Biochemical and Biophysical Research Communications 426 (2012) 480–485

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Green tea polyphenol epigallocatechin-3-gallate inhibits TLR4 signaling through the 67-kDa laminin receptor on lipopolysaccharide-stimulated dendritic cells

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ARTICLE INFO

Article history:
Received 14 August 2012
Available online 29 August 2012

Keywords:
Epigallocatechin-3-gallate
Dendritic cell
Toll-like receptor
67-kDa laminin receptor

ABSTRACT

Epigallocatechin-3-gallate (EGCG), a major active polyphenol of green tea, has been shown to down-regulate inflammatory responses in dendritic cells (DCs); however, the underlying mechanism has not been understood. Recently, we identified the 67-kDa laminin receptor (67LR) as a cell-surface EGCG receptor. In this study, we showed the molecular basis for the down-regulation of toll-like receptor 4 (TLR4) signal transduction by EGCG in DCs. The expressions of CD80, CD86, and MHC class I and II, which are molecules essential for antigen presentation by DCs, were inhibited by EGCG via 67LR. In addition, EGCG-treated DCs inhibited lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, and IL-6) and activation of mitogen-activated protein kinases (MAPKs), e.g., extracellular signal-regulated kinase 1/2 (ERK1/2), p38, c-Jun N-terminal kinase (JNK), and nuclear factor κB (NF-κB) p65 translocation through 67LR. Interestingly, we also found that EGCG markedly elevated the expression of the Tollip protein, a negative regulator of TLR signaling, through 67LR. These novel findings provide new insight into the understanding of negative regulatory mechanisms of the TLR4 signaling pathway and consequent inflammatory responses that are implicated in the development and progression of many chronic diseases.

1. Introduction

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that play a pivotal role in inducing primary immune responses [1]. Many studies regarding the function of DCs have mainly focused on the ability of these cells to express MHC class II and co-stimulatory molecules, including CD80 and CD86, which induce T cell proliferation [2,3]. Immature DCs have the ability to capture and internalize antigens (Ags) via pattern recognition receptors (PRRs), recognizing conserved molecular moieties that distinguish a broad variety of microbial products, the so-called pathogen-associated molecular patterns (PAMPs) [4]. Among various PRRs, toll-like receptors (TLRs) play a central role in the activation of the innate immune response and are pathogen recognition proteins that have important roles in detecting microbes and initiating inflammatory responses [5,6]. The intracellular signaling pathways activated by TLRs are mediated through several adaptor molecules, including myeloid differentiation factor 88 (MyD88) and toll/interleukin 1 receptor (IL-1R) domain-containing adaptor (TRIF), and ultimately lead to activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) [5]. These signaling processes enable DCs to become potent APCs to initiate robust innate immunity by up-regulating the expression of surface molecules, including CD80, CD86, and MHC class I and II, a number of cytokines such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α, and losing endocytic/phagocytic receptors [3,4]. As mentioned above, DCs play a pivotal role in controlling immune responses. Lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria, is one of the most powerful activators of TLR4 signaling.

Green tea (*Camellia sinensis* L.) is a popular beverage worldwide, and its possible health effects have been the subject of considerable attention [7]. The green tea polyphenols include catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG). Among the green tea polyphenols, EGCG has been focused on in recent years because of its anti-inflammatory and immunomodulatory activities [8–11]. Recently, EGCG has been reported to decrease LPS-induced TNF-α production in macrophages [9]. In addition,

Abbreviations: EGCG, epigallocatechin-3-gallate; 67LR, 67-kDa laminin receptor; DCs, dendritic cells; LPS, lipopolysaccharide; Tollip, toll-interacting protein; TLR, toll-like receptor; Iκ-B, inhibitor of κB; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin; MAPK, mitogen-activated protein kinase; SOCS1, suppressor of cytokine signaling 1; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation factor 88; TIR, toll/interleukin-1 receptor homology; TRIF, TIR domain containing adapter inducing interferon-β.

