAK1 (Thr^{184}/^{187}) clone 90C7, and anti-TAK1 clone D94D7 were all from Cell Signaling Technologies. Other Abs used were anti- IRAK1 clone H-273 and anti-TBK1 clone 6D603 (both from Santa Cruz); anti–β-actin clone AC-15, anti-Flag clone M2, and anti-c-Myc clone 9E10 (Sigma-Aldrich), and anti-TBK1 (pS172) clone J133-1171 (BD Pharmingen). Polyinosinic-polycytidylic acid [poly(I:C)] (high m.w.) was purchased from InvivoGen. IRAK1/4 inhibitor and the TAK1 inhibitor, (5Z)-7-oxozeaenol, were both purchased from Merck (Darmstadt, Germany). The IKKε and TBK1 inhibitor MRT67307 was obtained from the Medical Research Council Protein Phosphorylation Unit, University of Dundee (Dundee, Scotland, U.K.).

Reporter gene assays

TLR3-HEK293 cells or HEK293-I1A were seeded onto 96-well plates and grown for 24 h. Cells were then transfected using GeneJuice Transfection Reagent (Novagen) with expression constructs and luciferase reporter genes. Twenty-four hours posttransfection, cells were left untreated or were stimulated with poly(I:C) for 6 h, harvested in reporter lysis buffer (Promega), and assayed for firefly and *Renilla* luciferase activity using the luciferase assay system and coelenterazine (Promega). Each experiment was performed in triplicates; the mean fold induction (± SEM) relative to control levels was calculated in at least three independent experiments.

RNA interference/small interfering RNA gene silencing

Predesigned small interfering RNA (siRNA) targeting IRAK1 (sense: 5′-GGUUGUCCUUGAGUAAUAATT-3′; antisense: 5′-UUAUUACUCAAGGACAACCTG-3′), A20 (sense: 5′-AGUGUGUAUCGGUGCAUGGUU-3′; antisense: 5′-CCAUGCACCGAUACACACUUU-3′), and Lamin A/C (sense: 5′-GAAGGAGGGUGACCUGAUATT-3′; antisense: 5′-UAUCAGGUCACCCUCCUUCTT-3′) were purchased from Ambion. siRNAs targeting IKKε (IKKε siRNA 1 sense: 5′-GCGGAACAAGGAGAUCAUTT-3′; IKKε siRNA 1 antisense: 5′-AUGAUCUCCUUGUUCCGCCGT-3′; IKKε siRNA 2 sense: 5′-AGAUGAACUUCAUCUACAATT-3′; IKKε siRNA 2 antisense 5′-UUGUAGAUGAAGUUCAUCUTG-3′) were purchased from QIAGEN. Twenty-four hours later, TLR3-HEK293 cells were transfected with the specific siRNA oligonucleotides or negative control siRNA (IRAK1 or A20 at 15 nM final concentration; IKKε at 30 nM final concentration) using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies). The transfected cells were incubated for 35–40 h for IRAK1 and A20 experiments or ≥48 h for IKKε before DNA transfection. DNA was transfected using GeneJuice Transfection Reagent (Novagen), as described previously. Cells were treated with poly(I:C) for 6 h and then harvested and subjected to reporter gene assay. Knockdown was confirmed by Western blotting.

ELISA

Murine and human IL-6 ELISA Development Kits were purchased from PeproTech. Mouse IFN-β ELISA was obtained from R&D Systems. The cytokine levels were determined according to the manufacturers' instructions. Each experiment was performed in triplicates, and the average (± SEM) was calculated for at least three independent experiments

Real-time PCR

Total RNA was extracted from cell cultures using TRIzol Reagent (Invitrogen). First-strand cDNA synthesis was carried out according to the manufacturer's instructions using Moloney murine leukemia virus reverse-transcriptase RNase H minus (Metabion International, Martinsried, Germany). Quantitative real-time PCR was performed on a real-time PCR system (DNA Engine OPTICON system; MJ Research) using Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The data were analyzed using the $2^{-\Delta\Delta CT}$ method (21), with all samples normalized to *GAPDH*. All experiments were performed in triplicate.

Kinase assay

A total of 50 ng N-terminal 6xHis-tagged, recombinant human active IRAK1 (Millipore) was diluted in kinase buffer (40 mM HEPES [pH 7.4], 40 mM $MgCl_2$, 4 mM DTT, 0.1 mM EGTA) and preincubated with 1 μM the IRAK1/4 inhibitor or an equal volume of DMSO for 15 min at room temperature. The tubes were incubated for 30 min at 30°C in the presence of 20 μM cold ATP, boiled, and subjected to immunoblotting.

Western blot analysis and immunoprecipitation

HEK293 or HEK293I1A cells were seeded and transfected at ∼80% confluency using GeneJuice. Transfected cells were incubated at 37°C for 24 h. Cells were then lysed in lysis buffer containing 50 mM Tris-HCl [pH 7.5], 150 mM sodium chloride, 0.5% (v/v) IGEPAL, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, protease inhibitor mixture. The lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Whatman), and probed with the indicated Abs. BMDMs were seeded in 10-cm dishes, treated 24 h later with poly(I:C) for the indicated times, and harvested in lysis buffer. The indicated Abs were precoupled with Agarose A/G beads (Santa Cruz) overnight at 4°C and then cell lysates were added to the mix overnight. The resulting immune complexes were analyzed by SDS-PAGE and immunoblotting using the indicated Abs.

Statistical analysis

Data analysis was carried out using the unpaired Student *t* test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

IRAK1 regulates IFN-β and proinflammatory cytokine production downstream of TLR3

HEK293 cells stably expressing TLR3 were transiently transfected with the IFN-β luciferase (Fig. 1A), PRDIII-I luciferase (Fig. 1B), and PRDII luciferase (Fig. 1C) reporter plasmids in the presence of empty vector (EV), IRAK1, or kinase-inactive IRAK1 (IRAK1 KD; IRAK1 D340N) expression constructs. The cells were then stimulated with poly(I:C) for 6 h, and the luciferase activities were measured. IRAK1 inhibited poly(I:C)-induced activation of the IFN-β, PRDIII-I, and PRDII reporters compared with control EV-transfected cells (Fig. 1A–C). This inhibition was dependent on the kinase activity of IRAK1, because kinase–inactive IRAK1 failed to inhibit poly(I:C)-induced activation of these same reporters (Fig. 1A–C). Next, we knocked down the expression of IRAK1 in TLR3-expressing HEK293 cells through siRNA (Fig. 1D–F, Supplemental Fig. 1A–D). As a control, we silenced the expression of A20, a recognized negative regulator of TLR3 sig-

