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Ginkgo biloba extract suppresses endotoxin-mediated monocyte activation by inhibiting nitric oxide- and tristetraprolin-mediated toll-like receptor 4 expression

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Abstract

Monocytes expressing toll-like receptor 4 (TLR4) play a major role in regulating the innate immune response and are involved in systemic inflammation. Previous studies have shown that *Ginkgo biloba* extract (GBE) may act as a therapeutic agent for some cardiovascular and neurological disorders. The objective of this study was to determine whether GBE could modulate immunity in human cells. The monocytic cell line THP-1 was used. Enzyme-linked immunosorbent assay results showed that lipopolysaccharide (LPS) induces the expression of monocyte chemotactic protein-1 (MIP-1), tumor necrosis factor-α, stromal cell-derived factor-1, and MIP-1α, and this induction may be repressed by GBE treatment due to TLR4 blockade. The Griess reagent assay and western blot analysis showed that GBE-mediated inhibition of TLR4 expression was associated with the activation of mitogen-activated protein kinase and production of nitric oxide (NO). Actinomycin D chase experiments demonstrated that GBE decreased the TLR4 mRNA stability in cells. Confocal microscopy and real-time polymerase chain reaction showed that GBE induced the expression of intracellular tristetraprolin (TTP). Transfection with TTP siRNA reversed the effects of GBE in naïve or TLR4-overexpressing cells. Treatment with SNAP (an NO donor) may increase intracellular TTP expression in cells. Immunoprecipitation analysis showed that GBE mediates TTP activation and increases the interaction of TTP with the 3′ untranslated region (UTR) of TLR4 mRNA by regulating NO production. Our findings indicate that GBE could decrease the sensitivity of monocytes to LPS. Utilizing TTP to control TLR4 expression may be a promising approach for controlling systemic inflammation, and GBE may have potential applications in the clinical treatment of immune diseases.

Keywords: Toll-like receptor 4; *Ginkgo biloba* extract; Tristetraprolin; Nitric oxide

1. Introduction

Toll-like receptors (TLRs) are Type I transmembrane receptors that were identified as a *Drosophila* gene to administer ontogenesis and against microbial attach [1]. To date, more than 10 kinds of TLRs have been identified [2]. The intracellular domain of TLR4 is similar to that of interlukin-1 receptor (IL-1R) and is known as the Toll/IL-1R domain. Endotoxin-induced dimerization of TLR4 is followed by the recruitment of the IL-1R accessory protein, binding of MyD88, activation of interleukin-1 receptor-associated kinase and the phosphorylation of tumor necrosis factor receptor-associated factor 6 [3]. This pathway subsequently triggers the activation of nuclear factor-κB and the phosphorylation of mitogen-activated protein kinases (MAPKs), which regulate the induction of inflammation- and innate immune-related gene expression [4,5]. In addition to responding to endotoxins, TLR4 also recognizes a broad spectrum of microbes [6,7] such as *Saccharomyces cerevisiae*, *Candica albicans* and *Crytococcus neoformans*. TLR4 is also capable of responding to various endogenous ligands, including heat shock protein 60 [8,9], high mobility group box 1 [10], hyaluronate [11], minimally modified low-density lipoprotein [12] and heparin sulfate [13], in endothelial and epithelial cells as well as leukocytes. TLR4 recognizes various potential host-derived components that appear as key mediators of innate immunity [14]. It has been shown that TLR4 antagonists and TLR4 ligand-based therapies can be used to treat diseases and may serve as potential drug candidates.

TLR4 is abundantly expressed in monocytes and macrophages and mediates the expression of tumor necrosis factor-α (TNF-α) and

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manganese superoxide dismutase [15]. The regulation and expression of TLR4 may be involved in altering the actual immune capacity of monocytes and macrophages [16]. Monocytes express TLR4, which plays a key role in maintaining healthy function and differentiation [17], directly or indirectly. Correlation of TLR4 levels and mobility in patients with cardiovascular diseases indicated that monocytic TLR4 levels were elevated in patients with coronary artery disease [18], heart failure after acute myocardial infarction [19], and stable angina [18]. Recent investigations have revealed that in addition to its interactions with endotoxins and heat shock proteins from bacteria, TLR4 also interacts with endogenous factors and cytokines [20]. Therefore, up-regulated TLR4 may increase the cellular sensitivity to antigens and thereby mediate the progression of atherosclerosis.

Ginkgo biloba has been used in traditional Chinese medicine for thousands of years. Recently, *G. biloba* extract (GBE), a defined complex mixture extracted from *G. biloba* leaves and containing 24% Ginkgo flavone glycoside and 6% terpenlactones (ginkgolides and bilobalide), was used as a therapeutic agent for some cardiovascular and neurological disorders [21,22]. Several mechanisms have been proposed for the beneficial effects of GBE, including increased blood flow [23], inhibition of platelet aggregation [24], suppression of nitric oxide (NO) production [25], and potential antioxidant activity [26]. In addition, GBE provides protection in model systems of oxidative stress, including cardiac [23,27] and retinal [28] ischemia-reperfusion injury. In a recent study, we presented considerable evidence for the activation of nicotinamide adenine dinucleotide phosphate oxidase and MAPK signaling pathways as well as the stabilization of TLR4 mRNA in lipopolysaccharide (LPS)-stimulated human aortic smooth muscle cells (HASMCs) and endotoxin-induced peripheral arterial system injury [29,30]. GBE may down-regulate TLR4, thereby attenuating the progression of atherosclerosis or restenosis after angioplasty [31]. However, the effects of GBE on immunity are largely unknown. In this study, we investigated whether GBE regulates the immune capacity and sensitivity of monocytes to endotoxins by regulating the TLR4 levels. The results may provide a basis for future applications of GBE in the clinical treatment of immune diseases.

2. Materials and methods

2.1. Cell culture

THP-1 cells, a human promyelomonocytic cell line, were obtained from the American Type Culture Collection (ATCC, VA, USA) and grown in RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% antibiotic-antimycotic mixture. The cell density was maintained between 5×10^4 to 8×10^5 viable cells/ml, and the medium was renewed every 2–3 days. The GBE stock solution was purchased from Dr. Willmar Schwabe (Karlsruhe, Forschungszenstrum, Germany).

2.2. Cell viability assessment by the MTT assay and flow cytometry

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cell viability. Cells were grown in 96-well plates and incubated with various concentrations (12.5–400 μg/ml) of GBE for 2 or 24 h. Subsequently, 0.5 μg/ml of MTT was added to each well, and incubation was continued at 37°C for an additional 4 h. Dimethyl sulfoxide was added to each well, and the absorbance was recorded at 530 nm using a DIAS Microplate Reader (Dynex Technologies, Chantilly, VA, USA).

