

RESEARCH ARTICLE

Non-Canonical Role of IKK γ in the Regulation of STAT1 Phosphorylation in Antiviral Signaling

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Abstract

Non-self RNA is recognized by retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), inducing type I interferons (IFNs). Type I IFN promotes the expression of IFN-stimulated genes (ISGs), which requires the activation of signal transducer and activator of transcription-1 (STAT1). We previously reported that dsRNA induced STAT1 phosphorylation via a type I IFN-independent pathway in addition to the well-known type I IFN-dependent pathway. I κ B kinase (IKK γ) is involved in antiviral signaling induced by dsRNA; however, its role is incompletely understood. Here, we explored the function of IKK γ in RLR-mediated STAT1 phosphorylation. Silencing of IKK γ markedly decreased the level of IFN- α and STAT1 phosphorylation in HeH response to dsRNA. However, the inhibition of IKK γ did not alter the RLR signaling-mediated dimerization of interferon responsive factor 3 (IRF3) or the nuclear translocation of nuclear factor- κ B (NF- κ B). These results suggest a non-canonical role of IKK γ in RLR signaling. Furthermore, phosphorylation of STAT1 was suppressed by IKK γ knockdown in cells treated with a specific neutralizing antibody for the type I IFN receptor (IFNAR) and in IFNAR-deficient cells. Collectively, the dual regulation of STAT1 by IKK γ in antiviral signaling suggests a role for IKK γ in the fine-tuning of antiviral signaling in response to non-self RNA.

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Introduction

Microorganism invasion in a vertebrate host is initially recognized by pattern recognition receptors (PRRs), resulting in the activation of the innate immune system [1]. RIG-I-like receptors (RLRs) are PRRs that recognize non-self RNA in the cytoplasm. Following the recognition of non-self RNA, RIG-I undergoes a conformational change to interact with a downstream adaptor molecule, mitochondrial antiviral signaling protein (MAVS) [2], which is also known as virus-induced signaling adaptor (VISA) [3], interferon (IFN)-promoter stimulator-1 (IPS-1) [4], or caspase activation and recruitment domain adaptor inducing IFN- γ (Cardif) [5]. Subsequently, MAVS activates downstream signaling molecules to produce type I IFNs [6]. Type I IFNs are essential for mounting a robust host response against viral infection

[7]. The type I IFN family predominantly comprises IFN- α and IFN- β , which share a common cell surface receptor that is composed of two chains, the α -chain (IFNAR1) and the β -chain (IFNAR2) [8]. After type I IFN binds to these IFNARs, each subunit of the receptor activates the other (cross-phosphorylation), resulting in subsequent activation of Janus tyrosine kinases (JAK1 and Tyk2) [9]. JAK1 and Tyk2 in turn activate their downstream effectors, signal transducer and activator of transcription (STAT) 1 and STAT2 [7]. The activated STATs form heterodimers to induce the transcription of hundreds of IFN-stimulated genes (ISGs) [10]. Because ISGs are required for the antiviral response [11], STAT1 has a critical role in antiviral innate immunity. STAT1 activation in antiviral signaling is believed to be dependent on type I IFN [12, 13]. We previously showed that double-stranded RNA (dsRNA) induced STAT1 phosphorylation in a type I IFN-independent manner [14]. The regulation of this STAT1 phosphorylation in RLR signaling remains to be elucidated.

Nuclear factor- κ B (NF- κ B) is an important transcription factor that is activated by a variety of stimuli, including cytokines, stress, and pathogenic components [15]. In resting conditions, NF- κ B is sequestered by inhibitor of NF- κ B (I κ B). I κ B is phosphorylated by the I κ B kinase (IKK) complex composed of IKK α , IKK β , and IKK γ (also known as NF- κ B essential modulator; NEMO), leading to its degradation and the subsequent activation of NF- κ B [16]. IKK α and IKK β are essential for canonical NF- κ B activation [17]. In addition, IKK γ is required to activate interferon regulatory factor 3 (IRF3) in response to viral infection [18]. However, IKK γ is not required for the activation of the canonical NF- κ B pathway or IRF3, indicating a non-canonical role of IKK γ in cellular signal transduction, including antiviral signaling. Indeed, IKK γ was shown to be critical in Toll-like receptor (TLR) 7/9 signaling to produce type I IFN [19]. However, the TLR7/9-mediated signaling pathways are considerably different from RLR signaling; thus, the molecular role of IKK γ in RLR signaling remains to be elucidated.

