DFMG attenuates the activation of macrophages induced by co-culture with LPC-injured HUVE-12 cells via the TLR4/MyD88/NF-κB signaling pathway

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Abstract. 7-difluoromethoxy-5,4'-dimethoxy-genistein (DFMG) is a novel active chemical entity, which modulates the function and signal transduction of endothelial cells and macrophages (MPs), and is essential in the prevention of atherosclerosis. In the present study, the activity and molecular mechanism of DFMG on MPs was investigated using a Transwell assay to construct a non-contact co-culture model. Human umbilical vein endothelial cells (HUVE-12), which were incubated with lysophosphatidylcholine (LPC), were seeded in the upper chambers, whereas PMA-induced MPs were grown in the lower chambers. The generation of reactive oxygen species (ROS) and the release of lactate dehydrogenase (LDH) were measured using the corresponding assay kits. The proliferation and migration were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and wound healing assays, respectively. Foam cell formation was examined using oil red O staining and a total cholesterol assay. The protein expression levels of Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88) and nuclear factor (NF)-κB p65 were detected by western immunoblotting. The secretion of interleukin (IL)-1β was examined using an enzyme-linked immunosorbent assay. It was found that LPC significantly increased the generation of ROS and the release of LDH in HUVE-12 cells. The LPC-injured HUVE-12 cells activated MPs under co-culture conditions and this process was inhibited by DFMG treatment. LPC upregulated the expression levels of TLR4, MyD88 and NF-κB p65, and the secretion of IL-1β in the supernatant of the co-cultured HUVE-12 cells and MPs. These effects were reversed by the application of DFMG. Furthermore, CLI-095 and IL-1Ra suppressed the activation of MPs that was induced by co-culture with injured HUVE-12 cells. These effects were further enhanced by co-treatment with DFMG, and DFMG exhibited synergistic effects with a TLR4-specific inhibitor. Take together, these findings revealed that DFMG attenuated the activation of MP induced by co-culture with LPC-injured HUVE-12 cells. This process was mediated via inhibition of the TLR4/MyD88/NF-κB signaling pathway in HUVE-12 cells.

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Abbreviations: DFMG,7-difluoromethoxy-5,4'-dimethoxy-genistein; ECs, endothelial cells; MPs, macrophages; HUVE-12, human umbilical vein endothelial cell; LPC, lysophosphatidylcholine; ROS, reactive oxygen species; LDH, lactate dehydrogenase; AS, atherosclerosis; ECD, endothelial cell dysfunction; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; ox-LDL, oxidized low-density lipoprotein; LPS, lipopolysaccharide; GEN, genistein; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; THP-1, human acute monocytic leukemia cells; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; TC, total cholesterol ELISA, enzyme-linked immunosorbent assay

Key words: 7-difluoromethoxy-5,4'-dimethoxy-genistein, Toll-like receptor 4, human umbilical vein endothelial cells, macrophages, co-culture of cells

Introduction

Atherosclerosis (AS) is as a chronic inflammatory disease that exhibits severe complications, including myocardial infarction and ischemic stroke, which are considered two of the main causes of mortality worldwide (1). Macrophages (MPs) are the most abundant inflammatory cell to invade atherosclerotic lesions, and are essential during the atherogenic process (1,2). During initiation, inflammatory cytokines produced by MPs stimulate the generation of endothelial adhesion molecules, proteases and other mediators that enter the systemic circulation in soluble forms (3). Furthermore, MPs internalize

modified lipoproteins in order to produce foam cells, which are considered a hallmark event in the formation of early atherosclerotic lesions (4). During the advanced stage of the disease, MPs contribute to the plaque morphology, affecting the fibrous cap and necrotic core, which increase the pro-inflammatory responses and the apoptotic signals (5). Therefore, strategies focusing on the inhibition of MP activation may be beneficial for the prevention and treatment of AS.

AS is a multiphase process characterized by activation of the endothelium with the secretion of monocytic maturation factors (6). AS is initiated by endothelial injury due to oxidative stress associated with several cardiovascular risk factors (7). Endothelial cell dysfunction (ECD) manifested in lesion-prone areas of arterial vasculature recruits circulating monocytes from the blood into the tunica intima, which eventually differentiate into MPs (8-10). In turn, monocyte-derived MPs trigger an inflammatory cascade, including the production of pro-inflammatory cytokines, interleukin (IL)-1β, IL-12, tumor necrosis factor-α (TNF-α), and chemokines, including monocyte chemoattractant protein-1 (MCP-1). These mediators attract additional monocytes, which adhere to the endothelial cells (ECs) (1,11). In previous years, the interaction between MPs and ECs has been considered a significant feature of a majority of pathophysiological conditions (12). However, the precise signaling pathways between them remain to be fully elucidated.

Toll-like receptor 4 (TLR4) is expressed in MPs, ECs and smooth muscle cells. The receptor is mediated by myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (13,14). Nuclear factor-κB (NF-κB) is a transcription factor, which localizes to the cytoplasm and mainly exists as a heterodimer consisting of the p50 and p65 subunits (15). NF-κB signaling upregulates the expression levels of lectin-like oxidized low-density lipoprotein receptor 1, vascular adhesion molecule 1 and MCP-1 in ECs induced by oxidized low-density lipoprotein (ox-LDL) *in vitro* (16). The TLR4/MyD88/NF-κB signaling pathway is the main signaling pathway to be activated during TLR4-mediated inflammation (17). Activated TLR4 signaling upregulates the protein expression levels of TLR4 pathway-associated mediators, including upstream (TLR4, MyD88 and NF-κB) and downstream (IL-1β, IL-6 and TNF-α) factors (18). IL-1β belongs to the IL-1 cytokine family together with IL-1α, IL-1Ra, IL-18 and IL-33 (19,20). The generation of mature IL-1β is tightly controlled by a diverse class of cytosolic protein complexes, inflammasome components, and the K^+ efflux (19,21,22). However, the exact mechanism by which the activation of TLR4 affects the interaction between ECs and MPs, during the oxidative damage of ECs remains to be elucidated.

