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FNDC5 inhibits foam cell formation and monocyte adhesion in vascular smooth muscle cells via suppressing NFκB-mediated NLRP3 upregulation

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ABSTRACT

Foam cell formation and monocytes adhesion are key events in pathogenesis of atherosclerosis. Vascular smooth muscle cells (VSMCs) are an important origin of foam cells besides macrophages. Fibronectin type III domain containing protein 5 (FNDC5) is a protein, which induces browning of fat and attenuates glucose/lipid metabolic derangements in obese mice. The present study was designed to determine the roles of FNDC5 in inhibiting foam cell formation and monocyte adhesion in VSMCs and its underlying mechanisms. Oxidized low-density lipoprotein (oxLDL) was used to induce foam cell formation and monocyte adhesion in human aortic VSMCs. Foam cell formation was evaluated by intracellular lipid droplets, cholesterol contents, and mRNA levels of acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT-1) and ATP binding cassette transporter A-1 (ABCA-1). Monocyte adhesion was evaluated by the number of monocytes adhered to VSMCs and mRNA levels of monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1). FNDC5 inhibited oxLDL-induced foam cell formation, monocyte adhesion, ABCA-1 mRNA downregulation, and ACAT-1, MCP-1 and VCAM-1 mRNA upregulation in VSMCs. It inhibited oxLDL-induced p65-NFκB nuclear translocation, NLRP3 upregulation, caspase-1 and IL-1β production. Inhibition of NFκB with BMS-345541 or inhibition of NLRP3 inflammasome with MCC950 showed similar effects to FNDC5 in attenuating the oxLDL-induced foam cell formation, monocyte adhesion, and caspase-1 and IL-1β production. The oxLDL-induced NLRP3 upregulation was prevented by BMS-345541 rather than MCC950. These results indicate that FNDC5 inhibits oxLDL-induced foam cell formation and monocyte adhesion in VSMCs via suppressing NFκB-mediated NLRP3 upregulation and IL-1β production.

1. Introduction

Atherosclerosis is a chronic vascular disease involving plaque buildup and inflammation in arteries, and can lead to heart attack and stroke [1]. Foam cells are characterized by cholesterol esters and triglycerides accumulation in the cytoplasm [2], and the formation of foam cells is a critical step in the atherosclerosis and plays an essential role in destabilization, rupture and erosion of atherosclerotic plaque [3]. Foam cells are originated from macrophages and vascular smooth muscle cells (VSMCs) [4]. In advanced atherosclerosis lesions of rabbits, only 30% of foam cells displayed macrophage markers, whereas 45% have a VSMC phenotype [5]. Foam cell formation may be a new target for fighting atherosclerosis and cardiovascular disease [6]. Low-density lipoprotein (LDL) and its modified form oxidized-LDL (oxLDL) play a major role in the development of atherosclerosis and foam cell formation [7]. In the early of atherosclerosis, accumulation of highly oxLDL induces dysfunction of endothelial cells and VSMCs, leading to foam cell formation and production of proinflammatory cytokines that recruit monocytes [8]. The recruited monocytes become macrophages that take up oxLDL via scavenger receptors and transform into foam cells, which constitutes early atherogenic lesion and advanced atherosclerotic plaques [8]. Leukocyte recruitment is a crucial event in the pathogenesis of atherosclerosis and is a potential target for therapeutic approach of atherosclerosis [9].

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Chronic vascular inflammation is an important event in the initiation and progression of atherosclerosis, hypertension and several other vascular diseases [10–12]. Nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is a cytosolic protein complex for early inflammatory responses. It is composed of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. On activation, NLRP3 forms an inflammasome complex with ASC and causes a conversion of pro-caspase-1 to active caspase-1, which sequentially cleaves pro-interleukin (IL)-1β into its mature form IL-1β, and then triggers an inflammatory response [13]. Aortic IL-1β mRNA expression was increased in atherosclerosis [14]. NLRP3 inflammasome is involved in atherogenesis process [15].

Fibronectin domain-containing protein 5 (FNDC5) is a membrane protein (mouse and rat, 209 amino acids; human, 212 amino acids) comprising a short cytoplasmic domain, a transmembrane segment, and an ectodomain consisting of a fibronectin type III (FNIII) domain [16,17]. Irisin (112 amino acids), identified in 2012 [18], is released by the cleavage of the extracellular domain of FNDC5, being able to promote the browning of adipose tissue and improve metabolic diseases in both humans and mice [19,20]. Exercise-induced FNDC5/irisin expression in muscle is dependent on increased peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) [18]. Studies in our lab have shown that FNDC5 deficiency aggravates whereas FNDC5 overexpression prevents the high fat diet (HFD)-induced hyperlipemia, hepatic lipid accumulation, and impaired fatty acid β-oxidation (FAO) and autophagy in liver [21]. FNDC5 overexpression ameliorates hyperlipemia and enhances lipolysis in adipose tissues of obese mice [22]. More recently, we have shown that FNDC5 attenuates adipose tissue inflammation and insulin resistance in HFD-induced obese mice [23]. However, it is not known if FNDC5 would play beneficial roles in atherosclerosis. The present study was designed to determine the roles of FNDC5 in ox-LDL-induced foam cell formation and monocytes adhesion in VSMCs and its underline mechanisms.

2. Materials and methods

2.1. Mice with FNDC5 deletion and mice with atherosclerosis

Male WT and FNDC5$^{-/-}$ mice on a C57BL/6 background aged at 12 weeks were used in the experiment for primary VSMC culture. Male C57BL/6 mice at the age of 6 weeks were randomly divided into two groups, which were respectively fed a normal chow (Ctrl, 14.7 kJ/g, 13% of energy as fat) or a high fat diet (HFD, 21.8 kJ/g, 60% of energy as fat) for 16 weeks or 24 weeks to induce atherosclerosis. These mice were obtained from Nanjing BioMedical Research Institute of Nanjing University (Nanjing, China). All procedures were conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011), and performed in accordance with the standards in the guidelines of the Experimental Animal Care and Use Committee of Nanjing Medical University. The mice were housed in 12-h light/dark cycle with a free access to standard chow and tap water in a temperature-controlled room.

2.2. Cell culture

Human aortic VSMCs (HA-VSMCs, American Type Culture Collection, Rockville, MD, USA) were cultured as we previously reported [24]. Briefly, VSMCs were maintained in F12K Kaighn's modification medium containing 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C with 5% CO_2 in a humidified cell incubator. Cells between passages 3 and 8 were used for the experiments. After the cells were grown to 70%–80% confluence, the cells were serum-starved for 24 h prior to use [25].

