Original Paper

Epigallocatechin-3-Gallate Inhibits Matrix Metalloproteinase-9 and Monocyte Chemotactic Protein-1 Expression Through the 67- κ Da Laminin Receptor and the TLR4/MAPK/NF- κ B Signalling Pathway in Lipopolysaccharide-Induced Macrophages

Ya-Fei Li Hao Wang^a Yi Fan Hao-jie Shi Qi-Ming Wang Bing-rui Chen
Mohammad Reeaze Khurwolah Qing-qing Long Si-Bo Wang Ze-Mu Wang
Lian-Sheng Wang

Department of Cardiology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Key Words

Epigallocatechin-3-gallate • 67-kDa laminin receptor • MMP-9 • MCP-1 • TLR4 • Tollip • Atherosclerotic plaque stability

Abstract:

Background/Aims: Epigallocatechin-3-gallate (EGCG), a major catechin found in green tea, has been shown to prevent cardiovascular diseases. Previously, Matrix metalloproteinase-9 (MMP-9), monocyte chemotactic protein-1 (MCP-1) and toll-like receptor 4 (TLR4) were confirmed to play an important role in atherosclerosis and plaque instability. Both TLR4 and its negative regulator, Toll-interacting protein (Tollip), could be mediated by EGCG. The present study aimed to examine the effect of physiological concentration of EGCG (1 μ M) on the expression of MMP-9 and MCP-1 in lipopolysaccharide (LPS)-induced macrophages and the potential mechanisms underlying its actions. **Methods:** The RAW264.7 cell line was used. Western blot was used to determine MCP-1, TLR4, Tollip, Mitogen-activated protein kinase (MAPK) and Nuclear factor- κ B (NF- κ B) protein expression. MMP-9 activity was assayed by gelatine zymography. The mRNA expression of MMP-9 and MCP-1 was measured by real-time polymerase chain reaction (RT-PCR). **Results:** EGCG (1 μ M) significantly suppressed the expression of MMP-9 and MCP-1 and inhibited MAPK and NF- κ B in LPS-induced macrophages but was blocked by Tollip silencing. The expression of LPS-induced MMP-9 and MCP-1 and the phosphorylation of the ERK1/2, P38 and NF- κ B pathway proteins decreased after TLR4 siRNA treatment. Furthermore, EGCG mediated TLR4 and Tollip expression through binding

Y.-F. Li and H. Wang contributed equally to this work

Lian-Sheng Wang, MD, PhD

Department of Cardiology, the First Affiliated Hospital of Nanjing Medical University,
300 Guangzhou Road, Nanjing, 210029, Jiangsu Province, (China)
Fax/Tel. +86 25 83724440, E-Mail drslswang@njmu.edu.cn

to 67-kDa laminin receptor (67LR). **Conclusion:** The results of our study suggested that EGCG (1 μ M) suppresses the TLR4/MAPK/NF- κ B signalling pathway, decreases the expression of the plaque instability-mediating cytokines MMP-9 and MCP-1, and might prove to be effective in stabilizing atherosclerotic plaque.

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Introduction

Atherosclerotic plaques with thin fibrous caps are prone to rupture and have a higher rate of atherothrombosis, subsequent ischaemic events, and even target organ damage [1]. Current evidence demonstrates that both MMPs and inflammatory cytokines are critical factors contributing to atherosclerotic plaque instability [2, 3]. The degradation of the extracellular matrix (ECM) by MMPs is one of the key reasons for atherosclerotic plaque rupture [4]. MMP-9, one of the major MMPs destroying the ECM, is markedly expressed in vulnerable regions of atherosclerotic plaques, eventually leading to plaque instability [5, 6]. MCP-1, as an inflammatory factor, accelerates the progress of atherosclerosis and plaque instability through the recruitment of macrophages and monocytes, which can release a large number of inflammatory factors and MMPs into atherosclerotic lesions [7]. In contrast, in MCP-1-silenced mice, the vulnerable plaques turned out to be more stable, and plaque rupture decreased significantly [8].