Currently, the management of AS aims to reduce inflammation and adjust dyslipidemia. Therefore, research efforts have focused on the development of several natural and synthetic agents in order to attenuate inflammation at the molecular level. Resveratrol inhibits the IL-1β-induced expression of matrix metalloproteinase-13, IL-6 and TNF-α via the TLR4/MyD88-dependent signaling cascades (23,24), and it further attenuates neuronal autophagy and inflammatory injury in experimental traumatic brain injury (25). Diallyl trisulfide exerts anti-inflammatory effects via suppressing the TLR4/NF-κB signaling pathway in lipopolysaccharide (LPS)-stimulated Raw 264.7 macrophages and ischemic stroke-induced inflammation (26,27). In addition, (S,R)-3-phenyl-4,5-dihydro-5-isoxasole acetic acid inhibited LPS-induced NF-κB and p38 mitogen-activated protein kinase signaling pathways, and reduced the secretion of IL-1β, TNF-α and IL-10 from peritoneal cells *in vitro* and *ex vivo* (28,29). However, there are several disadvantages limiting the clinical application of these agents, including side effects and poor oral bioavailability (30). 7-difluoromethoxy-5,4'-dimethoxy-genistein (DFMG) is a novel active chemical entity, which is synthesized by the precursor genistein (GEN), and exhibits optimal fat-solubility, antioxidant and anti-inflammatory activities (31,32). DFMG is involved in the regulation of different inflammatory cytokines, including E-selectin, intercellular adhesion molecule 1 (ICAM-1), IL-6 and TNF-α (33). In addition, DFMG interacts with the inhibition of different signaling pathway activations, including mitochondrial apoptosis and TLR4 signaling (33-35).

The activation of MPs has been shown to be the critical step in the development of chronic inflammation. Therefore, the mechanism involving the activation of MPs is important for the development of preventive and therapeutic strategies for AS. The aim of the present study was to: i) identify the function of injured ECs with regard to the activation of MPs in a non-contact co-culture model and ii) to investigate the effect and molecular mechanism of DFMG treatment on the activation of MPs induced by co-culture with injured ECs.

Materials and methods

Reagents and antibodies. Phorbol 12-myristate 13-acetate (PMA), lysophosphatidylcholine (LPC) and oil red O powder were purchased from Sigma-Aldrich; EMD Millipore (Billerica, MA, USA). DFMG (purity >99%) was synthesized as previously reported (31) and dissolved in dimethyl sulfoxide (DMSO; Ameresco, Inc., Framingham, MA, USA). This compound was subsequently sterilized by filtration. CLI-095 was obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The IL-1 receptor antagonist (IL-1Ra) was purchased from Bioss (Beijing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Beyotime Institute of Biotechnology (Shanghai, China). Hematoxylin staining reagents were obtained from Servicebio Technology Co., Ltd. (Wuhan, China). Anti-TLR4 and NF-κB p65 antibodies were provided by ProteinTech Group, Inc. (Chicago, IL, USA, cat. nos. 19811-1-AP and 10745-1-AP). Anti-MyD88 and GAPDH antibodies were provided by Abgent, Inc. (San Diego, CA, USA cat. nos. ABO10975 and AM1020b). Goat anti-rabbit IgG and goat anti-mouse IgG were provided by ComWin Biotech Co., Ltd. (Beijing, China, cat. nos. CW0156S and CW0102S).

Cell culture and treatment. Human umbilical vein ECs (HUVE-12) and human acute monocytic leukemia cells (THP-1) were purchased from the China Center for Type Culture Collection (Wuhan, China) and Institute of Biochemistry and Cell Biology (Shanghai, China), respectively. The two cell lines were incubated in a humidified incubator at 37˚C with 5% CO_2 and were cultured in Roswell Park Memorial

Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The THP-1 cells ($1x10^6$ cells/ml) were seeded in 6-well plates and activated with 200 nM of PMA for 24 h, which allowed them to transform into MPs. All cell culture reagents were purchased from Biological Industries (Beit Haemek, Israel).

Reactive oxygen species (ROS) assay. The HUVE-12 cells were treated at 37˚C with various concentrations of LPC (10, 20, 30, 40 and 50 μM). Following incubation for 24 h, fresh medium with 10% FBS was used and the cells were cultured for another 24 h. Subsequently, ROS was determined using a ROS assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Fluorescence intensity was detected at excitation and emission wavelengths of 488 and 525 nm, respectively. Hematoxylin staining was used to quantify the number of cells under a light microscope (CX41RF, Olympus, Tokyo, Japan). The fluorescence intensity of each cell was obtained from the ratio of total fluorescence intensity to the total cell count.

Lactate dehydrogenase (LDH) assay. The HUVE-12 cells were incubated with various concentrations of LPC and subsequently treated as described in the aforementioned protocol. Subsequently, the supernatants were collected in order to determine the concentration of LDH using the LDH cytotoxicity assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The optical density (OD) was measured at 450 nm with a microplate reader (Elx800; BioTek Instruments, Inc., Winooski, VT, USA). The concentration of LDH was calculated according to the manufacturer's protocol.

Establishment of the co-culture model. The HUVE-12 cells, which had been stimulated by LPC were co-cultured with MPs in a Transwell chamber consisting of a 10-μm thick porous membrane with 0.4-μm pores in the culture inserts (Corning Incorporated, Corning, NY, USA). This methodology was used to construct an indirect co-culture system. Briefly, the MPs were seeded in the wells of a 6-well ($1x10^5$ cells/well) and/or a 24-well ($2.5x10^4$ cells/well) plate (lower layer) at 37˚C prior to co-culture with the HUVE-12 cells. The HUVE-12 cells were individually grown in the upper layer of the Transwell under the following treatment conditions: Pretreatment with LPC for 24 h, and/or pretreatment with DFMG for 1 h, followed by CLI-095 and IL-1Ra for 24 h, and subsequent treatment with LPC for another 24 h. The HUVE-12 cells in fresh medium containing the inserts were added to the upper chamber of the Transwell system, with the MPs located at the bottom.

Proliferation assay. The MTT assay was used to detect the viability of the cells. The MPs were co-cultured with the HUVE-12 cells, which had been pretreated for 24 h in 24-well plates, and 50 μl of MTT solution (MTT powder dissolved in PBS, 5 mg/ml) was added to each well. The plates were incubated at 37˚C for 4 h. Following removal of the medium, 750 μl of DMSO was added to each well for 10 min in order to fully dissolve the formazan crystals. The OD was measured on a microplate reader (BioTek Instruments, Inc.) at 490 nm. The viability of the cells was assessed in terms of OD values.