In this study, we investigated the role of IKK γ in RLR signaling, which is essential for antiviral innate immune responses in non-professional antigen-presenting cells, such as epithelial cells. We unexpectedly found a critical role of IKK γ in type I IFN-independent STAT1 activation in RLR signaling. Our data reveal that IKK γ contributes to both type I IFN-dependent and independent STAT1 activation in RLR signaling. These findings demonstrate a novel role of IKK γ in the antiviral innate immune system.

Materials and Methods

Cell Culture

HeLa cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Japan). Human IFNAR-deficient U5A cells and their parental 2fTGH cells were kindly provided by G. Stark (Cleveland Clinic Foundation Research Institute). The cells were maintained in a 5% CO₂ atmosphere at 37°C in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Perbio Science, Switzerland) and antibiotics (Life Technologies, Carlsbad, CA). The cells were treated with an IKK inhibitor (Merck Millipore, Darmstadt, Germany) when indicated.

Plasmids and siRNA

cDNA encoding full-length IKK γ was amplified from HeLa cell cDNA using Phusion DNA polymerase (Finnzymes, Keilaranta, Finland) and the primers Sall-IKK γ -F (5'-ATTCGgtcgacCATGGAGCGGCCCGGGGCT-3') and NotI-IKK γ -R (5'-TTCgcgccgcTCATTCTGTAAACCAACTCCA-3'). The amplified product was inserted into the Sall and NotI sites of a mammalian expression vector, pCMV-HA (Clontech, Mountain View, CA). The DNA construct was

analyzed by DNA sequencing. Plasmid DNA was purified using a plasmid purification column (Qiagen, Hilden, Germany).

To obtain the S176A mutant IKK expression vector, IKK S176A cDNA was generated using the overlapping PCR method. Two truncated IKK fragments were separately amplified by the primer pairs SalI-IKK⁻F and 5'-R (5'-AGCTCTGGGGCCAGATACTG-3') or 3'-R (5'-TGATCAAGGAgcTCTGTGT-3'), including the mutant bases, and NotI-IKK⁻R. Then, the full-length IKK S176A cDNA was generated by PCR using the primer pair SalI-IKK⁻F and NotI-IKK⁻R, and the two IKK fragments were simultaneously used as the templates. The full-length IKK S176A was cloned into the SalI-NotI site of pBlueScript II SK(+). Following confirmation of the DNA sequences, the full-length IKK S176A was transferred to pCMV-HA. The S180A mutant IKK expression vector was generated by site-directed mutagenesis using the IKK⁻-encoding vector pBlueScript II SK(+) as the template as well as the primer pair S180A-sense (5'-TCTGTGTACAgCTTTTGTGGG-3') and S180A-antisense (5'-CCCACAAAA GcGTGTACACAGA-3'). Then, the SalI-NotI cDNA fragment in the pBlueScript II SK(+)-IKK⁻-S180A vector was cloned into pCMV-HA.

siRNAs targeting IKK⁻ (SI02654659) and a non-silencing control siRNA were purchased from Qiagen. siRNAs targeting IKK⁻ (s7265) and IKK⁻ (s16186) were purchased from Life Technologies.

Transfection

Transient transfections of HeLa and U5A cells were performed as previously described [14]. Briefly, the cells were seeded at a density of 1.5×10^5 or 2.5×10^5 cells per well, respectively, in 12-well culture plates for 16 to 20 h prior to transfection and grown to 70%–80% confluency. The cells were transfected with a pCMV-HA-IKK⁻ vector using polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA) and incubated for 24 h for overexpression of exogenous IKK⁻. HeLa cells or U5A cells were transfected with a non-self dsRNA analog (poly I:C) using TransFectin (Bio-Rad, Hercules, CA) for the indicated period of time. RNA interference (RNAi) was performed via transfection with gene-specific siRNAs or control siRNA using Lipofectamine RNAiMAX (Life Technologies), following the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was extracted from the cells using an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Piscataway, NJ). Total RNA (500 ng) served as a template for single-stranded cDNA synthesis in a reaction using an oligo(dT)₁₈ primer and M-MLV reverse transcriptase (Life Technologies) under the conditions indicated by the manufacturer. A CFX96 real-time PCR detection system (Bio-Rad) was used for the quantitative analyses of IFN- β and 18S rRNA. The sequences of the primers were as follows:

IFN- β -F (5'-ACTGCCTCAAGGACAGGATG-3'),
 IFN- β -R (5'-AGCCAGGAGGTTCTCAACAA-3'),
 IL6-F (5'-AGGAGACTTGCCTGGTGAAA-3'),
 IL6-R (5'-CAGGGGTGGTTATTGCATCT-3'),
 18S rRNA-F (5'-ACTCAACACGGGAAACCTCA-3'), and
 18S rRNA-R (5'-AACCAGACAAATCGCTCCAC-3').

