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Suppression of MyD88- and TRIF-dependent signaling pathways of toll-like receptor by (−)-epigallocatechin-3-gallate, a polyphenol component of green tea☆

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TRIF, TIR domain-containing adapter inducing IFN-β
IKK, IκB kinase
TBK1, TANK-binding kinase 1
IRF3, IFN-regulatory factor 3

ABSTRACT

Toll-like receptors (TLRs) play an important role in recognition of microbial components and induction of innate immunity. The microbial components trigger the activation of two downstream signaling pathways of TLRs; MyD88- and/or TRIF-dependent pathways leading to activation of NF-κB. (−)-Epigallocatechin-3-gallate (EGCG), a flavonoid found in green tea, is known to inhibit NF-κB activation induced by many pro-inflammatory stimuli. EGCG was shown to inhibit the activity of IKKβ which is the key kinase in the canonical pathway for NF-κB activation in MyD88-dependent pathway of TLRs. However, it is not known whether EGCG inhibits TRIF-dependent pathway through which more than 70% of lipopolysaccharide (LPS)-induced genes are regulated. Therefore, we attempted to identify the molecular target of EGCG in TRIF-dependent pathways of TLR3 and TLR4. EGCG inhibited the activation of IFN regulatory factor 3 (IRF3) induced by LPS, poly[I:C], or the overexpression of TRIF. The inhibition of IRF3 activation by EGCG was mediated through the suppression of the kinase activity of TBK1. However, EGCG did not inhibit activation of IRF3 induced by overexpression of constitutively active IRF3. These results suggest that the molecular target of EGCG is TBK1 in TRIF-dependent signaling pathways of TLR3 and TLR4. Therefore, our results suggest that green tea flavonoids can modulate both MyD88- and TRIF-dependent signaling pathways of TLRs and subsequent inflammatory target gene expression.

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1. Introduction

Toll-like receptors (TLRs) recognize conserved pathogen associated molecular pattern (PAMP) and induce innate immune responses that are essential for host defense against invading microbial pathogens [1,2]. Currently, at least eleven TLRs in mammalian cells are identified with different types of agonists. TLR2 dimerized with TLR1 or TLR6 recognizes triacyl or diacyl lipopeptides. TLR4 and TLR3 are activated by LPS and double-stranded RNA, respectively [3–6]. TLRs can also be activated by endogenous agonists derived from non-microbial origin including heat shock protein 60 and 70, Type III repeat extra domain A of fibronectin, taxol, and saturated fatty acids [7–11].

In general, TLR activation triggers the activation of two downstream signaling pathways; MyD88-dependent and -independent pathways [12]. TLR4 activates both MyD88- and TRIF-dependent pathways while TLR2 and TLR3 activate primarily MyD88-dependent and TRIF-dependent signaling pathway, respectively. MyD88 is the immediate adapter molecule which is common to all mammalian TLRs except for TLR3. MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) and induces IRAK-4 phosphorylation. The phosphorylated IRAK-4 induces the phosphorylation of IRAK-1 leading to the degradation of IRAK-1. The phosphorylated IRAK-1 associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) leading to the activation of the canonical IKKα/β/γ complex resulting in the activation of NF-κB transcription factor. The activation of NF-κB leads to the induction of inflammatory gene products including cytokines. The activation of TLR3 and TLR4 recruits TIR domain-containing adapter inducing IFNβ (TRIF), which activates MyD88-independent signaling pathways. TRIF activates the downstream kinases, TBK1 and IKKε, leading to the activation of IFN regulatory factor 3 (IRF3) [13]. The activation of TRIF pathway also induces delayed activation of NF-κB possibly mediated through the association of TRIF with RIP1 [14,15]. The majority (more than 70%) of LPS-induced genes are known to be regulated through TRIF pathways [16]. The representative target genes regulated through TRIF signaling pathways of TLR4 include IFNβ and IFN-inducible genes such as iNOS and IP-10 [17,18].

The polyphenolic fraction of green tea is well known to have anti-inflammatory and chemopreventive effects. The green tea polyphenols include catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG). Of these, EGCG is known to possess the most potent anti-oxidative and chemopreventive properties. EGCG inhibited the activation of transcription factors, NF-κB and AP-1, induced by many pro-inflammatory stimuli such as UV, LPS, and IL-β [19–21] resulting in the decrease in the expression of inflammatory gene products including lipoxygenase [22], cyclooxygenase [23,24], nitric oxide synthase [25,26], and TNFα [20]. It has been shown that the activation of NF-κB was suppressed by EGCG possibly mediated through the suppression of the kinase activity of IκB kinase in macrophages and the intestinal epithelial cell line (IEC-6) as determined by the phosphorylation of IκBα-GST fusion protein [27,28]. However, direct targets of EGCG to exert anti-inflammatory effect have not been fully identified.

Enhanced inflammation is known to be a critical step in the cascade of events leading to the development of many inflammatory diseases including atherosclerosis, diabetes, rheumatoid arthritis, and cancer. Recent evidence suggests the involvement of TLRs in various chronic inflammatory diseases. Identifying the direct targets of polyphenols in TLR pathways would be important because the activation of TLRs by exogenous and endogenous agonists can induce inflammatory responses that are one of key etiological conditions for the development of many chronic inflammatory diseases. Therefore, we attempted to identify the molecular target of green tea polyphenols in MyD88-dependent and TRIF-dependent signaling pathways of TLRs.

