

# DFMG reverses proliferation and migration of vascular smooth muscle cells induced by co-culture with injured vascular endothelial cells via suppression of the TLR4-mediated signaling pathway

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**Abstract.** 7-Difluoromethoxy-5,4'-dimethoxy-genistein (DFMG) is a novel chemical compound synthesized using genistein. Previous studies have indicated that DFMG can reverse the apoptosis of vascular endothelial cells (VECs) by regulating the mitochondrial apoptosis pathway. The present study aimed to investigate the activity and molecular mechanism underlying DFMG-mediated protection of vascular smooth muscle cell (VSMCs) using a non-contact co-culture model established by using Transwell insert. Secretion of interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were measured by ELISA. Proliferation and migration of VSMCs were assessed using a Cell Counting kit-8 and wound healing assays, respectively. Toll-like receptor 4 (TLR4) mRNA and protein levels were detected by reverse transcription-quantitative polymerase chain reaction and western blotting analyses, respectively. In the present study, lysophosphatidylcholine (LPC) significantly increased the secretion of IL-6 and TNF- $\alpha$  in VECs. VECs treated with LPC markedly increased proliferation and migration of VSMCs, which were inhibited by DFMG. Transfection of either TLR4 short hairpin RNA (shRNA) or TLR4 cDNA in VECs inhibited and increased proliferation and migration of VSMCs, respectively. Furthermore, transfection of VECs with

TLR4 shRNA suppressed the proliferation and migration of VSMCs induced by co-culture with injured VECs, which was further enhanced by treatment with DFMG. By contrast, transfection of VECs with TLR4 cDNA enhanced proliferation and migration of VSMCs and this effect was inhibited by treatment with DFMG. Taken together, the results of the present study demonstrated that DFMG can reverse proliferation and migration of VSMCs induced by co-culture with injured VECs via suppression of the TLR4-mediated signaling pathway.

## Introduction

Vascular smooth muscle cells (VSMCs) are cellular components of the medial layer of the artery wall adjacent to vascular endothelial cells (VECs). VSMCs serve a number of roles under normal physiological conditions (1,2). Proliferation and migration of VSMCs occurs during the early stage of atherosclerotic lesion formation and post angioplasty restenosis (3). Numerous cell types are involved in atherogenesis and a complex network of transcription factors and proteins is involved in this process (4).

VECs can regulate the contractile phenotype of VSMCs through modulation of miR-206/ADP-ribosylation factor 6 and sodium/calcium exchanger 1/exosome (2). When stimulated by endothelial injury signals, VSMCs migrate into intima and undergo a proliferative phenotypic switch (5). Macrophages contribute to pathogenesis of restenosis via secretion of growth factors, cytokines and chemokines, which induce proliferation and activation of VSMCs (4). Endothelial dysfunction is an early contributor and predictor of atherosclerosis (AS) (6,7). Endothelial dysfunction results in the earliest detectable alterations during the development of atherosclerotic lesions (6,7). The VECs-VSMCs co-culture model has been widely adopted for the investigation of AS (2,8,9).

Data presented by a previous study supports the hypothesis that inflammation contributes to the initiation, progression and plaque rupture of AS (10). AS is dependent on innate immune responses and involves activation of Toll-like receptors (TLRs). TLRs are pattern-recognition receptors and a part of the interleukin-1 receptor/Toll-like receptor superfamily. Activation of TLRs leads to expression of inflammatory proteins that may

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**Abbreviations:** AS, atherosclerosis; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; IL-6, interleukin-6; LPC, lysophosphatidylcholine; TLR4, Toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VECs, vascular endothelial cells; VSMCs, vascular smooth muscle cells

**Key words:** 7-difluoromethoxy-5,4'-dimethoxy-genistein, TLR4, vascular smooth muscle cells, vascular endothelial cells, co-culture of cells, proliferation, migration

lead to acute coronary syndrome (11-13). Different subtypes of TLRs are involved in a number of aspects of the inflammatory response.

TLR4 exhibits a high level of expression in aortic tissues from patients who have undergone coronary artery bypass graft surgery and is positively correlated with Gensini score (14). Kapelouzou *et al* (15) demonstrated that the TLR4 mRNA expression level was markedly upregulated in a hyperlipidemic rabbit model and was associated with the progression of AS. Studies in knockout mice demonstrated that the TLR4/nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway contributes to chronic unpredictable mild stress-induced AS via activation of pro-inflammatory cytokines (16). The TLR4/NF- $\kappa$ B signaling pathway also contributes to the early stage intimal foam cell accumulation at lesion-prone aortic sites (12). In vitro, TLR4 mediates hyperglycemia-induced inflammation and dysregulation of the endothelial glycocalyx levels in human macrovascular aortic ECs (17). TLR4 inhibitor CLI-095 can suppress the progression of AS by reducing macrophage foam cell formation (18). Yang *et al* (19) hypothesized that TLR4 signaling promoted a pro-inflammatory phenotype in VSMCs. Positive feedback regulation of VSMCs proliferation is mediated by the TLR4/Rac family small GTPase 1/Akt serine/threonine kinase signaling pathway (20). Furthermore, the TLR4/NF- $\kappa$ B signaling pathway can increase the expression of matrix metalloproteinase-9 mRNA and protein levels in order to stimulate migration of human aortic VSMCs (21).

