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CCCTC-binding factor suppresses alpha-2-macroglobulin transcription to improve vascular endothelial cell functions in lower extremity arteriosclerosis obliterans

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Abstract. Vascular endothelial cell functions affect lower extremity arteriosclerosis obliterans (LEASO), while alpha-2-macroglobulin (A2M) and CCCTC-binding factor (CTCF) are closely related to the function of such cells. This paper aims to identify the influences of CTCF on vascular endothelial cells in LEASO by regulating A2M. A rat model of LEASO was established to measure intima-media ratio, blood lipid, and inflammatory factor levels. By constructing LEASO cell models, cell viability and apoptosis were assayed, while autophagy-related proteins, CTCF and A2M levels in femoral artery tissues and HUVECs were determined. The transcriptional regulation of CTCF on A2M was verified. In LEASO rat models, femoral artery lumen was narrowed and endothelial cells were disordered; levels of total cholesterol, IL-1, and TNF-α enhanced, and HDL-C decreased, with strong expression of A2M and low expression of CTCF. The viability of ox-LDL-treated HUVECs was decreased, together with higher apoptosis, lower LC3II/I expression, and higher p62 expression, which were reversed by sh-A2M transfection. Overexpression of CTCF inhibited A2M transcription, promoted the viability and autophagy of HUVECs, and decreased apoptosis. Collectively, CTCF improves the function of vascular endothelial cells in LEASO by inhibiting A2M transcription.

Key words: CCCTC-binding factor — Alpha-2-macroglobulin — Vascular endothelial cells — Lower extremity arteriosclerosis obliterans

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Abbreviations: A2M, alpha-2-macroglobulin; CTCF, CCCTC-binding factor; CCK-8, cell counting kit 8; ChIP, chromatin immunoprecipitation; HUVECs, human umbilical vein endothelial cells; HDL-C, high-density lipoprotein cholesterol; IL-1, interleukin-1; IGFBP5, insulin-like growth factor binding protein 5; LEASO, lower extremity arteriosclerosis obliterans; ox-LDL, oxidized low-density lipoprotein; PI, propidium iodide; TC, total cholesterol; TNF-α, tumor necrosis factor-α.

Introduction

Peripheral arterial occlusive disease is an arterial thrombosis-caused disorder of blood circulation, with the pathogenesis similar to that of atherosclerosis and a clinical sign of intermittent claudication (Yeh et al. 2022). The disease in most patients stems from atherosclerotic plaque accumulation in the lower limb arterial tree and even results in chronic limb threatening ischemia (Sucharitkul et al. 2021). Accumulation of fibrous elements and lipids in large arteries is a characteristic of lower extremity arteriosclerosis obliterans (LEASO) (Li et al. 2019). Once vessels encounter external damage, vascular endothelial injury will appear and subsequently cause proliferation and migration of smooth muscle cells, ultimately inducing luminal stenosis and vascular dysfunction (Gusev and Sarapultsev 2023). However, the explicit molecular mechanism behind LEASO is far from clear.

Alpha-2-macroglobulin (A2M) is a highly abundant protein in the peripheral blood circulation in humans and is generated mainly in the liver due to coordination between endothelial cells and hepatocytes (Yoshino et al. 2019). It has been shown to have a close association with vascular endothelial cell functions (Lehmann et al. 2022). In addition, the changes in A2M have been verified to be associated with atherosclerotic disease (Lin et al. 2022), with its mechanism unclear. With the aim to illustrate the mechanism of A2M in LEASO, we predicted possible genes interacting with A2M and found CCCTC-binding factor (CTCF) has a binding site in the promoter region of A2M. CTCT is a multifunctional insulator element that significantly affect chromatin organization (Agrawal et al. 2018). CTCF has been revealed to promote vascular development and block oxidative stress in endothelial cells (Roy et al. 2018). Other than the role in vascular endothelial cells, CTCF was found to suppress microRNA-383-5p expression by enriching in the promoter region and thereby enhancing HDAC9 expression, which lead to facilitated apoptosis in ischemic brain injury (Shen et al. 2022). These studies indicate the possible functions of CTCF in vascular endothelial cells in LEASO.

In plaque, low-density lipoprotein (LDL) cholesterol is transformed into oxidized LDL (ox-LDL) by oxidative process, and ox-LDL arouses endothelial injury and further spurs LEASO initiation and development (Cui et al. 2022). Atherosclerosis is a prevailing inflammatory disease related to endothelial dysfunction, and apoptosis and autophagy of endothelial cells greatly affect the progression of atherosclerosis (Zhang et al. 2018; Shan et al. 2021). Therefore, this study stimulated human umbilical vein endothelial cells (HUVECs) using ox-LDL to mimic LEASO initiation other than LEASO rat models, which has been validated to be effective by previous studies (Li et al. 2019; Jiang et al. 2020). This study aims to verify whether CTCF modulates A2M transcription to regulate vascular endothelial cell functions in LEASO.

Materials and Methods

LEASO rat models

Eight-week-old male Sprague Dawley rats (n = 16, 200–250 g weighing, all from Shanghai Lab. Animal Research Center) were kept at 23 ± 3°C and 40–70% humidity under a 12h/12h light/dark cycle and had free access to water and food. With the approval of the ethics committee of the First Affiliated Hospital of Fujian Medical University, animal experiments were implemented abiding by codes and operation specifications of laboratory animal management and complying with the relevant ethics requirements. After one week of feeding with high-fat diet, the left and right hind limbs of rats were anesthetized and sterilized, and the skin was incised from the groin to the place below the knee to identify the bifurcation of femoral artery at the popliteal artery. After the distal femoral artery was blocked with an artery clamp, 0.2 ~ 0.3 ml ddH_2O was infused to the blocked artery until the vessel was filling. Five minutes after the infusion, hemostasis and suture were carried out (Li et al. 2019).