Toll-like receptors (TLRs) play an important role in mediating the activation of the innate immune response and initiating inflammatory responses [9]. TLR4, the first identified member of the TLRs, has been studied widely in atherosclerosis and has been considered important in plaque destabilization [10-12]. LPS-stimulated TLR4 signalling can activate down-stream NF- κ B and MAPK (ERK1/2, P38 and JNK). In addition, the activation of TLR4 signalling is necessary for the LPS-induced expression of inflammatory factors and MMP-9 [13-15].

Epigallocatechin-3-gallate (EGCG) is a major catechin component of green tea that can be effective against variable drug-resistant cancers and can decrease the risk of cardiovascular diseases [16, 17]. Previous studies have shown that EGCG has a potential anti-inflammatory effect [18, 19] and reduces MMP-9 expression through a cell surface receptor, 67-kDa laminin receptor (67LR) [20]. Recently, it has been confirmed that 67LR is involved in the suppressive effect of EGCG on TLR4 expression in macrophages. Moreover, Tollip, a negative regulator of TLR4 signalling, plays an important role in mediating the anti-inflammatory effect of EGCG, and its expression can be uplified by EGCG through 67LR [18, 21].

In our previous studies, we demonstrated that the inhibitory effect of EGCG on MMP-9 and MCP-1 production was achieved at high concentrations (10-50 μ M) to demonstrate its biological activity [20, 22]. Whether EGCG still has this effect at the physiological concentration of 1 μ M is unknown. Furthermore, whether TLR4 and its negative regulator of Tollip are involved in the inhibitory action of EGCG on the up-regulation of MMP-9 and MCP-1 remains unclear. The aim of the current study was to confirm whether 1 μ M EGCG can inhibit the production of LPS-induced MMP-9 and MCP-1 through 67LR and to determine whether TLR4 and Tollip expression can be mediated by 1 μ M EGCG via 67LR. In addition, we further investigated the role of TLR4 and Tollip in the down-regulation of MMP-9 and MCP-1 by 1 μ M EGCG and researched their underlying molecular mechanisms.

Materials and Methods

Reagents and antibodies

EGCG and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO (Carlsbad, CA, USA). Anti-67LR antibody (MLuC5) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies against phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-NF- κ B and GAPDH were

purchased from Cell Signaling Technology (Boston, MA, USA). Antibodies against TLR4, Tollip, MCP-1, β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine-3000 and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). Trizol was obtained from Invitrogen (Carlsbad, CA, USA). Western blot chemiluminescence reagents were purchased from Thermo Scientific (Waltham, MA, USA).

Cell culture and treatment

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were incubated at 37°C with humidity in 5% CO₂. In some experiments, the cells were pretreated with EGCG (1-5 μ M), anti-67LR antibody (20 μ g/ml), and specific siRNAs (50 nM) and then activated with or without LPS (1 μ g/ml) for the indicated time periods to measure the expression of MMP-9, MCP-1, TLR4, and Tollip, the phosphorylation of NF- κ B and MAPK (ERK1/2, JNK, P38) and MMP-9 activity.

Small-interfering RNA transfection

RAW264.7 cells were transfected with small-interfering RNA (siRNA) for TLR4 and Tollip. The siRNA sequences of TLR4 and Tollip are shown in Table 1. The experimental siRNA and control siRNA were transfected into cells using Lipofectamine 3000 and Opti-MEM medium. The cells were cultured with siRNA for 36 hours prior to treatment with LPS or treated with EGCG first and then with LPS.

Real-time polymerase chain reaction (RT-PCR) analysis

The RAW264.7 cells were washed twice with ice-cold phosphate-buffered saline (PBS). Total cellular RNA was isolated from cells using Trizol reagent according to the manufacturer's instructions. The extracted RNA was used for reverse transcription using a Reverse Transcription Kit (Takara, Dalian, China). The SYBR green PCR Master Mix (Applied Biosystems, Foster, CA, USA) was used to perform PCR analysis. Specific RT-PCR primers are listed in Table 2. The resultant cDNA samples were used to perform RT-PCR under the following conditions: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and finally 58°C for 1 min. The mouse GAPDH gene expression value was used as an endogenous control for normalizing MMP-9 and MCP-1 gene expression.