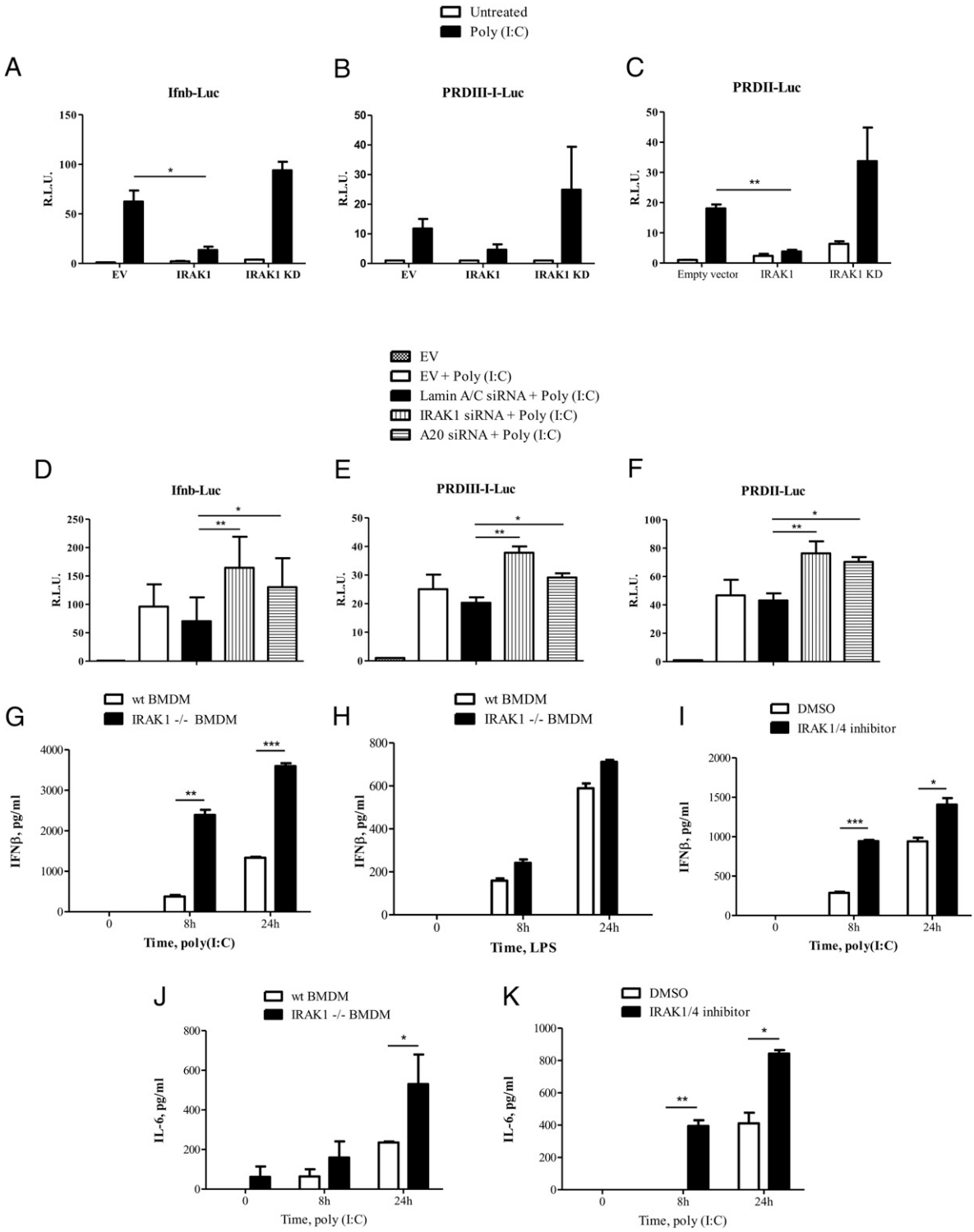

FIGURE 1. IRAK1 regulates poly(I:C)-induced IFN-β and IL-6 production. TLR3 HEK293 cells were transiently cotransfected with IFN-β promoter (80 ng; **A**), PRDIII-I (120 ng; **B**), or PRDII (80 ng; **C**) reporter constructs, together with EV pcDNA3.1, IRAK1, and IRAK1 KD expression plasmids (100 ng), as indicated. TK *Renilla* (20 ng) was cotransfected as an internal control. Twenty-four hours after transfection, cells were left untreated or were stimulated with poly(I:C) for 6 h and then harvested. Reporter gene activities were measured, and the values were normalized for transfection efficiency through *Renilla* luciferase expression. Data are presented as fold stimulation of luciferase expression relative to unstimulated cells. Data are the mean ± SEM ($n = 3$). (**D**–**F**) A total of 15 nM of siRNA against lamin A/C, IRAK1, or A20 was transfected in TLR3-HEK293 cells using Lipofectamine. After a 30-h incubation, the cells were cotransfected with the indicated reporter constructs, as detailed above. After 24 h, the cells were stimulated with poly(I:C) for 6 h, and the reporter gene activities were measured and normalized through *Renilla* luciferase expression. Data are the mean ± SEM ($n = 3$). WT or IRAK1$^{-/-}$ BMDMs were stimulated with 50 μg/ml poly(I:C) (**G**, **J**) or LPS (1 μg/ml) (**H**). Supernatants were collected at 8 and 24 h poststimulation, and IFN-β and IL-6 levels were measured by ELISA. (**I** and **K**) WT BMDMs were pretreated for 1 h with 1 μM IRAK1/4 inhibitor or an equal amount of DMSO and then stimulated with 50 μg/ml poly(I:C). Supernatants were collected after 8 and 24 h; IL-6 (K) and IFN-β (I) were measured by ELISA. Data are the mean (± SEM) of at least three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, unpaired Student t test.

naling (Fig. 1D–F) (22, 23). We found that knockdown of IRAK1 led to a significant increase in the activation of the IFN-β reporter relative to control siRNA (lamin A/C)-transfected cells (Fig. 1D), which was comparable to A20 knockdown cells. To our knowledge, increased activation of the IFN-β–PRDII reporter and the p65 reporter in IRAK1-knockdown cells (Fig. 1F, Supplemental Fig. 1B) provides the first evidence that endogenous IRAK1 may act as a negative regulator of the TLR3–NF-κB–signaling pathway. Previous work implicated IRAK1 as a negative regulator of TRIF-mediated activation of IRF-3 and IRF-7 (2). Our work supports this previously published study, because IRAK1-knockdown cells showed increased activation of the IFN-β–PRDIII-I reporter (Fig. 1E) and both the IRF3 (Supplemental Fig. 1A) and IRF7 (data not shown) reporters compared with control siRNA-treated cells following poly(I:C) stimulation. To address the role of IRAK1 in TLR3 signaling in immune cells, IRAK1$^{-/-}$ BMDMs were stimulated with poly(I:C) in parallel with WT BMDMs. IFN-β mRNA levels were assessed by quantitative real-time PCR, and IFN-β protein levels were measured by ELISA. IFN-β mRNA levels increased significantly in IRAK1$^{-/-}$ BMDMs compared with WT BMDMs; significance was evident at the 2-h time point (Supplemental Fig. 2A). Increased levels of IFN-β, as assessed by ELISA, were detected in the IRAK1$^{-/-}$ macrophages compared with WT macrophages at 8 and 24 post-poly(I:C) stimulation (Fig. 1G). In contrast, and in line with recently published work (24), no such increase in IFN-β levels was observed in the IRAK1$^{-/-}$ macrophages compared with WT macrophages following LPS stimulation (Fig. 1H). We used the IRAK1/4 inhibitor to assess the role of kinase activity of IRAK1 in the TLR3 pathway. WT BMDMs were pretreated with the IRAK1/4 inhibitor or the inhibitor diluent, DMSO, and the cells were stimulated with poly(I:C) for specific periods of time. The IRAK1/4 inhibitor enhanced IFN-β induction and type I IFN production in the IRAK1/4 inhibitor–pretreated cells compared with the DMSO-treated cells (Fig. 1I, Supplemental Fig. 2B). Because we observed an IRAK1-mediated inhibition of the PRDII reporter (Fig. 1C, 1F), we next measured IL-6 levels by ELISA, because NF-κB has a critical regulatory role in the transcriptional regulation of IL-6. Increased levels of IL-6 (Fig. 1J) were measured by ELISA in the IRAK1$^{-/-}$ macrophages relative to the WT macrophages at 8 and 24 h following poly(I:C) stimulation. The IRAK1/4 inhibitor enhanced IL-6 levels at 8 and 24 h following poly(I:C) stimulation (Fig. 1K). To address whether the findings in murine macrophages are representative of human immune cells, we analyzed the expression levels of IFN-β mRNA by quantitative real-time PCR and measured IL-6 by ELISA in the human microglial cell line CH3ME using the IRAK1/4 inhibitor (Supplemental Fig. 2C, 2D). Increased levels of IFN-β mRNA and IL-6 were found in IRAK1/4 inhibitor–pretreated, poly(I:C)-stimulated microglial cells compared with DMSO-treated cells.

IRAK1 limits activation of the TLR3-activated NF-κB– and IRF3-signaling pathways in macrophages

We next examined the activation status of IRF3 and p65. Our work has shown a novel role for IRAK1 in NF-κB regulation and previous work showed a role in IRF3 regulation. IRAK1$^{-/-}$ and WT BMDMs were stimulated with poly(I:C) for 0, 15, or 45 min or 1, 2, or 4 h, and the activation status of NF-κB and IRF3 was examined by Western blot analysis (Fig. 2A). We observed increased phosphorylation of IRF3 (Ser^{396}) and p65 (Ser^{536}) in IRAK1$^{-/-}$ macrophages compared with WT macrophages. This increase was evident after 45 min of poly(I:C) stimulation for both p65 and IRF3. The kinases IKKα/β, TBK1, and IKKε have all been implicated in p65 phosphorylation at serine residue 536, and TBK1 is the critical kinase involved in IRF3 activation (16, 17). A marked increase in phosphorylation of IKKβ (Ser^{177}; upper band) and TBK1 (Ser^{172}) was observed in IRAK1$^{-/-}$ macrophages compared with WT macrophages after 45 min of poly(I:C) stimulation. Recent work by Cohen's group (20) showed that the noncanonical kinases TBK1 and IKKε are substrates of the canonical kinases IKKα/β, which suggests that the increased IKKβ activation observed in IRAK1$^{-/-}$ macrophages may contribute to the increased phosphorylation of TBK1. We next decided to examine the signaling profile in WT BMDMs using the IRAK1/4 inhibitor (Fig. 2C). WT BMDMs were pretreated with the IRAK1/4 inhibitor or the inhibitor diluent DMSO, and the cells were stimulated with poly(I:C) for specific periods of time (0, 15, or 45 min or 1, 2, or 4 h). The IRAK1/4 inhibitor–treated cells exhibited a similar activation profile as the IRAK1$^{-/-}$ BMDMs. IKKβ and TBK1 were hyperactivated following poly(I:C) stimulation in the IRAK1/4 inhibitor–treated cells compared with DMSO-treated cells (Fig. 2C). This work suggests that the kinase activity of IRAK1 is required to limit the activation of IRF3 and NF-κB in the TLR3-signaling pathway.