Animal groups

Sixteen rats were randomly assigned into two groups: sham-operated group (sham group, n = 8) and LEASO model group (LEASO group, n = 8). Four weeks after operation, the rats were euthanatized by cervical dislocation for blood sampling after anaesthesia *via* intraperitoneal injection of pentobarbital sodium. Blood samples of tail vein (2.5 ml) were centrifuged at 4°C and 1000 × g for 15 min, with the supernatant collected and stored at –80°C. A fraction of rat femoral artery was obtained, washed with phosphate buffer

saline (PBS), and fixed at room temperature for 24 h in 4% paraformaldehyde.

Hematoxylin and eosin staining

After fixation in 4% paraformaldehyde, dehydration, clearing, paraffin penetration, and embedding, the rat femoral artery was sectioned into 4 μm-thick slices, placed in water at normal temperature for 60 min, and transferred to glass slides. At room temperature, the sections were stained in hematoxylin and eosin (Sigma, USA) for 5 min and 3 min, respectively. Visualization of the sections was carried out using an Eclipse 80i microscope (Nikon, Shanghai, China) and images were acquired and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics) to calculate the intima-media (I/M) value.

Determination of blood lipid level

An automatic serum biochemical analyzer (BK-200, BIOBASE) was utilized to detect the total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels in rat serum.

Detection of serum biochemical indices by enzyme-linked immunosorbent assay (ELISA)

Serum interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) levels were evaluated using IL-1 and TNF-α kits (Multi Sciences Biotechnology, Hangzhou, China) based on the manufacturer's instructions. The absorbance was measured and standard curve was plotted.

Cell cultures and LEASO cellular models

HUVECs (American Type Culture Collection, Manassas, VA, USA) were conventionally cultured in Dulbecco's modified Eagle medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C with 5% CO_2.

HUVECs were plated in a six-well plate (Corning, NY, USA) for establishment of LEASO cellular model. After they grew to 80% confluence, 100 μg/ml of ox-LDL (Yiyuan Biotech, Guangzhou, China) was added to the medium for 48-h incubation to induce *in vitro* atherosclerosis model (Zhu et al. 2019).

Cell transfection

HUVECs were plated in a 96-well plate and transduced with A2M interference vector (sh-A2M), its negative control (NC) (sh-NC), overexpression vectors of CTCF and A2M (oe-CTCF and oe-A2M) and its NC (oe-NC) (all from Hanbio, Shanghai, China) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as *per* the instructions. Forty-eight hours after transfection, the HUVECs were obtained for subsequent experiments.

Cell counting kit 8 (CCK-8) assay

CCK-8 (MedChemExpress, Monmouth Junction, NJ, USA) was used to determine cell viability. After cell counting on a counting plate, the HUVECs (3×10^3) were inoculated onto a 96-well plate and incubated with 10 μl CCK-8 solution *per* well at 37°C for 2 h in the dark, followed by absorbance determination at 450 nm with a microplate reader.

Flow cytometry

HUVEC apoptosis was detected using a fluorescein isothiocyanate (FITC) conjugated annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). HUVECs were detached with EDTA-free trypsin, collected, and washed three times with pre-cold PBS (Solarbio, Beijing, China). HUVECs (5×10^5) were suspended in 100 μl of binding buffer supplemented with 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) and incubated for 30 min in the dark before the addition of 400 μl of binding buffer. BD FACSCalibur (BD Biosciences, USA) instrument was utilized to determine the apoptotic rate of the stained cells.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

After total RNA isolation using RNAiso Plus (9109, Takara, Dalian, China), reverse transcription was performed for 2 μg of total RNA extracted from each sample using PrimeScrip RT Master Mix (RR036A, Takara) to convert RNA into cDNA. qRT-PCR was carried out in a fluorescence quantitative PCR instrument (ABI7500, ABI, Foster City, CA, USA) and the reaction system was as follows: SYBR Mix, 9 μl; forward primer, 0.5 μl; reverse primer, 0.5 μl; cDNA, 2 μl; RNase Free dH2O 8 μl; the reaction conditions were as follows: 95°C, 10 min; 95°C, 15 s; 60°C, 1 min; in a total of 40 cycles. Each sample had three replicates. The primers were synthesized by Shanghai Sangon Biotech (Shanghai, China) (primer sequences in Table 1). The Ct value of each well was recorded, and the relative expression of products was calculated using $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal reference.

Western blotting

Femoral arteries or HUVECs obtained from groups were lysed with protein extraction lysis buffer after washing with pre-cold PBS. Following the determination of protein concentration using the BCA kit (Thermo Fisher, Waltham, MA,

Table 1. Primer sequences used in qRT-PCR

Name of primer	Sequences (5-3)
CTCF-F (Rat)	ACGCAGATAACTGTGCTGGT
CTCF-R (Rat)	ACGCAGATAACTGTGCTGGT
CTCF-F (Human)	GAATTGGTTCGGCATCGTCG
CTCF-R (Human)	GCTGACTTCTACACTGGCGT
A2M-F (Rat)	GCTGCCATCAGACGTGGTAG
A2M-R (Rat)	AGCCATAGGGCATCTTGAGG
A2M-F (Human)	GAGGCAGAAGGACAATGGCT
A2M-R (Human)	ATAGGCGGAGAGGGTCACTT
GAPDH-F (Rat)	CCCTTAAGAGGGATGCTGCC
GAPDH-R (Rat)	ACTGTGCCGTTGAATTTGCC
GAPDH-F (Human)	TCCAAAATCAAGTGGGGCGA
GAPDH-R (Human)	AAATGAGCCCCAGCCTTCTC

F, forward; R, reverse.

