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Isoliquiritigenin Suppresses the Toll−Interleukin-1 Receptor Domain-Containing Adapter Inducing Interferon-β (TRIF)-Dependent Signaling Pathway of Toll-Like Receptors by Targeting TBK1

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Toll-like receptors (TLRs) play an important role in induction of innate immune responses. TLRs can trigger the activation of myeloid differential factor 88 (MyD88)- and Toll−interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways. Expression of more than 70% of lipopolysaccharide (LPS)-induced target genes is mediated through a TRIF-dependent signaling pathway. To evaluate the therapeutic potential of isoliquiritigenin (ILG), we examined its effect on signal transduction via the TRIF-dependent pathway of TLRs. ILG inhibited interferon regulatory factor 3 activation induced by LPS or polyinosinic−polycytidylic acid, as well as interferon-inducible genes, such as interferon-inducible protein-10. ILG attenuated ligand-independent activation of IRF3 induced by TRIF or TBK1. Furthermore, ILG inhibited TBK1 kinase activity *in vitro*. Together, these results demonstrate that TBK1 is the molecular target of ILG, resulting in the downregulation of the TRIF-dependent signaling pathways of TLRs.

KEYWORDS: Toll-like receptors; isoliquiritigenin; lipopolysaccharide; polyinosinic−polycytidylic acid; TRIF; TBK1

INTRODUCTION

Many polyphenols abundant in plants are widely known to exert anti-inflammatory and anticancer effects. Licorice root has been used as a traditional medicine to treat several diseases, such as asthma, allergy, and inflammation. It is known that licorice contains various flavonoids that include isoliquiritigenin (ILG), licochalcone, and glabridin. Of these, ILG, a flavonoid with a chalcone structure (4,2′,4′-trihydroxychalcone), has been evaluated for its various biological activities, including anti-allergy, anti-inflammatory, and antioxidant activities (*1*, *2*). ILG effectively inhibits LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by inhibiting the degradation and phosphorylation of nuclear factor-κB (NF-κB) inhibitor IκBα in RAW 264.7 macrophages (*3*, *4*). The anti-inflammatory effects of ILG are also accomplished by reducing NF-κB activation via the IκB kinase (IKK) signaling pathway (*5*). ILG inhibits TNFα-induced NF-κB activation but not activator protein-1 (AP-1) and octamer-binding (Oct-1) transcription factors (*5*). Thus, the inhibitory activity of ILG is specific to NF-κB.

The innate immune system is the first line of host defense against invading microbial pathogens. Toll-like receptors (TLRs) induce innate immune responses by recognizing diverse molecular products derived from all the major classes of microbes, including bacteria, viruses, yeast, and fungi (*6*, *7*). Currently, at least 13 mammalian TLRs are identified with different types of agonists. The stimulation of TLRs by their respective ligands recruits TIR domain-containing adapter molecules, such as myeloid differentiation protein-88 (MyD88) or Toll−interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF).

MyD88 is an essential molecule for signaling by all mammalian TLRs, except for TLR3 (*8*). It recruits IL-1 receptor-associated kinases (IRAK4 and IRAK1) to TLRs. IRAK4 and IRAK1 are activated by phosphorylation and associated with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), leading to the activation of the canonical IKK complex followed by the activation of the NF-κB transcription factor. NF-κB activation induces the expression of pro-inflammatory cytokine, including TNF-α, interleukin (IL)-6, IL-12, and IL-1β (*9*).

In addition to the pro-inflammatory signals, recognition of viral double-stranded RNA by TLR3 and bacterial lipopolysaccharide (LPS) by TLR4 induce type-I interferons (IFNs) in a TRIF-dependent pathway, which is an essential adapter molecule for the TLR3- and TLR4-mediated MyD88-independent pathway (*9*). TRIF activates two noncanonical IKKs, inducible IKK (IKK*i*) and IKKε, and TRAF family member associated NF-κB activator-binding kinase 1 (TBK1) (*10*). Activated TBK1 phosphorylates interferon regulatory factor (IRF3). Phosphorylated IRF3 is dimerized and subsequently translocated into the nucleus. Translocated IRF3 binds to consensus DNA sequences known as the IFN-stimulated response elements (ISREs) (*7*, *11*, *12*). The representative target genes regulated through the TRIF-dependent signaling pathway of TLRs include IFN-β, interferon

