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The proatherosclerotic function of BCAT1 in atherosclerosis development of aged-apolipoprotein E-deficient mice

Lili Tan [a, *, 1], Jie Lu [b, 1], Chunyang Zhang [a], Liang Meng [c], Qi Zhu [a]

[a] Department of Emergency Medicine, The Central Hospital Affiliated to Shenyang Medical College, Shenyang, Liaoning, People's Republic of China
[b] Department of Cardiology, The Fourth People's Hospital of Shenyang, Shenyang, Liaoning, People's Republic of China
[c] Department of Cardiology, The Central Hospital Affiliated to Shenyang Medical College, Shenyang, Liaoning, People's Republic of China

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ABSTRACT

Atherosclerosis (AS) is an inflammatory vascular disease. Branched-chain amino acid transaminase 1 (BCAT1) has been implicated in inflammatory diseases, while its role in AS is unclear yet. In ApoE$^{-/-}$ mice with a high fat diet (HDF), BCAT1 was highly up-regulated and more pronounced in aged than in young ApoE$^{-/-}$ mice, which was abundantly expressed in macrophages located in AS lesions. The function of BCAT1 in AS was explored using lentivirus-mediated BCAT1 overexpression. ApoE$^{-/-}$ mice fed a HFD with BCAT1 overexpression exhibited the worsening lipid deposition and pathological injury of aortic tissues, accompanied by aggravated hyperlipidemia as proved by increased serum triglyceride, total cholesterol, and low-density lipoprotein-cholesterol levels. Immunohistochemical staining of vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), and CD68 in the aortic root plaque suggested that BCAT1 overexpression could induce monocyte-endothelial cell adhesion and macrophages infiltration, thereby contributing inflammatory response by promoting TNF-α, IL-6, and IL-1β expression. Further, *in vivo* experiments, lipid accumulation, and inflammatory response induced by oxidized-LDL in RAW267.4 cells were also intensified or alleviated by BCAT1 overexpression or knockdown. Finally, BCAT1 overexpression aggravated AS development. These adverse effects of BCAT1 on hyperlipidemia, lipid accumulation, foaming cell formation, and inflammation suggested that the modulation of BCAT1 might be a potential approach to prevent AS disease.

1. Introduction

Atherosclerosis (AS), a typical inflammatory vascular disease around worldwide, is the leading cause of myocardial infarction, stroke, coronary heart disease, and peripheral disease [1]. It is mainly characterized by abnormal lipid metabolism, chronic inflammation and AS plaques formation [2]. At present, AS-associated cardiovascular diseases are still the major cause of mortality worldwide [3]. There is growing evidence that age is a vital risk factor for atherosclerosis development, and that aging promotes atherosclerosis [4]. The prevalence of coronary heart disease and cardiovascular disease in the 40- to 59-year-old age group was 10 times and 4 times higher, respectively than in the 20- to 39-year-old age group, with a linear increase in the elderly population [5]. The treatment of AS has been well developed, mainly centering on pharmacological treatments such as statins [6], PCSK9 inhibitors [7], and surgery in severe cases [8]. Given the unsatisfactory treatment outcome, a prime task to improve treatment is to explore the molecular pathogenesis and find more effective treatment targets for AS treatment. The pathogenesis of AS is multifaceted, including oxidative stress, inflammation, disturbed protein homeostasis, endothelial dysfunction and so on, while inflammation has been proved to be a key component in each stage of atherosclerosis [9]. Inflammation could accelerate AS through inflammatory cell infiltration of the vascular wall and endothelial dysfunction (ED) [10], in which macrophages are key players in coordinating the inflammatory response [11]. Multitudes of molecules are involved in the pathological transformation of macrophages during AS. The complex signaling network beneath macrophage inflammation in the context of AS is far from clear.

Branched-chain amino acid transaminase 1 (BCAT1) is the major isomer of BCAT and initiates the catabolism of branched-chain

* Corresponding author. Department of Emergency Medicine, The Central Hospital Affiliated to Shenyang Medical College, No. 5, Nanqi West Road, Tiexi District, Shenyang, Liaoning, People's Republic of China.
E-mail address: tanlili123@163.com (L. Tan).
[1] The authors contributed equally to this work.

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amino acids (BCAAs) [12]. Current studies on BCAT1 have mostly focused on its activity in tumor growth. Zhou et al. [13] reported the higher levels in nasopharyngeal carcinoma, and subsequently published studies showed the central effect of BCAT1 in tumor pathogenesis in ovarian cancer, breast cancer and glioblastomas [14–16]. A recent literature survey focused on the BCAT1 effect on inflammatory disease [12], and reported that BCAT1 had an important regulatory effect on chronic inflammatory disease largely via macrophages. In the patients with non-alcoholic fatty liver disease, BCAT1 was highly expressed and implicated in steatosis and disorder of lipid metabolism [17], while disorders of lipid metabolism are thought to be the key triggers for the development and progression of AS [18]. In addition, in the literature, BCAT1 was reported to be involved in the pathological changes that occur in pulmonary artery smooth muscle cells in a hypoxic environment through the regulation of autophagy [19]. The abnormal proliferation and migration of vascular smooth muscle cells have been implicated in intimal hyperplasia and further facilitate the progression of AS [20]. Inspired by the above studies, we may speculate that BCAT1 might be involved in the pathological mechanism regulating the development of AS. So far, no experimental evidence has been reported to support the above speculation.

