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Suppression of the TRIF-dependent signaling pathway of Toll-like receptors by luteolin

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ABSTRACT

Toll-like receptors (TLRs) play important roles in induction of innate immune responses for both host defense against invading pathogens and wound healing after tissue injury. Since dysregulation of TLR-mediated immune responses is closely linked to many chronic diseases, modulation of TLR activation by small molecules may have therapeutic potential against such diseases. Expression of the majority of lipopolysaccharide-induced TLR4 target genes is mediated through a MyD88-independent (TRIF-dependent) signaling pathway. In order to evaluate the therapeutic potential of the flavonoid luteolin we examined its effect on TLR-stimulated signal transduction via the TRIF-dependent pathway. Luteolin suppressed activation of Interferon regulatory factor 3 (IRF3) and NFκB induced by TLR3 and TLR4 agonists resulting in the decreased expression of target genes such as TNF-α, IL-6, IL-12, IP-10, IFNβ, CXCL9, and IL-27 in macrophages. Luteolin attenuated ligand-independent activation of IRF3 or NFκB induced by TLR4, TRIF, or TBK1, while it did not inhibit TLR oligomerization. Luteolin inhibited TBK1-kinase activity and IRF3 dimerization and phosphorylation, leading to the reduction of TBK1-dependent gene expression. Structural analogs of luteolin such as quercetin, chrysin, and eriodictyol also inhibited TBK1-kinase activity and TBK1-target gene expression. These results demonstrate that TBK1 is a novel target of anti-inflammatory flavonoids resulting in the down-regulation of the TRIF-dependent signaling pathway. These results suggest that the beneficial activities of these flavonoids against inflammatory diseases may be attributed to the modulation of TLR-mediated inflammatory responses.

1. Introduction

Toll-like receptors (TLRs) play a critical role in host defense by sensing invading microbial pathogens and initiating innate and adaptive immune responses. Activation of the immune system by bacterial or viral infection stimulates inflammatory responses that are necessary for host defense against invading pathogens. However, dysregulation of TLR-mediated cellular responses can lead to chronic inflammation, which in turn contributes to the development and progress of many inflammatory diseases. Accumulating evidence shows the close relationship between TLRs and various inflammatory diseases including septic shock, atherosclerosis, rheumatoid arthritis, diabetes and cancer [1,2]. Understanding how TLR activation can be modulated may thus provide new opportunities to develop effective therapeutics for chronic inflammatory diseases [3].

Broadly, TLRs can activate two branches of downstream signaling pathways, MyD88- and TRIF-dependent pathways culminating in the expression of inflammatory gene products including cytokines and chemokines. MyD88 is a common downstream adaptor leading to the activation of the IKKβ complex and the NFκB transcription factor [4]. TRIF, another adaptor molecule of TLRs, is mainly responsible for regulating MyD88-independent pathways [5]. TRIF activates the downstream kinases,

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Abbreviations: IFNβ, Interferon beta; IKK, IkappaB kinase; IRF3, Interferon regulatory factor 3; MyD88, myeloid differentiation primary response gene 88; TIR, Toll/interleukin-1 receptor; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TRIF, TIR-domain-containing adaptor inducing interferon-beta.

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TBK1 and IKKε, leading to the phosphorylation and activation of IRF3 and the consequent expression of type I IFNs and IFN-inducible genes [6]. IFNβ and IFN-inducible genes such as IP-10 and iNOS are critical inducers of endotoxic shock following LPS exposure. The survival rate of IFNβ- and iNOS-deficient mice was significantly greater than that of controls following induction of experimental sepsis [7–9]. Mice deficient in type I IFN receptors were also highly resistant to infection by the gram-positive bacterium, *Listeria monocytogenes* [10]. Since the expression of IFNβ is mainly dependent on activation of the TRIF-signaling pathway, these data suggest an important role for the TRIF-dependent signaling pathway in mediating inflammatory responses.

Flavonoids abundant in plants are widely known to exert various biological activities including anti-inflammatory and anti-cancer effects [11]. Luteolin, a flavonoid compound found in many herbal extracts including celery, green pepper, and chamomile, is known to have anti-inflammatory activity. Oral administration of luteolin to mice suppressed the inflammatory responses in acute and chronic inflammation animal models such as carrageenan-induced paw edema, air pouch models and the cotton pellet granuloma test [12]. Treatment of mice with luteolin alleviated inflammatory responses and decreased bacterial load in pulmonary infection with *Chlamydia pneumoniae* [13]. Intraperitoneal injection of luteolin greatly increased the survival rate of mice in a sepsis model induced by LPS challenge, and also reduced the level of inflammatory markers such as serum levels of TNF-α and IL-6, ICAM-1 expression in liver, and leukocyte infiltration in the lung and liver [14]. However, the molecular target of luteolin in TLR signaling pathways has not been fully identified.

Luteolin inhibits the activation of IKKβ [15]. This finding suggests a role for luteolin in MyD88-dependent signaling pathway since IKKβ is one of the major kinases downstreams of MyD88. However, it is not known if luteolin can regulate TRIF-dependent (MyD88-independent) signaling following TLR stimulation. Since the TRIF-dependent pathway is responsible for the expression of more than 70% of LPS-induced genes [16] and TRIF-dependent genes significantly contribute to endotoxin lethality [7], the modulation of the TRIF-dependent pathway of TLRs might be a useful and novel anti-inflammatory strategy. Therefore, we investigated whether flavonoids such as luteolin can modulate the TRIF-dependent signaling pathway of TLRs, and we identified their anti-inflammatory targets in this pathway. Our study will provide insight to understand how the dietary modulation of TLR activation can be an attractive strategy for reducing the risk of the inflammatory diseases.

2. Materials and methods

2.1. Cell culture

RAW264.7 cells and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml Penicillin, and 100 μg/ml Streptomycin (Invitrogen). Ba/F3 cells, a murine pro-B cell line, expressing TLR4 (Flag- or GFP-tagged), CD14, and MD2 were cultured in RPMI1640 medium as described previously [17]. Bone marrow cells isolated from wild type (C57BL/6) mice (Japan SLC, Inc., Japan) were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer, and 20% L929 cell conditioned medium for 6 days and adherent cells were used as macrophages [18]. Cells were maintained at 37 °C in a 5% CO_2/air environment.