Primary mouse VSMCs were isolated from thoracic aorta of WT and FNDC5$^{-/-}$ mice aged 12 weeks using a modified enzyme dispersal method. The VSMCs were used at passages 3–5 for the experiments [26].

2.3. Oil red O staining

Oil red O staining was used to show neutral triglycerides and lipids in VSMCs according to previous study [27]. Briefly, VSMCs were plated on a 24-well plate. After reaching to 70%–80% confluence, the cells were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 treatment for 24 h in serum-free media. Then, VSMCs were washed with 0.01 M PBS for three times, and stained with Oil Red O and Harris' hematoxylin. The images were captured under an optical microscope.

2.4. Measurement of intracellular total cholesterol

Cholesterol accumulation in cells is closely associated with the formation of foam cells. Intracellular total cholesterol level was determined with Cholesterol Assay Kit (Applygen Technologies, Beijing, China) as we previously reported [28]. Simply, VSMCs were collected and washed three times with PBS. Isopropylalcohol was used to extract the intracellular lipids by ultrasonication. Supernatant was used to determine the total cholesterol after centrifugation at 2000 × g for 5 min. Total protein levels were quantified by Bradford assay method. Cholesterol contents were expressed in nmol of cholesterol per milligram of cellular protein.

2.5. Monocyte adhesion assay

THP-1 (ATCC, Manassas, VA, USA) is a human monocytic cell line derived from the peripheral blood of a 1 year old male with acute monocytic leukemia, which was used to evaluate the monocyte adhesion to VSMCs. THP-1 cells were cultured in RPMI-1640 Medium supplemented with 10% FBS. VSMCs were incubated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 treatment (200 nM) for 24 h. The THP-1 cells (1×10^6 cells/well) were added to VSMC culture and incubated for 1 h at 10 rpm at 37 °C. Then, the medium was drained and washed twice with FBS to remove unattached monocytes. The VSMC layers with attached monocytes cells were fixed with 4% paraformaldehyde and measured with a microscope at × 200 filed. The attached monocytes were randomly counted in five areas per well from each independent experiments and averaged. The data of attached monocyte number were expressed as fold change of the control.

2.6. Real-time PCR

Total RNA was separated with Trizol reagent (Life Technologies, Gaithersburg, MD, USA) following the manufacturer's instructions. Reverse transcriptase reactions were performed using PrimeScript® RT reagent Kits (Takara, Otsu, Shiga, Japan). RT-PCR was done using Quantitative PCR with SYBR Premix Ex Taq TM (Takara, Otsu, Shiga, Japan) and ABI PRISM 7500 sequence detection PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was served as an internal control for mRNAs. The sequence-specific primers used were as follows. ACAT-1: AAGGCAGGCAGTATTGGGTG (forward), ACATCAGTTAGCC CGTCTTTTAC (reverse); ABCA-1: ACCCACCCTATGAACAACATGA (forward), GAGTCGGGTAACGGAAACAGG (reverse); MCP-1: CAGCCA GATGCAATCAATGCC (forward), TGGAATCCTGAACCCACTTCT (reverse); VCAM-1: CAGTAAGGCAGGCTGTAAAAGA (forward), TGGAGC TGGTAGACCCTCG (reverse); FNDC5: TGGAGGAGGATACGGAGTACA (forward), CCACATGAACAGGACCACGA (reverse); GAPDH: TGTTGCC ATCAATGACCCCTT (forward), CTCCACGACGTACTCAGCG (reverse).

2.7. Western blot

VSMCs were lysed in lysis buffer and incubated for 30 min on the ice, the supernatant was retained after centrifugation. Total protein was