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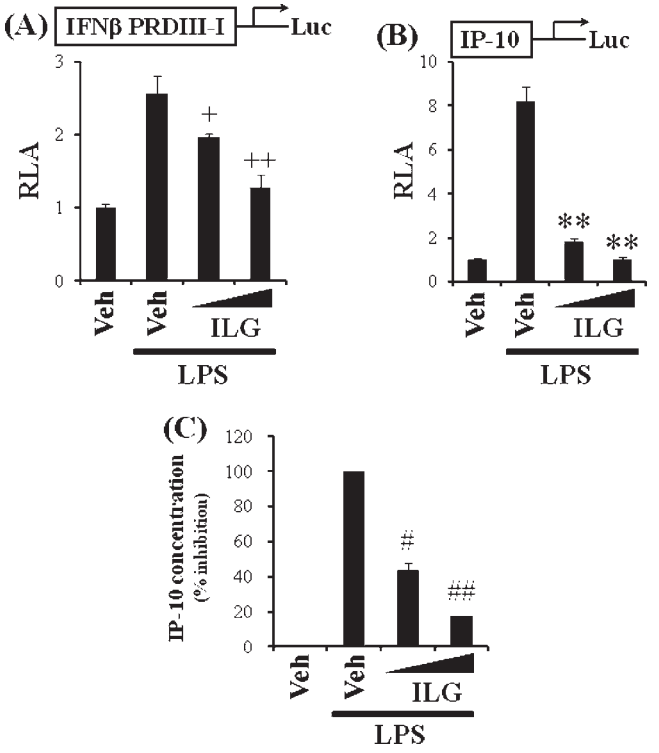

Figure 1. ILG inhibits LPS-induced IRF3 activation. (A and B) RAW 264.7 cells were transfected with the (A) IRF3-binding site (IFNβ PRDIII-I) or (B) IP-10 luciferase reporter plasmid, pretreated with ILG (50 and 100 μM) for 1 h, and then treated with LPS (10 ng/mL) for an additional 8 h. Cell lysates were prepared, and luciferase and β-galactosidase enzyme activities were measured, as described in the Materials and Methods. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean ± SEM (n = 3). (+) A result that is significantly different from LPS alone (A), $p < 0.05$ (+) and $p < 0.01$ (++). (*) A result that is significantly different from LPS alone (B), $p < 0.01$ (**). (C) RAW 264.7 cells were pretreated with ILG (50 and 100 μM) for 1 h and then treated with LPS (10 ng/mL) for an additional 8 h. Protein concentrations from cell culture supernatants were determined by ELISA as described in the Materials and Methods. Values are mean ± SEM (n = 3). (#) A result that is significantly different from LPS alone (C), $p < 0.05$ (#) and $p < 0.01$ (##). Veh, vehicle; ILG, isoliquiritigenin.