Migration assay. A wound healing assay was used to analyze the migratory activity of the cells. Prior to co-culture, the MPs were seeded in 6-well plates and scratched with a pipette tip (200-μl), followed by rinsing with PBS and incubation in RPMI-1640 medium in the absence of FBS. Images were captured at the 0 h time point. The pretreated HUVE-12 cells containing inserts were added to the upper chamber of the Transwell system and incubated at 37˚C. After 24 h, an additional image (24 h) was captured in the same position. The cell-free area was quantified using Adobe Photoshop CS6 software (Adobe Systems, Inc., San Jose, CA, USA).

Oil red O staining. The oil red O working solution was prepared by diluting the stock solution (0.05 g of oil red O powder dissolved in 10 ml of isopropanol) with distilled water (3:2), following which the solution was filtered. The MPs were co-cultured with HUVE-12 cells for 24 h and fixed in 4% paraformaldehyde for 15 min in order to prepare the samples for oil red O staining. The samples were washed with distilled water twice and were incubated with filtered oil red O working solution at room temperature for 10 min. Subsequently, the samples were washed twice with distilled water, and the cells were observed for image capture under an optical microscope (CX41RF, Olympus, Tokyo, Japan). The cells containing oil red O-positive fat droplets were considered as foam cells.

Assessment of total cholesterol (TC). The quantification of TC was performed according to the protocols provided by the manufacturer (Solarbio, Beijing, China). The MPs were co-cultured with HUVE-12 cells for 24 h, and the cells were collected and dissolved in isopropanol (1 ml/$4x10^4$ cells), followed by ultrasound treatment for 1 min. The samples were centrifuged at 4˚C at 8,000 x g for 10 min. The OD of the supernatants was detected at 500 nm using a spectrophotometer. The concentration of TC was calculated according to the manufacturer's protocol.

Western blot analysis. The pretreated HUVE-12 cells, which were co-cultured with MPs for 24 h, were digested and lysed in radioimmunoprecipitation assay buffer containing 1% phenylmethyl sulfonyl fluoride. The samples were centrifuged at 12,000 x g at 4˚C for 10 min. Protein quantitation was performed using the BCA protein assay kit (Solabio). A total of 25 μg of each protein lysate was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Solarbio), followed by transfer onto polyvinylidene difluoride membranes (EMD Millipore), which were blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween-20) at room temperature for 1 h. The membranes were incubated with antibodies against TLR4 (1:1,000), MyD88 (1:10,000), NF-κB p65 (1:2,500) and GAPDH (1:1,000) on a shaking platform at 4˚C overnight. Subsequently, the membranes were washed with PBST and incubated with goat anti-rabbit antibody (1:10,000) and goat anti-mouse antibody (1:10,000) for 1 h at room temperature, followed by washing in PBST three times. Finally, the protein bands were visualized using enhanced chemiluminescence. The results were analyzed using densitometry with GelPro 3.2 software (Media Cybernetics, Inc., Rockville, MD, USA).

Enzyme-linked immunosorbent assay (ELISA). Following co-culture for 24 h, the concentration of IL-1β in the supernatants was measured using a human IL-1β ELISA kit (MultiSciences Biotech Co., Ltd., Hangzhou, China) according to the manufacturer's protocol. The standards and/or samples were incubated in the wells at 37˚C for 1.5 h. Subsequently, the wells were washed and primary antibody was added, covered with an adhesive strip and incubated at 37˚C for 1 h. Following washing of the unbound biotinylated antibody, streptavidin-HRP was added for incubation at 37˚C for 30 min. A cycle of five washing steps (with PBST for 30 sec each time) was performed and substrate solution was added to the samples. Following incubation at dark for 30 min, the stop solution was added. The OD was detected at 450 nm on a microplate reader (BioTek Instruments, Inc.), and the concentration of IL-1β was calculated according to the manufacturer's protocol.

Statistical analysis. All experiments were performed independently at least three times, and the values are expressed as the mean ± standard deviation. SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. Student's paired t-test was used for the comparison of two samples, and one-way analysis of variance was used for multiple group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of the injured HUVE-12 cell-MP co-culture model. Initially, HUVE-12 cells were incubated with different concentrations (10, 20, 30, 40 and 50 μM) of LPC for 24 h, and the production of ROS and LDH were detected. The results indicated that LPC damaged the HUVE-12 cells in a concentration-dependent manner. LPC significantly promoted the generation of ROS at a concentration range of 20-50 μM (Fig. 1A and B). In terms of LDH activity, the effective concentration range of LPC was estimated to be between 30 and 50 μM (Fig. 1C). Therefore, 30 μM of LPC was selected in order to injure the HUVE-12 cells.

Subsequently, the capacity of MPs to proliferate, migrate and develop into foam cells following co-culture with injured HUVE-12 cells was detected. The HUVE-12 cells were incubated with LPC (30 μM) for 24 h and co-cultured with MPs in a Transwell system for another 24 h. A significant increase in the proliferation and migration of MPs was observed, which was caused by co-culture with injured HUVE-12 cells (Fig. 1D-F). In addition, co-culture with injured HUVE-12 cells resulted in abundant cytoplasmic lipid droplet accumulation in the MPs, as demonstrated by oil red O staining (Fig. 1G) and TC (Fig. 1H). Consequently, 30 μM of LPC was selected as the optimum concentration in order to construct the LPC-injured HUVE-12 cell-MP co-culture model.

LPC promotes the activation of TLR4/MyD88/NFκ-B signaling pathway in HUVE-12 cells co-cultured with MPs. It has been shown that the TLR4/MyD88/NF-κB transduction pathway is an important event in inflammation and in the subsequent secretion of IL-1β (36). Consequently, the change in the expression levels of TLR4 and its downstream signaling molecules was monitored in LPC-injured HUVE-12 cells. The supernatants collected from the MPs following co-culture with HUVE-12 cells for 24 h were obtained. It was noted that the expression levels of TLR4, MyD88 and NF-κB p65 in HUVE-12 cells were significantly upregulated (Fig. 2A and B) and the secretion of IL-1β in the co-culture supernatant was effectively increased (Fig. 2C). Taken together, these results suggested that LPC promoted the activation of the TLR4/MyD88/NF-κB signaling pathway in HUVE-12 cells. This pathway is essential in the cross-talk between LPC-injured-HUVE-12 cells and MPs.