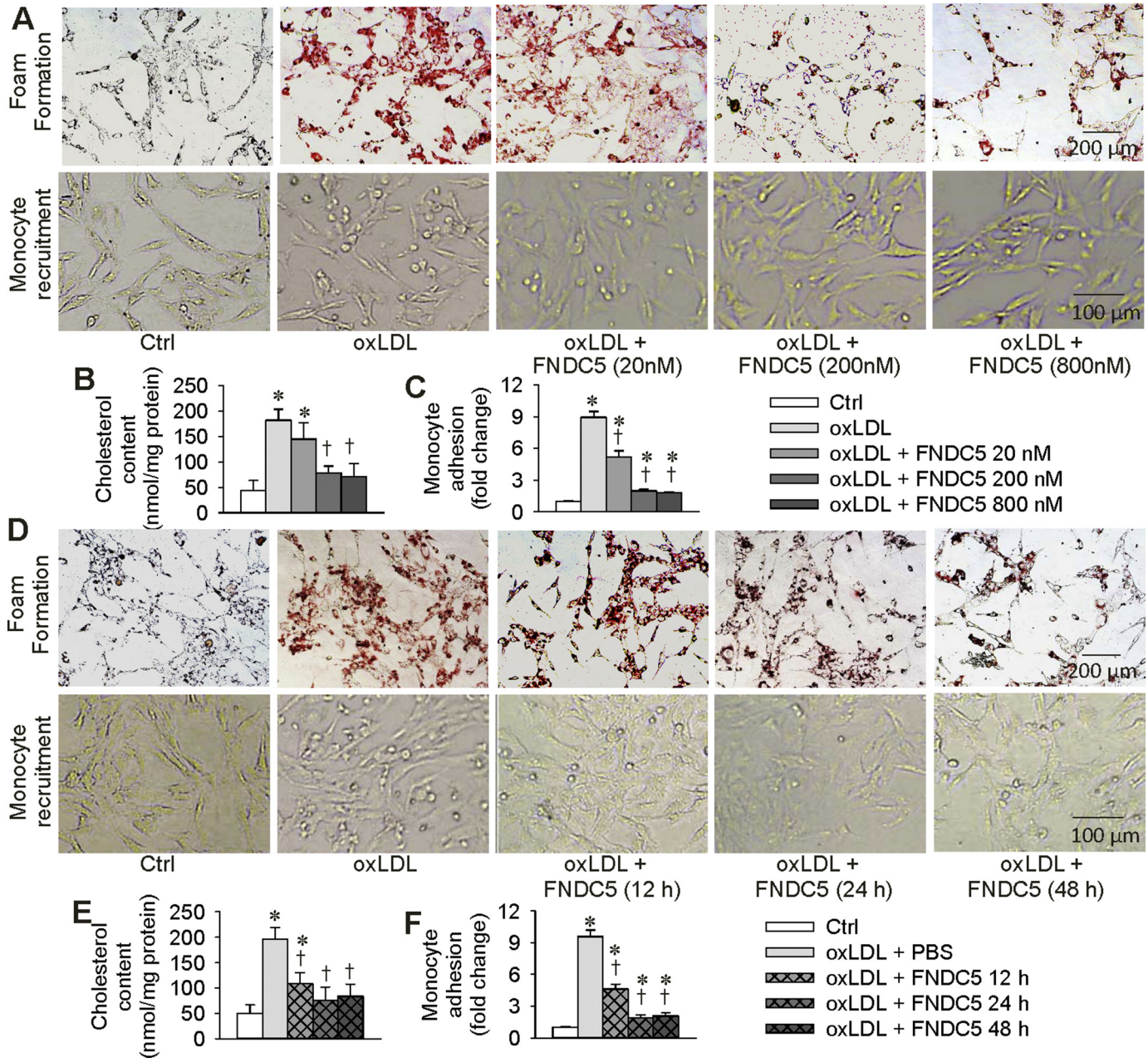

Fig. 1. Effects of FNDC5 on oxLDL-induced foam formation and monocyte adhesion in VSMCs. Foam formation was evaluated by Oil red O staining and intracellular cholesterol content in VSMCs. Red colour represents the neutral triglycerides and lipids in VSMCs. Monocyte adhesion was determined by the numbers of monocytes adhering to the VSMCs. The VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 treatment. A, representative images showing the dose effects of FNDC5 (0, 20, 200, or 800 nM for 24 h) on foam formation and monocyte adhesion. B and C. bar graph showing the dose effects of FNDC5 on intracellular cholesterol content and monocyte adhesion. D, representative images showing the time effects of FNDC5 (200 nM) for 0, 12, 24, or 48 h on foam formation and monocyte adhesion. E and F, bar graph showing the time effects of FNDC5 on intracellular cholesterol content and monocyte adhesion. Values are mean ± SE. $^{*}P < .05$ vs Ctrl. $^{\dagger}P < .05$ vs oxLDL alone. $n = 6$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measured with a protein assay kit (BCA; Pierce, Santa Cruz, CA, USA). Equal amounts of total protein were separated in sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% milk blocking buffer and incubated with primary antibodies overnight at 4 °C. Horseradish peroxidase-conjugated secondary antibodies were used for detection. The bands were visualized by enhanced chemiluminescence (Millipore, Billerica, MA, USA) and quantified by scanning densitometry.

2.8. Immunofluorescence staining

Cells were plated at 1.2×10^5 cells/mL on glass coverslips in 6-well plates and treated at 4% paraformaldehyde for 15 min. Cells were washed with 1× PBS and permeabilized with 0.2% Triton X-100 for 5 min. Fixed cells were blocked for 1 h in 1% BSA at room temperature, incubation with the primary anti-body against p65-NFκB (1:100). Cells were washed three times with PBS and incubated for 1 h with the secondary fluorescein anti-rabbit IgG (1:1000), followed by another 3 washes with PBS. The nucleus was stained with DAPI (Southern Biotech, Birmingham, AL). The immunofluorescence images were

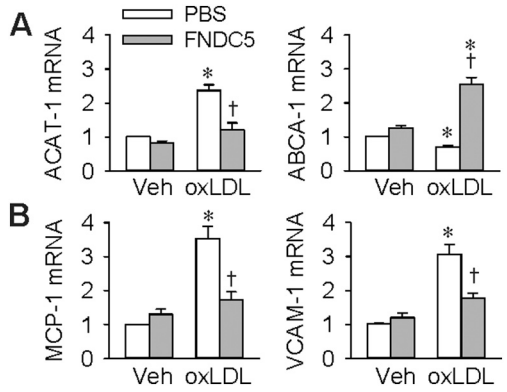

Fig. 2. Effects of FNDC5 on mRNA levels of ACAT-1, ABCA-1, MCP-1 and VCAM-1 in VSMCs. VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 (200 nM) for 24 h. A, mRNA levels of ACAT-1 and ABCA-1 which are respectively related to cholesteryl ester synthesis and cholesterol clearance. B, mRNA levels of MCP-1 and VCAM-1 which are involved in promoting cell adhesine. Values are mean ± SE. $^{*}P < .05$ vs vehicle (Veh). $^{\dagger}P < .05$ vs PBS. $n = 4$ per group.

acquired with an Olympus BX51 microscope (Olympus, Tokyo, Japan) coupled with an Olympus DP70 digital camera.

2.9. Antibodies and chemicals

Antibodies against p65, P-p65, pro-IL-1β, IL-1β, caspase-1 and were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against procaspase-1, p65-NFκB and CD36 was obtained from Proteintech Group (Rosemont, IL, USA). Antibodies against NLRP3, ASC and FNDC5 were purchased from Abcam (Cambridge, MA, USA). Antibodies against IκBα and P-IκBα were purchased from Bioworld Technology, Inc. (Minneapolis, MN, USA). Antibodies against Lamin B1 and GAPDH were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant FNDC5 expressed in *E. coli* was purchased from Sigma Inc. (St. Louis, MO, USA), and its extracellular domain has 100% identity between human, mouse and rat. Compound C was also purchased from Sigma Inc. OxLDL were obtained from YEASEN Biotech (Shanghai, China), and the endotoxin contents in the oxLDL solusion were measured with ToxinSensor TM Chromogenic LAL Endotoxin Assay Kit (Genscript Nanjing Co Ltd., Nanjing, China). BMS-345541 was purchased from Selleck Chemicals (Houston, TX, USA). MCC950 was acquired from MedChemexpress (Princeton, NJ, USA). RGDS was obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

2.10. Statistical analysis

Data were expressed as mean ± SE. One-way or two-way ANOVA followed by post hoc Bonferroni test was used for multiple comparisons. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. FNDC5 attenuates oxLDL-induced foam cell formation and monocyte recruitment in HA-VSMCs

VSMC foam cell formation and monocyte recruitment are crucial events in the pathogenesis of atherosclerosis [9,27]. OxLDL was used to induce foam cell formation and monocyte adhesion as previously reported [8]. The oxLDL used in the present study contained < 0.01 endotoxin units/mL of endotoxin. There were no significant difference in endotoxin contents among PBS, VSMC homogenate and the oxLDL (Supplementary Fig. 1), excluding the possibility that the effects of oxLDL were not caused by the endotoxin contained in the oxLDL solution (Supplementary Fig. 1). Foam formation was evaluated by Oil red O staining to show lipid accumulation in VSMCs, and intracellular cholesterol content in VSMCs. Monocyte adhesion was evaluated by the numbers of monocytes adhering to the VSMCs. FNDC5 reduced the oxLDL-induced accumulation of lipid droplets, intracellular cholesterol contents and monocyte adhesion in VSMCs dose-relatedly (Fig. 1A–C) and time-relatedly (Fig. 1D–F), and the maximal effects were observed at the concentration of about 200 nM of FNDC5 for 24 h. We further examined the FNDC5 expressions in aorta of mice with atherosclerosis induced by high fat diet for 16 weeks and 24 weeks. However, no significant difference in aortic FNDC5 expression was observed between control mice and atherosclerosis mice (Supplementary Fig. 2).