Using flow cytometry to measure the cytotoxicity of GBE. Prepare cell and resuspend cell at 2×10^6 cells/ml in PBS+0.1% bovine serum albumin (BSA). Add propidium iodide (PI) staining solution to cells, mix gently and incubate 1 min. PI fluorescence was analyzed by flow cytometry.

2.3. Enzyme-linked immunosorbent assay

THP-1 cells were seeded in 24-well plates at a density of 10^6 cells/ml/well, and these were then pretreated with various concentrations of GBE for 2 or 24 h followed by LPS (*E. coli* serotype 0127:B8; Sigma-Aldrich, MA, USA) stimulation (25 ng/ml). After 24 h, the culture medium was collected to quantify the levels of monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor alpha, stromal cell-derived factor-1 (SDF-1), and macrophage inflammatory protein-1 (MIP-1) alpha [MIP-1α ; using the DuoSet enzyme-linked immunosorbent assay (ELISA) development kits (R&D Biosystems, San Jose, CA, USA)].

2.4. Quantitative real-time polymerase chain reaction

Total RNA was isolated using the TRIZOL reagent kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using Superscript II reverse transcriptase. Quantitative real-time polymerase chain reaction (PCR) was performed using a FastStart DNA Master SYBR Green I kit and LightCycler (Roche, Pleasanton, CA, USA). The FastStart *Taq* DNA polymerase was activated by incubation at 95°C for 2 min. This was followed by 40 cycles at 95°C for 1 s, 60°C for 5 s and 72°C for 7 s. Fluorescence was measured at 86°C after the extension step at 72°C. Calculate the cross point values to detect the presence of the TLR4 mRNA and normalize TLR4 mRNA expression against an internal control, such as glyderaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The PCR primers used for the amplification of TLR4 and GAPDH were as follows. The TLR4 forward primer was 5′-AAG CCG AA GGT GAT TGT TG-3′, and the reverse primer: was 5′-CTG TCC TCC CAC TCC AGGTA-3′. The GAPDH forward primer was 5′-TGC CC CTC TGC TGA TGC C-3′, and the reverse primer was 5′-CCT CCG ACG CCT GCT TCA CA C-3′. All specific primers were synthesized by Sigma-Aldrich (St. Louis, MO, USA).

2.5. Flow cytometry analysis

To examine membrane TLR4 expression in THP-1 cells, the cells were incubated with a PE-conjugated mouse anti-hTLR4 antibody (Biolegend, San Diego, CA, USA) or with mouse IgM/IgG2a isotype controls (DakoCytomation, Hamburg, Germany). After washing with the staining buffer [phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% sodium azide], TLR4 expression was analyzed by flow cytometry. Sorting 10^4 cells and analyzing the cell surface fluorescent intensity. The mean fluorescent intensity in experimental groups were compared with that in untreated group and the data were represented as percentage of control.

2.6. Western blot analysis

Western blot analysis was used to determine the changes in the cytosolic activation of p38 MAPK, extracellular signal-regulated kinase 1 and 2 (ERK1/2), and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in THP-1 cells stimulated with GBE. Total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were transferred onto polyvinyl difluoride (PVDF) membranes. The PVDF membranes were first probed with the rabbit anti-p38 antibody, rabbit anti-phospho-p38 antibody, rabbit anti-SAPK/JNK antibody, rabbit anti-phospho-SAPK/JNK antibody, rabbit anti-p44/p42 MAPK antibody or mouse anti-phospho-p44/p42 MAPK antibody (all anti-MAPK antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:1000. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA).

2.7. Measurement of NO production

After incubation of THP-1 cells with GBE for 4–18 h, the nitrite levels of the conditioned media were measured using the Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine in 2% phosphoric acid], as described previously [32].

2.8. Actinomycin D chase experiments

To determine the steady-state dynamic balance between the rate of transcription and the message stability of TLR4 mRNA, an actinomycin D chase experiment [30] was conducted. Actinomycin D (20 μg/ml) was added to the cells for 1 h following treatment under various experimental conditions. Total RNA was extracted at 0, 30, 60, 120 and 240 min after the addition of actinomycin D, and quantitative real-time PCR was then performed. The mRNA decay curves were constructed, and the half-life (t1/2) was calculated from the curves.

2.9. TLR4 plasmid transfection

The full-length wild-type TLR4 plasmid was purchased from Origene (Cambridge Bioscience, Cambridge, UK). Exponentially growing THP-1 cells (3×10^6 cells) were transiently transfected with the plasmids using the Lipofectamine transfection reagent, according to the manufacturer's instructions (Sigma, MO, USA). The cells were seeded in six-well plates immediately after transfection for further experiments at 48 h after transfection.

2.10. Knockdown of gene expression by RNA interference

Knockdown of tristetraprolin (TTP) gene expression was performed by siRNA transfection. Cells (3×10^6) were suspended in 2.5 ml of serum-free medium, and 25 nM of TTP siRNA duplexes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were transfected, as described in the instruction manual. Silencer Validated siRNA

(negative control siRNA, Santa Cruz) was used to validate the knockdown. The cells were seeded in six-well plates immediately after transfection for further experiments at 48 h after transfection.

2.11. Cross-linking immunoprecipitation assay of RNA–protein interactions

To determine whether GBE regulates the interaction between TTP and the 3′ untranslated region (UTR) of TLR4 mRNA, immunoprecipitation and reverse transcriptase-PCR were carried out as described previously [29]. To induce cross-linking, cells in ice-cold PBS were directly irradiated with 4000 mJ of ultraviolet B light three times. The cells were lysed with cold cell lysis buffer, and the RNA-protein complexes were extracted by centrifugation. For immunoprecipitation, equivalent amounts of the cytoplasmic fraction (500 μg of RNA-protein complexes) was incubated with protein G-sepharose and 10 μg of an antibody that recognizes TTP. Western blot analysis was used to determine whether the TTP levels were equivalent in immunoprecipitated material. The membrane was probed with a rabbit anti-TTP antibody and an HRP-conjugated secondary antibody. The blot was developed using ECL detection reagents.