2. Materials and methods

2.1. Reagents

(−)-Epigallocatechin-3-gallate (EGCG) and catechin were purchased from Biomol (Plymouth Meeting, PA) and Sigma–Aldrich (St. Louis, MO), respectively, and dissolved in DMSO. Purified LPS was obtained from List Biological Lab. Inc. and dissolved in endotoxin-free water. Macrophage-activating lipopeptide 2-kDa (MALP-2) was purchased from Alexis Biochemical (San Diego, CA). Poly[I:C] was purchased from Amersham Biosciences (Piscataway, NJ). Antibody for IRAK-1 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies for phospho-IRF3 (S396) and IRF3 were obtained from Upstate Biotechnology (Waltham, MA) or Zymed Laboratories (San Francisco, CA), respectively. All other reagents were purchased from Sigma unless otherwise described.

2.2. Cell culture

RAW 264.7 cells (a murine monocytic cell line, ATCC TIB-71) 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, Invitrogen), 100 units/ml Penicillin, and 100 μg/ml Streptomycin (Invitrogen). Ba/F3 cells, an IL-3-dependent murine pro-B cell line, expressing TLR4 (Flag or GFP-tagged), CD 14, MD2 (Flag-tagged), and NF-κB luciferase reporter gene were described previously [29]. Cells were cultured in RPMI1640 medium containing recombinant murine IL-3 (70 U/ml), 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml Penicillin, and 100 μg/ml Streptomycin (GIBCO-BRL). Cells were maintained at 37 °C in a 5% CO_2 air environment.

2.3. Plasmids

NF-κB(2×)-luciferase reporter construct was provided by Frank 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 kind gift from David Dewitt (Michigan State University, East Lansing, MI). Heat shock protein 70 (HSP70)-β-galactosidase