2.2. Reagents and plasmids

Luteolin, chrysin, quercetin, eriodictyol, hesperetin, and naringenin were purchased from Sigma (St. Louis, MO). Purified LPS was obtained from List Biological Lab. Inc. (San Jose, CA) and dissolved in endotoxin-free water. Poly(I:C) was purchased from Amersham Biosciences (Piscataway, NJ). Anti-Flag antibody and anti-actin antibody were purchased from Sigma. Anti-GFP antibody was from Molecular Probes Inc. (Eugene, OR). Anti-phospho-IRF3 antibody was obtained from Cell Signaling (Boston, MA). Anti-IRF3 antibody was obtained from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma unless otherwise described. IFNβ PRDIII-I-luciferase plasmid, IP-10-promoter luciferase plasmid, IFNβ-promoter luciferase plasmid, and the expression plasmid of human TRIF or TBK1 were kind gifts from Katherine A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). Inducible nitric oxide synthase-promoter luciferase plasmid was from Christopher Glass (University of California, San Diego, CA). A NFκB(2x)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). Heat shock protein 70 (HSP70)-β-galactosidase plasmid was obtained from Robert Modlin (University of California, Los Angeles, CA). CD4-TLR4 was obtained from C.A. Janeway, Jr. (Yale University, New Haven, CT). MyD88(ΔToll) was provided by Jurg Tschopp (University of Lausanne, Switzerland). All DNA constructs were prepared in large scale using Endofree Plasmid Maxi kit (Qiagen, Valencia, CA) for transfection.

2.3. Transfection and luciferase assay

RAW264.7 cells and 293T cells were co-transfected with various plasmids and HSP70-β-galactosidase plasmid using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Luciferase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity.

2.4. Co-immunoprecipitation and immunoblot analysis

Supernatants from cell extracts were incubated with monoclonal anti-Flag antibody and 70 μl of 50% (v/v) protein A-agarose (Amersham Biosciences, Piscataway, NJ). Immune complexes were solubilized with Laemmli buffer after washing five times with lysis buffer. The solubilized immune complex was resolved in SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membranes were immunoblotted with anti-Flag or anti-GFP antibody, followed by secondary antibody coupled to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). The reactive bands were visualized with the ECL system (Amersham Biosciences).

2.5. Detection of IRF3 dimers

IRF3 dimerization was analyzed by native PAGE and immunoblotting with IRF3 antibody. Cell lysates (20 μg protein) from bone marrow-derived macrophages were applied to Tris-glycine gel in the absence of SDS and the proteins were transferred to polyvinylidene difluoride membrane. Membranes were probed with anti-mouse IRF3 and the secondary antibody.

2.6. RT-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and was reverse-transcribed with the UmProm-II^{TM} Reverse

Transcriptase (Promega, Madison, WI) according to the manufacturer's instruction. PCR was done with following primers: Ifnb, 5′-TCCAAGAAAGGACGAACATTCG-3′ and 5′-TGCGGACATCTCCCACGTCAA-3′; Actin, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and 5′-TTGCGGTGCACGATGGAGGGGCCGGA-3′; Tnfa, 5′-AAAATTCGAGTGACAAGCCTGTAG-3′ and 5′-CCCTTGAAGAGAACCTGGGAGTAG-3′; Il6, 5′-TTCCTCTCTGCAAGAGACT-3′ and 5′-TGTATCTCTCTGAAGGACT-3′; Cxcl9, 5′-TCCTTTTGGGCATCATCTTC-3′ and 5′-TGAACGACGACGACTTTGG-3′; Il12p40, 5′-GAAGTTCAACATCAAGAGCAGTAG-3′ and 5′-AGGGAGAAGT AGGAATGGGG-3′; Il27, 5′-CTCTGCTTCCTCGCTACCAC-3′ and 5′-GGGGCAGCTTCTTTTCTTCT-3′. For real-time quantitative PCR analysis, one microgram of total RNA was reverse-transcribed and amplified with a Chromo4 (MJ research) using the SYBR Premix Ex Taq™. The specificity of the amplified PCR products was assessed by a melting curve analysis. The fold induction of gene expression was calculated as previously described [18].

2.7. In vitro TBK1 kinase assay

TBK1 kinase activity was analyzed with Chemiluminescent MBP Assay kit according to the manufacturer's instructions (Upstate Biotechnology, Billerica, MA). Test compounds and 50 ng of recombinant active TBK1 (Upstate Biotechnology) were added to MBP-coated wells containing Magnesium/ATP buffer. After incubation at 30 °C for 40 min, the wells were washed with PBS and blocked with blocking buffer. HRP-conjugated anti-phospho-MBP in blocking buffer was added to the wells. After washing with PBS and washing buffer, LumiGLO Chemiluminescent substrate was added. Luminescent intensity was determined with luminometer.

2.8. ELISA

For IP-10 measurement, cells were treated with LPS or poly(I:C) for 24 h. The levels of IP-10 were determined from culture medium using IP-10 ELISA kit according to the manufacturer's instruction (R&D Systems, Minneapolis, MN). The concentration range for standard curve is from 31.2 to 2000 pg/ml and the minimum detectable dose (MDD) ranged from 1.2 to 4.2 pg/ml.

2.9. Nitrite production

Cells were pre-treated with luteolin for 1 h, and then stimulated with LPS (100 ng/ml) for additional 24 h. To measure the concentration of nitrite, 50 μl supernatant was collected and mixed with 50 μl Griess reagent (Sigma) in the 96-well plates. After incubation for 10 min at room temperature, optical density was measured with a microplate reader at 540 nm. The concentration of nitrite was determined by a standard curve with sodium nitrite.

2.10. Statistical analysis

Data were presented as the means and standard errors of means for all treatment groups. Difference between each group was examined using Student's *t*-test (significant when $p < 0.05$).

3. Results

3.1. Luteolin suppresses the activation of TLR3 and TLR4

To investigate if luteolin modulates TRIF-dependent signaling pathway of TLRs, we first determined whether luteolin affects the activation of TLR3 and TLR4 which have TRIF as an adaptor molecule. Luteolin inhibited IRF3-reporter gene expression induced by LPS (TLR4 agonist) or poly(I:C) (TLR3 agonist) in mouse macrophage cell line, RAW264.7 (Fig. 1A and B). In addition, NFκB-reporter gene expression induced by LPS or poly(I:C) was prevented by luteolin (Fig. 1C and D). Furthermore, luteolin blocked the expression of TLR3 or TLR4-target genes including TNFα, IL-6, IL-12, IP-10, IFNβ, CXCL9, and IL-27 in bone marrow-derived macrophages (Fig. 2). These results showed that luteolin suppressed activation of transcription factors and expression of target genes induced by TLR3 or TLR4 agonist.