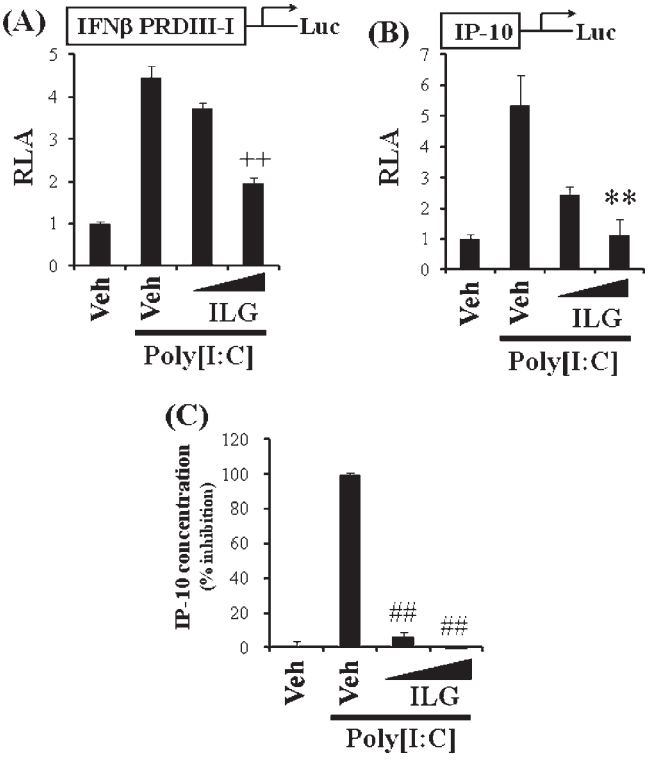

Figure 2. ILG inhibits poly[I:C]-induced IRF3 activation. (A and B) RAW 264.7 cells were transfected with the (A) IRF3-binding site (IFNβ PRDIII-I) or (B) IP-10 luciferase reporter plasmid and pretreated with ILG (50 and 100 μM) for 1 h and then treated with poly[I:C] (10 μg/mL) for an additional 8 h. Cell lysates were prepared, and luciferase and β-galactosidase enzyme activities were measured as described in the Materials and Methods. RLA was determined as described in the caption of **Figure 1**. Values are mean ± SEM (n = 3). (+) A result that is significantly different from poly[I:C] alone (A), $p < 0.01$ (++). (*) A result that is significantly different from poly[I:C] alone (B), $p < 0.01$ (**). (C) RAW 264.7 cells were pretreated with ILG (50 and 100 μM) for 1 h and then treated with poly[I:C] (10 μg/mL) for an additional 8 h. Protein concentrations from cell culture supernatants were determined by ELISA as described in the Materials and Methods. Values are mean ± SEM (n = 3). (#) A result that is significantly different from poly[I:C] alone (C), $p < 0.01$ (##). Veh, vehicle; ILG, isoliquiritigenin.

inducible protein-10 (IP-10), and regulated on activation normal T-cell expressed and secreted (RANTES) (*7*, *13*, *14*). The activation of the TRIF pathway also leads to the delayed activation of NF-κB mediated through receptor-interacting protein-1 (RIP1) (*8*).

It is already known that ILG inhibits the MyD88-dependent signaling pathway of TLRs by targeting IKKβ, which is one of the major kinases downstream of MyD88 (*5*). However, the molecular target of ILG in the TRIF-dependent signaling pathway has not been identified. Because more than 70% of LPS-induced genes are regulated through the TRIF-dependent pathway, we attempted to identify the molecular target of ILG in the TRIF-dependent signaling pathway.

MATERIALS AND METHODS

Reagents. ILG was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide prior to use. Purified LPS was purchased from List Biologicals (San Jose, CA) and dissolved in endotoxin-free water. Polyinosinic−polycytidylic acid (poly[I:C]) was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were purchased from Sigma-Aldrich unless otherwise described.

Cell Culture. RAW 264.7 murine monocytic cells (TIB-71; American Type Culture Collection, Manassas, VA) and 293T human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2.

Plasmids. NF-κB (2×)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). Heat-shock protein 70 (HSP70)-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). IP-10-luciferase reporter constructs were from Dr. Daniel Hwang (University of California, Davis, CA). All DNA constructs were prepared in large scale for transfection using an EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA).

Transfection and Luciferase Assays. The assays were performed as described previously (*15*, *16*). Briefly, RAW 264.7 cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using a SuperFect transfection reagent (Qiagen) according to the instructions of the manufacturer. Luciferase and β-galactosidase enzyme activities were determined using a commercial luciferase assay and β-galactosidase enzyme systems (Promega, Madison, WI) according to the instructions of the manufacturer. Luciferase activity was normalized to β-galactosidase activity.