Therefore, in this project, we aim to investigate the role of BCAT1 using a model of ApoE knockout (ApoE$^{-/-}$) mice fed with a high fat diet (HFD) and RAW264.7 cells stimulated by oxidized-low-density lipoprotein (ox-LDL), and offer possible valuable therapeutic targets for AS.

2. Materials and methods

2.1. Animals

The ApoE$^{-/-}$ mice (C57BL/6, male) at 8 weeks or 52 weeks of age were purchased from Skbex Biotechnology (Henan, China) and allowed to acclimate at the animal facility for a week prior to experiments. All mice were given libitum assess to standard rodent chow and water, and housed in a facility with sentinel monitoring at standard temperature (21–23 °C) and humidity (45–55%) on a 12/12 h light/dark cycle. All animal study protocols were approved by the Ethics Committee of Shenyang Medical College. For atherosclerotic lesion formation experiments, the ApoE$^{-/-}$ mice were fed with HFD (Harlan Teklad TD.88137: 21% fat and 0.2% cholesterol) or normal chow diet for 12 weeks [21] and then sacrificed. The aortic root and aorta were isolated, and the serum was obtained from blood samples.

One week before AS modeling, the ApoE$^{-/-}$ mice at 8 weeks of age were injected with negative control lentivirus or BCAT1 lentivirus via tail vein with a total lentivector dose of 1×10^8 TU/mL per mouse. Subsequent AS modeling was conducted as mentioned above. The lentivirus injection was conducted once a month [2,3]. After 12 weeks, the mice were sacrificed and the aortic root, aorta, and serum were obtained.

2.2. Pathology observation

The pathology of AS lesions was observed using hematoxylin and eosin (H&E) and oil red O staining. Dehydrated aortic root tissue was fixed in optimal cutting temperature (OCT) embedding agent prior to H&E staining. The blocks were cut into 10 μm thick, and then sections were subjected to H&E as well as oil red O (Sigma) staining as described [22]. The pathological changes were visualized using a light microscope (Olympus, Japan).

2.3. Immunofluorescence (IF) and immunohistochemistry (IHC)

Aortic root tissues were frozen in OTC, and cut into 10 μm sections, followed by 4% paraformaldehyde fixing. The expression of CD68, monocyte chemoattractant protein-1 (MCP-1), and vascular cell adhesion molecule-1 (VCAM-1) in aortic root was accessed by IHC. The 3% hydrogen peroxide (Sinopharm, China) and 1% BSA solution were utilized to repair antigen and block non-specific antigen. The sections were incubated with primary and secondary antibodies. 3, 3′-diaminobenzidine (DAB) (Maxinm Biotech, China) and hematoxylin were used for visualization and observed under a microscope. For VCAM-1, the primary antibody anti-VCAM-1 (ABclonal, China, CAS. A0279) and secondary antibody HRP-conjugated goat-anti rabbit IgG (Thermo Scientific, USA, CAS. #31460) were used. For MCP-1, the primary antibody anti-MCP-1 (ABclonal, China, CAS. A7277) and secondary antibody HRP-conjugated goat-anti-rabbit IgG (Thermo Scientific, USA, CAS. #31460). For CD68, the primary antibody anti-CD68 (Santa Cruz, USA, CAS. SC-17832) and secondary antibody HRP-conjugated goat-anti-mouse IgG (Thermo Scientific, USA, CAS. #31430). All primary antibodies were at 1:100 dilution and secondary antibodies were at 1:500 dilution. The staining slides were observed and photographed under microscopy.

The 10 μm aortic root tissues sections were blocked in 1% BSA solution (Sangon, China) for 15 min before incubation with primary antibody solution containing anti-BCAT1 (Proteintech Group, Inc., China) and anti-CD68 for double-staining, and labeled with secondary antibody solution containing Cy3-conjugated anti-rabbit IgG (Invitrogen, USA) and FITC-conjugated anti-mouse IgG (Abcam, UK). The cell climbing slides were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 (Beyotime, China) for 30 min. The slides were blocked in 1% BSA solution (Sangon, China) for 15 min before incubation with primary antibody solution containing anti-BCAT1 (Proteintech Group, Inc., China), and labeled with secondary antibody solution containing Cy3-conjugated anti-rabbit IgG (Invitrogen, USA). The slides were washed 3 times in PBS solution, and counterstained with DAPI for nucleus staining. The fluorescence of cell images was captured using microscopy.

2.4. Cell culture and experiment design

RAW264.7 were purchased from iCell Bioscience Inc. (China) and maintained in DMEM medium (Procell, China) at 37 °C with 5% CO_2. RAW264.7 cells were stimulated with ox-LDL to establish an atherosclerotic cell model. Cells were stimulated with 0, 25, 50, or 100 μg/mL ox-LDL (Peking Union-Biology Co. Ltd., China) for 24 h [23] to measure the BCAT1 expression.

RAW264.7 cells were seeded in 24-well plates and co-transfected with lentiviral particles for BCAT1 overexpression, small interfering (si) BCAT1 or their negative control by Lipofectamine 3000 at a multiplicity of infection (MOI) of 50 for 48 h. Subsequently, the transfected cells were stimulated with 50 ng/mL ox-LDL for 24 h for further experiments. The lipid accumulation in RAW267.4 cells under different processing was detected by oil red O staining. The cells were fixed in ORO fixative for 15–25 min, stained with ORO dye solution (Leagene Biotechnology, China) for 15 min, and then photographed through a microscope. The red staining indicated lipid accumulation. The quantification of ORO staining was accessed using a microplate reader at an optical density of 490 nm with isopropanol-extracted ORO dye.

