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ApoE [−/−] CA1-overexpressing knock-in mice aggravated atherosclerosis by increasing M1 macrophages

Jinbao Zong [a,1], Changyuan Wang [b,1], Hongji Zhou [c,d], Yu Song [c], Kehua Fang [e,*,1], Xiaotian Chang [c,**,1]

[a] Clinical Laboratory and Qingdao Key Laboratory of Immunodiagnosis, Qingdao Hiser Hospital Affiliated of Qingdao University (Qingdao Traditional Chinese Medicine Hospital), Renmin Road 4, Qingdao, 266000, PR China
[b] Department of Dermatology, Qingdao Hospital, University of Health and Rehabilitation Sciences(Qingdao Municipal Hospital), Dengyun Road 369, Qingdao, 266000, PR China
[c] Medical Research Center, The Affiliated Hospital of Qingdao University, Wutaishan Road 1677, Qingdao, 266000, PR China
[d] Department of Cardiology, Fushun Municipal Central Hospital, Xincheng Road 5, Fushun, Liaoning, 113006, PR China
[e] Clinical Laboratory, The Affiliated Hospital of Qingdao University, Wutaishan Road 1677, Qingdao, Shandong, 266000, PR China

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ABSTRACT

Background: Carbonic anhydrase I (CA1) has been reported to be a diagnostic and therapeutic target for atherosclerosis (AS). This study aimed to verify the essential role of CA1 in AS progression in CA1-overexpressing mice.

Methods: A ApoE [−/−] CA1-overexpressing knock-in mouse model was constructed via CRISPR/Cas9-mediated genome engineering. AS was then induced in these transgenic mice via the administration of a high-fat diet, and a second group simultaneously received treatment with methazolamide (MTZ), a carbonic anhydrase inhibitor.

Results: Compared with ApoE [−/−] mice without CA1 overexpression, CA1-overexpressing mice had a greater average body weight, regardless of whether their treatment with MTZ or their AS induction status. Sudan IV, hematoxylin and eosin and Oil Red O staining revealed more plaques and fat deposits in the cardiac aortas of CA1-overexpressing mice than in those of ordinary ApoE−/− mice when AS was induced. Moreover, the atherogenic index; low-density lipoprotein, total cholesterol and triglyceride levels were significantly elevated, and high-density lipoprotein levels were declined in the peripheral blood of CA1-overexpressing mice than in that of ordinary ApoE [−/−] mice, regardless of whether these animals were induced to AS. Immunohistochemistry, *Von Kossa* staining and fluorescence immunohistochemistry revealed increases in CA1 expression, calcium deposition and M1 macrophages in the aortic tissues of CA1-overexpressing mice with AS. MTZ treatment significantly suppressed AS pathologies in the above experiments.

Conclusion: These findings revealed aggravated AS in ApoE [−/−] CA1-overexpressing mice and suggest that CA1 aggravates AS by increasing M1-type macrophages, a proinflammatory macrophage subtype.

1. Introduction

Coronary artery calcification is a well-known clinical and pathological indicator of atherosclerosis (AS) and is a potential risk marker for this disease [1–3]. Calcifications in the cardiac aorta involve cholesterol, lipoproteins, hydroxyapatite, triglycerides, albumin, calcium carbonate, calmodulin and other proteins in the plaque precursor matrix, which plays an important role in atherogenesis [4]. Carbonic anhydrases are a group of isoenzymes that can convert carbon dioxide into bicarbonate and can either directly facilitate calcium carbonate deposition to promote calcium phosphate mineralization or indirectly mediate γ-glutamyl-carboxylase to activate proteins involved in calcification. These

* Corresponding author. Clinical Laboratory, The Affiliated Hospital of Qingdao University, Wutaishan Road 1677, Qingdao, Shandong, 266000, PR China
** Corresponding author. Medical Research Center, The Affiliated Hospital of Qingdao University, Wutaishan Road 1677, Qingdao, 266000, PR China.
E-mail addresses: zongjb@163.com (J. Zong), changyuanwang2008@163.com (C. Wang), zhouhongjira@163.com (H. Zhou), ssongyura@163.com (Y. Song), kehua.fang@163.com (K. Fang), changxt@126.com (X. Chang).
[1] Kehua Fang and Xiaotian Chang have equal contribution to this study as corresponding authors. Jinbao Zong and Changyuan Wang have equal contribution to this study as the first authors.

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enzymes are also involved in regulating acid balance to mediate metabolism in the body [5,6]. The relationship between carbonic anhydrase I (CA1), a member of the carbonic anhydrase family, and AS has attracted attention in recent years. Ando et al. detected higher concentrations of anti-CA1 autoantibodies in abdominal aortic aneurysm patients than in healthy donors, indicating that autoimmunity to CA1 is involved in AS pathogenesis [7]. Argan et al. reported that drugs that are preferred for AS treatment can effectively inhibit human CA1 and carbonic anhydrase II (CA2) activity *in vitro* [8]. Baragetti et al. reported that CA1 is the best predictor of subclinical AS [9]. We previously detected high CA1 expression in the aortic lesions of ApoE [−/−] mice with induced AS and reported that methazolamide (MTZ), a carbonic anhydrase inhibitor and a drug used for glaucoma treatment, can significantly alleviate AS pathology by inhibiting calcification. Furthermore, a high level of CA1 expression was detected in the human atherosclerotic aorta [10]. A decreased level of low-density lipoprotein (LDL), a biochemical measure of AS, was detected in AS patients with glaucoma after MTZ was used to cure their glaucoma, indicating the key role of CA1 in AS development [11]. We recently reported that M1 macrophages secrete TNF-α to increase CA1 and CA2 expression in vascular smooth muscle cells (VSMCs) to promote calcification [12]. The above studies suggest that CA1 is involved in AS progression and that CA1 can promote calcification in atherosclerotic aortic tissues. Moreover, MTZ can potentially treat AS by inhibiting CA1-mediated calcification. However, an animal model with CA1 overexpression is needed to verify the essential role of CA1 in AS pathology and the treatment of MTZ in this disease. With CA1-overexpressing animal models, we can further explore the pathogenic mechanism of AS.