Impaired phosphorylation of IKKε and enhanced phosphorylation of TBK1 in IRAK1$^{-/-}$ BMDMs following poly(I:C) stimulation

A key event in the activation of the IKK-related kinases TBK1 and IKKε is their phosphorylation at Ser^{172}, which is located in the activation loop of these kinases. Ectopic expression of TBK1 and IKKε leads to their autoactivation. While probing the IRAK1-mediated regulation of the TLR3 pathway, we found that phosphorylation at Ser^{172} was not detected for flag-tagged IKKε in IRAK1-deficient HEK2931IA cells, whereas it could be clearly seen in parental HEK293 cells (Fig. 3A). In contrast, immunoblotting showed comparable levels of phosphorylation at Ser^{172} for TBK1 in HEK293I1A cells and the parental HEK293 cells (Fig. 3A). We found that overexpression of kinase-inactive IRAK1 in the HEK2931IA cells did not lead to the phosphorylation of IKKε at Ser^{172} (Fig. 3A). This work suggests that the kinase activity of IRAK1 is required for the activation of IKKε. IRAK1 and IKKε interacted in unstimulated macrophages, as well as following ligand stimulation, as assessed by endogenous immunoprecipitations (Fig. 3B). Given the findings in HEK2931IA cells and because the p-TBK1 Ser^{172} Ab used in Western blots cross-reacts with p-IKKε, we sought to define the activation status of TBK1 and IKKε, separately, in IRAK1$^{-/-}$ BMDMs. Endogenous TBK1 and IKKε were immunoprecipitated from IRAK1$^{-/-}$ BMDMs [after 0 and 1 h of poly(I:C) stimulation], and the activation status of the noncanonical kinases was assessed by Western blot analysis using the p-IKKε/TBK1 (Ser^{172}) Ab. We found impaired phosphorylation of IKKε in both unstimulated cells and following poly(I:C) stimulation in IRAK1$^{-/-}$ macrophages (Fig. 3C). Interestingly, the opposite profile was observed for TBK1 in IRAK1$^{-/-}$ macrophages (Fig. 3D), showing increased phosphorylation of TBK1 in both unstimulated cells and following poly(I:C) stimulation. We next sought to address whether the kinase activity of IRAK1 is involved in mediating the activation of IKKε. WT BMDMs were pretreated with the IRAK1/4 inhibitor or DMSO and then left untreated or stimulated with poly(I:C) for 45 min, 1 h, or 2 h. Endogenous IKKε was immunoprecipitated from cell lysates, and the phosphorylation of Ser^{172} was analyzed by immunoblotting. Use of the IRAK1/4 inhibitor led to reduced phosphorylation of IKKε in both unstimulated cells and following poly(I:C) stimulation (Fig. 3E). To our knowledge, this is the first study to implicate IRAK1 in the activation of IKKε. At the same time, this study showed that IRAK1 limits the activation of TBK1.

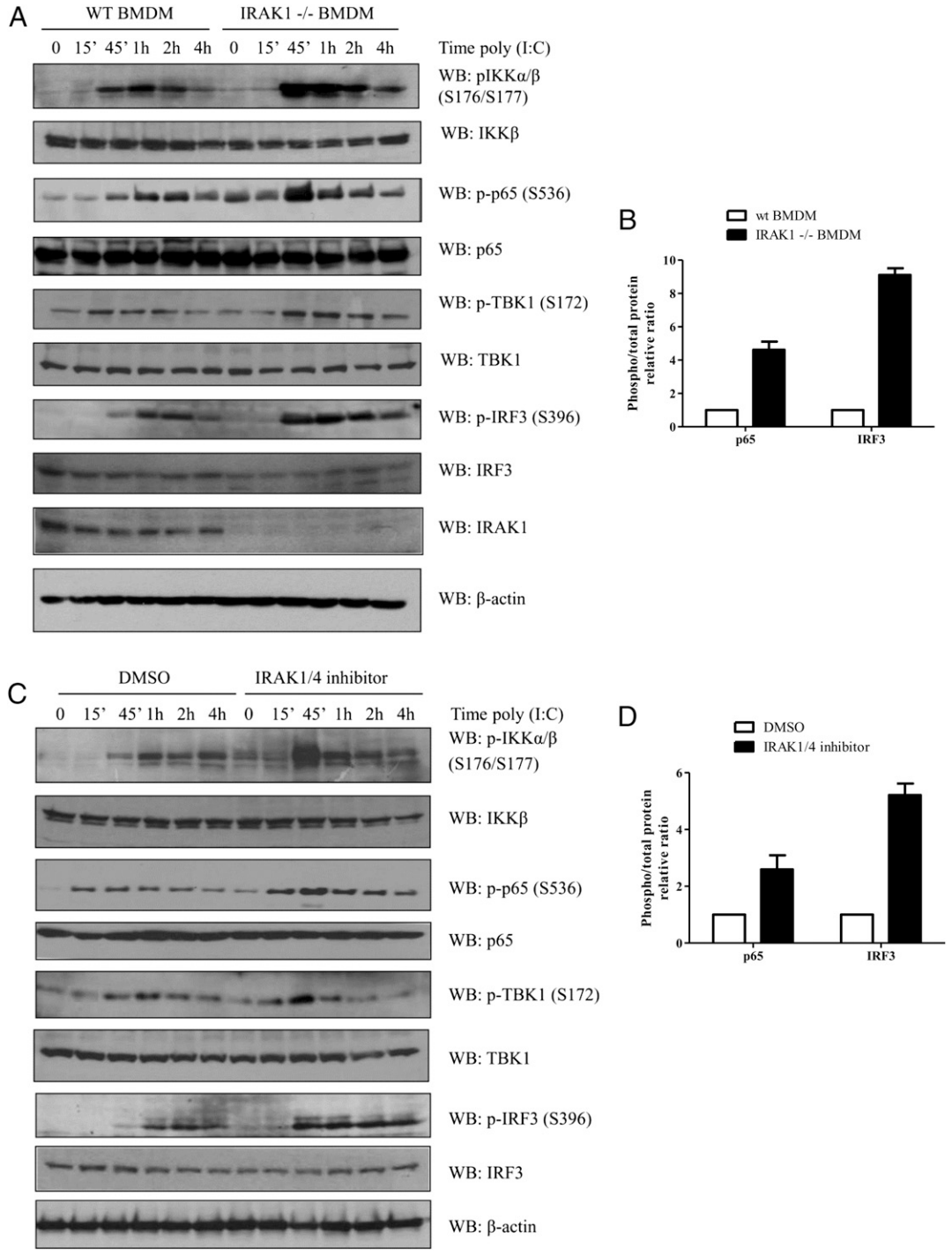

FIGURE 2. Increased activation of IKKβ and TBK1 in IRAK1$^{-/-}$ macrophages and in cells treated with the IRAK1/4 inhibitor. (**A**) WT or IRAK1$^{-/-}$ BMDMs were stimulated with poly(I:C) and harvested at the indicated times (0–4 h). Cell lysates were subjected to Western blot analysis. The membranes were probed with the indicated Abs. Similar results were obtained in three independent experiments. (**B**) Relative ratio of phospho/total p65 and IRF3 in WT and IRAK1$^{-/-}$ BMDMs at 45 min following poly(I:C) stimulation, as determined by densitometric analysis of three independent experiments. (**C**) IRAK1/4 inhibitor– or DMSO-pretreated WT BMDMs were stimulated with poly(I:C) and harvested at the indicated times (0–4 h). Cell lysates were resolved by SDS-PAGE and subjected to Western blot using the indicated Abs. Similar results were obtained in three independent experiments. (**D**) Relative ratio of phospho/total p65 and IRF3 in WT BMDMs treated with DMSO or the IRAK1/4 inhibitor at 45 min following poly(I:C) stimulation, as determined by densitometric analysis of three independent experiments.