The amplification reactions were performed with SsoAdvancedTM Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's specifications. The amplification conditions were as follows: 30 s at 98°C, followed by heating consecutively at 98°C for 10 s, 58°C for 5 s each for 40 cycles. After the amplification was complete, a melting curve was generated by slowly heating from 65°C to 95°C in 0.5°C increments at 5 s per step, with continuous monitoring of the fluorescence. Quantitative analysis of the data was performed using a CFX manager (Bio-Rad).

ELISA

Conditioned culture medium was collected at the indicated times and was centrifuged at 12,000 × g for 5 min at 4°C to remove cell debris. The IFN- γ and IL6 concentrations in the culture medium were then measured using a human IFN- γ ELISA kit (Kamakura Techno-Science, Japan) or a Quantikine Human IL6 ELISA kit (R&D Systems, Minneapolis, MN), respectively.

Immunoprecipitation

Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM MgCl₂, 0.5% NP-40] containing 0.2% protease inhibitors. The lysates were centrifuged at 12,000 rpm for 5 min at 4°C to remove cell debris. Protein G Dynabeads (Life Technologies) were preincubated with an anti-STAT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a non-immune control antibody (Santa Cruz Biotechnology) for 10 min at room temperature, followed by incubation with protein lysates at 4°C for 1 h. After 3 washes with PBS-0.1% Tween 20, the immunoprecipitates were eluted with 3×SDS sample buffer at 95°C for 10 min. Anti-HA antibody beads (Wako, Japan) were incubated with protein lysates overnight at 4°C to pull down exogenous IKK α . After 5 washes using PBS-0.1% Tween 20, the immunoprecipitates were eluted with 3×SDS sample buffer at 95°C for 10 min. Then, the immunoprecipitates were subjected to SDS-PAGE.

Immunoblot Analyses

After two washes with phosphate-buffered saline (PBS; pH 7.4), cells were lysed in hypotonic lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM MgCl₂, and 0.5% NP-40] containing 0.2% protease inhibitors. The cell lysates were cleared by centrifugation at 12,000 rpm for 5 min at 4°C and 10 μ g of the lysate was subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), which were subsequently blocked for 1 h at room temperature in TBST buffer [20 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk (blocking buffer). Next, the membranes were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-IKK α , rabbit anti-IKK β , rabbit anti-IKK γ , rabbit anti-JAK1, rabbit anti-Tyk2, rabbit anti-phospho-JAK1, rabbit anti-phospho-Tyk2, rabbit anti-p50, rabbit anti-p52, rabbit anti-Rel B, rabbit anti-I κ B α , rabbit anti-phospho-STAT2, and rabbit anti-STAT2 (Cell Signaling), rabbit anti-IRF3 (IBL, Japan), rabbit anti- β -actin (Sigma-Aldrich), mouse anti-HA (Covance), rabbit anti-STAT1, mouse anti-phospho-STAT1, mouse anti-HSP90, mouse anti-p65, or mouse anti-histone (Santa Cruz Biotechnology). After the membranes were washed five times with TBST, they were further incubated for 1 h at room temperature with a bovine anti-rabbit (Santa Cruz Biotechnology) or a ZyMax anti-mouse IgG antibody (Life Technologies) coupled to horseradish peroxidase (HRP) at a 1:10,000 dilution in blocking buffer. The washes were repeated using TBST, and the immunoreactive bands were then visualized using Luminata Crescendo Western HRP Substrate (Millipore).

For native PAGE analysis, the cells were harvested in native lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl]. After centrifugation at 12,000 rpm for 10 min at 4°C, the lysates were subjected to native PAGE as previously reported [20].

Nuclear Fractionation

Following cell lysis with hypotonic lysis buffer and centrifugation at 2,000 rpm for 15 min at 4°C, the supernatant was collected (cytoplasmic fraction), and the pellet (nucleus) was resuspended in

RIPA buffer and passed through a 26 G needle-attached syringe. Then, the lysate was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant (nuclear fraction) was collected. Both the cytoplasmic and nuclear fractions were further subjected to SDS-PAGE.