Receptor oligomerization is one of the initial steps during TLR activation following ligand stimulation [19]. Previous studies reported that certain phytochemicals inhibited oligomerization of TLR4 resulting in the decrease of TLR signaling [17,20]. However, luteolin did not affect LPS-induced oligomerization as determined by co-immunoprecipitation study using Ba/F3 cells expressing GFP-TLR4 and Flag-TLR4 (Fig. 3A). These results indicate that luteolin does not block interaction of LPS with TLR4 or the resulting aggregation of the TLR4 monomers to form the oligomeric receptor complex.

Luteolin was able to suppress ligand-independent activation of IRF3 and NFκB induced by constitutively active TLR4 or TRIF (Fig. 3B and C). The results further support the suppressive effects of luteolin on TRIF-signaling pathway and suggest that the target of luteolin lies downstream of the receptor and TRIF.

3.2. Luteolin inhibits the kinase activity of TBK1 and the expression of TBK1-dependent target genes

TBK1 is a major downstream kinase which phosphorylates and activates IRF3 in TRIF-signaling pathways. Therefore, we investigated whether the inhibitory effect of luteolin on IRF3 activation is mediated through the inhibition of TBK1. Luteolin reduced IRF3 activation induced by TBK1 overexpression (Fig. 4A). An in vitro kinase assay using recombinant, active TBK1 showed that luteolin suppressed the kinase activity of TBK1 in a dose-dependent manner (Fig. 4B). To further confirm the inhibition of TBK1 kinase activity by luteolin, we assessed phosphorylation and dimerization of IRF3. Luteolin inhibited LPS-induced phosphorylation and dimerization of IRF3 in bone marrow-derived macrophages (Fig. 4C). These results suggest that TBK1 is a novel target of luteolin, resulting in the reduced phosphorylation of IRF3 that is normally induced by TLR3 and TLR4 stimulation.

The suppression of IRF3-reporter gene expression induced by overexpression of constitutively active IRF3 by luteolin was observed at 50 μM which was relatively higher than the concentration shown in other experiments as effective (Fig. 4D). In most of other experiments, luteolin showed inhibitory effects at lower concentrations, such as 10 or 20 μM. These results indicate that the suppressive effects of luteolin at lower concentrations correlate with the inhibition of TBK1 kinase activity but that the inhibitory activity for IRF3 requires a higher concentration of luteolin.

The activation of IRF3 through TRIF–TBK1 complexes culminates in the expression of IFNβ and IFN-inducible genes such as IP-10 and iNOS. The expression of IFNβ and IP-10 was almost completely abolished in TBK1-deficient cells as compared with wild-type cells when stimulated with LPS or poly(I:C) [21]. Since luteolin suppressed TBK1 kinase activity, we determined if luteolin modulated the expression of IFNβ and IFN-inducible genes. The expression of IFNβ was down-regulated by luteolin treatment as demonstrated by IFNβ-promoter reporter gene assay and quantitative real-time PCR analysis (Fig. 5A and B). The expression of IP-10 induced by LPS or poly(I:C), was also attenuated by luteolin dose-dependently as demonstrated by IP-10 promoter reporter assay and ELISA (Fig. 5C and D). In addition to IP-10, IFNβ induces the expression of iNOS in an autocrine and paracrine manners [22]. LPS-induced iNOS expression and nitric oxide production were

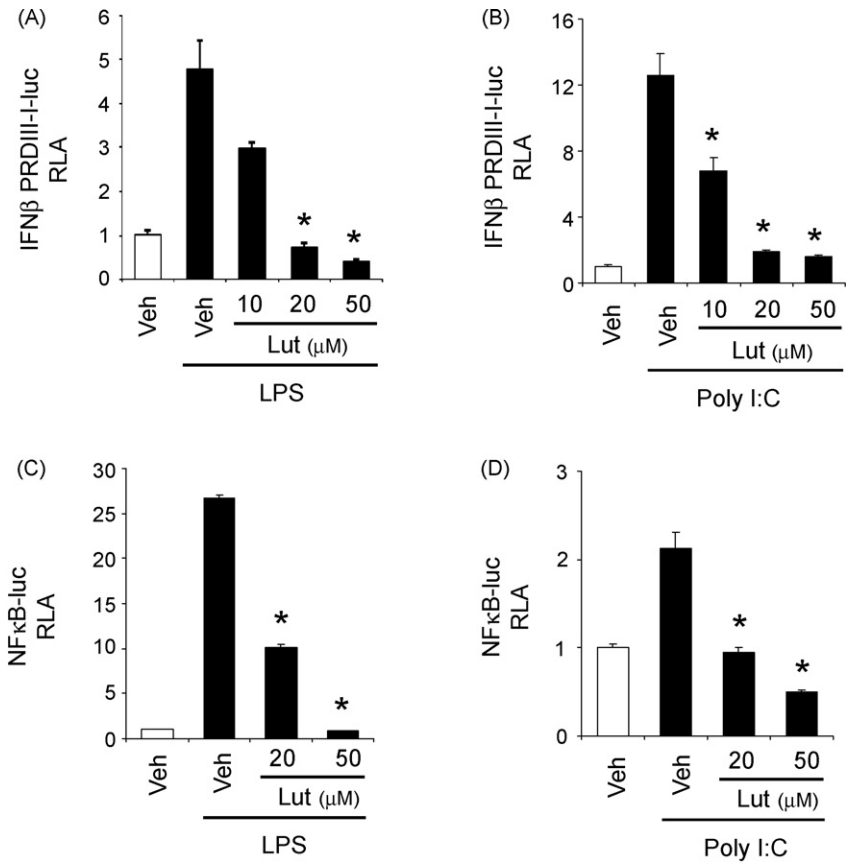

Fig. 1. Luteolin suppresses activation of IRF3 and NFκB induced by TLR3 and TLR4 agonist. (A, B and D) RAW 264.7 cells were transfected with a luciferase reporter plasmid containing (A and B) a partial domain of IFNβ promoter containing IRF3 binding site (IFNβ PRDIII-I) or (D) NFκB binding site (2X) and an expression plasmid of β-galactosidase as an internal control. Cells were pre-treated with luteolin (Lut) for 1 h, and then stimulated with LPS (10 ng/ml) or poly(I:C) (30 μg/ml) for additional 8 h. (C) Ba/F3 cells stably expressing TLR4, CD14, MD2, and a luciferase gene containing NFκB binding site were pre-treated with luteolin for 1 h, and then stimulated with LPS (1 ng/ml) for 8 h. Cell lysates were analyzed for luciferase enzyme activities as described in Section 2. Relative luciferase activity (RLA) was calculated after normalization with β-galactosidase activity (A, B and D) or with protein concentration of cell lysates (C). Values are mean ± SEM ($n = 3$). *, significantly different from LPS or poly(I:C) alone, $p < 0.05$. Representative results from more than three separate experiments are shown. Veh is vehicle.