DFMG inhibits the activation of MPs induced by co-culture with injured HUVE-12 cells. To determine whether DFMG was able to attenuate the activation of MPs induced by co-culture with LPC-oxidative damaged HUVE-12 cells, the HUVE-12 cells were treated with various concentrations (0.3, 1.0 and 3.0 μM) of DFMG for 1 h prior to LPC (30 μM) treatment. DFMG effectively inhibited the proliferation of MPs at a concentration range of 0.3-3.0 μM (Fig. 3A), and treatment at concentrations of 1.0-3.0 μM DFMG effectively suppressed the migration (Fig. 3B and C) and the formation of foam cells (Fig. 3D and E) in a dose-dependent manner.

DFMG inhibits the TLR4/MyD88/NF-κB signaling pathway in HUVE-12 cells. As mentioned above, activation of the TLR4/MyD88/NF-κB signaling pathway in HUVE-12 cells was associated with the cross-talk between HUVE-12 cells and MPs. The molecular mechanism underlying the effect of DFMG, in relation to the activation of MPs induced by co-culture with injured HUVE-12 cells, was examined by investigating the expression levels of TLR4, MyD88 and NF-κB p65 in the HUVE-12 cells co-cultured with MPs. In addition, the concentration of IL-1β in the co-culture supernatant was monitored using ELISA. The data indicated that DFMG (1.0 and/or 3.0 μM) effectively reduced the expression levels of TLR4, MyD88 and NF-κB p65 (Fig. 4A-D) and the secretion of IL-1β (Fig. 4E). These observations suggested that DFMG attenuated the activation of MPs induced by co-culture with injured HUVE-12 cells, at least partly, via inhibition of the TLR4/MyD88/NF-κB signaling pathway in the LPC-injured HUVE-12 cells. Accordingly, 3.0 μM was selected as the optimum concentration of DFMG to be used for the following experiments.

TLR4 signaling pathway is involved in the activation of MPs induced by co-culture with injured HUVE-12 cells. To further confirm the effect of the TLR4 signaling pathway on the activation of MPs induced by co-culture with injured HUVE-12 cells, the HUVE-12 cells were pre-incubated with DFMG (3.0 μM) for 1 h, and CLI-095 (5 μg/ml) or IL-1Ra (10 μg/ml) were added for 24 h. Subsequently, LPC (30 μM) was added and incubated for another 24 h, followed by co-culture with MPs for 24 h. The results indicated that DFMG, CLI-095 and IL-1Ra significantly decreased the proliferation and migration of MPs (Fig. 5A-C) and the formation of foam cells (Fig. 5D and E), which were induced by co-culture with injured HUVE-12 cells. In addition, DFMG with CLI-095 or IL-1Ra exhibited synergistic effects in promoting the stability

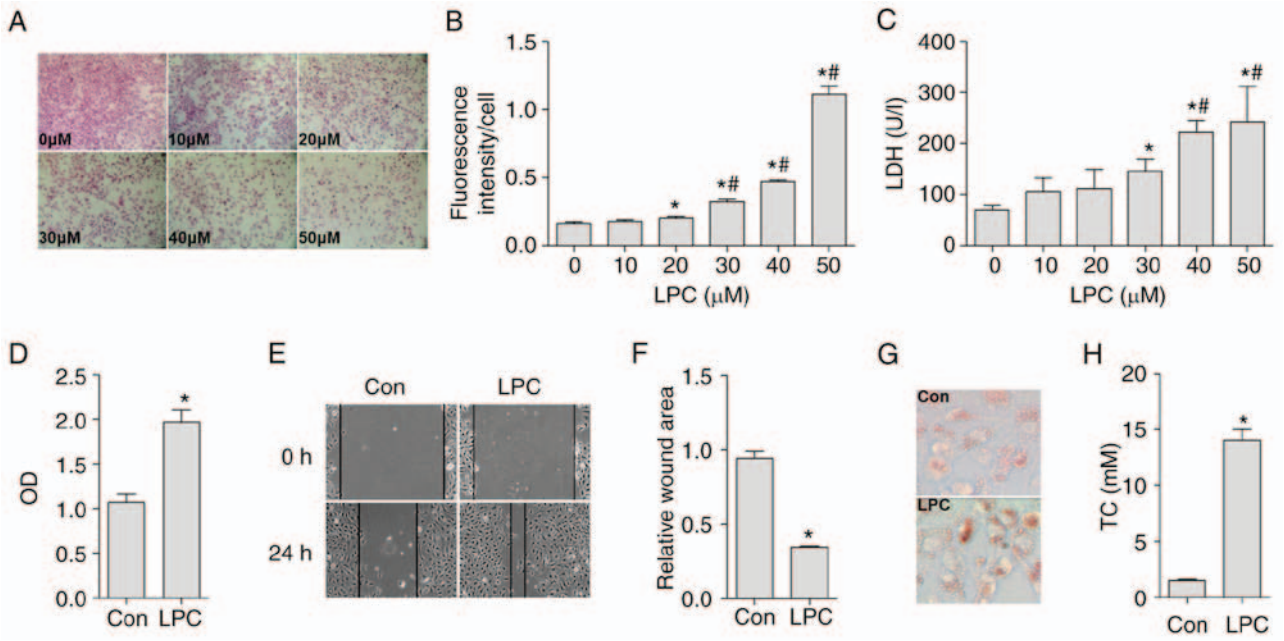


Figure 1. Effects of LPC on the generation of ROS and LDH in HUVE-12 cells, and activation of MPs induced by co-culture with LPC-injured HUVE-12 cells. (A) Representative images of hematoxylin and eosin staining of LPC-injured HUVE-12 cells. The number of stained cells was counted for each group across four areas at 200x magnification and the mean was estimated. (B) LPC (20, 30, 40 and 50 μM) significantly enhanced the fluorescence intensity of each cell in a concentration-dependent manner. (C) LPC (30, 40 and 50 μM) significantly increased the release of LDH in a concentration-dependent manner. LPC (30 μM) effectively increased the (D) cell viability, and decreased the relative wound area of MPs co-cultured with LPC-injured HUVE-12 cells, as shown in (E) images (magnification, x100) and (F) graph. (G) Representative images of foam cell formation. LPC (30 μM) effectively increased lipid accumulation in MPs (magnification, x200). (H) LPC (30 μM) effectively increased the TC content. Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, vs. 0 μM LPC and/or Con (not treated with LPC) group, $^{\#}$P<0.05, vs. 20 μM and/or 30 μM LPC group. LPC, lysophosphatidylcholine; ROS, reactive oxygen species; LDH, lactate dehydrogenase; MPs, macrophages; Con, control; OD, optical density.