Immunofluorescence Analyses

HeLa cells that were grown on glass coverslips were transfected with rhodamine-labeled poly I:C (InvivoGen) and then fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked with 3% BSA for 1 h. The cells were then incubated for 1 h with mouse monoclonal anti-IKK α (Millipore) and rabbit polyclonal anti-STAT1 (Santa Cruz Biotechnology) antibodies. After a washing step, the cells were incubated with Alexa 488-conjugated anti-mouse IgG and Alexa 555-conjugated anti-rabbit IgG. The cells were mounted in ProLong Gold antifade reagent (Life Technologies), and the subcellular localizations of IKK α and STAT1 were visualized by confocal laser scanning microscopy (C1si, Nikon, Japan).

Statistics

Statistical significance was analyzed using a one-way ANOVA followed by a post hoc Fisher's protected least significant difference test. All probability (*P*) values were based on two-tailed tests, and *P* < 0.05 was considered to be significant.

Results

Role of IKK β in RLR-mediated antiviral signaling

We initially determined whether IKK β is involved in the expression of IFN- β , a type I IFN produced from a variety of cells, including epithelial cells, in response to activation of RLR signaling. We previously reported that transfection of polyinosinic-polycytidylic acid (poly I:C), a synthetic viral dsRNA analog, activates RLR signaling in HeLa cells [20]. As expected, poly I:C markedly stimulated the expression of IFN- β mRNA and protein (Fig 1A and 1B). IKK α , not IKK β or IKK γ , was shown to be the most important IKK in the complex for antiviral signaling [18]. Consistent with this report, silencing of IKK α decreased the expression of IFN- β in response to poly I:C (Fig 1A). We found that silencing of IKK β in addition to IKK α substantially decreased the expression of IFN- β . Although suppression of IFN- β was also observed following knockdown of IKK γ , this effect was limited compared with those of IKK α and IKK β silencing. Activation of STAT1 is required to induce ISGs [21]; therefore, we next examined the role of IKKs in the RLR signaling-mediated STAT1 phosphorylation. We found that silencing of IKK α or IKK β inhibited the phosphorylation of STAT1 in response to poly I:C transfection (Fig 1C). In contrast, this effect was not observed when IKK γ was silenced by RNAi. Thus, we hypothesized that IKK β has an important role in RLR signaling-mediated antiviral innate immunity. To explore the detailed mechanisms by which IKK β regulates RLR signaling, we focused on the effect of IKK β on RLR signaling-mediated STAT1 phosphorylation. Time-dependent phosphorylation of STAT1 induced by poly I:C was notably inhibited by IKK β knockdown (Fig 1D).

IKK β is essential for activation of tumor necrosis factor (TNF)-mediated intracellular signal transduction [22]. Therefore, we examined whether IKK β was activated when the cells were exposed to dsRNA. Concentration-dependent STAT1 phosphorylation was observed in response to poly I:C (Fig 2A). However, poly I:C failed to stimulate phosphorylation of IKK β , while TNF rapidly activated IKK β phosphorylation. Furthermore, a time-course experiment showed that poly I:C did not activate IKK β phosphorylation (Fig 2B). The IKK β residues Ser-176 and Ser-180 are essential for activation of downstream signaling, especially for NF- κ B-mediated signaling [23]; thus, we analyzed the effect of mutations in these residues on STAT1

Fig 1. Effects of IKKs on IFN- β expression in response to poly I:C. (A, C) HeLa cells were transfected with siRNA targeting IKK α , IKK β , and IKK γ or control siRNA. Forty-eight hours after the transfection, the cells were further transfected with poly I:C (50 ng/well) for an additional 4 h. (A) The expression of IFN- β mRNA was analyzed by qRT-PCR. Data are presented as the mean \pm SD of three independent experiments. * P <0.01. (B, D) Following IKK RNAi silencing, the cells were transfected with poly I:C (50 ng/well) for 8 h (B) or up to 8 h (C). (B) IFN- β levels were determined by ELISA. All data are shown as the mean of three independent experiments. * P <0.01. (C, D) Protein levels were analyzed by immunoblotting. The results are representative of three independent experiments.

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phosphorylation. Overexpression of wild type (WT) IKK γ did not induce STAT1 phosphorylation (Fig 2C), indicating that the amount of IKK γ does not affect STAT1 phosphorylation. IKK γ mutations at either Ser-176 or Ser-180 did not alter the phosphorylation of STAT1. The levels of poly I:C-induced STAT1 phosphorylation were unchanged by the introduction of exogenous WT IKK γ or IKK γ mutated at Ser-176 and Ser-180. These results suggest that the phosphorylation of IKK γ is not required for RLR-mediated STAT1 activation.