IKKε limits activation of the TLR3–NF-κB– and IRF3-signaling pathways similarly to IRAK1

Given that IRAK1 is required for the activation of IKKε, we next investigated the functional role of IKKε in the TLR3-signaling pathway. Surprisingly, poly(I:C)-stimulated IKKε$^{-/-}$ BMDMs resulted in enhanced induction of IFN-β mRNA, as assessed by quantitative real-time PCR, and increased production of IFN-β protein compared with WT BMDMs (Fig. 4A, Supplemental Fig. 3A). In contrast, no significant difference in IFN-β levels was observed in IKKε$^{-/-}$ BMDMs compared with WT BMDMs following LPS stimulation (Fig. 4B). We performed similar experiments in the TBK1-deficient BMDMs (TBK1$^{-/-}$ TNFR$^{-/-}$

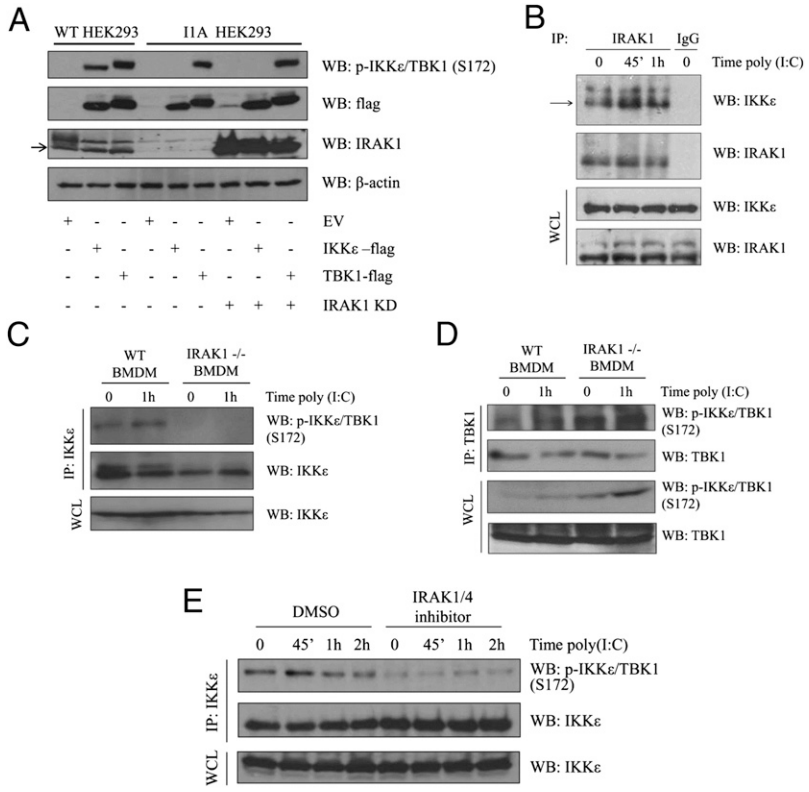

FIGURE 3. IRAK1 is involved in the activation of IKKε. (**A**) WT and IRAK1-deficient (I1A) HEK293 cells were transfected with EV pcDNA3.1 or expression constructs for flag-tagged IKKε or flag-tagged TBK1. IRAK1-deficient (I1A) HEK293 cells were reconstituted with kinase inactive IRAK1 (IRAK1 KD; 2 μg) together with EV pcDNA3.1 or flag-tagged IKKε or flag-tagged TBK1 expression constructs. After 24 h, cells were lysed, and whole-cell lysates were analyzed by Western blotting (WB) with anti–phospho-S172, anti-flag, anti-IRAK1, and anti–β-actin Abs. Similar results were obtained in three independent experiments. (**B**) WT BMDMs were treated with poly(I:C) for the indicated times and then harvested. Cell lysates were subjected to immunoprecipitation (IP) with the anti-IRAK1 Ab or, as a control, rabbit IgG. The IPs were run on SDS-PAGE and probed with an anti-IKKε Ab, as well as anti-IRAK1 as a control for equal IP. The expression levels of endogenous IKKε and IRAK1 were also examined in whole-cell lysates (WCL). Similar results were obtained in two independent experiments. (**C** and **D**) WT or IRAK1$^{-/-}$ BMDMs were treated for 1 h with 50 μg/ml poly(I:C) and then harvested. Lysates were immunoprecipitated with anti-IKKε (C) or anti-TBK1 (D) and subjected to SDS-PAGE and Western blot for p-S172 TBK1/IKKε. Whole cell lysates were also probed for anti-IKKε (C) and TBK1 (D), as indicated. Similar results were obtained in three independent experiments. (**E**) WT BMDMs were pretreated for 1 h with 1 μM IRAK1/4 inhibitor or an equal amount of DMSO and then stimulated with 50 μg/ml poly(I:C) for 45 min or 1 or 2 h. Cells were lysed and subjected to IP using anti-IKKε Ab. The samples were subjected to SDS-PAGE and Western blot and probed for anti-p-Ser172 TBK1/IKKε and total IKKε, as indicated. Whole-cell lysates were probed for IKKε. Similar results were obtained in four independent experiments.

BMDMs) and the corresponding control cell line (TBK1$^{+/+}$ TNFR$^{-/-}$ BMDMs). We found that TBK1 is critical for IFN-β production, because reduced IFN-β mRNA and cytokine levels were observed following poly(I:C) stimulation, in line with published work (25, 26) (Fig. 4C, Supplemental Fig. 3B). Poly(I:C) stimulation of IKKε$^{-/-}$ BMDMs also resulted in enhanced IL-6 production compared with WT BMDMs (Fig. 4D). Interestingly, we found reduced IL-6 production in the TBK1$^{-/-}$ macrophages at the earlier time point of 8 h, whereas no significant difference was observed at 24 h (Fig. 4E). These data suggest that TBK1 has a positive role in the TLR3–NF-κB–signaling pathway. We next examined the phosphorylation/activation status of p65, IRF3, IKKα/β, and TBK1 and found enhanced phosphorylation of p65 and IRF3, together with enhanced phosphorylation of their upstream kinases IKKβ and TBK1, in the IKKε$^{-/-}$ BMDMs compared with WT macrophages (Fig. 4F). In agreement with our data on IL-6, TBK1-deficient BMDMs showed reduced phosphorylation of p65 compared with matching BMDMs (Fig. 4F). This work highlights a novel positive role for TBK1 in TLR3–NF-κB signaling and adds to the increasing evidence for the divergent roles of these two kinases, TBK1 and IKKε. The cytokine and immune-signaling profile found in IKKε$^{-/-}$ BMDMs mirrors that of IRAK1$^{-/-}$ BMDMs, providing further evidence of a tightly linked TLR3-IRAK1/IKKε–signaling axis.

IRAK1 and IKKε are involved in limiting the activation of TAK1 in the TLR3-activated NF-κB pathway

Activation of MAPKs plays pivotal roles in the expression of various cytokines, including TNF-α, IL-8, IL-6, and IL-12 (27). Furthermore, JNK was shown to be involved in the phosphorylation of IRF3 (Ser^{173}), acting as a positive regulator of IRF3 activation (28). Therefore, we addressed whether IRAK1 and IKKε had a functional role in MAPK activation downstream of TLR3, as assessed by Western blot analysis (Fig. 5A, 5B). Interestingly, IRAK1- and IKKε-deficient BMDMs showed enhanced phosphorylation of the MAPKs JNK and p38 compared with WT cells (Fig. 5A, 5B). We next assessed whether the kinase activity of IRAK1 is involved in the modulation of MAPK activation. We observed enhanced activation of JNK and p38 in cells treated with the IRAK1/4 inhibitor (Fig. 5C). The use of the IRAK1/4 inhibitor does not exclude the possibility that IRAK4 has a regulatory role in TLR3-MAPK activation. We next examined the activation status of TAK1, because it has a critical role in NF-κB activation and a regulatory role in JNK activation in the TLR3-signaling pathway (7). First, to assess more comprehensively the role of TAK1 in TLR3-mediated MAPK and NF-κB activation, BMDMs were treated for 1 h with DMSO or the TAK1 inhibitor (5Z)-7-oxozeaenol and then stimulated with poly(I:C) for