Equivalent amounts of the immunoprecipitated material were used in quantitative real-time PCR reactions to detect the presence of the 3′ UTR of TLR4 mRNA. The mRNA was reverse-transcribed using a Reverse-iT 1st Strand Synthesis Kit (ABgene, Epsom, UK) followed by quantitative real-time PCR to measure the 3′ UTR transcript levels. The PCR primers designed for the 3′ UTR of TLR4 mRNA were 5′-GAA CTG GGT GTT CAC TTT TTC C-3′ and 5′-ATC CCA GCC ATC TGT GTC TC-3′. Cross-point values were calculated to detect the presence of the 3′ UTR of TLR4 mRNA.

2.12. Immunofluorescence staining

The THP-1 cells (1×10^5) were fixed with 4% paraformaldehyde and quickly aliquoted into the appropriate wells of a Shandon CytoSpin III Cytocentrifuge (GMI, Ramsey, MN, USA). They were then plated onto coverslips by centrifugation at 1000 *g* for 3 min. Cell membranes were fenestrated with 0.4% Triton X-100-PBS, and nonspecific binding sites were blocked with 2% BSA-PBS-Tween 20 (0.1%, v/v). The cells were incubated with rabbit anti-TTP, human antigen R, or AU-binding factor 1 (AUF1) antibodies (all antibodies were purchased from Chemicon, Temecula, CA, USA) and then with the fluorescein isothiocyanate (FITC) conjugated secondary antibody. 4′-6-diamidino-2-phenylindole (DAPI) was used to detect the nuclei of THP-1 cells. All slides were examined by confocal microscopy.

2.13. Statistical analyses

The values are expressed as the means±S.E.M. Data were analyzed using Student's *t* test and one- or two-way analysis of variance followed by the Dunnett test. Probability values (*P*) that were <.05 were considered to be significant.

3. Results

3.1. Cytotoxicity of GBE in THP-1 cells

The cytotoxicity of GBE was assessed by the MTT assay and using propidium iodine in flow cytometry. It was shown that the dose of GBE used in the following experiments did not affect the viability of THP-1 cells (Fig. 1A and B).

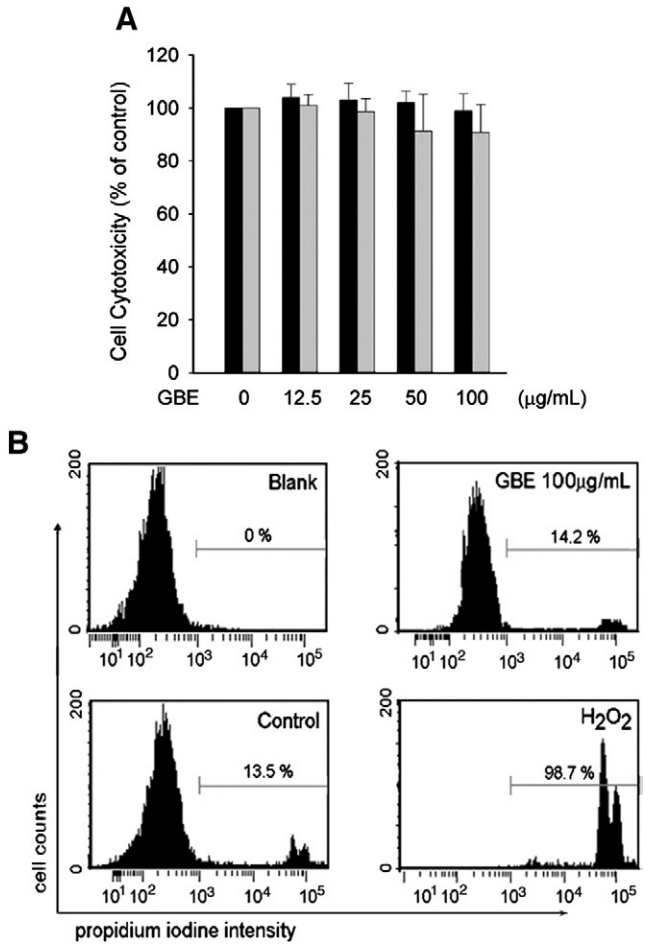

Fig. 1. GBE does not impair the viability of THP-1 cells. (A) THP-1 cells were treated with GBE (25–100 μg/ml) for 2 (black square) or 24 (gray square) h. The cytotoxicity of GBE was analyzed using the MTT assay. (B) THP-1 cells were treated with GBE (100μg/ml) or H_2O_2 (100μM) for 24 h. The cell viability was analyzed with propidium iodine in flow cytometry.

3.2. GBE decreases the production of cytokines in LPS-activated THP-1 cells

To determine whether GBE suppresses the activation of THP-1 cells by LPS, ELISA was used to examine the production of MCP-1, TNF-α, SDF-1 and MIP-1α. THP-1 cells were pretreated for 1 or 24 h with 25–100 μg/ml of GBE prior to the addition of 25 ng/ml LPS. Long-duration (24 h) treatment with 25, 50 or 100 μg/ml GBE significantly inhibited the LPS-induced expression of MCP-1 (499.0±77.3, 428.5±40.4 and 430.1±50.9 vs. control: 943.2±113.0 pg/ml, respectively), TNF-α (208.8±58.2, 191.4±30.4 and 121.8±10.9 vs. control: 580.1±53.1 pg/ml, respectively), SDF-1 (61.7±6.8, 49.18±6.1 and 52.8±6.1% vs. control: 89.4±9.9 pg/ml, respectively), and MIP-1α (4143.3±672.1, 3524.5±504.6 and 3572.2±509.4 vs. control: 7832.2±833.7 pg/ml, respectively) in THP-1 cells (Fig. 2A, B–D). Short-duration GBE treatment (1 h) only decreased MCP-1 production (467.8±63.2 pg/ml) in the samples treated with 100 μg/ml GBE (Fig. 2A) and decreased TNF-α production (377.2±85.4, 365.5±79.9, and 348.8±63.2 pg/ml, respectively) in the samples treated with 25–100 μg/ml GBE (Fig. 2B).