Western blot analysis

RAW264.7 cells were washed in ice-cold PBS, collected and lysed in 100 μ l lysis buffer containing 1 μ l of phosphatase inhibitor, 0.1 μ l of protease inhibitor, and 0.5 μ l of phenylmethylsulfonyl fluoride (PMSF). The amount of protein was measured using a BCA Protein Assay Kit (Takara, Dalian, China). Protein extracts were electrophoresed through 10% or 15% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in 5% BSA for 2 hours and were incubated overnight at 4°C with primary antibodies for MCP-1, TLR4, Tollip, 67LR, p-JNK, p-P38, p-ERK1/2, JNK, ERK1/2, P38, p-NF- κ B, GAPDH, and β -actin. After the membranes were washed 3 times for 10 min with TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 90 min. The specific bands were detected by chemiluminescence (Thermo Scientific, Waltham, MA, USA). The band intensity was detected and quantified using the ChemiDoc XRS Imaging System (BioRad, Hercules, CA, USA).

Gelatin zymography assay

Culture supernatants of RAW264.7 cells with or without specific treatment were collected from 6-well plates. Samples were mixed with 5% Tris-glycine SDS sample buffer and loaded onto 10% polyacrylamide

Table 1. Small-interfering RNA of TLR4 and Tollip

Target	Small-interfering RNA	
TLR4	Sense:	5'-GAAAUGAGCUGGUAAGAATT-3'
	Antisense:	5'-UUCUUUACCAGCUCAUUUCTT-3'
Tollip	Sense:	5'-GCAAAGAAUUAUGGCAUGATT-3'
	Antisense:	5'-UCAUGCCAUAAUUCUUUGCTT-3'

Table 2. Primers of MCP-1, MMP-9 and GAPDH for real-time PCR

Target	Primers	
MCP-1	Sense:	5'-GCTGACCCCAAGAAGGAA-3'
	Antisense:	5'-GCTTGAGGTGTTGTGGAA-3'
MMP-9	Sense:	5'-GAGTCCGGCAGACAATCCTT-3'
	Antisense:	5'-CCCTGTAATGGGCTTCCTCTA-3'
GAPDH	Sense:	5'-CAGCAAGGACACTGAGCAAG-3'
	Antisense:	5'-GGGTGCAGCGAACTTATTG-3'

gels with 1 mg/ml gelatine. Following 90 min of electrophoresis at 100 V, gels were washed with 2.5% Triton X-100 for 40 min twice to remove SDS and then incubated for 11 hours at 37°C in developing buffer (10 mM Tris Base, 40 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl₂, 0.02% Brij-35). Gels were then stained using 0.5% Coomassie Blue R-250 for 2 hours and then destained in 50% methanol, and 10% glacial acetic acid in 40% water. Areas of MMP-9 activity, appearing as transparent bands, were detected against the blue background. Zymograms were photographed using the ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Significant differences among experimental groups were tested for statistical significance using one-way analysis of variance (ANOVA) or Student's t-test. All values are expressed as the mean±standard deviation (SD). Statistics were calculated using SPSS statistical software version 17.0 (SPSS Inc., Chicago, IL). A P value less than 0.05 was considered statistically significant.

Results

EGCG inhibits LPS-induced production of MMP-9 and MCP-1 through 67LR in RAW264.7 cells

To examine whether EGCG at a physiological concentration still has an inhibitory effect on the up-regulation of MMP-9 and MCP-1 via 67LR, macrophages were treated with or without anti-67LR antibody (20 µg/ml) for 1 hour. The cells were then either pretreated with 1 µM EGCG for 1 hour prior to a 24-hour incubation with 1 µg/ml LPS to measure MMP-9 gene expression and activity or pretreated with 1 µM EGCG for 1 hour prior to a 6-hour incubation with 1 µg/ml LPS to measure MCP-1 gene and protein expression. As shown in Fig. 1A-1D, the LPS-induced MMP-9 and MCP-1 production and the LPS-induced MMP-

Fig. 1. Inhibitory action of EGCG on LPS-induced MMP-9 and MCP-1 expression is mediated by 67LR. (A) Real-time PCR results of MMP-9 mRNA expression. (B) Gelatin zymography results of MMP-9 activity. (C) Real-time PCR results of MCP-1 mRNA expression. (D) MCP-1 protein expression was determined by Western blot. Data represent the mean±SD of 3 independent measurements. *: P < 0.05.

