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TLN1 synergizes with ITGA5 to ameliorate cardiac microvascular endothelial cell dysfunction

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Background: *The complex process of atherosclerosis is thought to begin with endothelial cell dysfunction, and advanced atherosclerosis is the underlying cause of coronary artery disease (CAD). Uncovering the underlying mechanisms of CAD-related endothelial cell injury may contribute to the treatment.*

Materials and methods: *Cardiac microvascular endothelial cells (CMVECs) were treated with oxidised low-density lipoprotein (ox-LDL) to mimic an injury model. The involvement of Talin-1 (TLN1) and integrin alpha 5 (ITGA5) in the proliferation, apoptosis, angiogenesis, inflammatory response, and oxidative stress in CMVECs were assessed.*

Results: *TLN1 overexpression assisted CMVECs in resistance to ox-LDL stimulation, with alleviated cell proliferation and angiogenesis, reduced apoptosis, inflammatory response, and oxidative stress. TLN1 overexpression triggered increased ITGA5, and ITGA5 knockdown reversed the effects of TLN1 overexpression on the abovementioned aspects. Together, TLN1 synergized with ITGA5 to ameliorate the dysfunction in CMVECs.*

Conclusions: *This finding suggests their probable involvement in CAD, and increasing their levels is beneficial to disease relief.* (Folia Morphol 2024; 83, 1: 92–101)

Key words: Talin-1, integrin, coronary artery disease, cardiac microvascular endothelial cells, atherosclerosis

INTRODUCTION

Coronary artery disease (CAD), a common cardiovascular disease, is one of the primary threats to human health worldwide [15]. Advanced atherosclerosis is the underlying cause of CAD and heritability estimates for CAD vary from 40% to 70%, indicating a strong contribution of genes to disease pathology [13]. Genome-wide association studies have revealed that approximately one-third of CAD-related loci are associated with traditional risk factors, such as lipids, blood pressure, body mass index, diabetes, and smoking behaviour. Genes at the remaining loci have been implicated in vascular wall-related risk mechanisms [1]. Cardiac microvascular endothelial cells (CMVECs), the most abundant cells in the myocardium [22], orchestrate cardiogenesis during development, regulate

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adult cardiac function, and modulate the pathological processes in heart failure [31, 32, 36]. CAD is always accompanied by endothelial cell damage [21], and uncovering the underlying mechanisms of CAD-related endothelial cell injury may contribute to the treatment of the disease.

The protein encoded by the Talin-1 (TLN1) gene, a major component of the extracellular matrix (ECM), mediates the adhesion of integrins to the ECM [3, 18], and both bioinformatics and experimental analyses identified downregulated expression of TLN1 in CAD samples [9]. Overexpressed TLN1 may cooperate with β-oestradiol to stimulate endometrial stromal cell proliferation and neovascularisation in adenomyosis, synergistically promoting the growth and survival of ectopic lesions [24]. TLN1 expression was significantly downregulated in aortic dissection samples, and downregulation of TLN1 expression was associated with increased proliferation and migration of vascular smooth muscle cells in aortic dissection [26]. However, the specific role and regulatory mechanism of TLN1 in CAD have not been reported yet. According to the String website [19], TLN1 was found to have a potential interaction with integrin alpha 5 (ITGA5), and ITGA5 is involved in promoting endocardial differentiation and cardiac morphogenesis [17].

This study utilised oxidised low-density lipoprotein (ox-LDL) to induce CMVECs to mimic an injury model [29], with the intention of defining the association existing between TLN1 and ITGA5, and exploring their roles in CMVECs. Exploring the mechanism of endothelial cell injury is beneficial to the development of CAD treatment.

MATERIALS AND METHODS

Cell culture and treatment

Cardiac microvascular endothelial cells (Procell, Wuhan, China) were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco) along with 10% fetal bovine serum (Gibco), 30 μg/mL endothelial cell growth supplement (ScienCell), 1 U/mL heparin, and penicillin-streptomycin mixture (Gibco) [4]. CMVECs were maintained at 37°C in a 5% CO_2 atmosphere. CMVECs were stimulated with ox-LDL (100 μg/mL, Yeasen, Shanghai, China) for 24 h to mimic CAD [29].

Cell transfection

Cells underwent transfection to promote TLN1 overexpression or ITGA5 knockdown. X-tremeGENE transfection reagent (Roche, Shanghai, China) mixed with plasmids or short hairpin RNAs (HanBio, Shanghai, China) were added to the CMVECs [23] and incubated at 37°C for 6 h before replacing with fresh medium. After 48 h, transfection efficacy was assessed.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Cardiac microvascular endothelial cells were added with TRIzol® Reagent (Invitrogen), followed by chloroform, and the lysate was centrifuged at 10,000×g for 15 min at 4°C. Isopropanol precipitated the RNA in the upper aqueous phase and then RNA was reverse transcribed to generate cDNA using Evo M-MLV RT Kit (Accurate, Changsha, China). QuantiTect SYBR Green PCR Kit (Qiagen, Shanghai, China) was used to perform quantitative polymerase chain reaction (qPCR) according to the instructions. Relative mRNA levels were measured using the ΔΔCt method after normalization to actin [37].