3.3. GBE-mediated inhibition of TLR4 expression in THP-1 cells occurs through the MAPK pathways

As showed in the Supplemental Data that LPS-induced release of cytokines in THP-1 cells is mediated by TLR4. We hypothesized that TLR4 suppression may be involved in the GBE-mediated inhibition of LPS-induced cytokine production. Therefore, we examined the effects of GBE on TLR4 mRNA and protein expression by employing quantitative real-time PCR and Western blot analysis, respectively. Treatment of THP-1 cells with 25–100 μg/ml of GBE for 2 h did not inhibit TLR4 mRNA expression. Naïve THP-1 cells showed significantly decreased TLR4 mRNA expression upon treatment with 50 or 100 μg/ml of GBE for 24 h; GBE treatment resulted in TLR4 mRNA expression levels that were 65.2±7.8% and 57.2±11.1% of the control value, respectively, (Fig. 3A). Interestingly, treatment of the cells with 50–100 μg/ml GBE for 2 h or with 25–100 μg/ml GBE for 24 h could effectively inhibit the constitutive expression of TLR4 (Fig. 3B). The results suggest that GBE decreases TLR4 expression in THP-1 cells and that this is mediated by the suppression of TLR4 mRNA production and inhibition of the presentation of membrane TLR4. To determine the role of MAPKs in TLR4 expression in naïve THP-1 cells, we pretreated cells with 10μM of MAPK inhibitors for

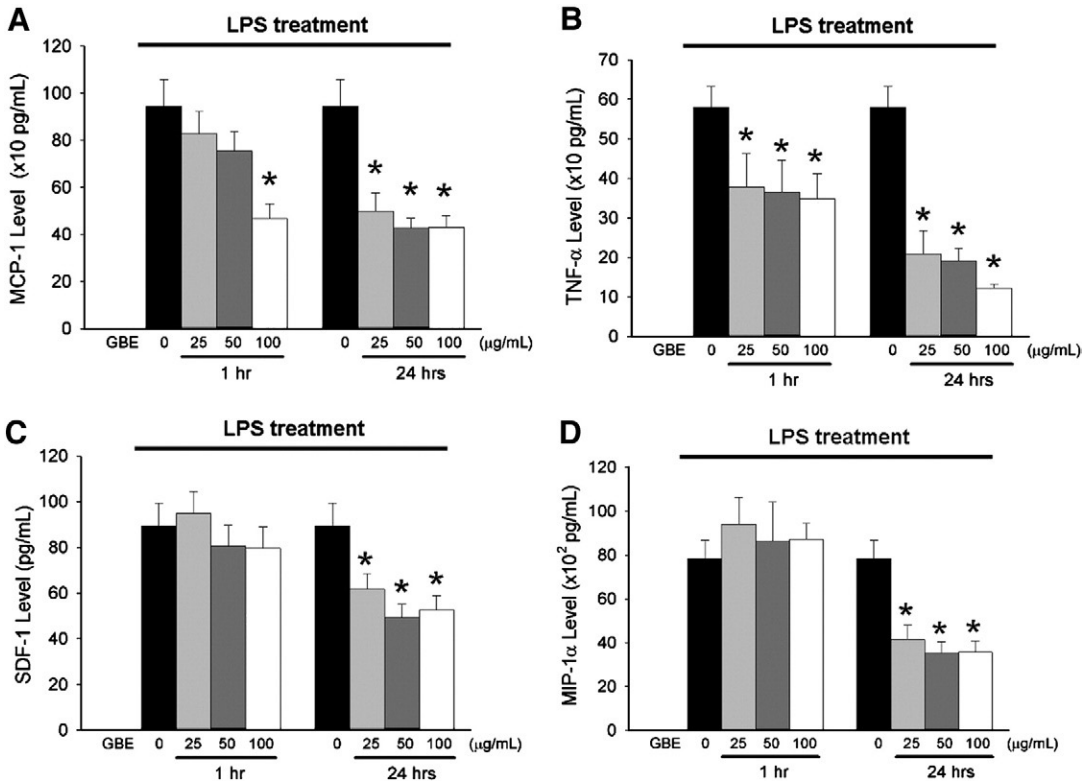

Fig. 2. GBE inhibits LPS-induced cytokine production. (A–D) THP-1 cells were pretreated with or without GBE (25–100μg/ml) for 1 or 24 h followed by 25 ng/ml of LPS (8 h for MCP-1 and TNF-α and 24 h for SDF-1 and MIP-1α). The cytokine levels in the culture medium were analyzed using a specific ELISA kit. The data represent the results of three independent experiments (means±S.E.M.; *P<.05, in comparison with LPS-stimulated cells).

24 h. Real-time PCR demonstrated that TLR4 mRNA expression was reduced by PD98059 (an extracellular signal-regulated kinase ERK1/2 inhibitor) but not by SP600125 (a stress-activated protein kinase SAPK/JNK inhibitor) and SB203580 (a p38 MAPK inhibitor) (Fig. 3C). This suggests that ERK1/2 plays more significant roles than p38 MAPK and SAPK/JNK in the transcriptional regulatory signaling pathway of naïve TLR4 mRNA expression. We next investigated whether GBE affects TLR4 expression via ERK1/2 signaling in THP-1 cells. The Western blot results (Fig. 3D) showed that GBE inhibits spontaneous ERK1/2 activation and induces p38 MAPK and SAPK/JNK phosphorylation in a dose-dependent manner. These results suggest that GBE curtails TLR4 expression through transcriptional regulation and that this effect is mediated by the ERK1/2 signaling pathway.

3.4. NO mediates the GBE-induced inhibition of TLR4 expression

The potential involvement of NO-, p38 MAPK- and SAPK/JNK-related mechanisms in the manifestation of the effects of GBE on THP-1 cells was also examined. After incubation with GBE for 4–18 h, NO production in the culture medium, as determined by the Griess reagent analysis, was significantly increased in cultures with 25 to 100 μg/ml of GBE (Fig. 4A).

Incubation with 100 μM of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) or *S*-nitrosocysteine (SNOC) significantly ameliorated naïve membrane TLR4 expression in THP-1 cells (Fig. 4B). To explore whether GBE and the NO donor also affect TLR4 overexpression, the full-length, wild-type human TLR4 plasmid-transfected cells were incubated with GBE or SNAP. The flow cytometry results showed that the full-length wild-type human TLR4 plasmid-transfected cells expressed larger amounts of TLR4 than naïve cells. Preincubation with SNAP, SNOC or GBE for 24 h may inhibit TLR4 overexpression (Fig. 4C). Simultaneous incubation with GBE and SB203580 but not with SP600125 may significantly decrease the enhanced effects of GBE on NO production in THP-1 cells (Fig. 4D). These data indicate that GBE may down-regulate membrane TLR4 expression by modulating NO production and through p38 MAPK-related mechanisms.

