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# Specific Inhibition of MyD88-Independent Signaling Pathways of TLR3 and TLR4 by Resveratrol: Molecular Targets Are TBK1 and RIP1 in TRIF Complex<sup>1</sup>

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TLRs can activate two distinct branches of downstream signaling pathways. MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$  (TRIF) pathways lead to the expression of proinflammatory cytokines and type I IFN genes, respectively. Numerous reports have demonstrated that resveratrol, a phytoalexin with anti-inflammatory effects, inhibits NF- $\kappa$ B activation and other downstream signaling pathways leading to the suppression of target gene expression. However, the direct targets of resveratrol have not been identified. In this study, we attempted to identify the molecular target for resveratrol in TLR-mediated signaling pathways. Resveratrol suppressed NF- $\kappa$ B activation and cyclooxygenase-2 expression in RAW264.7 cells following TLR3 and TLR4 stimulation, but not TLR2 or TLR9. Further, resveratrol inhibited NF- $\kappa$ B activation induced by TRIF, but not by MyD88. The activation of IFN regulatory factor 3 and the expression of IFN- $\beta$  induced by LPS, poly(I:C), or TRIF were also suppressed by resveratrol. The suppressive effect of resveratrol on LPS-induced NF- $\kappa$ B activation was abolished in TRIF-deficient mouse embryonic fibroblasts, whereas LPS-induced degradation of I $\kappa$ B $\alpha$  and expression of cyclooxygenase-2 and inducible NO synthase were still inhibited in MyD88-deficient macrophages. Furthermore, resveratrol inhibited the kinase activity of TANK-binding kinase 1 and the NF- $\kappa$ B activation induced by RIP1 in RAW264.7 cells. Together, these results demonstrate that resveratrol specifically inhibits TRIF signaling in the TLR3 and TLR4 pathway by targeting TANK-binding kinase 1 and RIP1 in TRIF complex. The results raise the possibility that certain dietary phytochemicals can modulate TLR-derived signaling and inflammatory target gene expression and can alter susceptibility to microbial infection and chronic inflammatory diseases. *The Journal of Immunology*, 2005, 175: 3339–3346.

Toll-like<sup>0</sup> receptors induce innate immune responses by recognizing invading microbial pathogens leading to the activation of adaptive immune responses (1, 2). Currently, at least 13 TLRs in mammalian cells are identified with different types of agonists (3, 4). The TLR agonists include LPS for TLR4, bacterial lipopeptides, and peptidoglycan for TLR2, dsRNA for TLR3, flagellin for TLR5, and ssRNA and bacterial unmethylated CpG DNA for TLR7 and TLR9, respectively (5–12). It was reported that TLR4 signaling pathways can be activated by nonbacterial agonists such as heat shock protein 60, fibronectin, Taxol,

respiratory syncytial virus fusion protein, and saturated fatty acids (13–18). This fact points to the possibility that TLRs are involved in inflammatory responses induced by molecules with noninfectious origins.

Broadly, the stimulation of TLRs by agonists can trigger the activation of two downstream signaling pathways: MyD88-dependent and -independent pathways (3). MyD88 is the immediate adaptor molecule that is common to all TLRs, with the exception of TLR3. MyD88 recruits IL-1R-associated kinase and TNFR-associated factor 6 (TRAF6)<sup>5</sup> leading to activation of the canonical I $\kappa$ B kinase (IKK) $\alpha\beta\gamma$  complex. IKK $\beta$  phosphorylates I $\kappa$ B $\alpha$  resulting in the subsequent degradation of I $\kappa$ B $\alpha$  leading to the nuclear translocation and DNA binding of NF- $\kappa$ B (19–22). LPS- or poly(I:C)-induced activation of Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$  (TRIF; TICAM-1), an adaptor molecule that functions independently of MyD88, leads to the delayed activation of NF- $\kappa$ B (23, 24). TRIF also induces the activation of the transcriptional regulator, IFN regulatory factor (IRF)3 and the expression of IFN- $\beta$  and IFN-inducible genes through the activation of TANK-binding kinase (TBK)1 and IKK $\epsilon$  (25, 26). TLR3 activates primarily TRIF pathway, whereas TLR4 activates both MyD88- and TRIF-dependent pathways.

Deregulated activation of TLRs can lead to the development of severe systemic inflammation including septic shock with high

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<sup>5</sup> Abbreviations used in this paper: TRAF6, TNF receptor-associated factor 6; iNOS, inducible NO synthase; COX, cyclooxygenase; MALP-2, macrophage-activating lipopeptide of 2 kDa; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$ ; IKK, I $\kappa$ B kinase; TBK, TANK-binding kinase; MEF, mouse embryonic fibroblast; IRF, IFN regulatory factor.

mortality. Moreover, chronic inflammation is known to be an important etiological condition for various chronic diseases including atherosclerosis, diabetes, and cancer. Recent evidence suggests the involvement of TLRs in these chronic diseases (27–29). Identifying molecular targets by which pharmacological or dietary factors modulate TLR-mediated signaling pathways and target gene expression would provide new opportunity to manage the deregulation of TLR-mediated inflammatory responses leading to acute and chronic inflammatory diseases.

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenol found in grapes and other plants. Resveratrol has been known to protect plants against fungal infections and to provide the resistance against a variety of plant diseases (30, 31). In mammalian systems, resveratrol has been shown to possess anti-inflammatory and chemopreventive properties (32, 33). Resveratrol suppressed the macrophage activation and also inhibited the growth and tumorigenic potential of various cancer cell lines including prostate, breast, and colon cancer cells (34). Resveratrol inhibited the expression of proinflammatory markers including cyclooxygenase (COX)-2 and inducible NO synthase (iNOS) in both macrophages and cancer cell lines (35). NF- $\kappa$ B activation is critically linked to inflammatory responses and other chronic diseases (36). Despite numerous reports demonstrating the inhibitory effects of resveratrol on NF- $\kappa$ B activation and target gene expression induced by various proinflammatory stimuli (37, 38), the direct molecular targets and the mechanisms for such inhibition are not known. The inhibition by resveratrol of NF- $\kappa$ B activated by different agonists suggests that the targets of resveratrol are likely to be downstream signaling components responsible for the activation of the transcription factor, rather than the receptors themselves. NF- $\kappa$ B can be stimulated by multiple signaling components derived from the activation of different types of receptors (39).