2.5. Western blot

Whole-cell lysates were prepared in RIPA buffer (Solarbio, China) plus 1 mM PMSF (Solarbio, China) as protease inhibitor on ice, and the total protein concentration was quantified using a BCA kit (Solarbio, China) after centrifugation at 10000g for 5 min. The samples were denatured by boiling in 6 × loading buffer and subjected to 12% polyacrylamide gel (Solarbio, China). Gels were transferred onto the PVDF membrane (Millipore, USA) for blotting. The membranes were blocked in 5% skim milk powder (Sangon, China) and subsequently incubated with primary antibodies (anti-BCAT1, Proteintech, China, CAS. 13640-1-AP; anti-GAPDH, Proteintech, China, CAS. 60004-1-Ig) overnight on a shaker. HRP-conjugated secondary antibodies (goat-anti-mouse IgG, Proteintech, China, CAS. SA00001-1; goat-anti-rabbit IgG, Proteintech, China, CAS. SA00001-2) and ECL kit (Solarbio, China) were used for protein band detection.

2.6. RT-qPCR

Aorta tissues and *in vitro* cultured RAW264.7 cells were subjected to RNA extraction using TRIpure reagent (BioTeke, China) together with chloroform extraction approach. Reverse transcription was processed in the presence of BeyoRT II M-MLV Reverse Transcriptase (Beyotime, China). Predesigned PCR primers for BCAT1, TNF-α, IL-6, and IL-1β were used, and the relative mRNA expression was evaluated using SYBR Green Master Mix (Solarbio, China) on Exicycler 96 (Bioneer, Korea). Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. Primer sequences: BCAT1 (forward: 5′-TAA CTG AAG TAG GCA CAA TGA A-3′; reverse: 5′-GAG ACT GGG CAG ACA ACG-3′); TNF-α (forward: 5′-CAG GCG GTG CCT ATG TCT CA-3′; reverse: 5′-GCT CCT CCA CTT GGT GGT TT-3′); IL-6 (forward: 5′-ATG GCA ATT CTG ATT GTA TG-3′; 5′-GAC TCT GGC TTT GTC TTT CT-3′); IL-1β (forward: 5′-CTC AAC TGT GAA ATG CCA CC-3′; 5′-GAG TGA TAC TGC CTG CCT GA-3′).

2.7. Commercial kit detection

Triglyceride (TG), Total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) levels in serum were detected using the commercial kit (Jiancheng Bioengineering Institute, China), respectively. The TNF-α, IL-6, and IL-1β levels in cell supernatant were detected using the corresponding ELISA kit (MultiSciences Biotech, China). All procedures were conducted as per manufacturers' instruction unless otherwise noted.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. One-way ANOVA analysis was used for statistical analysis. Each experiment was repeated at least 3 times. *P* value below 0.05 was regarded as significant.

3. Results

3.1. BCAT1 expression in young vs. old ApoE$^{-/-}$ mice with atherosclerotic lesions

In view of the fact that age is usually an independent risk factor for cardiovascular disease and that inflammation usually increases with age, we evaluated the BCAT1 expression in the young (8 weeks) and old (52 weeks) ApoE$^{-/-}$ mice. Higher BCAT1 levels were found in aged ApoE$^{-/-}$ mice under HFD compared with normal chow diet, as well as in aged ApoE$^{-/-}$ mice versus young ApoE$^{-/-}$ mice (Fig. 1a and b). Under normal dietary conditions, BCAT1 gene and protein levels in aortic tissue had no significant change in aged mice compared to young mice (Fig. 1a and b). Under HFD conditions, a significant increase in BCAT1 gene and protein levels in the aortic tissues of ApoE$^{-/-}$ mice was observed in aged mice compared to young ApoE−/− mice (Fig. 1a and b). A similar trend was observed in IF results (Fig. 1c). IF staining of the aortic tissues showed a strong immunoreactivity of BCAT1 in the AS lesion of HFD-induced aged ApoE$^{-/-}$ mice than in the d young ApoE$^{-/-}$ mice. Besides, the increased BCAT1 expression was noticed to be mainly localized in the lesion-infiltrating macrophages, which was authenticated by CD68 staining (Fig. 1c).

3.2. BCAT1 overexpression aggravated atherosclerosis development in ApoE$^{-/-}$ mice

To further understand the involvement of upregulated BCAT1 in the AS progression, the BCAT1 expression was overexpressed by lentivirus vector in ApoE$^{-/-}$ mice and fed with normal diet or HFD for 12 weeks starting at 8 weeks of age. TC, TG, LDL-C, and HDL-C are typical clinical atherogenic factors. The results revealed that supplement with HFD significantly increased the concentrations of these factors in serum compared to normal administration (Fig. 2a), and these increases were further exacerbated by LV-BCAT1 injection except HDL-C levels (Fig. 2a). Correspondingly, the aortic tissues and aortic root tissues from the HFD group exhibited significant increases in lipid content in plaques relative to the normal group (Fig. 2b and c) as visualized by oil red O staining. Furthermore, LV-BCAT1 treatment resulted in lipid formation in AS plaque more obvious and densely packed in ApoE$^{-/-}$ mice than in vector expressing ApoE$^{-/-}$ mice (Fig. 2b and c). H&E staining results showed the pronounced arterial wall thickening and atherosclerotic lesions in overexpressed-BCAT1 ApoE$^{-/-}$ mice relative to control mice (Fig. 2d). Collectively, these results demonstrated that BCAT1 overexpression aggravated AS lesions in ApoE$^{-/-}$ mice.