7-Difluoromethoxy-5,4'-dimethoxy-genistein (DFMG) is a novel active chemical molecule. Our previous studies indicated that DFMG was more efficient compared with genistein (GEN) in reducing the risk of cardiovascular disease by inhibition of oxidative damage and/or inhibition of the TLR4 signaling pathway (22,23). The authors of the present study, also previously reported that DFMG could protect VECs through inhibition of the mitochondrial apoptotic pathway (24,25). Furthermore, the preventive effect of DFMG was more apparent compared with the therapeutic effect within apolipoprotein E<sup>-/-</sup> mice induced by a cholesterol-rich diet (26).

The present study investigated whether TLR4 has a role in abnormal proliferation and migration of VSMCs induced by lysophosphatidylcholine (LPC)-injured VECs. The mechanism underlying DFMG-mediated protection of VSMCs from VECs-stimulated dysfunction in vitro has also been investigated. The present study aimed to determine the activity of DFMG on the proliferation and migration of VSMCs using a non-contact co-culture model with LPC-injured VECs.

## Materials and methods

**Reagents and plasmids.** LPC was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and dissolved in PBS. DFMG (purity, >99%) was synthesized as previously described (27) and dissolved in dimethyl sulfoxide (Amresco, LLC, Solon, OH, USA). DFMG was subsequently filter-sterilized. Human interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) ELISA kits were purchased from Shanghai ExCell Biology, Inc. (EH004-96 and EH009-96; Shanghai, China). The Cell Counting kit-8 (CCK-8) was purchased from Vazyme (Piscataway, NJ, USA). MegaTran1.0 transfection reagent, p-green fluorescent protein (GFP)-V-RS-TLR4-short

hairpin (sh)RNA and pCMV6-AC-GFP-TLR4-cDNA plasmids were obtained from OriGene Technologies, Inc. (Rockville, MD, USA). Anti-TLR4 and anti- $\beta$ -actin antibodies were obtained from Abgent, Inc. (ASC10194 and AM1021B, San Diego, CA, USA). All secondary antibodies were supplied by ComWin Biotech Co. Ltd. (CW0156S and CW0102S; Beijing, China).

**Cell culture.** Human umbilical vein EC and human aortic SMC cell lines were obtained from the China Center for Type Culture Collection (Wuhan, China). Both cell lines were incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub> and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G and 100  $\mu$ g/ml streptomycin. Cell culture reagents were obtained from Biological Industries Israel Beit-Haemek Ltd. (Beit HaEmek, Israel).

**Determination of IL-6 and TNF- $\alpha$  levels.** VECs were pretreated with LPC (10, 20, 30, 40 and 50  $\mu$ M) at 37°C for 24 h; the levels of IL-6 and TNF- $\alpha$  in supernatants were quantified using human IL-6 and TNF- $\alpha$  ELISA kits (aforementioned) according to the manufacturer's protocol. A total of 100  $\mu$ l serially-diluted standard samples and/or supernatant samples were added into the microplate and incubated at 37°C for 1 h. The plate was further incubated with 100  $\mu$ l antibody solution against IL-6 or TNF- $\alpha$  at 37°C for an additional 1 h. Subsequently, 100  $\mu$ l horseradish peroxidase-conjugated secondary antibodies were added and the plate was incubated at 37°C for 30 min. The samples were washed four times with 100  $\mu$ l PBS containing Tween-20 (PBST) prior to incubation with secondary antibodies. Finally, the plates were incubated with 100  $\mu$ l substrate in the dark at 37°C for 15 min. The absorbance was measured at a wavelength of 450 nm with a spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Establishment of the co-culture model.** Co-culture of VECs and VSMCs was performed using a Transwell chamber with a 10  $\mu$ m-thick porous membrane. The membrane contained 0.4  $\mu$ m pores in a Transwell cell culture insert (Corning Incorporated, Corning, NY, USA). This prevented cells from direct contact. Briefly, VSMCs (1 $\times$ 10<sup>5</sup> cells/well) were seeded in 6-well plates and cultured for 24 h prior to co-culture with pretreated and/or transfected VECs. VECs (1 $\times$ 10<sup>5</sup> cells/well) were individually cultured on the insert filters for 24 h, then pretreated with DFMG (0.3, 1.0 and 3.0  $\mu$ M) for 12 or 24 h prior to treatment with LPC for 24 h, or pretreated following transfection for 48 h. Subsequently, VECs (1 $\times$ 10<sup>5</sup> cells/well) with inserts were added to the upper chamber of the Transwell system (VSMCs at the bottom). Both cells were maintained in DMEM with 10% FBS at 37°C for 24 h. A light microscope was used to observe migratory cells in lower chamber; magnification,  $\times$ 200.