Therefore, we attempted to identify the molecular target of resveratrol in relatively well defined downstream signaling pathways and target gene expression induced by the activation of TLRs.

## Materials and Methods

### Reagents

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) and *trans*-stilbene were purchased from Sigma-Aldrich and were dissolved in DMSO. Purified LPS was obtained from List Biological Laboratories. Macrophage-activating lipopeptide of 2 kDa (MALP-2) was purchased from Alexis Biochemical. Poly(I:C) was purchased from Amersham Biosciences. Unmethylated CpG DNA (ODN1668) was purchased from TIB MolBiol. Polyclonal Ab for COX-2 or GAPDH was prepared as previously described (40). Ab for I $\kappa$ B $\alpha$  or iNOS was obtained from Santa Cruz Biotechnology. Ab for phospho-IRF3 (S396) or IRF3 was obtained from Upstate Biotechnology or Zymed Laboratories, respectively. All other reagents were purchased from Sigma-Aldrich unless otherwise described.

### Cell culture

RAW 264.7 cells (a murine monocytic cell line; American Type Culture Collection no. TIB-71) and human embryonic kidney (HEK)293T cells were cultured in DMEM containing 10% (v/v) heat-inactivated FBS (Invitrogen Life Technologies), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Invitrogen Life Technologies). Mouse embryonic fibroblast (MEF) from wild-type and TRIF-deficient mice were prepared at the Department of Host Defense, Research Institute for Microbial Diseases, Osaka University (Osaka, Japan) as previously described (41). MEFs were cultured in DMEM containing 20% (v/v) FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Immortalized macrophages derived from MyD88-deficient or wild-type mice were established by infecting primary bone marrow cells with the J2 recombinant retrovirus at the Laboratory of Experimental Immunology, National Cancer Institute (Frederick, MD), according to the procedure as previously described (42). Cells were maintained at 37°C in a 5% CO<sub>2</sub>/air environment.

**Plasmids** NF- $\kappa$ B(2 $\times$ ) luciferase reporter construct was provided by F. Mercurio (Signal Pharmaceuticals, San Diego, CA). The luciferase reporter plasmid (pGL2) containing the promoter region of the murine COX-2 gene

(–3.2 kb) was a gift from D. Dewitt (Michigan State University, East Lansing, MI). Heat shock protein 70- $\beta$ -galactosidase reporter plasmid was from R. Modlin (University of California, Los Angeles, CA). A wild type of MyD88 was provided by J. Tschopp (University of Lausanne, Lausanne, Switzerland). The wild-type IKK $\beta$  was obtained from M. Karin (University of California, San Diego, CA). The wild-type IRF3 was obtained from G. Cheng (University of California, Los Angeles, CA). A constitutively active form of IRF3 (IRF3–5D) was from J. Hiscott (McGill University, Montreal, Quebec, Canada) (43). The wild type of p65 was obtained from J. Ye (Pennington Biomedical Research Center, Baton Rouge, LA). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen) for transfection.

### Transfection and luciferase assay

These were performed as described in our previous studies (7, 16). Briefly, RAW264.7 or HEK293T cells were cotransfected with a luciferase plasmid and heat shock protein 70- $\beta$ -galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. Various expression plasmids or corresponding empty vector plasmids for signaling components were cotransfected. The total amount of transfected plasmid was equalized by supplementing with the corresponding empty vector to eliminate the experimental error from the transfection itself. Luciferase and  $\beta$ -galactosidase enzyme activities were determined using the Luciferase Assay System and  $\beta$ -galactosidase Enzyme System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized by  $\beta$ -galactosidase activity.

### Immunoblotting

These were performed essentially the same as previously described (18, 44, 45). Equal amounts of cell extracts were resolved on SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membranes were blocked with PBS containing 0.1% Tween 20 and 3% nonfat dry milk and were blotted with the indicated Abs and secondary Abs conjugated to HRP (Amersham). The reactive bands were visualized with the ECL system (Amersham Biosciences). To reprobe with different Abs, the membrane was stripped in stripping buffer at 55°C for 1 h.

### Real-time RT-PCR analysis of IFN- $\beta$ expression

Total RNAs were extracted using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's instruction. Five micrograms of total RNAs were reverse-transcribed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) and amplified with a LightCycler (Roche Applied Science) using the LightCycler FastStart DNA Master SYBR Green I kit. The primers used to detect mouse IFN- $\beta$  are as follows: forward primer 5'-TCCAAGAAAGGAC GAACATTCG-3', reverse primer 5'-TGAGGACATCTCCCACGTCAA-3'. The primers for mouse  $\beta$ -actin (used as an internal control) are as follows: forward primer 5'-TCATGAAGTGTGACGTTGACATCCGT-3', reverse primer 5'-CCTAGAAGCATTTCGGTGACGATG-3'. The following program was used: denaturation at 95°C for 5 min and 45 cycles consisting of denaturation at 95°C for 10 s, annealing at 56°C for 5 s, and extension at 72°C for 13 s. The specificity of the amplified PCR products was assessed by a melting curve analysis. The fold induction of IFN- $\beta$  expression by real-time PCR was measured three times in duplicate relative to vehicle control and calculated as previously described (46).