Western blotting

Proteins were isolated from CMVECs after treatment with RIPA lysis buffer (Solarbio, Beijing, China) and quantified using a Nano 3000 protein detector (YPH-Bio, Beijing, China). Proteins were then separated using SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Roche) [6]. Membranes were incubated sequentially with skimmed milk, primary antibodies, and HRP-conjugated secondary antibody. The antibodies involved in the study were all from Invitrogen or Abcam. Blots were visualised after ECL reagent (Millipore) treatment and semi-quantified using ImageJ software.

Cell counting Kit-8 (CCK8)

Transfected CMVECs were seeded in 96-well plates and treated with ox-LDL for 24 h. The incubation was continued for 2 h after CCK8 solution (Beyotime, Shanghai, China) was supplemented into each well [11]. Optical density was recorded at 450 nm using a microplate reader (Thermo Fisher Scientific).

5-ethynyl-2'-deoxyuridine (EdU) assay

Following the transfected CMVECs were treated with ox-LDL for 24 h, they were incubated with 100 μL EdU reagent (Ribobio, Guangzhou, China) for 4 h [12]. Then CMVECs were washed twice with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde (Chemegen, Shanghai, China) for 15 min, and stained with DAPI (Beyotime) for 10 min.

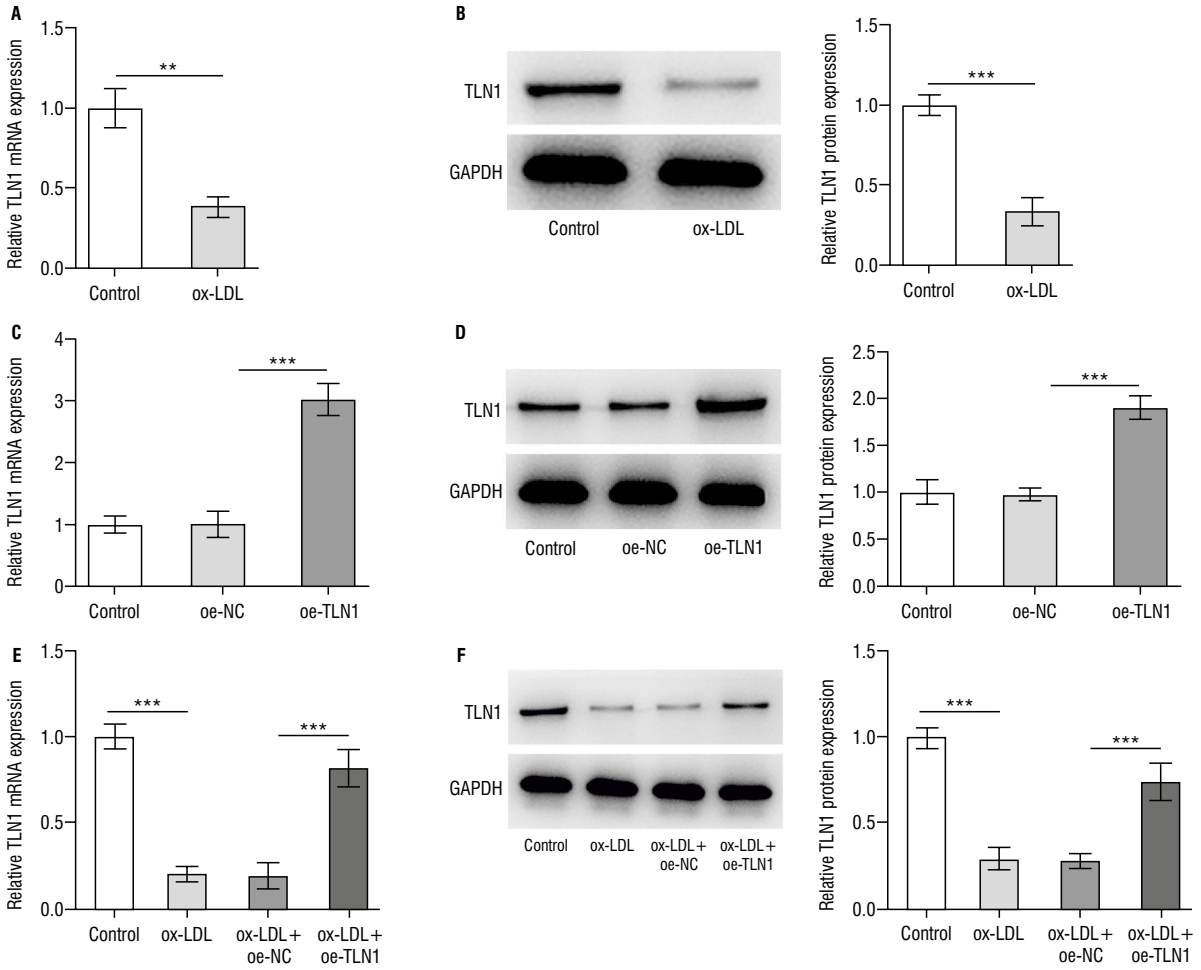

Figure 1. Talin-1 (TLN1) level in cardiac microvascular endothelial cells (CMVECs) (**A**) CMVECs were treated with oxidised low-density lipoprotein (ox-LDL) and the expression level of TLN1 was determined using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and (**B**) western blotting; TLN1 