

## Original Article

# MEF2C protects against the development of atherosclerosis via inhibiting TLR/NF- $\kappa$ B activation

Le He<sup>1,2</sup>, Tong Wang<sup>3</sup>, Feng-Min Lu<sup>2</sup>, Jing Xu<sup>2</sup>, Hong-Liang Cong<sup>2</sup>

<sup>1</sup>Tianjin Medical University, Tianjin 300070, China; <sup>2</sup>Department of Cardiology, Tianjin Chest Hospital, Tianjin 300051, China; <sup>3</sup>Metabolic Diseases Hospital, Tianjin Medical University, Tianjin 300070, China

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**Abstract:** Increasing evidences have suggested vascular endothelial inflammatory processes are the initiator of atherosclerosis. The aim of this study was to investigate the effect of MEF2C in regulating atherosclerosis and the underlying molecular mechanism. We first demonstrated that MEF2C is expressed in endothelial cells and decreased after tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) treatment. Moreover, we found that overexpression of MEF2C in the mouse model of atherosclerosis by adenovirus-mediated gene transfer significantly attenuated the expression of pro-inflammatory factors including TNF- $\alpha$ , IL-6 and MCP-1. In addition, the adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, were also inhibited by MEF2C overexpression. In addition, overexpression of MEF2C significantly decreased the protein levels of toll-like receptor (TLR) 2, TLR4 and phosphorylated p65 (p-p65). Additionally, the results of luciferase reporter assay exhibited that MEF2C overexpression was capable of inhibiting the activation of nuclear factor-kappa B (NF- $\kappa$ B). Taken together, our results demonstrated that up-regulation of MEF2C relieves the development of atherosclerosis that may be associated with regulating the TLR/NF- $\kappa$ B pathway.

**Keywords:** MEF2C, TLR/NF- $\kappa$ B, inflammatory, atherosclerosis

## Introduction

Inflammation contributes broadly to a number of human diseases, including cardiovascular diseases. During the pathological progression of inflammatory diseases, vascular inflammation plays a critical role, especially in vascular diseases such as atherosclerosis [1]. Atherosclerosis has been regarded as a systemic inflammatory and immune vascular disease, and inflammatory events [2], the generation of inflammatory cytokines [3], and the involvement of TLRs as well as NF- $\kappa$ B [4] in atherosclerotic lesions participate in its initiation, development and progression.

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is a major pro-inflammatory transcription factor activated in endothelial cells by inflammatory cytokines and plays a central role in upregulating the expression of adhesion molecules and other pro-inflammatory genes (e.g. VCAM-1, E-selectin, ICAM-1, IL-8) [5]. There is much less known regarding transcription factors that play an

anti-inflammatory role in endothelial cells. Furthermore, the cascade in TLR-signaling induces activation of transcription factor, NF- $\kappa$ B, leading to the production of a broad range of pro-inflammatory agents [6]. In essence, atherosclerosis is a continuous response of inflammation; therefore, inhibiting TLR/NF- $\kappa$ B signaling pathway is a good biological target for anti-atherosclerosis by reducing inflammation in the vascular wall.

The myocyte enhancer factor (MEF2) family of transcription factors plays a critical role in diverse developmental programs [7]. Of the MEF2 transcription factors, MEF2C plays a critical role in vascular cells [7]. MEF2C is involved in regulating endothelial integrity and survival. It has been reported that MEF2C regulates endothelial cell angiogenesis; mice with endothelial-cell specific ablation of MEF2C exhibited marked increase in vascular regrowth under stress conditions [8]. Knockdown of MEF2C in endothelial cells regulated endothelial cell survival and tube formation, further confirming the

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role of this transcription factor in the regulation of angiogenesis [8]. Consistent with this, MEF2C was recently reported as a negative regulator of angiogenic sprouting of endothelial cells [9]. To date, the effects of MEF2C on atherosclerosis are rarely mentioned.

In the present study, we aimed to investigate the role of MEF2C in atherosclerosis. We found that the overexpression of MEF2C markedly inhibited gene expression of inflammatory mediators and adhesion molecules. Furthermore, the overexpression of MEF2C inhibited the process of atherosclerosis via the TLR/NF- $\kappa$ B signaling pathway. Taken as a whole, we identified that MEF2C might be an effective molecular target for interventional therapy for atherosclerosis.