ApoE [−/−] mice are usually used to establish an AS animal model through the administration of a high-fat diet. In the present study, we constructed CA1-overexpressing C57BL/6 J mice. The CA1-overexpressing knock-in (KI) mice were then crossed with ApoE [−/−] mice to obtain CA1-overexpressing ApoE [−/−] mice. These CA1-overexpressing mice were then induced to express AS characteristics by being fed a high-fat diet. Some of these transgenic mice were treated with MTZ after AS characteristics were induced (MTZ-treated group). Additionally, some CA1-overexpressing mice were fed a high-fat diet and simultaneously treated with MTZ (MTZ-preventive group). AS pathological characteristics were measured via a series of laboratory examinations.

AS is a progressive inflammatory disorder of the arterial vessel wall characterized by substantial infiltration of macrophages [13]. Macrophages can polarize toward proinflammatory M1 or anti-inflammatory M2 phenotypes [14]. An imbalance between these two kinds of macrophages has a pronounced effect on the development of AS [15]. In this study, we also examined M1 and M2 macrophage subtypes in mouse aortic tissues to analyze the relationship between CA1 expression and macrophage polarization in AS.

The present study revealed that ApoE [−/−] CA1-overexpressing KI mice presented more severe AS features than did ordinary ApoE [−/−] mice. This study further suggested that CA1 aggravated AS by increasing M1-type macrophages.

2. Materials and methods

2.1. Generation of CA1-overexpressing knock-in ApoE [−/−] mice via CRISPR/Cas9 gene editing

Transgenic CA1-overexpressing mice were generated via CRISPR/Cas-mediated genome engineering via a commercial service from Cyagen Biosciences (China). A complete mouse CA1 cDNA encoding region 792 bp in length was generated via PCR and subsequently inserted into a targeting vector. For construction of the targeting vectors, two arms homologous to intron 1 of the mouse Rosa26 locus were used for PCR amplification. These two guide RNA (gRNA) target sequences were designed via online software (http://crispor.tefor.net/). The sequence of gRNA1 was GGCAGGCTTAAAGGCTAACC-TGG, and the sequence of gRNA2 was CTCCAGTCTTTCTAGAAGAT-GGG. Cas9 mRNA and gRNA were generated via *in vitro* transcription. Cas9 and gRNA were coinjected into fertilized eggs with the targeting vectors for KI mouse production. The fertilized eggs were collected from C57BL/6 J mice, and the transgenic embryos were planted into pseudopregnant recipients. The CAG promoter-Kozak-mouse CA1 CDS-rBG pA cassette was subsequently cloned and inserted into intron 1 of the Rosa26 gene. Founder lines with successful insertion of the CA1 gene were identified via PCR with mouse tail DNA as a template. The positive female founder mice and wild-type C57BL/6 J male mice were bred to obtain F1 CA1-overexpressing heterozygote mice. The male CA1-overexpressing heterozygous mice and the female CA1-expressing heterozygous mice were then crossed to produce CA1-overexpressing homozygous mice.

To establish CA1-overexpressing ApoE [−/−] mice, Rosa26 [KI/KI] mice with CA1 overexpression were crossed with ApoE [−/−] mice on a C57BL/6 J background. The genotypes of the newborn mice were identified, and offspring with the Rosa26 [KI/KI] ApoE [−/−] genotype were retained. Finally, sixty (Rosa26 [KI/KI] ApoE [−/−]) male mice aged 8 weeks with systemic CA1 overexpression were obtained and delivered to our laboratory. The DNA templates were extracted from peripheral blood collected from the mouse tail. The genotypes of the transgenic mice were examined via PCR. The sequence of primer set 1 (F1: 5′-GGCAACGTGCTGGTTATTGTG-3′,

R1: 5′-TTCAGAACAGATTGGTTGCTACT-3′) covered the CAG promoter of the targeting vectors. The expected PCR product size was 296 bp if CA1 cDNA was inserted into the genomic DNA of the KI mice. The sequence of primer set 2 (F2: 5′-CACTTGCTCTCCCAAAGTCGCTC-3′, R2: 5-ATACTCCGAGGCGGATCACAA-3’;

The internal control PCR primer F (5′-CATGCCAATGGTTCACTC-TAAGGT-3′) and the internal control PCR primer R (5′-TCTCTATGTCCCAAAGTGCAGACAC-3′) targeted the homology arms of the vectors. The internal control PCR product size was 335 bp. In KI mice, PCR with a DNA template from homozygotes can produce one band that is 335 bp long, and PCR with a DNA template from heterozygotes can produce two bands that are 335 bp and 453 bp long. In mice with the wild-type allele, PCR can produce two bands that are 335 bp and 453 bp long. The sequence of primer set 3 (primer-1: 5′-GCCTAGCCGAGGGAGAGCCG-3′, primer-2: 5′-GCCGCCCCGACTG-CATCT-3′, primer-3: 5′-TGTGACTTGGGAGCTCTGCAGC-3′) targeted the ApoE gene. PCR can produce a 245 bp band in KI ApoE [−/−] mice, whereas PCR can produce a 155 bp band in mice with the wild-type allele.