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http://dx.doi.org/10.1016/j.bbrc.2012.08.096

EGCG inhibits activation of MAPK and NF-κB, which are induced by pro-inflammatory stimuli such as LPS in DCs [12]. Moreover, EGCG profoundly inhibited CD80, CD86, and MHC class I and II expression on DCs [12]. Although some mechanisms for the anti-inflammatory activities of EGCG have been proposed, the molecular mechanism for the inhibitory action of EGCG in TLR4 ligand-stimulated DCs has not yet been clarified.

The 67-kDa laminin receptor (67LR) is a non-integrin cell-surface receptor with high affinity for laminin [13]. Its role as a laminin receptor makes it an important molecule in cell adhesion to the basement membrane and metastasis of tumor cells [14]. Recently, it was identified 67LR as a cell-surface EGCG receptor that mediates the anti-cancer action of EGCG at physiologically achievable concentrations (0.1–1 μM) [15]. Others showed that RNAi-mediated silencing of 67LR results in abrogation of EGCG-induced apoptosis in multiple myeloma cells [16]. Furthermore, this receptor has also been shown to be responsible for the inhibitory action of EGCG on degranulation in basophils [17,18]. In macrophages, EGCG inhibits LPS-induced TLR4 signaling and peptidoglycan-induced TLR2 signaling through 67LR [19]. However, the relationship of 67LR and anti-inflammatory activity of EGCG in LPS-stimulated DCs remains unclear.

Therefore, in this study, we tried to elucidate the molecular basis for the down-regulation of TLR4 signal transduction by EGCG on DCs. Here, we show that 67LR is essential for mediating the anti-inflammatory action of EGCG in LPS-stimulated DCs.

2. Materials and methods

2.1. Antibodies and reagents

Recombinant mouse granulocyte–macrophage colony stimulating factor (GM-CSF), recombinant IL-4, and the fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) kit were purchased from R&D Systems (Minneapolis, MN). EGCG was purchased from Sigma–Aldrich (St. Louis, MO). LPS from *Escherichia coli* O111:B4 was purchased from Sigma and Invivogen (San Diego, CA). Anti-67LR monoclonal antibody (mAb) (MLuC5) was purchased from Neo-Markers (Fremont, CA). Anti-TLR4 polyclonal Ab, anti-CD14 polyclonal Ab, anti-Tollip mAb, and anti-67LR (F-18) polyclonal Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphorylated ERK1/2 mAb, anti-phosphorylated JNK mAb, anti-phosphorylated p38 mAb, anti-NF-κB (p65) polyclonal Ab, anti-phosphorylated inhibitor of κB (IκB)-α mAb, and anti-lamin B polyclonal Ab were obtained from Santa Cruz Biotechnology, Inc. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG Ab and HRP-conjugated anti-rabbit Ab were obtained from Calbiochem (San Diego, CA), and anti-β-actin mAb (AC-15) was purchased from Sigma–Aldrich. FITC-conjugated mAb to CD11c, phycoerythrin (PE)-conjugated mAb to CD80, CD86, MHC class I, and MHC class II were purchased from eBioscience (San Diego, CA). IL-6, IL-1β, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences (San Diego, CA).

2.2. Generation and culture of DCs

Murine bone marrow-derived DCs were prepared and cultured as recently described [20]. To obtain highly purified populations (>95% cell purity) in some experiments, the DCs were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by positive selection on paramagnetic columns (LS columns; Miltenyi Biotech) according to the manufacturer's instructions.

2.3. Anti-67LR Ab treatment

Bone marrow-derived DCs (BMDCs) were seeded on plates and incubated at 37 °C in 5% CO_2 for 24 h before any treatment. Then, cells were incubated with anti-67LR Ab, MLuC5 (20 μg/mL), or isotype-matched control mouse IgM (20 μg/mL) at 37 °C in 5% CO_2 for 1 h before the addition of EGCG or LPS.