### Materials and methods

#### *Animals and cell culture*

A total of 60 male apolipoprotein E (Apo E)-deficient mice (3 weeks old, weighing 20-25 g) were purchased from Beijing Biocytogen (Beijing, China). The mice were housed in a temperature-controlled (24°C) facility with a 12-h light/dark cycle and fed a high-fat diet (20% fat, 20% sugar, and 1.25% cholesterol) with free access to water. All studies were carried out in accordance with the guidelines of the Institutional Animal Care.

For isolation of mouse aortic endothelial cells (MAECs), aorta was collected from anesthetized mice. MAECs were collected according to a previously reported method [10]. They were maintained in endothelial basal medium-2, 2% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37°C in a 5% CO<sub>2</sub> incubator (Life Technologies, USA).

#### *Quantitative real-time PCR*

Total RNA from cells was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Single-stranded cDNA was synthesized using a PrimeScript™ 1st Strand cDNA Synthesis kit (Takara, Dalian, China) according to the supplier's instructions. The level of RNA expression was measured by Real-time PCR analysis performed with a SYBR PrimeScript PT-PCR kit (Takara, Dalian, China). The data obtained were

calculated by 2<sup>- $\Delta\Delta$ Ct</sup>. The target gene was normalized to the internal reference gene GAPDH.

#### *Western blot analysis*

Tissues from the right common carotid arteries were frozen in liquid nitrogen and treated with an ice cold lysis buffer. The protein in cell lysis was separated by 12% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was then blocked at room temperature in TBS containing 2.5% non-fat dry milk and incubated in primary antibody blocking solution overnight at 4°C. Primary antibodies included anti-VCAM-1, anti-ICAM-1, anti-E-selectin, and anti-GAPDH (Santa Cruz, CA, USA), as well as anti-phosphor-p65, anti-TLR2, and anti-TLR4 (Cell Signaling Technology, Danvers, MA, USA). Next, the membrane was further incubated in HRP labeled secondary antibodies at 37°C in the blocking buffer for 1 h. The target protein was visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

#### *Adenovirus preparation and infection*

Replication-deficient adenovirus expressing mouse full-length MEF2C (Ad-MEF2C) and green fluorescent protein (GFP, Ad-GFP) were provided as previously described [11]. MAECs were seeded in 12-well plate or 10 cm dish and were 75% confluent by the next day. Cells were incubated with Ad-GFP and Ad-MEF2C at 300 infection unit (IFU) per cell for 48 h. Before TNF- $\alpha$  treatment, cells were washed with PBS, and cultured in EGM2 without FBS overnight.

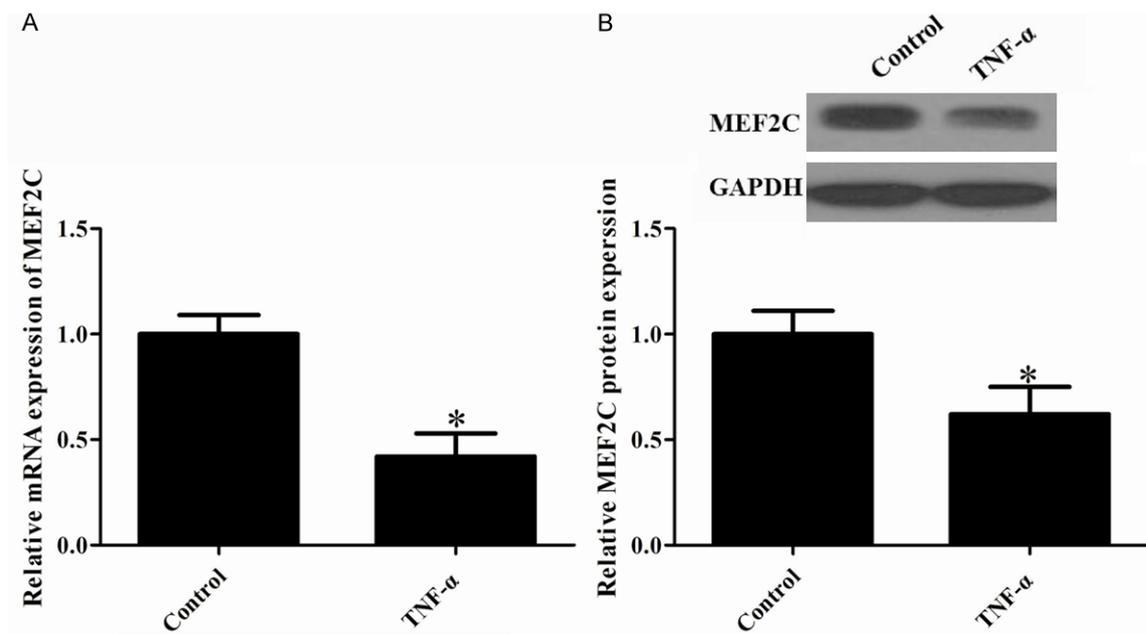
#### *Enzyme-linked immunosorbent assay (ELISA)*