3.3. BCAT1 overexpression aggravated inflammation response in ApoE$^{-/-}$ mice

Inflammatory mediators have been implicated in AS progression [24]. VCAM-1 is an important adhesion molecule that initiates the AS process by promoting endothelial cell activation and inducing the recruitment of leukocytes to atherosclerotic plaques [25]. IHC results showed an obvious increase in VCAM-1 expression in ApoE$^{-/-}$ mice with BCAT1 overexpression relative to control mice (Fig. 3a). MCP-1 plays a role in monocyte migration and promotes monocyte differentiation [26]. We observed a trend toward increased MCP-1 expression in BCAT1-overexpressed mice, indicating increased monocyte recruitment to the arterial wall (Fig. 3b). Meanwhile, the increased CD68 expression by IHC assay proved the pronounced macrophage accumulation in the arterial wall of AS lesions induced by overexpressed BCAT1 (Fig. 3c). Besides, we also noticed an obvious increase in aortic pro-inflammatory factors mRNA levels, including TNF-α, IL-6, and IL-1β induced in ApoE$^{-/-}$ mice with BCAT1 overexpression compared to vector (Fig. 3d). All these data suggested that BCAT1 overexpression promoted inflammatory response in HFD-administrated ApoE$^{-/-}$ mice.

3.4. BCAT1 overexpression aggravated atherosclerosis development in ox-LDL-treated RAW267.4 cells

To establish an appropriate *in vitro* model of AS, the RAW267.4 cells were stimulated by different concentrations of ox-LDL (25, 50, and 100 ng/mL), and the BCAT1 expression was detected by RT-qPCR and Western blot. The BCAT1 levels were significantly increased under all concentration stimulation (Fig. 4a–c). Based on our results and published literature, the concentration of 50 mg/mL was selected for the following experiment. The expression levels of BCAT1 in response to knockdown or overexpression of BCAT were verified and the results were depicted in Fig. 4d and e Simultaneously, ox-LDL significantly increased the