**VSMCs viability assay.** The CCK-8 assay was used in order to detect cell viability of VSMCs. VSMCs were co-cultured with VECs treated with different interventions for 24 h. VSMCs were digested and cultured in 100  $\mu$ l medium in 96-well plates (1 $\times$ 10<sup>4</sup> cells/well) with four replicates for 24 h. Subsequently,

the medium was replaced and 10  $\mu$ l CCK-8 solution was added to each well for an additional 2 h incubation at 37°C. The absorbance was measured at a wavelength of 450 nm. Cell viability was calculated based on the optical density values of each group.

**VSMCs wound healing (migration) assay.** The wound healing assay was carried out as previously described (28). Prior to co-culture, VSMCs were seeded on 6-well plates and scratched using a 200  $\mu$ l pipette tip in order to generate a cell-free area. PBS was used to rinse cell debris three times and cells were incubated in FBS-free media in order to prevent proliferation. Phase contrast images of cells were captured at 0 h. Furthermore, pretreated and/or transfected VECs with inserts were added to the upper chamber of the Transwell system. Following 24 h incubation at 37°C, images of the same plates and cells were captured. Cell-free areas were quantified using Adobe Photoshop CS6 software (Adobe Systems, Inc., San Jose, CA, USA). The migration was expressed as percentage coverage.

**VECs transfection.** Transfection of VECs with TLR4 shRNA and cDNA was performed out MegaTran 1.0, a non-lipid polymer-based transfection reagent with relatively low toxicity on the transfected cells. VECs were seeded on 6-well plates and cultured until 50-70% confluence prior to transfection. 3  $\mu$ g TLR4 shRNA, cDNA and corresponding negative control (only with GFP gene) plasmid (3  $\mu$ g) was diluted in 300  $\mu$ l DMEM and vortexed gently. A total of 9  $\mu$ l MegaTran 1.0 was added to the diluted DNA and vortexed immediately for 10 sec. Samples were incubated for 10 min at room temperature and the MegaTran 1.0/DNA mixture was added to each well (already containing 2 ml DMEM). The plate was shaken gently in order to achieve an even distribution of the complexes and incubated at 37°C for 4 h. The expression of TLR4 was detected at 24 to 48 h following transfection.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. To quantify the mRNA, 500 ng RNA from each sample were reverse transcribed into complementary DNA (cDNA) at 50°C for 15 min, then 85°C for 5 sec using HiScript Q RT SuperMix for qPCR (Vazyme Biotech Co., Ltd., Nanjing, China). qPCR was carried out using SYBR Premix ExTaq™ (Vazyme) and a 7500 fast qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cycling conditions consisted of an initial incubation at 95°C for 5 min, then 40 cycles of 10 sec at 95°C and 30 sec at 60°C, final extension for 15 sec at 95°C and 1 min at 60°C. The samples were run in triplicate. The  $2^{-\Delta\Delta C_q}$  method was used to analyze relative gene expression (29). GAPDH was used as an endogenous housekeeping gene control. The qPCR primer sequences are listed in Table I.

**Western blotting.** Cells were digested and subsequently lysed on ice using radioimmunoprecipitation assay lysis buffer containing 1% phenylmethyl sulfonyl fluoride. Samples were centrifuged at 12,000 x g at 4°C for 15 min. Protein

Table I. Primer sequences used for qPCR.

Gene	Primer sequence (5'-3')	Product size (bp)
GAPDH	F: CAGGAGGCATTGCTGATGAT R: GAAGGCTGGGGCTCATTT	138
TLR4	F: CCGAGGCCATTATGCTATGT R: TCCCTTCCTCCTTTTCCCTA	141

concentration was determined with a Bicinchoninic Acid protein assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). A total of 25  $\mu$ g of each protein lysate was separated by 10% SDA-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and blocked with 5% skimmed milk in PBST (PBS containing 0.05% Tween-20) at 37°C for 1 h. The membranes were incubated with rabbit anti-TLR4 antibody (1:1,000 dilution) and mouse anti- $\beta$ -actin antibody (1:10,000 dilution) on a shaking platform at 4°C overnight. Following washing of the membranes with PBST, goat anti-rabbit antibody (1:10,000 dilution) and goat anti-mouse antibody (1:10,000 dilution) were incubated with the membranes for 1 h at room temperature. The membranes were washed with PBST three times and protein expression was visualized by Pierce ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). The protein expression was quantified using densitometry and analyzed using GelPro software (version 3.2; Media Cybernetics, Inc., Rockville, MD, USA).  $\beta$ -actin expression was used as an internal control.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation from three independent experiments. SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) and Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used for all statistical analyses. Differences between groups were examined by Student's t-test for two groups and one-way analysis of variance followed by a least significant difference test for multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Establishment of an injured VECs-VSMCs co-culture model.** Initially different concentrations of LPC were used in order to treat VECs and detect the secretion of IL-6 and TNF- $\alpha$ . The results indicated that LPC induced VECs injury in a dose-dependent manner. LPC (20-50  $\mu$ M) significantly promoted the secretion of IL-6 and TNF- $\alpha$  (Fig. 1A and B). An increase in proliferation and migration of VSMCs was observed that was caused by co-culture with LPC-injured VECs (Fig. 1C-E). Consequently, 30  $\mu$ M LPC was selected in order to produce the injured VECs-VSMCs co-culture model.