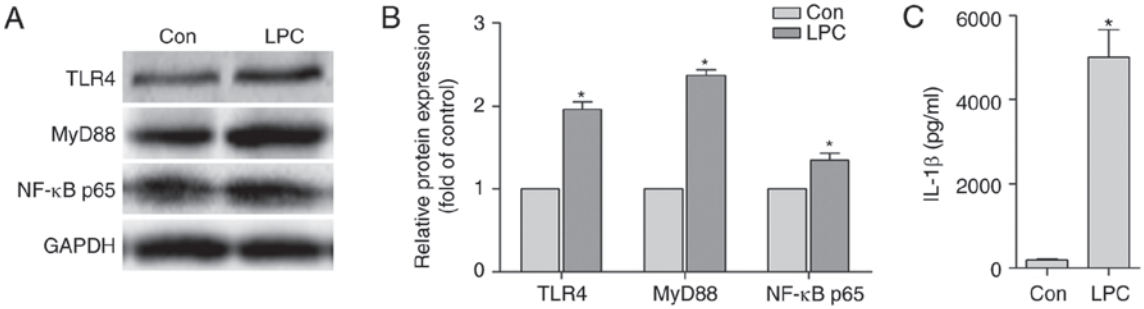


Figure 2. LPC activates the TLR4/MyD88/NF-κB pathway in HUVE-12 cells and increases the secretion of IL-1β in the co-culture supernatant. (A) Representative western blots of protein expression of TLR4, MyD88 and NF-κB p65 in HUVE-12 cells treatment with or without LPC (30 μM) following co-culture with macrophages. (B) Densitometric analysis was used to quantify the protein levels of TLR4, MyD88 and NF-κB p65, which were markedly upregulated in LPC-injured HUVE-12 cells. (C) Concentration of IL-1β in the co-culture supernatant of LPC-injured HUVE-12 cells was increased. The data are presented as the mean ± standard deviation of three separate experiments. *P<0.05 vs. Con (not pretreated with LPC) group. LPC, lysophosphatidylcholine; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; IL-1β, interleukin-1β; Con, control.

of MPs. These data indicated that the inhibition of TLR4 or its downstream targets in HUVE-12 cells effectively attenuated the activation of MPs induced by co-culture with LPC-injured HUVE-12 cells. The results also confirmed that the TLR4 signaling pathway in LPC-injured HUVE-12 cells was associated with the activation of MPs induced by co-culture.

DFMG attenuates activation of the TLR4/MyD88/NF-κB signaling pathway in LPC-injured HUVE-12 cells co-cultured with MPs. DFMG (3.0 μM), CLI-095 (5 μg/ml), IL-1Ra (10 μg/ml) and LPC (30 μM) were incubated with HUVE-12 cells, as described above, in order to investigate the molecular mechanism underlying the effect of DFMG on the inhibition of the activation of MPs induced by co-culture with LPC-injured HUVE-12 cells. The results indicated that DFMG, CLI-095 and IL-1Ra significantly downregulated the expression levels of TLR4, MyD88 and NF-κB p65 (Fig. 6A-D) in the HUVE-12 cells and decreased the secretion of IL-1β (Fig. 6E) in the co-culture supernatant. Furthermore, DFMG and CLI-095 or IL-1Ra exhibited synergistic effects in the inhibition of the TLR4 signaling pathway. Consequently, DFMG suppressed activation of the TLR4/MyD88/NF-κB signaling pathway in LPC-injured HUVE-12 cells following co-culture with MPs.

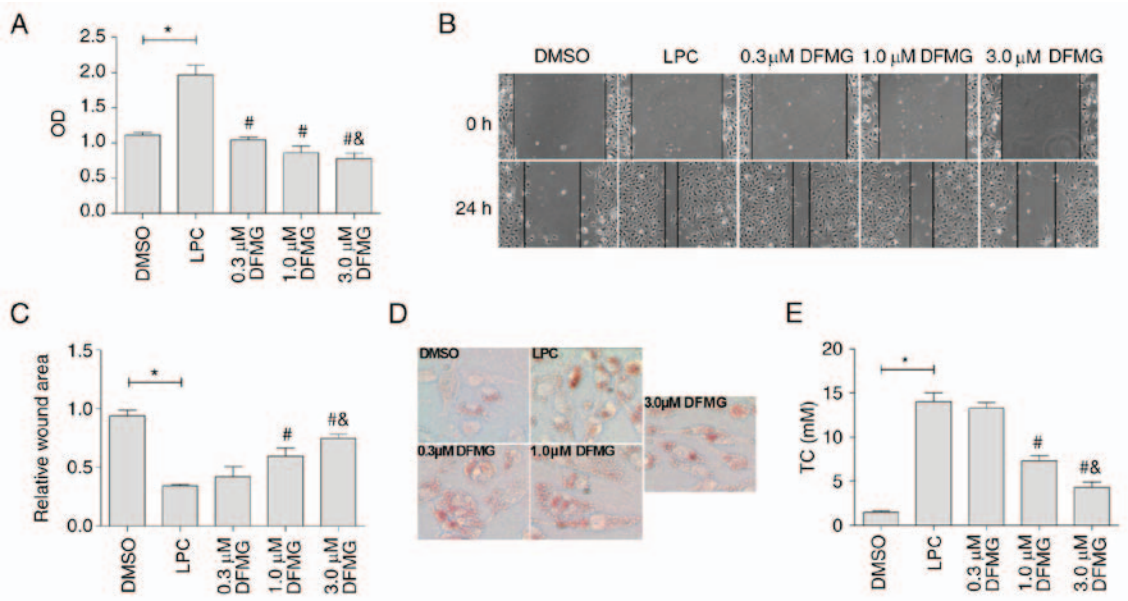


Figure 3. DFMG attenuates the activation of MPs induced by co-culture with LPC-injured HUVE-12 cells in a dose-dependent manner. (A) DFMG (0.3, 1.0 and 3.0 μM) effectively decreased cell viability of MPs co-cultured with LPC-injured HUVE-12 cells. (B) Images and (C) quantification showing that DFMG (1.0 and 3.0 μM) effectively increased the relative wound area of MPs (magnification, x100). (D) Images and (E) graph show that DFMG (1.0 and 3.0 μM) significantly reduced the lipid accumulation in MPs (magnification, x200). The data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, vs. DMSO group, $^{\#}$P<0.05, vs. LPC group, $^{\&}$P<0.05, vs. 0.3 or 1.0 μM DFMG group. LPC, lysophosphatidylcholine; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; MPs, macrophages; DMSO, dimethyl sulfoxide.