9 activity were significantly decreased by 1 μ M EGCG treatment. However, the inhibitory action of EGCG was not observed in the anti-67LR antibody-treated cells. Moreover, the inhibitory action of EGCG on the expression of MMP-9 and MCP-1 was not observed in the LPS-untreated cells. Therefore, the results showed that EGCG (1 μ M) performs its inhibitory role in the expression of LPS-induced MMP-9 and MCP-1 through binding to 67LR.

EGCG suppresses TLR4 protein production through 67LR

To investigate whether EGCG inhibits the expression of TLR4 through 67LR in macrophages, cells were treated with the anti-67LR antibody (20 μ g/ml) or control mouse IgM (20 μ g/ml) for 1 hour before exposure to 1 μ M EGCG for 24 hours. Western blot was used to measure the expression of TLR4 protein. As shown in Fig. 2C, the expression of TLR4 protein was significantly suppressed by 1 μ M EGCG in control cells, whereas the inhibitory effect of EGCG was not observed in the anti-67LR antibody treated cells. These results indicated that 67LR is essential for the suppressive effect of EGCG on the reduction of TLR4 expression.

Fig. 2. EGCG suppresses TLR4 protein expression via 67LR. In addition, TLR4 down-regulation decreases the production of LPS-induced MMP-9 and MCP-1. **A.** TLR4 protein expression in macrophages transfected with TLR4 siRNA and control siRNA for 36 hours was detected by Western blot. **B.** Levels of Tollip and 67LR protein expression were measured by Western blot in TLR4 down-regulated cells and control cells. **C.** TLR4 protein expression was detected by Western blot. **(D)** Real-time PCR results of MMP-9 mRNA expression. **(E)** MMP-9 activity was measured by gelatine zymography. **(F)** Real-time PCR results of MCP-1 mRNA expression. **(G)** Western blot results of MCP-1 protein expression. Data represent the mean $\pm$ SD of 3 independent measurements. *: $P < 0.05$; **: $P < 0.01$.

TLR4 mediates LPS-induced MMP-9 and MCP-1 expression

To explore whether TLR4 mediates the LPS-induced production of MMP-9 and MCP-1, we used siRNA to silence TLR4 expression in macrophages. RAW264.7 cells were treated with TLR4 siRNA (50 nM) or control siRNA for 36 hours. The siRNA treated cells were then either pretreated with 1 μ M EGCG for 1 hour prior to a 24-hour incubation with 1 μ g/ml LPS to measure MMP-9 gene expression and activity or pretreated with 1 μ M EGCG for 1 hour prior to a 6-hour incubation with 1 μ g/ml LPS to measure MCP-1 gene and protein expression. Western blot analysis showed that TLR4 siRNA silenced TLR4 expression without affecting the expression of 67LR and Tollip (Fig. 2A, 2B). In addition, the analysis showed that the expression of TLR4 protein in macrophages was not affected by stimulation with LPS (data not provided). Compared with control cells, the expression of LPS-induced MMP-9 and MCP-1 was significantly decreased in TLR4 siRNA macrophages (Fig. 2D, 2F, 2G). In addition, the activity of LPS-induced MMP-9 in the TLR4 siRNA cells was markedly lower than in the control cells (Fig. 2E). Interestingly, EGCG in the control cells significantly suppressed the LPS-induced MMP-9 and MCP-1 gene expression and MMP-9 activity, but this effect of EGCG was not observed in TLR4 siRNA cells (Fig. 2D-2G). Together, these data indicated that TLR4 signalling is involved in the LPS-induced expression of MMP-9 and MCP-1. Moreover, the results suggested that TLR4 signalling is indispensable for EGCG inhibition of the production of MMP-9 and MCP-1 in LPS-activated macrophages.