3.2. FNDC5 affects ACAT-1, ABCA-1, MCP-1 and VCAM-1 mRNA levels in HA-VSMCs

Acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) promotes foam cell formation by increasing intracellular cholesteryl ester synthesis [27]. ATP binding cassette transporter A-1 (ABCA-1) serves as a gatekeeper for modulating flux of tissue cholesterol into the reverse cholesterol transport pathway, and drugs that induce ABCA1 in mice

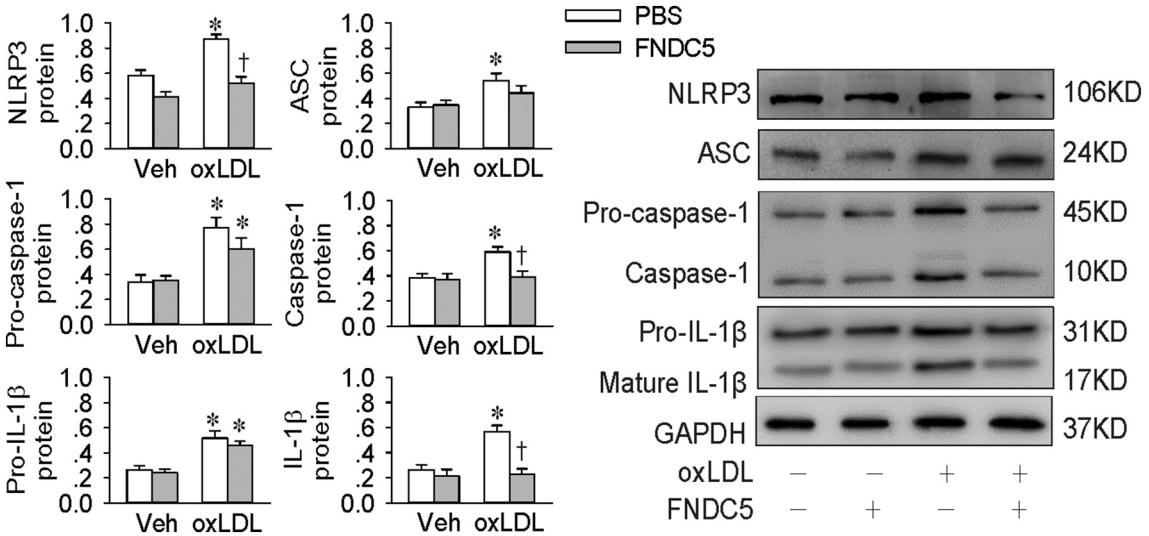

Fig. 3. Effect of FNDC5 on the oxLDL-induced upregulation of NLRP3 inflammasome components and IL-1β in VSMCs. VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 (200 nM) for 24 h. Values are mean ± SE. $^{*}P < .05$ vs Veh. $^{\dagger}P < .05$ vs PBS. $n = 4$ per group.