### In vitro TBK1 kinase assay

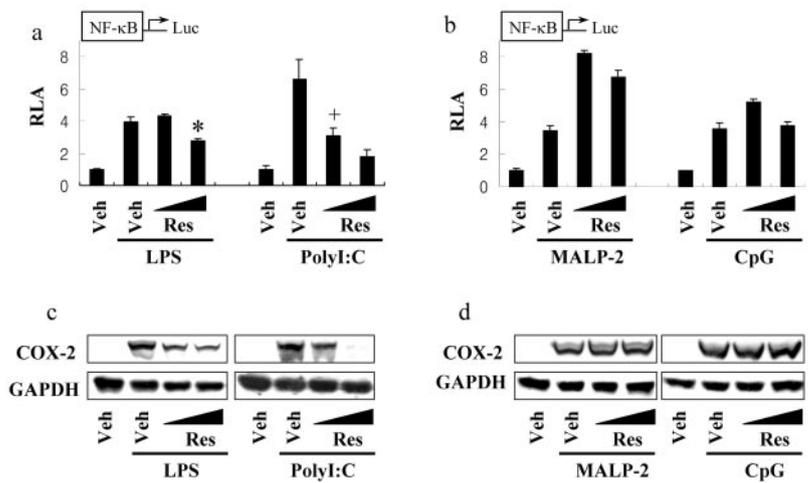
TBK1 kinase assay was conducted with recombinant human TBK1 (catalogue no. 12-628; Upstate Biotechnology) according to the manufacturer's instruction. Briefly, active TBK1 was incubated with IRF3 protein (0.5  $\mu$ g) in kinase buffer containing 50  $\mu$ M ATP and 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 10 min at 30°C. A 20- $\mu$ l aliquot was transferred onto the phosphocellulose paper. After more than four times of washing, the radioactivity was determined by scintillation counter. Purified IRF3 (aa 173–427) (47) was obtained from K. Lin (University of Massachusetts Medical School, Worcester, MA).

## Results

### Resveratrol inhibits the activation of NF- $\kappa$ B and the expression of COX-2 induced by LPS and poly(I:C), but not by MALP-2 and CpG DNA in macrophages

Broadly, the major downstream signaling pathways of TLRs consist of MyD88-dependent and –independent pathways. The activation of TLR4 triggers the activation of both MyD88-dependent and –independent (TRIF-dependent) signaling pathways. TLR3

**FIGURE 1.** Resveratrol inhibits the activation of NF- $\kappa$ B and the expression of COX-2 induced by TLR4 and TLR3 agonist (LPS and poly(I:C), respectively), but not by TLR2 and TLR9 agonist (MALP-2 and CpG DNA, respectively) in macrophages. *a* and *b*, RAW264.7 cells transfected with NF- $\kappa$ B(2 $\times$ ) binding site luciferase reporter plasmid were treated with resveratrol (30, 50  $\mu$ M) for 1 h. Cells were further stimulated with LPS (3 ng/ml), poly(I:C) (2  $\mu$ g/ml), MALP-2 (1 ng/ml), or CpG DNA (ODN1668, 0.2  $\mu$ M) for 18 h. Cell lysates were prepared and luciferase and  $\beta$ -galactosidase enzyme activities were measured as described in *Materials and Methods*. Relative luciferase activity (RLA) was determined by normalization with  $\beta$ -galactosidase activity. Values are mean  $\pm$  SEM ( $n = 3$ ). Significantly different (\*,  $p < 0.05$ ) from LPS alone. Significantly different (+,  $p < 0.05$ ) from poly(I:C) alone. *c* and *d*, RAW264.7 cells were treated with resveratrol (30, 50  $\mu$ M) for 1 h and then further stimulated with LPS (5 ng/ml), poly(I:C) (10  $\mu$ g/ml), MALP-2 (2 ng/ml), or CpG DNA (ODN1668, 0.2  $\mu$ M) for 18 h. Cell lysates were analyzed for COX-2 and GAPDH immunoblots. Representative data are from more than three independent experiments. Veh, vehicle; Res, resveratrol.



only activates the TRIF-dependent pathways, whereas TLR2 and TLR9 lead to the activation of the MyD88-dependent and not the TRIF-dependent pathway. Because both MyD88 and TRIF signaling pathways can lead to NF- $\kappa$ B activation, NF- $\kappa$ B is the common downstream signaling component for all TLRs. COX-2 is one of the target genes induced by various TLR agonists, which is primarily regulated through the activation of NF- $\kappa$ B in macrophages (44). Therefore, to investigate whether resveratrol modulates TLR-mediated signaling pathways, the activation of NF- $\kappa$ B and the expression of COX-2 induced by TLR agonists were used as a read-out for the activation of TLRs.

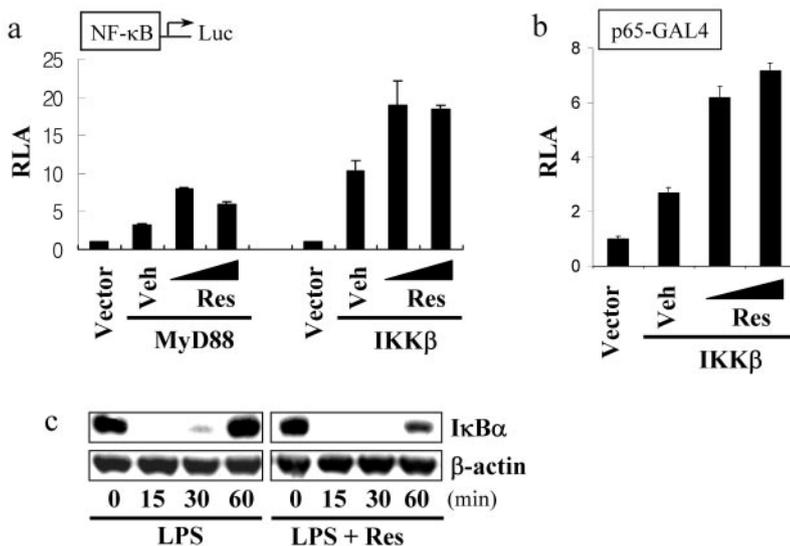
Resveratrol suppressed LPS-induced (TLR4 agonist) and poly(I:C)-induced (TLR3 agonist) activation of NF- $\kappa$ B in a dose-dependent manner as determined by the luciferase reporter gene assay (Fig. 1*a*). In contrast, NF- $\kappa$ B activation induced by MALP-2 (TLR2 agonist) or unmethylated CpG DNA (TLR9 agonist) was not inhibited by resveratrol (Fig. 1*b*). NF- $\kappa$ B activation induced by MALP-2 or CpG DNA was not affected by even prolonged pretreatment with resveratrol up to 8 h (data not shown). Similarly, resveratrol inhibited the expression of COX-2 induced by LPS or poly(I:C), but not by

MALP-2 or CpG DNA as determined by COX-2 immunoblotting (Fig. 1, *c* and *d*). The inhibitory effect of resveratrol on NF- $\kappa$ B activation and COX-2 expression induced by TLR3 agonist was more pronounced than the inhibitory effect on TLR4 activation.