Effect of 1 μ M EGCG and TLR4 siRNA treatment on the MAPK/NF- κ B signalling pathway

Western blot analysis was used to determine the protein expression of NF- κ B and MAPK (JNK, ERK1/2 and P38). As shown in Fig. 3A-3D, LPS-activated phosphorylation of these kinases was significantly attenuated by treatment with 1 μ M EGCG in control siRNA cells.

Fig. 3. The LPS-induced activation of NF- κ B and MAPK (ERK1/2, JNK and P38) was significantly decreased by treatment with 1 μ M EGCG. Moreover, NF- κ B, ERK1/2 and P38 pathways induced by LPS were markedly attenuated in TLR4-silenced cells. Macrophages were incubated with either TLR4 siRNA or control siRNA for 36 hours. The cells were then pretreated with 1 μ M EGCG for 1 hour prior to exposure to LPS (1 μ g/ml) for 45 minutes. Western blot analysis was performed using specific antibodies to p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38, and p-NF- κ B. Data represent the mean $\pm$ SD of 3 independent experiments. *: $p < 0.05$; **: $p < 0.01$.

In addition, LPS-induced phosphorylation of ERK1/2, P38 and NF- κ B pathway proteins in TLR4 gene-silenced cells was significantly lower than in the control siRNA cells. However, the expression of LPS-activated phosphorylation of the JNK pathway was not affected in TLR4 gene-silenced cells compared with control cells. Moreover, in addition to the JNK pathway, LPS-induced phosphorylation of the ERK1/2, P38 and NF- κ B pathway proteins in TLR4 gene-silenced cells was not further decreased by 1 μ M EGCG. These results showed that TLR4 signalling is involved in LPS-activated ERK1/2, P38 and NF- κ B pathways. In addition, the TLR4/MAPK/NF- κ B signalling pathway is mediated by the inhibitory action of EGCG on the expression of MMP-9 and MCP-1 induced by LPS.

EGCG elevates Tollip protein production through 67LR

To confirm whether EGCG mediates Tollip protein expression, macrophages were treated with anti-67LR antibody (20 μ g/ml) or control mouse IgM (20 μ g/ml) for 1 hour

Fig. 4. EGCG elevates Tollip protein expression through 67LR. Moreover, Tollip mediates the suppressive effect of EGCG on the production of LPS-induced MMP-9 and MCP-1 expression. A. Tollip protein expression in the macrophages treated with EGCG (1–5 μ M) was detected by Western blot. B. The non-LPS-activated macrophages were treated with 1 μ M EGCG for the indicated time periods. Western blot was then used to detect Tollip protein expression. C. Tollip protein expression in the macrophages transfected with either Tollip siRNA or control siRNA was detected by Western blot. D. Levels of TLR4 and 67LR protein expressions were measured by Western blot in Tollip-silenced cells and control cells. E. Tollip protein expression in either anti-67LR antibody or control antibody cells treated with 1 μ M EGCG was measured by Western blot. F. TLR4 protein expression in either Tollip siRNA- or control siRNA-silenced cells treated with 1 μ M EGCG was measured by Western blot. (G). Real-time PCR results of MMP-9 mRNA expression. (H) Gelatin zymography results of MMP-9 activity. (I). Real-time PCR results of MCP-1 mRNA expression. (J) MCP-1 protein expression was measured by Western blot. Data represent the mean $\pm$ SD of 3 independent measurements. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

before exposure to 0–5 μ M EGCG for 2 hours. We found that EGCG up-regulates Tollip protein expression in a time- and concentration-dependent manner in LPS untreated macrophages (Fig. 4A, 4B). In addition, EGCG elevated Tollip protein expression in the control antibody cells; however, this effect of EGCG was not observed in anti-67LR antibody cells (Fig. 4E). The results indicated that the 67LR antibody prevents the effect of EGCG on Tollip protein expression.