Cell culture supernatant and serum were analyzed for mouse ICAM-1, VCAM-1 and E-selectin using ELISA kits (Abcam, MA, USA). The concentration of chemokine (MCP-1) and pro-inflammatory cytokines in cell lysates and serum were determined by mouse TNF- $\alpha$ , and IL-6 ELISA kits (R&D Systems, Minnesota, USA). All measurements were performed according to the manufacturer's instructions.

#### *Luciferase reporter assay*

MAECs seeded on 24-well plates (2 × 10<sup>5</sup> per well) were co-transfected with NF- $\kappa$ B promoter-

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**Figure 1.** TNF- $\alpha$  decreased MEF2C expression in MAECs. (A) Quantitative PCR and (B) western blot analysis of MEF2C expression in MAECs isolated from mice after treatment with TNF- $\alpha$  (10 ng/ml). GAPDH was used as a control. \* $P < 0.05$  vs. Control (untreated group).

luciferase vector (Promega, Madison, WI, USA) and adenovirus vector carrying GFP or MEF2C. The transfected cells were treated with TNF- $\alpha$  (2 ng/ml) for 6 h and then incubated for 48 h. MAECs were washed with PBS and then lysed in a reporter lysis reagent. The luciferase activity in cell lysate was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

### Statistical analysis

Values are described as the means  $\pm$  SD. Statistical significance of differences between groups was determined using the Student's t-test or the ANOVA by SPSS 22.0 software (SPSS, Inc., Chicago, USA). A  $P$  value of  $< 0.05$  was considered the criterion of a statistically significant difference.

## Results

### TNF- $\alpha$ impaired endogenous MEF2C expression in MAECs

TNF- $\alpha$  is one of the most important pro-inflammatory molecules that increases the expression of adhesion molecules and induces endothelial inflammatory response [12]. Here, we examined the effect of TNF- $\alpha$  on MEF2C expression in MAECs. As **Figure 1** shown, the mRNA