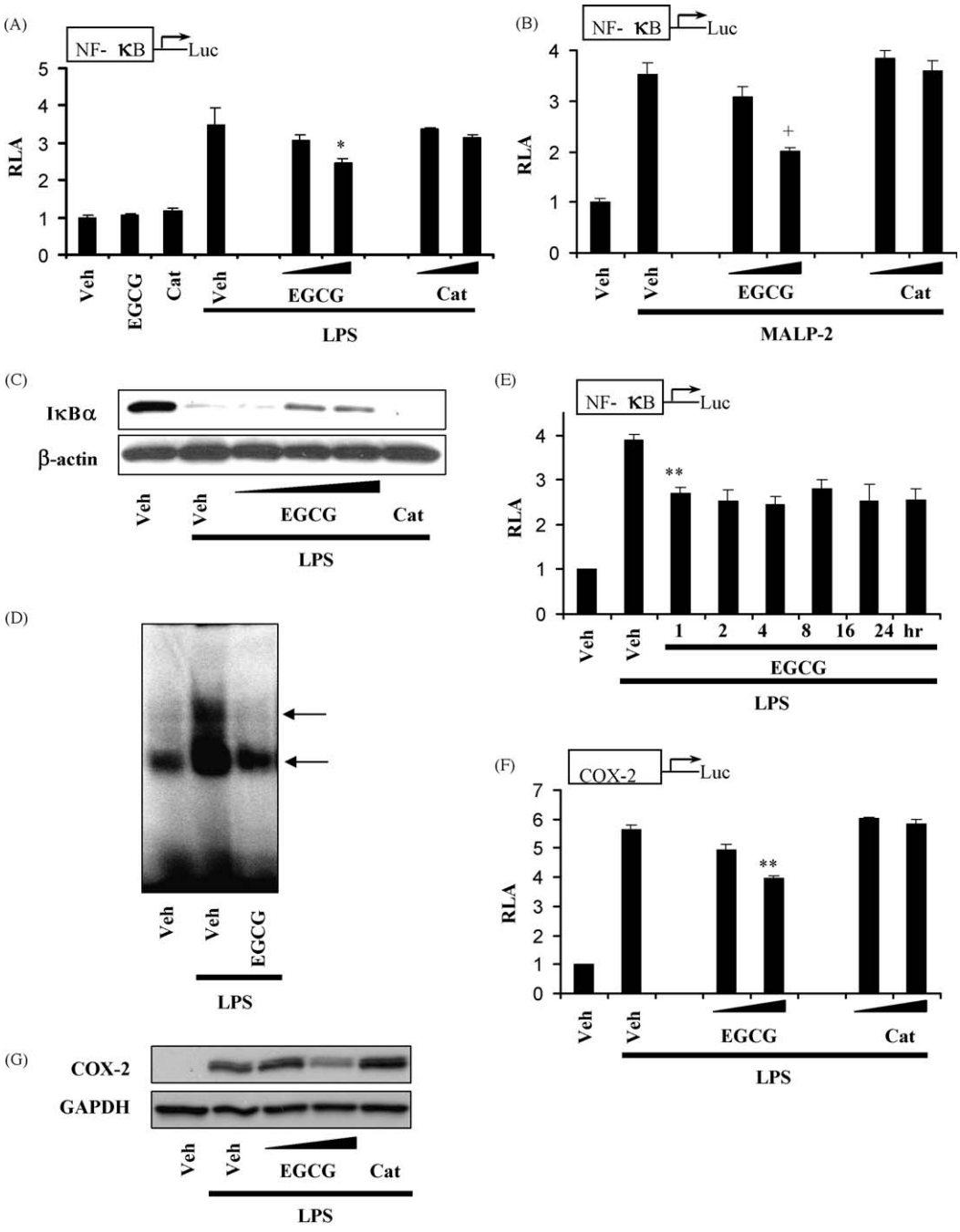

Fig. 1 – EGCG inhibits the activation of NF-κB and the expression of cyclooxygenase-2 (COX-2) induced by TLR4 and TLR2 agonist (LPS and MALP-2, respectively) in macrophages. (A) RAW264.7 cells were transfected with luciferase reporter plasmid containing NF-κB binding site (2×). After cells were pretreated with EGCG (20, 30, 50 μM) or catechin (20, 50 μM) for 1 h, cells were further stimulated with LPS (5 ng/ml) for 6 h. For EGCG or catechin alone group, cells were treated with EGCG or catechin for 7 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in Section 2. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). Significantly different from LPS alone, $^{*}p < 0.05$. (B) RAW264.7 cells transfected with NF-κB binding site (2×)-luciferase reporter plasmid were pretreated with EGCG or catechin (20, 50 μM) for 1 h and further stimulated with MALP-2 (1 ng/ml) for 6 h. Significantly different from MALP-2 alone, $^{+}p < 0.0l$. (C) RAW264.7 cells were pretreated with EGCG (20, 50, 100 μM) or catechin (100 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 15 min. Cell lysates were analyzed for IκBα, and β-actin protein by immunoblotting. (D) Gel shift analyses of NF-κB were performed with nuclear extracts prepared from RAW264.7 cells treated with LPS (0.1 μg/ml) or LPS plus EGCG (50 μM) for 20 min. All lanes contained 10 μg of nuclear extracts and the labeled NF-κB DNA consensus sequence. Arrow indicates the NF-κB DNA complex. (E, F) RAW264.7 cells were transfected with luciferase reporter plasmid containing NF-κB binding site (2×) (E) or COX-2 promoter (F). Cells were pretreated with EGCG (50 μM) for indicated time period (E) or EGCG (20, 50 μM) or catechin

reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). The wild-type IKKβ was obtained from Michael Karin (University of California, San Diego, CA). A constitutively active form of IRF3 was from John Hiscott (McGill University, Canada). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

2.4. Transfection and luciferase assay

These were performed as described in our previous studies [30,31]. Briefly, RAW264.7 or 293T cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Various expression plasmids or corresponding empty vector plasmids for signaling components were co-transfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector in order to eliminate the experimental error from transfection itself. Luciferase and β-galactosidase 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.

2.5. Immunoblotting

These were performed essentially the same as previously described [32,33]. Equal amounts of cell extracts were resolved on SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membranes were blocked with phosphate-buffered saline containing 0.1% Tween 20 and 3% nonfat dry milk and were blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). The reactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences). To reprobe with different antibodies, the membrane was stripped in the stripping buffer at 55 °C for 1 h.

2.6. Immunoprecipitation

Protein extracts from Ba/F3 cells expressing TLR4 (Flag or GFP-tagged), CD 14, MD2 (Flag-tagged), and NF-κB luciferase reporter gene for immunoprecipitation were prepared as described [29]. Mouse-GFP antibodies (Molecular Probes Inc., Eugene, OR) were bound to protein A before immunoprecipitation. The samples were immunoprecipitated with mouse-GFP antibody/protein A for overnight. The solubilized immune complex was resolved on 8% SDS-PAGE. The membranes were immunoblotted with Flag antibodies (Sigma–Aldrich, St. Louis, MO) and reprobed with rabbit GFP antibodies (Molecular Probes Inc., Eugene, OR).

2.7. In vitro TBK1 kinase assay

These were performed essentially the same as previously described [34]. TBK1 kinase assay was conducted with recombinant human TBK1 (Upstate, cat#12-628) according to the manufacturer's instruction. Briefly, active TBK1 was incubated with IRF3 protein (0.5 μg) in kinase buffer containing 50 (M of ATP and 1 μCi of [γ-^{32}P]-ATP for 10 min at 30 °C. A 20 μl 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) was obtained from Kai Lin (University of Massachusetts Medical School, Worcester, MA).

2.8. Quantitative real-time reverse transcription (RT)-PCR analysis of interferon-beta (IFNβ) expression

These were performed essentially the same as previously described [34]. Briefly, total RNAs were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Five micrograms of total RNAs were reverse-transcribed and amplified through PCR. The specificity of the amplified PCR products was assessed by a melting curve analysis. The fold induction of IFNβ expression by real-time PCR was calculated as previously described [30].

2.9. Electromobility shift assay (EMSA)

These were performed essentially same as previously described [35]. A double-stranded oligonucleotide containing the consensus sequence for NF-κB binding site was used (5′-GCGGACCAGGACAAAGGTCACGTTC-3′) after end-labeling with [γ-^{32}P]-ATP by T4 polynucleotide kinase (Promega, Madison, WI). The reaction mixture contained 4 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC and 50 mM Tris–Cl (pH 7.5), 10 ng of nuclear extracts and sterile water in a total volume of 20 μl. Incubations were carried out at room temperature for 20 min by addition of 1 μl probe (10^6 cpm), following 10 min preincubations. Samples were loaded onto 5% polyacrylamide gels at 100 V. The gels were removed and dried, followed by autoradiography.