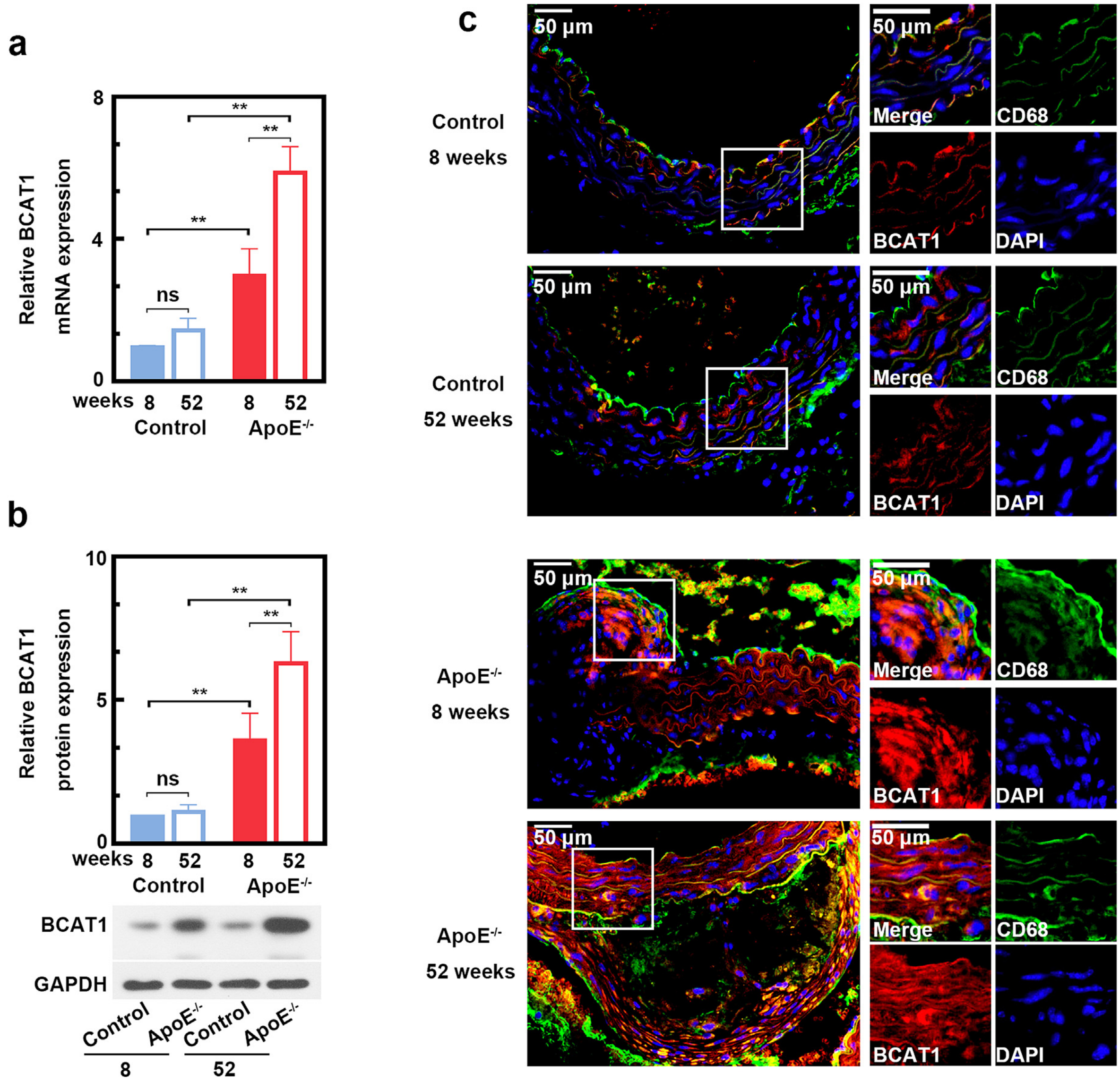

Fig. 1. BCAT1 expression in young vs. old ApoE−/− mice with atherosclerotic lesions a-b. Relative Branched-chain amino acid transaminase 1 (BCAT1) gene (a) and protein (b) expression in aortic tissue of mice fed a western diet for 12 weeks at 8 and 52 Weeks. c. Representative images of aorta tissue stained for BCAT1, CD68 and DAPI in8 and 52 week sold mice fed a Western diet for 12 weeks. Results were mean and standard deviation. ***P* < 0.01; ns, no significance, as assessed with one-way ANOVA.

lipid accumulation, foam cell formation (Fig. 4f) as well as the release of pro-inflammatory factors (engorged macrophages) (Fig. 4f and g). BCAT1 overexpression further remarkably promoted lipid accumulation (Fig. 4f) and increase TNF-α, IL-6, and IL-1β levels (Fig. 4f and g). We also determined whether BCAT1 knockdown could present an opposite effect to BCAT1 overexpression and obtained the expected results (Fig. 4e–g).

4. Discussion

AS in the aged population has far surpassed such other age-related diseases, such as chronic lung disease and cancer, as a cause of morbidity and mortality in the elderly. Aging has evolved to become the strongest independent risk factor for the development of AS [27]. Middle-aged mice (64- week-old), equivalent to ≈54-year-old humans [28], were used to explore the BCAT1 expression in AS with age. The data from our research firstly reported the upregulated BCAT1 expression in AS progression, and the increase in age was accompanied by upregulation of BCAT1 expression in the aorta tissues of ApoE$^{-/-}$ mice. In addition, IF analysis of mice aortae proved that BCAT1 was highly expressed in AS plagues, mainly located in lesion-infiltrated macrophages. These suggested that BCAT1 might contribute to the age-related progression of AS, as well as a major role possibly through macrophages.

Subsequently, we further explored the possible role of BCAT1 in AS. HFD treatment increased serum lipid levels and lipid accumulation in ApoE$^{-/-}$ mice, and these changes were further expanded by BCAT1 overexpression. The H&E staining results further demonstrated that overexpressed-BCAT1 deteriorated the formation of lipid droplets and aortic plaque in aortic root tissues. Thus, BCAT1 might play a pro-atherogenic role in AS. Elevated-lipid levels is a critical risk factor for AS [29]. It is characterized by increased levels of TC, TG LDL-C, and decreased levels of HDL-C [30]. All these phenomena were shown in our ApoE$^{-/-}$ mice with HDF administration. The increased serum lipid levels caused the accumulation of lipid in blood vessels and formed the early AS plaque [31], which was confirmed in our study by oil red O and H&E staining. Serum