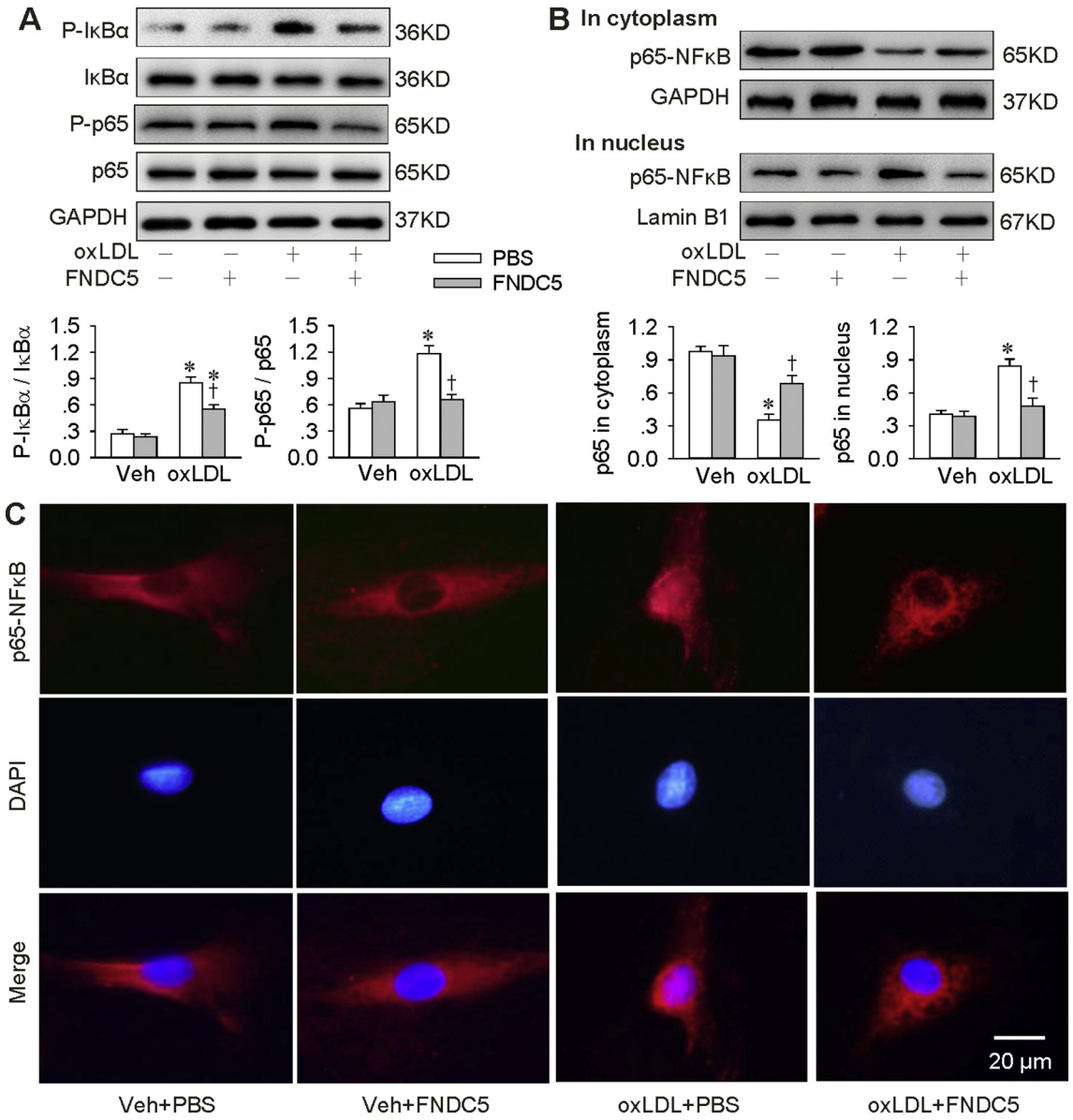

Fig. 4. Effects of FNDC5 on oxLDL-induced p65-NFκB activation in VSMCs. VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 (200 nM) for 24 h. A, phosphorylation of IκBα and p65. B, p65-NFκB in cytoplasm and nucleus. C, representative images of Immunofluorescence staining showing p65-NFκB nuclear translocation in VSMCs. Red, p65-NFκB; Blue, DAPI used as a nuclear counterstain in fluorescence microscopy. Values are mean ± SE. $^{*}P < .05$ vs Veh. $^{\dagger}P < .05$ vs PBS. $n = 4$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase clearance of cholesterol [29]. FNDC5 inhibited oxLDL-induced ACAT-1 mRNA upregulation, and reversed ABCA-1-induced mRNA downregulation (Fig. 2A), suggesting that ACAT-1 downregulation and ABCA-1 upregulation are involved in the beneficial roles of FNDC5 in attenuating foam cell formation. Monocyte chemotactic protein-1 (MCP-1) is also called chemokine (C−C motif) ligand 2 (CCL2), recruits monocytes and macrophages to the sites of inflammation [30]. Vascular cell adhesion molecule-1 (VCAM-1) is an important cell adhesion molecule for leukocyte recruitment in the development of atherosclerosis [31]. VSMCs with oxLDL treatment showed higher MCP-1 and VCAM-1 mRNA levels than the control VSMCs, which were inhibited by FNDC5 (Fig. 2B), suggesting the inhibitory effects of FNDC5 on MCP-1 and VCAM-1 expressions may contribute its role in attenuating monocyte adhesion.

3.3. FNDC5 inhibits oxLDL-induced NLRP3 and IL-1β upregulation in HA-VSMCs

It is known that oxLDL induces NLRP3 inflammasome activation, which leads to IL-1β release, and then, triggers vascular inflammatory responses, foam cell formation and progression of atherosclerosis [32,33]. We found that FNDC5 prevents the oxLDL-induced upregulation of NLRP3, caspase-1 and IL-1β, but had no significant effects on oxLDL-induced ASC, pro-caspase-1 and pro-IL-1β upregulation (Fig. 3).

3.4. FNDC5 prevents oxLDL-induced NFκB activation in HA-VSMCs

A recent study in our lab have shown that nuclear factor-κB (NFκB) activation in hypertension contributes to NLRP3 inflammasome activation [34]. It is interesting to know whether NFκB is involved in the effects of FNDC5. Treatment of VSMCs with oxLDL promoted the phosphorylation of inhibitor κBα (IκBα) and p65 subunit of NFκB, which were attenuated by FNDC5 (Fig. 4A). Moreover, oxLDL-induced

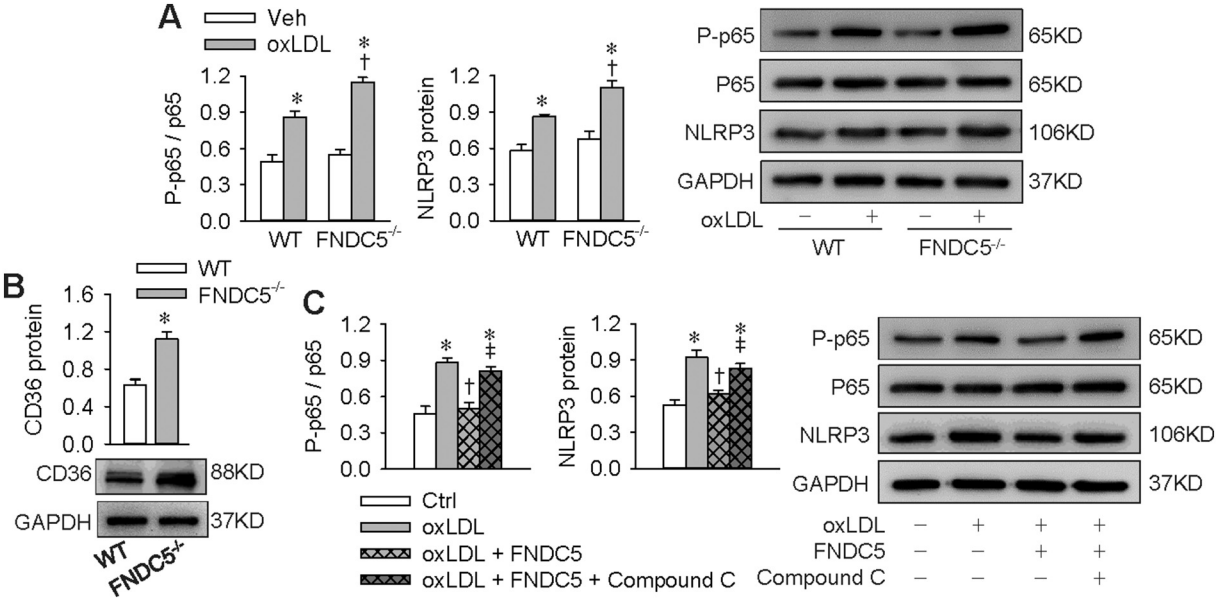

Fig. 5. Effects of FNDC5 knockout and AMPK inhibitor on oxLDL-induced p65-NFκB phosphorylation and NLRP3 expression in VSMCs. A, VSMCs from WT mice and FNDC5$^{-/-}$ mice were treated with oxLDL (50 μg/mL) for 24 h. $^{*}P < .05$ vs Veh. $^{\dagger}P < .05$ vs WT. B, CD36 protein expression in VSMCs of WT and FNDC5 knockout mice. $^{*}P < .05$ vs WT. C, VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 (200 nM) plus compound C (1 μM) for 24 h. Values are mean ± SE. $^{*}P < .05$ vs Ctrl. $^{\dagger}P < .05$ vs oxLDL. $^{\ddagger}P < .05$ vs oxLDL + FNDC5. $n = 4$ per group.

p65-NFκB nucleus translocation were prevented by FNDC5 treatment evidenced by both Western blotting (Fig. 4B) and immunofluorescence (Fig. 4C). These results indicated that FNDC5 prevents NFκB activation in VSMCs.