USA), a corresponding volume of protein was mixed with 5×loading buffer (Sangon Biotech) and heated in a boiling water bath for 3 min to denature protein. The electrophoresis was carried out at 80 V for 30 min, and the voltage was switched to 120 V for 1–2 h of electrophoresis once bromophenol blue reached the separation gel. The protein was passed onto the membrane in an ice bath at 300 mA for 60 min. After the transferring, the membrane was washed in a washing solution for 1–2 min and blocked in a blocking solution at room temperature for 60 min or at 4°C overnight. At room temperature, the membrane was incubated at a shaking table for 1 h with anti-GAPDH (CB-100034, 1:10000, Cameback, Changsha, China), anti-CTCF (ab128873, 1:1000, Abcam), anti-A2M (ab109422, 1:1000, Abcam), anti-LC3B (ab192890, 1:2000, Abcam), and anti-SQSTM1/p62 (ab109012, 1:10000, Abcam) before washing three times with the washing solution for 10 min each, and the membrane was transferred to secondary antibody anti-IgG for 1 h of incubation at room temperature and washed three times for 10 min each. After the developing solution was added to the membrane, the chemiluminescence was detected with the chemiluminescence imaging system (Tanon, Shanghai, China).

Chromatin immunoprecipitation (ChIP)

According to the manufacturer's protocol of the ChIP kit (26156, Thermo Fisher), chromatin was cross-linked and digested with micrococcal nuclease. Anti-CTCF (#3418, CST, Beverly, MA, USA) or anti-IgG (ab182931, Abcam) was added to the reaction system for IP and the sediments were washed several times to remove nonspecific binding substance. After de-crosslinking, the DNA sample was purified and the enrichment of certain genomic region was determined using qRT-PCR. IgG served as the negative control and A2M promoter primer therein is as follows: F: 5'-GAAACAACCACCAGCCTCTG-3'; R: 5'-AACATCATGCCCTGTGAGTCAT-3'.

Luciferase reporter assay

The binding of the transcription factor CTCF and the promoter of A2M was predicted by JASPAR database and the wild sequence (wt-A2M) and designed mutated sequence (mut-A2M) of the binding site were inserted into luciferase reporter vector (pGL3-Basic) and the vectors were transfected into HEK293T cells (Qingqi (Shanghai) Biotechnology Development) with oe-CTCF or oe-NC. Forty-eight hours after the transfection, a Luciferase Assay System kit (E2920, Promega, Madison, WI, USA) was used to determine luciferase activity.

Statistics analysis

GraphPad prism7 software was applied for statistics analysis and all data were represented as mean ± standard deviation. For samples conforming to normal distribution, the data between two groups were compared using *t* test and those among multiple groups were compared using one-way analysis of variance, followed by Tukey's multiple comparisons test for *post-hoc* analysis. For sample not conforming to normal distribution, two independent samples were compared using Mann-Whitney U test and multiple independent samples were compared using Kruskal-Wallis. $p < 0.05$ is considered to have statistical significance.

Results

A2M strongly expressed in LEASO model rats

Image-Pro Plus detected the I/M value in rats, which showed an increased I/M value in the LEASO group *versus* the sham group (Fig. 1A). The detection of blood lipid levels displayed that rats in the LEASO group had substantially increased TC level and decreased HDL-C level compared to rats in the sham group (Fig. 1B,C). As ELISA revealed, the serum levels of IL-1 and TNF-α were enhanced in the LEASO group compared to in the sham group (Fig. 1D,E). The above results demonstrated the successful establishment of the LEASO rat model. The expression of A2M, detected by qRT-PCR and Western blotting, was found to be higher in the LEASO group than in the sham group (Fig. 1F,G).

Silencing A2M restores HUVEC viability and autophagy but suppressed the apoptosis

qRT-PCR and Western blotting demonstrated an increase in A2M expression in the ox-LDL group *versus* the control