2.4. Cytotoxicity analysis

EGCG was added to cultures of isolated BMDCs in 12-well plates (0.5×10^6 cells/mL). To investigate the cytotoxic effect of EGCG on BMDCs, the cell death pattern of BMDCs was analyzed after treatment with EGCG. After 24 h of treatment, harvested DCs were washed with phosphate-buffered saline (PBS) and stained by FITC-annexin V/PI (BD Biosciences). Thereafter, cytotoxicity against DCs was analyzed by using FACSCanto flow cytometry (BD Biosciences).

2.5. Measurement of cytokines

ELISA was used for detecting IL-6, IL-1β, and TNF-α in culture supernatants as described previously [20].

2.6. Analysis of the expression of surface molecules by flow cytometry

On day 6, BMDCs were harvested, washed with PBS, and re-suspended in fluorescence-activated cell sorter washing buffer (2% FBS and 0.1% sodium azide in PBS). The cells were preincubated with 0.5% BSA in PBS for 30 min and washed with PBS. The cells were stained with PE-conjugated anti-H-2Kb (MHC class I), anti-I-Ab (MHC class II), anti-CD80, and anti-CD86 along with FITC-conjugated anti-CD11c for 45 min at 4 °C. Cells were washed 3 times with PBS and re-suspended in 500 μL PBS. The fluorescence was measured by flow cytometry and the data were analyzed using CellQuest data analysis software.

2.7. Immunoblotting analysis

BMDCs 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 Na_4PO_7, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μg/mL aprotinin, and 1 mM pervanadate. Whole-cell lysate samples were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The membranes were blocked in 5% skim milk and incubated with the respective Ab for 2 h, followed by incubation with HRP-conjugated secondary Ab for 1 h at room temperature. Epitopes on target proteins, including MAPKs and NF-κB, recognized specifically by the used Abs were visualized using the ECL advance kit (GE Healthcare, Little Chalfont, UK).

2.8. Nuclear extract preparation

Nuclear extracts from cells were prepared as follows: DCs were treated with 100 μL lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5 mM PMSF) on ice for 10 min. Following centrifugation at 4000 rpm for 5 min, the pellet was re-suspended 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. After centrifugation at 12,000 rpm for 10 min, the supernatant containing nuclear extracts was collected and stored at −80 °C until required.

2.9. Statistical analysis

All experiments were repeated at least 3 times with consistent results. The levels of significance for comparison between samples were determined by Tukey's multiple comparison test distribution using statistical software (GraphPad Prism Software, version 4.03; GraphPad Software, San Diego, CA). The data in the graphs are expressed as mean ± SEM. Each value of $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ was considered to be statistically significant.

3. Results

3.1. Cytotoxicity of EGCG-treated DCs

TLR4 has been shown to have a crucial role in LPS-induced inflammatory responses, and CD14 is required as a co-receptor for TLR4 to recognize LPS. We evaluated the expression of TLR4 and CD14 in anti-67LR Ab-treated cells and control cells. Immunoblot analysis indicated that the expression of TLR4 and CD14 in anti-67LR Ab-treated cells was not altered compared with control cells (Fig. 1A). Furthermore, in control cells, the protein level of 67LR and TLR4 were not affected by treatment with 10 μM EGCG for 24 h (Fig. 1B). These results suggest that EGCG treatment did not affect the expression of 67LR, and that the binding of LPS to TLR4 may be equal in both anti-67LR Ab-treated cells and control cells. We also examined the EGCG-induced cytotoxicity in DCs. Cells were treated with 10 μM EGCG for 24 h and stained with anti-CD11c, annexin V, and PI to assess cell viability. As shown in Fig. 1C, treatment with EGCG (up to 10 μM) did not result in any cellular toxicity against DCs. This finding suggests that EGCG is not cytotoxic to DCs and does not contain significant amounts of endotoxin that would potentially interfere with our studies using concentrations below 10 μM.

