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TLR4 Signaling Inhibitory Pathway Induced by Green Tea Polyphenol Epigallocatechin-3-Gallate through 67-kDa Laminin Receptor

Eui Hong Byun,* Yoshinori Fujimura,† Koji Yamada,* and Hirofumi Tachibana*,†,‡

Epigallocatechin-3-gallate (EGCG), a major active polyphenol of green tea, has been shown to downregulate inflammatory responses in macrophages; however, the underlying mechanism has not been understood. Recently, we identified the 67-kDa laminin receptor (67LR) as a cell-surface EGCG receptor that mediates the antiinflammatory action of EGCG at physiologically relevant concentrations (0.1–1 μM). In this study, we show the molecular basis for the downregulation of TLR4 signal transduction by EGCG at 1 μM in macrophages. Anti-67LR Ab treatment or RNA interference-mediated silencing of 67LR resulted in abrogation of the inhibitory action of EGCG on LPS-induced activation of downstream signaling pathways and target gene expressions. Additionally, we found that EGCG reduced the TLR4 expression through 67LR. Interestingly, EGCG induced a rapid upregulation of Toll-interacting protein (Tollip), a negative regulator of TLR signaling, and this EGCG action was prevented by 67LR silencing or anti-67LR Ab treatment. RNA interference-mediated silencing of Tollip impaired the TLR4 signaling inhibitory activity of EGCG. Taken together, these findings demonstrate that 67LR plays a critical role in mediating anti-inflammatory activity of a physiologically relevant EGCG, and Tollip expression could be modulated through 67LR. These results provide a new insight into the understanding of negative regulatory mechanisms for the TLR4 signaling pathway and consequent inflammatory responses that are implicated in the development and progression of many chronic diseases. The Journal of Immunology, 2010, 185: 33–45.

Toll-like receptors are important in the activation of the innate immune response and are pathogen recognition proteins that have important roles in detecting microbes and initiating inflammatory responses (1). Recognition of microbial components by TLRs plays a central role in the immune system’s decision to respond or not to a particular microbial infection (1). The intracellular signaling pathways activated by TLRs are mediated through the Toll/IL-1R homology (TIR) domains. Activation of signaling through TIR domains results in recruitment of the adaptor molecule MyD88 and ultimately leads to degradation of IkB and translocation of NF-κB to the nucleus (1). LPS, a major component of the outer membrane of Gram-negative bacteria, is one of the most powerful activators of TLR4 signaling and is also well known to induce production of inflammatory mediators, such as TNF-α, the β form of pro-IL-1, IL-6, and NO, and activation of the MAPK signaling pathway and NF-κB, leading to death from endotoxemic shock in animal models (2–4).

Green tea (Camellia sinensis L.) is a popular beverage worldwide, and its possible health effects have been the subject of considerable attention (5). Among the green tea polyphenols, epigallocatechin-3-gallate (EGCG) constitutes ~60% of the catechins in tea. EGCG has a variety of biological and pharmacological properties including cancer-preventive, antiallergic, antioxidative stress, and anti-inflammatory activities (6–9). Recently, EGCG has been shown to protect mice against lethal endotoxemia induced by LPS and rescue mice from lethal sepsis (10). For the RAW 264.7 murine macrophages, EGCG inhibits activation of NF-κB and AP-1, which are induced by proinflammatory stimuli, such as UV radiation and LPS (7, 11), resulting in the decrease in the expression of inflammatory gene products including lipoxigenase (12), cyclooxygenase (COX) (13), NO synthase (14), and TNF-α (15). Moreover, EGCG inhibits MyD88-dependent signaling pathways and TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent signaling pathways of TLRs in RAW264.7 cells, which suppresses inflammatory responses (16). Although some mechanisms for the anti-inflammatory activities of EGCG have been proposed, most experiments have been conducted at high concentrations (10–50 μM) of EGCG to demonstrate these biological activities, even though the peak plasma concentration of EGCG is <1 μM (6). It is still not clear which EGCG-induced molecular events are responsible for its anti-inflammatory activity at physiologically relevant concentrations.

The 67-kDa laminin receptor (67LR) is a nonintegrin cell-surface receptor for laminin with high affinity (17). Its role as a laminin receptor makes it a crucial molecule in cell adhesion to the basement membrane and the metastasis of tumor cells (18). An increase in the expression of 67LR as compared with the corresponding normal tissue has been found in a variety of common cancers.
(19, 20). 67LR acts as a receptor for Sindbis virus and appears to be important for entry of the virus into mammalian cells (21). The 67LR protein also interacts specifically with the major Dengue virus serotypes (22). Furthermore, the protein has been shown to be crucial in bacterial infections, such as bacterial meningitis development and the internalization of the *Escherichia coli* K1 strain into brain endothelial cells (23).

Recently, we identified 67LR as a cell-surface EGCG receptor that mediates the anticancer action of physiologically achievable concentrations of EGCG (0.1–1 μM) (24). Indeed, expression of 67LR confers EGCG responsiveness to tumor cells. Others showed that RNA interference (RNAi)-mediated silencing of 67LR results in abrogation of EGCG-induced apoptosis in multiple myeloma cells (25). Furthermore, this receptor has also been shown to be responsible for antiallergic effects of EGCG in basophils (26, 27). In preadipocytes, EGCG has been reported to mediate anti-insulin signaling via the 67LR pathway (28). However, it is not clear whether EGCG action through 67LR involves the negative regulatory effect of EGCG for inflammatory responses. In this study, we tried to illuminate the molecular basis for the downregulation of TLR4 signal transduction by a physiologically relevant concentration of EGCG. In this paper, we show that a negative regulator of TLR signaling, Toll-interacting protein (Tollip), is indispensable for mediating the anti-inflammatory action of EGCG, and its protein expression level is upregulated by EGCG through 67LR. Additionally, we found that EGCG can reduce the expression of TLR4 via the laminin receptor.