overexpression in CMVECs by transfection was confirmed using RT-qPCR (**C**) and western blotting (**D**); Following ox-LDL treatment, TLN1 in the transfected CMVECs was confirmed using RT-qPCR (**E**) and western blotting (**F**); $^{**}p < 0.01$, $^{***}p < 0.001$; oe — overexpression; NC — negative control.

Stained CMVECs were visualized and imaged under a fluorescent microscope (Olympus, Japan).

Flow cytometry

Cardiac microvascular endothelial cells were washed twice with cold PBS and suspended in binding buffer. 100 μL of cell suspension was transferred to culture tubes and incubated with Annexin V FITC and propidium iodide (Elabscience, Wuhan, China) for 15 min at room temperature in the dark [25]. Apoptosis was analysed using flow cytometry (BD FACSCanto, USA) and FlowJo software.

Angiogenesis assay

Matrigel (BD Biosciences) was diluted 1:1 with cold endothelial cell growth medium, starved CMVECs were seeded on Matrigel and incubated at 37°C for 6 h [35]. The structure of the capillary was observed using a microscope (Olympus).

ELISA

Secreted levels of tumour necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1β [10] were determined in CMVECs using corresponding ELISA kits (Elabscience) according to the manufacturer's instructions. Optical density was recorded at 450 nm using a microplate reader.

Co-immunoprecipitation (Co-IP)

Cardiac microvascular endothelial cells were lysed on ice for 10 min, centrifuged at 13,000×g for 10 min at 4°C, and the supernatant was collected. 2.5 μg of TLN1 or ITGA5 antibody (Invitrogen) was added to the lysate (500 μg/IP) along with 10 μL of