3.2. EGCG suppresses LPS-induced production of inflammatory cytokines in DCs through 67LR

A previous report identified 67LR as a cell-surface EGCG receptor that mediates the anti-cancer action of EGCG at physiologically relevant concentrations (0.1–1 μM) [15]. However, it is unknown whether EGCG affects an inflammatory response in DCs through 67LR. To examine the effect of EGCG mediated through 67LR on the production of inflammatory cytokines from DCs, cells were treated with either isotype-matched control Ab or anti-67LR Ab which has been shown to be able to block the binding of EGCG to the cell surface 67LR [15]. Ab-treated cells were pretreated for 1 h with EGCG prior to exposure to LPS. As shown in Fig. 1D, levels of IL-6, IL-1β, and TNF-α in the culture supernatant from DCs were increased upon treatment with LPS, and the LPS-induced production of these inflammatory mediators was inhibited by EGCG

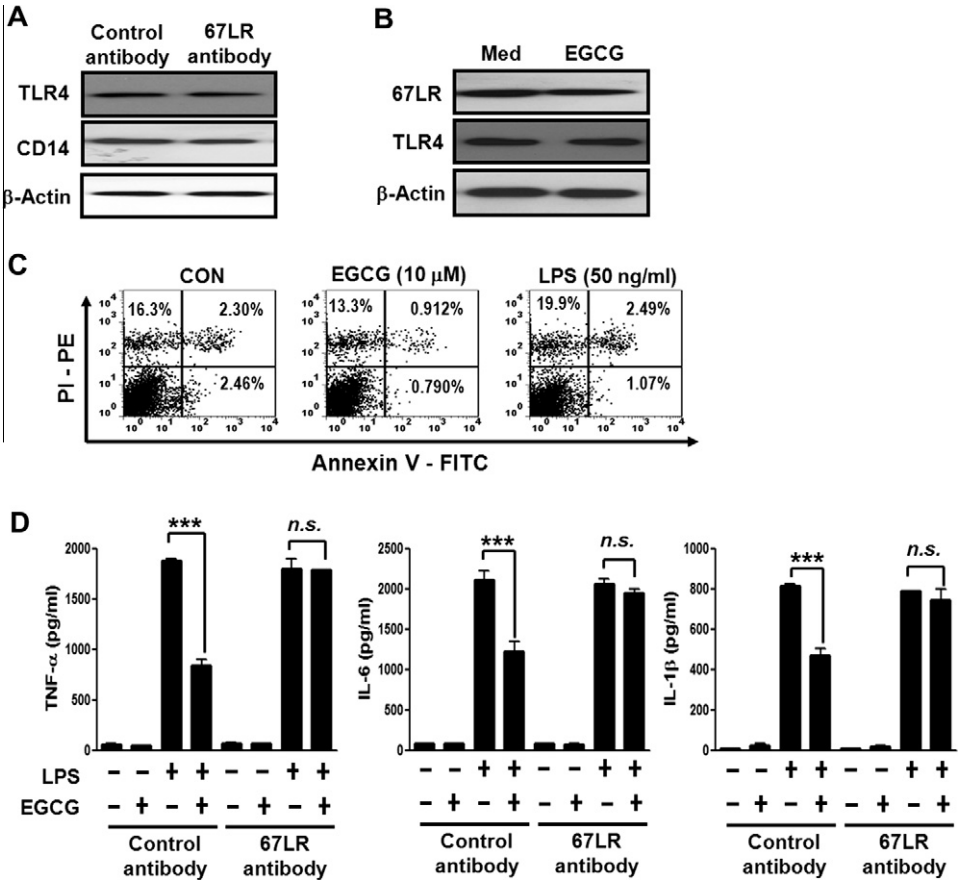

Fig. 1. Anti-inflammatory action of EGCG-treated DCs is mediated by 67LR. (A) DCs were incubated with either anti-67LR antibody (Ab) or control Ab for 24 h. The expression levels of TLR4 and CD14 in anti-67LR Ab-treated cells and control cells were measured by immunoblot analysis using specific Abs. (B) DCs were treated with EGCG (10 μM) for 24 h, and protein expression of 67LR and TLR4 was determined by immunoblot analysis using specific Abs. (C) EGCG was added on day 6, and cultures were harvested 24 h later. Cell viability of EGCG-treated