### Materials and Methods

#### Reagents

EGCG was purchased from Sigma-Aldrich (St. Louis, MO). LPS, anti–β-actin polyclonal Ab, and HRP-conjugated anti-rabbit Ab were obtained from Sigma-Aldrich. Anti-67LR mAb (MLuCS) was purchased from NeoMarkers (Fremont, CA). Anti-67LR (F-18) polyclonal Ab, anti-TLR4 (L-14) polyclonal Ab, anti-MyD88 polyclonal Ab, anti-CD14 polyclonal Ab, anti-inducible NO synthase (iNOS) polyclonal Ab, anti-COX-2 polyclonal Ab, anti-phosphorylated ERK1/2 Ab, anti-ERK1/2 polyclonal Ab, anti-phosphorylated JNK Ab, anti-JNK polyclonal Ab, anti-phosphorylated c-Jun Ab, anti-c-Jun polyclonal Ab, anti-phosphorylated p38 Ab, anti-p38 polyclonal Ab, anti-phosphorylated IκB-α Ab, anti-IκB-α mAb, anti–NF-κB (p65/p50) polyclonal Ab, anti-suppressor of cytokine signaling 1 (SOCS1) polyclonal Ab, anti-siRNA, anti-single Ig IL-1-related receptor (SIGIRR), polyclonal Ab, anti-IL-1-associate kinase (IRAK) M Ab, anti-Tollip mAb, HRP-conjugated anti-goat donkey IgG Ab, PE-conjugated TLR4 (MTSS10) Ab, and PE-conjugated isotype control (normal rat IgG) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-conjugated anti-Rat IgG2a Ab, HRP-conjugated anti-mouse IgG Ab, HRP-conjugated streptavidin, and anti-mouse IgM Ab were obtained from Zymed Laboratories (San Francisco, CA). HRP-conjugated anti-rabbit Ab was purchased from ICN Pharmaceuticals (Aurora, OH). An anti-mouse TNF-α Ab was obtained from Endogen (Woburn, MA). Biotinylated anti-mouse TNF-α Ab was obtained from BioSource International (Camarillo, CA). IL-6 ELISA kit was obtained from eBioscience (San Diego, CA). Thioglycollate-elicited peritoneal macrophages were obtained from specific pathogen-free male BALB/c mice at 6–8 wk of age by injection of 1 ml sterile 3% thioglycollate solution (Difco, Detroit, MI) for 4 d prelavage with 10 ml PBS. The peritoneal macrophages were washed once with RPMI 1640 (without phenol red) supplemented with 10% endotoxin-free heat-inactivated FBS (Intergen), 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were resuspended in RPMI 1640 at a density of 2 × 10⁶ cells/ml. Viability was >95% as determined by trypsin blue dye exclusion. The cells were plated and incubated for 4 h at 37°C in a humidified incubator containing 5% CO₂ to allow macrophage adherence. The plates were then washed once with warm RPMI 1640 to remove nonadherent cells.

#### Anti-67LR Ab treatment

Thioglycollate-elicited peritoneal macrophages were seeded on the plates and incubated at 37°C in 5% CO₂ for 24 h before any treatments. Then cells were incubated with anti-67LR Ab, MLuCS (20 μg/ml), or isotype-matched control mouse IgM (20 μg/ml) for 1 h before the addition of EGCG or LPS.

#### Construction of 67LR-suppressed cells

Target sequences for short hairpin RNA (shRNA) for 67LR and nonspecific control are as follows: shRNA for 67LR, 5'-GGAGGAGTTTACGGTTAA-3'; shRNA for nonspecific control, 5'-GACATATGCGCTACCTAGCAT-3'. The annealed shRNA inserts were cloned into the pHSNA-IhH1neo shRNA expression vector (for 67LR shRNA) (Inovogen, San Diego, CA) according to the manufacturer’s protocol.

#### Construction of Tollip-suppressed cells

The Tollip shRNA expression vector was purchased from Santa Cruz Biotechnology. shRNA plasmids consist of three of five lentiviral transactivation plasmids each encoding target-specific 19–25 nt (plus hairpin) shRNAs designed to knock down gene expression. For each transfection, we added 0.8 ml shRNA plasmid transfection medium to well and then incubated the cells for 7 h. We further performed the neomycin selection for obtaining stably transfected cells.

#### WST-1 assay for cell viability

To measure cell viability, the WST-1 assay was performed. RAW264.7 cells were mechanically scraped, seeded in 96-well plates at 5 × 10⁵ cells/ml, and incubated for 24 h. Postincubation, the cells were treated with EGCG at concentration of 0, 1, or 5 μM for 24 h. The tetrazolium salt 2-(4-iodophenyl)-3-(4-nitropheno)-5-(2,4-disulfophenyl)-2H-tetrazolium, known as WST-1 (Roche, Mannheim, Germany), was used to detect the loss of viability. The supernatant medium was replaced by WST-1 and incubated for 2 h. The colored supernatants without particles were transferred into a clean 96-well plate and measured at 450 nm in a multwell plate reader. The results are given as relative percentage to the untreated control.