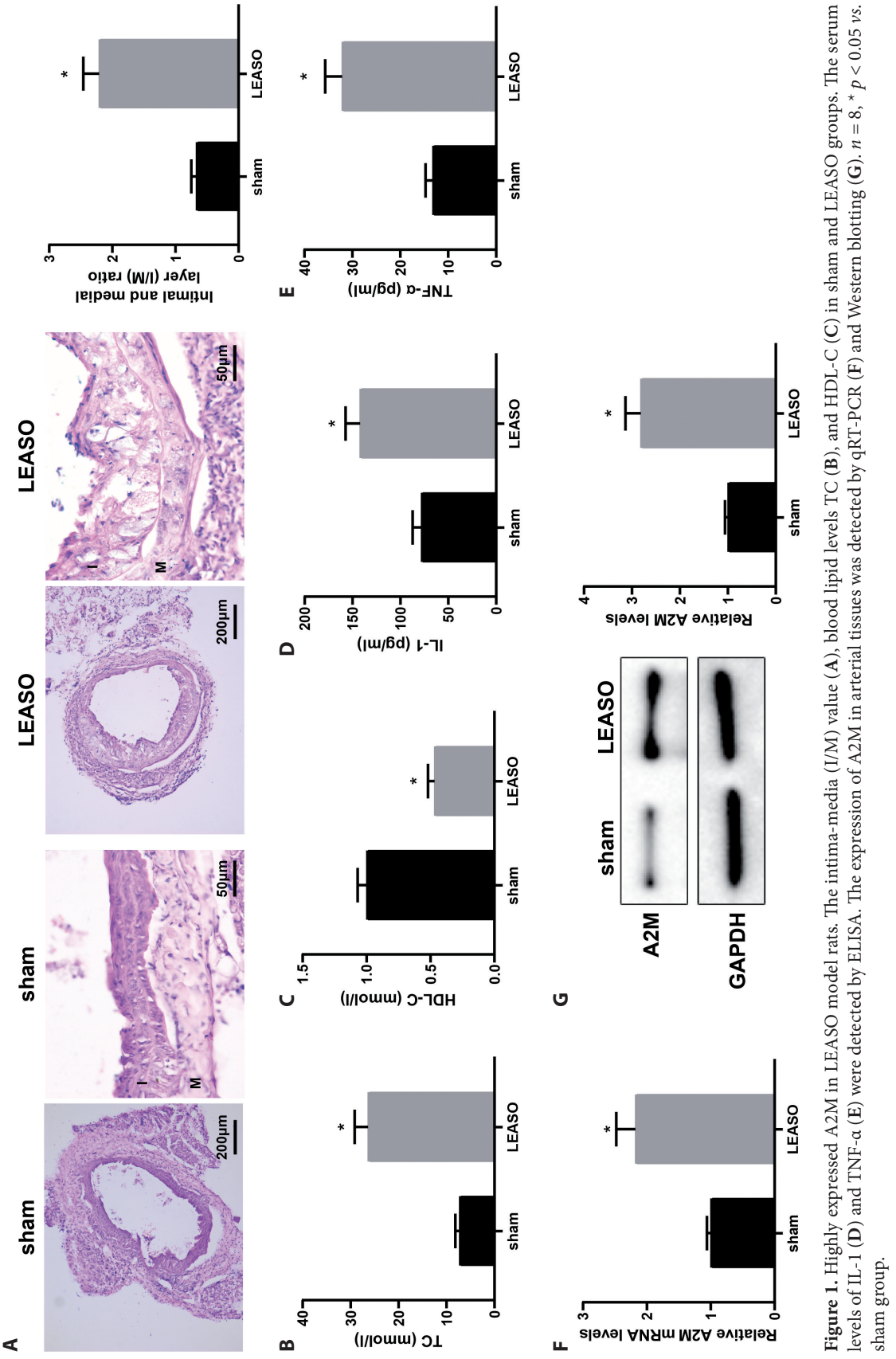

Figure 1. Highly expressed A2M in LEASO model rats. The intima-media (I/M) value (**A**), blood lipid levels TC (**B**), and HDL-C (**C**) in sham and LEASO groups. The serum levels of IL-1 (**D**) and TNF-α (**E**) were detected by ELISA. The expression of A2M in arterial tissues was detected by qRT-PCR (**F**) and Western blotting (**G**). $n = 8$, * $p < 0.05$ *vs.* sham group.

group (Fig. 2A,B). HUVECs were transfected with sh-A2M or its NC to investigate the influences of A2M on HUVEC functions. The results of qRT-PCR and Western blotting verified the successful transfection (Fig. 2A,B). The viability of HUVECs, shown by the CCK-8 assay, was suppressed in the ox-LDL group compared with the control group but restored in the ox-LDL+sh-A2M group in comparison with the ox-LDL+sh-NC group (Fig. 2C). Flow cytometry assay displayed that the apoptotic rate was elevated in the ox-LDL group in comparison with the control group and decreased in the ox-LDL+sh-A2M group compared with the ox-LDL+sh-NC group (Fig. 2D). The results of Western blotting showed downregulated LC3II/I expression and facilitated p62 expression in the ox-LDL group compared with the control group; the ox-LDL+sh-A2M group had higher LC3II/I expression and lower p62 expression than those in the ox-LDL+sh-NC group (Fig. 2E). Moreover, previous studies stated that ox-LDL can induce oxidative stress and inflammation in HUVECs (Chen et al. 2019; Jia et al. 2022). Here, we designed ELISA to test the inflammatory factors in the supernatant, and found that the ox-LDL group had higher levels of IL-1 and TNF-α than the control group, while opposite levels of these factors were noted in the ox-LDL+sh-A2M group compared with the ox-LDL+sh-NC group (Fig. 2F,G).

Overexpression of CTCF inhibits A2M transcription

qRT-PCR and Western blotting detected the expression of CTCF in rat femoral artery tissues and HUVECs. The results showed that CTCF levels in the LEASO group were lower than that in the sham group (Fig. 3A,B); in the ox-LDL group, CTCF levels were decreased compared with the control group (Fig. 3C,D). In response to the transfection of oe-CTCF into HUVECs, levels of CTCF mRNA and protein were elevated in the ox-LDL+oe-CTCF group in comparison with the ox-LDL+oe-NC group; however, A2M protein and mRNA levels were reduced (Fig. 3E.F).