DCs was analyzed by flow cytometry. DCs were stained with anti-CD11c, annexin V, and PI. The percentage of positive cells (annexin V- and PI-stained cells) in each quadrant is indicated. The results are representative of the results of three experiments. (D) DCs were incubated with either an anti-67LR Ab or control Ab for 1 h. Then, the cells were then pretreated with EGCG (10 μM) for 1 h before exposure to LPS (50 ng/mL) for 24 h. The concentrations of TNF-α, IL-6, and IL-1β in the culture medium were measured by ELISA. All data are expressed as the mean ± SD ($n = 3$) and statistical significance ($^{***}p < 0.001$) is shown for the treatments compared to the controls. The value of *n.s.* was defined as no significant effect.

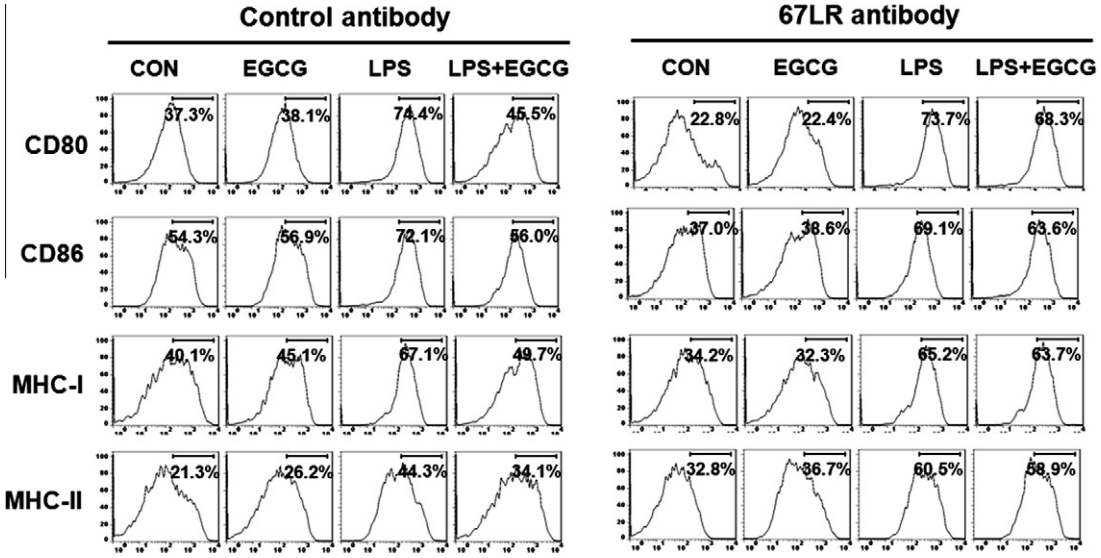

Fig. 2. EGCG suppresses the maturation of DC through 67LR. DCs were incubated with either an anti-67LR Ab or control Ab for 1 h. The cells were then pretreated with EGCG (10 μM) for 1 h before exposure to LPS (50 ng/mL) for 24 h. The cells were gated for CD11c. DCs were stained with anti-CD80, anti-CD86, or anti-MHC class I or anti-MHC class II. The percentage of positive cells is shown for each panel.

treatment. However, in anti-67LR Ab-treated cells, the inhibitory effects of EGCG were significantly lower than those observed in control Ab-treated cells (Fig. 1D). These results indicate that the ability of EGCG to reduce the production of inflammatory cytokines is mediated through its binding to the 67LR.