These results demonstrate that resveratrol specifically inhibits the activation of TLRs that can stimulate the TRIF-dependent pathway (TLR3 and TLR4), but not the activation of TLRs that can stimulate only the MyD88-dependent pathway (TLR2 and TLR9). Therefore, the results suggest that resveratrol inhibits TRIF-dependent signaling pathways, but not MyD88-dependent pathways. The greater inhibition by resveratrol of the activation of TLR3 than TLR4 may be due to the fact that NF- $\kappa$ B activation by TLR3 agonist is mostly dependent on TRIF, whereas NF- $\kappa$ B activation by TLR4 agonist is mediated through both MyD88 and TRIF.

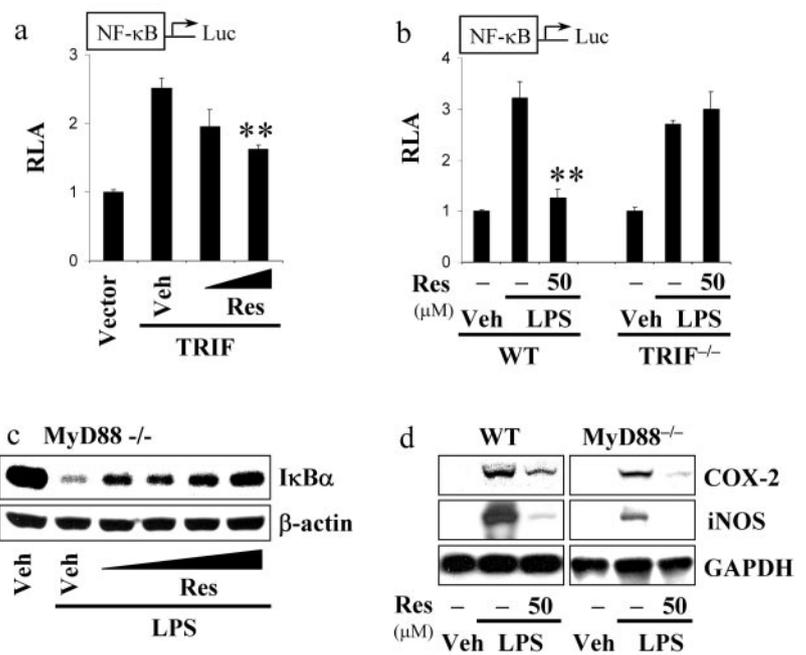
*Resveratrol suppresses TRIF-dependent, but not MyD88-dependent signaling pathways of TLR3 and TLR4*

We further investigated the differential regulation of MyD88- or TRIF-dependent signaling pathways by resveratrol. Resveratrol did not suppress the agonist-independent activation of NF- $\kappa$ B



**FIGURE 2.** Resveratrol does not suppress the activation of NF- $\kappa$ B mediated through MyD88- $\text{IKK}\beta$  signaling pathway in macrophages. RAW264.7 cells were transfected with NF- $\kappa$ B(2 $\times$ ) binding site luciferase reporter plasmid (*a*) or the p65-GAL4 plasmid (*b*) containing p65 fused with the DNA binding domain of GAL4 transcription factor and the plasmid containing the GAL4 responsive element-luciferase reporter gene. Cells were cotransfected with the expression plasmid of MyD88,  $\text{IKK}\beta$ , or an empty vector. Cells were further treated with resveratrol (30, 50  $\mu$ M) for 18 h. Relative luciferase activity (RLA) was determined as described for Fig. 1. Values are mean  $\pm$  SEM ( $n = 3$ ). *c*, RAW264.7 cells were treated with resveratrol (50  $\mu$ M) for 1 h and then stimulated with LPS (10 ng/ml) for the indicated time periods. Cell lysates were analyzed for  $\text{I}\kappa\text{B}\alpha$  and  $\beta$ -actin immunoblots. Representative data are from more than three independent experiments. Veh, vehicle; Res, resveratrol.

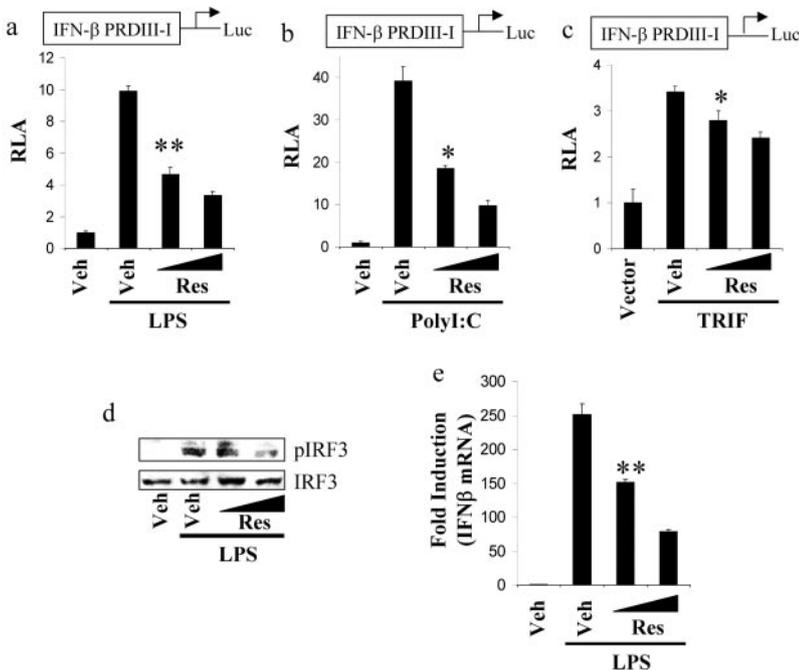
**FIGURE 3.** Resveratrol suppresses the activation of NF- $\kappa$ B mediated through TRIF signaling pathway in macrophages. *a*, RAW264.7 cells were transfected with NF- $\kappa$ B(2 $\times$ ) binding site luciferase reporter plasmid and the expression plasmid of TRIF or an empty vector. Cells were further treated with resveratrol (30, 50  $\mu$ M) for 18 h. *b*, TRIF-deficient (TRIF<sup>-/-</sup>) or wild-type MEFs were transfected with NF- $\kappa$ B(2 $\times$ ) binding site luciferase reporter plasmid. Cells were treated with resveratrol for 1 h and further stimulated with LPS (100 ng/ml) for 18 h. Relative luciferase activity (RLA) was determined as described for Fig. 1. Values are mean  $\pm$  SEM ( $n = 3$ ). Significantly different (\*\*,  $p < 0.01$ ) from TRIF plus vehicle (*a*) or LPS alone (*b*). *c* and *d*, MyD88-deficient (MyD88<sup>-/-</sup>) or wild-type (WT) macrophages were treated with resveratrol (30, 50, 75, 100  $\mu$ M (*c*) and 50  $\mu$ M (*d*)) for 1 h and further stimulated with LPS (50 ng/ml (*c*) and 10 ng/ml (*d*)) for 1 h (*c*) and 18 h (*d*). Cell lysates were analyzed for I $\kappa$ B $\alpha$ ,  $\beta$ -actin, COX-2, iNOS, and GAPDH immunoblots. The film exposure time was longer for the immunoblotting results of MyD88-deficient macrophages than for the results of wild-type macrophages. Representative data are from more than three independent experiments. Veh, vehicle; Res, resveratrol.