Inhibition of RLR-mediated STAT1 phosphorylation by an IKK inhibitor

The 2-benzamido-pyrimidines are IKK inhibitors. The IC₅₀ analysis for one IKK inhibitor, IKK VII, showed that it inhibited IKK α activation at 40 nM, formation of the IKK complex at 70 nM, and IKK β activation at 200 nM [24]. When the cells were pretreated with varying concentrations of the IKK inhibitor, poly I:C-induced IFN- β expression was significantly

Fig 2. Effects of silencing IKK on STAT1 phosphorylation in response to dsRNA transfection. (A) HeLa cells were transfected with poly I:C at the indicated concentrations for 4 h. (B) HeLa cells were transfected with poly I:C (50 ng/well) for up to 6 h or treated with TNF for 10 min. (C) HeLa cells transiently overexpressing WT IKK, IKK S176A, or IKK S180A were transfected with poly I:C for 4 h. Protein levels were analyzed by immunoblotting. The results are representative of three independent experiments.

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inhibited, even at 40 nM of the IKK inhibitor (Fig 3A). In contrast, no suppression of STAT1 phosphorylation was observed following pretreatment of the cells with 40 nM or 70 nM of the IKK inhibitor (Fig 3B), suggesting a type I IFN- and IKK-independent STAT1 activation pathway in RLR signaling. However, pretreatment of the cells with 200 nM of the IKK inhibitor markedly inhibited the RLR-mediated STAT1 phosphorylation, indicating an IKK-dependent pathway for STAT1 phosphorylation in RLR signaling. We observed that the IKK inhibitor was active in HeLa cells because it abolished TNF-induced degradation of I β in a concentration-dependent manner (S1 Fig).

Involvement of IKK in type I IFN-independent STAT1 phosphorylation

To further confirm whether IKK is involved in type I IFN-independent STAT1 phosphorylation, we used an anti-type I IFN receptor (IFNAR)-neutralizing antibody to block type I IFN-mediated STAT1 phosphorylation. Pretreatment of the cells with the neutralizing antibody partially inhibited the phosphorylation of STAT1 in response to poly I:C (Fig 4A, lane 4). These results indicate that RLR signaling can activate STAT1 phosphorylation at least partly in a type I IFN-independent manner. We noted that this neutralizing antibody almost completely inhibited the phosphorylation of STAT1 in response to IFN γ , whose concentration was much greater than that produced by poly I:C transfection (Fig 4A, lane 10). Silencing IKK drastically suppressed the phosphorylation of STAT1 (Fig 4A, lane 6). Furthermore, in the presence of the type I IFNAR-neutralizing antibody, poly I:C failed to induce STAT1 phosphorylation in IKK-depleted conditions (Fig 4A, lane 8). These results suggest that IKK mediates both type I IFN-dependent and independent STAT1 phosphorylation in RLR signaling. Introduction of poly I:C in IFNAR-deficient U5A cells resulted in type I IFN-independent STAT1 phosphorylation (Fig 4B), as shown previously [14]. Silencing IKK suppressed the level of phosphorylated STAT1 in response to poly I:C in U5A cells, indicating the involvement of IKK in type I IFN-independent STAT1 phosphorylation activated by RLR signaling. In type I IFN signaling, STAT1 and STAT2 heterodimers associate with IRF9 to form the ISGF3 complex that translocates to the nucleus [25]. Then, we confirmed the effect of IKK on phospho-STAT2 levels. In IFNAR-expressing 2FTGH cells, STAT2 was phosphorylated in response to poly I:C (S2 Fig). IKK silencing partially suppressed the STAT2 phosphorylation as well as STAT1 phosphorylation. In contrast, no phosphorylation of STAT2 was observed in IFNAR-deficient U5A cells, indicating that activation of STAT2 in response to poly I:C is type I IFN-dependent.

Various cytokines have been shown to directly activate STAT1, and IL6 is one of the best-known cytokines that activates STAT1 [26]. Therefore, we confirmed the involvement of IKK in IL6 expression by RLR signaling. As expected, transfection with poly I:C induced both mRNA expression and protein secretion of IL6 (S3 Fig). Silencing of IKK significantly but partially inhibited the induction of IL6, suggesting a partial contribution of IL6 to IKK-mediated type I IFN-independent STAT1 activation by RLR signaling.

Effects of IKK on activation of IRF3 and NF- κ B in response to dsRNA

Because coordinated activation of NF- κ B and IRF3 was essential for induction of type I IFN [27], we next investigated the role of IKK in the activation of NF- κ B and IRF3 in response to

Fig 3. Effect of an IKK inhibitor on STAT1 phosphorylation in response to dsRNA. HeLa cells were pretreated with an IKK inhibitor at the indicated concentrations for 1 h and then transfected with poly I:C (50 ng/well) for 4 h. (A) The expression of IFN- β was analyzed by qRT-PCR. Data are presented as the mean \pm SD of three independent experiments. * $P < 0.01$. (B) Cell extracts were subjected to SDS-PAGE and then analyzed by immunoblotting. The results are representative of three independent experiments.