3.3. EGCG suppresses LPS-induced maturation of DCs through 67LR

To investigate whether EGCG induces DC maturation through 67LR, we measured the expression of DC maturation markers such as CD80, CD86, and MHC classes I and II. DCs were incubated with either an anti-67LR Ab or control Ab for 1 h, then treated with EGCG for 1 h, and analyzed for the expression of surface markers. LPS was used as positive control. EGCG was found to down-regulate the LPS-induced expression of the DC maturation markers CD80, CD86, and MHC classes I and II (Fig. 2). However, in anti-67LR Ab-treated cells, this effect of EGCG was not observed (Fig. 2). These results are consistent with the hypothesis that 67LR mediates the suppressive effect of EGCG on LPS-induced maturation of DCs.

3.4. Effect of 67LR down-regulation on EGCG-induced inactivation of the MAPK signal pathway

MAPKs are important targets for investigating anti-inflammatory activity [21]. We examined whether EGCG suppresses LPS-induced MAPK activation through 67LR. 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 treatment with EGCG (Figs. 3A). However, in anti-67LR Ab-treated cells, the inhibitory effect of EGCG on LPS-induced up-regulation of phosphorylation was attenuated (Fig. 3A). These results indicate that EGCG inhibits the MAPK pathway in DCs through 67LR.

3.5. 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 [22]. In this study, we investigated whether EGCG inhibits phosphorylation of IκB-α. As shown in Fig. 3B, EGCG inhibited LPS-induced phosphorylation of IκB-α in control cells, whereas the inhibitory effect of EGCG was attenuated in anti-67LR Ab-treated cells, suggesting that EGCG inhibits LPS-induced phosphorylation and degradation of IκB-α through 67LR. Next, we examined the effect of EGCG on nuclear translocation of p65 induced by LPS. In parallel with the phosphorylation of IκB-α, treatment of the cells with LPS increased nuclear translocation of p65. We found that EGCG significantly suppressed LPS-induced nuclear translocation of p65 in control cells, whereas in anti-67LR Ab-treated cells, this effect of EGCG was not observed (Fig. 3C). These results indicate that EGCG inhibits LPS-induced NF-κB activation through 67LR by suppressing IκB-α phosphorylation, resulting in the inhibition of nuclear translocation of p65.

3.6. EGCG up-regulates Tollip protein expression through 67LR

Overexpression of Tollip causes suppression of TLR-mediated NF-κB activation, indicating that Tollip negatively regulates TLR signaling [23]. In addition, it has recently been demonstrated that EGCG induced a rapid up-regulation of Tollip through 67LR in macrophages [18]. Therefore, we examined whether EGCG has a similar effect in DCs. DCs were incubated with either an anti-67LR Ab or control Ab for 1 h, and then treated with EGCG. We found that EGCG elevated the expression of Tollip in a time-dependent manner in LPS-untreated DCs (Fig. 4A). Furthermore, EGCG was found to up-regulate the expression of Tollip in control cells, whereas in anti-67LR Ab-treated cells, this effect of EGCG was not observed (Fig. 4B). Considering this result, the anti-inflammatory actions of EGCG mediated through 67LR may be due to, in part, the up-regulation of Tollip protein expression.

4. Discussion

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

67LR has been reported to be a dominant laminin-binding protein expressed in neutrophils, macrophages, and monocytes. In addition, this receptor has been implicated in laminin-induced