3.5. GBE reduces TLR4 expression in THP-1 cells by posttranscriptional regulation

Control of mRNA stability may modulate gene expression and efficiently adjust inflammatory responses. THP-1 cells were treated with 100 μg/ml of GBE for 24 h and then with actinomycin D for 40 min. The $t_{1/2}$ of the mRNA indicated that GBE actually decreased the stability of TLR4 mRNA (the $t_{1/2}$ of the TLR4 mRNA in the GBE group was 77.9±10.9 min, whereas that of the control group was more than 240 min) (Fig. 5A). mRNA stability is often modulated by RNA-binding proteins. Confocal microscopy revealed that HuR was predominantly present in the nucleus in naïve THP-1 cells, and its distribution remained unchanged following GBE incubation (Fig. 5B). In contrast, treatment with 100 μg/ml of GBE and 100 μM of SNAP resulted in a marked accumulation of cytoplasmic TTP (Fig. 5C). Flow cytometry analysis revealed effective reduction of TTP in the TTP siRNA-transfected group in comparison with the negative control siRNA-transfected group and the naïve control group (Fig. 5D). GBE-mediated inhibition of TLR4 mRNA expression was completely blocked by TTP siRNA. This effect was not observed with the negative control siRNA, suggesting the critical role of TTP in the regulation of TLR4 mRNA. In full-length wild-type human TLR4 plasmid-transfected cells, cotransfection with TTP siRNA, not with the negative control siRNA, may reverse the phenomenon of TLR4 overexpression (Fig. 5E). Based on the cytoplasmic localization of TTP in GBE-treated THP-1 cells and the specific region of

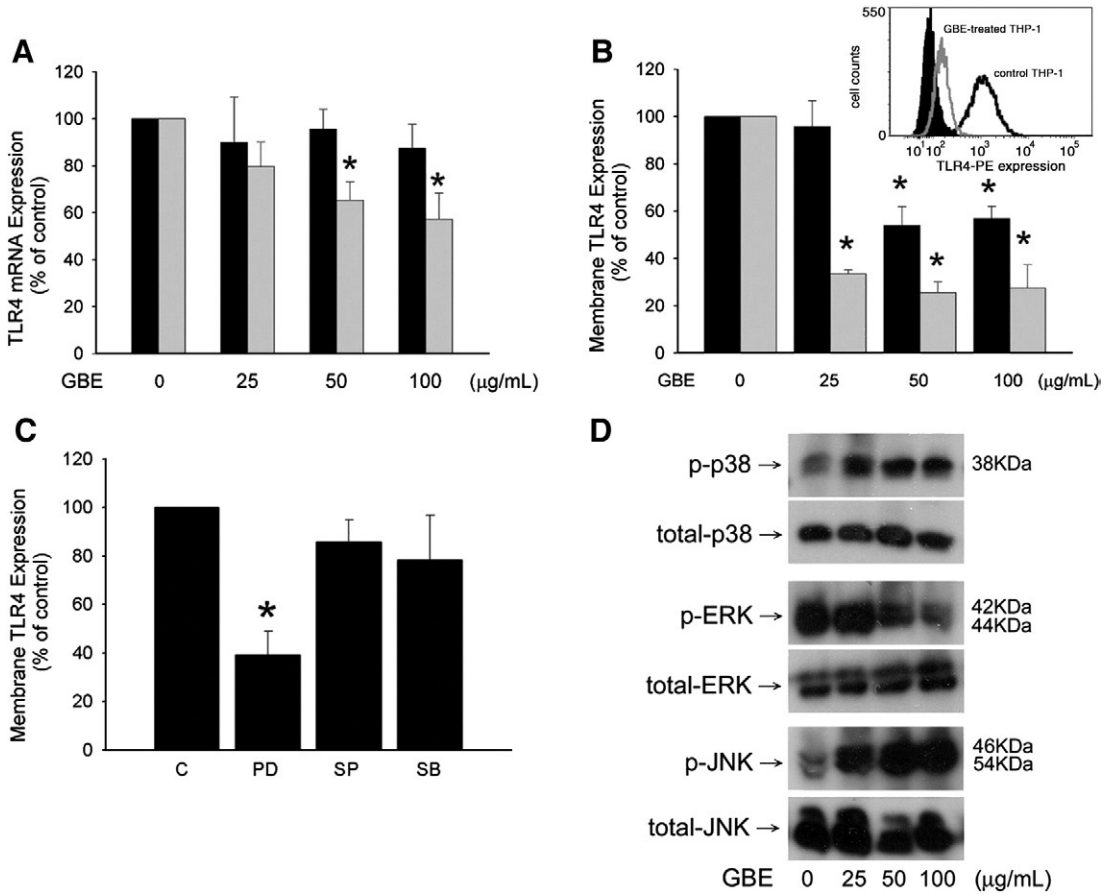

Fig. 3. GBE decreases ERK1/2 activation, which mediates TLR4 expression in THP-1 cells. (A) THP-1 cells were treated with 25–100 μg/ml of GBE for 2 (black square) or 24 (gray square) h. TLR4 mRNA was evaluated by quantitative real-time PCR. (B) THP-1 cells were treated with 25–100μg/ml of GBE for 2 (black square) or 24 (gray square) h. Membrane TLR4 was analyzed by flow cytometry. Additionally, the graph showed an example of the flow cytometry analysis. Negative control analyses were performed in the absent of specific PE-conjugated anti-TLR4 antibody (black graph); treated with 100 μg/ml GBE for 24 h may down-regulate the TLR4 expression (gray outline-hollow graph) in naïve THP-1 cells (black outline-hollow graph). (C) THP-1 cells were treated with 10 μM of PD98059 (PD), SP600125 (SP) or SB203580 (SB) for 24 h. Membrane TLR4 was analyzed by flow cytometry. (D) THP-1 cells were treated with 25–100 μg/ml of GBE for 24 h. Phosphorylation of p38 MAPK, ERK1/2, and SAPK/JNK was analyzed by western blotting. Total p38 MAPK, ERK1/2 and SAPK/JNK was used as the loading control. Data represent the results of three independent experiments (means±S.E.M.; *P<.05, in comparison with the unstimulated group).

ARE recognized by TTP, we postulate that GBE might alter the interaction of TTP with the 3′ UTR of TLR4 mRNA. We assessed this by performing immunoprecipitation and quantitative real-time PCR studies. The protein fractions were immunoprecipitated with the anti-TTP antibody or control pre-immune rabbit serum and then subjected to PAGE. The anti-TTP antibody was efficient in immunoprecipitation. Treatment with GBE, SNAP and SNOC markedly increased the interaction of TTP with the 3′ UTR of TLR4 mRNA (Fig. 5F). These findings indicate the critical role of TTP in the regulation of TLR4 mRNA and suggest that GBE increases TTP expression and also enhances the interaction of TTP with the 3′ UTR of TLR4 mRNA in THP-1 cells. In addition, GBE-induced NO production may play an important role in the signaling pathway.