Tollip mediates the inhibitory effect of EGCG on LPS-induced MMP-9 and MCP-1 production and TLR4 signalling

Macrophages were treated with Tollip siRNA (50 nM) or control siRNA for 36 hours. Then, the siRNA-treated cells were either pretreated with 1 μ M EGCG for 1 hour prior to a 24-hour incubation with 1 μ g/ml LPS to measure MMP-9 gene expression and activity or pretreated with 1 μ M EGCG for 1 hour prior to a 6-hour incubation with 1 μ g/ml LPS to measure MCP-1 gene and protein expression. Western blot analysis showed that Tollip siRNA silenced the Tollip protein expression level without inhibiting the expression of TLR4 and 67LR (Fig. 4C, 4D). Moreover, as shown in Fig. 4G–4J, the suppressive effect of EGCG on the LPS-induced MMP-9 and MCP-1 expression and LPS-induced MMP-9 activity was blocked by Tollip silencing. Furthermore, TLR4 protein expression was not affected by 1 μ M EGCG treatment for 24 hours in Tollip-silenced cells (Fig. 4F). The results suggested that the suppressive action of EGCG on TLR4 signalling and LPS-induced MMP-9 and MCP-1 expression is mediated through the up-regulation of Tollip.

EGCG suppresses LPS-activated phosphorylation of MAPK through Tollip

Western blot analysis was used to determine whether Tollip mediates the inhibitory effect of EGCG on LPS-activated phosphorylation of NF- κ B and MAPK. We found that the suppressive action of EGCG on LPS-induced phosphorylation of NF- κ B, ERK1/2, JNK and

Fig. 5. EGCG suppresses LPS-activated activation of the NF- κ B and MAPK signalling pathways via Tollip. RAW264.7 cells were incubated with either Tollip siRNA (50 nM) or control siRNA for 36 hours. The cells were then pretreated with 1 μ M EGCG for 1 hour and induced with 1 μ g/ml LPS for 45 min. Western blot analysis was carried out using specific antibodies to p-p38, P38, p-ERK1/2, ERK1/2, p-JNK, JNK, and p-NF- κ B. Data represent the mean $\pm$ SD of 3 independent experiments. *: $p < 0.05$.

P38 pathway proteins was blocked in Tollip-silenced cells (Fig. 5A-5D). Our results indicated that the LPS-activated phosphorylation of NF- κ B and MAPK (ERK1/2, JNK and P38) was suppressed by 1 μ M EGCG via Tollip.

Discussion

Atherosclerosis, a progressive inflammatory disease, is closely related to the over-expression of inflammatory cytokines and MMPs, such as MCP-1 and MMP-9 in macrophages/monocytes [5, 7]. Our present research showed that a physiological concentration of EGCG (1 μ M) significantly decreased the production of MMP-9 and MCP-1 in LPS-induced macrophages. In addition, the suppressive effects of EGCG were mediated by 67LR and were achieved through the inhibition of the TLR4/MAPK/NF- κ B signalling pathway. Finally, we found that Tollip indeed mediated the inhibitory action of EGCG on TLR4 signalling and on the LPS-induced expression of MMP-9 and MCP-1.