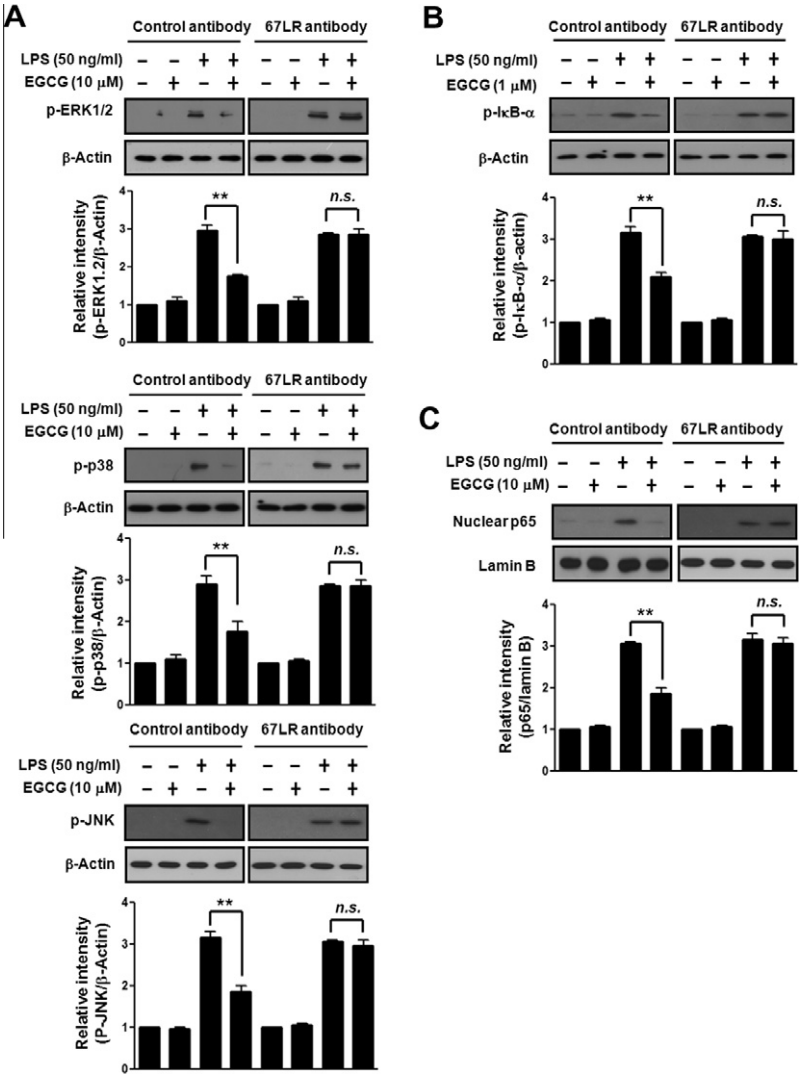

Fig. 3. EGCG inhibits LPS-induced activation of MAPK and NF-κB signal pathway through 67LR. DCs were incubated with either an anti-67LR Ab or control Ab for 1 h. The cells were then pretreated with the indicated concentrations of EGCG (10 μM) for 1 h before exposure to LPS (50 ng/mL) for 45 min. Cells lysates were subjected to SDS–PAGE, and immunoblot analysis was performed using specific Abs to phospho-p38 (p-p38), phospho-ERK1/2 (p-ERK1/2), phospho-JNK (p-JNK), phospho-IκB-α (p-IκB-α), and p65 NF-κB. β-Actin and lamin B were used as loading controls for cytosolic and nuclear fractions, respectively. Relative band intensity of each protein was expressed as percentage when compared to the value of untreated controls. The results shown are representative of three experiments conducted under each condition. Data are shown as mean ± SD ($n = 3$) and statistical significance ($^{**}p < 0.01$) is indicated for treatments versus untreated DCs.

tumor cell attachment and migration, as well as in tumor angiogenesis, invasion, and metastasis [24]. These findings suggest that 67LR may play an essential role in the regulation of cell adherence via the basement membrane laminin and in signal transduction following the binding event [25]. Previously, it has been reported that the inhibitory effect of EGCG on tumor cell proliferation is exerted through its binding to 67LR as a cell-surface receptor [15]. Moreover, EGCG suppresses the expression of the high-affinity IgE receptor FcεRI and histamine release through its binding to 67LR [17,26]. It has recently been demonstrated that 67LR as a cell-surface EGCG receptor mediates the anti-inflammatory action of EGCG in LPS-stimulated macrophages [18]. Nonetheless, the relationship between 67LR and anti-inflammatory activity of EGCG in LPS-stimulated DCs has not yet been established.

All inhibitory actions of EGCG shown in this study were mediated through 67LR, suggesting that 67LR has a pivotal role as a cell-surface receptor that mediates the inhibitory action of EGCG in TLR4-triggered signaling in DCs.