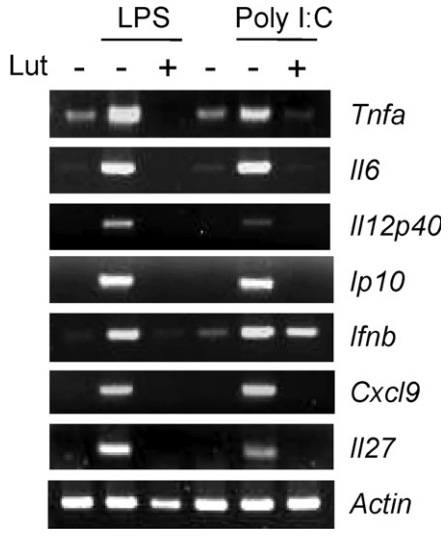

Fig. 2. Luteolin reduces TLR3 or TLR4 agonist-induced target gene expression in bone marrow-derived macrophages. Bone marrow-derived macrophages were pre-treated with luteolin (Lut; 50 μM) for 1 h, and further stimulated with LPS (100 ng/ml) or poly(I:C) (30 μg/ml) for additional 4 h. Total RNAs were extracted and each gene expression was determined by RT-PCR analysis.

also decreased by luteolin in a dose-dependent manner (Fig. 5E and F). The results further suggest that TLR-mediated pro-inflammatory genes of which expression is dependent on TRIF–TBK1 can be down-regulated by luteolin through the suppression of TBK1 activity.

3.3. The effects of structural analogs of luteolin on TRIF pathway of TLRs

To determine the structural motifs in luteolin which confer the inhibitory activity for TBK1 kinase function, we compared the effects of structural analogs of luteolin on TBK1 kinase activity. Flavonoid analogs (quercetin, chrysin, eriodictyol, naringenin, and hesperetin) are structurally similar with luteolin, differing in hydroxylation patterns in B ring and a C_2–C_3 double bond in carbonyl-containing C-ring (structures shown in Fig. 6A). TBK1 kinase activity was suppressed by quercetin, chrysin, and eriodictyol in a dose-dependent manner, whereas naringenin and hesperetin did not affect kinase activity to the same extent as luteolin (Fig. 6B). In addition, quercetin, chrysin, and eriodictyol were also able to attenuate IRF3 activation while naringenin and hesperetin did not show the inhibitory effects (Fig. 6C). Luteolin, quercetin, and chrysin all have a C_2–C_3 double bond in the carbonyl-containing C-ring, suggesting that this structure is important for TBK1 inhibition. Interestingly, among the

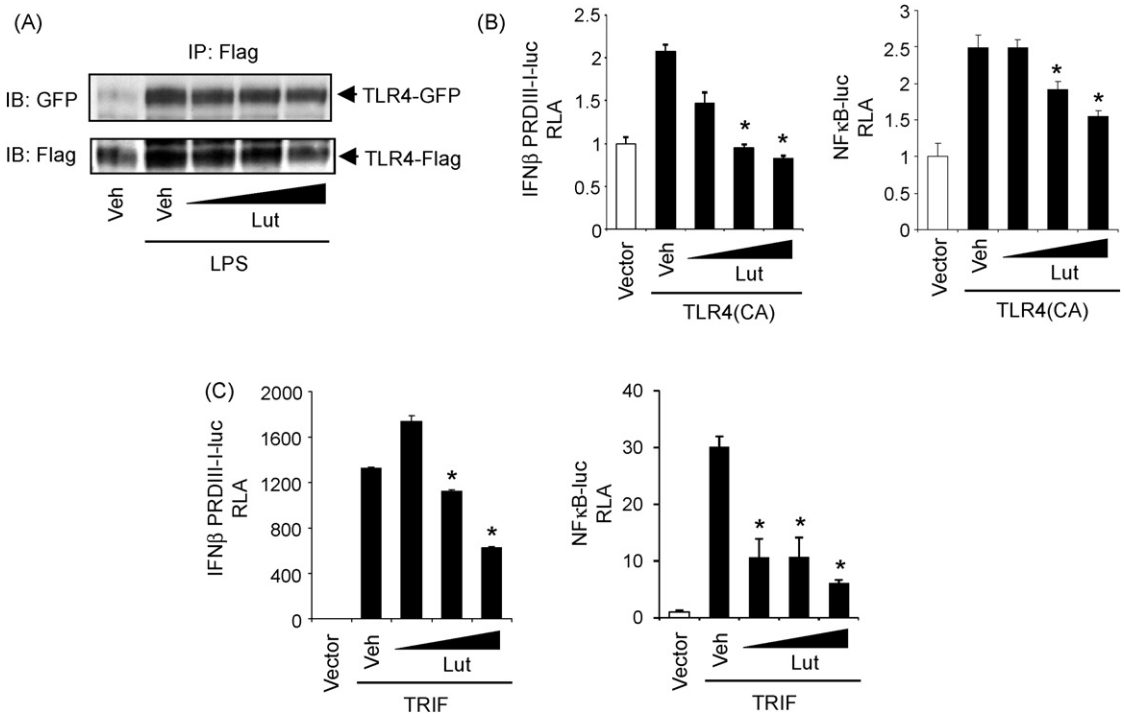

Fig. 3. Luteolin inhibits constitutively active TLR4- or TRIF-induced activation of IRF3 and NFκB, but not TLR4 oligomerization. (A) Ba/F3 cells stably expressing Flag-tagged TLR4, GFP-tagged TLR4, CD14, and MD2 were pre-treated with luteolin (Lut; 10, 20, 50 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 20 min. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody and immunoblotting with anti-GFP (upper) or anti-Flag (lower) antibody (B and C). (B) RAW 264.7 cells or (C) 293T cells were transfected with a luciferase reporter plasmid containing IFNβ PRDIII-I or NFκB binding site and the expression plasmid of constitutively active (CA) TLR4 or TRIF. Cells were treated with luteolin (10, 20, 50 μM) for 8 h. Relative luciferase activity (RLA) was determined. Values are mean ± SEM ($n = 3$). *, significantly different from (B) TLR4(CA) alone or (C) TRIF alone, $p < 0.05$. Veh is vehicle.