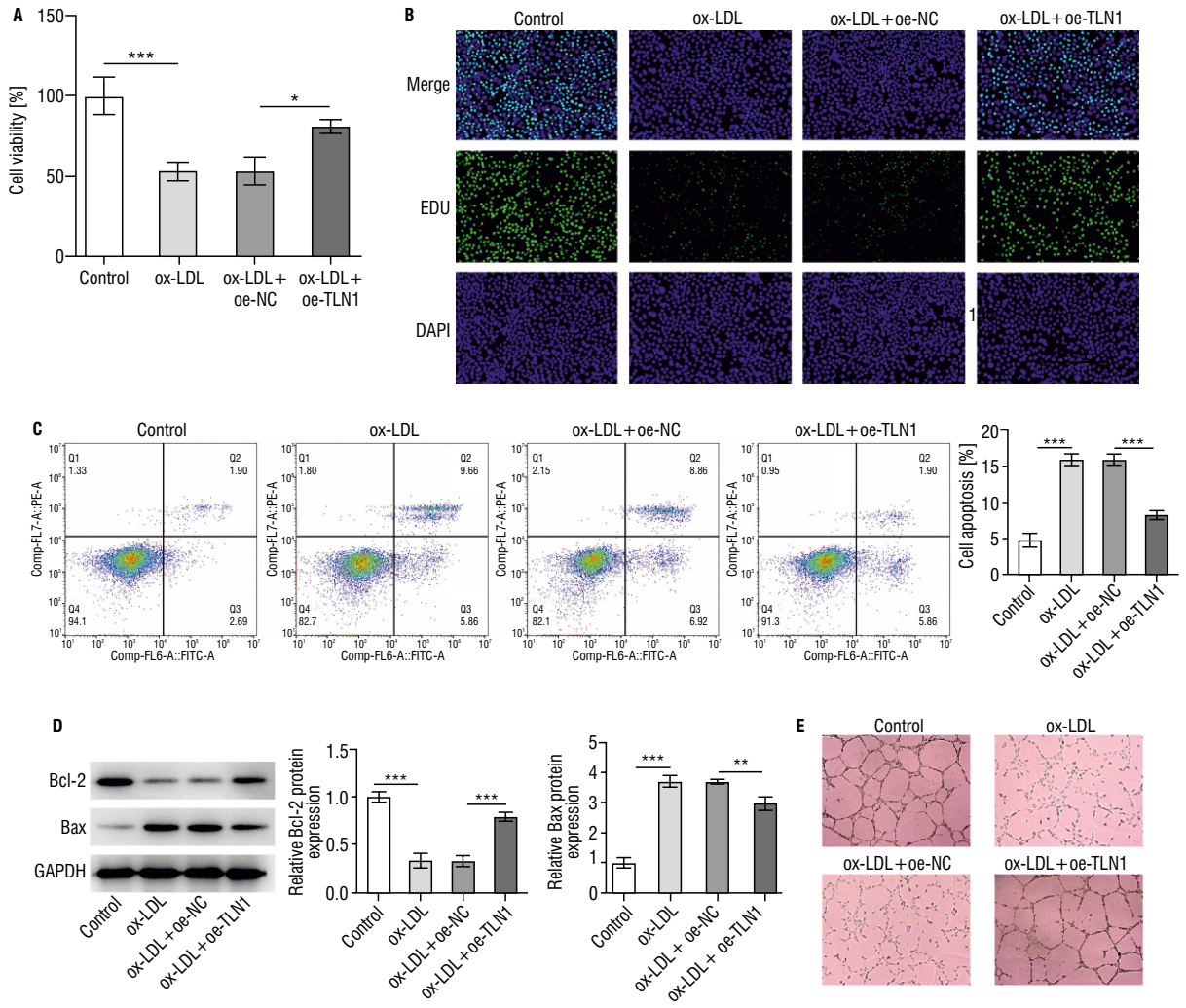

Figure 2. Talin-1 (TLN1) in apoptosis and angiogenesis. The viability and proliferation in each group was determined using cell counting Kit-8 (CCK8) (**A**) and 5-ethynyl-2′-deoxyuridine (EdU) assays (**B**). Cell apoptosis was determined using flow cytometry (**C**) and western blotting (**D**); **E.** The matrigel-based angiogenesis assay was used to detect the angiogenic capacity; *p < 0.05, **p < 0.01, ***p < 0.001; ox-LDL — oxidised low-density lipoprotein; oe — overexpression; NC — negative control.

protein A+G magnetic beads (Beyotime), followed by gentle rotation for 2 h at room temperature. The supernatant was removed magnetically and the beads together with SDS sample buffer were boiled at 95°C for 5 min prior to routine western blot analysis [37].

Statistical analysis

Statistical analysis was performed using SPSS 19.0. Data are presented as mean ± standard deviation, and statistical differences between groups were analysed using two tailed, unpaired Student's t-test (two groups), and one-way ANOVA followed by Tukey's post hoc test (multiple groups) [14]. $P < 0.05$ was considered a significant difference.

RESULTS

TLN1 in apoptosis and angiogenesis

In CMVECs, TLN1 mRNA and protein levels declined in response to the ox-LDL treatment (Fig. 1A, B). To discover the specific roles of TLN1, the overexpression of TLN1 in CMVECs was confirmed (Fig. 1C, D). Following ox-LDL treatment, TLN1 in the ox-LDL + oe-TLN1 group elevated compared with the ox-LDL + oe-NC group (Fig. 1E, F). Cell viability (Fig. 2A) and proliferation (Fig. 2B) were decreased upon ox-LDL treatment and TLN1 overexpression reversed these decline. Ox-LDL treatment increased the proportion of apoptotic cells, accompanied by a decline in Bcl-2 and an increase in Bax. Nevertheless, TLN1 overex-