2.2. Establishment of an AS mouse model

To establish the AS mouse model, 60 eight-week-old healthy male (Rosa26 [KI/KI] ApoE [−/−]) mice with CA1 overexpression were identified by PCR and randomly divided into four groups: A, B, C and D (n = 15 for each group). The animals in Group A were fed a normal diet, while those in Groups B, C and D were fed a high-fat diet (1 % cholesterol and 10 % fat, and the remainder was standard chow) for a total of 24 weeks. Group A was the normal control and was given normal saline. Group B included the AS model and was treated with normal saline. Group C was treated with normal saline from weeks 1–12 and with MTZ from the 13th week to the 24th week to create the MTZ-treated group. Group D was treated with MTZ for 24 weeks as the MTZ-preventive treatment group. Normal saline was intragastrically administered at a dose of 0.1 mL/10 g, and MTZ dissolved in normal saline was intragastrically administered at a dose of 25 mg/kg. MTZ was obtained from Hangzhou Aoyipollen Pharmaceutical (China), and the animals were treated once every other day. The dose used was determined on the basis of previous experimental results and protocols [10]. Moreover, 60 eight-week-old healthy male C57BL/6 J ApoE [−/−] mice without further gene modification were subjected to the above protocol. These mice were also divided into four groups, which were named E, F, G and

H (n = 15 for each group). The E group was subjected to the same protocol as the A group, the F group was the B group, the G group was the C group, and the H group was the D group. Mouse cardiac aorta tissues and peripheral blood were collected for subsequent measurements. The changes in body weight in each group were recorded every week.

Among the 15 mice in each subgroup, five aortic samples were collected from the mice and then fixed and embedded in paraffin for hematoxylin and eosin (HE) staining, immunohistochemistry and von Kossa staining. All three techniques require deparaffinization and hydration, and as such, the same blocks were able to be used for all three techniques. Another 5 samples were used for Oil red O staining, and the other 5 samples were used for Sudan IV staining. Five mice were randomly selected from the 15 total mice in each group, and peripheral blood was collected from the selected mice for biochemical tests.

All animals were housed under specific pathogen-free conditions and handled in accordance with the Helsinki Convention on Animal Protection and EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental design was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (QYFY WZLL 27438).

2.3. Hematoxylin and eosin (HE) staining

The thoracic aorta was isolated and fixed overnight with 4 % formalin at 4 °C. These tissues were routinely embedded in paraffin, sectioned at a thickness of 6 μm, and mounted on silanized slides. The tissue sections were differentiated in an ethanol series from 100 % to 30 % and finally hydrated in distilled water. The sections were stained with hematoxylin and eosin solution (Solabio, China), dehydrated with an ethanol series, cleared with xylene, and mounted. The extent and composition of the aortic lesions were quantified, and the size of lesions were determined by the area of these lesions accounting for the total cross-sectional area of the aortic intima.

2.4. Sudan IV staining

To observe lipid accumulation in the mouse aorta, Sudan IV staining was performed. The whole aorta was collected from the mouse model. The mouse aorta samples were stained in Sudan IV solution (Solabio, China), rinsed with water and differentiated in 80 % ethanol. The aortic tissue was photographed via an anatomical microscope. The distribution and extensiveness of lipid deposition were semiquantified by calculating lipid accumulation in the whole aorta area (red color) via ImageJ software (Wayne Rasband, USA).

2.5. Oil red O staining

To observe the distribution and extensiveness of oil droplets in mouse aortic tissues, Oil Red O staining was performed. Cryostat sections of mouse aortic tissues were prepared, and the sections were air dried. The samples were fixed in formalin and briefly washed with running tap water for 1–10 min. The tissue sections were rinsed with 60 % isopropanol and stained with freshly prepared Oil Red O working solution (Solabio, China) for 15 min. The sections were rinsed with 60 % isopropanol, washed in distilled water and then counterstained with hematoxylin. Following a rinse with distilled water, the atherosclerotic tissue structure was observed and photographed under a light microscope. The total number and extensiveness of the oil droplets in the cardiac tissues were semiquantified by calculating oil droplets (red color) on the whole aorta via ImageJ software.

2.6. Biochemical examination

To determine the successful establishment of the AS mouse model, biochemical examination of mouse peripheral blood was performed. The mouse blood samples were collected in a heparin blood collection tube, and the supernatants were obtained via centrifugation. The levels of high-density lipoprotein (HDL), LDL, total cholesterol (TC) and triglycerides (TG) in the mouse plasma were measured via an automatic biochemical analyzer (Mindray, China). Atherogenic index (AI) = [TC-HDL]/HDL. AI is a novel metabolic biomarker of AS and can predict carotid atherosclerosis [16].

2.7. Immunohistochemistry

To localize CA1 expression in cardiac aorta tissues, immunohistochemistry was performed. Paraffin sections (6 μM) of the mouse aortic tissues were deparaffinized in ethanol in a routine way and finally hydrated in water. The sections were incubated with an anti-CA1 polyclonal antibody (Abcam, USA) overnight at 4 °C. The antibody was raised from rabbits by immunization with recombinant mouse CA1 protein. The sections were then rinsed with water, treated with diaminobenzidine (DAB; Zhongshan Golden Bridge Biotechnology) and counterstained with hematoxylin.

2.8. Von Kossa staining

To observe calcification in the cardiac aorta tissues, *von Kossa* staining was performed. After routine deparaffinization and hydration, the sections were incubated with a *von Kossa* silver solution (Solabio, China) under bright light for 15 min and then treated with a sodium thiosulfate solution for 2 min. The tissue sections were counterstained with hematoxylin and eosin solution. The sections were then dehydrated, cleared and mounted with neutral balsam. Calcium deposition was observed under a light microscope.

2.9. Immunofluorescent immunohistochemistry

To determine the tissue distribution and proportion of macrophages, the cardiac aorta tissues embedded in paraffin were cut into sections with a thickness of 15 μM. The tissue sections were incubated with SpGreen-labeled anti-mouse CD86 antibody (BioLegend, USA) and Cy5-labeled anti-mouse CD163 antibody (BioLegend). CD86 is a routine marker of M1-type macrophages, and CD163 is a marker of M2-type macrophages, which are shown in green and yellow, respectively, by immunofluorescence. After being washed with PBS, the sections were stained with a DAPI solution. Images were taken with a PANNORAMIC panoramic tissue section scanner (3DHISTECH, Hungary). CaseViewer 2.4 software (3DHISTECH, Hungary) was used to analyze the microscopy images. The number of positive cells and the total number of cells in each section were semiquantified via the INDICALABS-HIPPLEX FL V3.1.0 module in Halo v3.0.311.314 analysis software (Indica Labs, USA), and the percentage (%) of positive target cells was calculated.