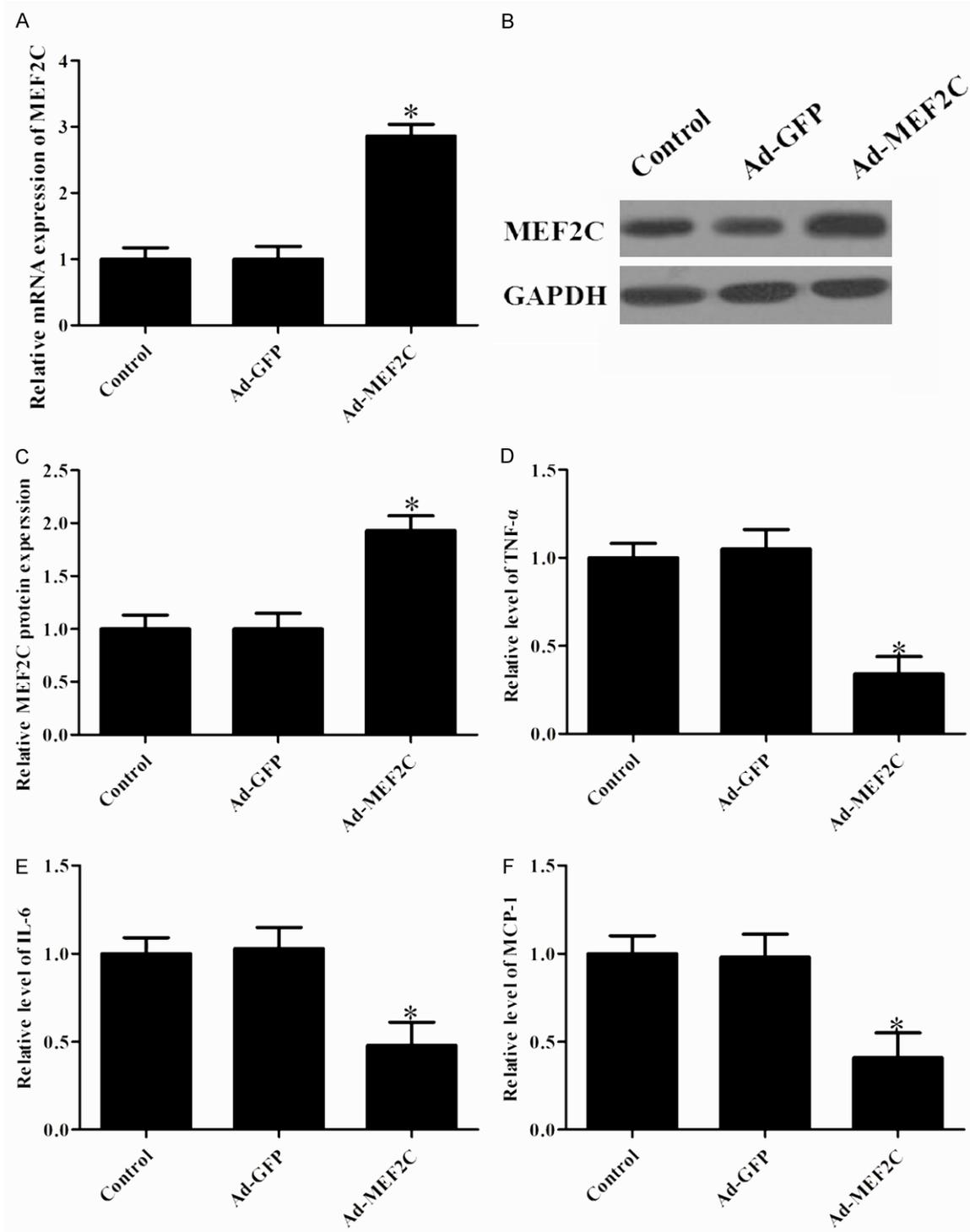
(**Figure 1A**) and protein (**Figure 1B**) levels of MEF2C were decreased significantly after TNF- $\alpha$  treatment in MAECs.