#### ELISA

The amount of TNF-α and IL-6 in culture medium was measured by sandwich ELISA. Ab solution diluted at 500 times by PBS was added to a 96-well plate and incubated overnight at 4°C. After washing with 0.05% Tween 20-PBS three times, each well was blocked with 1% BSA-PBS for 2 h at 37°C. Then wells were washed again three times with wash buffer. Following blocking reaction, each well was treated with 50 μl culture supernatant for 1 h at 37°C. The wells were then treated with biotinylated anti-mouse TNF-α or IL-6 Abs for 1 h at 37°C. HRP-conjugated streptavidin solution was added to each well at 100 μl. Then, 0.6 mg/ml of 2,2′-azinobis (ethylbenzothiazoline-6-sulfonic acid diamonium salt) dissolved in 0.03% H₂O₂-0.05 M citrate buffer (pH 4) was added to the well at 100 μl, and the absorbance at 405 nm was measured after the addition of 1.5% oxalic acid to terminate the color reaction at 100 μl.

#### Measurement of NO

The concentration of NO in culture supernatant was determined as nitrite by the Griess reagent (1% sulfanilamide/0.1% naphthylethylenediame dihydrochloride in 2.5% H₃PO₄ (Sigma Aldrich). Cells were seeded into a six-well plate at 3 × 10⁵ cells/well in 3 ml complete medium per well and incubated for 24 h at 37°C. The cells were pretreated with 1 μM EGCG for 1 h and stimulated with LPS (50 ng/ml) for 24 h. Next, supernatant of cell culture medium was collected and assayed for NO production using the Griess reagent. Culture medium (100 μl) was incubated with 100 μl Griess reagent. Absorbance of the mixture was then measured at 535 nm. The concentration of nitrite was converted into sodium nitrite concentration as a standard.
RT-PCR

Cells were harvested by centrifugation and washed twice in PBS. Total cellular RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Ten micrograms total RNA was denatured at 70 °C with 1 μl oligo-dT20 primer (0.5 μg/ml) in 13.8 μl final volume. Primers-templates were then cooled on ice for 10 min, and then 2 μl deoxynucleoside 5′-triphosphate (10 mM), 0.1 μl Moloney murine leukemia virus reverse transcriptase (20 U), 0.1 μl RNAse inhibitor (10 U), and 4 μl 5 × buffer were added in 20 μl final volume. The resultant cDNA samples were subjected to 37 cycles of PCR amplification in the presence of specific sense and antisense primers. Mouse β-actin cDNA was amplified as an internal control. Each cycle consisted of denaturation at 94 °C for 1 min (β-actin, IL-6, and TNF-α), and DNA synthesis at 72 °C for 1 min. Sequences for the PCR primers are as follows: for the β-actin, sense 5′-GGATGCTCCTGTCACTGATTCAAAC-3′ and antisense 5′-TAAAGCCGTCGTAGAAGACCTGCGG-3′; for the TNF-α, sense 5′-TCTCCTAAAGGATGAGAATGTTG-3′ and antisense 5′-TACATTACAGGTTGAGCCTCA-3′; and for the IL-6, sense 5′-AAGTGTTAATCTCAGAGACTACA-3′ and antisense 5′-AAGTGCTCATGCGTTGGTTACATATA-3′. These primer sets yielded PCR products of 276, 443, and 661 bp for TNF-α, IL-6, and β-actin, respectively. Samples were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed.

Real-time quantitative RT-PCR

Total RNA from cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA was treated with RNase-free DNase I (Takara Bio) to remove any contaminating DNA. The RNA concentration was quantified using a spectrophotometer at 260 and 280 (Amersham Biosciences, Piscataway, NJ) and stored at −80 °C preanalysis. Real-time quantitative PCR was performed using the relative standard curve method to quantify the target gene expression. In brief, 1 μl cDNA product was used as the template for RT-PCR, which was performed on the Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq (Takara Bio) according to the manufacturer’s instructions. Sequences for the PCR primers are as follows: for the Tollip, sense 5′-GGTGAAGGTGTGTGCTGAAA-3′ and antisense 5′-TCACTGCTTAGTAGTAC-A3′ for the TollR4, sense 5′-GGGCTCTAACCAAGTCTGTGTTG-3′ and antisense 5′-GGCGGCGTAAGGCACTG-3′; for the TNF-α, sense 5′-ATGCGTACGTTGCTGATTTA-3′ and antisense 5′-AGTGTTACGACCAAGGGCATC-3′. The mRNA levels were obtained from the value of cycle threshold for each specific gene and normalized against the cycle threshold of β-actin.

Immunoblot analysis

Cells were lysed in 100 μl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, 50 mM NaF, 30 mM Na3P2O7, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, and 1 mM pervaean. Whole-cell lysate samples were separated by 8% SDS-PAGE and electrotransferred to nitrocellulose membranes. The nitrocellulose membranes were blocked in 2.5% BSA and incubated with primary Abs for 1 h, followed by incubation with HRP-conjugated secondary Abs for 1 h at room temperature. Equal loading was determined by Ponceau staining of membranes. The bands were visualized using ECL advance kit (GE Healthcare, Little Chalfont, U.K.). Band intensities were quantified using National Institutes of Health (NIH) ImageJ software (Bethesda, MD).

Nuclear extract preparation

Nuclear extracts from cells were prepared as follows. Cells were treated with 100 μl lysis buffer [10 mM HEPES (pH 7.9), 0.1 M KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithioether, 0.5 mM PMSF] on ice for 10 min. Postcentrifugation at 4000 rpm for 5 min, the pellet was resuspended in 100 μl extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF] and incubated on ice for 30 min. Postcentrifugation at 12,000 rpm for 10 min, the pellet containing nuclear extracts was collected and stored at −80 °C until required.