3.6. GBE reduces TLR4 expression via multiple modification pathways

The schematic diagram shown in Fig. 6 summarizes the results and possible mechanisms. GBE inhibits spontaneous ERK1/2 signaling, which is involved in naïve TLR4 expression. Furthermore, GBE-mediated inhibition of TLR4 expression was associated with the production of NO. GBE decreased the TLR4 mRNA stability via inducing the expression of intracellular TTP. GBE mediates TTP activation and increases the interaction of TTP with the 3′ UTR of TLR4 mRNA by regulating NO production. AUF1 is predominantly present in the nucleus of naïve monocytes. Although GBE results in a marked accumulation of cytoplasmic AUF1 over time, it is still unclear whether AUF1 plays a role in decreasing the TLR4 levels.

4. Discussion

This study focused on monocytic cells because these cells are known to be involved in phagocytosis and microbial defense processes, which also involve in eliciting immune responses in humans. These data showed that GBE decreases TLR4 expression and increases TTP expression in both naïve and TLR4-overexpressing monocytes. The p38 MAPK-dependent NO production contributed to TLR4 down-regulation and TLR4 mRNA stabilization in GBE-treated THP-1 cells. These data may be evidence of the direct involvement of TTP in GBE-mediated TLR4 expression in THP-1 cells, which may contribute to the activation of endotoxin-stimulated responses.

It can be seen in Fig. 2A and B that short-duration treatment with GBE may reduce membrane TLR4 expression but not TLR4 mRNA expression. Therefore, we speculate that short-duration treatment may affect the stability of the protein and accelerate the rate at which membrane TLR4 is degraded. Indeed, addition of 50 mM *N*-benzyloxycarbonyl-Leu-Leu-leucinal (MG132; Calbiochem, CA, USA), a proteasome inhibitor, to cells actually reverses the effects of GBE on TLR4 expression. In contrast, treatment with 100 mM

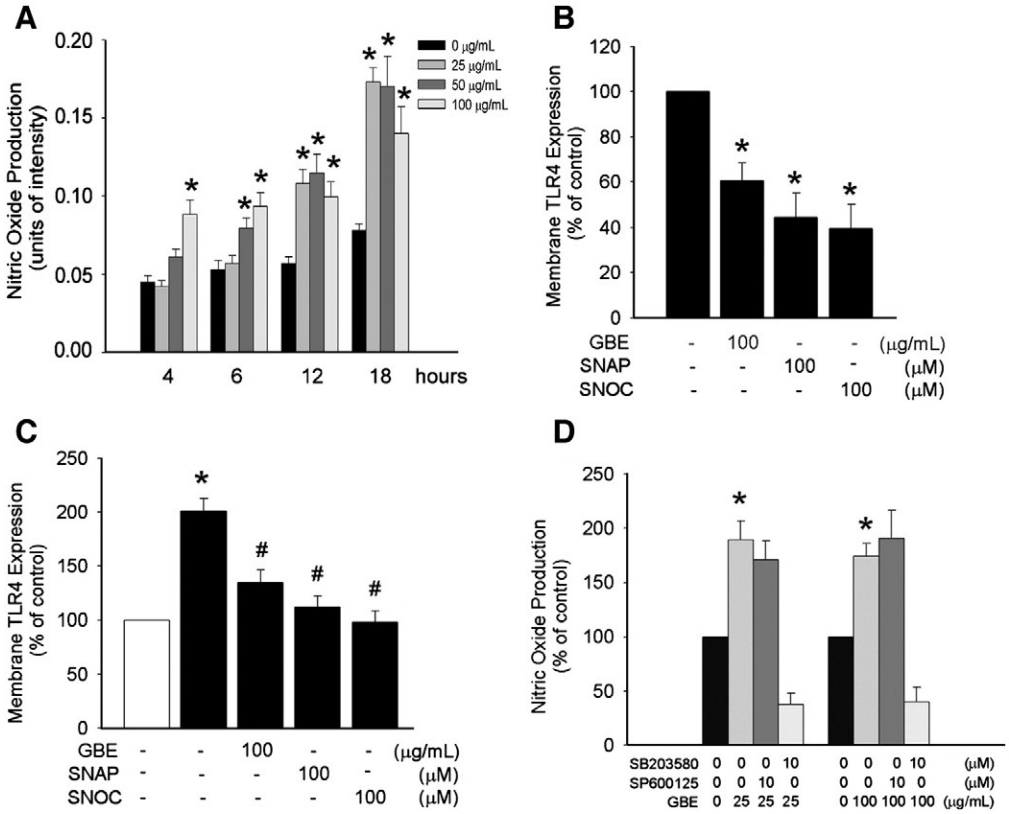

Fig. 4. GBE increases NO production, which mediates TLR4 expression in THP-1 cells. (A) THP-1 cells were treated with 25–100 μg/ml of GBE for 4–18 h. NO production in the culture medium was analyzed using the Griess reagent. (B) Naïve THP-1 cells were treated with SNAP, SNOC or GBE for 24 h, and membrane TLR4 was analyzed by flow cytometry. (C) Full-length wild-type human TLR4 plasmid-transfected THP-1 cells (■) were treated with SNAP, SNOC or GBE for 24 h, and membrane TLR4 was analyzed by flow cytometry. (D) Naïve THP-1 cells were pretreated with 10 μM of SB203580 (SB) or SP600125 (SP) for 1 h prior to treatment with GBE for 24 h. NO production in the culture medium was analyzed. Data represent the results of three independent experiments [means±S.E.M.; *P<.05, in comparison with the untreated group or naïve group (□); #P<.05, in comparison with the untreated-TLR4 plasmid-transfected group or the only GBE-treated group].

chloroquine (Sigma, CA, USA), a lysosome inhibitor, could not reverse the effects of GBE (Supplemental Fig 2), suggesting that GBE increases membrane TLR4 degradation by increasing proteosome activity. GBE rapidly induces intracellular proteosome activity. In fact, GBE contains water-soluble components and has low permeability in cells. Methods to increase the permeability of GBE in cell membranes require further investigation. Whether GBE interacts with TLR4 and competes with the binding sites of LPS is also an issue that needs to be clarified.