3. Results

3.1. EGCG inhibits NF-κB activation and COX-2 expression induced by the agonist of TLR2 or TLR4

To investigate whether EGCG modulates TLR-mediated signaling pathways, we compared the effect of EGCG on the activation of TLR2 and TLR4. TLR2 activates only MyD88-dependent signaling pathway leading to NF-κB activation while TLR4 activates both MyD88-dependent and -independent signaling

(20, 50 μM) for 1 h (F). Cells were further stimulated with LPS (5 ng/ml) for 6 h. Significantly different from LPS alone, $^{**}p < 0.01$. (G) RAW264.7 cells were pretreated with EGCG (20, 50 μM) and catechin (50 μM) for 1 h and then further stimulated with LPS (5 ng/ml) for 6 h. Cell lysates were analyzed for COX-2 and GAPDH protein by immunoblotting. The panels are representative data from more than three independent experiments. Veh, vehicle; EGCG, (−)-epigallocatechin-3-gallate; Cat, catechin.

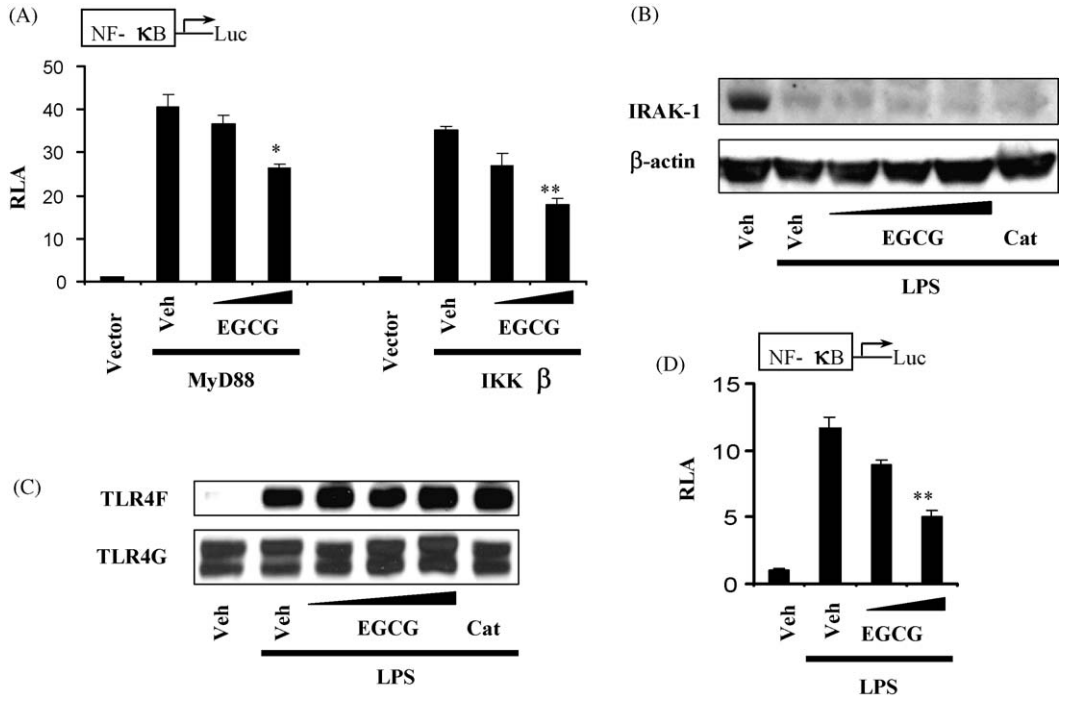

Fig. 2 – EGCG suppresses the activation of NF-κB induced by IKKβ in MyD88-dependent signaling pathways. (A) 293T cells were transfected with NF-κB binding site (2×)-luciferase reporter plasmid and the expression plasmid of MyD88 or IKKβ. After 24 h, cells were further treated with EGCG (20, 50 μM) or catechin (20, 50 μM) for 6 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ± S.E.M. ($n = 3$). Significantly different from MyD88 plus vehicle, $^{*}p < 0.05$. Significantly different from IKKβ plus vehicle, $^{**}p < 0.05$. (B) RAW264.7 cells were pretreated with EGCG (20, 50, 100 μM) or catechin (100 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 30 min. Cell lysates were analyzed for IRAK-1 and β-actin by immunoblotting. (C) Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag, and CD14 were pre-treated with EGCG (20, 50, 100 μM) or catechin (100 μM) for 1 h and then treated with LPS (50 ng/ml) for 20 min. Cells were then subjected to immunoprecipitation with anti-mouse GFP antibody and immunoblotted with anti-Flag (upper) or anti-rabbit GFP (lower) antibody. The panels are representative data from more than three independent experiments. (D) Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag, CD 14, and luciferase reporter containing NF-κB binding site were treated with EGCG (20, 50 μM) for 1 h. Cells were further stimulated with LPS (5 ng/ml) for 18 h. Veh, vehicle; EGCG, (−)-epigallocatechin-3-gallate; Cat, catechin.

pathways. NF-κB is the common downstream signaling component for all TLRs. Therefore, the activation of NF-κB induced by TLR agonists was used as readout for the activation of TLRs.