DNA microarray

Cells were pretreated with 1 μM EGCG for 1 h and then stimulated with LPS (50 ng/ml) for 6 h. Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was then amplified using the AmpliChip Biotin Amel Amplification Kit (approved Biosystems, Tokyo, Japan), according to the manufacturer’s instructions, and column purified. Biotinylated RNA (5 μg) was fragmented using fragmentation reagents (Applied Biosystems) and then incubated at 90°C for 7.5 min. The fragmentation reaction was terminated by the addition of stop solution. Hybridization was carried out with a DNA microarray (Genopat, Mitsubishi Rayon, Tokyo, Japan) in 150 μl hybridization buffer (0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20) and 5 μg fragmented biotinylated RNA at 65°C overnight. Posthybridization, the DNA microarray was washed twice in 0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20 at 65°C for 20 min followed by washing in 0.12 M Tris-HCl/0.12 M NaCl for 10 min. The DNA microarray was then labeled with streptavidin-Cy5 (GE Healthcare Bio-Science KK, Tokyo, Japan). The fluorescent labeled-DNA microarray was washed for 5 min four times in 0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20 at room temperature. Hybridization signal acquisition was performed using a DNA microarray reader adopting multibeam excitation technology (Yokogawa Electric, Tokyo, Japan). The DNA microarrays were scanned at multiple exposure times ranging from 0–10 s. Then the intensity values with the best exposure condition for each spot were selected. Data sets of intensities of 209 probe sets per array were analyzed using a Multi Experiment Viewer MA TIGR software (Genopat, Mitsubishi Rayon).

Primary microarray data

The microarray data are available in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE12320.

Flow cytometric analysis

RAW264.7 cells were seeded in six-well plates at 1 × 105 cells/ml and incubated for 24 h. Postincubation, the cells were treated with EGCG (1 μM) for 1, 12, or 24 h. The cell-surface expression of TLR4 was assayed by flow cytometry (FACSCalibur, BD Biosciences, Sunnyvale, CA). The cells were preincubated with 0.5% BSA in PBS for 30 min and washed with PBS. The cells were stained with PE-conjugated TLR4 Ab or isotype control Ab for 45 min at 4°C. Cells were then washed three times with PBS and resuspended in 500 μl PBS. Cells were then analyzed by flow cytometry, and the data were analyzed using CellQuest software (BD Biosciences). The extent of TLR4 expression is represented as the mean fluorescence intensity.

Statistical analysis

All data were expressed as the mean ± SD. Statistical significance was analyzed by Student t test. Each value of p < 0.05, **p < 0.01, or ***p < 0.001 was considered to be statistically significant.

Results

EGCG suppresses LPS-induced production of inflammatory mediators in peritoneal macrophages through 67LR

Recently, we identified 67LR as a cell-surface EGCG receptor that mediates the anticancer action of EGCG at physiologically relevant concentrations (0.1–1 μM) (24). However, it is unknown whether EGCG affects an inflammatory response in macrophage through 67LR. To examine the effect of 1 μM EGCG mediated through 67LR on the production of inflammatory mediators from murine peritoneal macrophages, the cells were treated with either anti-67LR Ab MLuC5 or isotype-matched control Ab. We have previously reported that anti-67LR Ab MLuC5 can block the binding of EGCG to the cell-surface 67LR, thereby inhibiting actions of EGCG (24). Based on this fact, in the current study, a treatment with anti-67LR Ab was performed for blocking the interaction between EGCG and 67LR. These Ab-treated cells were pretreated for 1 h with 1 μM EGCG prior to exposure to LPS. As shown in Fig. 1, production of TNF-α, IL-6, and NO in culture supernatant of murine peritoneal macrophages was significantly increased upon treatment with LPS, and the LPS-induced production of these inflammatory mediators was significantly inhibited by 1 μM EGCG treatment in control Ab-treated cells. However, in anti-67LR Ab-treated cells, the inhibitory effects of EGCG were not observed (Fig. 1). Furthermore, the inhibitory effect of EGCG on the production of inflammatory mediators was not observed in LPS-untriggered cells. These observations suggest that EGCG can act specifically to LPS-triggered inflammatory responses and does not affect resting macrophages. Taken together, these results indicate that the ability
of EGCG to decrease the production of inflammatory mediators was mediated through binding to the 67LR.

**Effect of 67LR downregulation on the expression of LPS signaling mediators and anti-inflammatory action of EGCG**

To explore whether the effects of EGCG on cellular responses to LPS stimulation could be mediated through 67LR, RAW264.7 macrophages were stably transfected with shRNA expression vector to reduce 67LR expression. Total cellular 67LR expression was lowered in RAW264.7 cells transfected with shRNA for 67LR (Fig. 2A). TLR4 has been shown to have a crucial role in LPS-induced inflammatory response. TLR4 activation triggers the activation of two downstream signaling pathways: MyD88-dependent and -independent pathways (1, 4). CD14 is also required as a coreceptor for TLR4 to recognize LPS (29). We evaluated the amounts of TLR4, CD14, and MyD88 expressions in 67LR-downregulated cells and control cells. Immunoblot analysis indicated that the expression of TLR4, CD14, and MyD88 in 67LR-downregulated cells was not altered as compared with the control cells (Fig. 2B). This result suggested that the silencing of 67LR did not affect the expression of TLR4, CD14, and MyD88 and that the binding of LPS to TLR4 may be equal in both 67LR-downregulated cells and control cells. We also examined the EGCG cytotoxicity in both 67LR-downregulated cells and control cells. The cells were treated with 1 and 5 μM EGCG for 24 h and subjected to WST-1 assay to assess the cell viability. As shown in Fig. 2C, EGCG up to 5 μM did not display any cellular toxicity against RAW264.7 cells. Thus, the effects of EGCG were not attributable to cytotoxic effects.