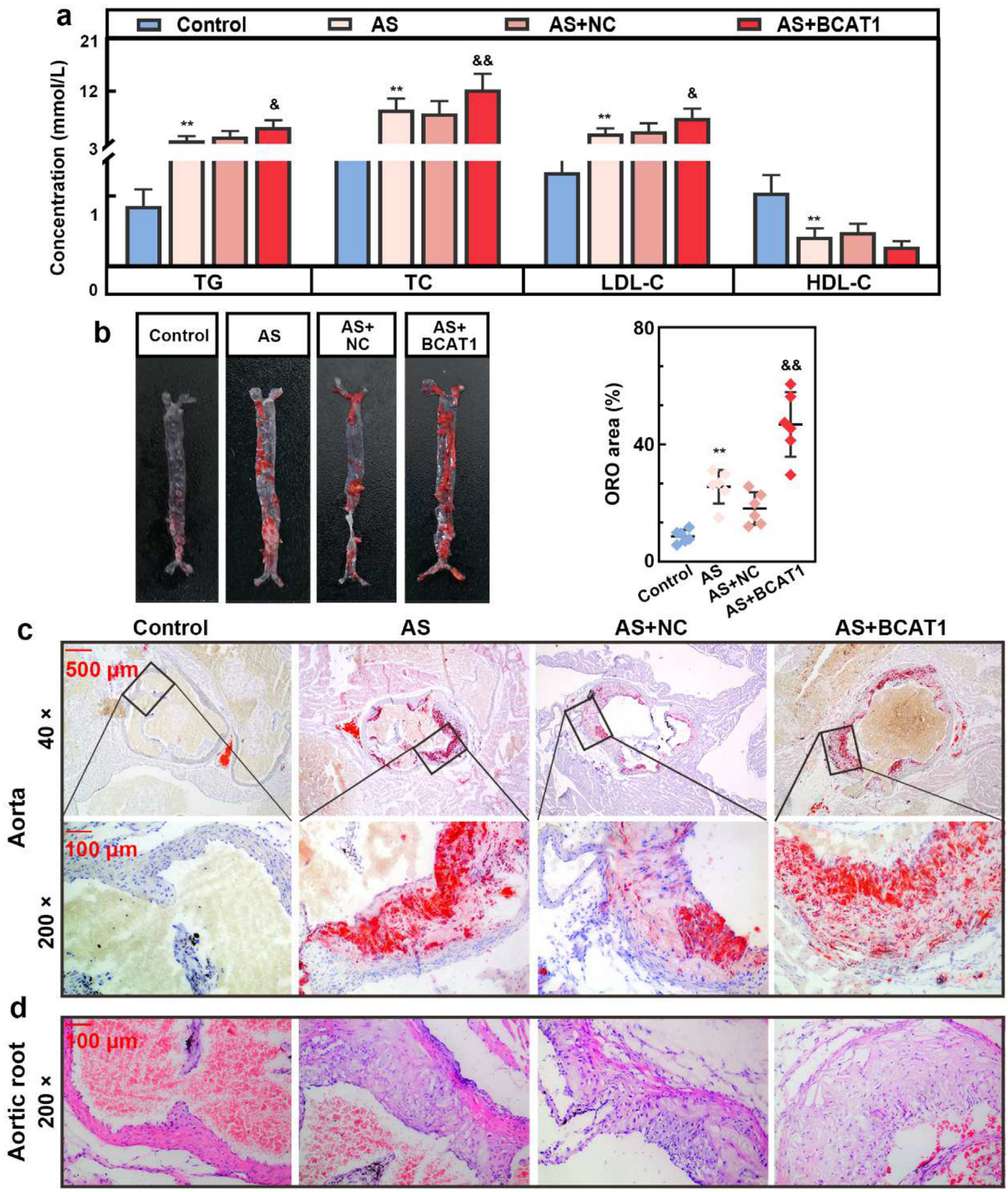

Fig. 2. BCAT1 overexpression aggravated atherosclerosis development in ApoE−/− mice a. Serum triglyceride (TG), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C) and low density lipoprotein-cholesterol (HDL-C) concentrations was detected by corresponding commercial kit. b. Oil red O staining of whole aortas (left panel), and expressed as a percentage (%) of total aortic area stained positively for Oil red O (right panel). c. Representative images of aortic root sections stained with Oil red O at different magnification. d. Representative images of aortic root sections stained with hematoxylin and eosin. Results were mean and standard deviation. Positive staining was indicated by bright red color. $^{**}P < 0.01$ vs control, $^{\&\&}P < 0.01$ vs. AS + NC, as assessed with one-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

TC, TG and LDL-C levels were reported to be correlated with AS and considered to be important factors for AS initiation and promotion [9,32]. HDL-C plays an anti-atherosclerotic role by transporting cholesterol from the peripheral tissues to the liver for catabolism, thereby reducing cholesterol deposition in the peripheral blood vessel walls [33]. Intriguingly, in our study, overexpressed-BCAT1 led to increased serum TC, TG, and LDL-C levels, but HDL-C levels had slight decreases. These phenomena indicated that BCAT1 might alter lipid profile mainly TC, TG, and LDL-C to drive lipid deposition, thereby worsening AS development in ApoE$^{-/-}$ mice fed with HFD.

In the early stage of AS, the accumulation of LDL-C in the aortic wall was oxidized into ox-LDL. The accompanying increase in blood pressure causes endothelial cell activation, the expression of adhesion molecule growth and monocyte recruitment in the