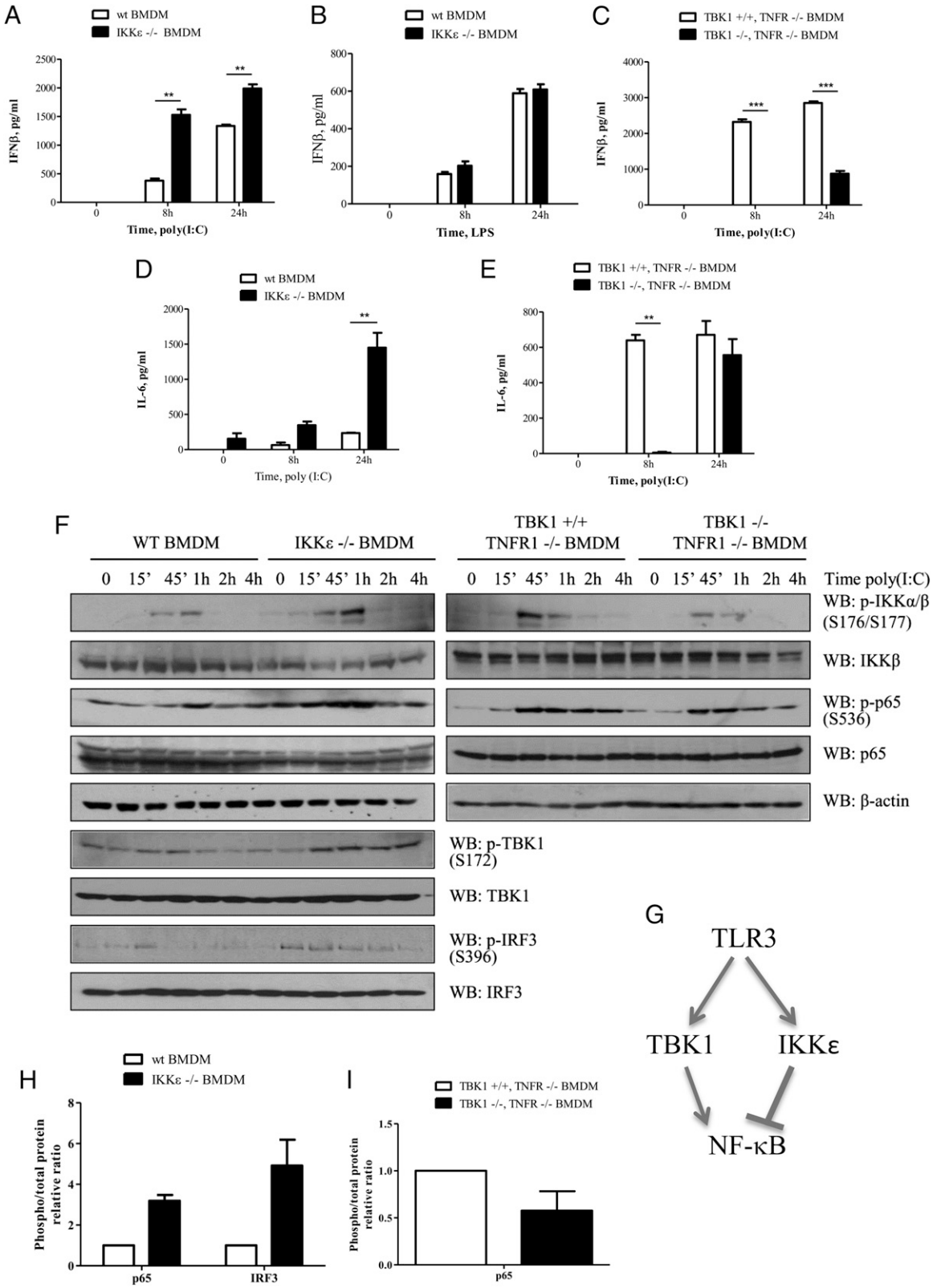

FIGURE 4. IKKε limits activation of the TLR3–NF-κB and -IRF3–signaling pathways similarly to IRAK1. WT and IKKε$^{-/-}$ BMDMs (**A**, **B**, **D**) or TBK1$^{+/+}$ TNFR1$^{-/-}$ BMDMs and TBK1$^{-/-}$ TNFR1$^{-/-}$ BMDMs (**C**, **E**) were stimulated with 50 μg/ml poly (I:C) (A, D, C, E) or LPS (1 μg/ml) (B) for the indicated times; IFN-β and IL-6 were measured by ELISA. Data are the mean ± SEM of at least three experiments. (**F**) WT and IKKε$^{-/-}$ BMDMs or TBK1$^{+/+}$ TNFR1$^{-/-}$ BMDMs and TBK1$^{-/-}$ TNFR1$^{-/-}$ BMDMs were stimulated with 50 μg/ml poly(I:C) and harvested at the indicated times (0–4 h). Whole-cell lysates were resolved by SDS-PAGE and subjected to Western blot using the indicated Abs. Similar results were obtained in at least three independent experiments. (**G**) Scheme showing the role of TBK1 and IKKε in the TLR3–NF-κB pathway. (**H**) Relative ratio of phospho/total p65 and IRF3 in WT and IKKε$^{-/-}$ BMDMs at 45 min following poly(I:C) stimulation, as determined by densitometric analysis of three independent experiments. (**I**) Relative ratio of phospho/total p65 in TBK1$^{+/+}$ TNFR1$^{-/-}$ BMDMs and TBK1$^{-/-}$ TNFR1$^{-/-}$ BMDMs after 45 min of poly(I:C) treatment, as determined from densitometric analysis of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired Student t test.

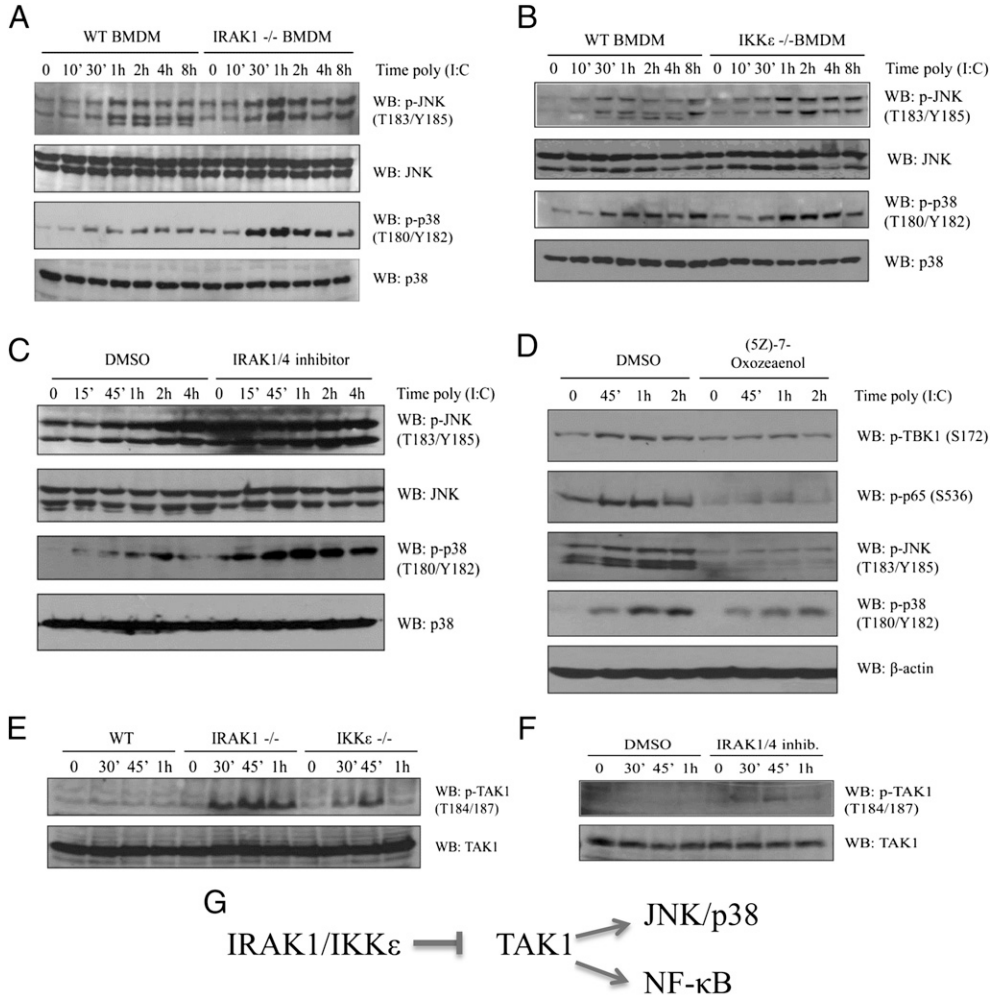

FIGURE 5. IRAK1 and IKKε negatively regulate the TAK1-JNK/p38 MAPK–signaling axis. WT and IRAK1$^{-/-}$ BMDMs (**A**) or WT and IKKε$^{-/-}$ BMDMs (**B**) were not treated or were stimulated with 50 μg/ml poly(I:C) and analyzed by Western blot using the indicated Abs. Representative data of three independent experiments are shown. (**C**) WT BMDMs were pretreated for 1 h with 1 μM IRAK1/4 inhibitor or an equal amount of DMSO and then stimulated with poly(I:C), as indicated. The phosphorylation of the MAPKs was monitored by Western blotting. (**D**) WT BMDMs were treated for 1 h with 1 μM (5Z)-7-oxozeaenol prior to stimulation with 50 μg/ml poly(I:C) for the indicated times. Whole-cell lysates were immunoblotted with the indicated Abs. Representative data of three independent experiments are shown. (**E**) WT, IRAK1$^{-/-}$, and IKKε$^{-/-}$ BMDMs were treated in parallel with 50 μg/ml poly(I:C) for the indicated times. Whole-cell lysates were subjected to SDS-PAGE and Western blot analysis using p-TAK1 and total TAK1 Abs. Representative data of three independent experiments are shown. (**F**) WT BMDMs were treated for 1 h with 1 μM IRAK1/4 inhibitor or an equal amount of DMSO and then stimulated with 50 μg/ml poly(I:C) for the indicated times. Cell lysates were subjected to immunoblotting with phospho- and total-TAK1 Abs. Representative data of three independent experiments are shown. (**G**) Scheme showing the role of IRAK1 and IKKε in the regulation of the MAPK pathway.