EGCG inhibited the activation of NF-κB induced by both LPS (TLR4 agonist) and MALP-2 (TLR2 agonist) in RAW264.7 cells as determined by the luciferase reporter gene assay (Fig. 1A and B). EGCG also inhibited IκBα degradation induced by LPS (Fig. 1C). In contrast, catechin which is the same subfamily (*flavan-3-ol*) of EGCG in flavonoid class did not inhibit NF-κB activation induced by LPS or MALP-2 suggesting that the galloyl groups in EGCG, which is not present in catechin, are important for the inhibitory effects of EGCG on TLR activation. This is consistent with previous report that the galloyl groups on EGCG are important for its anti-inflammatory property [26]. To further confirm whether EGCG inhibits NF-κB activation, we performed gel shift assay using a radiolabeled NF-κB consensus sequence. RAW264.7 cells were treated with LPS or LPS plus EGCG and nuclear extracts were prepared. EGCG suppressed the formation of NF-κB DNA complex (Fig. 1D). The results further support that EGCG inhibited the activation of NF-κB induced by TLR4 activation.

After EGCG was pretreated for 1, 2, 4, 8, 16, 24 h, cells were stimulated with LPS. The results showed that the inhibitory effect of EGCG was maximally observed at 1 h and similar throughout different time periods (Fig. 1E). LPS-induced expression of COX-2, one of the target genes regulated by NF-κB activation, was weakly suppressed by EGCG, but not by catechin as determined by COX-2 promoter luciferase reporter assay and COX-2 immunoblotting (Fig. 1F and G).

3.2. *EGCG inhibits the downstream signaling components including IKKβ, but not the upstream signaling components of IRAK-1 including TLR4 itself or MyD88*

To further investigate the regulation of MyD88-dependent signaling pathways by EGCG, NF-κB activation was induced by the overexpression of MyD88 or its downstream kinase, IKKβ, in 293T cells. IKKβ is one of the key kinases for NF-κB activation induced by TLR agonists. EGCG suppressed agonist-independent activation of NF-κB induced by MyD88 or IKKβ in 293T cells (Fig. 2A) demonstrating that EGCG suppresses MyD88/IKKβ-dependent signaling pathways. This is well correlated with

previous reports suggesting that EGCG inhibits the kinase activity of IKKβ as determined by IKK immunocomplex kinase assay or *in vitro* phosphorylation of IκBα-GST, a substrate of IKKβ in TNF-stimulated cytosolic extracts [27,28,36].

The stimulation of TLR4 with agonists induces the homodimerization of TLR4 leading to the recruitment of MyD88. MyD88 induces the phosphorylation of IRAK-4, which in turn phosphorylates IRAK-1 leading to the degradation of IRAK-1 and the activation of downstream signaling molecules including IKKβ. Thus, IRAK-1 degradation was used as an additional readout for the activation of TLRs. Both EGCG and catechin did not inhibit LPS-induced the degradation of IRAK-1 (Fig. 2B). MyD88 is the upstream component which induces the phosphorylation and degradation of IRAK-1. Therefore, our results suggest that EGCG does not inhibit MyD88.

To investigate whether EGCG directly inhibits TLR4, we determined the dimerization of TLR4 induced by LPS using Ba/F3 cells stably transfected with murine TLR4-Flag, TLR4-GFP, MD2, and CD14. EGCG and catechin did not inhibit LPS-induced dimerization of TLR4 (Fig. 2C) although the activation of NF-κB in Ba/F3 cells stimulated with LPS was still suppressed by EGCG (Fig. 2D). These results demonstrate that EGCG does not inhibit the interaction of LPS with the receptor nor receptor dimerization after agonist engagement.

These results further suggest that the molecular target of EGCG is not the upstream signaling components of IRAK-1