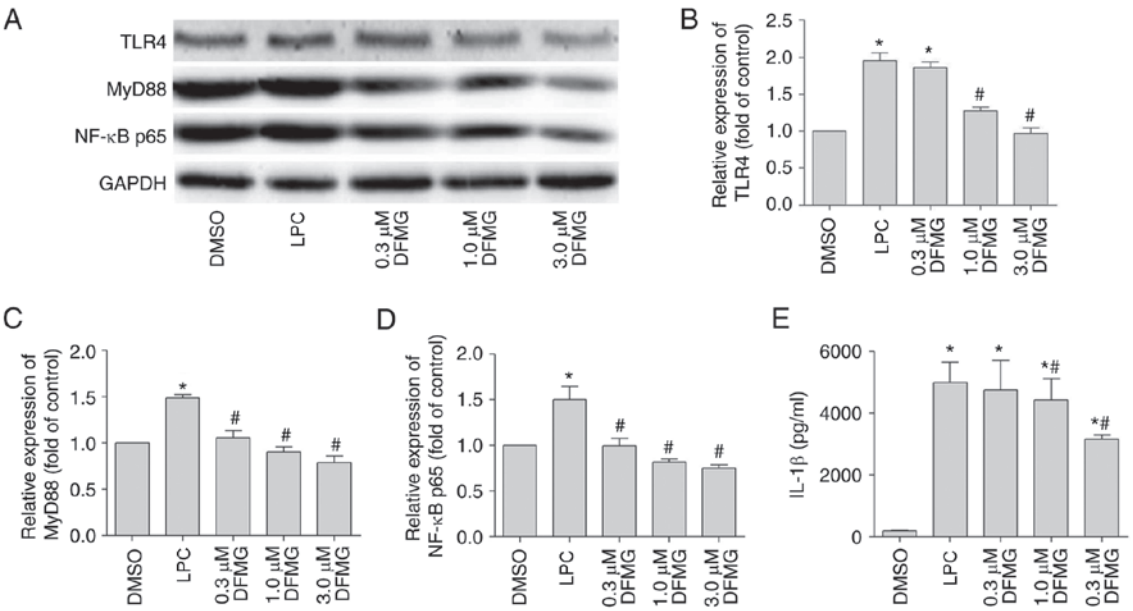


Figure 4. DFMG represses activation of the TLR4/MyD88/NF-κB pathway in LPC-injured HUVE-12 cells co-cultured with MPs, and reduces the generation of IL-1β in the co-culture supernatant. (A) Representative western blots of protein expression levels of TLR4, MyD88 and NF-κB p65 in HUVE-12 cells co-cultured with MPs. Densitometric analysis was used to quantify the protein levels of (B) TLR4, (C) MyD88 and (D) NF-κB p65. DFMG (0.3, 1.0 and 3.0 μM) downregulated the expression of these proteins. (E) DFMG (1.0 and 3.0 μM) decreased the concentration of IL-1β in the co-culture supernatant. The data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, vs. DMSO group, $^{\#}$P<0.05, vs. LPC group. LPC, lysophosphatidylcholine; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; MPs, macrophages; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; IL-1β, interleukin-1β; DMSO, dimethyl sulfoxide; OD, optical density.

Discussion

MP dysregulation is considered a risk factor for several inflammatory complications, including AS and cancer (37). The process of atherogenesis involves several types of cells, notably ECs, smooth muscle cells, monocytes and macrophages. AS initiates from the endothelium of the arterial wall (7). ECD is a vital contributor to the pathobiology of atherosclerotic cardiovascular disease (8). Previous studies have shown that ECD induces the functional changes of MPs through the mutual cross-talk between cells in the vascular microenvironment (12,38). The *in vitro* Transwell co-culture model can be