3.5. Effects of FNDC5 deletion on oxLDL-induced NFκB activation and NLRP3 upregulation in VSMCs of mice

VSMCs were isolated from the aorta of WT and FNDC5$^{-/-}$ mice. The primary mouse VSMCs were treated with oxLDL for 24 h. FNDC5 deletion had no significant effects, but aggravated the oxLDL-induced p65 phosphorylation and NLRP3 upregulation (Fig. 5A). The results suggest that endogenous FNDC5 play beneficial roles in attenuating oxLDL-induced NFκB activation and NLRP3 upregulation. It is known that CD36 is a receptor of oxLDL and is involved in atherosclerotic lesion formation [35]. We found that CD36 protein was upregulated in FNDC5$^{-/-}$ mice (Fig. 5B), suggesting that the increased CD36 expression in FNDC5 knockout mice may be at least partially responsible for the oxLDL-induced NFκB activation and NLRP3 upregulation.

3.6. Effects of AMPK inhibitor and integrin inhibitor on oxLDL-induced NFκB activation and NLRP3 upregulation in HA-VSMCs

Previous studies have shown that AMPK mediates the effects of FNDC5 on adipose tissue inflammation in obese mice [23]. It is interesting to determine whether AMPK is involved in the effects of FNDC5 on NLRP3 expression in VSMCs. The VSMCs were treated with oxLDL for 24 h followed by FNDC5 plus compound C for 24 h. Inhibiting AMPK with compound C prevented the beneficial roles of FNDC5 in attenuating the oxLDL-induced p65 phosphorylation and NLRP3 expression (Fig. 5C). The results indicate that AMPK mediates the beneficial effects of FNDC5 on oxLDL-induced NFκB activation and NLRP3 upregulation. On the other hand, it has been found that integrins are irisin receptors in osteocytes and adipose tissues [36]. We found that inhibition of integrin with RGDS abolished the beneficial roles of FNDC5 in attenuating the oxLDL-induced p65 phosphorylation and NLRP3 up-regulation in VSMCs, suggesting that integrins are involved in the effects of FNDC5 in the present study (Supplementary Fig. 3).

3.7. Effects of FNDC5 plus BMS-345541 or MCC950 on oxLDL-induced foam formation and monocyte adhesion in HA-VSMCs

Inhibition of NFκB with BMS-345541 or inhibition of NLRP3 inflammasome with MCC950 reduced the oxLDL-induced accumulation of lipid droplets and intracellular cholesterol levels in VSMCs, but the effects of BMS-345541 or MCC950 were significantly weaker than those of FNDC5. The inhibitory effects of FNDC5 alone, or FNDC5 + BMS-345541, or FNDC5 + MCC950 on the oxLDL-induced accumulation of lipid droplets and intracellular cholesterol levels in VSMCs were greater than BMS-345541 or MCC950 alone (Fig. 6A and B). The inhibitory effects of BMS-345541 or MCC950 on oxLDL-induced monocyte adhesion to VSMCs were similar to that of FNDC5, but the effects of FNDC5 + BMS-345541, or FNDC5 + MCC950 were not greater than those of FNDC5, BMS-345541 or MCC950 alone (Fig. 6A and C). The roles of BMS-345541 or MCC950 in inhibiting oxLDL-induced ACAT-1 upregulation and ABCA-1 mRNA downregulation were significantly weaker than those of FNDC5 (Fig. 6D), while the inhibitory effects of BMS-345541 or MCC950 on oxLDL-induced changes in MCP-1 and VCAM-1 mRNA levels were similar to those of FNDC5 (Fig. 6E).

3.8. Effects of FNDC5 plus BMS-345541 or MCC950 on oxLDL-induced on NLRP3 expression

Inhibition of NFκB with BMS-345541 attenuated the oxLDL-induced NLRP3, caspase-1 and IL-1β upregulation, but had no significant effects on ASC, pro-caspase-1 and pro-IL-1β expressions. The effects of FNDC5 + BMS-345541 were not greater than those of FNDC5 or BMS-345541 alone. Inhibition of NLRP3 inflammasome with MCC950 attenuated the oxLDL-induced caspase-1 and IL-1β upregulation, but had no significant effects on NLRP3, ASC, pro-caspase-1 and pro-IL-1β expressions. The effects of FNDC5 + MCC950 were not greater than those of MCC950 alone (Fig. 7A). On the other hand, oxLDL, BMS-345541, MCC950 had no significant effects on FNDC5 expression (Fig. 7B).

4. Discussion

Foam cell formation plays crucial roles in the early stage atherosclerotic lesions [3]. VSMCs and macrophages are the main source of