TNF-α, IL-6, and NO as well as iNOS or COX-2 are major mediators involved in systemic inflammation, and these mediators are induced readily in response to LPS (1). Generally, the effect on LPS-triggered production of inflammatory mediators, such as IL-6, TNF-α, and NO, was investigated mainly at 24 h posttreatment with LPS (2). In contrast, iNOS and COX expressions were induced prior to the secretion of inflammatory mediators, thereby investigating these expressions at 12 h poststimulation of LPS (4). To test whether the 67LR silencing affects LPS-induced inflammatory responses, we stimulated 67LR-downregulated RAW264.7 cells with LPS. The downregulation of 67LR did not affect LPS-induced production or expression of these mediators (Fig. 2D–H). We also examined the involvement of 67LR in the inhibitory effect of 1 μM EGCG in LPS-stimulated RAW264.7 cells. 67LR-downregulated cells and control cells were pretreated for 1 h with 1 μM EGCG prior to exposure to LPS. As shown in Fig. 2D–H, production of TNF-α, IL-6, and NO and expression of iNOS and COX-2 proteins were significantly inhibited upon treatment with 1 μM EGCG. However, this inhibitory effect was not observed in 67LR-downregulated cells, suggesting that EGCG at physiological levels suppresses the production or expression of LPS-induced inflammatory mediators through 67LR.

**EGCG suppresses LPS-induced activation of target gene mRNA expression through 67LR**

We examined an involvement of 67LR in the inhibitory effects of EGCG on mRNA expression of inflammatory mediators induced by LPS. Usually, gene expression of inflammatory mediators was known to be induced at ~6 h of LPS stimulation (4). As shown in Fig. 3A, a 6-h LPS treatment markedly upregulated the mRNA expression of TNF-α and IL-6, and 1 μM EGCG significantly inhibited the LPS-induced gene expressions in control cells. However, in 67LR-downregulated cells, the inhibitory effects of 1 μM EGCG on the expression of the aforementioned LPS-induced inflammatory mediators (Fig. 2B, 2C) was not observed (Fig. 3A, 3B).

Next, we investigated the 67LR-mediated inhibitory effects of EGCG on the expression of inflammatory-related genes in LPS-stimulated RAW264.7 cells. Both 67LR-downregulated and control cells were pretreated with 1 μM EGCG for 1 h prestimulation with 50 ng/ml LPS for 6 h. Gene expression was determined using a fibrous DNA microarray carrying 209 genes that were related to inflammation, immunity, and housekeeping (30). Expression of 67 genes among the 209 genes on the DNA microarray was induced by LPS in both control and 67LR-downregulated cells (Supplemental Fig. 1). As shown in Fig. 3C, among the 67 genes induced by LPS in the control cells, EGCG reduced the expression of 21 inflammatory-related genes. However, EGCG did not affect the expression of the aforementioned 21 genes in the 67LR-downregulated cells treated with LPS. These results are consistent with a suggestion that 67LR mediates the suppressive effect of a physiologically relevant EGCG on the expression of LPS-induced inflammatory-related genes.

**Effect of 67LR downregulation on EGCG-induced inactivation of MAPK signal pathway**

MAPKs are a family of proteins, including ERK1/2, p38, and JNK (31). These pathways convert a large variety of extracellular signals, leading to a wide range of cellular responses, such as growth, differentiation, inflammation, and apoptosis (32, 33). Furthermore, mRNA expressions of inflammatory cytokines, such as TNF-α and...
IL-6, are increased by activation of MAPKs (2). Thus, MAPKs are important targets for anti-inflammatory activity. Previous studies demonstrated the suppressive effect of high doses of EGCG on the LPS-induced MAPK pathway, but the influence of physiologically relevant levels of EGCG is entirely unknown (34). Therefore, we examined whether 1 μM EGCG suppresses LPS-induced MAPK activation through 67LR (Fig. 4). The phosphorylation of MAPKs including ERK1/2, p38, and JNK was measured by immunoblot analysis using specific Abs. LPS-induced phosphorylation of ERK1/2, p38, and JNK was inhibited by EGCG (Fig. 4A–C). However, in 67LR-ablated cells, the inhibitory effect of EGCG on LPS-induced upregulation of phosphorylation was attenuated (Fig. 4A–C).

c-Jun, a major component of AP-1, is activated through double phosphorylation by the JNK pathway (35). In control cells, 1 μM EGCG caused ~50% reduction of LPS-induced phosphorylation of c-Jun, whereas in 67LR-downregulated cells, EGCG did not...