### Overexpression of MEF2C decreased the levels of inflammatory cytokines

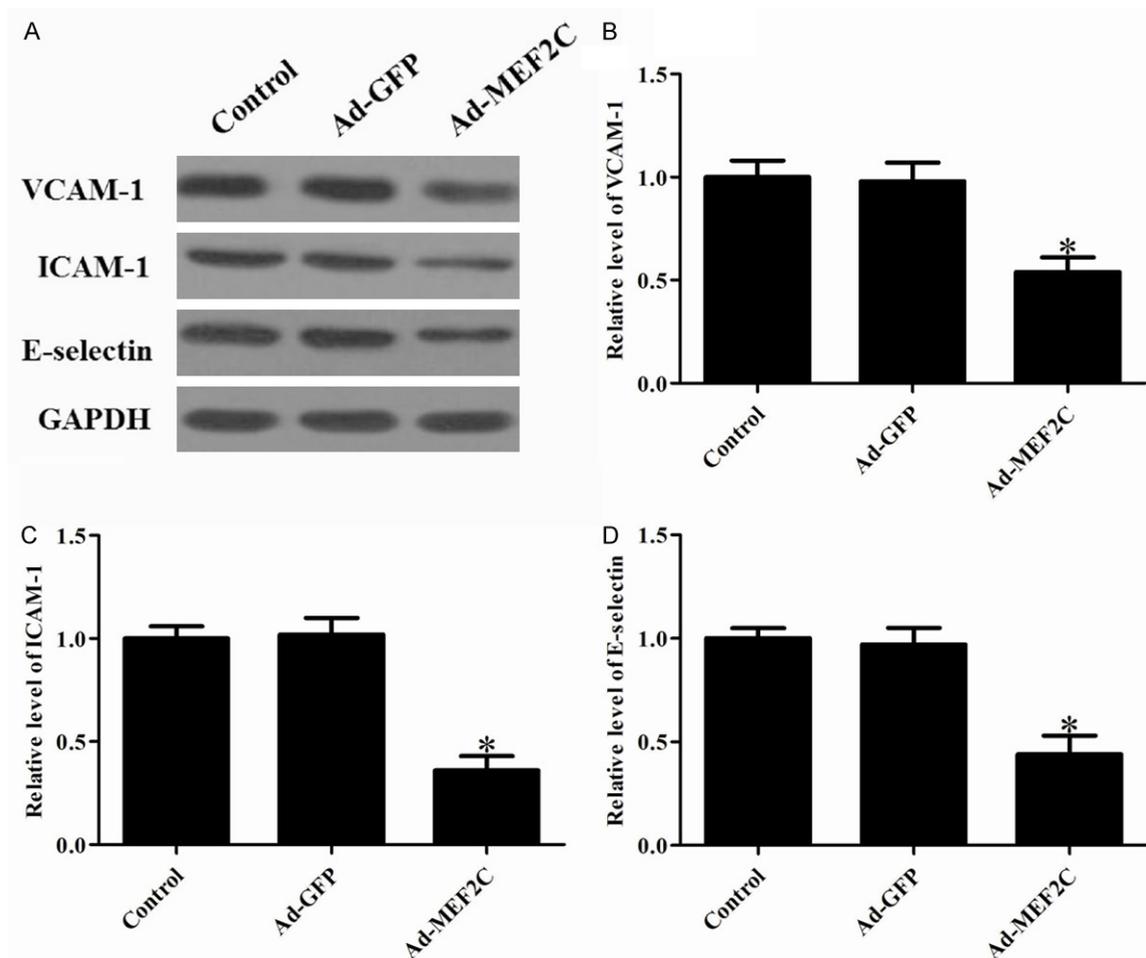
To investigate whether overexpression of MEF2C alleviates atherosclerosis, we overexpressed MEF2C in Apo E-deficient mice by the infection of recombinant adenovirus expressing. The expression of MEF2C was measured by RT-PCR and Western blot analysis. The results showed that the levels of MEF2C mRNA and protein in the Ad-MEF2C group were significantly increased compared with those in the Apo E-deficient mice (the control group) or in the Ad-GFP group (**Figure 2A-C**). Overexpression of MEF2C notably downregulated the expression levels of TNF- $\alpha$ , IL-6 and MCP-1 in the Ad-MEF2C mice compared to those in the control or Ad-GFP groups (**Figure 2D-F**). These findings suggest that MEF2C has a regulatory effect on serum inflammatory cytokines in atherosclerotic mice.

### Overexpression of MEF2C decreased the expression of adhesion molecules

The high expression of adhesion molecules in vascular endothelial cells, including VCAM-1,



**Figure 2.** Overexpression of MEF2C inhibited the inflammatory cytokines in Apo E-deficient mice. (A) The expression of MEF2C mRNA in Apo E-deficient mice transfected recombinant adenovirus containing MEF2C gene. (B and C) The expression level of MEF2C protein in Apo E-deficient mice transfected recombinant adenovirus containing MEF2C gene. Overexpression of MEF2C decreased the levels of the inflammatory cytokines (D) TNF- $\alpha$ , (E) IL-6 and (F) MCP-1. Control: Apo E-deficient mice without treatment; Ad-GFP: Apo E-deficient mice received infection of an adenovirus vector containing GFP; Ad-MEF2C: Apo E-deficient mice received infection of an adenovirus vector containing MEF2C. \* $P < 0.05$  vs. Control and Ad-GFP.



**Figure 3.** The effects of overexpression of MEF2C on adhesion molecules in Apo E-deficient mice. A. Overexpression of MEF2C decreased the levels of adhesion molecules. B. VCAM-1. C. ICAM-1. D. E-selectin. \* $P < 0.05$  vs. Control and Ad-GFP.

ICAM-1, and E-selectin, is one of the important events during the initiation and development of atherosclerosis [13]. To verify whether MEF2C overexpression decreased the expression of adhesion molecules in vascular endothelial cells in Apo E-deficient mice, we measured the protein levels of VCAM-1, ICAM-1, and E-selectin by Western blot analysis (Figure 3A). Compared with the control or Ad-GFP groups, overexpression of MEF2C inhibited the expression levels of VCAM-1, ICAM-1, and E-selectin in the Ad-MEF2C group (Figure 3B-D), suggesting that the effects of MEF2C on atherosclerosis may be involved in the decrease in the expression of adhesion molecules.