Western Blotting. These were performed the same as previously described (*17*). Equal amounts of extracts were resolved by 8% sodium

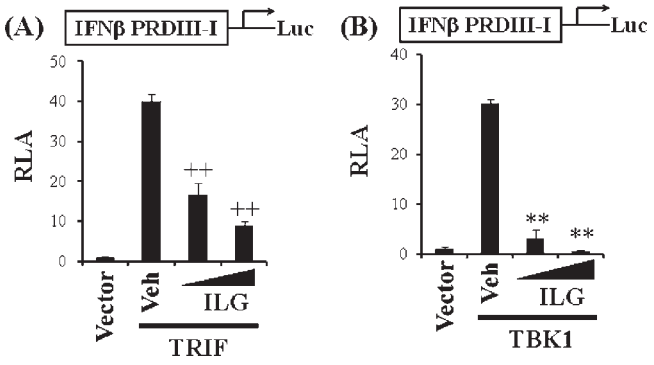

Figure 3. ILG inhibits IRF3 activation induced by TRIF or TBK1. (A and B) 293T cells were transfected with IRF3-binding site (IFNβ PRDIII-I)-luciferase reporter plasmid and the expression plasmid of (A) TRIF or (B) TBK1. Cells were further treated with ILG (50 and 100 μM) for 18 h. RLA was determined as described in the caption of **Figure 1**. Values are mean $\pm$ SEM ($n = 3$). (+) A result that is significantly different from TRIF plus vehicle (A), $p < 0.01$ (++). (*) A result that is significantly different from TBK1 plus vehicle (B), $p < 0.01$ (**). Veh, vehicle; ILG, isoliquiritigenin.

dodecyl sulfate−polyacrylamide gel electrophoresis, and the separated proteins were electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween-20 and 3% nonfat dry milk and blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (HRP; Amersham, Arlington Heights, IL). The reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). To reprobe with different antibodies, the membrane was stripped in 0.2 N NaOH at room temperature for 10 min.

Enzyme-Linked Immunosorbent Assay (ELISA). For the measurement of the IP-10 concentration, cells were pretreated with ILG (50 and 100 μM) for 1 h and then treated with LPS (10 ng/mL) or poly[I:C] (10 μg/mL) for an additional 8 h. The levels of IP-10 were determined with culture medium using an IP-10 ELISA kit according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN).

***In Vitro* TBK1 Kinase Assay.** TBK1 kinase activity was conducted with recombinant human TBK1 (Upstate Biotechnology, Lake Placid, NY) and a chemiluminescence detection myelin basic protein (MBP) assay kit (Upstate Biotechnology) according to the instructions of the manufacturer. The assay kit is designed to measure the phosphotransferase activity of numerous kinases, including TBK1, c-Jun kinase, and mitogen-activated protein kinase based on phosphorylation of MBP using a non-radioactive magnesium/ATP cocktail as a phosphate donor. The mixture of reagents, magnesium/ATP, and recombinant TBK1 were added to MBP-coated wells. After incubation of the wells for 60 min at 30 °C, the wells were blocked with blocking buffer. HRP-conjugated antiphospho-MBP was added to the wells. After washing with washing buffer, luminescent intensity was determined with the LumiGLO chemiluminescent system (Upstate Biotechnology).

Data Analysis. All data were expressed as mean $\pm$ standard error of the mean (SEM) of at least three independent experiments performed in triplicate.

RESULTS AND DISCUSSION

ILG Suppresses LPS- or Poly[I:C]-Induced IRF3 Activation. TLR signaling pathways trigger the activation of IRF3 mediated through TRIF-dependent pathways. This pathway induces the activation of downstream kinase, TBK1, and then phosphorylates IRF3, leading to the IRF3 activation (*10*). Therefore, IRF3 activation was used as the readout for the TRIF-dependent pathway. ILG inhibited IRF3-reporter gene expression induced by LPS in RAW 264.7 macrophages (**Figure 1A**). To further identify the inhibition of the TRIF-dependent pathway by ILG, the expression of IP-10 associated with the TRIF-dependent