Through the transcription factor JASPAR database (http://jaspar.genereg.net), we predicted the binding sites between CTCF and A2M promoter, and found that there was a binding site between the transcription factor CTCF and the A2M promoter (Fig. S1 in Supplementary materials), indicating that A2M transcription may be regulated by CTCF. To investigate whether A2M is directly controlled by CTCF, we performed ChIP to detect the binding of CTCF to A2M, and the results showed that the A2M promoter bound by the CTCF antibody was more stronger than that bound by the IgG antibody (Fig. 3G), indicating that CTCF was enriched in the transcriptional regulatory region of the A2M gene. The luciferase reporter gene assay further verified that the luciferase activity of the oe-CTCF group inserted with the mut-A2M sequence was not different from that of the oe-NC group, while the luciferase activity of the oe-CTCF group inserted with the wt-A2M sequence was lower than that in the oe-NC group (Figure 3H). Based on the above, the transcription factor CTCF can regulate the transcription of A2M gene.

CTCF improves the functions of vascular endothelial cells in LEASO by inhibiting the transcription of A2M

HUVECs treated with ox-LDL were subjected to transfection with oe-CTCF and oe-A2M to further investigate the impacts of CTCF in LEASO. Shown by qRT-PCR and Western blotting assays, CTCF expression was higher in the oe-CTCF group than in the oe-NC group; A2M expression in the oe-CTCF+oe-A2M group was higher than in the oe-CTCF+oe-NC group (Fig. 4A,B). Functionally, HUVEC viability in the oe-CTCF group was restored compared with that in the oe-NC group; the viability of ox-LDL-treated HUVECs was suppressed in response to A2M overexpression after CTCF overexpression (Fig. 4C). The apoptotic rate in the oe-CTCF group was lower than in the oe-NC group, while the rate in the oe-CTCF+oe-A2M group was increased in comparison with the oe-CTCF+oe-NC group (Fig. 4D). Besides, LC3II/I showed an increase in the oe-CTCF group *versus* the oe-NC group and a decrease in the oe-CTCF+oe-A2M group *versus* the oe-CTCF+oe-NC group, and p62 displayed a decrease in the oe-CTCF group compared with the oe-NC group and an elevation in the oe-CTCF+oe-A2M group compared with the oe-CTCF+oe-NC group (Fig. 4E). As for the results from ELISA, the levels of IL-1 and TNF-α were markedly decreased in the oe-CTCF group in comparison with the oe-NC group, and were elevated by further oe-A2M transfection (Fig. 4F,G). Overall, overexpression of CTCF can promote the viability and autophagy of HUVECs, and reduce the apoptosis and inflammatory response, and on this basis, overexpression of A2M can reverse these effects.

Discussion

LEASO is the stem of peripheral artery disease that is related to atherosclerosis of other vascular beds (Barnes et al. 2020). In this study, a LEASO rat model was established in which A2M was highly abundant in femoral arteries and CTCF was modestly expressed. In ox-LDL-induced HUVECs, CTCF was shown to suppress A2M transcription to alleviate vascular endothelial cell functions in LEASO.

Atherosclerotic occlusion is involved in endothelial cell dysfunction and migration, thrombus formation, and vascular remodeling with resultant local inflammation and cytokine release (Qi et al. 2021). The high-fat diet-induced LEASO rats showed high levels of TC, IL-1, and TNF-α,