#### *MEF2C inhibited the expression of TLR-2 and TLR-4 in vivo*

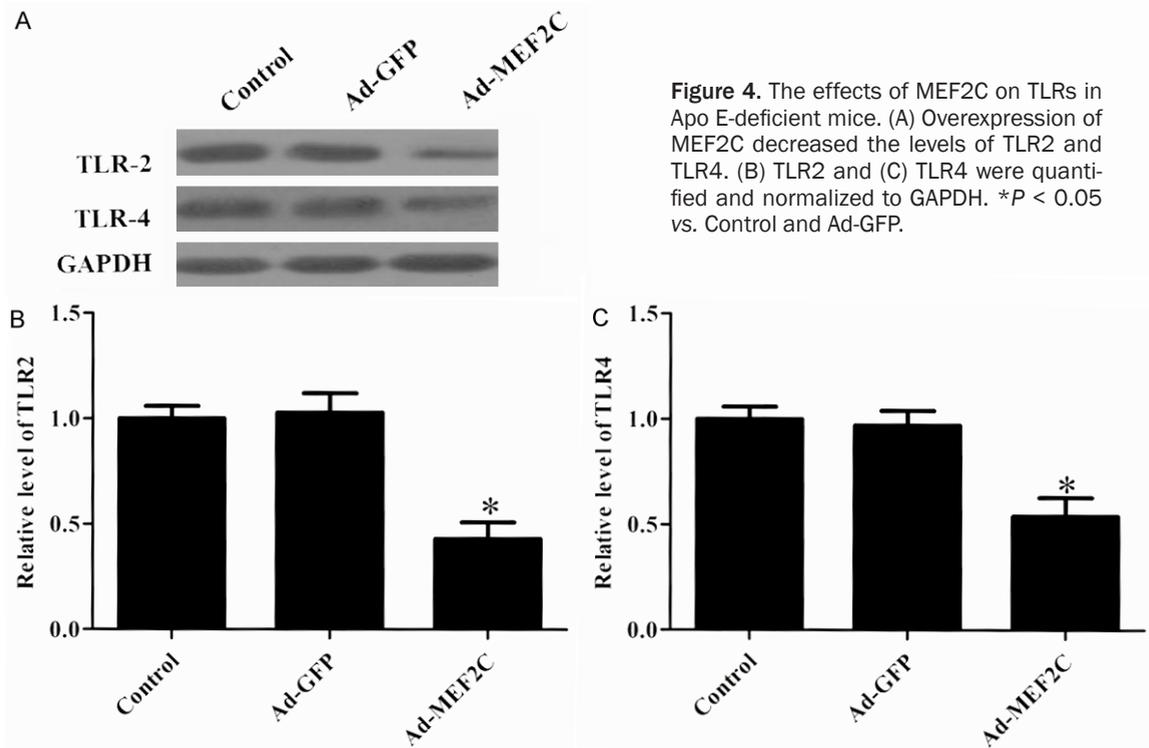
TLRs in atherosclerotic lesions are bound up with the initiation and progression of athero-

sclerosis [14]. NF- $\kappa$ B is an important composition of TLRs downstream pathway [15]. We measured the expression levels of TLR-2 and TLR-4 in aortic tissues using Western blot analysis (Figure 4A). As shown in Figure 4B, the relative levels of TLR-2 and TLR-4 were decreased in the Ad-MEF2C group compared with those in the control or Ad-GFP groups.

#### *Overexpression of MEF2C decreased NF- $\kappa$ B activity*

The role of NF- $\kappa$ B in inflammatory responses via transcriptional regulation of pro-inflammatory genes is well known [16]. To investigate whether MEF2C regulates the extent of NF- $\kappa$ B activation, we detected the expression level of p-p65 to assess the activation status of NF- $\kappa$ B (Figure 5A). As shown in Figure 5B, the expression levels of p65 in the control and Ad-GFP

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**Figure 4.** The effects of MEF2C on TLRs in Apo E-deficient mice. (A) Overexpression of MEF2C decreased the levels of TLR2 and TLR4. (B) TLR2 and (C) TLR4 were quantified and normalized to GAPDH. \* $P < 0.05$  vs. Control and Ad-GFP.