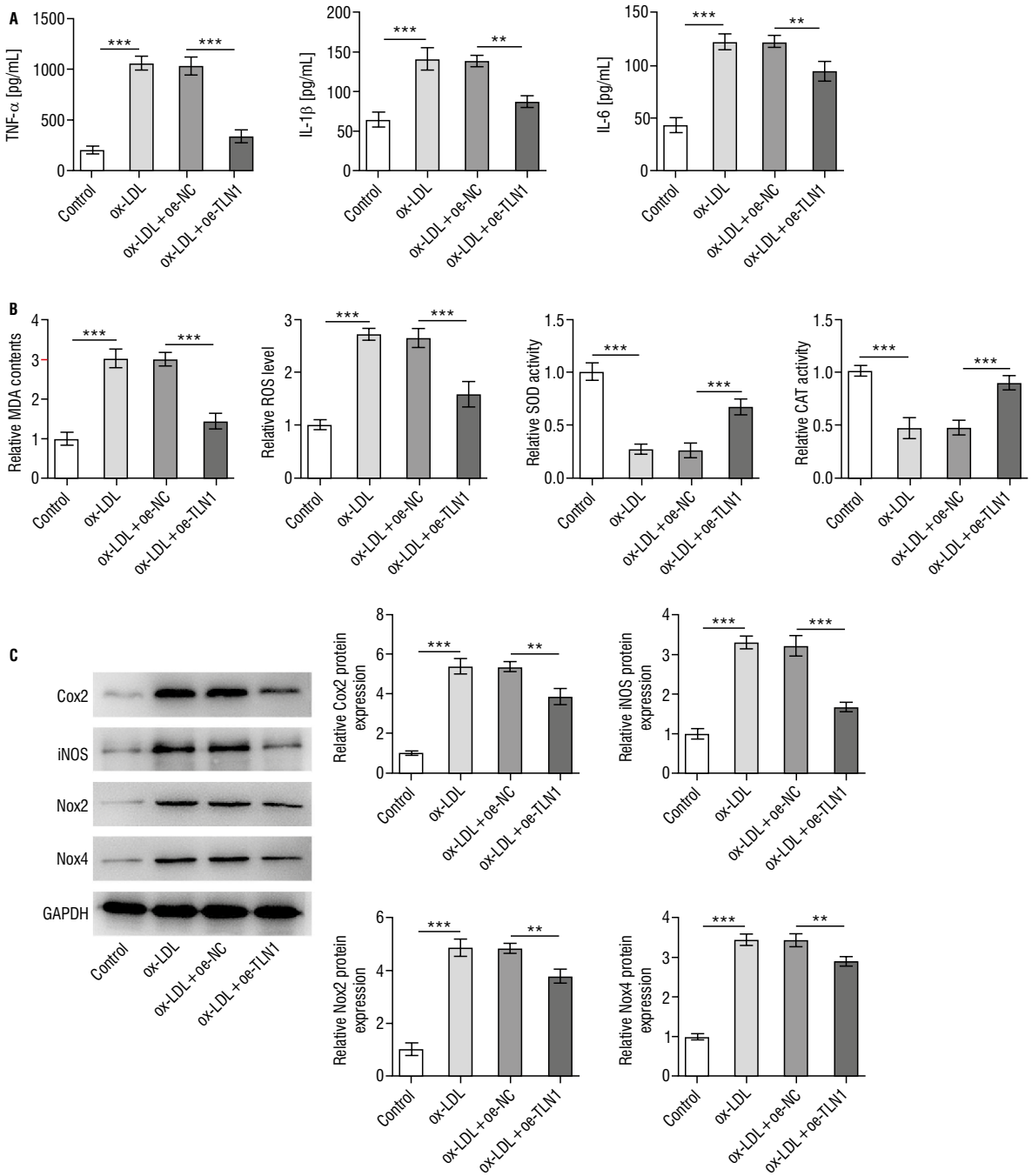

Figure 3. Talin-1 (TLN1) in inflammation and oxidative stress; **A.** ELISA kits were used to measure cellular inflammatory factor levels; **B.** Oxidative stress was evaluated based on the levels of malondialdehyde (MDA), reactive oxygen species (ROS), superoxide dismutase (SOD), and catalase (CAT); **C.** Western blotting was used to detect the enrichment of proteins associated with inflammation and oxidative stress; $^{**}p < 0.01$; $^{***}p < 0.001$; ox-LDL — oxidised low-density lipoprotein; oe — overexpression; NC — negative control.

pression reduced ox-LDL-induced apoptosis (Fig. 2C, D). Moreover, ox-LDL treatment attenuated the angiogenic capacity of cells, whereas TLN1 overexpression alleviated this impact (Fig. 2E).

TLN1 in inflammation and oxidative stress

TNF-α, IL-1β, and IL-6 levels in CMVECs were increased upon ox-LDL treatment, and TLN1 overexpression reduced the impacts of ox-LDL on these