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dsRNA. Following activation by poly I:C, NF- κ B translocated to the nucleus (Fig 5A). Knockdown of IKK α or IKK β , but not IKK γ , inhibited the translocation of NF- κ B, suggesting an essential role for IKK α and IKK β and a dispensable role for IKK γ in RLR-mediated NF- κ B activation. RLR-mediated IRF3 dimerization was reduced by IKK α silencing, but knockdown of IKK β or IKK γ had no effect on dimerization (Fig 5B). We further examined the involvement of IKK α in the nuclear translocation of p50, p52, and Rel B, other NF- κ B subunits. TNF- α , which was used as a positive control, induced the nuclear translocation of p50, p52, and Rel B in HeLa cells (S4 Fig). IKK α silencing partially suppressed the nuclear translocation of p50.

Fig 4. Role of IKK in type I IFN-dependent and independent STAT1 phosphorylation induced by dsRNA.
(A) HeLa cells were transfected with siRNA targeting IKK or control siRNA for 48 h. Then, the cells were treated

with an anti-IFNAR neutralizing antibody for 1 h followed by transfection with poly I:C (50 ng/well) for an additional 4 h. HeLa cells were also treated with IFN- γ (200 IU/ml) for 15 min as a control to confirm the neutralizing activity of the anti-IFNAR antibody. (B) IFNAR-null U5A cells were transfected with siRNA targeting IKK γ or control siRNA for 48 h, and the cells were then further transfected with poly I:C (500 ng/well) for 10 h. Cell extracts were analyzed by immunoblotting. The results are representative of three independent experiments.

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Activation of RLR signaling by poly I:C transfection only enhanced the nuclear translocation of Rel B, and IKK γ silencing did not inhibit the nuclear localization. We found that knock-down of IKK γ enhanced nuclear accumulation of p52 and Rel B by an unknown mechanism. We confirmed that this observation was a non-specific effect by IKK γ siRNA because we observed similar results using other IKK γ -specific siRNAs (data not shown). These results indicate that IKK γ is not involved in canonical RLR signaling, which includes NF- κ B and IRF3.

Effects of IKK γ on Janus tyrosine kinase phosphorylation

Janus tyrosine kinases (JAKs) are upstream of STAT1 in the IFNAR-JAK-STAT axis. We therefore examined whether JAK activation was required for IKK γ -mediated STAT1 phosphorylation. Poly I:C induced phosphorylation of JAK1 and Tyk2, both of which serve as kinases for STAT1 [28], in HeLa cells (Fig 6A). Silencing of IKK γ suppressed phosphorylation of JAK1 and Tyk2 in response to poly I:C. IFNAR-deficient U5A cells exposed to poly I:C did not show phosphorylation of JAK1 and Tyk2, while poly I:C was able to induce STAT1 phosphorylation in these cells (Fig 4B). These results suggest that 1) activation of JAKs is entirely IFN-dependent; 2) IKK γ mediates phosphorylation of JAKs via the expression of type I IFN; and 3) IFNAR-independent STAT1 phosphorylation is IKK γ -dependent and JAK-independent.

No direct interaction between IKK γ and STAT1

We next determined whether IKK γ directly regulates STAT1. Immunoprecipitation analysis showed that neither endogenous nor exogenous IKK γ interacted with the STAT1 protein in the presence or the absence of poly I:C treatment in the cells (Fig 7A and 7B). In addition, immunofluorescence analyses did not find a physical interaction between IKK γ and STAT1, even in poly I:C-transfected cells (Fig 7C).