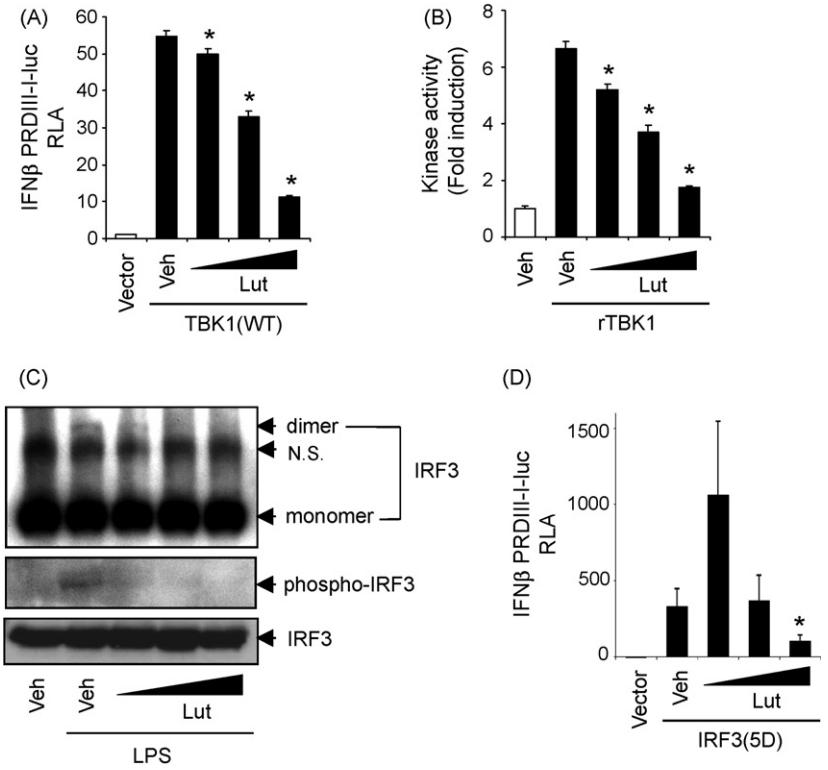

Fig. 4. Luteolin suppresses functional kinase activity of TBK1. (A and D) 293T cells were co-transfected with the expression plasmid of TBK1(WT) or IRF3(5D) and an IFNβ PRDIII-I luciferase reporter plasmid. Cells were further treated with luteolin (Lut; 10, 20, 50 μM) for 8 h. Relative luciferase activity (RLA) was determined. (B) In vitro kinase assay for TBK1 activity using recombinant active TBK1 (rTBK1) was performed in the absence or presence of luteolin (10, 20, 50 μM). Values are mean ± SEM ($n = 3$). *, significantly different from (A) TBK1(WT) alone, (B) rTBK1 alone, or (D) IRF3(5D) alone, $p < 0.05$. (C) Bone marrow-derived macrophages were pre-treated with luteolin (10, 20, 50 μM) for 1 h and then stimulated with LPS (10 ng/ml) for 30 min. IRF3 monomers and dimers were analyzed in native gel with anti-IRF3 antibody. Phosphorylated IRF3 was determined by immunoblotting with anti-phospho-IRF3 antibody. Veh is vehicle; WT is wild-type.