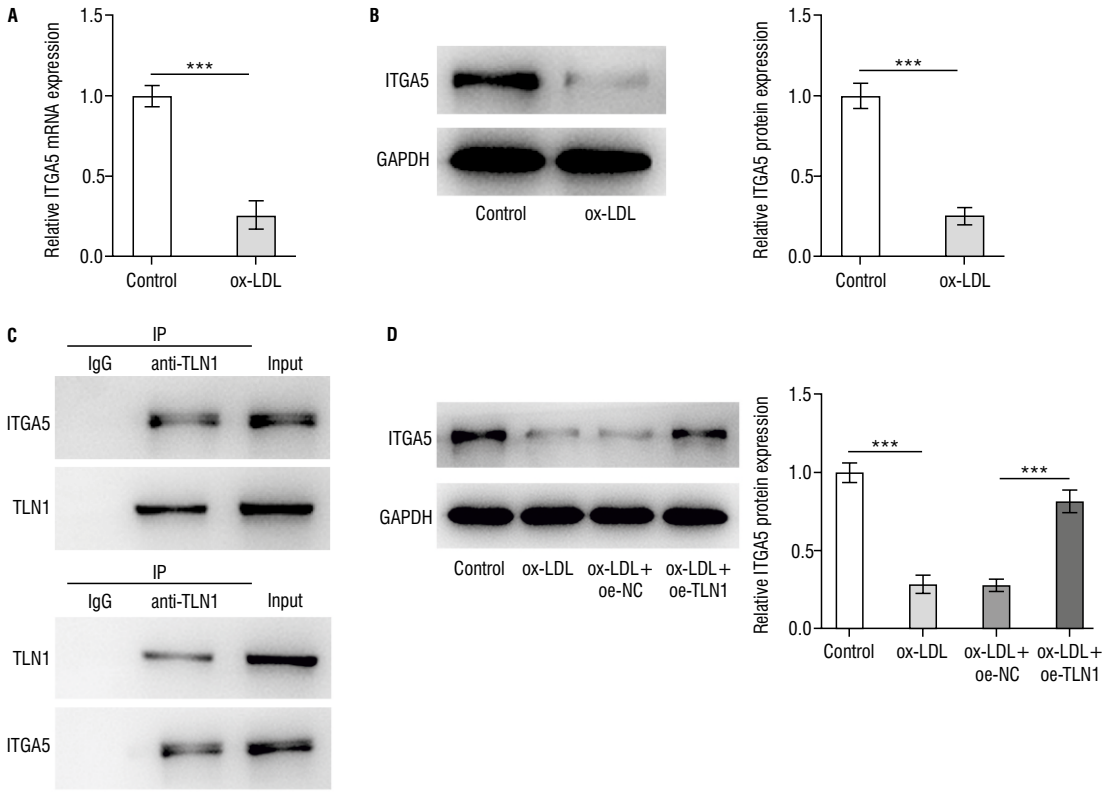

Figure 4. The interaction between talin-1 (TLN1) and integrin alpha 5 (ITGA5). Following oxidised low-density lipoprotein (ox-LDL) treatment, ITGA5 level in cardiac microvascular endothelial cells (CMVECs) was confirmed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) (**A**) and western blotting (**B**). **C.** Co-immunoprecipitation (Co-IP) assay was used to verify the interaction between TLN1 and ITGA5; **D.** The level of ITGA5 upon TLN1 overexpression was determined using western blotting; ***$p < 0.001$; oe — overexpression; NC — negative control.

factors (Fig. 3A). In terms of oxidative stress, ox-LDL treatment triggered the increase in malondialdehyde (MDA) and reactive oxygen species (ROS), accompanied by the decrease in superoxide dismutase (SOD) and catalase (CAT). TLN1 overexpression likewise reduced the alterations in these indicators resulted from ox-LDL treatment (Fig. 3B). The enrichments of proteins associated with inflammation (Cox2 and iNOS) and oxidative stress (Nox2 and Nox4) were all elevated after ox-LDL treatment, and partially fell back due to TLN1 overexpression (Fig. 3C).

The interaction between TLN1 and ITGA5

The expression level of ITGA5 was found to be declined in response to the ox-LDL treatment (Fig. 4A, B). According to Co-IP results, ITGA5 protein enrichment could be detected in TLN1 antibody-bead complexes, vice versa (Fig. 4C). Moreover, TLN1 overexpression increased the level of ITGA5 (Fig. 4D).

Modulation of ITGA5

Following ITGA5 was identified to be knocked down (Fig. 5A, B), additional ITGA5 knockdown was found to reduce cell viability and proliferation, partly reversing the effects of TLN1 overexpression (Fig. 5C, D). ITGA5 knockdown likewise promoted the apoptosis of CMVECs, along with dropped Bcl-2 and elevated Bax protein expression (Fig. 5E, G). The angiogenesis of CMVECs was weakened by the influence of ITGA5 knockdown (Fig. 5H). In addition, ITGA5 knockdown enhanced the secretion of inflammatory factors and promoted oxidative stress (Fig. 6A–C).

DISCUSSION

Atherosclerotic plaque builds up in the blood vessels that supply the heart with oxygen and nutrients [20, 33]. The complex process of atherosclerosis begins early and is thought to begin with dysfunction of coronary endothelial cells [2]. To prevent CAD,