CA1 expression was also detected via immunofluorescent immunohistochemistry via a similar protocol. Rabbit anti-mouse CA1 antibody was obtained from Servicebio (China). The antibody was labeled with SpOrange, which is shown in red by immunofluorescence. The quantified signal was normalized to the total cellularized area.

2.10. Statistical analysis

The blood biochemistry data, aortic plaque semiquantification data, oil droplet semiquantification data and flow cytometry data were analyzed via GraphPAD Prism 8.0. If the data were normally distributed, one-way ANOVA was used to analyze the significant differences among multiple groups, and the LSD-T test was used for comparisons between two groups. If the data did not fit a normal distribution or homogeneity of variance, the Kruskal–Wallis test was applied to analyze significant differences among multiple groups. If the P value was less than 0.05, the differences were considered statistically significant.

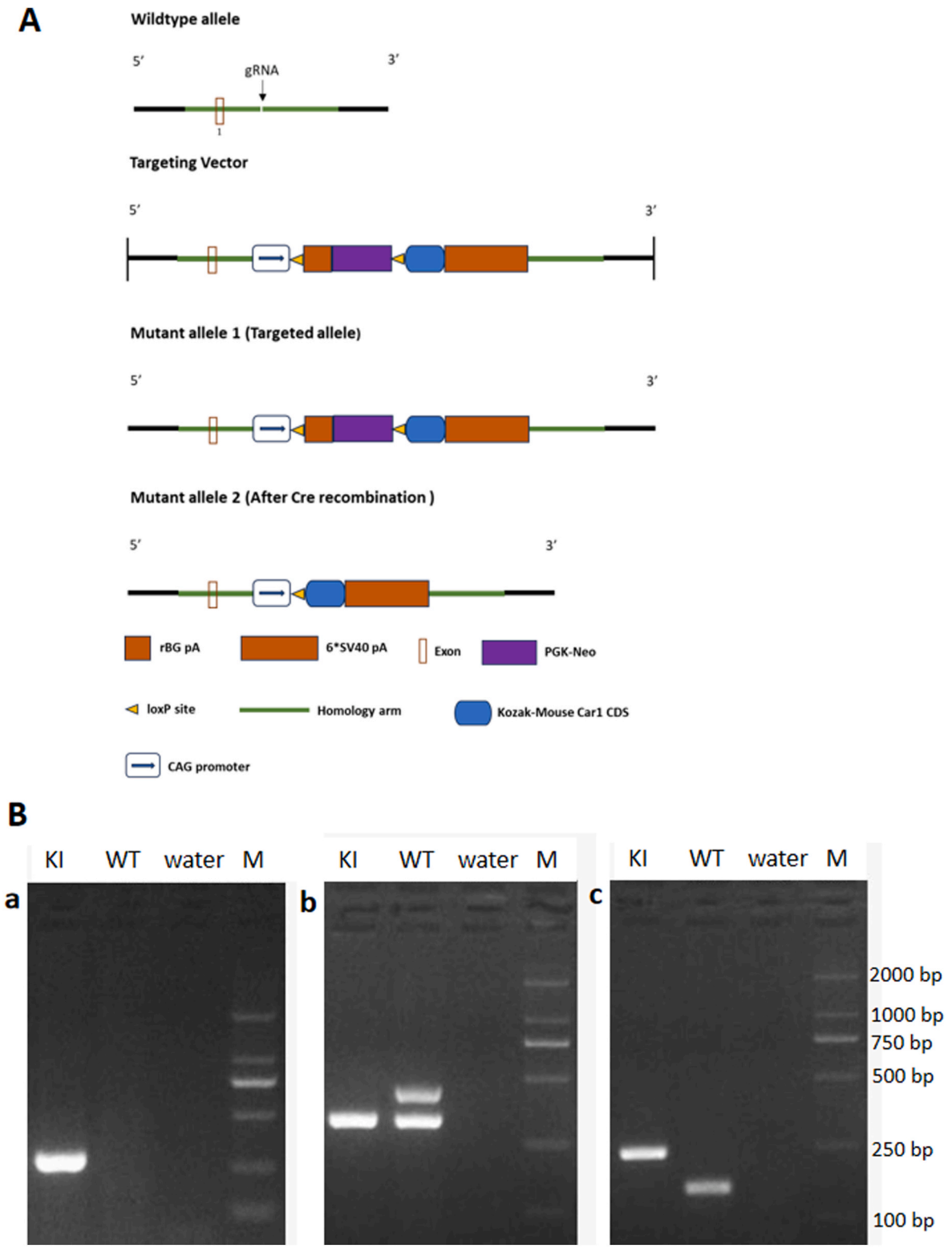

Fig. 1. Establishment of CA1 KI ApoE [−/−] C57BL/6 mice via the CRISPR/Cas9 gene re-editing technique. A. Diagram of the establishment of KI mice with CA1 cDNA inserts. An expression cassette with a CAG promoter and CA1-encoding gene was inserted into intron 1 of the Rosa26 locus in the C57BL/6 mouse chromosome via the CRISPR/Cas9 system. **B.** Representative PCR images showing the successful establishment of CA1-overexpressing KI mice. **a.** PCR with primer set 1 yielded one band of 296 bp in KI mice. **b.** PCR with primer set 2 yielded one band of 335 bp in KI mice. **c.** PCR with primer set 3 yielded one band of 245 bp in the KI mice. KI: knock-in mice; WT: wild type; M: molecular size marker of DNA.