Discussion

IKK γ has been well-characterized as a regulator of canonical NF- κ B signaling. In normal conditions, NF- κ B is sequestered in the cytoplasm by I κ B family proteins [16]. The IKK complex consists of IKK α and IKK β , which function as the catalytic subunits, and IKK γ , which serves as a bridge for the IKKs and interacts with upstream signaling molecules [29]. A variety of stimuli, including cytokines and LPS, induce the IKK complex to undergo a conformational change to phosphorylate I κ B, resulting in degradation of I κ B and nuclear translocation of NF- κ B. Although the phosphorylation of specific serine residues in either IKK α or IKK β can activate the IKK complex, a study revealed that IKK α alone is sufficient for canonical NF- κ B activation [30]. A variety of stimuli activate the major NF- κ B p65 subunit (RelA). NF- κ B p65 is phosphorylated in response to pro-inflammatory stimuli [31]. I κ B degradation and NF- κ B activation were undetectable in IKK γ -deficient mouse embryonic fibroblasts (MEFs), suggesting an essential role for IKK γ in the classical NF- κ B pathway [32]. Collectively, IKK α and IKK β were shown to be indispensable for canonical NF- κ B activation, but the role of IKK γ is unclear. In the canonical pathway, NF- κ B p65 is phosphorylated at Ser-276, leading to an increase in its transcriptional activity [33]. In contrast, IKK γ could recognize NF- κ B but not I κ B as a substrate and phosphorylated the protein at Ser-536 in response to TNF- α [34]. Indeed, our

Fig 5. Effects of IKK α on translocation of the NF- κ B p65 subunit and dimerization of IRF3 in response to dsRNA. IKK α , IKK β , or IKK γ in HeLa (A) and U5A (B) cells were silenced as described above. Then, the cells were transfected with poly I:C for 4 h. Cell extracts were subjected to SDS-PAGE or native-PAGE (IRF3) followed by immunoblotting. The results are representative of three independent experiments.

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present study indicated that poly I:C did not alter the phosphorylation of NF- κ B p65 at Ser-536 (data not shown) or the nuclear translocation of NF- κ B p65 (Fig 5A). The amino acid sequences of IKK α and IKK β are structurally similar. However, IKK α is restricted to the cytosolic domain, whereas IKK β can shuttle from the cytoplasm to the nucleus following stimulation [35]. Our data showed that RLR signaling did not affect the intracellular distribution of IKK α (Fig 7C). These results indicate a complicated and unique role of IKK α in the non-canonical NF- κ B signaling pathway.

In NF- κ B signaling, both canonical and alternative pathways were reported to be involved in the innate immune reaction induced by viruses. Viruses have evolved multiple mechanisms to activate or inhibit these pathways to promote replication or to maintain infection, partly by manipulating IKK α . For example, the Molluscum contagiosum virus (MCV) inhibited the NF- κ B cascade via degradation of IKK α by the viral protein MC160, although MC160 did not interact with IKK α directly [36]. In contrast to MCV, herpes simplex virus (HSV)-1 replication was dependent on the activation of the NF- κ B cascade. An obvious loss of HSV virus yield and reduced NF- κ B nuclear translocation were observed in IKK α -deficient MEFs [37]. Additionally, IKK α is involved in hepatitis C virus (HCV)-induced lipogenesis and viral assembly independent of NF- κ B activation. IKK α was activated by the interaction of DDX3X with the HCV 3' untranslated region and then induced CBP/p300-mediated lipogenic gene transcription,

Fig 6. Influence of IKK α knockdown on JAK1 and Tyk2 activation in HeLa and U5A cells. Following knockdown of IKK α in HeLa (A) and U5A cells (B), poly I:C was introduced for an additional 4 h and 10 h, respectively. HeLa cells were treated with IFN- γ (10 ng/ml) for 20 min to confirm phosphorylation of JAK1 and Tyk2 as a positive control. Cell extracts were analyzed by immunoblotting. The results are representative of three independent experiments.

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Fig 7. No interaction of IKK and STAT1. (A) HeLa cells were transfected with poly I:C for 4 h. (B) Twenty-four hours after transfection with a HA-tagged IKK expression plasmid or an empty plasmid (mock), HeLa cells were transfected with poly I:C for 4 h. Immunoprecipitation (IP) was performed using an anti-STAT1, anti-IgG (A) or anti-HA beads (B), followed by immunoblotting using anti-IKK, anti-STAT1 or anti-HA antibody. (C) The cells were fixed with 4% paraformaldehyde and incubated with anti-IKK and anti-STAT1 antibodies. IKK and STAT1 were then detected separately using a secondary antibody coupled to Alexa 488 (green) or Alexa 555 (red).

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leading to core-associated lipid droplet formation, which facilitates viral assembly [38]. In addition, IKK was required for functional maturation of dendritic cells and acquired immunity to *Listeria monocytogenes* infection [39]. These findings clearly show the essential NF- κ B signaling-independent role of IKK in antiviral innate immunity. In fact, a recent study found that IKK regulates IRF3-mediated transcription [39].