**DFMG reverses the proliferation and migration of VSMCs induced by co-culture with injured VECs.** To determine whether DFMG protects against proliferation and migration of VSMCs induced by co-culture with LPC-injured VECs,



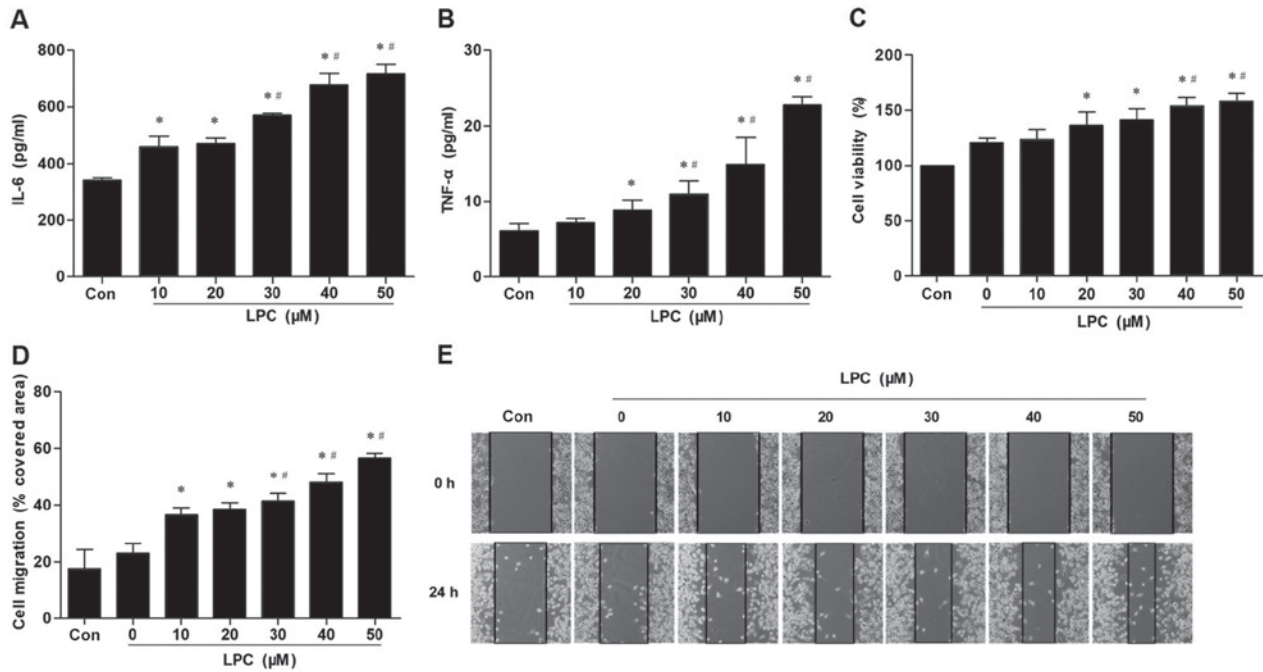


Figure 1. Effect of LPC on secretion of IL-6 and TNF- $\alpha$  in VECs, and proliferation and migration of VSMCs induced by co-culture with injured VECs. LPC (20, 30, 40 and 50  $\mu$ M) significantly increased the levels of (A) IL-6 and (B) TNF- $\alpha$  in VECs-conditioned medium, in a concentration-dependent manner. Pre-treatment of VECs with LPC (20, 30, 40 and 50  $\mu$ M) significantly enhanced VMSC (C) survival and (D) migratory ability demonstrated by (E) wound healing induced by co-culture with injured VECs. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05 vs. Con. \*\* $P$ <0.05 vs. 10 and 20  $\mu$ M LPC. LPC, lysophosphatidylcholine; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Con, control.

VECs were pretreated with various concentrations of DFMG for various durations prior to treatment with LPC (30  $\mu$ M). A significant decrease in the proliferation and migration of VSMCs was observed (Fig. 2). Proliferation and migration of VSMCs decreased following treatment with  $\geq 1.0$   $\mu$ M DFMG (Fig. 2A and C). Cell viability of the control group was set as 100%. VECs were pretreated with 1.0  $\mu$ M DFMG and the relative cell viability of VSMCs was  $\sim 86.7\%$ , whereas the corresponding healing rate was  $\sim 30.73\%$  (Fig. 2A and C). Proliferation and migration of VSMCs was markedly reduced following pretreatment of VECs for 12 h with 1.0 or 3.0  $\mu$ M DFMG (Fig. 2B and D). The longer incubation time (24 h) demonstrated no significant effect on migration and proliferation of VSMCs (Fig. 2B and D). Thus, 1.0  $\mu$ M DFMG was selected as the optimum concentration for the pretreatment of VECs for 12 h.