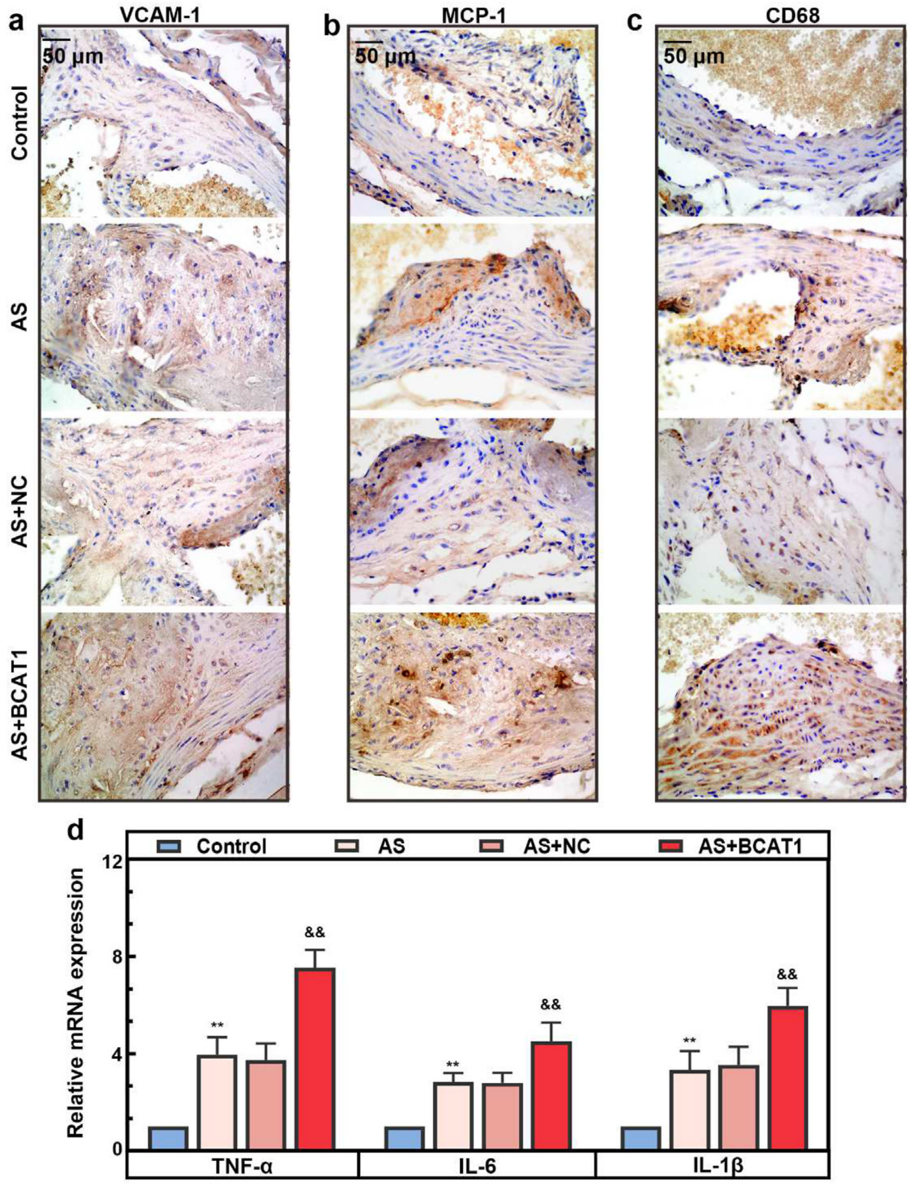

Fig. 3. BCAT1 overexpression aggravated inflammation response in ApoE−/− mice a-c. The expression and distribution of vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1) and CD68 in the aortic root plaque was evaluated by immunohistochemistry. d. The mRNA levels of TNF-α, IL-6 and IL-1 β in aortic tissues was determined by RT-qPCR. Results were mean and standard deviation. $^{**}P < 0.01$ vs control, $^{\&\&}P < 0.01$ vs. AS + NC, as assessed with one-way ANOVA.

intima. Subsequently, monocytes differentiate into macrophages and intake ox-LDL, resulting in the formation of lipid-filled foam cells, which is the marker of AS [34]. Aging was reported to be related to augmented atherosclerotic lesions with increased macrophage infiltration [35]. Activated macrophages produce inflammatory cytokines such as TNF-α and interleukins, which promote the other immune cell infiltration and potentiate the arterial wall inflammation and plaque formation [36]. VCAM-1 is a regulator in endothelial activation that promotes AS. The activated endothelial cells produce MCP-1 which further recruits monocytes and macrophages [37]. Besides, the increased VCAM-1 could further prove monocyte and macrophage adhesion [38]. In our study, the migration and infiltration of monocytes and macrophages were approved by increased VCAM-1 and MCP-1 levels in aortic tissues in ApoE$^{-/-}$ mice with HFD, which was similar to other published researches [39,40]. HFD induced upregulation of markers indicating M1-like macrophages presence and inflammatory response promotion, such as IHC for CD68 and the increases in TNF-α, IL-6, and IL-1β secretion. While overexpressed-BCAT1 further aggravated the above-mentioned phenomenon. As the effect of immune cell infiltration and anti-inflammatory effect of BCAT1 have been reported [12], suggesting that BCAT1 could effectively reduce the migration and infiltration of monocytes and macrophages to inhibit the inflammatory response, thereby alleviate AS development.

It is well known that macrophages play an important role in the biochemical cascade of AS. The crucial step for AS plaque lesions development is the formation of foaming cells to promote inflammatory response which is formulated by macrophages with ox-LDL up-taking [41]. The foaming cell formation and inflammatory cytokines released in RAW267.4 cells were affected by BCAT1 overexpression or knockdown in our research. These proved the pro-inflammatory effect of BCAT1 on macrophages of AS probably by lipid accumulation and foaming cell formation. BCAT1 is an enzyme