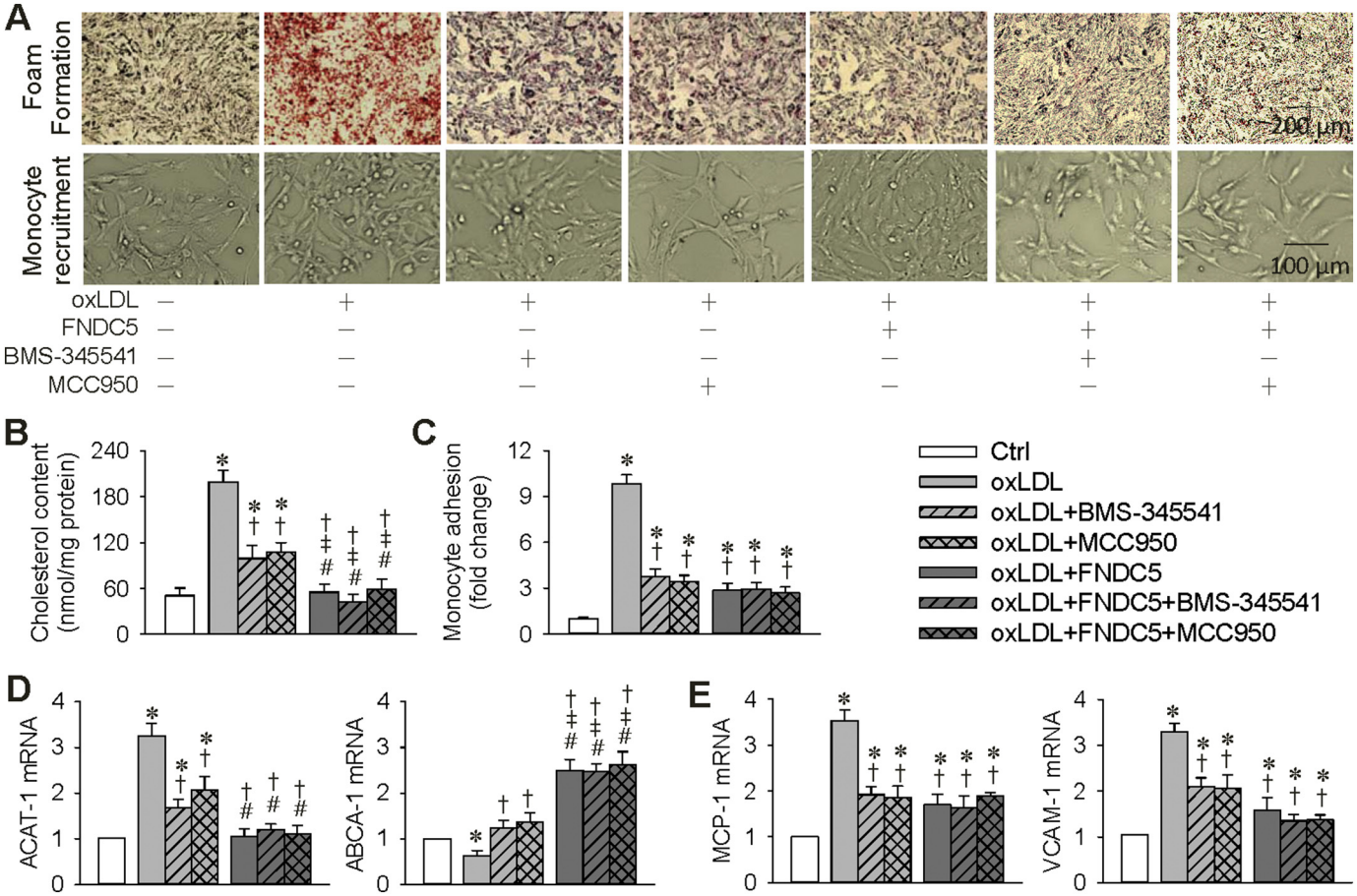

Fig. 6. Effects of FNDC5 plus BMS-345541 (a NFκB inhibitor) or MCC950 (a NLRP3 inflammasome inhibitor) on the oxLDL-induced foam formation and monocyte adhesion in VSMCs. Foam formation was evaluated by Oil red O staining and intracellular cholesterol content in VSMCs. Red colour represents the neutral triglycerides and lipids in VSMCs. Monocyte adhesion was determined by the numbers of monocytes adhering to the VSMCs. VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 (200 nM), BMS-345541 (5 μM), MCC950 (50 μM), FNDC5 + BMS-345541 or FNDC5 + MCC950 for 24 h. A, representative images showing the foam formation and monocyte adhesion. B and C, bar graph showing intracellular cholesterol content and monocyte adhesion. D, relative values of ACAT-1 and ABCA-1 mRNA. E, relative values of MCP-1 and VCAM-1 mRNA. Values are mean ± SE. $^{*}P < .05$ vs Ctrl. $^{\dagger}P < .05$ vs oxLDL alone. $^{\ddagger}P < .05$ vs oxLDL + BMS-345541. $^{\#}P < .05$ vs oxLDL + MCC950. $n = 6$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

foam cell formation [4]. ACAT-1 is a key enzyme for the synthesis of intracellular cholesterol ester [37]. Excessive cholesterol ester causes accumulation of cytoplasmic lipid droplets and triggers foam cell formation [38]. ABCA1 mediates the transport of cholesterol and phospholipids across cellular membranes, where they are removed from cells by lipid-poor high-density lipoprotein (HDL) apolipoprotein [39]. Reduced ABCA1 activity contributes to foam cell formation human VSMCs [40]. We found that FNDC5 inhibited intracellular lipid droplets and cholesterol accumulation, ACAT-1 upregulation and ABCA-1 downregulation in oxLDL-treated VSMCs. The findings indicate that FNDC5 attenuates oxLDL-induced foam cell formation in VSMCs. ACAT-1 upregulation and ABCA-1 downregulation are involved in the beneficial effects of FNDC5 on foam cell formation.

Monocyte recruitment in the wall of arteries accelerates chronic inflammation and atherosclerosis [41]. MCP-1 is strongly expressed in the cells of macrophage-rich regions of human and rabbit atherosclerotic lesions, and promotes recruitment of monocyte-macrophages into developing lesions [42]. VCAM-1 are important for the migration of monocytes and lymphocytes into the vessel wall [43]. We found that FNDC5 inhibited the monocyte adhesion to VSMCs and the upregulation of MCP-1 and VCAM-1 in oxLDL-treated VSMCs. MCP-1 and VCAM-1 downregulation are involved in the beneficial effects of FNDC5 on monocyte adhesion to VSMCs.

Atherosclerosis is considered as a chronic vascular inflammatory disease, and inflammatory process participates in all stages of atherosclerosis [44]. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals [45]. NLRP3 expression level is considered as a limiting step in inflammasome activation [46,47]. FNDC5 prevented oxLDL-induced NLRP3 upregulation, and thereby reduced caspase-1 and IL-1β production, suggesting that FNDC5 inhibits NLRP3 inflammasome and inflammation, which at least partially contributed to the roles of FNDC5 in attenuating foam cell formation and monocyte adhesion to VSMCs. The results were further supported by the findings that inhibition of caspase-1 and IL-1β production with MCC950 attenuated oxLDL-induced foam cell formation and monocyte adhesion.