**Proliferation and migration of VSMCs co-cultured with VECs is inhibited by transfection with TLR4 shRNA.** To further investigate the role of TLR4, transfection of TLR4 shRNA was used in order to silence TLR4 gene expression in VECs. VECs transfected with TLR4 shRNA exhibited significantly decreased expression of TLR4 mRNA and protein levels compared with those transfected with the GFP gene (Fig. 3A-C). TLR4 shRNA significantly inhibited the proliferation and migration of VSMCs co-cultured with VECs compared with the GFP control (Fig. 3D-F).

**Proliferation and migration of VSMCs co-cultured with LPC-injured VECs is inhibited by DFMG treatment and TLR4 shRNA transfection.** The effects of DFMG in combination with TLR4 shRNA transfection of VECs were investigated in

order to determine whether DFMG could reverse the effects of LPC-injured VECs on the proliferation and migration of VSMCs. The results indicated that DFMG and TLR4 shRNA suppressed proliferation and migration of VSMCs, and the combination of these interventions was more effective than TLR4 knockdown alone (Fig. 4). DFMG and TLR4 shRNA exhibited a synergistic effect in promoting the stability of VSMCs.

**TLR4 overexpression in VECs promotes proliferation and migration of VSMCs induced by co-culture with VECs.** TLR4 cDNA significantly increased the expression of TLR4 mRNA and protein in VECs compared with the cells transfected with the GFP gene (Fig. 5A-C). Furthermore, transfection of the cells with TLR4 cDNA significantly enhanced the proliferation and migration of VSMCs in the co-culture model compared with the GFP control (Fig. 5D-F).

**TLR4 cDNA transfection of VECs enhances injured VEC-induced proliferation and migration of VSMCs, which in turn attenuates the protective effect of DFMG.** Combined treatment with DFMG and TLR4 cDNA transfection of VECs was performed in order to investigate whether TLR4 was involved in the DFMG mediated anti-migratory and anti-proliferative effect in VSMCs. The results indicated that TLR4 cDNA transfection significantly attenuated the inhibitory effects of DFMG on the proliferation and migration of VSMCs (Fig. 6).

## Discussion

During the pathogenesis of atherosclerosis, the endothelial dysfunction is considered to be an initial step. Various risk

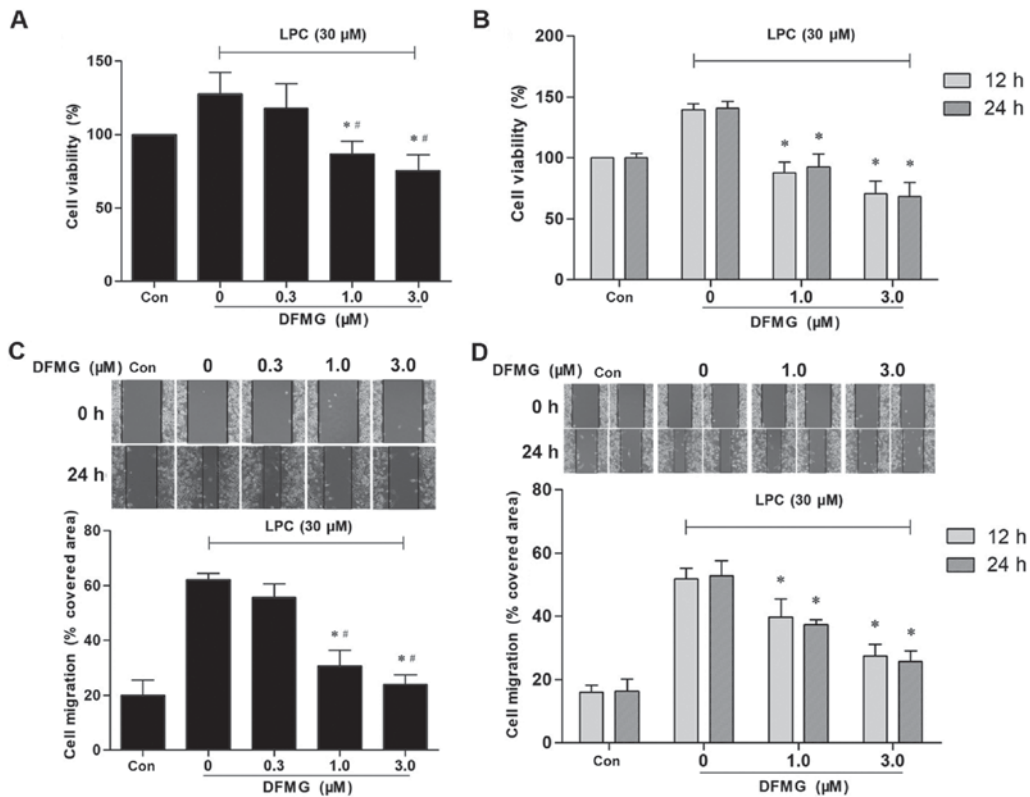


Figure 2. Effect of DFMG with different concentrations and pretreatment time periods on the proliferation and migration of VSMCs induced by co-culture with injured VECs. (A) DFMG at 1.0 and 3.0  $\mu$ M significantly inhibited the viability and migratory ability of VSMCs induced by co-culture with injured VECs. (B) The viability and migratory ability of VSMCs induced by co-culture with injured VECs were significantly decreased in VECs pretreated with DFMG (1.0 and 3.0  $\mu$ M) for 12 h. Wound healing experiment involving (C) VECs treated with different concentration of DFMG (D) for 12 or 24 h. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05, vs. 0  $\mu$ M DFMG. # $P$ <0.05 vs. 0.3  $\mu$ M DFMG. LPC, lysophosphatidylcholine; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; Con, control.