induced by MyD88 or IKK $\beta$  in macrophages (RAW264.7 cells) (Fig. 2*a*). In addition, resveratrol did not suppress IKK $\beta$ -induced transactivation of p65 as determined by the p65 transactivation assay that uses the p65/GAL4 plasmid containing p65 fused with the DNA binding domain of GAL4 transcription factor and the plasmid of the GAL4 responsive element-luciferase reporter gene (Fig. 2*b*). Furthermore, resveratrol did not inhibit the degradation of I $\kappa$ B $\alpha$  induced by LPS, which reflects mostly, if not completely, the kinase activity of IKK $\beta$  in RAW264.7 cells (Fig. 2*c*). These results show that resveratrol does not inhibit the canonical pathway for NF- $\kappa$ B activation, which is activated through the MyD88-IKK $\beta$  pathway. This finding is consistent with the results that resveratrol did not

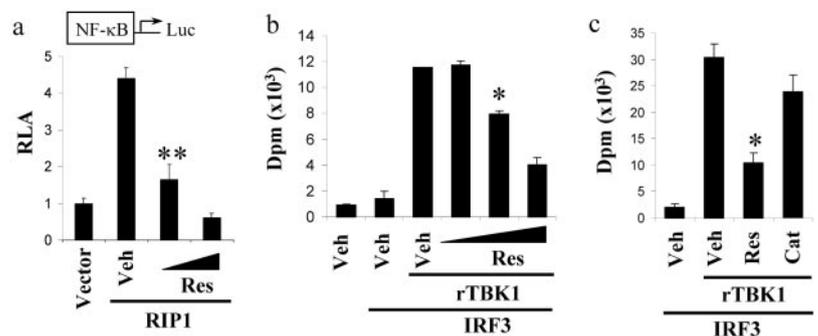
inhibit NF- $\kappa$ B activation induced by TLR2 and TLR9 agonists that activate only MyD88-dependent signaling pathways.

In contrast, resveratrol suppressed the agonist-independent activation of NF- $\kappa$ B induced by TRIF in RAW264.7 cells (Fig. 3*a*). The suppressive effects of resveratrol on LPS-induced NF- $\kappa$ B activation were abolished in TRIF-deficient MEFs, whereas resveratrol inhibited the NF- $\kappa$ B activation in wild-type MEFs (Fig. 3*b*). The degradation of I $\kappa$ B $\alpha$  induced by LPS was suppressed by resveratrol in MyD88-deficient macrophages (Fig. 3*c*) in contrast to the results obtained from RAW264.7 cells (Fig. 2*c*). The suppression of LPS-induced expression of COX-2 and iNOS by resveratrol was still observed in MyD88-deficient macrophages as well as wild-type macrophages (Fig.



**FIGURE 4.** Resveratrol suppresses the activation of IRF3 and the expression of IFN- $\beta$  induced by LPS, poly(I:C), or TRIF in macrophages. *a-c*, RAW264.7 cells were transfected with IRF3 binding site (IFN $\beta$  PRDIII-I) luciferase reporter plasmid. Cells were treated with resveratrol (30, 50  $\mu$ M) for 1 h and further stimulated with LPS (*a*) (3 ng/ml) or poly(I:C) (2  $\mu$ g/ml) (*b*), or cotransfected (*c*) with the expression plasmid of TRIF or an empty vector for 18 h. Relative luciferase activity (RLA) was determined as described for Fig. 1. *d*, RAW264.7 cells were treated with resveratrol (30, 50  $\mu$ M) for 1 h and further stimulated with LPS (5 ng/ml) for 1.5 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 immunoblots. *e*, RAW264.7 cells were treated with resveratrol (30, 50  $\mu$ M) for 1 h and further stimulated with LPS (5 ng/ml) for 18 h. Total RNAs were extracted and the levels of IFN- $\beta$  expression were determined by quantitative real-time RT-PCR analysis. IFN- $\beta$  expression was normalized with  $\beta$ -actin (internal control) expression. The results were presented as fold inductions compared with the vehicle control. Values are mean  $\pm$  SEM ( $n = 3$ ). Significantly different (\*\*,  $p < 0.01$ ) from LPS alone (*a* and *d*). Significantly different (\*,  $p < 0.05$ ) from poly(I:C) alone (*b*) and TRIF plus vehicle (*c*). Veh, vehicle; Res, resveratrol.

**FIGURE 5.** Resveratrol suppresses the functional activity of TBK1 and RIP1. *a*, RAW264.7 cells were transfected with NF- $\kappa$ B(2 $\times$ ) binding site luciferase reporter plasmid and the expression plasmid of RIP1, or a corresponding empty vector. Cells were further treated with resveratrol (30, 50  $\mu$ M) for 18 h. Relative luciferase activity (RLA) was determined as described for Fig. 1. Values are mean  $\pm$  SEM ( $n = 3$ ). *b* and *c*, In vitro TBK1 kinase assay was performed using recombinant active TBK1 (rTBK1) and IRF3 as a substrate as described in *Materials and Methods*. TBK1 kinase activity was determined in the presence of resveratrol (20, 50, 100  $\mu$ M (*b*); 100  $\mu$ M (*c*)) or catechin (100  $\mu$ M). Values are mean  $\pm$  SE ( $n = 2$ ). Significantly different (\*,  $p < 0.05$ ) from vehicle plus rTBK1 plus IRF3 (*b* and *c*). Significantly different (\*\*,  $p < 0.01$ ) from RIP1 plus vehicle (*a*). Representative data are from more than three independent experiments. Veh, vehicle; Res, resveratrol; Cat, catechin.