Transcription factor NFκB activation contributes to inflammation associated with the onset of atherosclerosis [48]. Our previous study have shown that NFκB activation promotes NLRP3 expression in VSMCs [34]. In the present study, FNDC5 attenuates oxLDL-induced NFκB activation in VSMCs, which were similar to the effects of a NF-κB inhibitor BMS-345541. The inhibitory effect of FNDC5 on NFκB activation at least partially contributes to its roles in attenuating the NLRP3-mediated inflammation and the following foam cell formation and monocyte adhesion. Integrins are irisin receptors in osteocytes and adipose tissues [36]. In the present syudy, inhibition of integrin with RGDS prevented the beneficial effects of FNDC5 on the oxLDL-induced p65 phosphorylation and NLRP3 up-regulation in VSMCs, indicating that integrins

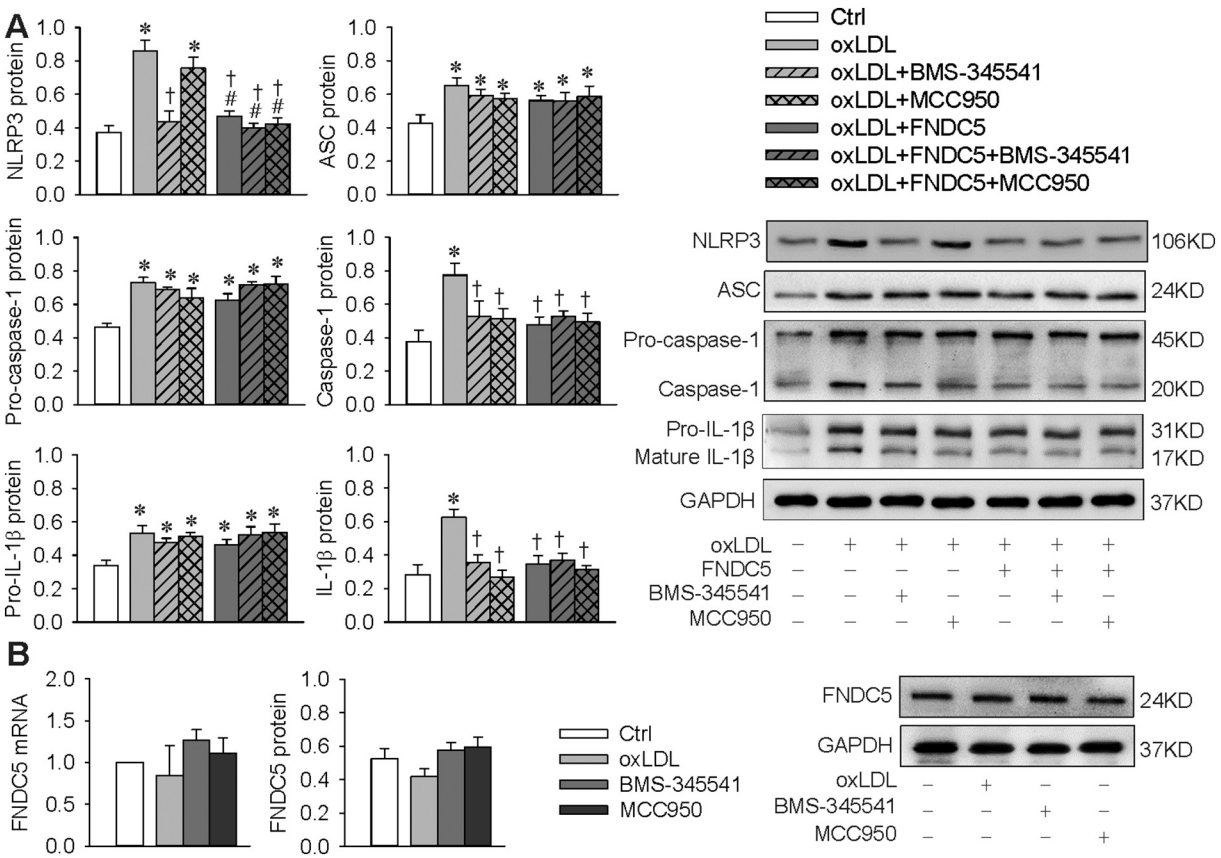

Fig. 7. Effects of FNDC5 plus BMS-345541 or MCC950 on the oxLDL-induced upregulation of NLRP3 inflammasome components in VSMCs. VSMCs were treated with oxLDL (50 μg/mL) for 24 h followed by FNDC5 (200 nM), BMS-345541 (5 μM), MCC950 (50 μM), FNDC5 + BMS-345541 or FNDC5 + MCC950 for 24 h. A, NLRP3 inflammasome activation. B, FNDC5 expression. Values are mean ± SE. $^{*}P < .05$ vs Ctrl. $^{\dagger}P < .05$ vs oxLDL alone. $^{\ddagger}P < .05$ vs oxLDL + BMS-345541. $^{\#}P < .05$ vs oxLDL + MCC950. $n = 3$ per group.

are involved in the effects of FNDC5. However, it is not known that the effects of FNDC5 are caused by directly acting on integrin or indirectly via converting FNDC5 to irisin.

FNDC5 normalized oxLDL-induced foam cell formation in VSMCs. However, BMS-345541 and MCC950 greatly reduced, but could not completely abolish oxLDL-induced foam cell formation. These results suggest that FNDC5 prevents foam cell formation mainly via inhibiting NFκB and NLRP3 inflammasome activation. Our previous study have shown that FNDC5 enhances lipolysis and ameliorates glucose/lipid metabolic derangements in adipose tissues [22], and FNDC5 overexpression prevents HFD-induced hyperlipemia, hepatic lipid accumulation, and impaired fatty acid oxidation and autophagy in mice liver [21]. Thus, we proposed that the roles of FNDC5 in ameliorating glucose/lipid metabolic derangements may partially contributes to the inhibitory effects of FNDC5 on oxLDL-induced foam cell formation. In the present study, there was no significant difference in aortic FNDC5 expression between control mice and atherosclerosis mice. A limitation in the present study was that the effects of FNDC5 on atherosclerosis were not examined in vivo, which need further investigation. It is noted that FNDC5 exists in other cells apart from VSMCs, and FNDC5 gene is predominantly expressed in muscle. Circulating irisin is detected in all subjects studied, whereas circulating FNDC5 is only a distinct minority of subjects [49], suggesting that FNDC5 in other cells hardly directly affect VSMCs, but there is a possibility that FNDC5 may indirectly affect VSMCs via its cleaved hormone, irisin.

In summary, FNDC5 inhibited oxLDL-induced foam cell formation and monocyte adhesion in VSMCs primarily via suppressing NFκB activation and NLRP3 upregulation in VSMCs.

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Conflicts of interest

The authors declare no competing or financial interests.

Appendix A. Supplementary data

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