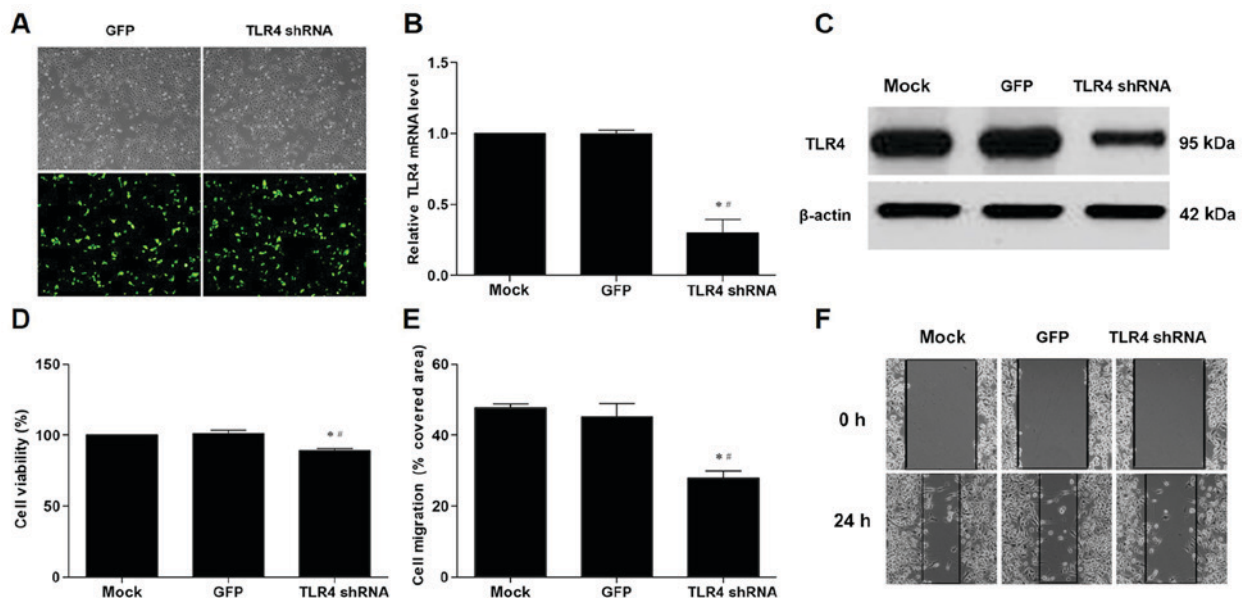


Figure 3. Effect of TLR4 shRNA transfection of VECs on the proliferation and migration of VSMCs induced by the co-culture model. (A) VECs transfected with GFP and TLR4 shRNA were captured under phase contrast and fluorescence microscopy. The expression of TLR4 (B) mRNA and (C) protein were markedly downregulated in the shRNA group. (D) viability and (E) migratory ability of VSMCs induced by the co-culture model were markedly decreased. (F) Wound healing experiment. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05 vs. mock. # $P$ <0.05 vs. GFP. TLR4, Toll-like receptor 4; shRNA, short hairpin RNA; GFP, green fluorescent protein empty vector.

factors lead to structural and functional alteration in the vascular endothelium, including lipid deposition into the

intima, infiltration of inflammatory cytokines (IL-6, IL-8, TNF- $\alpha$ ), proliferation and migration of VSMCs, synthesis