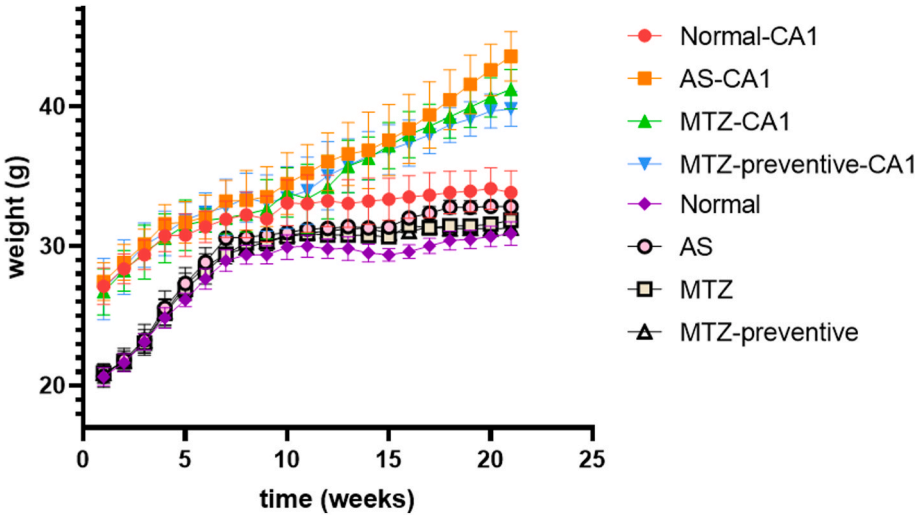

Fig. 2. Changes in the body weights of the AS model mice. CA1-overexpressing ApoE [−/−] mice and ordinary ApoE [−/−] mice were induced to AS with high-fat food.
These patients were divided into the following groups: normal, AS model, AS model with MTZ treatment and AS model with MTZ preventive-treatment. Each group included 15 mice. Mice with CA1 overexpression generally had greater body weights than did those without CA1 overexpression. The weight of the mice with AS was generally greater than that of the mice without AS, and the weight of the mice with AS was reduced following MTZ treatment and MTZ-preventive treatment.

3. Results

3.1. CA1-overexpressing KI ApoE [−/−] mice were successfully generated

To generate KI mice with CA1 overexpression, a construct containing the mouse full-length CA1 encoding cDNA was inserted into intron 1 of the Rosa26 locus in C57BL/6 mouse zygotes through homologous recombination via CRISPR/Cas9 gene re-editing. The Rosa26-CA1-expressing mice were then crossed with ApoE [−/−] C57BL/6 mice, which constitute a strain that has a background identical to that of ApoE [−/−] C57BL/6 mice. The strategy for generating the transgenic mice is shown in Fig. 1A. PCR with primer set 1 covering the CAG promoter and CA1 insert yielded one band of 296 bp in the genomic DNA from the KI mice, but this band was not detected in the sample from the wild-type mice, indicating successful insertion of the CA1-encoding gene in the KI mice (Fig. 1B–a). Another PCR with primer set 2, which targeted homology arms, yielded one band of 335 bp in the genomic DNA from the KI mice and two bands of 335 bp and 453 bp in the genomic DNA from the wild-type ApoE [−/−] mice, indicating successful insertion of the CA1-encoding gene into C57BL/6 mice and the establishment of CA1 [KI/KI] homozygotes (Fig. 1B–b). PCR with primer set 3, which targets the ApoE gene, yielded one band of 245 bp in the genomic DNA from the KI mice and one weak band of 155 bp in the sample from the wild-type ApoE [−/−] mice, indicating successful insertion of the CA1 gene into ApoE [−/−] mice and the establishment of CA1 [KI/KI] ApoE [−/−] mice with a C57BL/6 background (Fig. 1B and c).

3.2. ApoE [−/−] mice with CA1 overexpression presented more severe AS pathology

To determine the effect of CA1 expression on AS pathological progress, ApoE [−/−] mice with CA1 overexpression and normal ApoE [−/−] mice were fed a high-fat diet to induce AS for 24 weeks (B, C, D, F, G and H groups). Some of these animals with AS were treated with MTZ from 13 weeks to 24 weeks when AS was established (C and G groups), and other AS animals were treated with MTZ from the first week to the 24th week as the MTZ-preventive treatment group (D and H groups). The changes in body weight and general health in each group were recorded every week. The mice with CA1 overexpression generally had greater body weights than did those without CA1 overexpression. Additionally, the weights of the AS model mice were generally greater than those of the normal control mice (A and E groups), and the weights of the AS model mice were reduced after MTZ treatment. The body weight changes are shown in Fig. 2.

Cardiac aorta tissues from the model mice were collected and examined by Sudan IV staining. Atherosclerotic lipid accumulation was semiquantified by calculating the number and extent of stained signals. Compared with those in the aortic wall of control mice without AS induction (A and E groups), increased atherosclerotic lipid droplets were observed in the aortas of AS model mice (B and F groups), and the number and extent of lipid accumulation significantly decreased after MTZ treatment (C and G groups) and MTZ-preventive treatment (D and H groups), regardless of whether these mice were ApoE [−/−] mice or ApoE [−/−] mice with CA1 overexpression. Furthermore, atherosclerotic lipid accumulation was much greater in ApoE [−/−] mice with CA1 overexpression than in ordinary ApoE [−/−] mice, especially in those with induced AS (Fig. 3). HE staining revealed that the aortic wall of the AS mice was significantly thickened, as determined by calculating the surface plaque area across the entire aorta area, and that numerous atherosclerotic plaques and high levels of foam cells were present in the thoracic aorta tissues compared with those of the mice without induced AS. Moreover, the smooth muscle layer of the aortic tissue was relaxed and structurally disrupted, and typical atherosclerotic plaques protruded from the intima in the mice with AS. After MTZ treatment or MTZ-preventive treatment, the number and extent of atherosclerotic plaques in aortic tissues significantly decreased, and the tissue structure became orderly. Most importantly, compared with control ApoE [−/−] mice, CA1-overexpressing mice presented much greater aortic thickness, regardless of whether AS was induced and treated with MTZ (Fig. 4). Oil Red O staining revealed strong red staining of substantial fat deposits in the aortic intima of the AS group compared with those of the healthy group and the MTZ-treated group, with and without CA1 overexpression. Furthermore, the number and extensiveness of fat deposits in CA1-overexpressing mice with AS were much greater than those in AS mice without CA1 overexpression according to a semiquantitative calculation (Fig. 5). Compared with the healthy control group, the AS group presented elevated AI, LDL, TC and TG levels and decreased HDL levels in the peripheral blood, and the AS group treated with MTZ presented decreased AI, TC, TG and LDL levels and increased HDL levels. Furthermore, sera from CA1-overexpressing mice presented much higher levels of AI, LDL, TC and TG and lower levels of HDL than did