3*d*). Together, these results convincingly demonstrate that resveratrol suppresses TRIF-dependent signaling pathways, but not MyD88-dependent pathways.

Although NF- $\kappa$ B activation is common to both MyD88 and TRIF signaling pathways, the activation of IRF3 and the expression of target genes, including type I IFNs (IFN- $\alpha$ , IFN- $\beta$ ), is dependent on TRIF, but not MyD88, in TLR3 and TLR4 signaling (23–25). Therefore, the activation of IRF3 and the expression of IFN- $\beta$  were used as readouts for the activation of TRIF-dependent signaling pathways. Resveratrol suppressed the activation of IRF3 induced by LPS, poly(I:C), or by overexpression of TRIF as determined by the luciferase reporter gene assay with IFN- $\beta$  promoter that contains binding site for IRF3 but not for NF- $\kappa$ B (IFN $\beta$  PRDIII-I-luc) (Fig. 4, *a–c*). In addition, the phosphorylation of IRF3 induced by LPS was also inhibited by resveratrol (Fig. 4*d*). Furthermore, resveratrol inhibited LPS-induced expression of endogenous IFN- $\beta$  as determined by the quantitative RT-PCR assay for IFN- $\beta$  mRNA (Fig. 4*e*). These results demonstrated that resveratrol suppresses TRIF-specific signaling pathways and target gene expression derived from TLR3 and TLR4 activation.

#### Resveratrol inhibits the functional activity of TBK1 and RIP1

The next question is which signaling molecules in TRIF pathways are the targets of resveratrol. To identify the target molecules, we determined whether the downstream signaling components of TRIF pathway are inhibited by resveratrol. TBK1 and RIP1 are known to interact with TRIF and mediate downstream signaling pathways to IRF3 and NF- $\kappa$ B, respectively. TBK1 directly phosphorylates and activates IRF3. RIP1 is important for NF- $\kappa$ B activation. It is still unclear how RIP1 leads to the activation of NF- $\kappa$ B (25, 43, 48, 49). We therefore sought to determine whether resveratrol affected either TBK1 or RIP1 function. Resveratrol sup-

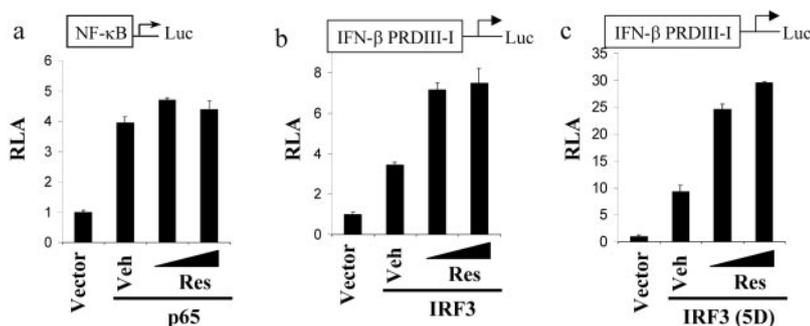
pressed RIP1-induced NF- $\kappa$ B activation as determined by the reporter gene assay (Fig. 5*a*). We next investigated whether resveratrol affected TBK1 kinase activity by monitoring the phosphorylation of IRF3. The results from in vitro kinase assay showed that resveratrol suppressed the kinase activity of TBK1 in a dose-dependent manner (Fig. 5*b*). In contrast, catechin, a flavonoid, did not affect TBK1 kinase activity (Fig. 5*c*). This was consistent with the result that catechin did not inhibit LPS-induced IRF3 activation as determined by the reporter gene assay (data not shown).

Finally, it was determined whether resveratrol directly inhibits transcriptional activity of NF- $\kappa$ B and IRF3. Resveratrol did not inhibit the expression of luciferase gene containing NF- $\kappa$ B or IRF3 binding site induced by the transfection of an expression plasmid of p65 (a subunit of NF- $\kappa$ B) or IRF3, respectively (Fig. 6). These results suggest that the transcription factors, p65 and IRF3, are not the direct target of resveratrol.

A structural analog of resveratrol, stilbene, suppressed the activation of IRF3 induced by LPS (TLR4 agonist) and poly(I:C) (TLR3 agonist) and the expression of IFN- $\beta$  expression induced by LPS (Fig. 7). These results show that stilbene, similar to resveratrol, has suppressive effects on TRIF-dependent signaling pathways and target gene expression of TLR3 and TLR4.

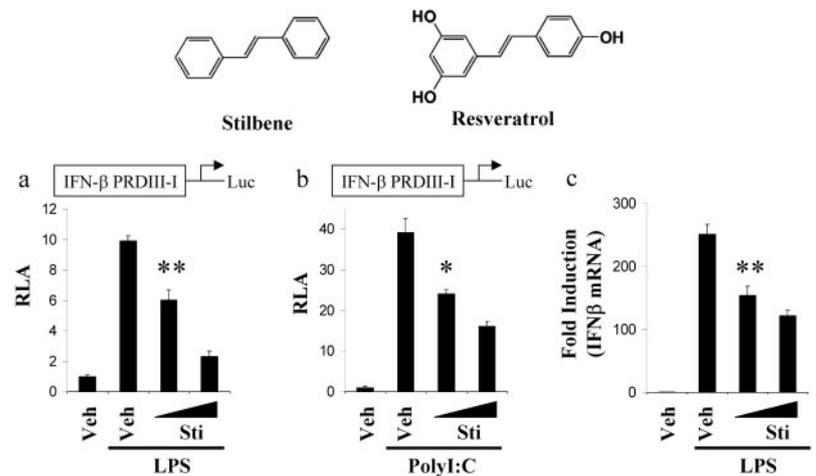
## Discussion

Our study demonstrates that resveratrol suppresses MyD88-independent, but not MyD88-dependent signaling pathways of TLR3 and TLR4. TBK1, but not IRF3 was the target of inhibitory effect of resveratrol. In addition to TBK1, RIP1-induced NF- $\kappa$ B activation was also inhibited by resveratrol. Because RIP1 and TBK1 are associated with TRIF, resveratrol may inhibit the function of the TRIF-TBK1-RIP1 signaling complex. The suppression of TRIF-dependent pathways of TLRs by resveratrol was accompanied by