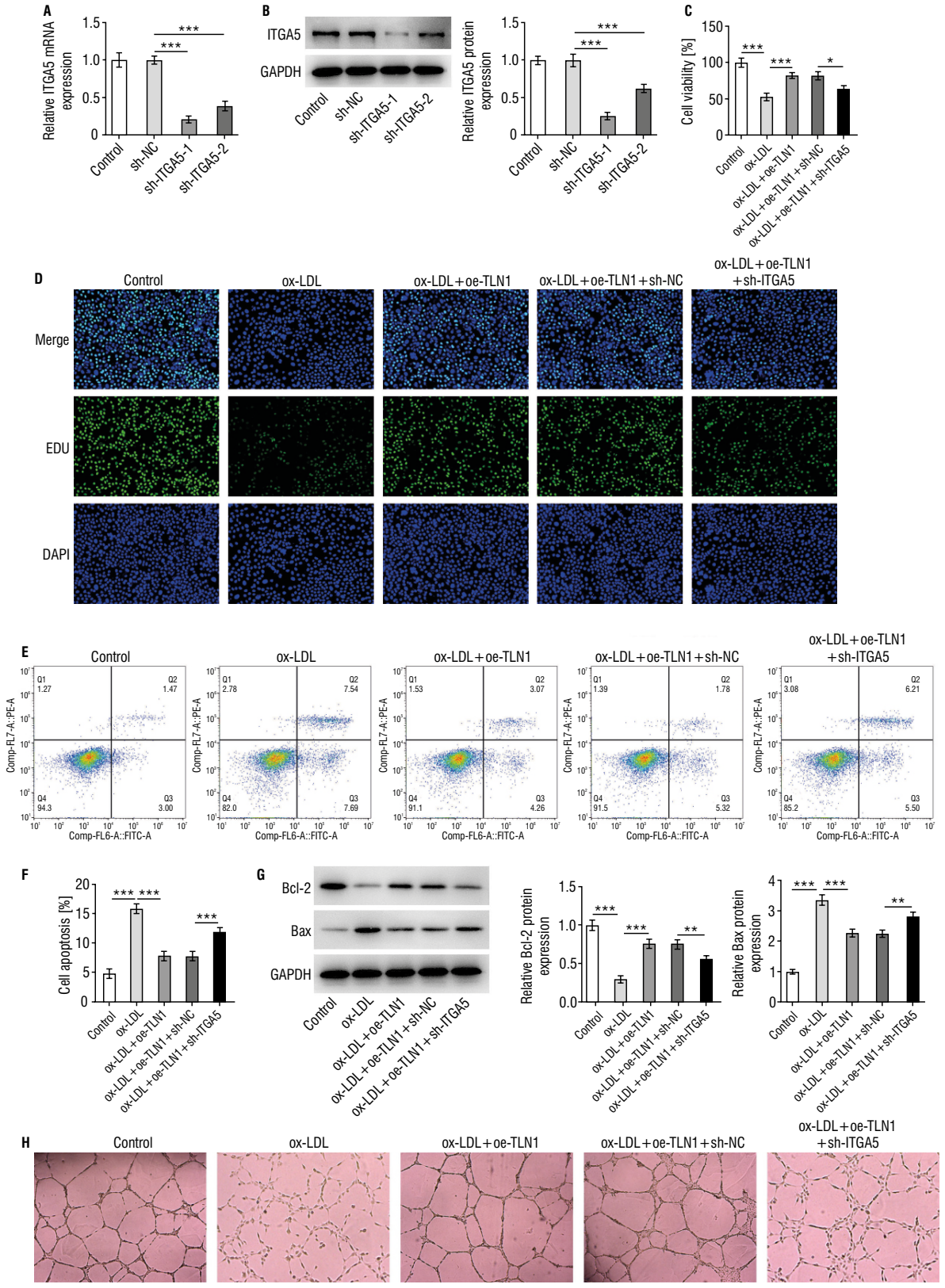

Figure 5. Modulation of integrin alpha 5 (ITGA5) in apoptosis. ITGA5 knockdown in CMVECs by transfection was confirmed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) (**A**) and western blotting (**B**); The impacts of ITGA5 knockdown on the viability and proliferation of cardiac microvascular endothelial cells (CMVECs) was assessed using cell counting Kit-8 (CCK8) (**C**) and 5-ethynyl-2′--deoxyuridine (EdU) assays (**D**); The impact of ITGA5 knockdown on the apoptosis was determined using flow cytometry (**E**, **F**) and western blotting (**G**); **H.** The impact of ITGA5 knockdown on the angiogenesis was detected. $^{**}p < 0.01$; $^{***}p < 0.001$; ox-LDL — oxidized low-density lipoprotein; sh — short hairpin RNA; oe — overexpression; NC — negative control.