0 or 45 min or 1 or 2 h (Fig. 5D). Reduced phosphorylation of JNK and p38 MAPK was evident in inhibitor-treated cells and was notably more pronounced for JNK. A significant reduction in the phosphorylation of p65 (Ser^{536}) was also observed in cells pretreated with the TAK1 inhibitor compared with DMSO-treated cells. A slight reduction in the phosphorylation of TBK1 was also observed. Altogether, these data confirm a positive role for TAK1 in TLR3-induced JNK and p38 activation, as well as p65 activation. A marked increase in the activation status of TAK1 was detected in both IRAK1$^{-/-}$ and IKKε$^{-/-}$ macrophages following poly(I:C) stimulation [as early as 30 min following poly(I:C) stimulation] compared with WT macrophages (Fig. 5E). This increase in TAK1 activation could explain the observed increase in both JNK and p38 activation in the IRAK1$^{-/-}$ and IKKε$^{-/-}$ macrophages. To address whether the kinase activity of IRAK1 is involved in limiting the activation of TAK1 in TLR3 signaling, we again used the IRAK1/4 inhibitor. We found increased activation of TAK1 in BMDMs that were pretreated with IRAK1/4 inhibitor and stimulated with poly(I:C) (Fig. 5F). To our knowledge, this is the first study to implicate IRAK1 and IKKε in the signaling events upstream or at the level of TAK1 in the TLR3-signaling cascade.

IRAK1 and IKKε are involved in activation of ERK1/2 downstream of TLR3

While examining the role of IRAK1 and IKKε in MAPK activation, we found that the activation of ERK1/2 was abolished following poly(I:C) stimulation in IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs (Fig. 6A, 6B). In cells treated with the IRAK1/4 inhibitor, ERK1/2 activation was also impaired following poly(I:C) stimulation (Fig. 6C). To investigate whether the kinase activity of IKKε is also required for the activation of ERK1/2, we treated WT BMDMs with a TBK1/IKKε inhibitor (MRT67307) for 1 h prior to poly(I:C)

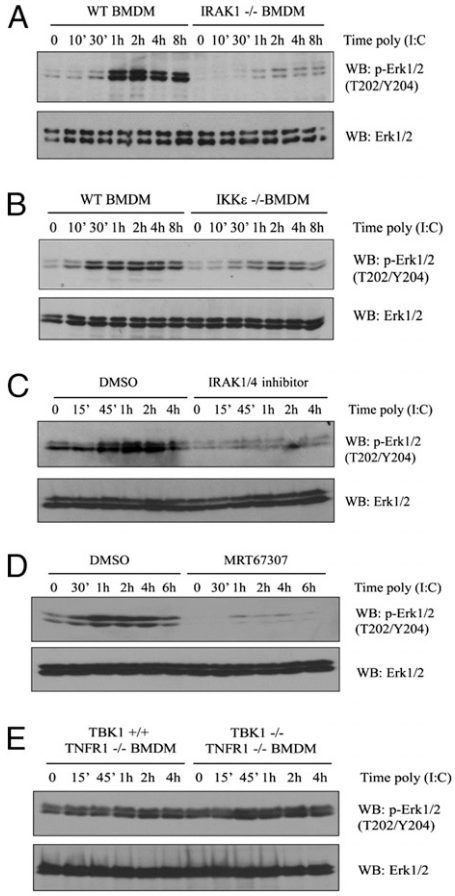

FIGURE 6. IRAK1 and IKKε are involved in the activation of ERK1/2 downstream of TLR3. WT and IRAK1$^{-/-}$ BMDMs (**A**) or WT and IKKε$^{-/-}$ BMDMs (**B**) were not treated or were stimulated with 50 μg/ml poly(I:C) and analyzed by Western blot using the indicated Abs. Representative data of three independent experiments are shown. (**C**) WT BMDMs were treated for 1 h with 1 μM IRAK1/4 inhibitor or an equal amount of DMSO and then stimulated with poly(I:C), as indicated. The phosphorylation of ERK1/2 was assessed by Western blotting. (**D**) WT BMDMs were treated for 1 h with 2 μM MRT67307 or an equal amount of DMSO and then stimulated with 50 μg/ml poly(I:C) for the indicated times. Cell extracts were immunoblotted with phospho- and total-ERK1/2, as indicated. (**E**) TBK1$^{+/+}$ TNFR1$^{-/-}$ BMDMs and TBK1$^{-/-}$ TNFR1$^{-/-}$ BMDMs were stimulated with 50 μg/ml poly(I:C) for the indicated times. Whole-cell lysates were analyzed by immunoblotting with anti–phospho- and total-ERK1/2. Representative data from three independent experiments are shown.

stimulation. The phosphorylation of ERK1/2 was severely impaired in the presence of MRT67307, indicating that the kinase activity of TBK1 and/or IKKε is involved in the activation of ERK1/2 (Fig. 6D). The activation status of ERK1/2 was assessed in TBK1-deficient BMDMs (TBK1$^{-/-}$ TNFR$^{-/-}$ BMDMs) compared with the corresponding control cells (TBK1$^{+/+}$ TNFR$^{-/-}$ BMDMs). We found that ERK1/2 was activated following poly(I:C) stimulation in TBK1-deficient cells (Fig. 6E), indicating that the effect observed with MRT67307 resulted from the inhibition of IKKε. Taken together, these findings indicate that IRAK1 and IKKε are novel positive regulators of ERK1/2 activation in the TLR3-signaling pathway.

The TAK1 inhibitor (5Z)-7-oxozeaenol reduces IFN-β mRNA levels in IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs following poly(I:C) stimulation

To gain further insight into the mechanism by which IRAK1 and IKKε modulate IFN-β production downstream of TLR3, WT, IRAK1$^{-/-}$, and IKKε$^{-/-}$ BMDMs were treated for 1 h with DMSO or the TAK1 inhibitor (5Z)-7-oxozeaenol and then stimulated with poly(I:C) for 2 h (Fig. 7A, 7B). IFN-β mRNA levels were measured by quantitative RT-PCR in the WT, IRAK1$^{-/-}$, and IKKε$^{-/-}$ BMDMs (Fig. 7A, 7B). Inhibition of TAK1 led to a slight reduction in the levels of IFN-β following poly(I:C) treatment in WT BMDMs, although this difference was not significant (Fig. 7A). This is in line with recent data indicating that NF-κB has a minor role in IFN-β induction following viral infection (18). Increased IFN-β mRNA was observed in the DMSO-pretreated, poly(I:C)-stimulated IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs compared with the DMSO-treated WT cells, as seen previously (Fig. 7A, 7B, Supplemental Figs. 2A, 3A). Pretreatment of the IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs with the TAK1