As an important pathogen recognition receptor, TLR4 has been shown to play a significant role in the progression of atherosclerosis [9]. Previous studies have manifested that TLR4 production is markedly elevated in inflammatory cells and vascular smooth muscle cells of atherosclerotic vessels [11, 12]. The over-expression of TLR4 has been reported to decrease the ECM, subsequently resulting in plaque rupture by stimulating macrophages to produce MMPs [23]. Besides, gene knockout of TLR4 in mice has resulted in significantly reduced atherosclerosis development and more stable plaques by altering plaque composition [24]. It has recently been confirmed that the expression of Tollip in human failing hearts and murine hypertrophic hearts is down-regulated and that the over-expression of Tollip protects hearts from cardiac remodelling [25]. A previous study also showed that TLR4-activated NF- κ B and MAPK signalling pathways were suppressed by the up-regulation of Tollip [26]. Moreover, down-regulation of Tollip leads to abrogation of the suppressive effect of EGCG on TLR4-triggered inflammatory responses [18]. These results indicated that Tollip plays a pivotal role in the negative regulation of TLR4 signalling. Consistent with this notion, we found that EGCG reduces TLR4-triggered production of MMP-9 and MCP-1 through an increase in Tollip expression. Interestingly, our study showed that TLR4 protein expression was not affected by 1 μ M EGCG treatment in Tollip-silenced cells, which is contradictory to the findings of Byun et al. (Fig. 8E) but is consistent with the description in their results section [18]. Therefore, further analysis is necessary and will be performed in our later *in vivo* studies. Here, we showed for the first time that the down-regulation of Tollip prevented EGCG from its suppressive action on TLR4-triggered MMP-9 and MCP-1 production in LPS-activated macrophages. These results demonstrated that EGCG may play an important role in plaque stability by mediating the expression of TLR4 and Tollip. In addition, Tollip may be a critical factor responsible for EGCG-mediated stabilization of atherosclerotic plaques.

A dominant laminin-binding protein for neutrophils, macrophages and monocytes, 67LR has been identified as a cell-surface receptor binding to EGCG, making EGCG exert anti-inflammatory and anti-tumour effects [18, 27]. Previously, we reported that 67LR expression is not affected by EGCG treatment and that 67LR expression confers EGCG an inhibitory effect on the dysregulated up-regulation of MMP-9 and MCP-1 [20, 22]. Furthermore, numerous studies have shown that EGCG suppresses the expression of TLR4 signalling through binding to the 67LR [18, 28]. In our present research, we proved that EGCG (1 μ M) mediated the expression of TLR4 and Tollip in macrophages through 67LR. Upon 67LR inhibition, the mediatorial action of EGCG on TLR4 and Tollip expression was blocked, and the expression of MMP-9 and MCP-1 induced by LPS could not be decreased. These results suggested that 67LR has a pivotal role as a cell-surface receptor that confers the plaque stabilizing effects of EGCG.

In our research, the LPS-induced phosphorylation of the NF- κ B, ERK1/2 and p38 pathway proteins in TLR4 siRNA-treated cells was reduced, and the results indicated that down-regulation of TLR4 specifically suppressed the LPS-induced NF- κ B and MAPK signalling

pathways. A recent study revealed that the LPS-activated phosphorylation of ERK1/2 and P38 pathway proteins in TLR4 siRNA-silenced mouse macrophages was down-regulated [29], which is consistent with our results. Although the underlying molecular mechanisms of TLR4 siRNA on down-regulation of MMP-9 and MCP-1 expression remain unclear, inhibition of the activation of NF- κ B, ERK1/2 and P38 pathways is likely to be involved. In this study, we confirmed that the suppressive action of EGCG on the LPS-activated up-regulation of NF- κ B and MAPK (JNK, ERK1/2 and P38) activation was not observed in Tollip-silenced macrophages. Our data indicated that Tollip is indispensable for the inhibitory effect of EGCG on the LPS-triggered phosphorylation of NF- κ B and MAPK signalling (JNK, ERK1/2 and P38).

Conclusion

We demonstrated that the green tea polyphenol EGCG (1 μ M) inhibits the expression of MMP-9 and MCP-1 in LPS-induced macrophages through 67LR and the TLR4/MAPK/NF- κ B signalling pathway and that Tollip is indispensable for this EGCG action. Our study reveals a novel mechanism underlying the down-regulation of MMP-9 and MCP-1 by EGCG. In addition, our findings not only demonstrate the potential role of physiological concentrations of EGCG (1 μ M) in stabilizing atherosclerotic plaque but also provide new insight into better prevention of plaque instability by mediating TLR4 and Tollip. Nevertheless, further in-vivo studies are necessary in order to fully elucidate the role of EGCG, TLR4 and Tollip in the stabilization of atherosclerotic plaque.

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Disclosure Statement

There are no conflicts of interest.

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