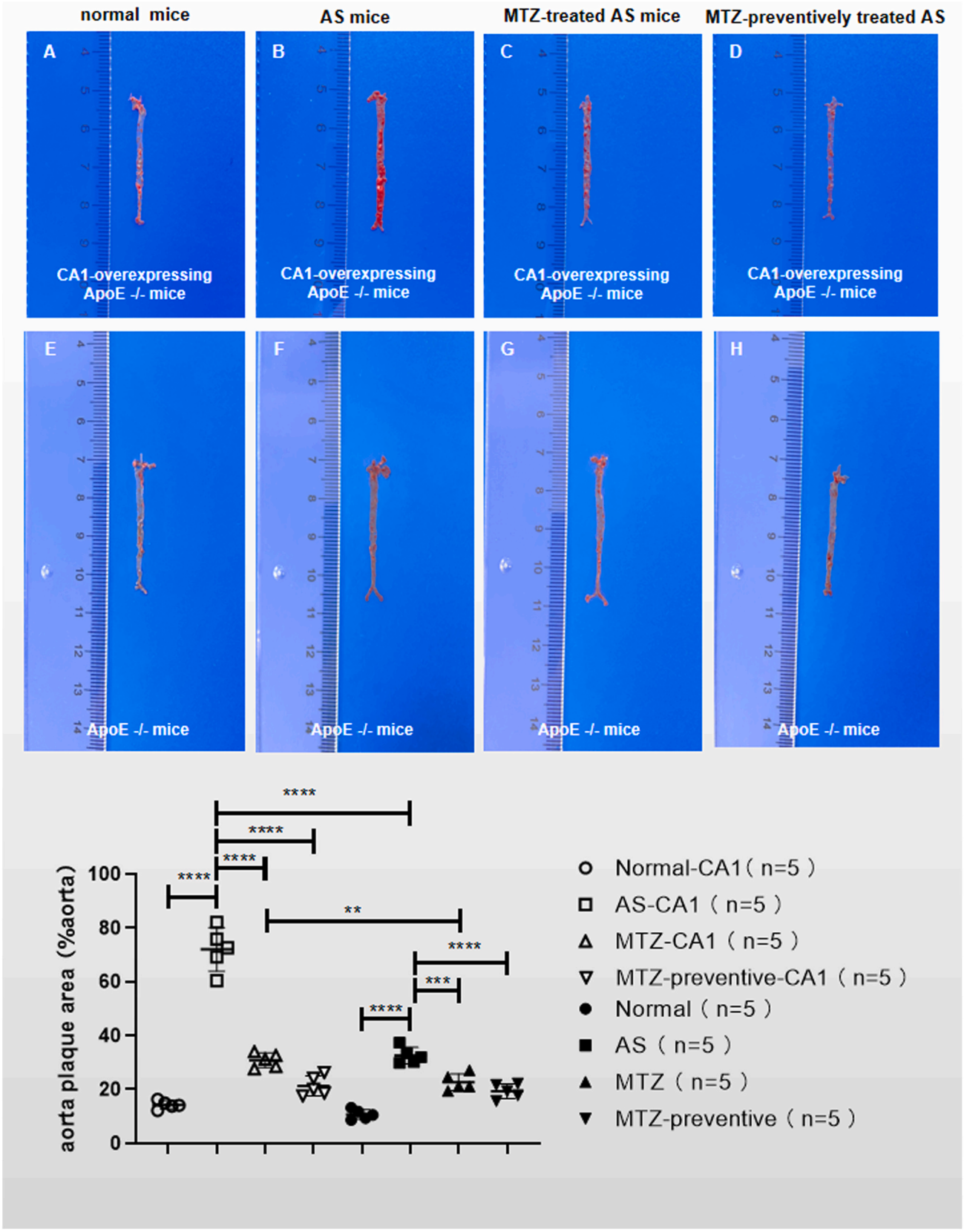

Fig. 3. Sudan IV staining of mouse cardiac aorta tissues. The number and extent of accumulated lipids in the aorta were semiquantitatively analyzed by calculating lipid accumulation in the whole aorta. The lipid accumulation in the CA1-overexpressing mice was much greater than that in the mice without CA1 overexpression. MTZ treatment significantly decreased the quantity and volume of lipid accumulation. ** indicates $P < 0.01$, *** indicates $P < 0.001$, and **** indicates $P < 0.0001$.