Currently, a review of the literature on this topic shows that GBE may improve decreased peripheral immune functions in schizophrenia [33] and increase phagocytic functions in stressed rats [34]. We demonstrated that GBE might decrease cytokine production in monocytes by suppressing TLR4 expression, suggesting that GBE may function as an immunomodulator. Discovery and development of a TLR4 antagonist may be a potential means of modulating TLR4-mediated diseases. GBE has been regarded as an immunomodulator in autoimmune disorders [35]. Until now, less has known the authentic effects of GBE on other TLRs expression. In our recent data show that GBE inhibits GroEL (a heat shock protein 60 of *chlamydia pneumonia*)-induced TLR2 expression (unpublished data) in human coronary endothelial cells. We speculate that GBE may regulate not only TLR4 but also other receptors expression on cell surface. In the future, sufficient studies are essential to prove it.

Among the cytokines released, it was observed that GBE treatment of LPS-stimulated THP-1 cells affected the expression of TNF-α to a greater extent than that of MCP-1, MIP-1α and SDF-1; this may be attributed to the characteristics of proinflammatory cytokines, which are usually released during the initial period of inflammation and are induced very rapidly in response to amplified inflammation [36,37]. Long-duration treatment with GBE resulted in the inhibition of MCP-1, SDF-1 and MIP-1α in LPS-stimulated THP-1 cells, suggesting that GBE may act through multiple intracellular signaling pathways but may not interact directly with TLR4 to alter the expression of these cytokines. The anti-inflammatory cytokines, such as IL-4, IL-6, IL-10, IL-11, IL-12 and IL-13 are a series of immunoregulatory molecules that control the responses of inflammatory cytokines [38]. Less has been known about the influences on anti-inflammatory cytokines expression. Jiao et al. showed that GBE may increase IL-10 expression in U937-derived foam cells [39]. Jiao YB et al. also showed that the protein and mRNA level of IL-10 and IL-10 receptor in the brain were markedly higher in GBE-treated atherosclerotic rats than non-treated atherosclerotic rats [40]. Similar to these reports, we discovered that GBE may increase the production of IL-10 and IL-1R antagonist (IL-1ra) in cultured THP-1 cells (unpublished data). Whether the inducing of IL-10 and IL-1ra by GBE plays a critical role in physiological activity on THP-1 cells remained to be elucidated. Although the immunemodulatory properties and anti-inflammatory effects of GBE could exert a beneficial effect in inflammatory diseases, it was probable that complex mechanisms were also involved.

NO plays an important part in human physiology. NO is synthesized by nitric oxide synthase (NOS), namely neuronal (n-), inducible (i-), and endothelial (e-) NOS, from L-arginine [41].