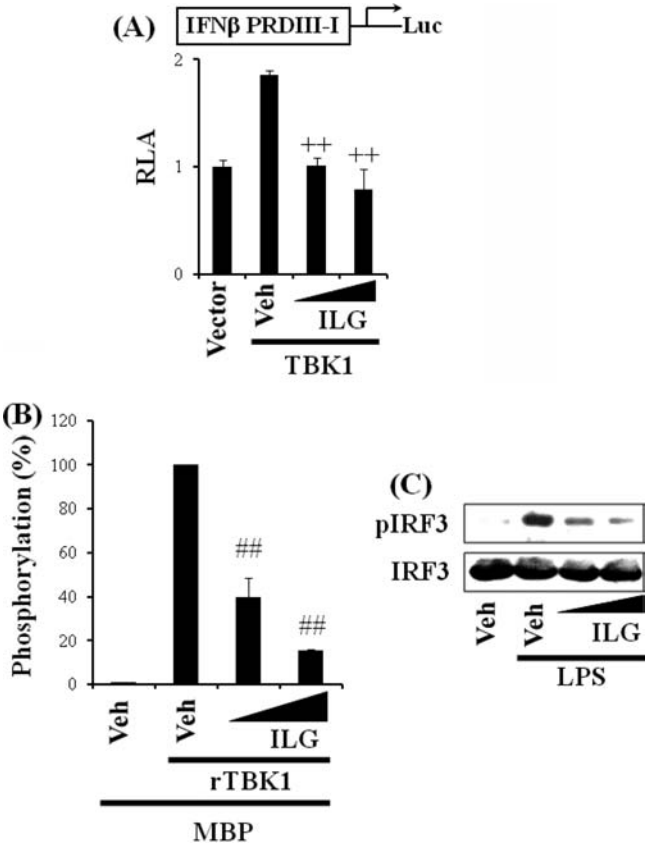

Figure 4. ILG suppresses the functional activity of TBK1. (A) RAW 264.7 cells were transfected with IRF3-binding site (IFNβ PRDIII-I) luciferase reporter plasmid and the expression plasmid of TBK1. Cells were further treated with ILG (50 and 100 μM) for 8 h. RLA was determined as described in the caption of **Figure 1**. Values are mean $\pm$ SEM ($n = 3$). (+) A result that is significantly different from TBK1 plus vehicle (A), $p < 0.01$ (++). (B) *In vitro* TBK1 kinase assay was performed using recombinant active rTBK1 and MBP as a substrate. The TBK1 kinase activity was determined in the presence of ILG (50 and 100 μM). Values are mean $\pm$ SE ($n = 3$). (#) A result that is significantly different from vehicle plus rTBK1 plus MBP (B), $p < 0.01$ (##). (C) RAW 264.7 cells were pretreated with ILG (50 and 100 μM) for 1 h and then further stimulated with LPS (50 ng/mL) for 2 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 proteins by Western blotting. Veh, vehicle; ILG, isoliquiritigenin.

pathways was measured. ILG inhibited LPS-induced IP-10 expression by a luciferase reporter gene assay (**Figure 1B**) and ELISA (**Figure 1C**).

Although the TLR4 signaling pathway can use both MyD88- and TRIF-dependent pathways to activate NF-κB and IRF3, TLR3 uses only the TRIF-dependent pathway to activate NF-κB and IRF3. Therefore, IRF3 activation by poly[I:C] (a TLR3 agonist) can be used as the readout for the TRIF-dependent pathway. ILG inhibited poly[I:C]-induced IRF3 activation and poly[I:C]-induced IP-10 expression as determined by a luciferase reporter gene assay (panels **A** and **B** of **Figure 2**, respectively) and ELISA (**Figure 2C**). These results suggest that ILG inhibits the TRIF-dependent signaling pathway of TLR3 and TLR4 for the inhibition of IRF3.

ILG Inhibits the Kinase Activity of TBK1. We next sought to determine which signaling molecules in the TRIF-dependent pathway of TLRs are the targets of ILG. To further identify the molecular targets of ILG for the inhibition of the TRIF-dependent pathway, IRF3 activation was induced by the overexpression of TRIF or TBK1 in 293T cells. ILG inhibited TRIF- or