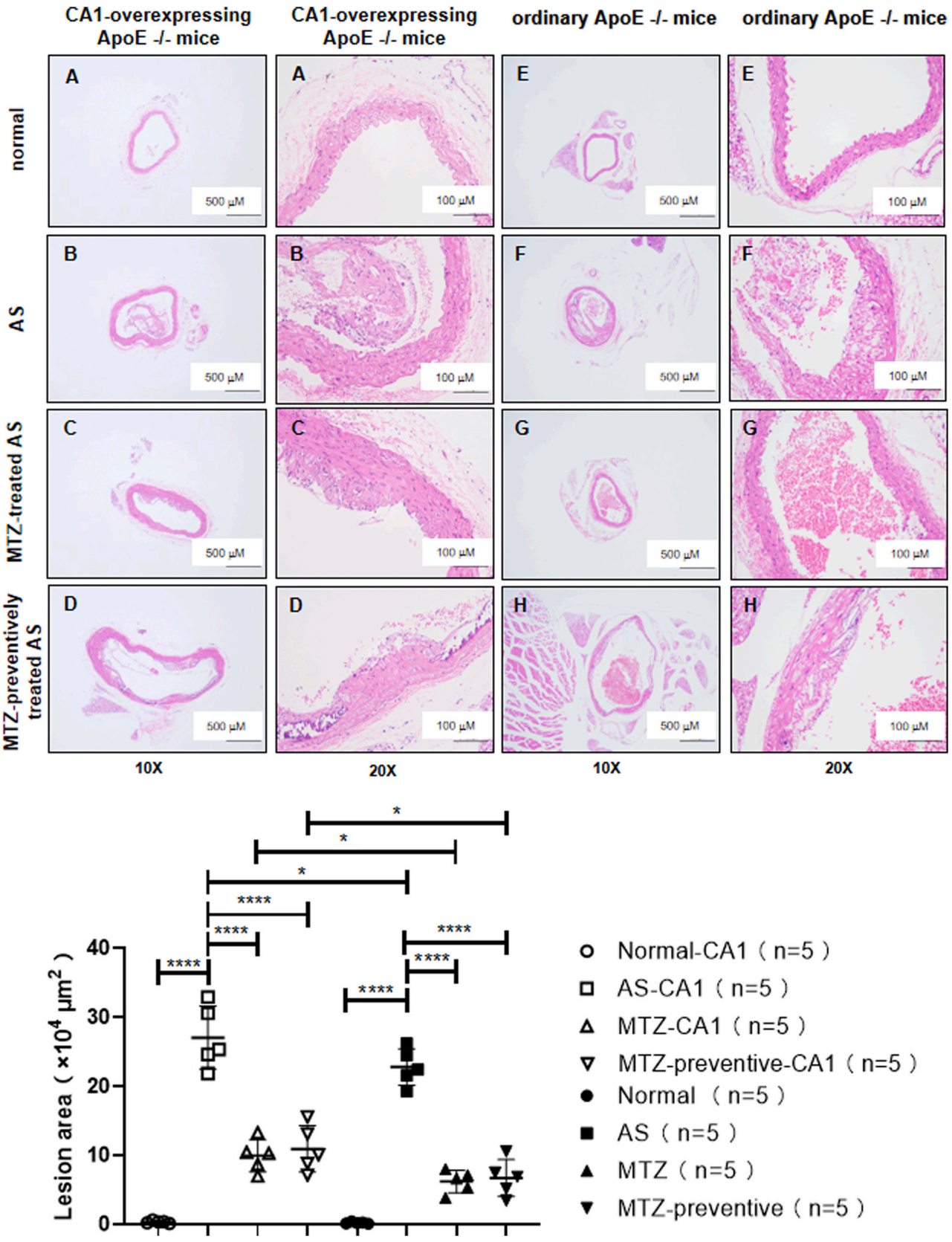

Fig. 4. HE staining of mouse cardiac aorta tissues. The extent and composition of the aortic lesions were semiquantified by calculating the surface plaque area across the entire aortic area. The number of aortic plaques and wall thickness in CA1-overexpressing mice were greater than those in mice without CA1 overexpression. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$.