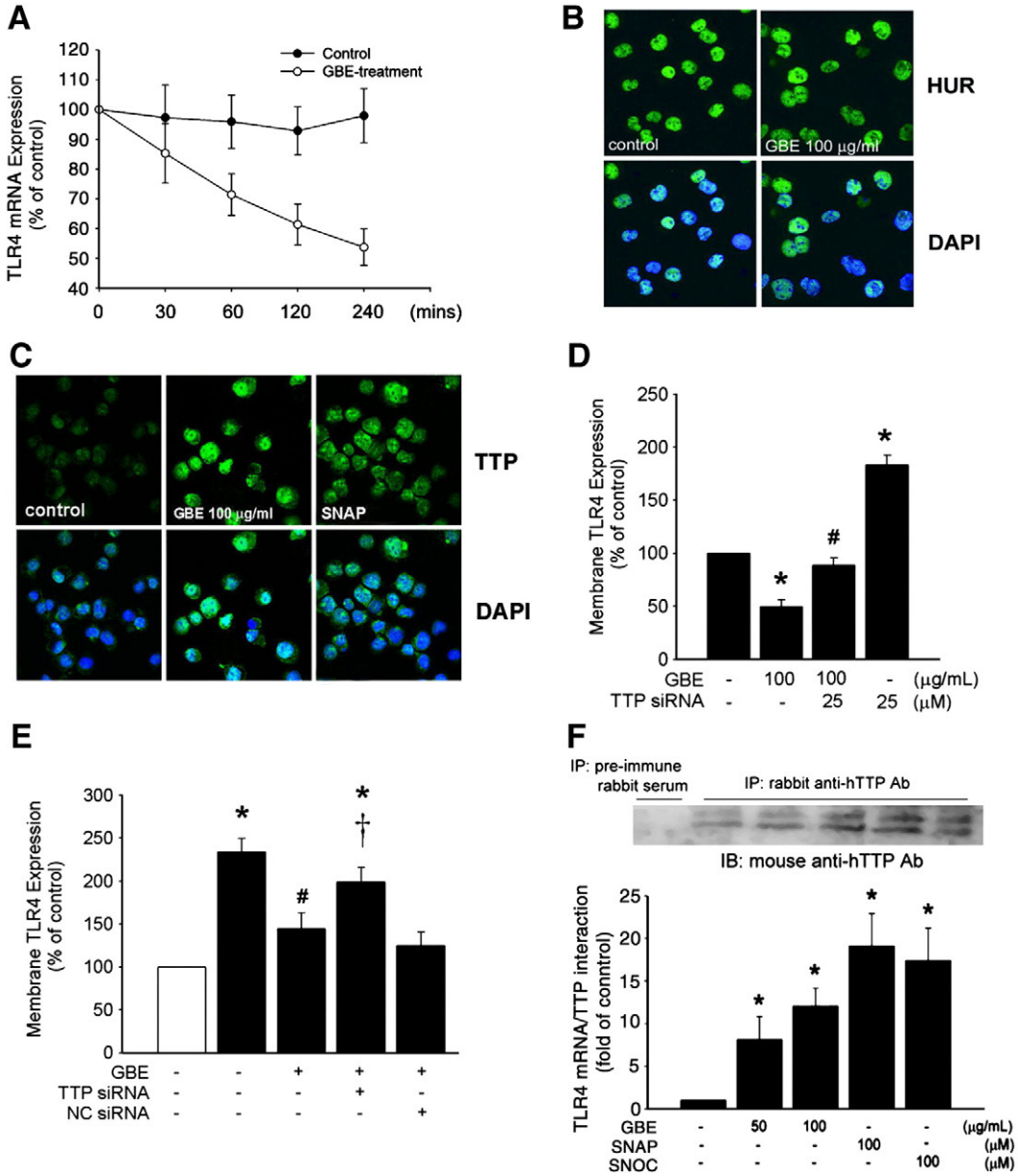

Fig. 5. GBE decreases TLR4 mRNA stability mediated by TTP induction. (A) An actinomycin D chase experiment was performed to evaluate TLR4 mRNA stability. Cells were pretreated with 100 μg/ml GBE for 4 h prior to actinomycin D treatment for 40 min. Total RNA was extracted at various time points, and quantitative real-time PCR was performed. The half-life of TLR4 mRNA was calculated according to the following formula: $t_{1/2}=0.693/\kappa$, where $\kappa=\ln(N_0/N_t)/t$. N_0 represents the cross-point of real-time PCR at $t=0$, and N_t represents the cross-point at time t. (B and C) THP-1 cells were treated with GBE, SNAP or SNOC for 12 h. Subcellular distribution of HuR and TTP in THP-1 cells was detected by immunofluorescence and observed by confocal microscopy. DAPI was used to stain the nuclei of THP-1 cells. (D) THP-1 cells were transfected with 25 nM of TTP siRNA prior to GBE treatment. Membrane TLR4 expression was analyzed by flow cytometry. (E) The human TLR4 plasmid/TTP siRNA cotransfected THP-1 cells were treated with GBE. Membrane TLR4 expression was detected by flow cytometry. Silencer Validated siRNA (NC siRNA) was used to validate the knockdown. (F) THP-1 cells were treated with GBE, SNAP or SNOC, and the interaction between the 3′ UTR of TLR4 mRNA and TTP was analyzed by cross-linking immunoprecipitation experiments and quantitative real-time PCR studies. The data represent the results of three independent experiments (means±S.E.M.; * and †$P<.05$, in comparison with the untreated group or naïve group (□); #$P<.05$, in comparison with the only GBE-treated group or the untreated-TLR4 plasmid-transfected group (■).

Unsimilar to constitutively expressed nNOS and eNOS, iNOS is induced by inflammation or cytokine activation, etc. [42]. The iNOS-synthesized NO plays important roles in immunity and inflammation [43]. During inflammation, a metabolitic pathway, known as respiratory burst, may be activated in leukocytes. The purpose of respiratory burst is the production of microbicidal oxidants through the reduction of oxygen. Unfortunately, high amount or excess of NO is always produced in inflammation which might inhibit antioxidant enzymes and induce elevated hydrogen peroxide (H_2O_2) as well as peroxynitrite ($ONOO^-$) production [44,45]. Simultaneously, the H_2O_2 and $ONOO^-$ may inhibit glutathione reductase [46] and aconitase [47] as well as lead to cell damage. Several evidences showed that LPS may induce large amount of NO production in macrophages, monocytes and animals [43,48–50]. We admire Dr. Marcocci's finding about the NO-scavenging properties of GBE in acellular system [25] in vitro. Nevertheless, in intracellular circumstance, GBE modulates the NO production in inflammation might via multiple and complex signaling pathways. Similar to these evidences [43,48–50], pretreatment of THP-1 cells with 100 μg/ml of GBE for 18 h before LPS stimulation significantly inhibited LPS-induced NO production. (Supplemental Fig 4). In the meanwhile, the NO level in only GBE-treated THP-1 cells also higher than that in

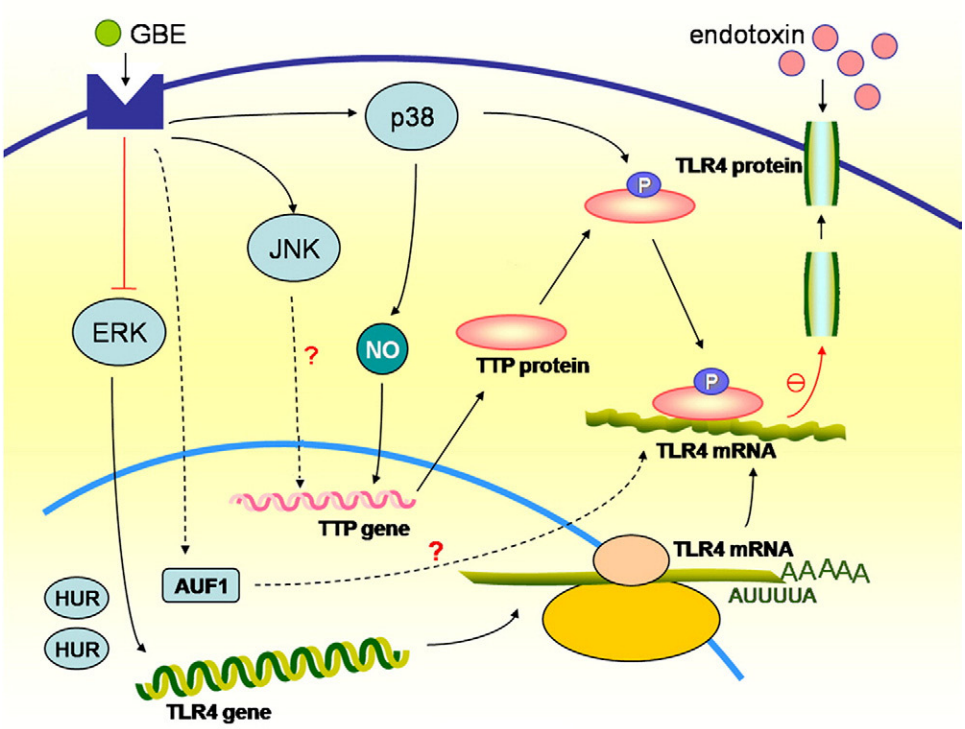

Fig. 6. The schematic diagram summarizes possible mechanisms that GBE reduces TLR4 expression in monocytes via multiple modification pathways.

naïve THP-1 cells but lower than that in LPS-stimulated THP-1 cells. We conjecture and believe that adequate amount of NO in cell may play critical roles for intracellular signaling pathways. To preserve an acceptable level of NO in cell is significant. THP-1 cells cannot express eNOS [43], therefore, iNOS is the only enzyme which can enhance NO production in our study. Embarrassingly, the actual and adequate level of NO for cell is unknown. It is essential to prove by sufficient studies in the future.

In conclusion, we found that TLR4 expression in THP-1 cells is mediated by MAPKs activation and mRNA stabilization. This expression is blocked by GBE, suggesting that the anti-inflammatory ability of GBE is responsible for some of its beneficial effects. Regulation of the sensitivity of monocytes to endotoxins and a therapy directed against TLR4 expression using anti-inflammatory agents such as GBE may be a promising way to prevent inflammatory and immune disturbances. In the future, it would be necessary to further explore the effects of the major components of GBE, i.e., flavonoid glycosides and terpenlactone, on systemic immune functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.03.002.

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