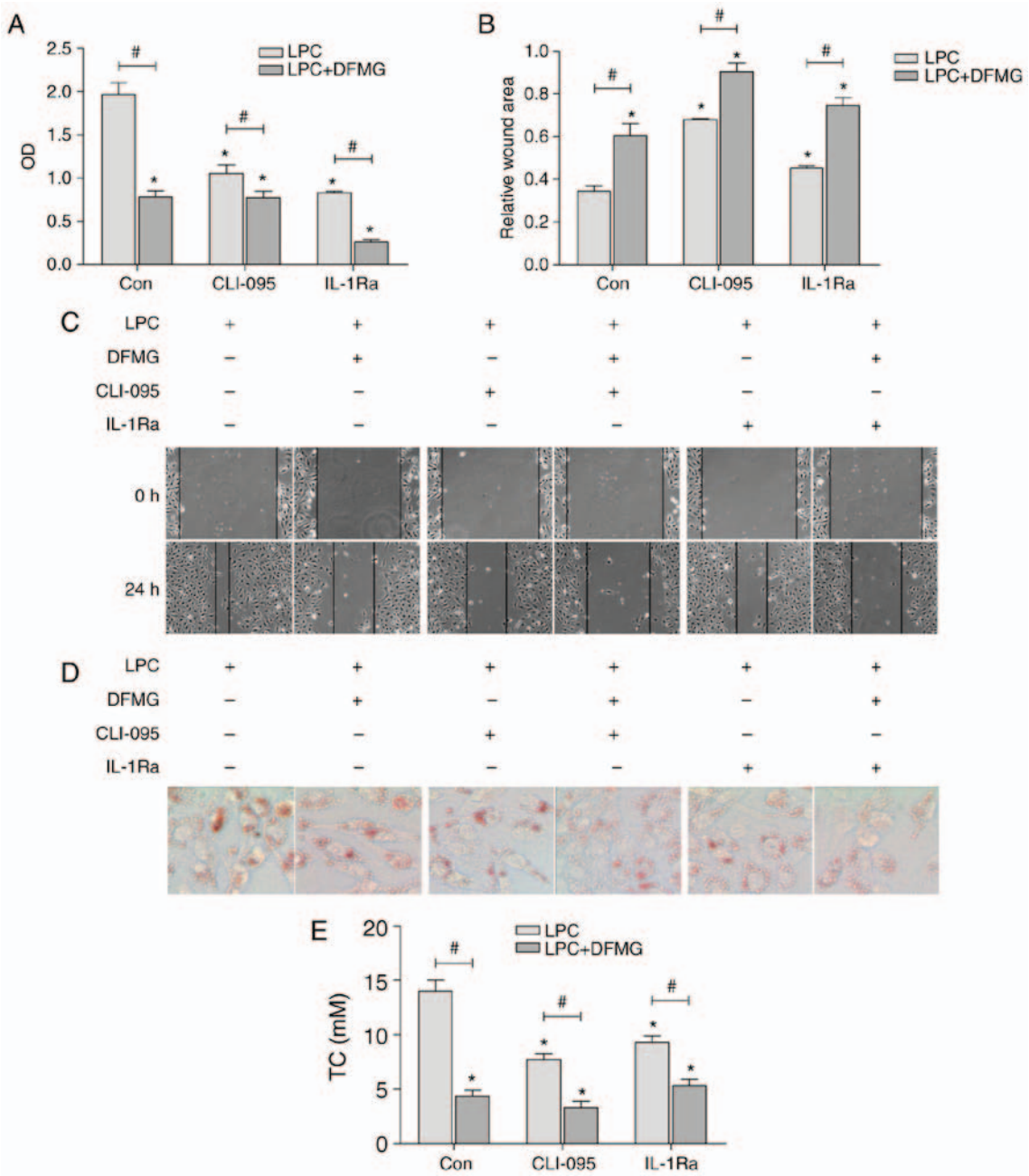


Figure 5. TLR4/MyD88/NF-κB pathway is involved in the activation of MPs induced by co-culture with LPC-injured HUVE-12 cells. (A) CLI-095 and IL-1Ra effectively reduced the cell viability of MPs co-cultured with LPC-injured HUVE-12 cells, which were enhanced by co-treatment with DFMG (magnification, x200). (B) Graph and (C) images of wound healing assay showed that CLI-095 and IL-1Ra effectively increased the relative wound area of MPs, which were promoted by DFMG (magnification, x100). (D) Images and (E) quantification showed that CLI-095 and IL-1Ra effectively reduced lipid accumulation in MPs co-cultured with LPC-injured HUVE-12 cells, which were enhanced by co-treatment with DFMG (magnification, x200). The data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, vs. Con (without DFMG pretreatment) group, #P<0.05, vs. adjacent group. LPC, lysophosphatidylcholine; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; MPs, macrophages; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; IL-1Ra, interleukin-1 receptor antagonist; OD, optical density.

used to examine the effects of ECD on MPs. Oxidative stress is key in the progression of AS (39). LPC is a major component of ox-LDL, which can cause AS (40). In the present study, LPC stimulated the generation of ROS and the release of LDH (cell oxidative damage index) in ECs, but promoted the activation of MPs induced by co-culture with EC. The results also indicated that LPC increased the expression levels of TLR4, MyD88 and NF-κB p65 in the ECs co-cultured with THP-1-derived MPs. LPC also increased the secretion of IL-1β in the co-culture supernatant. These findings demonstrated that LPC exhibited oxidative effects on ECs. In addition to these observations, ECD promoted the activation of MPs, which may be associated with activation of the TLR4/MyD88/NF-κB signaling pathway in ECs.

AS, the main cause of cardiovascular disease, is characterized by the accumulation of inflammatory cells in the artery wall (41). TLRs are crucial in the initiation of an innate immune response through activating the inflammatory cells (42). To confirm that the TLR4 signaling pathway was involved in the activation of macrophages induced by co-culture