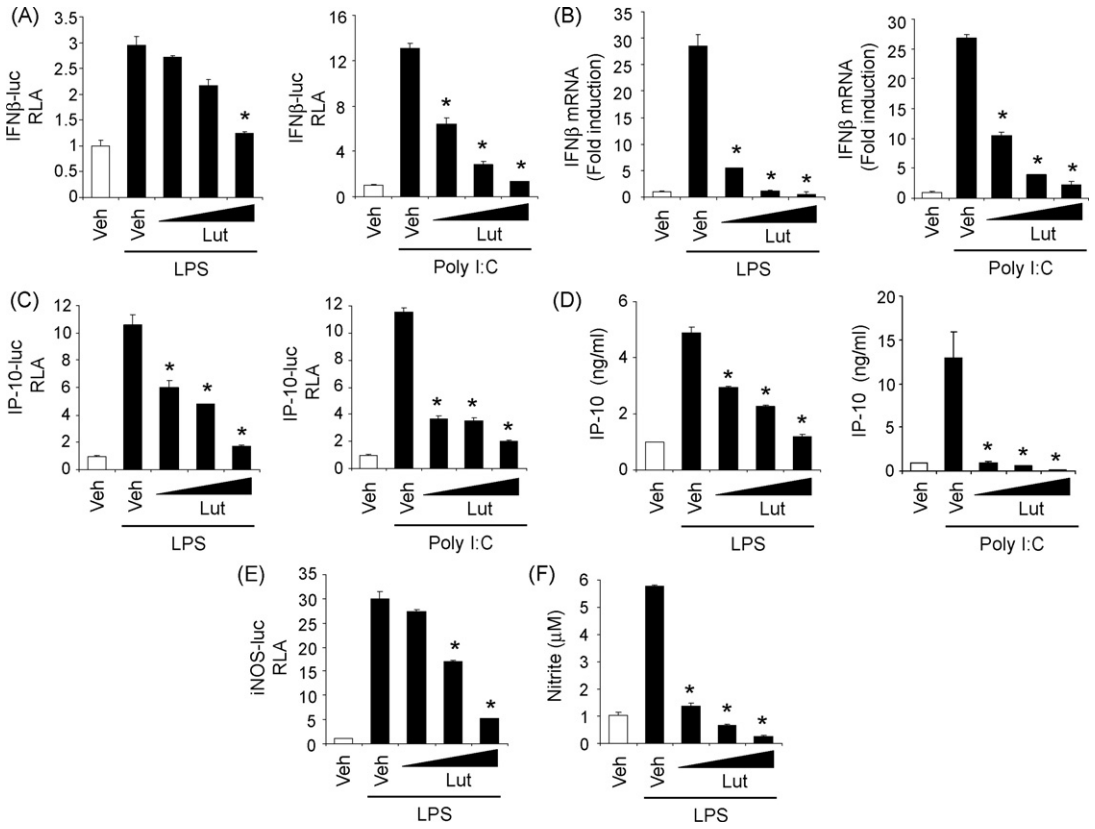

Fig. 5. Luteolin prevents expression of TBK1-dependent genes induced by TLR3 or TLR4 agonist. (A, C and E) RAW 264.7 cells were transfected with a luciferase reporter plasmid containing (A) IFNβ promoter, (C) IP-10 promoter, or (E) iNOS promoter. Cells were pre-treated with luteolin (Lut; 10, 20, 50 μM) for 1 h, and then stimulated with LPS (10 ng/ml) or poly(I:C) (30 μg/ml) for additional 8 h. Relative luciferase activity (RLA) was determined. (B) RAW 264.7 cells were pre-treated with luteolin (10, 20, 50 μM) for 1 h, and further stimulated with LPS (10 ng/ml) or poly(I:C) (30 μg/ml) for additional 4 h. Total RNAs were extracted and the levels of IFNβ expression were determined by quantitative real-time PCR analysis. IFNβ expression was normalized with β-actin (internal control) expression. The results were presented as fold inductions compared with vehicle control. (D) RAW 264.7 cells were pre-treated with luteolin luteolin (10, 20, 50 μM) for 1 h, and then stimulated with LPS (10 ng/ml) or poly(I:C) (30 μg/ml) for additional 24 h. Culture medium was collected and analyzed for IP-10 secretion by ELISA. (F) RAW 264.7 cells were pre-treated with luteolin (10, 20, 50 μM) for 1 h, and then stimulated with LPS (100 ng/ml) for 24 h. Culture medium was collected and analyzed for nitrite level to determine nitric oxide production. Values are mean ± SEM ($n = 3$). *, significantly different from LPS or poly(I:C) alone, $p < 0.05$. Representative results from more than three separate experiments are shown. Veh is vehicle.

compounds which do not have a C_2–C_3 double bond in the C-ring (eriodictyol, naringenin, and hesperetin), eriodictyol showed inhibitory activity whereas naringenin and hesperetin did not. Eriodictyol has two hydroxyl groups at 3′ and 4′ of the B-ring while naringenin and hesperetin have only one hydroxyl groups in the B-ring. Although a more extensive study for structure–activity relationship analysis may be required, these results suggest that a C_2–C_3 double bond in carbonyl-containing C-ring or two hydroxyl groups in the B-ring are important to confer the inhibitory effects of flavonoids on TBK1 activity. The inhibitory potency of different flavonoid analogs for TBK1 kinase activity was well correlated with the inhibitory activities for the expression of TBK1-dependent target genes, IFNβ and IP-10 induced by LPS or poly(I:C) (Fig. 7).

Collectively, these results suggest that anti-inflammatory activity of certain flavonoids is mediated through the suppression of TBK1 activity resulting in the decreased expression of inflammatory genes.

4. Discussion

Accumulating evidence now suggests the importance of TRIF-dependent signaling pathway of TLRs in inflammatory responses and development of certain chronic diseases. It was reported that the expression of more than 70% of LPS-induced genes was modulated in a TRIF-dependent manner suggesting the significant contribution of TRIF-signaling to TLR4-mediated immune responses [16]. Especially, the expression of IFNβ and IFN-inducible genes that are critical mediators of endotoxic shock [7] was dependent on TRIF and TBK1 in mouse embryonic fibroblasts stimulated with poly(I:C) or LPS, or infected with viruses [21,23]. TBK1 is also implicated in angiogenesis contributing to tumor growth [24]. TBK1 expression was elevated in malignant tissues of colon and breast cancers as compared with nonmalignant tissues. Elevated expression of TBK1 correlated with increase of VEGF expression under hypoxic condition and proliferation of human umbilical vein endothelial cells (HUVECs). Human cytomegalovirus increased TBK1 activity resulting in the induction of proatherosclerotic genes in vascular smooth muscle cells suggesting the involvement of TBK1 in inflammatory diseases such as atherosclerosis [25]. Our results demonstrated that certain flavonoids can suppress TBK1 kinase activity resulting in the down-regulation of TRIF-dependent inflammatory gene expression in TLR3- and TLR4-mediated signaling pathways. The results identify a novel target for inhibition of TLR activation and provide new insights into the mechanism underlying the anti-inflammatory activity of flavonoids. The results from our previous studies showed that (−)-epigallocatechin-3-gallate (EGCG) and resveratrol, which are well-known anti-inflammatory agents inhibited IRF3 activation mediated through the suppression of the kinase activity of TBK1 [26,27]. These results suggest that the TRIF and TBK1 pathways can be important molecular targets for control of inflammatory diseases.

It is well known that IFNβ production is the first line of host defense against viral infection. In addition, recent studies have