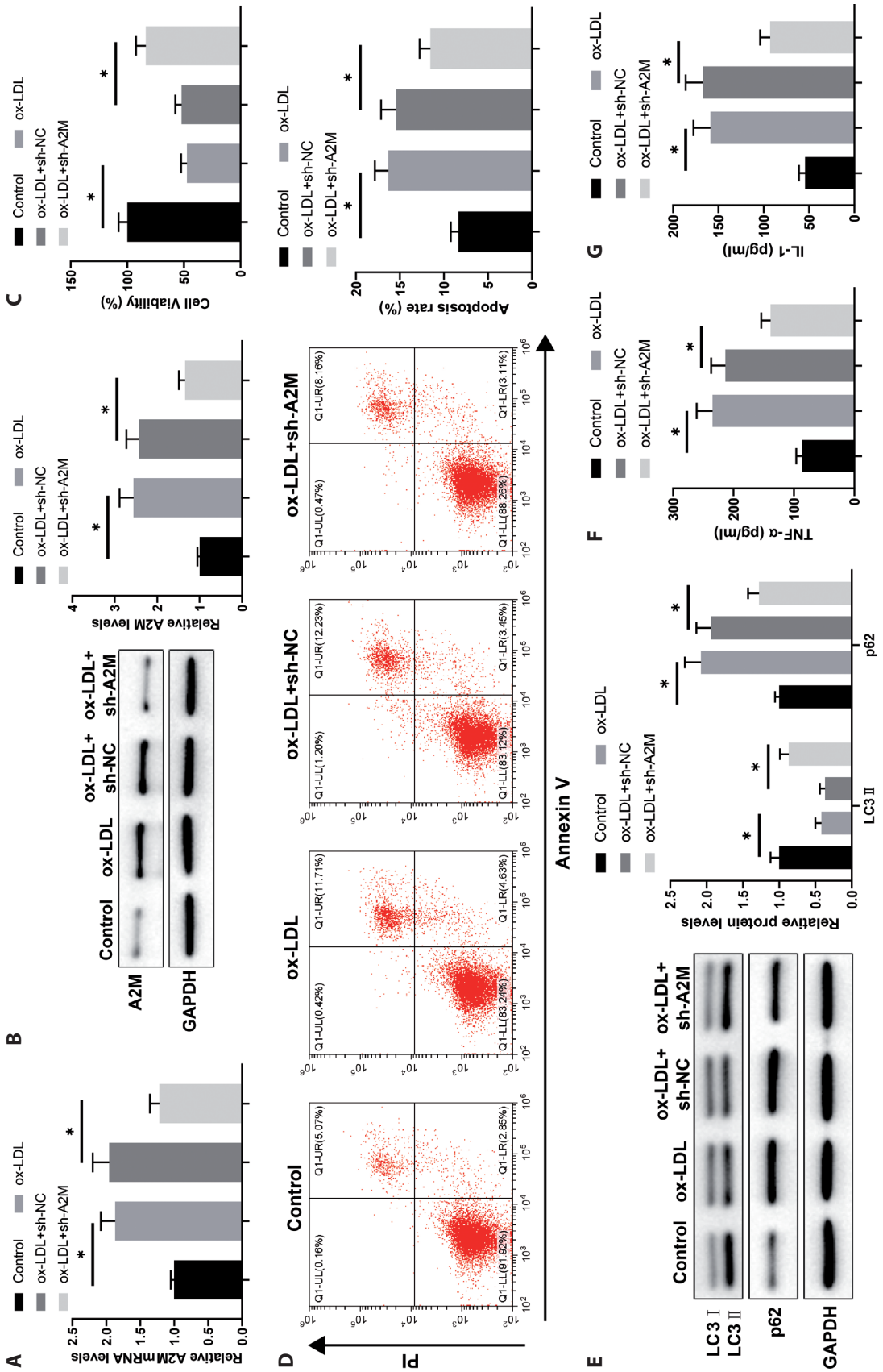

Figure 2. Silencing A2M facilitates HUVEC viability and autophagy and blocks the apoptosis. The level of A2M in HUVECs was detected by qRT-PCR (**A**) and Western blotting (**B**). **C.** The viability of HUVECs determined using CCK-8 assay. **D.** The apoptotic rate of HUVECs evaluated by flow cytometry. **E.** The levels of autophagy-related proteins measured by Western blotting. The levels of IL-1 (**F**) and TNF-α (**G**) in the supernatant were tested by ELISA. * $p < 0.05$ *vs.* control or ox-LDL+sh-NC group. Each experiment had three replicates.

and low level of HDL-C, as well as high A2M expression. Excessive apoptosis of endothelial cells is preliminary in atherosclerosis, and inhibiting apoptosis may prevent and treat atherosclerosis (Duan et al. 2021). Molecular endothelial signatures identified that A2M is a major downstream regulator of endothelial cell phenotype (Menon et al. 2020). A2M is synthesized in response to simulation of pro-inflammatory cytokines and elevates in the process of chronic or acute inflammatory diseases; apart from inflammation, it can trap many proteinases related to coagulation and fibrinolysis (Lagrange et al. 2022). Previously, serum A2M level was considered to indicate endothelial dysfunction assessed by flow-mediated dilation in patients with chronic stroke or cardiovascular risk factors (Shimomura