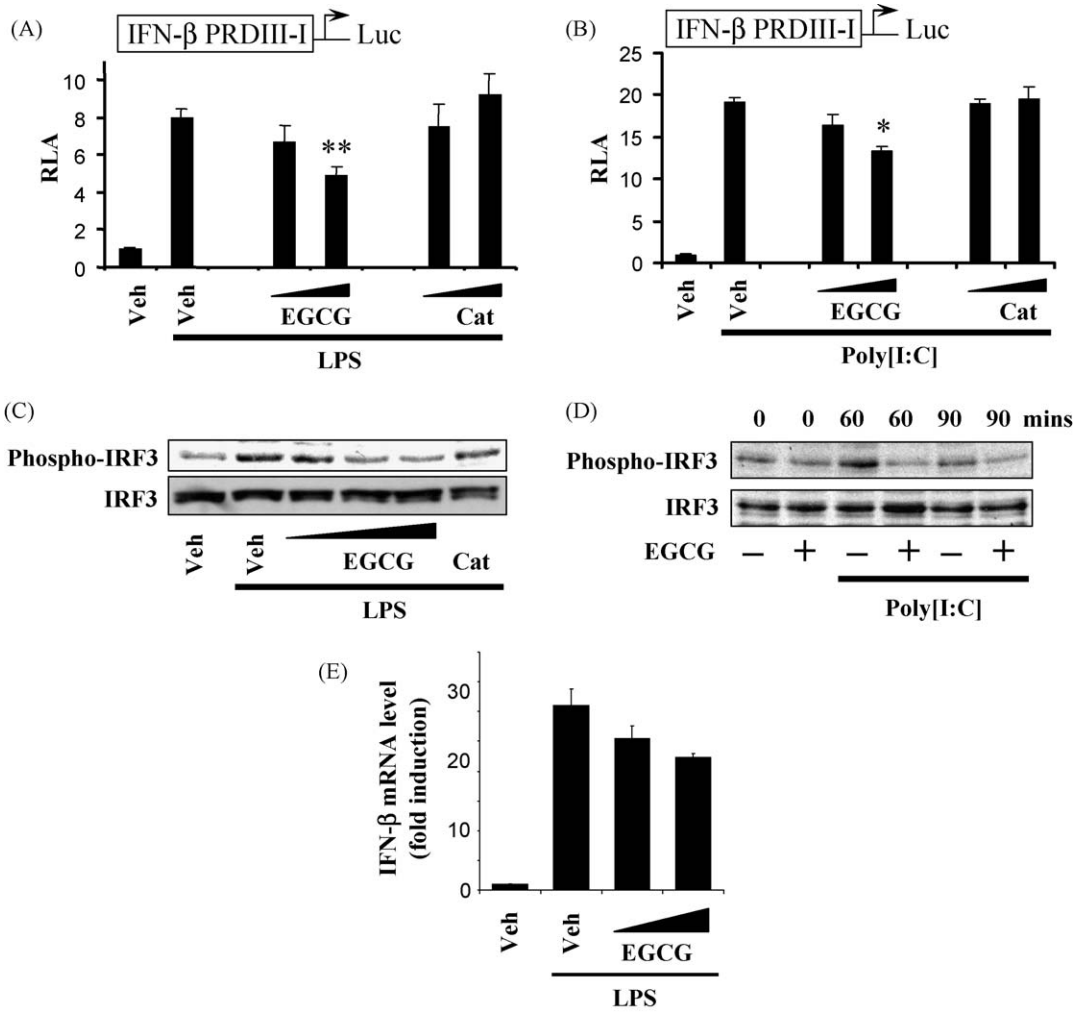

Fig. 3 – EGCG suppresses the activation of IRF3 induced by LPS or poly[I:C] in macrophages. RAW264.7 cells were transfected with IRF3 binding site (IFNβ PRDIII-I)-luciferase reporter plasmid. Cells were treated with EGCG (20, 50 μM) or catechin (20, 50 μM) for 1 h and further stimulated with LPS (5 ng/ml) (A) or poly[I:C] (10 μg/ml) (B) for 6 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ± S.E.M. ($n = 3$). Significantly different from LPS alone, $^{**}p < 0.01$. Significantly different from poly[I:C] alone, $^{*}p < 0.05$. (C) RAW264.7 cells were treated with EGCG (20, 50, 100 μM) or catechin (100 μM) for 1 h and further stimulated with LPS (50 ng/ml) for 1.5 h. Cell lysates were analyzed for phospho-IRF3 (S396) and IRF3 immunoblots. (D) RAW264.7 cells were treated with EGCG (50 μM) for 1 h and further stimulated with poly[I:C] (20 ng/ml) for the indicated time periods. (E) RAW264.7 cells were treated with EGCG (20, 30 μM) for 1 h and further stimulated with LPS (5 ng/ml) for 4 h. Total RNAs were extracted and the levels of IFNβ expression were determined by the Quantitative RT-PCR analysis. IFNβ expression was normalized with β-actin (internal control) expression and presented as fold inductions compared with the vehicle control. Values are mean ± S.D. ($n = 2$). The panels are representative data from more than three independent experiments. Veh, vehicle; EGCG, (−)-epigallocatechin-3-gallate; Cat, catechin.

including TLR4 itself and MyD88, but the downstream signaling components including IKKβ.

3.3. EGCG suppresses TRIF-dependent signaling pathways of TLR3 and TLR4

Next, we determined whether EGCG inhibits TRIF-dependent (MyD88-independent) signaling pathways of TLRs. TLR4 and TLR3 have TRIF-dependent downstream signaling pathways. TRIF induces the activation of downstream kinases including TBK1 which phosphorylates IRF3 leading to the activation of IRF3 and the expression of type I IFNs [13,37,38]. Therefore, the activation of IRF3 was used as readout for the activation of TRIF-dependent signaling pathways. EGCG suppressed activation of IRF3 induced by LPS (TLR4 agonist) or poly[I:C] (TLR3 agonist) as determined by reporter gene assay using IFNβ promoter domain containing IRF3 binding site (IFNβ PRDIII-I) and the phosphorylation of IRF3 (Fig. 3A–D). However, catechin did not affect the activation of IRF3.

To investigate the consequence of the inhibition of IRF3 by EGCG, we determined the expression of IRF3-target genes such as IFNβ. Quantitative RT-PCR analysis showed that IFNβ production in LPS-stimulated RAW264.7 cells was decreased by EGCG treatment (Fig. 3E). These results demonstrate that EGCG suppresses TRIF-dependent signaling pathways and target gene expression derived from TLR3 and TLR4 activation.