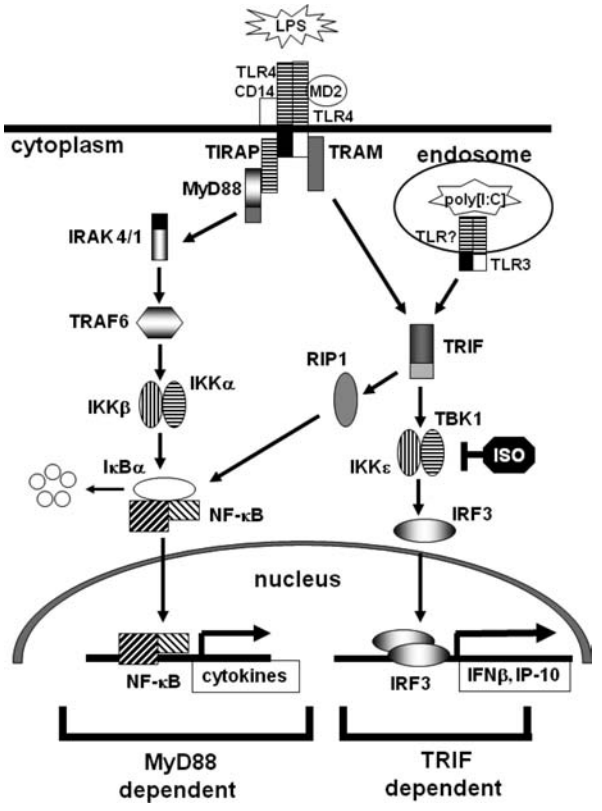

Figure 5. Molecular targets of ILG in TLR signaling pathways. TBK1 is the molecular target of ILG.

TBK1-induced IRF3 activation as determined by an IRF3-binding site (IFNβ PRDIII-I) reporter gene assay (panels **A** and **B** of **Figure 3**).

TBK1 is a major downstream kinase of the TRIF-dependent signaling pathway and phosphorylates and activates IRF3. Therefore, we investigated whether the inhibitory effect of ILG on IRF3 activation is mediated through the inhibition of TBK1. ILG inhibited IRF3 activation induced by overexpression of TBK1 in RAW 264.7 cells (**Figure 4A**). An *in vitro* kinase assay using recombinant TBK1 showed that ILG suppressed the TBK1 kinase activity in a dose-dependent manner (**Figure 4B**). To further confirm the inhibition of TBK1 kinase activity by ILG, we assessed phosphorylation of IRF3. ILG inhibited LPS-induced phosphorylation of IRF3 as determined by Western blotting (**Figure 4C**). All of the data suggested that TBK1 is the molecular target of ILG in the TRIF-dependent signaling pathway that is normally induced by TLR3 and TLR4 stimulation (**Figure 5**).

All TLR signaling pathways culminate in NF-κB activation, which induces cytokine expression, leading to pro-inflammatory responses. NF-κB activation induced by microbial components involves the signal-induced phosphorylation and subsequent degradation of IκBα protein through the canonical IKK complex composed of two catalytic kinase subunits, IKKα and IKKβ (*18*−*20*). In contrast to NF-κB activation, IRF3 activation occurs directly through its phosphorylation by two additional IKKs: TBK1 and IKKε (*21*). These modifications induce the homodimerization of IRF3 and subsequent translocation of IRF3 into the nucleus (*22*). IRF3 translocated into the nucleus binds to consensus DNA sequences known as the ISREs found in the promoter regions of genes, such as those encoding IFNβ, IP-10, and RANTES (*7*, *11*, *12*).

Growing evidence now suggests that the TRIF-dependent signaling pathway of TLRs is important in inflammatory responses and development of certain chronic diseases. It was reported that more than 70% of LPS-inducible genes are regulated through the TRIF-dependent pathway (*7*). Therefore, the modulation of the TRIF-dependent pathway of TLRs might be a useful anti-inflammatory strategy. Our results showed that ILG suppressed IFNβ production induced by TLR3 or TLR4 agonists and the transcriptional activation of IRF3 by targeting TBK1 of the TRIF-dependent signaling pathway.

Supporting Information Available: RAW 264.7 cells treated with ILG (50 and 100 μM) for 8 h (Supplemental Figure 1) and RAW 264.7 cells treated with ILG (50 and 100 μM) and LPS (10 ng/mL) for 4 h (Supplemental Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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