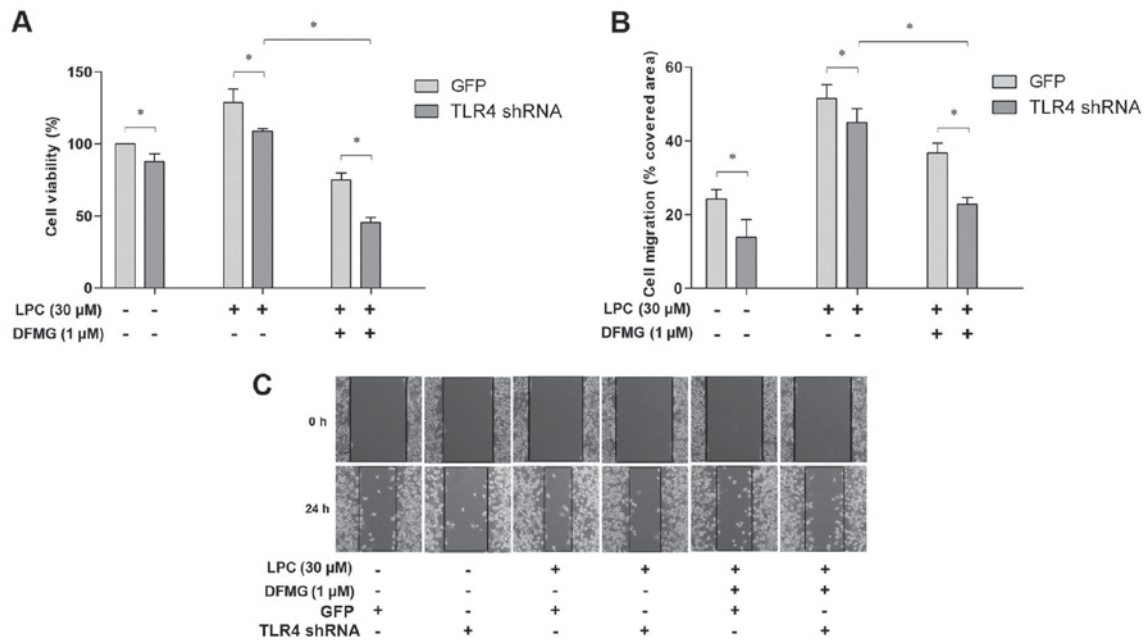


Figure 4. Combined effect of DFMG and TLR4 shRNA transfection of VECs on the proliferation and migration of VSMCs induced by co-culture with LPC-injured VECs. (A) Cell viability. (B) Cell migration. (C) Wound healing assay. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05. LPC, lysophosphatidylcholine; DFMG, 7-difluoromethoxy-5,4'-dimethoxy-genistein; TLR4, Toll-like receptor 4; shRNA, short hairpin RNA.

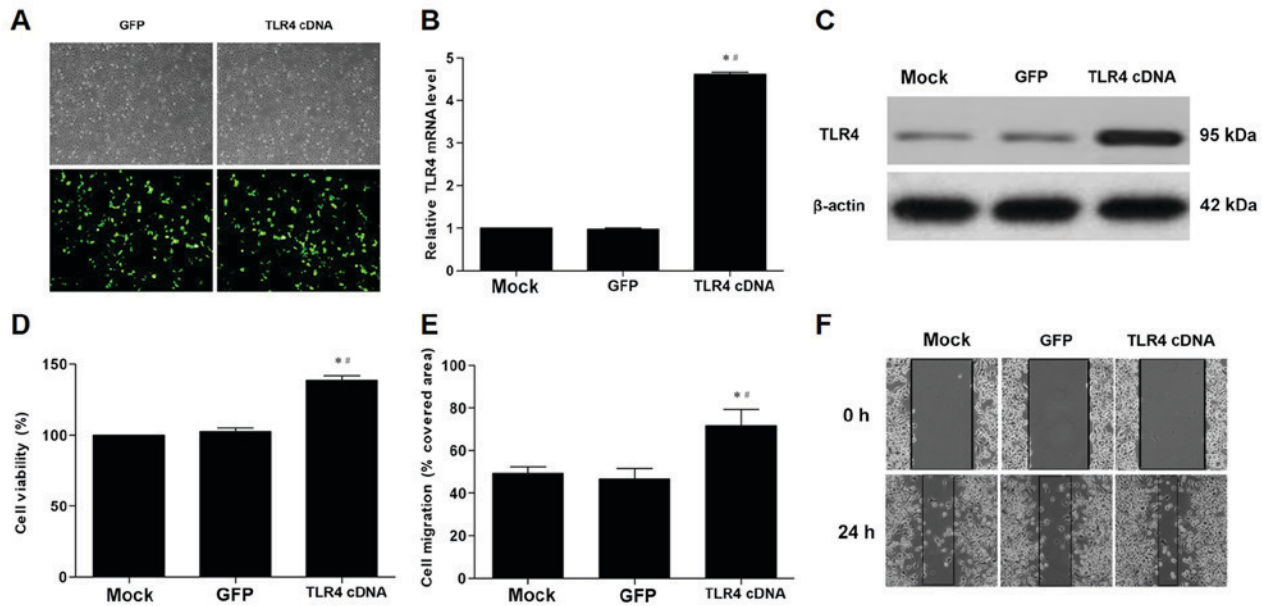


Figure 5. Effect of TLR4 cDNA transfection of VECs on proliferation and migration of VSMCs induced by the co-culture model. (A) VECs transfected with GFP and TLR4 cDNA were captured under phase contrast and fluorescence microscopy. Expression of TLR4 (B) mRNA and (C) protein were significantly upregulated. (D) Cell viability. (E) Cell migration. (F) Wound healing experiment. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05 vs. the mock group. \*\* $P$ <0.05 vs. the GFP group.

of extracellular matrix, and the formation of foam cells and plaques (30). Oxidation and enzymatic modification of low-density lipoproteins (LDL) leads to synthesis of LPC (31). LPC is a component of oxidized-LDL (ox-LDL) that can stimulate ox-LDL to cause AS (32). In the present study, LPC stimulated secretion of IL-6 and TNF- $\alpha$  in VECs, and promoted the proliferation and migration of VSMCs in a co-culture model. These processes are crucial to neointima formation (33).