**FIGURE 2.** 67LR is indispensable for anti-inflammatory action of EGCG in RAW264.7 cells. A, Expression of 67LR in the cells transfected with the 67LR-shRNA vector was measured by immunoblot analysis using 67LR Ab. B, Amounts of TLR4, CD14, and MyD88 expressions in the 67LR-downregulated and control cells were measured by immunoblot analysis using each specific Ab. In both A and B, the left panel displays protein levels from the same filter blotted again with the anti-β-actin Ab. The band intensities quantified using NIH ImageJ software. C, Cells were treated with EGCG (0, 1, and 5 μM) for 24 h, and cell viability was assessed by a WST-1 assay. The results were shown as relative cell number to untreated control. D–F, RAW264.7 cells were incubated with EGCG (1 μM) for 1 h and then treated with LPS (50 ng/ml) for 24 h. D and E, The amount of TNF-α and IL-6 in culture medium was measured by ELISA. F, NO level in the culture medium was measured by the Griess assay. G and H, RAW264.7 cells were incubated with EGCG (1 μM) for 1 h and then treated with LPS (50 ng/ml) for 12 h. Whole-cell lysates were used for analysis of the amount of iNOS (G), COX-2 (H), and β-actin by immunoblotting using each specific Ab, as described in Materials and Methods. Relative band intensity of each protein was normalized for β-actin. All data were expressed as the mean ± SD (n = 3). Statistical significance was analyzed by Student t test. *p < 0.05; **p < 0.01; ***p < 0.001.
inhibit LPS-induced phosphorylation of c-Jun (Fig. 4D). Taken together, these results indicate that EGCG at 1 μM inhibits MAPKs pathway through 67LR.

FIGURE 3. EGCG suppresses LPS-induced target gene mRNA expression through 67LR. RAW264.7 cells were pretreated with 1 μM EGCG for 1 h and then stimulated with LPS (50 ng/ml) for 6 h. A and B, Total RNA were collected from each cells under the indicated conditions, and mRNA expression levels of TNF-α, IL-6, and β-actin were measured by RT-PCR. Relative band intensity of each gene was normalized for β-actin. C, Total RNA was collected from each cell under the indicated conditions, and inflammation-relating gene expression levels were measured by cDNA macroarray. All data were expressed as the mean ± SD (n = 3). Statistical significance was analyzed by Student t test. *p < 0.05; **p < 0.01; ***p < 0.001.

EGCG inhibits LPS-induced NF-κB activation through 67LR

NF-κB is one of the most important transcription factors in the production of inflammatory mediators and has a critical role in the
induced phosphorylation and degradation of IκBα. Activation of NF-κB factors, p50 and p65, which are activated by LPS in macrophages such as innate immunity, cellular proliferation, and survival (4). The regulation of a wide variety of genes important in cellular responses, shown as mean ± SD (n = 3). Statistical significance was analyzed by Student’s t test. **p < 0.01.

**FIGURE 4.** EGCG inhibits LPS-induced activation of MAPK signal pathway through 67LR. RAW264.7 cells were pretreated with 1 μM EGCG for 1 h and then stimulated with LPS (50 ng/ml) for 45 min. Cell lysates were subjected to SDS-PAGE, and immunoblot analysis was performed using each specific Ab to phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-JNK (p-JNK), JNK, phospho-p38 (p-p38), p38, phospho-c-Jun (p-c-Jun), and c-Jun. Band intensities were quantified using NIH ImageJ software. The total MAPK levels were used as an internal control, and relative band intensity was expressed as a percentage compared with the value of untreated control. The results shown are typically of three experiments done under each condition. The data are shown as mean ± SD (n = 3). Statistical significance was analyzed by Student’s t test. **p < 0.01.

**EGCG downregulates TLR4 protein expression through 67LR**

TLRs are pathogen recognition molecules that activate the immune system as part of the innate immune response. Among several kinds of TLR, TLR4 is activated by LPS and is required for LPS-induced NF-κB and AP-1 activation and chemokine expression (1). To investigate whether EGCG suppresses the expression of TLR4 via 67LR, both control and 67LR-downregulated cells were treated with 1 μM EGCG. The cell-surface expression and total protein level of TLR4 were measured by flow cytometric analysis and immunoblot analysis, respectively. In addition, we also performed real-time PCR assay to ascertain the TLR4 gene expression level in control-shRNA and 67LR-shRNA samples. In both 67LR-downregulated cells and control cells, protein level and cell-surface expression of TLR4 were not affected under a 1-h EGCG treatment (Fig. 6A, 6B). In contrast, when the cells were incubated with 1 μM EGCG for 24 h, total cellular protein expression of TLR4 was markedly reduced in control-shRNA cells (Fig. 6C, 6D), which is consistent with the result that TLR4 mRNA expression level was decreased by EGCG treatment for 24 h (Fig. 6E). However, the suppressive effect of EGCG was not observed in 67LR-downregulated cells (Fig. 6D, 6E). Furthermore, in control-shRNA cells, the cell-surface expression of TLR4 was suppressed by treatment with EGCG for 24 h, but the cell-surface expression was not affected at 12 h of EGCG treatment (Fig. 6F). In 67LR-ablated cells, the suppressive effect of EGCG at 24 h was not observed (Fig. 6F). These results indicate that EGCG downregulates the TLR4 expression at mRNA level, and 67LR is essential for the effect of EGCG on TLR4.