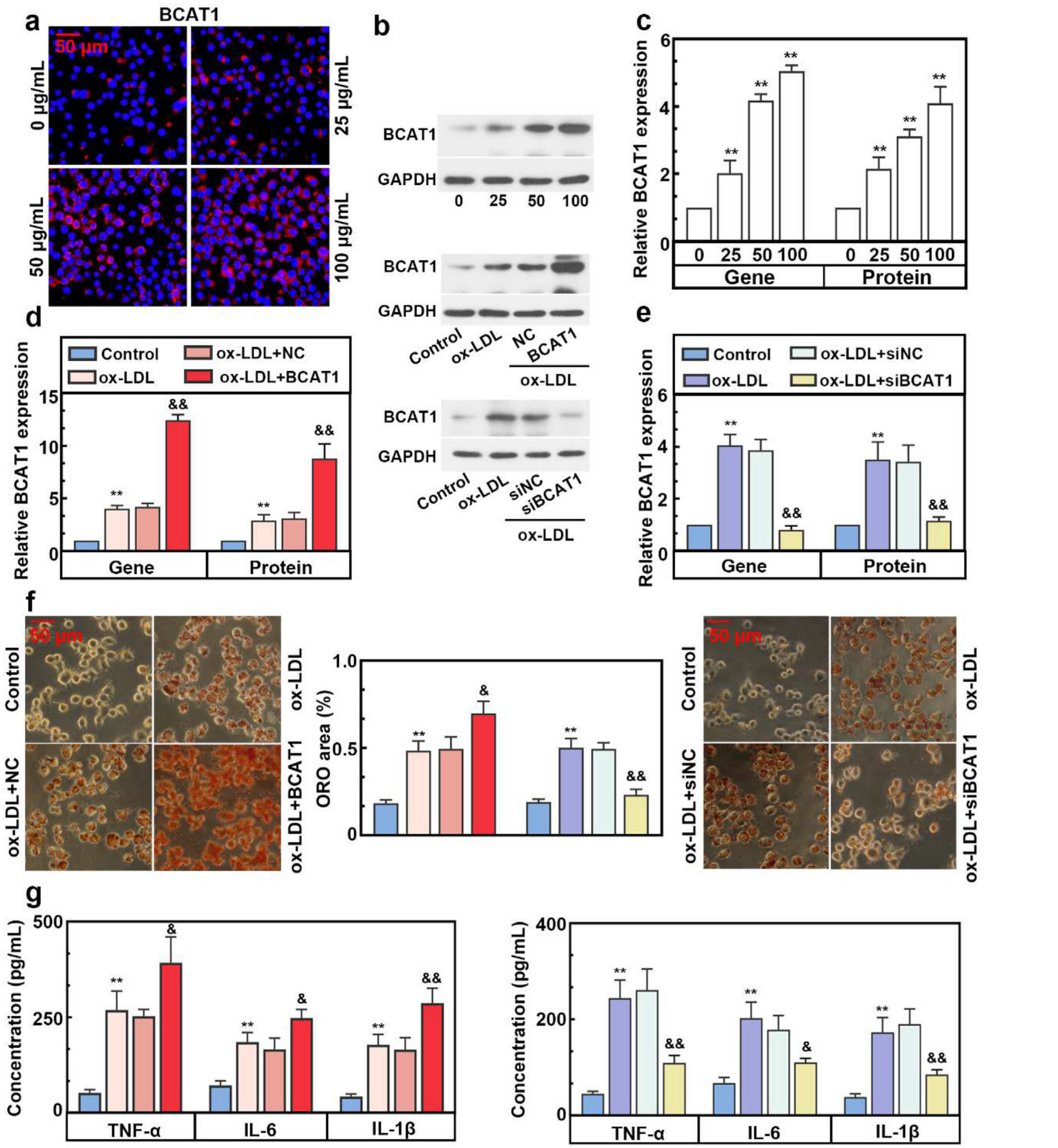

Fig. 4. BCAT1 overexpression aggravated atherosclerosis development in ox-LDL-treated RAW267.4 cells a. Immunofluorescence staining of BCAT1 (red color) in RAW267.4 cells in different group at different concentrations of oxidized-low-density lipoprotein (ox-LDL). b. Representative images of immunoblots for all experiment. c. The gene and protein expression of BCAT1 in RAW267.4 cells in different group at different concentrations of ox-LDL. Results were mean ± standard deviation. $^{**}P < 0.01$ vs 0, as assessed with one-way ANOVA. d-e. Western blot and RT-qPCR analysis confirming BCAT1 overexpression and BCAT1 knockdown. Results were mean ± standard deviation. $^{**}P < 0.01$ vs control, $^{\&\&}P < 0.01$ vs. ox-LDL + NC/ox-LDL + siNC, as assessed with one-way ANOVA. f. Oil red O staining (dark red staining) was utilized to test lipid accumulation in macrophages and foamy macrophages formation, and relative absorbance of Oil red O eluted by isopropanol was measured at 490 nm. Results were mean ± standard deviation. $^{**}P < 0.01$ vs control, $^{\&}P < 0.05$ vs. ox-LDL + NC, $^{\&\&}P < 0.01$ vs. ox-LDL + siNC, as assessed with one-way ANOVA. g. The release of TNF-α, IL-6 and IL-1 β in cell supernatant was detected by corresponding commercial kit. Results were mean and standard deviation. $^{**}P < 0.01$ vs control, $^{\&}P < 0.05$ vs. ox-LDL + NC/ox-LDL + siNC, $^{\&\&}P < 0.01$ vs. ox-LDL + NC/ox-LDL + siNC, as assessed with one-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to catalyze branched-chain amino acids (BCAA). The deficiency of BCAA has been reported to be associated with lipid metabolism impairment including TG and LDL-C [42]. BCAA is also suggested to be involved in dyslipidemiain of type 2diabetes. Therefore, increased BCAT1 might affect lipid metabolism in macrophage foaming cells via BCAA catabolism in AS, but the appropriate mechanism could be further explored.

5. Conclusion

In conclusion, our study proved that BCAT1 was upregulated in AS, and the expression was increased with aging. Overexpressed-

BCAT1 aggravated the pathological changes in the aortic tissues accompanied by increased serum lipid levels and lipid accumulation in aorta. Further, the results showed that the overexpression of BCAT1 promoted inflammatory response in macrophages probably via lipid accumulation and foaming cell formation. These findings underscore that the inhibition of BCAT1 might serve as a therapeutic target in addressing inflammation in AS.

Ethics approval

The study was approved by the Ethics Committee of Shenyang Medical College.

Author contribution

Lili Tan: Conceptualization, Methodology, Validation, Funding acquisition, supervision, Writing- Reviewing and Editing. Jie Lu: Conceptualization, Methodology, Visualization, formal analysis, Writing-Original draft preparation. Chunyang Zhang: Conceptualization, Data curation, Visualization,. Liang Meng: Visualization, formal analysis. Qi Zhu: Data curation, formal analysis. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The experimental data will be available on the request.

Declaration of competing interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Acknowledgments

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.09.041.

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