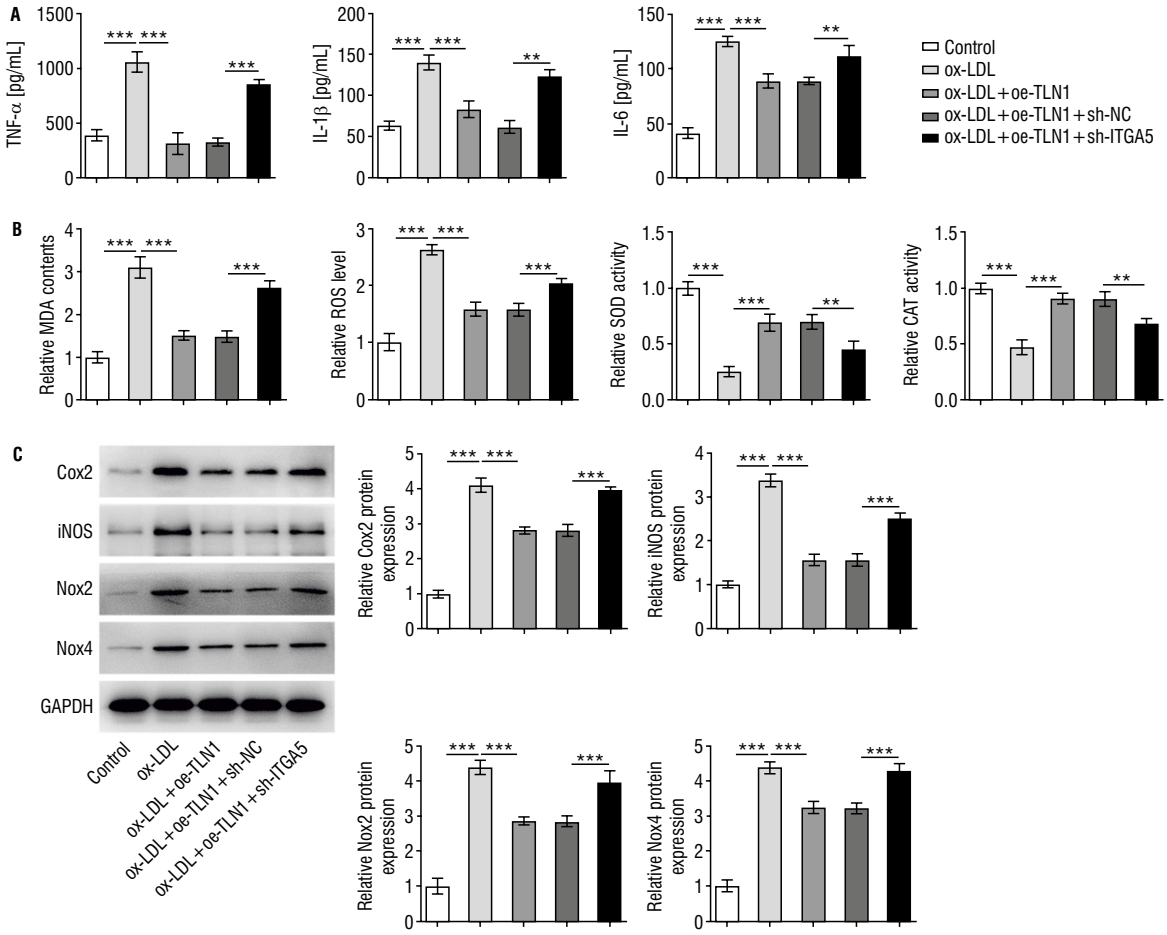

Figure 6. Modulation of integrin alpha 5 (ITGA5) in inflammation and oxidative stress; **A.** ELISA kits were used to measure cellular inflammatory factor levels; **B.** The impact of ITGA5 knockdown on oxidative stress was evaluated; **C.** The impact of ITGA5 knockdown on the enrichment of proteins associated with inflammation and oxidative stress were assessed using western blotting; $^{**}p < 0.01$; $^{***}p < 0.001$; TLN1 — talin-1; ox-LDL — oxidised low-density lipoprotein; MDA — malondialdehyde; ROS — reactive oxygen species; SOD — superoxide dismutase; CAT — catalase; sh — short hairpin RNA; oe — overexpression; NC — negative control.

the population is encouraged to adhere to a healthy lifestyle, such as not smoking, avoiding obesity, eating a healthy diet, and exercising regularly [13]. High-risk groups benefit from drugs to lower LDL, blood pressure, or prevent blood clots [8, 28]. As with most complex diseases, an individual's risk for developing CAD is modulated by the interplay between genetic and lifestyle factors [15]. In this study, TLN1 was found to positively cooperate with ITGA5 to suppress ox-LDL-induced inflammation, oxidative stress, and enhance angiogenesis in CMVECs. The involvement of TLN1 in CAD has been revealed for the first time.

The integrin family to which ITGA5 belongs is closely related to the occurrence and development of atherosclerosis [5, 16]. Alterations in integrin signalling affect multiple aspects of atherosclerosis, from the earliest induction of inflammation to the development of advanced fibrotic plaques [7]. In a previous study, the expression of miR-92a was upregulated in neointimal hyperplastic lesions after vein transplantation, and TGF-β1 induced a significant increase of microRNA-92a in human umbilical vein endothelial cells and induced endothelial-mesenchymal transition. ITGA5 is a potential target gene involved in the development of neointima formation in these vein grafts [34]. MicroRNA-92a was also elevated in LPS-induced pulmonary microvascular endothelial cells, and inhibition of miR-92a negatively regulated ITGA5 to improve LPS-induced (LPS, lipopolysaccharide) endothelial barrier dysfunction [27]. In view of the penetration of ITGA5 in the development of atherosclerosis [30], it is suggested that regulating ITGA5 can eradicate the occurrence of CAD from the early stage.

CONCLUSIONS

In summary, this study reveals for the first time that TLN1 and ITGA5 are involved in alleviating CMVECs injury, and suggests their probable involvement in CAD, and increasing their levels is beneficial to disease relief. It is hoped that the findings of the present study will provide a theoretical basis for further research.

Conflict of interest: None declared

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