**EGCG upregulates Tollip protein expression through 67LR**

Although the downregulation of TLR4 expression was observed in the cells treated with EGCG for 24 h, the expression was not affected under a 1-h EGCG treatment. Because these results did not correlate with the inhibitory effect of EGCG on MAPK inhibition shown in Fig. 4, we investigated another inhibitory target responsible for the anti-inflammatory action of EGCG. TLR signaling pathways are tightly regulated by endogenous regulators at multiple levels. Inhibitory proteins, such as SOCS1, IRAKM, SIGIRR, and Tollip, selectively suppress IRAK function by targeting various stages of
the TLR signaling pathways (36). To elucidate additional mechanisms underlying EGCG-mediated anti-inflammatory action, we examined whether EGCG induces the negative regulators of TLRs. Both 67LR-downregulated cells and control cells were treated with 1 μM EGCG for 1 h, and then the expressions of negative regulators of TLRs were measured by immunoblot analysis. Intriguingly, we found that the protein level of Tollip in control-shRNA cells was upregulated by 1 μM EGCG treatment, whereas the other protein levels were not affected (Fig. 7A). In 67LR-ablated cells, such an effect of EGCG was not observed (Fig. 7A). Furthermore, we found that EGCG elevated the expression of Tollip in a time- or dose-dependent manner in LPS-untreated control-shRNA cells (Fig. 7B, 7C). We also examined whether EGCG has a similar effect in primary macrophages. Murine peritoneal macrophages were incubated with either anti-67LR Ab or control Ab for 1 h, then treated with EGCG (1 μM) for 1 h. EGCG was found to upregulate the expression of Tollip. However, in anti-67LR Ab-treated cells, such an effect of EGCG was not observed (Fig. 7D). To further elucidate the mechanism of EGCG on Tollip expression, the effect of EGCG on mRNA expression of Tollip was investigated. In control-shRNA cells without the stimulation of LPS, Tollip gene expression was significantly increased by EGCG treatment for >30 min, whereas the elevation was not observed in 67LR-ablated cells (Fig. 7E). Considering this result and the fact that an increase in the expression of Tollip protein was observed after a 60-min treatment (Fig. 7B), it is suggested that 1 μM EGCG transcriptionally enhances the expression of Tollip, a negative regulator of TLR4, and the anti-inflammatory actions of EGCG mediated through 67LR may be due in part to the upregulation of Tollip protein.

**Discussion**

Cell-surface interactions are one of the most important events for the initiation of biological responses to extracellular stimuli. In this paper, we demonstrate that EGCG action through the cell-surface receptor 67LR can negatively regulate TLR4 signaling.

67LR has been shown to be a dominant laminin-binding protein for neutrophils, macrophages, and monocytes. Moreover, this receptor has been implicated in laminin-induced tumor cell attachment (37), migration (38), and shear stress-dependent endothelial NO synthase expression (39). 67LR has a role as a receptor for viruses, such as Sindbis virus and Dengue virus, as well as prion protein (21, 22). These findings suggest that 67LR may play a significant role in the regulation of cell adherence via the basement membrane laminin and in signal transduction following the binding event (40). Previously, we have reported for the first time that the inhibitory effect of EGCG on tumor cell proliferation is exerted through its binding to the 67LR as a cell-surface receptor (24). We also demonstrated that EGCG inhibits cell growth by reducing the myosin regulatory L chain phosphorylation mediated through 67LR (25). Moreover, we found that EGCG suppresses the expression of FcεRI and histamine release through its binding to 67LR (26, 27). Nonetheless, the relationship between 67LR and anti-inflammatory activity of EGCG in LPS-stimulated macrophages is still unknown.
In this study, all inhibitory actions of EGCG were shown to be mediated through 67LR, suggesting that 67LR has a pivotal role as a cell-surface receptor that confers the inhibitory effect of EGCG on TLR4-triggered signaling in macrophages.

In previous studies, EGCG was found to have an enhanced inhibitory effect on TNF-α release from BALB/3T3 cells treated with okadaic acid (41). EGCG was shown to inhibit the degradation of IRAK induced by IL-1β in A549 cells (42). This polyphenol was also demonstrated to inhibit LPS-induced activation of MAPK pathways, including ERK1/2, p38, and JNK (34). It has been described that EGCG suppresses LPS-induced activation of NF-κB by blocking the degradation of IκB-α following IκB-α phosphorylation (7). In addition, EGCG was found to inhibit the production of inflammation mediators, such as TNF-α, IL-6, and IL-8, through the inhibition of the intracellular Ca2+ level (43). Furthermore, 50 μM EGCG was reported to be able to modulate both MyD88- and TRIF-dependent signaling pathways of TLRs (16). Although this research has proposed various different mechanisms for the anti-inflammatory action of EGCG, it is still not clear which EGCG-induced molecular events are responsible for the inhibition of

**FIGURE 6.** EGCG downregulates TLR4 protein expression through 67LR. A and B, RAW264.7 cells were treated with 1 μM EGCG for 1 h. A, Cell lysates were subjected to SDS-PAGE, and total TLR4 protein expression was determined by immunoblotting with specific anti-TLR4 Ab. Band intensities were quantified using NIH ImageJ software. Relative band intensity was expressed as a percentage compared with the value of untreated control. The data are shown as mean ± SD (n = 3). B, Cell-surface TLR4 expression was determined by flow cytometry using a specific Ab. C, Control-shRNA cells were treated with 1 μM EGCG for the indicated time periods. Cell lysates were subjected to SDS-PAGE, and total TLR4 protein expression was determined by immunoblotting with specific anti-TLR4 Ab. D, RAW264.7 cells were treated with 1 μM EGCG for 24 h, and then cell lysates were subjected to SDS-PAGE and total TLR4 protein expression determined by immunoblotting with specific anti-TLR4 Ab. E, RAW264.7 cells were treated with 1 μM EGCG for the indicated time periods, and then TLR4 mRNA expression was determined by real-time PCR analysis. mRNA levels were normalized to the levels of β-actin mRNA. The data are shown as mean ± SD (n = 3). F, RAW264.7 cells were treated with 1 μM EGCG for 12 or 24 h, and then the cell-surface TLR4 expression was determined by flow cytometry using a specific Ab. Statistical significance was analyzed by Student t test. *p < 0.05.
inflammation because the concentrations of EGCG (10–50 μM) shown to have an inhibitory effect in most previous works are much higher than the concentrations observed in the plasma or tissues of animals or in human plasma (usually lower than 1 μM) after tea ingestion (6). EGCG is the only known polyphenol presented in plasma in a large proportion (77–90%) of a free form, although the other catechins are highly conjugated with glucuronic acid and/or sulfate group (44). Based on these circumstances, the activities observed at 1 μM EGCG are relevant to the in vivo situations.