In this study, we report a role of IKK in both type I IFN-dependent and independent phosphorylation of STAT1 in response to the activation of RLR signaling. Although we did not identify a role of IKK in poly I:C-induced IRF3 dimerization, silencing of IKK markedly inhibited the transcription of IFN- β mRNA in response to poly I:C. These results indicate that IKK regulates antiviral signaling via an IRF3-independent mechanism. The molecules that are involved in IKK-mediated antiviral signaling remain to be elucidated. In RLR signaling, few reports have shown involvement of the transcription factor AP-1, which mediates gene regulation of a variety of cytokines [40]. Indeed, AP-1 regulates the expressions of IFN- β [41] and IL6 [42]. A recent study identified a possible interaction of IKK with AP-1 [43]. This may explain the type I IFN-independent and IKK-dependent STAT1 activation in RLR signaling. We previously reported that RLR signaling can activate type I IFN-independent STAT1 phosphorylation [14]. Interestingly, activation of the type I IFN-independent STAT1 is unrelated to the expression of ISGs. We also observed that knockdown of IKK did not alter the level of ISGs in response to poly I:C in IFNAR-deficient cells (data not shown). RLR

Fig 8. Comprehensive roles of IKK β in RLR signaling-mediated STAT1 activation. In addition to participating in the IKK complex to activate the canonical antiviral signaling pathway, IKK β mediates type I IFN expression (a long dashed dotted line: A). IKK β could directly activate STAT1 phosphorylation in a type I IFN-independent manner (a dashed line: B). IKK β -mediated cytokine expression likely activates STAT1 via a type I IFN-independent positive feedback loop (red line: C).

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signaling can induce a variety of cytokines, including CCL5 [44] and IL6 [45]. In the present study, we demonstrated that IL6 can at least partially contribute to type I IFN-independent and IKK β -dependent STAT1 phosphorylation in RLR signaling. Our results using IFNAR-deficient cells showed that dsRNA activates STAT1 with delayed kinetics (8 h after dsRNA transfection). This indicates that synthesized proteins, including cytokines, may be required to activate STAT1 in a type I IFN-independent manner. Collectively, these results combined with our previous data suggest that the type I IFN-independent STAT1 phosphorylation is regulated by IKK β , and activated STAT1 may be involved in signaling pathways with functions other than the expression of ISGs.

In summary, we investigated the function of IKK β in RLR signaling (Fig 8). RLR signaling-mediated IKK β did not affect NF κ B or IRF3, both of which are known as classical antiviral signaling molecules. However, IKK β altered the expression of IFN β in response to dsRNA; therefore, IKK β induces IFN β via an unknown non-canonical pathway [Fig 8, a long dashed dotted line (A)]. In this pathway, STAT1 was phosphorylated in a type I IFN-dependent manner. In addition, IKK β also phosphorylated STAT1 in a type I IFN-independent manner [Fig 8, a dashed line (B)]. Cytokines induced by dsRNA can drive a positive feedback loop in type I IFN-independent STAT1 phosphorylation [Fig 8, red line: (C)]. Although these pathways can phosphorylate STAT1 at Tyr-701, the role of type I IFN-independent STAT1 phosphorylation differs from that of type I IFN-dependent STAT1 phosphorylation. This novel role of IKK β in STAT1 phosphorylation may be essential for antiviral innate immunity.

Supporting Information

S1 Fig. Effect of an IKK β inhibitor on I κ B degradation by TNF α . HeLa cells were pretreated with an IKK inhibitor at the indicated concentrations for 1 h, and then, cell extracts were analyzed by immunoblotting. The results are representative of three independent experiments. (TIF)

S2 Fig. Effect of IKK β on dsRNA-induced STAT2 phosphorylation. Following knockdown of IKK β in HeLa (A) and U5A cells (B), poly I:C was introduced for an additional 4 h and 10

h, respectively. Cell extracts were analyzed by immunoblotting. The results are representative of three independent experiments.

(TIF)

S3 Fig. Involvement of IKK in IL6 induction in response to dsRNA. Following knockdown of IKK, HeLa cells were transfected with poly I:C for an additional 48 h (A, B). IL6 mRNA (A) or protein (B) expression was examined. All data are shown as the mean of three independent experiments. $P < 0.01$.

(TIF)

S4 Fig. Effects of IKK on translocation of NF- κ B p50, p52, and Rel B subunits and dimerization of IRF3 in response to dsRNA. IKK in HeLa cells was described previously. Then, the cells were transfected with poly I:C for 4 h or stimulated with TNF- α (5 ng/mL) for 3 h. Cell extracts were subjected to SDS-PAGE followed by immunoblotting. CBB staining of the transferred membrane was used as a loading control. The results are representative of three independent experiments.

(TIF)

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Author Contributions

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Funding acquisition: FX TM.

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Methodology: FX TM.

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