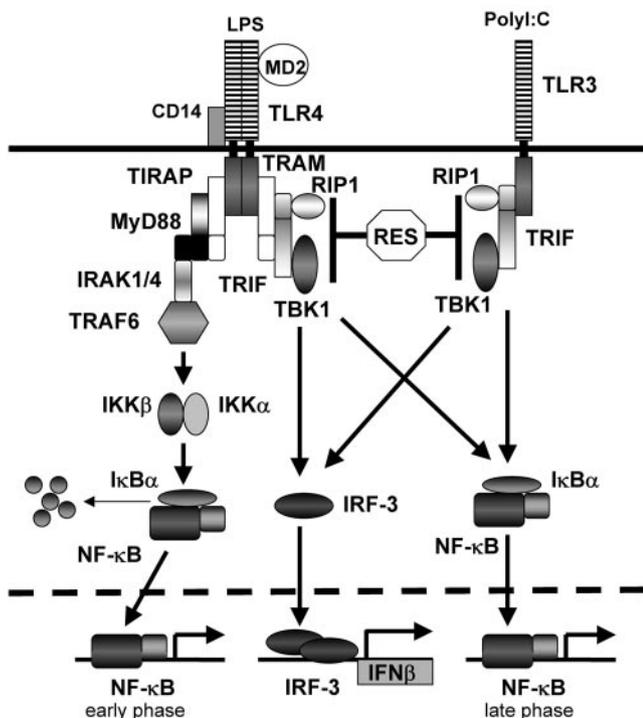


**FIGURE 6.** Resveratrol does not inhibit the transcriptional activity of p65 and IRF3. *a*, RAW264.7 cells were transfected NF- $\kappa$ B(2 $\times$ ) binding site luciferase reporter plasmid and the expression plasmid of p65. *b* and *c*, RAW264.7 cells were transfected with IRF3 binding domain (IFN $\beta$  PRDIII-I) luciferase reporter plasmid and the expression plasmid of wild-type or constitutively active IRF3 (IRF3 (5D)). Cells were further treated with resveratrol (30, 50  $\mu$ M) for 18 h. Relative luciferase activity (RLA) was determined as described for Fig. 1. Values are mean  $\pm$  SEM ( $n = 3$ ). Veh, vehicle; Res, resveratrol.

**FIGURE 7.** Stilbene suppresses the activation of IRF3 and the expression of IFN- $\beta$  induced by LPS and poly(I:C) in macrophages. *a* and *b*, RAW264.7 cells were transfected with IRF3 binding site (IFN $\beta$  PRDIII-I) luciferase reporter plasmid. Cells were treated with stilbene (30, 50  $\mu$ M) for 1 h and further stimulated with LPS (3 ng/ml) or poly(I:C) (2  $\mu$ g/ml) for 18 h. Relative luciferase activity (RLA) was determined as described for Fig. 1. *c*, RAW264.7 cells were treated with stilbene (30, 50  $\mu$ M) for 1 h and further stimulated with LPS (5 ng/ml) for 18 h. Total RNAs were extracted, and the levels of IFN- $\beta$  expression were determined by the quantitative RT-PCR analysis as described for Fig. 4. IFN- $\beta$  expression was normalized with  $\beta$ -actin (internal control) expression. The results were presented as fold induction compared with the vehicle control. Values are mean  $\pm$  SEM ( $n = 3$ ). Significantly different (\*\*,  $p < 0.01$ ) from LPS alone (*a* and *c*). Significantly different (\*,  $p < 0.05$ ) from poly(I:C) alone (*b*). Veh, vehicle; Sti, stilbene.



the down-regulation of the activation of NF- $\kappa$ B and IRF3 and of the expression of their target genes including COX-2, iNOS, and IFN- $\beta$ . The schematic representation of the inhibitory effect of resveratrol on TLR3 and TLR4 signaling pathways is depicted in Fig. 8. It is also known that  $>70\%$  of LPS-induced genes are derived from TRIF pathway (50). These facts suggest that the inhibition of TRIF/TBK1 signaling complex and the consequent down-regulation of IRF3 activity by resveratrol can significantly suppress the target gene expression of TLR3 and TLR4. IFN- $\beta$  production is another important mediator for endotoxic shock induced by LPS exposure, as IFN- $\beta$ -deficient mice are highly resistant to endotoxic shock induced by LPS challenge (51). Our results identify a new target of resveratrol in inhibition of TLR activation and provide new insight to understand the mode of action of resveratrol for its anti-inflammatory effects.