In the current study, we showed for the first time that 1 μM EGCG significantly inhibited the expression of inflammatory mediators, such as TNF-α, IL-6, iNOS, and COX-2, by blocking the activation of MAPK and NF-κB pathways through 67LR in LPS-induced RAW264.7 cells. These findings demonstrate that 67LR is responsible for mediating the anti-inflammatory action of EGCG and participates critically in the cell signaling pathway and cell-surface interaction. EGCG at a physiologically relevant concentration may contribute to regulation of inflammation in people drinking green tea as a daily beverage.

TLRs play a crucial role in both the innate and adaptive immune responses of a host to microbial pathogens (1, 4). It has been reported that EGCG blocked the interaction of LPS with TLR4 by creating a sealing effect (7). However, the concentrations of EGCG used in the study are much higher than those observed in the blood or tissues. In this study, we found that 1 μM EGCG can reduce the cell-surface expression and protein level of TLR4, and the suppressive effect of EGCG is mediated through 67LR. In a 24-h treatment with EGCG, there was 25% reduction of total cellular protein expression of TLR4, and the reduction rate of mRNA expression of TLR4 was also ∼25%, suggesting that EGCG at least downregulates the TLR4 expression at the transcription level. Furthermore, the reduction rate of the protein level...
was enhanced to 40% by longer treatment duration up to 60 h (Fig. 6C). In contrast, the reduction rate of the cell-surface expression was 55% at 24-h treatment (Fig. 6F). The diminish rate of the cell-surface expression of TLR4 by EGCG was higher than that of total cellular expression, implicating the presence of a posttranslational mechanism. In previous studies, the proper surface expression of TLR4 has been suggested to be regulated by either enhancing or diminishing the retention of TLR4 in the Golgi (45). In addition, TLR4 has been shown to recycle rapidly between the plasma membrane and the Golgi apparatus in the absence of ligand (46). Therefore, a traffic regulation may be involved in the difference between the cell-surface expression and total cellular expression of TLR4. To date, there is little information regarding potential regulatory factors of TLR4 expression. At present, we cannot provide the conclusive mechanism for the action of EGCG on TLR4 expression in the present data alone. Further research is needed to resolve the complexity of regulation of TLR4 expression.

TLR4-mediated activation of NF-κB and MAPK was known to be mediated via the MyD88-dependent IRAK-1 pathway or MyD88-independent TRIF pathway (4, 16). Overexpression of Tollip impairs TLR4-triggered NF-κB and MAPK signaling pathways (47). Tollip also interacts with IRAK-1 prior to stimulation and suppresses IRAK-1’s kinase activity (48), indicating that Tollip negatively regulates TLR4 signaling. In addition, it was recently reported that

FIGURE 8. Tollip mediates the TLR4 signaling inhibitory action of EGCG. A. RAW264.7 cells were transfected with the Tollip-shRNA vector. Protein levels of Tollip were detected by immunoblotting. B. Cells were treated with 1 μM EGCG for 1 h. Total cellular proteins were resolved by SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes and detected with anti-Tollip Ab. C. Cells were incubated with 1 μM EGCG for 1 h prior to a 24-h treatment with 50 ng/ml LPS. The amount of TNF-α and IL-6 in culture medium was measured by ELISA. The data are expressed as the mean ± SD (n = 3). D. Cells were pretreated with 1 μM EGCG for 1 h, and then stimulated with 50 ng/ml LPS for 6 h. Total RNA was prepared, and mRNA expression levels of IL-6, TNF-α, and β-actin were measured by RT-PCR. Relative band intensity of each gene was normalized by β-actin. E. Cells were treated with 1 μM EGCG for 24 h. Cell lysates were processed as in A, and total TLR4 protein expression was determined by immunoblotting with specific anti-TLR4 Ab. Band intensities were quantified using NIH ImageJ software. Relative band intensity was expressed as a percentage compared with the value of untreated control. The data are shown as mean ± SD (n = 3). Statistical significance was analyzed by Student t test. *p < 0.05; **p < 0.01; ***p < 0.001.
mice lacking Tollip caused an abrogation of inflammatory responses (49), suggesting that Tollip also possesses a potential role in proinflammatory signaling. In this study, we showed that 1 μM EGCG upregulated the expression of Tollip through 67LR, and the elevation was regulated at the transcriptional level. Taken together, our findings that EGCG suppresses TLR4 signaling through the 67LR-mediated upregulation of Tollip expression suggest that the action of EGCG may be due to the inhibition of the MyD88-dependent pathway.

Chemoprevention by edible phytochemicals is now considered to be an inexpensive, readily applicable, and accessible approach to inflammation control and management (50); however, little is known about the mechanism of the chemopreventive action of most phytochemicals, including EGCG. In the current study, we showed for the first time that 67LR and Tollip are indispensable for anti-inflammatory action of a physiological concentration of EGCG, and these proteins mediate EGCG-triggered unique signaling for negative regulation of TLR4 signaling. Our results not only illuminate the mechanisms for the inflammation inhibitory activity of EGCG, but also should help in the design of new strategies to prevent inflammatory diseases. Therefore, strategies for upregulation of Tollip and downregulation of TLR4 expressions via 67LR may prove effective in treating both chronic and acute inflammatory diseases.

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Disclosures
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References