Previously, it was reported that MAPK activation followed by NF-κB translocation from the cytosol to the nucleus was particularly important in the LPS-induced secretion of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β [5]. In addition, it has been demonstrated that NF-κB and MAPK signaling play a central role in the activation of DCs [4]. In previous studies, EGCG was reported to inhibit LPS-induced activation of MAPK pathways, including ERK1/2, p38, and JNK [18]. It has also been described that EGCG suppresses LPS-induced activation of NF-κB by blocking IκB-α phosphorylation [18]. In addition, EGCG inhibits LPS-induced maturation of DCs through inhibition of MAPK and NF-κB [12]. Although these researches have proposed different mechanisms for the anti-inflammatory action of EGCG, the direct targets of EGCG to exert anti-inflammatory effect on TLR4 ligand-stimulated DCs have not been fully identified. Here, we showed for the first time that EGCG significantly inhibited the expression of the inflammatory cytokines TNF-α, IL-6, and IL-1β by blocking the activation of MAPK and NF-κB pathways through 67LR in LPS-induced DCs. These findings demonstrate that 67LR is responsible for mediating the anti-inflammatory action of EGCG in DCs and participates critically in the cell-signaling pathway and cell-surface interaction.

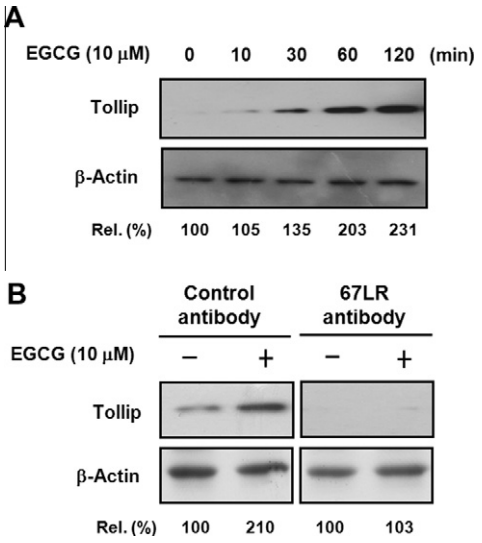

Fig. 4. EGCG up-regulates Tollip protein expression through 67LR. (A) DCs were treated with EGCG (10 μM) for the indicated periods. (B) DCs were incubated with either an anti-67LR Ab or control Ab for 1 h and then treated with EGCG (10 μM) for 1 h. Protein levels of Tollip were measured by immunoblotting. Relative band intensity of each protein was expressed as percentage when compared to the value of untreated controls.

Recently, negative regulators of TLRs have become a popular topic in the study of the innate immune system. Several studies have reported that TLR-mediated overactivation of the host immune response is regulated by several intracellular negative regulators of TLRs, including suppressor of cytokine signaling 1 (SOCS1), Tollip, and IL-1R-associated kinase (IRAK) M [27]. Among the negative regulators of TLRs, overexpression of Tollip impairs TLR2- and TLR4-triggered NF-κB and JNK signaling pathways [28]. This protein also interacts with IRAK-1 prior to stimulation and suppresses IRAK-1's kinase activity [23], indicating that Tollip negatively regulates TLR4 signaling. In addition, it was recently reported that Tollip regulates the activation of LPS-induced pro-inflammatory responses [29]. Moreover, it has been reported that Tollip is indispensable for mediating the anti-inflammatory action of EGCG in TLR4 signaling in macrophages, and its protein expression level is up regulated by EGCG through 67LR [18]. Here, we showed that EGCG significantly enhances the expression of Tollip, a negative regulator of TLR4, through 67LR expressed on the surface of DCs.

Therefore, our results provide a new insight into the immune-pharmacological role of EGCG in the TLR4 signaling pathway in DCs, suggesting a novel approach for the manipulation of DCs in the development and progression of many chronic diseases.

Acknowledgment

The authors thank Prof. Byun Myung-Woo for her helpful support in the cell experiments.

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