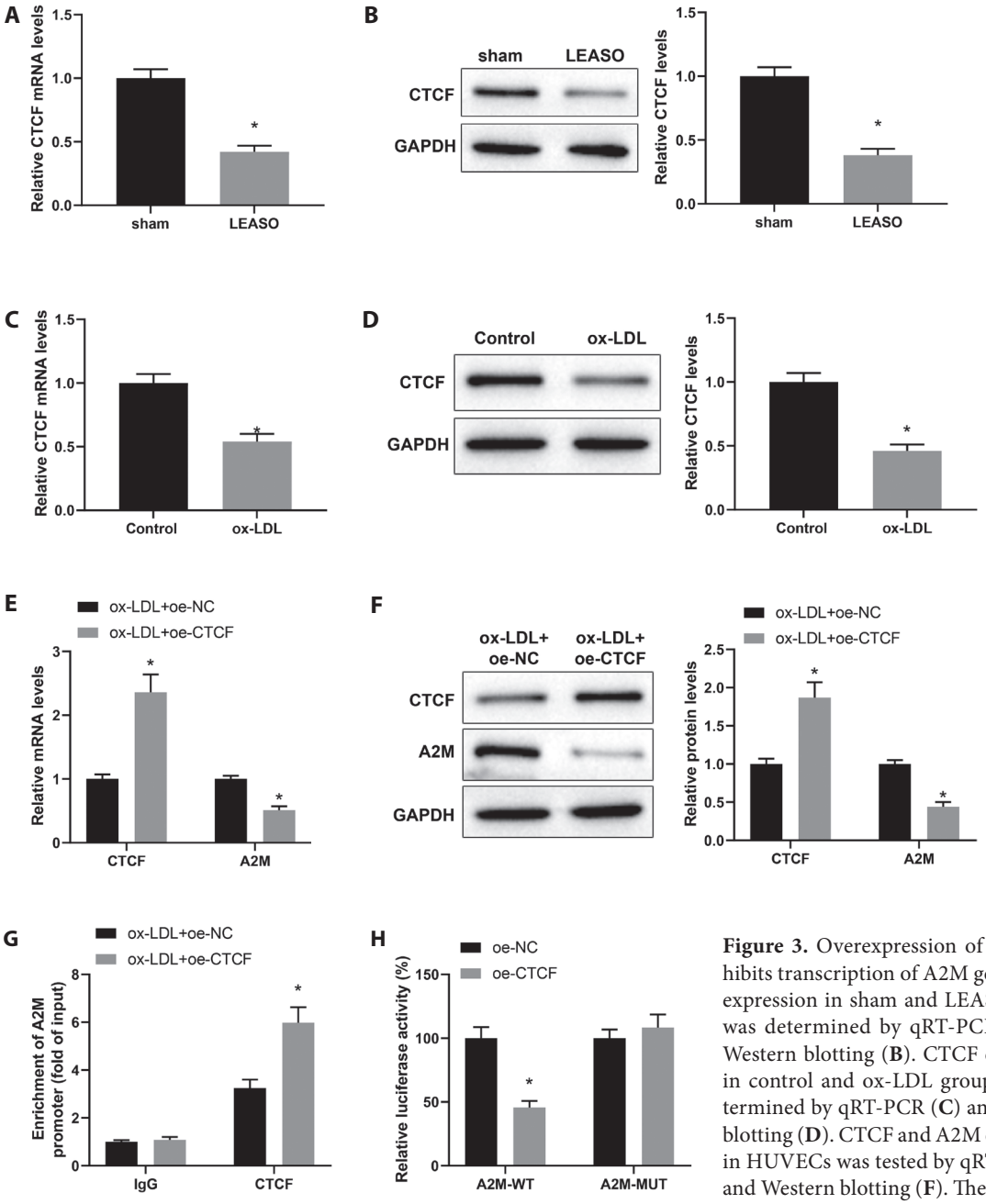

Figure 3. Overexpression of CTCF inhibits transcription of A2M gene. CTCF expression in sham and LEASO groups was determined by qRT-PCR (**A**) and Western blotting (**B**). CTCF expression in control and ox-LDL groups was determined by qRT-PCR (**C**) and Western blotting (**D**). CTCF and A2M expression in HUVECs was tested by qRT-PCR (**E**) and Western blotting (**F**). The transcriptional regulation of CTCF on A2M was evaluated by ChIP (**G**) and luciferase reporter gene assays (**H**). * $p < 0.05$ *vs.* sham group, control group, ox-LDL+oe-NC group, or oe-NC group. The experiment was set up with 3 independent repetitions.

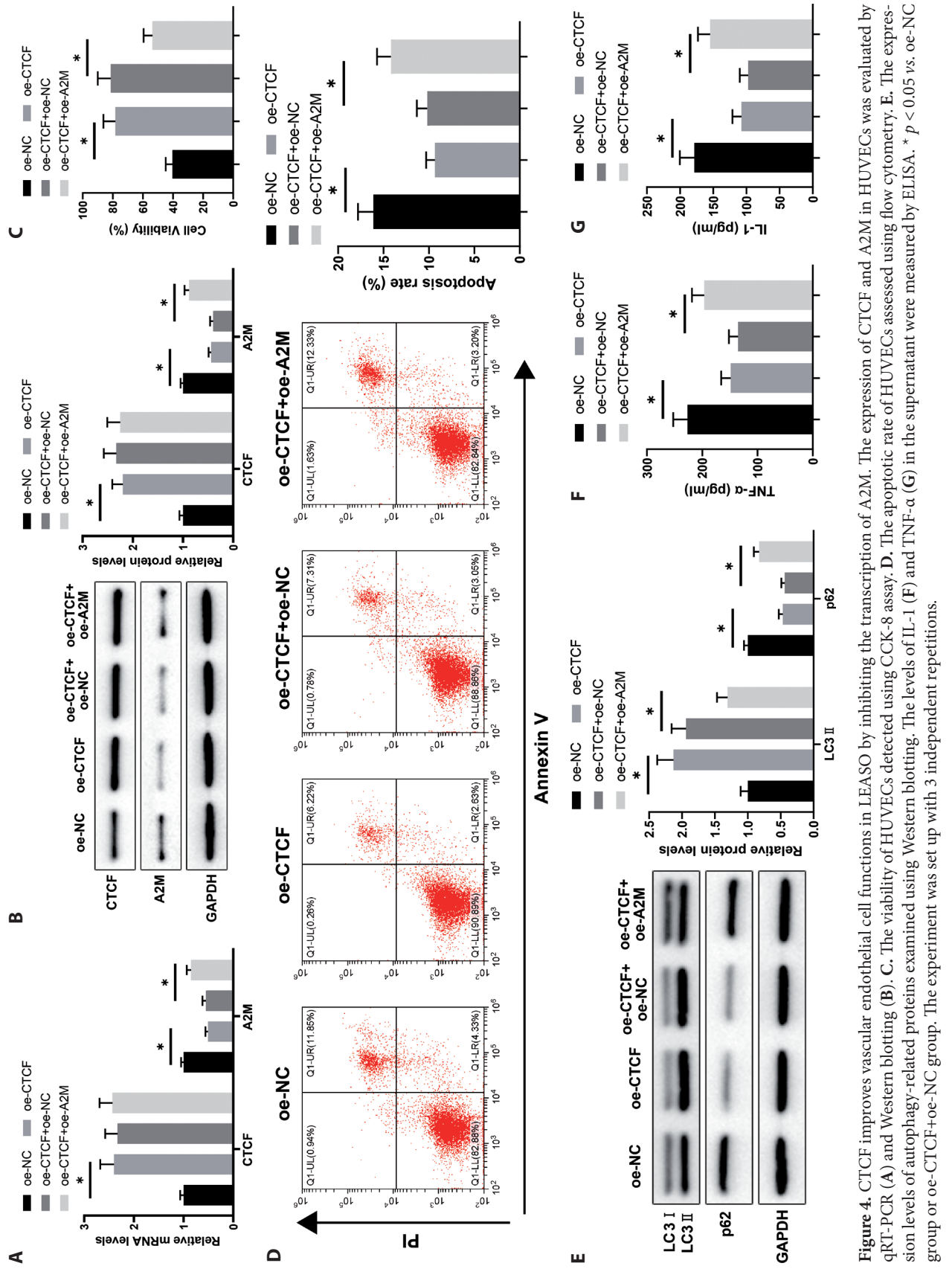

Figure 4. CTCF improves vascular endothelial cell functions in LEASO by inhibiting the transcription of A2M. The expression of CTCF and A2M in HUVECs was evaluated by qRT-PCR (**A**) and Western blotting (**B**). **C.** The viability of HUVECs detected using CCK-8 assay. **D.** The apoptotic rate of HUVECs assessed using flow cytometry. **E.** The expression levels of autophagy-related proteins examined using Western blotting. The levels of IL-1 (**F**) and TNF-α (**G**) in the supernatant were measured by ELISA. * $p < 0.05$ *vs.* oe-NC group or oe-CTCF+oe-NC group. The experiment was set up with 3 independent repetitions.