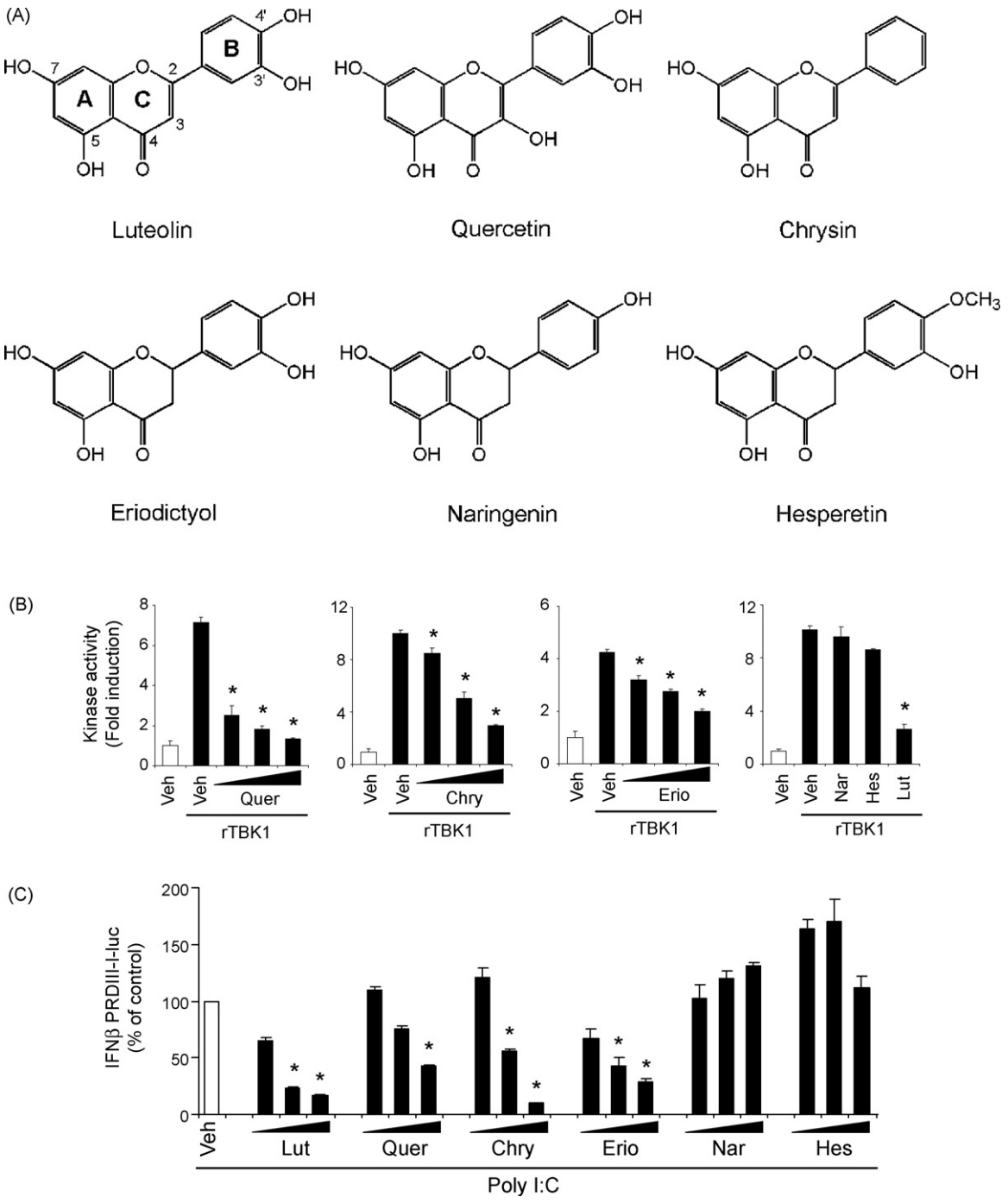

Fig. 6. The effects of flavonoid analogs of luteolin on TBK1 activity and IRF3 activation. (A) The structure of various flavonoids. (B) In vitro kinase assay for TBK1 activity was performed in the absence or presence of flavonoids; quercetin, chrysin, eriodictyol (Quer, Chry, Erio; 10, 20, 50 μM) and naringenin, hesperetin, luteolin (Nar, Hes, Lut; 50 μM). (C) RAW 264.7 cells were transfected with an IFNβ PRDIII-I luciferase reporter plasmid. Cells were pre-treated with Lut, Quer, Chry, Erio, Nar, Hes, (10, 20, 50 μM) for 1 h, and then stimulated with poly(I:C) (30 μg/ml) for additional 8 h. Relative luciferase activity (RLA) was determined. Values are mean ± SEM ($n = 3$). *, significantly different from (B) rTBK1 alone or (C) poly(I:C) alone, $p < 0.05$. Veh is vehicle.

shown that TRIF-dependent IFNβ production in innate immune cells limits the generation of inflammatory Th17 cells through decreased osteopontin-i expression and increased IL-27 expression [28,29]. It has been shown that excessive Th17 cell responses upon infection may result in inflammatory tissue damage and chronic autoimmune disease. Deficiency in TRIF and IFNAR led to the reduction of IL-27 level and the generation of Th17 lineage cells. These suggest that TRIF/IFNβ pathway plays important roles in regulation of development of autoimmune diseases. Therefore, blockade of TRIF pathway and subsequent reduction in IFNβ and IL-27 levels by luteolin may be accompanied by unwanted adverse effects such as increased susceptibility to viral infection and enhanced risk of autoimmune disease. Luteolin also reduced the expression of IL-6 which together with TGFβ induces the differentiation of Th17 cells. Thus, the immune-modulatory pattern by luteolin in physiological condition would be more complex since luteolin suppressed the expression of both IL-27 and IL-6 which have opposing effects on Th17 cell differentiation.

In addition to the TRIF pathway, luteolin modulated the MyD88 pathway as it suppressed NFκB reporter gene expression