TLR4 is involved in the inflammatory reaction. Recently, the interaction between the TLR4 transduction pathway and AS has been extensively investigated (34-37). In the present study, a transient transfection was used in order to modulate the expression of TLR4 mRNA and protein in VECs. The results of the present study indicated that TLR4 cDNA promoted the proliferation and migration of VSMCs in a co-culture model, while TLR4 shRNA exerted the opposite effect. Furthermore, the results of the present study further supported the role of



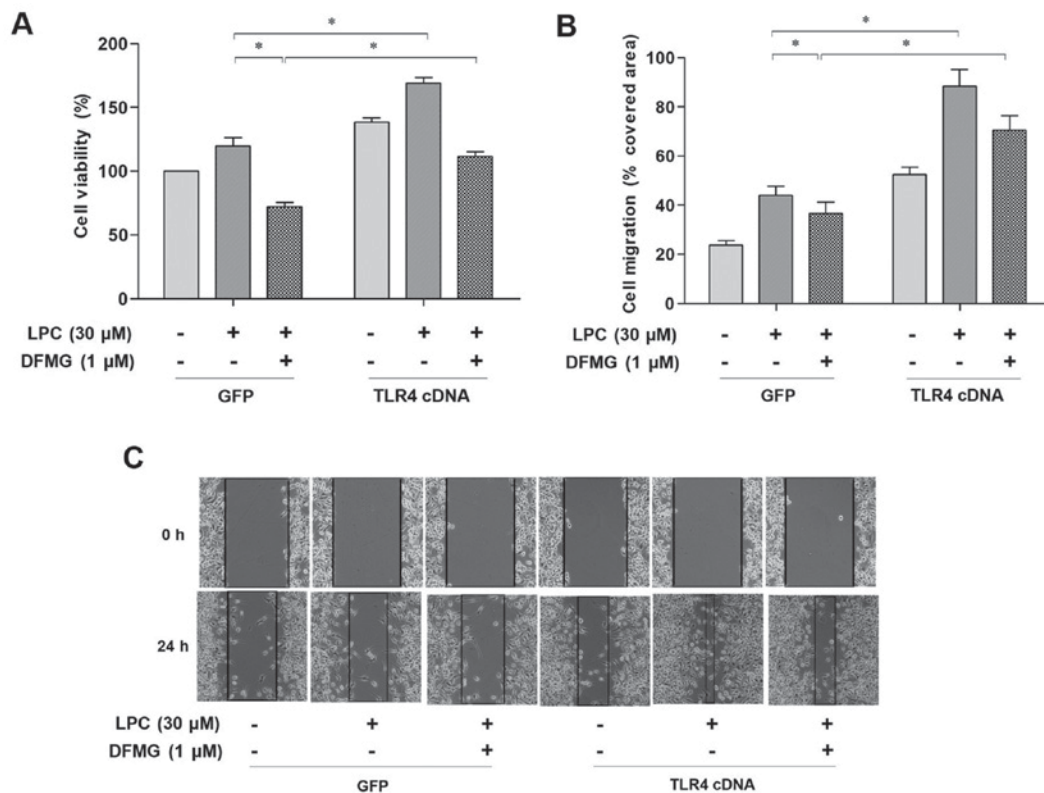


Figure 6. Combined effect of DFMG and TLR4 cDNA transfection of VEC on the proliferation and migration of VSMCs induced by co-culture with LPC-injured VECs. (A) Cell viability. (B) Cell migration. (C) Wound healing assay. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ .

TLR4 signaling in the proliferation and migration of VSMCs induced by co-culture with LPC-injured VECs.

Genistein, the lead compound of DFMG, is a natural isoflavone present in legumes and dentate plants. Genistein can alleviate inflammatory damage by altering TLR4/NF- $\kappa$ B signaling (22,23). In the present study, VECs were pretreated with DFMG prior to treatment with LPC and subsequently co-cultured with VSMCs. The results demonstrated that the proliferation and migration of VSMCs were decreased, which was dependent on the concentration of DFMG. In addition, the combination of treatment with DFMG and TLR4 shRNA transfection of VECs significantly suppressed the proliferation and migration of VSMCs induced by the co-culture with LPC-injured VECs. Inhibition of TLR4 signaling in VECs restrained abnormal proliferation and migration of VSMCs. TLR4 cDNA transfection of VECs enhanced proliferation and migration of VSMCs that were induced by co-culture with injured VECs, and attenuated the effects of DFMG.

In conclusion, the results of the present study demonstrated that DFMG effectively inhibited proliferation and migration of VSMCs induced by co-culture with LPC-injured VECs via regulation of the TLR4 signaling pathway. DFMG may be useful as a novel agent for the prevention of AS. However, the present study has certain limitations, including that the effects of DFMG on VSMCs have not been investigated *in vivo*. Furthermore, regulation of the TLR4/myeloid differentiation primary response 88 and TLR4/Toll-like receptor adaptor molecule 1 pathways by DFMG during this process has not

been investigated. Consequently, understanding of detailed effects of DFMG on the TLR4 signaling pathway in AS requires further investigation.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

XF designed the study and prepared the manuscript. LC and HH performed the experiments. YZ analysed the data. JC contributed to the interpretation of some of the data and 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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