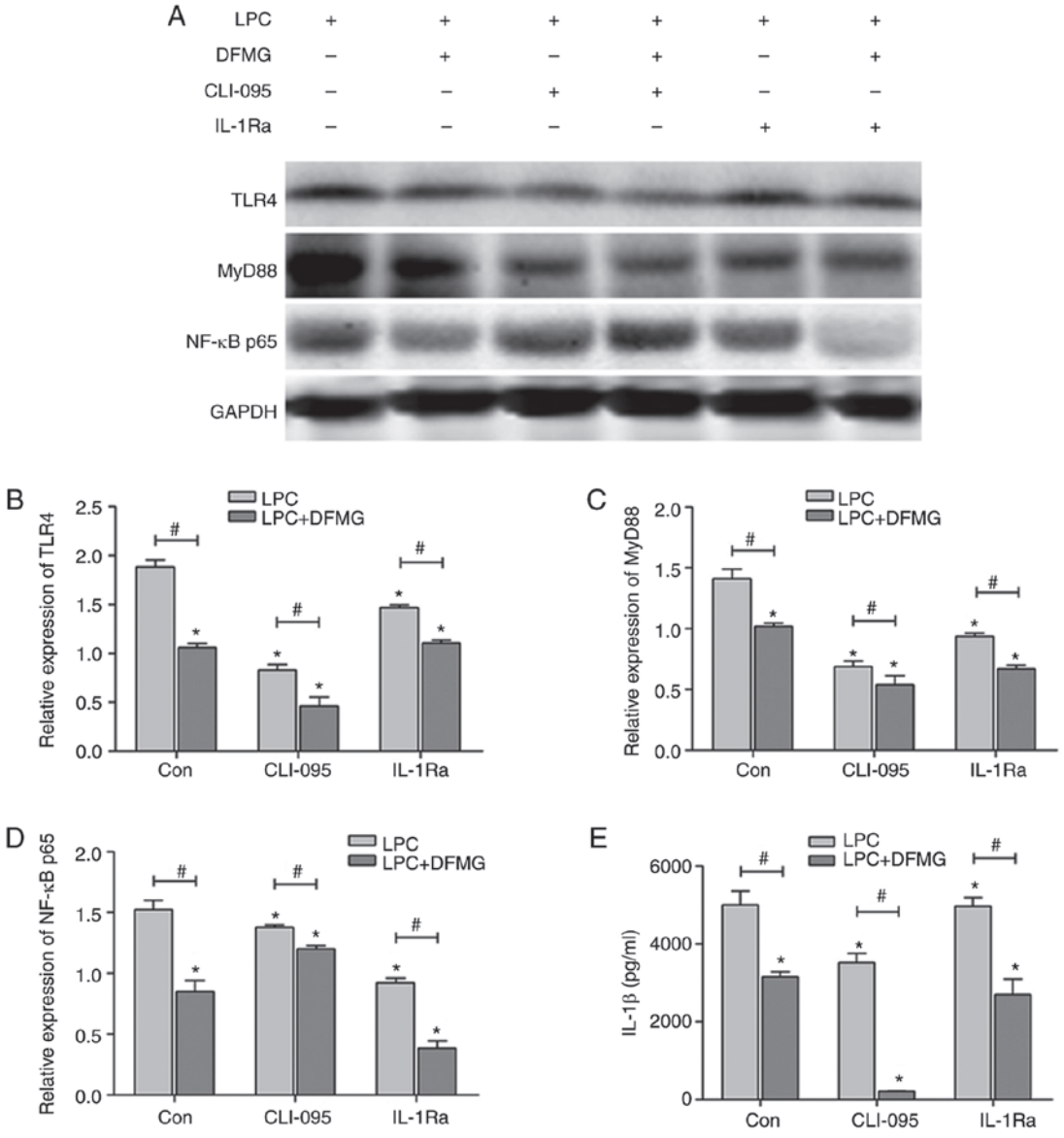


Figure 6. DFMG inhibits the TLR4/MyD88/NF-κB pathway in LPC-injured HUVE-12 cells co-cultured with MPs, and inhibits the production of IL-1β in the co-culture supernatant. (A) Representative western blots of protein expression of TLR4, MyD88 and NF-κB p65 in LPC-injured HUVE-12 cells co-cultured with MPs. Densitometric analysis and ELISA were used to quantify the expression levels of (B) TLR4, (C) MyD88, (D) NF-κB p65 and the (E) concentration of IL-1β in the co-culture supernatant. CLI-095 and IL-1Ra downregulated the expression of these proteins and reduced the generation of IL-1β, which was promoted by co-treatment with DFMG. The data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, vs. Con (without DFMG pretreatment), #P<0.05, vs. adjacent group. LPC, lysophosphatidylcholine; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; MPs, macrophages; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; IL-1Ra, interleukin-1 receptor antagonist.

with LPC-injured HUVE-12 cells, specific inhibitors of the proteins and the mediators acting on this pathway were used. CLI-095, also known as TAK-242, is a novel cyclohexene derivative, which selectively suppresses TLR4 signaling, potently suppressing ligand-dependent and ligand-independent signaling of TLR4 (43,44). IL-1 belongs to the innate immune system and is important in the initiation of the immunoinflammatory cascade reaction (45). IL-1Ra is a specific and pure antagonist of the IL-1 receptor family (46), which has the capacity to inhibit inflammasome activation and control the balance between the pro-inflammatory and anti-inflammatory response (47). As expected, treatment of LPC-injured EC with CLI-095 and IL-1Ra significantly impaired the proliferation and migration of MPs, and the formation of foam cells induced by co-culture with injured ECs. Taken together, the data demonstrated that activation of the TLR4 signaling pathway in ECs led to the proliferation, migration and lipid accumulation of MPs in the co-culture model.

GEN, a primary soy isoflavone, has received considerable attention as a protein kinase inhibitor (48). In addition, studies have demonstrated that GEN possesses atheroprotective effects via regulation of the TLR4/NF-κB signaling pathway (49,50). GEN exhibits low bioavailability and intestinal absorption *in vivo*, and its clinical application is limited (51). DFMG is synthesized using GEN as a precursor. DFMG exhibits improved bioavailability and absorption. Our previous studies indicated that DFMG was more efficient than GEN in reducing the risk of cardiovascular disease by the inhibition of oxidative damage, mitochondrial apoptosis and/or inhibition of the TLR4 signaling pathway (33-35). In the present study,

DFMG was shown to effectively attenuate the activation of THP-1-derived MPs induced by co-culture with LPC-injured ECs. Furthermore, inhibition of the TLR4 signaling pathway using specific inhibitors (CLI-095 and IL-1Ra) increased the anti-inflammatory potential of DFMG. These observations indicated that DFMG may exert its anti-inflammatory activity through inhibition of the TLR4 signaling pathway.

In view of the aforementioned results, the expression levels of TLR4 were further assessed. In the present study, DFMG significantly downregulated the protein expression levels of the aforementioned indicators (TLR4, MyD88 and NF-κB p65) in ECs, and the levels of IL-1β in the co-culture supernatant. CLI-095 and IL-1Ra markedly reduced the activation of TLR4, which was markedly enhanced by DFMG treatment. The endogenous IL-1Ra has been shown to regulate the extent of TLR9-induced liver damage (52), whereas IL-1Ra deficiency can promote TLR4-dependent arthritis (53). The present study hypothesized that DFMG may act by upregulating the expression of endogenous IL-1ra in order to inhibit the activity of TLR4.

Considering the above findings, it was suggested that the atheroprotective effects of DFMG against the activation of MPs induced by co-culture with LPC-injured ECs was mediated via regulation of the TLR4/MyD88/NF-κB signaling pathway in ECs. However, the effects of DFMG on MPs were not examined *in vivo*. Consequently, the detailed effects of DFMG on the TLR4 signaling network in AS require further investigation using animal knockout models. TLR4 signaling is also crucial in other diseases, including systemic lupus erythematosus (29), non-small cell lung cancer (54), idiopathic pulmonary fibrosis (55), myocardial inflammation (56) and type 2 diabetes (57). According to the present study, it was hypothesized that DFMG may prevent and/or treat these diseases via inhibiting the TLR4 signaling pathway.

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Availability of data and materials

All data generated or analysed during this study were included in this published article.

Authors' contributions

XF designed the study and prepare the manuscript. LC and SY performed the experiments. YZ analysed the data. JC revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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