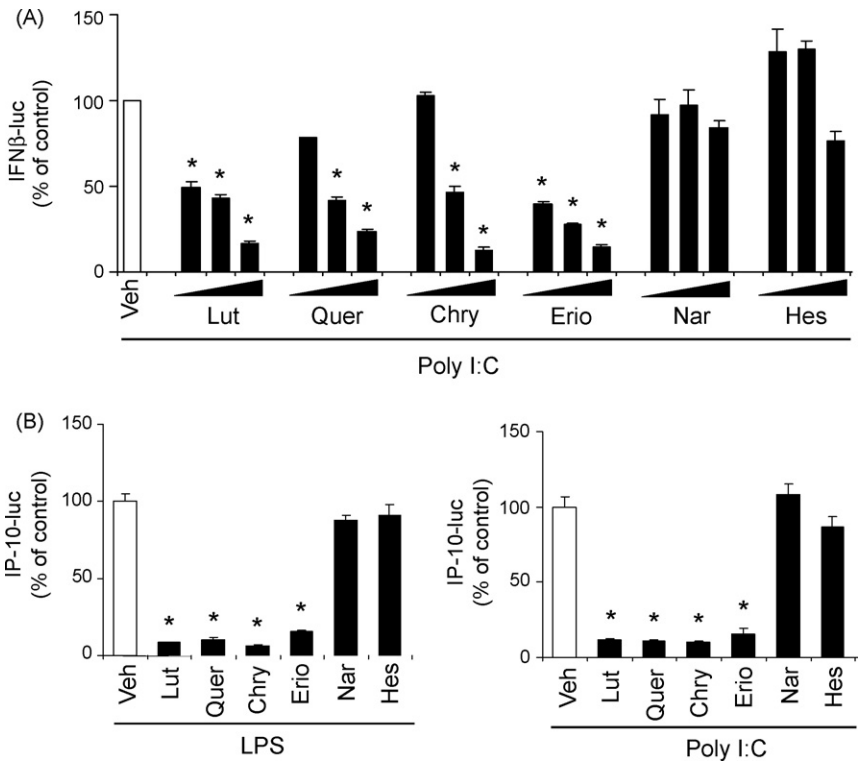

Fig. 7. The effects of flavonoids on TBK1-dependent genes induced by TLR3 or TLR4 agonist. (A) RAW 264.7 cells were transfected with a luciferase reporter plasmid containing IFNβ promoter. Cells were pre-treated with luteolin, quercetin, chrysin, eriodictyol, naringenin, or hesperetin (Lut, Quer, Chry, Erio, Nar, Hes; 10, 20, 50 μM) for 1 h, and then stimulated with poly(I:C) (30 μg/ml) for additional 8 h. (B) RAW 264.7 cells were transfected with a luciferase reporter plasmid containing IP-10 promoter. Cells were pre-treated with Lut, Quer, Chry, Erio, Nar, or Hes (50 μM) for 1 h, and then stimulated with LPS (10 ng/ml) or poly(I:C) (30 μg/ml) for additional 8 h. Relative luciferase activity (RLA) was determined. Values are mean ± SEM ($n = 3$). *, significantly different from LPS or poly(I:C) alone, $p < 0.05$. Veh is vehicle.

induced by MyD88 overexpression in 293T cells (Supplemental Fig. 1A). In addition, the inhibitory effect of luteolin on COX-2 expression was not observed in MyD88-deficient macrophages while LPS-induced COX-2 expression was markedly attenuated by luteolin in wild-type macrophages (Supplemental Fig. 1B). COX-2 expression was rather potentiated by luteolin alone or luteolin with LPS in MyD88-deficient macrophages. These results suggest that luteolin modulates the MyD88-dependent signaling pathway in addition to the TRIF-dependent pathway. Furthermore, luteolin reduced NFκB activation induced by MALP-2 (a TLR2 agonist) and CpG1668 (a TLR9 agonist) (Supplemental Fig. 1C and D). Since TLR2 and TLR9 have MyD88 as a major adaptor molecule, these results suggest that luteolin affects the activation of MyD88-dependent signaling pathway. Activation of the MyD88 pathway is represented by the activation of IKKβ and MAPKs. However, luteolin did not inhibit LPS-induced IκBα degradation suggesting that luteolin did not affect IKKβ activity (Supplemental Fig. 2A). The phosphorylation of JNK and ERK that reflects the activation of MAPKs was not suppressed by luteolin in LPS-treated RAW264.7 cells (Supplemental Fig. 2B). While luteolin did not affect the activation of IKKβ and MAPKs, luteolin decreased phosphorylation of Akt induced by LPS time- and dose-dependently (Supplemental Fig. 2C and D). Akt phosphorylation is dependent on PI3K activity that is known to be associated with MyD88 [30]. Akt is also reported to be linked to NFκB activation through the phosphorylation of p65 to enhance transcriptional activity of NFκB [31,32]. Furthermore, Akt is positively involved in COX-2 expression in TLR4 pathway [33]. Therefore, it is possible that the inhibitory effects of luteolin on MyD88-pathway-dependent activation of NFκB and expression of COX-2 may result from the suppression of PI3K-Akt pathway. Other studies also showed the effect of luteolin on IKKβ, MAPKs, and Akt although the results seemed controversial depending on cell type and stimulus. Luteolin inhibited kinase activities of IKKβ, ERK, p38, and JNK as demonstrated by immune-complex kinase assay using TNFα-stimulated A549 alveolar epithelial carcinoma cells [15]. Luteolin enhanced VEGF-induced phosphorylation of p38 while it did not inhibit VEGF-induced phosphorylation of ERK in HUVECs [34]. Luteolin inhibited VEGF-induced PI3K activity as demonstrated with immune-complex kinase assay and VEGF-induced Akt phosphorylation in HUVECs [34]. However, luteolin did not inhibit JNK phosphorylation in LPS-stimulated macrophages (RAW264.7) [35]. In addition, Xagorari et al. did not observe the correlation between inhibition of Akt and reduction of TNFα release by luteolin in LPS-stimulated macrophages (RAW264.7) [35]. Collectively, these suggest that luteolin may have different kinases as targets in different context to maximize its anti-inflammatory effects. Another flavonoid compound, quercetin, was reported to inhibit the phosphorylation activity of pp60src, a viral gene product, by competing for ATP binding suggesting that luteolin-mediated inhibition of various kinases may be mediated through the competitive inhibition of ATP for kinase activity [36]. However, it remains to be determined whether flavonoids inhibit different kinases by the same or different mechanism, and whether this inhibition is responsible for the anti-inflammatory effects reported for each flavonoid.

In general, a C_2–C_3 double bond in carbonyl-containing C-ring and two hydroxyl groups at positions 3′ and 4′ in B-ring are commonly considered as critical structural motifs for biological activities of flavonoids [15,37–40]. However, different reports have shown slightly different biological activities and potencies of various flavonoids depending on target kinases and cellular

responses evaluated. Although more extensive study for structure–activity relationship analysis may be required, our results using structural derivatives of luteolin suggest that a C_2–C_3 double bond in carbonyl-containing C-ring and two hydroxyl groups at positions 3′ and 4′ in B-ring are important in the inhibitory effects of flavonoids tested in this study on TBK1 activity. Interestingly, the presence of either a C_2–C_3 double bond in carbonyl-containing C-ring or two hydroxyl groups at positions 3′ and 4′ in B-ring is sufficient for the inhibitory activity. The inhibitory pattern of flavonoids tested for TBK1 activity was well correlated with their effects on IRF3 activation and target gene expression.

Several lines of evidence indicate that modulation of the immune response through TLRs can be an effective therapeutic strategy for inflammatory diseases. The results from our study suggest that beneficial effects of dietary phytochemicals on chronic inflammatory diseases are mediated through modulation of TLRs and their downstream signaling components such as TBK1. Furthermore, the study will provide critical information on identifying anti-inflammatory target in TLR signaling and help to understand the mechanism of TLR activation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.01.009.

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