3.4. EGCG inhibits the functional activity of TBK1

To further identify the molecular target of EGCG in the inhibition of TRIF-dependent signaling pathways, IRF3 activation was induced by the overexpression of TRIF or IRF3 in 293T cells. EGCG inhibited the activation of IRF3 induced by TRIF as determined by IRF3 binding site (IFNβ PRDIII-I)-reporter gene assay while catechin did not (Fig. 4A). In contrast, EGCG did not inhibit the activation of IRF3 induced by overexpression of constitutively active IRF3 (Fig. 4B). These results suggest that transcription factor IRF3 is not the direct target of EGCG and that the molecular targets of EGCG lie between TRIF and IRF3.

TBK1 is the downstream kinase of TRIF and phosphorylates IRF3 resulting in the activation of IRF3 [13]. EGCG inhibited the activation of IRF3 induced by overexpression of TBK1 as determined by IRF3 binding site (IFNβ PRDIII-I) reporter gene assay (Fig. 4C). Consistently, the results from *in vitro* kinase assay showed that the kinase activity of TBK1 was suppressed by EGCG dose-dependently, but not by catechin (Fig. 4D).

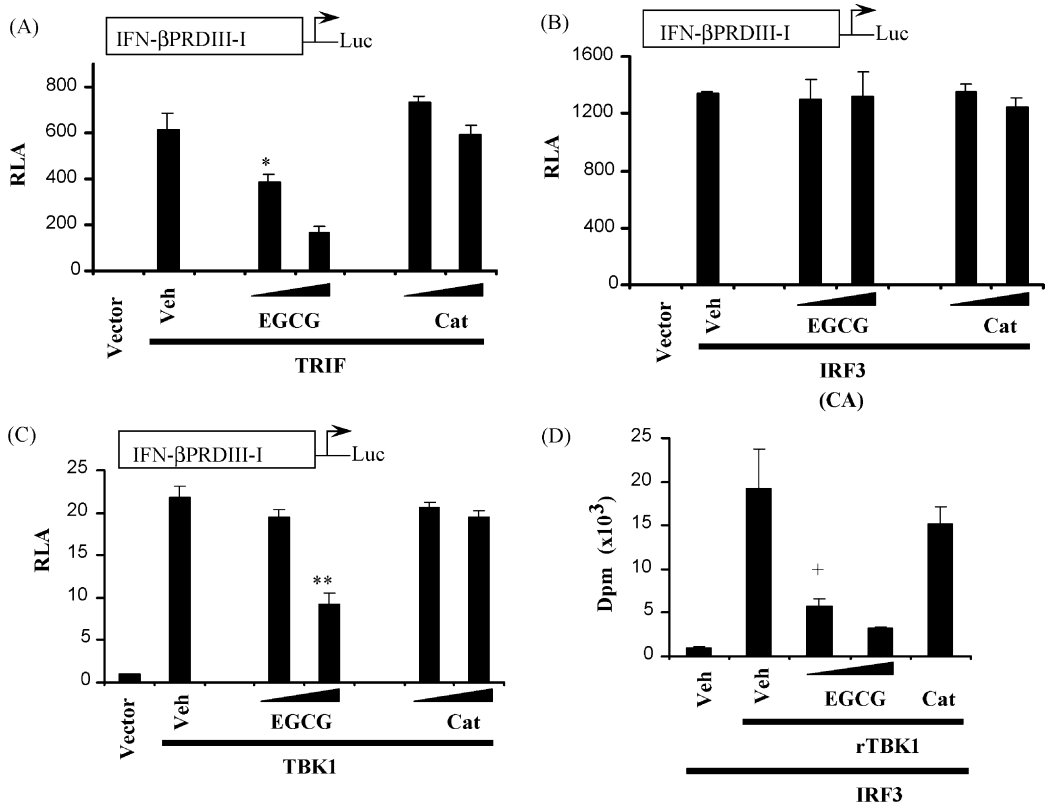

Fig. 4 – EGCG suppresses the functional activity of TBK1. 293T cells were transfected with IRF3 binding site (IFNβ PRDIII-I)-luciferase reporter plasmid and the expression plasmid of TRIF (A), constitutively active IRF3 [IRF3(CA)] (B), or TBK1 (C). Cells were further treated with EGCG (20, 50 μM) or catechin (20, 50 μM) for 6 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ± S.E.M. ($n = 3$). Significantly different from TRIF plus vehicle, $^{*}p < 0.05$. Significantly different from TBK1 plus vehicle, $^{**}p < 0.01$. (D) *In vitro* TBK1 kinase assay was performed using recombinant active TBK1 (rTBK1) and IRF3 as a substrate as described in Section 2. TBK1 kinase activity was determined in the presence of EGCG (20, 50 μM) or catechin (100 μM). Values are mean ± S.E. ($n = 2$). Significantly different from vehicle plus rTBK1 plus IRF3, $^{+}p < 0.05$. The panels are representative data from more than three independent experiments. Veh, vehicle; EGCG, (−)-epigallocatechin-3-gallate; Cat, catechin.

These results demonstrated that TBK1 is the molecular target of EGCG in TRIF-dependent signaling pathways of TLRs resulting in the decrease of IRF3 activation.