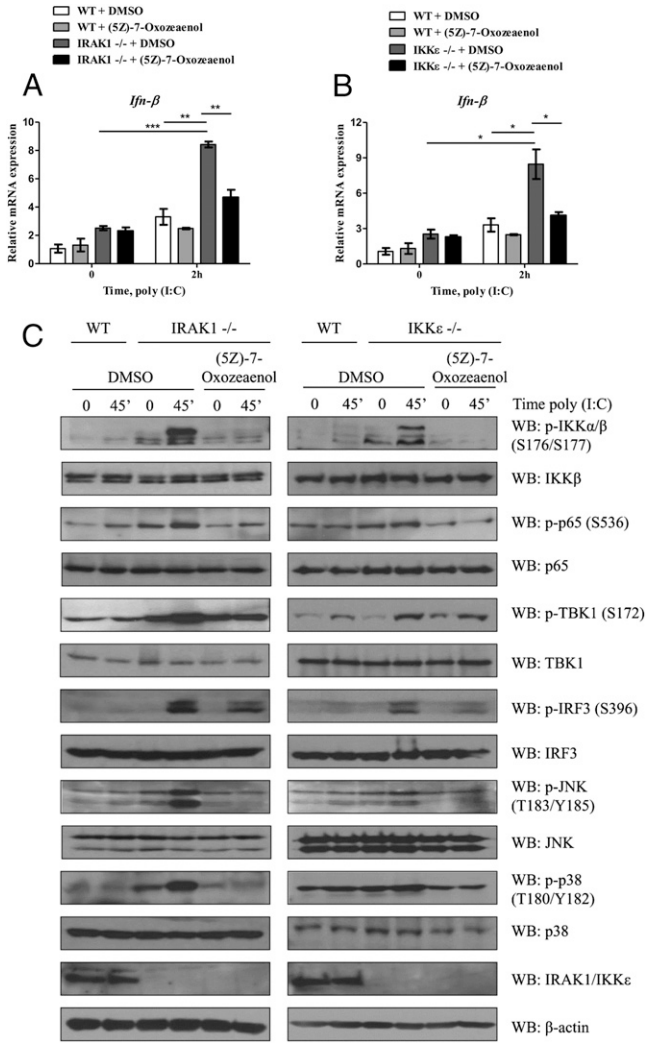

FIGURE 7. The TAK1 inhibitor, (5Z)-7-oxozeaenol, significantly reduces the elevated IFN-β responses in IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs. IRAK1$^{-/-}$ BMDMs (**A**) or IKKε$^{-/-}$ BMDMs (**B**) were treated for 1 h with 1 μM (5Z)-7-oxozeaenol or DMSO and then stimulated with 50 μg/ml poly (I:C) for 2 h. WT BMDMs were treated in parallel with DMSO and then stimulated with poly(I:C) for 2 h. The transcriptional level of *Ifnβ* was determined by real-time PCR and normalized against *GAPDH* mRNA (mean ± SEM; $n = 3$). (**C**) IRAK1$^{-/-}$ BMDMs and IKKε$^{-/-}$ BMDMs were treated for 1 h with DMSO or 1 μM (5Z)-7-oxozeaenol and then stimulated with 50 μg/ml poly(I:C) for 45 min. WT BMDMs were treated with DMSO and stimulated with poly(I:C) for 45 min. Whole-cell extracts were subjected to Western blot analysis with the indicated Abs. Representative data of three independent experiments are shown. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired Student t test.

inhibitor led to a significant reduction in IFN-β induction following poly(I:C) stimulation compared with DMSO-treated IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs (Fig. 7A, 7B). In Western blots, reduced phosphorylation of p65 and IRF3 was detected in IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs that were pretreated with TAK1 inhibitor versus DMSO. This indicates that hyperactivation of TAK1 is largely responsible for the increased level of TBK1 activation and, consequently, IRF3 activation in IRAK1$^{-/-}$ and IKKε$^{-/-}$ BMDMs. Interestingly, the level of activation of TBK1 and IRF3 in the IRAK1$^{-/-}$ TAK1 inhibitor–treated macrophages remained higher compared with WT DMSO-treated macrophages. The same was not true for the IKKε$^{-/-}$ TAK1 inhibitor–pretreated BMDMs. Preliminary work suggests that IRAK1 inhibits the TLR3-activated IRF3 and IRF7–signaling axis; hence, IRAK1 acts at multiple levels to control the magnitude of the type I IFN response downstream of TLR3.

IRAK1 does not inhibit the TLR3–NF-κB–signaling axis in the absence of IKKε

Given our findings that IRAK1 and IKKε interact and that both limit activation of the TLR3–NF-κB–signaling pathway, we investigated whether modulation of this pathway by IRAK1 is IKKε dependent. First, we silenced the expression of IKKε in TLR3-HEK293 cells using two independent siRNA oligonucleotides. In agreement with our data on murine macrophages, knockdown of IKKε led to a significant increase in both the activation of the IFN-β promoter and PRDII reporters relative to control siRNA (lamin A/C)-transfected cells (Fig. 8A, 8B). Increased activation of the IFN-β–PRDII reporter in IKKε-knockdown cells (Fig. 8B) provided evidence that endogenous IKKε limits activation of the TLR3–NF-κB–signaling pathway in human cells. Both siRNA oligonucleotides gave comparable results and reduced the expression of IKKε by ~70 and ~90%, as assessed by Western blot analysis (Fig. 8C, 8D). Next, we addressed whether IRAK1 acts through IKKε in limiting activation of the TLR3–NF-κB–signaling pathway. To address this central question, we transfected IRAK1 or EV expression constructs 48 h following the siRNA transfection against IKKε or lamin A/C, stimulated the cells with poly(I:C), and compared the results from the control siRNA versus the IKKε siRNA reporters. Ectopic expression of IRAK1 in IKKε-knockdown cells inhibited poly(I:C)-stimulated activation of the IFN-β reporter (Fig. 8A). Examining the IFN-β–PRDII luciferase reporter gave us mechanistic insight into the IRAK1–IKKε signaling relationship. IRAK1 failed to inhibit poly(I:C)-stimulated activation of the IFN-β–PRDII luciferase reporter in the absence of IKKε. This result indicates that IRAK1 requires IKKε to mediate the negative regulation of the TLR3–NF-κB–signaling axis (Fig. 8B). IRAK1 inhibits activation of the IFN-β luciferase reporter following poly(I:C) stimulation in IKKε-silenced cells. This suggests that IRAK1 can also act independently of IKKε in the regulation of TLR3-mediated IRF3 and/or -7 activation. We present a summary of our research findings in Fig. 9.

Discussion

IRAK1 was previously reported to inhibit TRIF- and RIG-I–mediated IFN-β induction; however, limited mechanistic insight into this inhibition was uncovered (2). That study suggested that IRAK1 inhibits the activation of IRF3 and IRF7 (2). Contrary to this work, a later study reported that IRAK1 is required for IFN-β induction in the RIG-I–signaling pathway in murine macrophages (29).

Our work shows that IRAK-1 limits the activation of TAK1 in the TLR3-signaling pathway. We found increased TAK1, IKKβ, and TBK1 activation in IRAK-1–deficient macrophages following poly(I:C) stimulation. Increased IKKβ and TBK1 activity most likely contributes to the increased IL-6 and IFN-β cytokine levels following TLR3 stimulation in IRAK1$^{-/-}$ macrophages compared with WT macrophages. The increased IKKβ activation may contribute to the observed increase in TBK1 activity and, consequently, the increased IRF3 activation. Our understanding of the

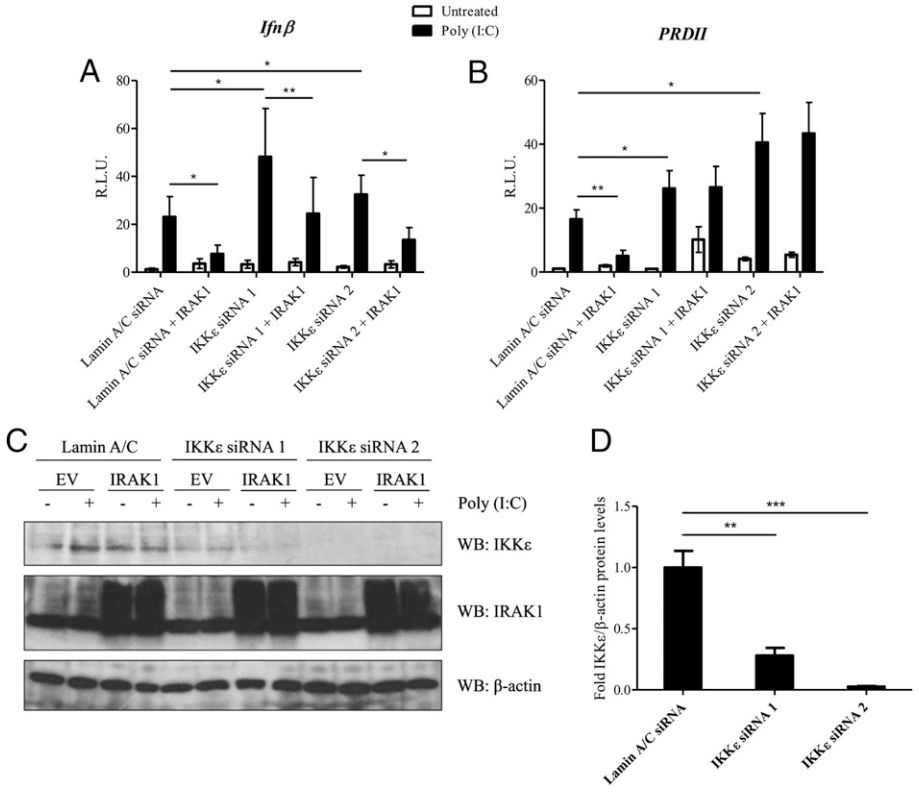

FIGURE 8. IRAK1 inhibits the TLR3–NF-κB pathway through an IKKε-dependent mechanism. (**A** and **B**) IKKε was silenced in TLR3 HEK293 cells using two siRNAs (IKKε siRNA 1 and IKKε siRNA 2) at a final concentration of 30 nM. siRNA and lamin A/C (30 nM) were used as control. After 48 h, the cells were transfected, with the IFN-β promoter (A) and PRDII (B) reporter constructs as previously described, and a plasmid encoding for IRAK1 (100 ng) or EV (100 ng), as shown. After 24 h, the cells were stimulated with poly(I:C) for 6 h, and the reporter gene activities were normalized by *Renilla* luciferase expression. Data are presented as fold stimulation of luciferase expression relative to unstimulated cells. Data are shown as mean ± SEM (n = 4). (**C**) The knockdown of IKKε was confirmed by Western blot analysis, using siRNA against lamin A/C as a control. (**D**) Plot shows reduced densitometry of IKKε protein levels by IKKε siRNA 1 and IKKε siRNA 2 transfection compared with lamin A/C siRNA (n = 4/group). Values were normalized to β-actin. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, unpaired Student t test.