groups were not statistically significant. However, overexpression of MEF2C markedly decreased the level of p-p65 in the Ad-MEF2C group compared to the control or Ad-GFP groups (Figure 5C). To verify the inhibitory effect of MEF2C on NF- $\kappa$ B, we assessed the effect of MEF2C on the TNF- $\alpha$ -induced activation status of NF- $\kappa$ B *in vitro* by luciferase reporter assay. TNF- $\alpha$  induced the activation of NF- $\kappa$ B in endothelial cells compared to untreated cells. Nevertheless, MEF2C overexpression attenuated the TNF- $\alpha$ -induced activation status of NF- $\kappa$ B *in vitro* (Figure 5D). Collectively, these data suggest that MEF2C overexpression has an inhibitory effect on NF- $\kappa$ B in atherosclerotic mice.

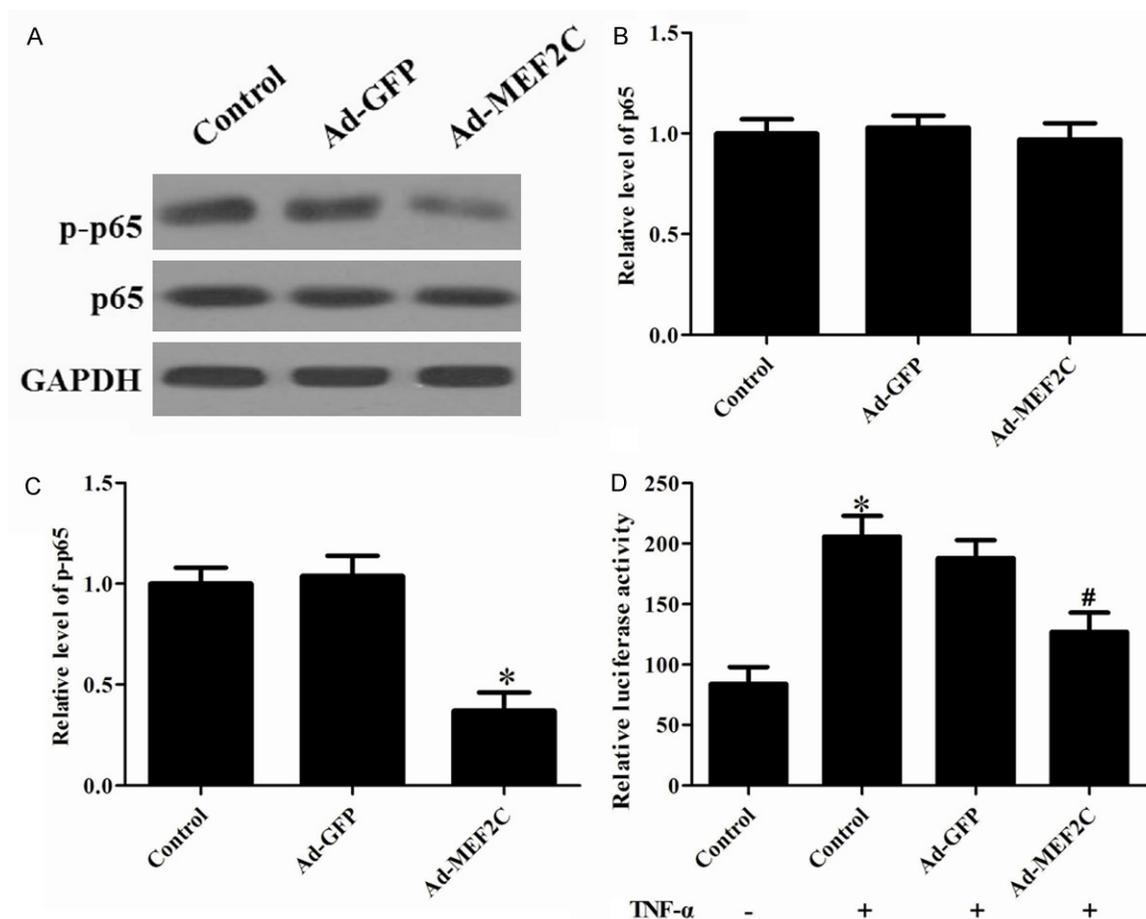
### Discussion

Atherosclerosis is a chronic inflammatory disorder in which various pro-inflammatory mediators and events have crucial roles in the onset and progression of the disease [17]. Pro-inflammatory cytokines and cell adhesion molecules with a wide range of functions are capable of orchestrating events such as the activation of immune cells, induction of other cytokines and pro-coagulant changes in the endothelium. These activities facilitate the migration