4. Discussion

Our results demonstrated that the molecular target of EGCG (a flavonoid found in green tea) is TBK1 in TRIF signaling pathways of TLR3 and TLR4. In contrast, catechin did not affect agonist-induced activation of TLR signaling pathways. The activation of TRIF-dependent signaling pathways of TLR3 and TLR4 leads to the activation of TBK1 kinase that phosphorylates IRF3 resulting in the translocation into the nucleus [13]. Our results showed that EGCG inhibited the activation of IRF3 induced by the transfection of TBK1 expression plasmid. In addition, the results from *in vitro* kinase assay demonstrated that EGCG inhibits the kinase activity of TBK1. Since TLR4 ligand-induced inflammatory cytokine production was impaired in TRIF-deficient mice [39], both TRIF- and MyD88-dependent pathways may be required for the maximum expression of cytokines. The predominant portion (>70%) of LPS-induced genes is known to be regulated through TRIF-dependent pathways [16]. This fact suggests that the inhibition of TRIF-dependent pathway mediated through TBK1 and the consequent downregulation of IRF3 activity by EGCG can lead to the significant downregulation of the target genes induced by the activation of expression of TLR3 and TLR4.

It was shown that EGCG also inhibits TNF-induced NF-κB activation [28]. TBK1 has been implicated to be involved in TNF-induced activation of NF-κB independently of IκBα degradation and NF-κB DNA binding [40]. Our results that EGCG suppresses the kinase activity of TBK1 suggest that TBK1 may be the common target of inhibition by EGCG at least in TNF and TLR signaling pathways.

EGCG suppresses NF-κB activation induced by pro-inflammatory stimuli including LPS [27,28,36]. NF-κB is one of the key positive regulators for COX-2 expression in murine macrophages exposed to LPS [32]. Therefore, the inhibitory effect of EGCG on NF-κB activation is correlated with the inhibitory effect on COX-2 expression. The inhibitory potency of EGCG on COX-2 expression was weak. This may be due to the requirement of other transcription factors involved in COX-2 expression such as AP-1 activated by MAPK pathway that may be EGCG-insensitive. Park et al. reported that EGCG does not inhibit LPS-induced COX-2 expression [41]. This discrepancy may be due to the difference in experimental conditions. In their studies, 1 mg/ml of LPS was used to stimulate macrophages, whereas we used 5 ng/ml of purified LPS (List Biological Lab.). The source of LPS was not indicated in their report. It is well recognized that unpurified LPS is contaminated with other components that can activate TLRs other than TLR4.

Lin and Lin [26] postulated that EGCG blocked the interaction of LPS with its receptor (TLR4) by creating a ''sealing effect''. If EGCG blocks the interaction of LPS with the receptor, it should inhibit the dimerization of TLR4 and the degradation of IRAK-1. In addition, Wheeler et al. [21] showed that EGCG inhibited the degradation of IRAK induced by IL-1β in A549 cells. However, our results showed that EGCG did not inhibit LPS-induced dimerization of TLR4 in Ba/F3 cells stably transfected with TLR4 (Fig. 2C). In addition, the degradation of IRAK-1 induced by LPS was not inhibited by EGCG in macrophages (Fig. 2B). Our results suggest that EGCG does not inhibit the interaction of LPS with TLR4. Therefore, the target of EGCG is not the component in the upstream of IRAK-1, TLR itself or MyD88, but the downstream components such as IKKβ.

Our results demonstrate that EGCG inhibits not only MyD88-dependent signaling pathways, but also TRIF-dependent signaling pathways of TLRs. IKKβ and TBK1 are the targets of EGCG in MyD88- and TRIF-dependent signaling pathways, respectively. Our finding suggests that green tea polyphenols can modulate TLR activation and subsequent inflammatory responses.

In most luciferase reporter assays, the inhibitory effect of EGCG on the activation of NF-κB and IRF3 was significant at 50 μM. However, for TBK1 *in vitro* kinase assay, the inhibitory effect of EGCG was more pronounced. This may be because *in vitro* TBK1 kinase assay does not involve the process for cellular uptake of EGCG. The results from the cellular uptake kinetic study showed that the cytosolic concentrations of EGCG were linearly increased (915.3–6851.6 μg/g) after incubation of 20–600 μM of EGCG in human colon carcinoma cells [42]. EGCG is unstable in culture medium of KYSE150 cells or A431 cells with half-life of 30 min and the presence of 5% serum prolongs the half-life of EGCG to 1 h [43]. Although we did not measure the stability of EGCG in the macrophages, we used 10% serum which is expected to increase the half-life of EGCG. Our results also showed that SOD treatment diminished the inhibitory effects of EGCG on IKKβ-induced NF-κB activation and TBK1-induced IRF3 activation (data not shown). These results suggest that oxidation products of EGCG formed during incubation are involved in the inhibition of TLR signaling. Hou et al. reported that EGCG dimer (Theasinensin A), GCG, and hydrogen peroxide are generated from EGCG oxidation and that oxidation products of EGCG were involved in its inhibitory effect on EGFR activation [43]. The cellular uptake kinetics and the formation of oxidation products of EGCG in macrophages remain to be determined in future study.

In summary, our results revealed that TBK1 kinase in TRIF pathways of TLR3 or TLR4 is the molecular target of EGCG. These results suggest that anti-inflammatory effects of EGCG in green tea are at least in part mediated through the inhibition of TRIF signaling pathways.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.06.021.

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