et al. 2018). Additionally, A2M appears to modulate the release of proangiogenic microvesicles by binding to its main receptor LDL receptor-related protein 1 (Laberge et al. 2021). Herein, our findings revealed that silencing of A2M restored the viability and autophagy of ox-LDL-stimulated HUVECs, and repressed the apoptosis and inflammation. Based on previous findings, A2M not only regulates the functions of vascular endothelial cells but may also affect the accumulation of fibrous proteins and lipids. The influences of A2M in LEASO require more extensive investigations to obtain a more intensive understanding of A2M in the disease.

In this work, CTCF was demonstrated as a transcription factor of A2M. CTCF was at a low level in ox-LDL-induced HUVEC and CTCF could inhibit A2M transcription by enriching in the promoter region. CTCF overexpression alone suppressed ox-LDL-induced HUVEC apoptosis and enhanced the viability and autophagy; however, CTCF overexpression-induced changes in HUVEC functions were reversed by A2M overexpression. Similarly, CTCF activates the FXN promoter, so as to restore FXN level in damaged endothelial cells, thereby improving vascular development and limiting oxidative stress (Roy et al. 2018). CTCF facilitated H3K27me3 modification in the promoter region of insulin-like growth factor binding protein 5 (IGFBP5) by recruiting EZH2, leading to downregulation of IGFBP5 and alleviating colonic mucosal epithelial cell injury in ulcerative colitis (Gu et al. 2023). In combination with previous studies and our findings, a conclusion was drawn that CTCF also has anti-apoptotic roles in endothelial cells. Concerning atherosclerosis, CTCF was revealed to have a binding site in the CDKN28 promoter region and CDKN28-AS1 promoted the formation of atherosclerotic plaques by recruiting CTCF and EZH2 to the CDKN28 promoter (Murdoch and Showman 1988). This study seems to indicate a promoting effect of CTCF in plaque formation. At the same time, CTCF binding sites have been believed to be highly mutated across cancers, especially when they are within loop anchor points (Kaiser and Semple 2018). The mutation of CTCF binding sites may be an explanation for the difference between the results of our study and that of previous study. In addition, depletion of CTCF in a liver-specific manner triggered hepatic steatosis and inflammation (Choi et al. 2021). The stress-induced apoptotic response in cells requires downregulation of CTCF, suggesting that CTCF may be an important downstream target of stress-induced signaling pathways (Li and Lu 2007). The anti-inflammation effects of CTCF and the possible pathways should be explored in our future studies.

This study mainly focused on the interaction between CTCF and A2M and their effects on HUVEC functions. However, the interpretation of the results shall be cautious due to the limitations of the present study. For instances, the regulatory effects of A2M and CTCF on vascular endothelial cell functions are validated only in the ox-LDL-induced cellular model. LEASO development *in vivo* cannot be completely mimicked by the cellular model. The specific mechanism of CTCF-mediated transcription inhibition of A2M should be explored, for example, whether CTCF recruits a certain gene to modify the methylation of the A2M promoter. The current work verified that CTCF transcriptionally suppressed A2M expression, thus suppressing the apoptosis of HUVECs and restoring their viability. Overall, this study helps to deepen the understanding behind LEASO initiation and progression and provides interesting targets for treating the disease.

Conflicts of interests. The authors report no relationships that could be construed as a conflict of interest.

Author contribution. TH, MY, PC and SF conceived the ideas. TH, MY and HZ designed the experiments. TH, MY and HZ performed the experiments, analyzed the data, provided critical materials, and wrote the manuscript. PC and SF supervised the study. All the authors have read and approved the final version for publication.

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Availability of data and materials. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate. With the approval of the ethics committee of the First Affiliated Hospital of Fujian Medical University, animal experiments were implemented abiding by codes and operation specifications of laboratory animal management and complying with the relevant ethics requirements.

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Supplementary Material

CCCTC-binding factor suppresses alpha-2-macroglobulin transcription to improve vascular endothelial cell functions in lower extremity arteriosclerosis obliterans

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Supplementary Figure

Matrix ID	Name	Score	Relative score	Sequence ID	Start	End	Strand	Predicted sequence
MA0531.1	MA0531.1.CTCF	6.2267017	0.8160075364603584	NC_000012.12:c9118229-9116129	593	607	-	TGGGAAGATGGAGTA
MA0531.1	MA0531.1.CTCF	5.83319	0.810903169766731	NC_000012.12:c9118229-9116129	75	89	+	GAAACAGATGTTTCC
MA0531.1	MA0531.1.CTCF	5.346674	0.8045924155882906	NC_000012.12:c9118229-9116129	2021	2035	+	ACCATAAAAGGCGTT

Figure S1. JASPAR database prediction for the binding site of CTCF and A2M gene promoter.