**FIGURE 8.** Schematic illustration of the inhibitory effect of resveratrol on TLR3 and TLR4 signaling pathways.

It has been controversial whether resveratrol inhibits IKK $\beta$  activity. It was reported that resveratrol inhibited IKK activity resulting in the decreased phosphorylation and degradation of I $\kappa$ B $\alpha$  induced by TNF in human monocytic cells (THP-1) (37). Tsai et al. (52) also reported that resveratrol inhibited TNF-induced IKK activity in human endothelial cells (ECV304) as determined by immune-complex kinase assay. In contrast, resveratrol did not inhibit TNF-induced phosphorylation and degradation of I $\kappa$ B $\alpha$ , which reflects IKK $\beta$  kinase activity, in human lymphoma cells (U937) (38). Murakami et al. (53) also showed that I $\kappa$ B $\alpha$  degradation induced by LPS plus IFN- $\gamma$  was not inhibited by resveratrol in macrophages (RAW264.7 cells). These results suggest that the suppression of NF- $\kappa$ B activation by resveratrol is not mediated through the inhibition of IKK $\beta$  activity. This discrepancy may be in part due to the cell type-specific variation in downstream signaling machinery for the same receptor. Therefore, it remains to be further clarified whether the inhibitory effect of resveratrol on NF- $\kappa$ B activation is mediated through the suppression of IKK $\beta$  in different cell types. Our results suggest that resveratrol does not inhibit IKK $\beta$  in RAW264.7 cells with several reasons. First, resveratrol did not inhibit the LPS-induced degradation of I $\kappa$ B $\alpha$ , which is mostly dependent on IKK $\beta$  kinase activity, in macrophages (RAW264.7 cells). Secondly, NF- $\kappa$ B activation as well as transactivation of p65, a subunit of NF- $\kappa$ B, induced by transfection of IKK $\beta$  expression plasmid was not inhibited by resveratrol in RAW264.7 cells. Thirdly, IKK $\beta$  is also the downstream kinase of MyD88. If resveratrol inhibits IKK $\beta$ , MyD88-induced and thus TLR2 or TLR9 ligand-induced NF- $\kappa$ B activation should be inhibited by resveratrol. However, our results showed that MyD88-, TLR2 ligand-, or TLR9 ligand-induced NF- $\kappa$ B activation was not suppressed by resveratrol. Therefore, our results collectively suggest that IKK $\beta$  is not the direct target of resveratrol in the inhibition of TLR activation in RAW264.7 cells.

Interestingly, our results show that LPS-induced I $\kappa$ B $\alpha$  degradation was inhibited by resveratrol in the absence of MyD88. It was suggested that I $\kappa$ B $\alpha$  degradation induced by TRIF pathway is mediated through the interaction between TRIF and TRAF6 thereby leading to the activation of IKK $\beta$  because TRAF6 was shown to associate with the N-terminal part of TRIF (54, 55). In addition, poly(I:C)-induced NF- $\kappa$ B activation was completely abolished in TRAF6-deficient MEFs (55). In contrast, Gohda et al. (56) showed that poly(I:C)-induced I $\kappa$ B $\alpha$  degradation and cytokine production was not affected in macrophages derived from TRAF6-deficient

mice. Therefore, it is still unclear whether TRAF6 is involved in NF- $\kappa$ B activation mediated through the TLR3-TRIF pathway. Because RIP1 also associates with TRIF, it is possible that RIP1 is also important for TRIF-induced NF- $\kappa$ B. Nevertheless, our results demonstrate that resveratrol inhibited I $\kappa$ B $\alpha$  degradation mediated through TRIF-dependent signaling pathway.

TBK1 has clearly been shown to function as the IRF3 kinase (25). Our results from in vitro kinase assay demonstrated that TBK1 kinase activity was suppressed by resveratrol. In addition, the results showed that resveratrol suppresses the activation of IRF3 induced by the transfection of TBK1 expression plasmid (data not shown). These results demonstrate that TBK1 is the molecular target of resveratrol in inhibiting TLR3 and TLR4 downstream signaling pathways and target gene expression. TBK1 has also been implicated in the activation of NF- $\kappa$ B, although the target of TBK1 in the NF- $\kappa$ B pathway is still unclear. TBK1 has been implicated in TNF-induced activation of NF- $\kappa$ B independently of I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B DNA binding (57). Indeed, it was reported that resveratrol suppressed TNF-induced NF- $\kappa$ B activation in human lymphoma cells (U937) and human monocytic cells (THP-1) (37, 38). Therefore, these results suggest that TBK1 may be the common target of inhibition by resveratrol at least in TNF, TLR3, and TLR4 signaling pathways, and further suggests that the stimulus or agonist that activates TBK1 can be inhibited by resveratrol.

Our results demonstrate that TLR2 or TLR9 ligand-induced transcriptional activity of the NF- $\kappa$ B promoter reporter gene was not inhibited by resveratrol. These results suggest that resveratrol does not inhibit the DNA binding of NF- $\kappa$ B in RAW264.7 cells. If it does, NF- $\kappa$ B reporter gene activity induced by the activation of any upstream signaling components (e.g., IKK $\beta$ , MyD88, TLR2, TLR9) should be inhibited by resveratrol. These are consistent with the results that resveratrol did not inhibit the DNA binding ability of NF- $\kappa$ B, whereas it suppressed TNF-induced phosphorylation and nuclear translocation of p65 (a subunit of NF- $\kappa$ B) in U937 cells (38).

Resveratrol was also shown to stimulate Sirt1 histone deacetylase activity (58). It was shown that Sirt1 physically interacts with RelA/p65 subunit of NF- $\kappa$ B and inhibits the transcription by deacetylating RelA/p65, and that resveratrol, an agonist of Sirt1, inhibits NF- $\kappa$ B-regulated gene expression in lung cancer cell lines (59). If such inhibition of NF- $\kappa$ B mediated by resveratrol-induced Sirt1 activation occurs in RAW264.7 cells, resveratrol should inhibit the activation of NF- $\kappa$ B and target gene expression induced by all upstream signaling components of NF- $\kappa$ B (e.g., IKK $\beta$ , MyD88, TLR2, TLR9). Again our results that TLR2 or TLR9 agonist-induced NF- $\kappa$ B activation and COX-2 expression were not inhibited by resveratrol suggest that either resveratrol does not activate Sirt1 or that activated Sirt1 somehow does not lead to the inhibition of NF- $\kappa$ B in RAW264.7 cells.

The structural analogue, stilbene showed similar inhibitory effects on TLR3 and TLR4 activation. It was reported that the hydroxyl groups on stilbene backbone are critical for the inhibitory effect on NF- $\kappa$ B activation induced by TNF in leukemic cell line (KBM-5) (60). Another report, which demonstrated that resveratrol suppressed TGF- $\beta$ -induced COX-2 promoter-dependent transcriptional activity in colon cancer cells, suggested that the resorcin moiety is important for the inhibitory activity (61). However, our results demonstrate that the main backbone structure of resveratrol is effective to suppress the activation of TRIF pathways of TLRs in macrophages.

In summary, our results demonstrate specific inhibition of MyD88-independent signaling pathways and target gene expression by resveratrol. The molecular targets of the inhibition by res-

veratrol are TBK1 and RIP1 in TRIF complex. These results suggest that certain plant polyphenols can modulate TLR-mediated inflammatory responses and the risk of chronic diseases associated with exaggerated TLR activation.

## Disclosures

The authors have no financial conflict of interest.

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