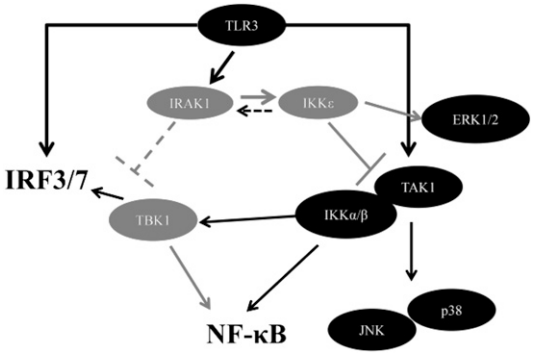

FIGURE 9. Schematic representation of the TLR3-signaling pathway building on our findings presented in this article. Following TLR3 stimulation, the NF-κB– and IRF3/7-signaling pathways become activated, which then lead to the production of proinflammatory cytokines and IFN-β. Mechanisms are in place to prevent hyperactivation of these signaling pathways. Our work shows that IRAK1 activates IKKε following TLR3 stimulation, which then limits activation of the TAK1–IKKβ–NF-κB–signaling axis. IRAK1 also negatively regulates the TLR3–IRF3/7–controlled IFN-β response in separate pathways, but the mechanisms have yet to be uncovered.

IKKβ–TBK1 link comes from work showing that IKKα and IKKβ directly phosphorylate TBK1 and IKKε at serine 172 in the activation loop (20). In TLR3 signaling, a second pathway, not yet defined, leads to the autoactivation of TBK1 and IKKε (20).

To assess whether the kinase activity of IRAK1 is critical in mediating this negative regulation of the TLR3-signaling pathway, we used an IRAK1/4 dual-specific inhibitor, because an IRAK1-specific inhibitor is not available commercially. The use of the IRAK1/4 inhibitor on WT macrophages reproduced our findings with the IRAK1-deficient macrophages showing increased IL-6 and IFN-β production following TLR3 stimulation. Previous work had already addressed the role of IRAK4 and its kinase activity in TLR3 signaling. Comparable levels of IL-6, TNF-α, and IFN-α were detected in IRAK4$^{-/-}$ and WT macrophages following TLR3 stimulation (30). Furthermore, inactivation of the kinase activity of IRAK4 failed to modulate the TLR3–IL-6 response (30, 31). Therefore, our data suggest that the kinase activity of IRAK1, rather than IRAK4, is required to mediate this negative regulation of the TLR3–NF-κB pathway. Recently, differences in the requirements for IRAK1/4 kinase activity in IL-1R signaling have emerged between human and mouse immune cells, indicating that the kinase activity is dispensable in IL-1R signaling in human cells (32). We found that blocking the kinase activity of IRAK1 and IRAK4 through the use of the dual IRAK1/4 kinase inhibitor limits the activation of the TLR3 pathway in human cells, because increased IL-6 and IFN-β levels were found in human microglial cells, which were pretreated with the IRAK1/4 inhibitor, following poly(I:C) stimulation.

Emerging data indicate divergent functional roles for the kinases TBK1 and IKKε, together with differences in their cellular localization and activation mechanisms (17, 33–36). Previous work used a dual-specific inhibitor of TBK1/IKKε to determine that the noncanonical kinases act in a negative-feedback loop to limit the activity of the canonical IKKs (35). Our work showed that TBK1 is involved in the positive regulation of NF-κB downstream of TLR3. In contrast, we report that IKKε is not involved in the positive regulation of this same pathway, highlighting their differences at the functional level in the TLR3 pathway. Because TBK1$^{-/-}$ mice are embryonic lethal, investigators have used embryonic fibroblasts, siRNA targeting TBK1, TNFR1$^{-/-}$ TBK1$^{-/-}$ double-knockout mice, and, more recently, TBK1-mutant mice that encode a mutant TBK1 protein lacking catalytic activity. Previous studies established a positive role for TBK1 in TNF- and IL-1–induced transcriptional activity of NF-κB in embryonic fibroblasts (37). In contrast to our findings of reduced IL-6 production following poly(I:C) stimulation, other investigators reported no difference in mouse embryonic fibroblasts using siRNA against TBK1 or in macrophages from the TBK1-mutant mice (38, 39). Perhaps the conflicting results arise from functional differences for TBK1 that are dependent on cell type, or it may be that the scaffold function, rather than the catalytic activity of TBK1, is important for TLR3-induced NF-κB activation. We show that IKKε is involved in the negative-feedback loop of the TLR3–NF-κB pathway. No significant difference in poly(I:C)-induced IL-6 production was detected in BMDMs from TBK1-mutant mice (38), which provides further support that IKKε is the key kinase negatively regulating the TLR3–NF-κB axis. Future work will identify whether IKKε and/or TBK1 is the kinase limiting IKK activity downstream of TLR2, -4, and -9. IKKε was reported to limit type I IFN production in the RIG-I–signaling pathway, acting at the level of MAVS and leading to the dissociation of TRAF3 from MAVS (34). The kinase activity of IKKε was found not to be required for the disruption of the RIG-I/MAVS/TRAF-3 interactome (34). Our work advances knowledge on TLR3 signaling, implicating IKKε as a novel regulator of the signaling pathways regulating the production of IFN-β and proinflammatory cytokines.

Impaired activation of IKKε in IRAK1-deficient macrophages following poly(I:C) stimulation provides, to our knowledge, the first evidence that IRAK1 is involved in the activation of IKKε. We also demonstrated that IKKε activation is inhibited using an IRAK1/4 kinase inhibitor. The availability of specific IRAK1 and IRAK4 kinase inhibitors will more fully address the role of IRAK1 and/or IRAK4 in the activation of IKKε. TANK-deficient cells also show impaired activation of IKKε, together with reduced TBK1 activity following TLR-2, -4, and -9 signaling (35). TANK-deficient macrophages show increased NF-κB activation in response to TLR ligands. The interaction between the IKK-related kinases and the canonical IKKs is reduced in TANK-deficient cells, preventing the IKK-related kinases from negatively regulating the canonical IKKs (35). It would be interesting to assess the interaction between IKKε and IKKβ in IRAK1-deficient macrophages. Our siRNA work suggests that IRAK1 acts through IKKε to mediate its negative-regulatory effect on the TLR3–NF-κB–signaling pathway. Importantly, we identified IKKε as the key signaling molecule regulating the TLR3-stimulated inflammatory cytokine response, limiting the activation of NF-κB. The identity of the target(s) of IKKε will be the focus of future work in this field. We show increased TAK1 activity in IKKε-deficient macrophages following TLR3 stimulation. Pharmacological inhibition of TAK1 in IRAK1- and IKKε-deficient macrophages led to reduced IFN-β responses to poly(I:C), comparable to WT macrophages. These findings suggest that IKKε is targeting a signaling molecule upstream of the IKK complex. Interestingly, TANK$^{-/-}$ macrophages, which have impaired activation of IKKε, show enhanced E3 ligase activity of TRAF6 following TLR7 stimulation (40).

The importance of MAPKs in the regulation of cytokine production prompted us to examine the activation status of JNK, p38, and ERK following poly(I:C) stimulation in the IRAK1- and IKKε-deficient macrophages. We detected increased activation of JNK and p38 and impaired phosphorylation of ERK1/2 following poly(I:C) stimulation in both the IRAK-1– and IKKε-deficient BMDMs. The increased activation of JNK and p38 results from increased TAK1 activity. We found that the kinase activity of

IKKε is required for ERK1/2 activation. To our knowledge, this is the first report showing that IRAK1 and IKKε are required for ERK1/2 activation downstream of TLR3. Future work will address whether the kinase activity of IRAK1 is required for ERK1/2 activation and examine how IRAK1 and IKKε facilitate ERK1/2 activation downstream of TLR3.

This work has provided mechanistic understanding of IRAK1's negative regulation of the TLR3–IFN-β and proinflammatory cytokine response. We show a novel role for IRAK1 and IKKε in regulating the intensity of the TLR3–NF-κB–signaling pathway, uncovering an IRAK1–IKKε–TAK1–directed regulatory mechanism. Importantly, IRAK1 has emerged as a novel positive upstream regulator of IKKε. Finally, we show that IRAK1 regulates the intensity of IFN-β production acting at multiple levels, which highlights the complexity of signaling regulation in the TLR3 pathway.

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Disclosures

The authors have no financial conflicts of interest.

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