of neutrophils, monocytes and lymphocytes into the developing atherosclerotic lesion [18]. As a key transcription factor in inflammatory response, NF- $\kappa$ B is involved in the regulation of many inflammatory genes, including chemokines, cytokines, growth factors and cell adhesion molecules, thus it affects the progress of atherosclerosis [16].

In endothelial cells, TNF- $\alpha$  induces the expression of genes associated with inflammation, which appears to be a classic inflammatory model [19]. In the current study, we noticed the expression of MEF2C was significantly decreased after TNF- $\alpha$  stimulated by quantitative PCR and western blot. These results strongly suggest that MEF2C is involved in endothelial inflammation.

The increased expression of adhesion molecules and chemokines is the earliest important events during the pathogenesis of inflammation [20]. Moreover, some key chemokines and pro-inflammatory cytokines such as MCP-1, TNF- $\alpha$  and IL-6 have been reported to play a crucial role in inflammatory injury. ICAM-1, VCAM-1 and E-selectin are adhesion molecules, which recruits immune cells to the vascular endothelium, a characteristic of inflammation



**Figure 5.** The effects of MEF2C on NF- $\kappa$ B in Apo E-deficient mice. A. The levels of p65 and p-p65 were detected by using Western blot. B. The level of p65 in the control, Ad-GFP, and Ad-MEF2C showed no significant difference. C. Overexpression of MEF2C decreased the level of p-p65. D. Overexpression of MEF2C decreased the TNF- $\alpha$ -induced NF- $\kappa$ B transcriptional activity. \* $P < 0.05$  vs. Control and Ad-GFP.

[21]. In this study, we found that overexpression of MEF2C significantly inhibited the secretion of TNF- $\alpha$ , IL-6 and MCP-1, as well as the expression of VCAM1, ICAM1 and E-selectin. Therefore, we concluded that overexpression of MEF2C inhibits atherosclerosis by attenuating the level of inflammatory responses in atherosclerotic mice.

TLRs are key orchestrators of the atherosclerotic disease process, and they have also been implicated to play roles in the pathogenesis of cardiovascular risk factors [22]. Increasing evidence indicates that TLR4 affects atherosclerosis in multiple ways. TLR4 is overexpressed in human atherosclerotic lesions and the activation of TLR4 evokes the production of cytokines and chemokines [15]. Therefore, it is recently recognized that suppressing TLR4 activation as well as its downstream inflammatory pathways

could inhibit the initiation or progression of atherosclerosis [23]. Our study demonstrated that overexpression of MEF2C could significantly decrease the TLR2 and TLR4 protein expression in aortic tissues of atherosclerotic mouse models. In response to pro-inflammatory stimulation, NF- $\kappa$ B pathway is involved in the inflammatory responses in endothelial cells [24]. Activated NF- $\kappa$ B has been identified upon inflammatory stimulation, and a variety of adhesion molecules and chemokines have been reported to be the direct targets of NF- $\kappa$ B [25, 26]. In our study, overexpression of MEF2C markedly decreased the expression of NF- $\kappa$ B. Furthermore, overexpression of MEF2C strongly blocked TNF- $\alpha$  induction of NF- $\kappa$ B activity. These data indicated that MEF2C inhibits atherosclerosis in Apo E-deficient mice through the TLRs/NF- $\kappa$ B pathway.

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Taken as a whole, these observations showed that overexpression of MEF2C in Apo E-deficient mice ameliorated atherosclerotic symptoms via inhibition of the TLRs/NF- $\kappa$ B pathway. This work reveals a novel role of MEF2C in the pathogenesis of endothelial inflammation, suggesting MEF2C may be a new strategy to prevent inflammatory diseases.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Hong-Liang Cong, Department of Cardiology, Tianjin Chest Hospital, Tianjin 300051, China. Tel: +86-22-23147100; Fax: +86-22-23147100; E-mail: cong\_hongliang@sina.com

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