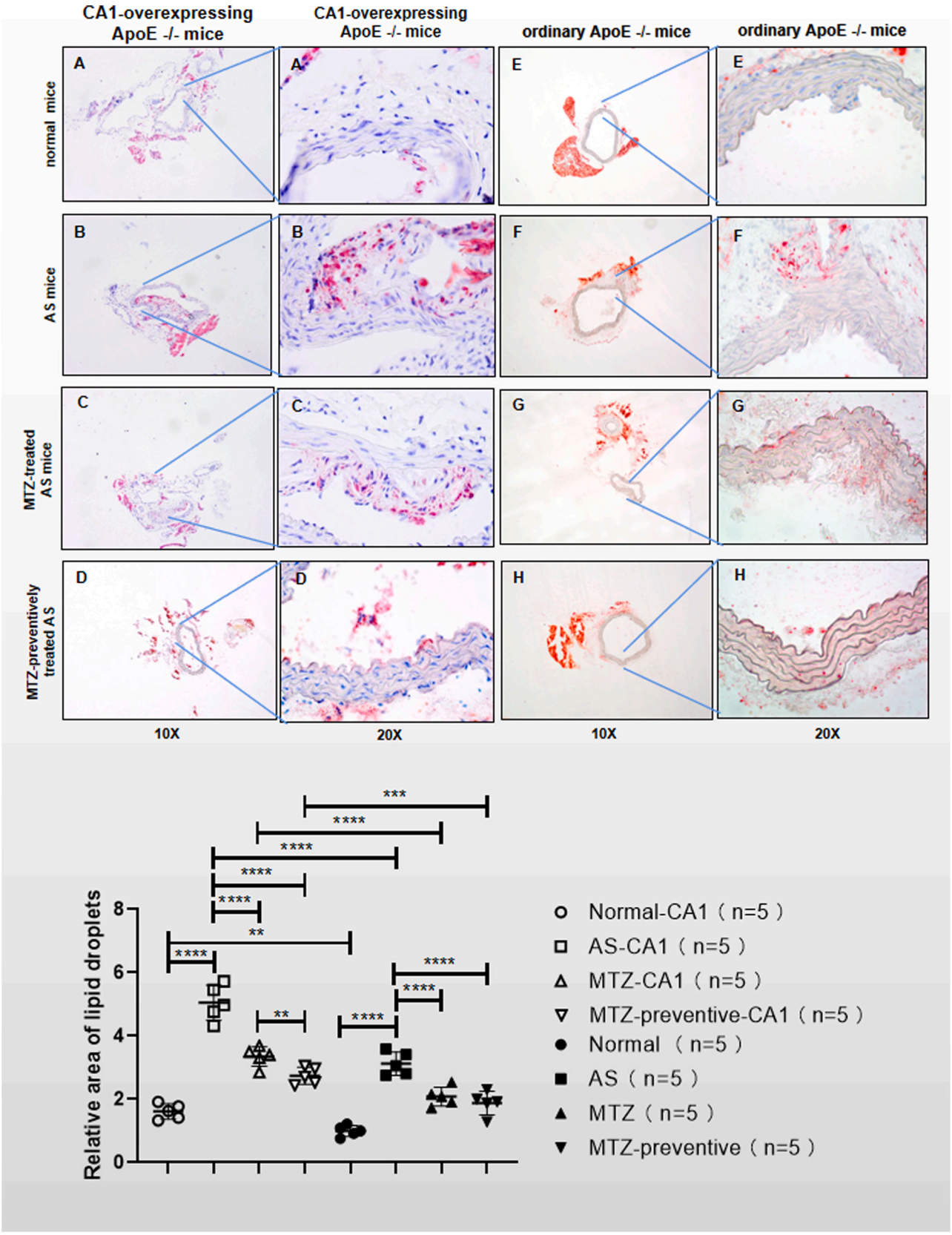

Fig. 5. Oil Red O staining of mouse cardiac aorta tissues. The number and size of fat deposits were semiquantitatively analyzed. Fat deposition in cardiac aorta tissues was greater in CA1-overexpressing mice with AS than in mice with AS without CA1 overexpression. MTZ treatment significantly decreased the volume and number of fat deposits. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$.

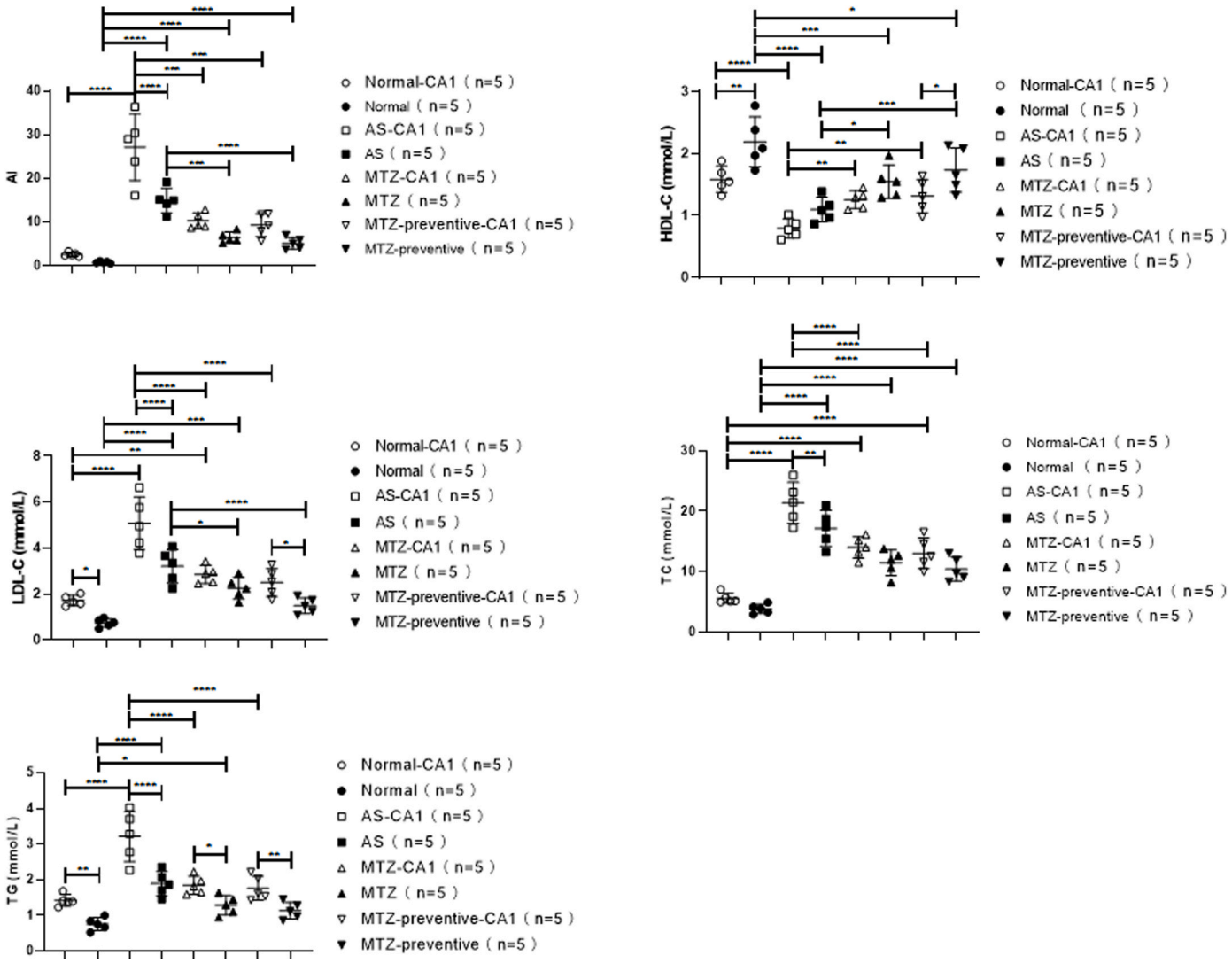

Fig. 6. Biochemical examination of mouse peripheral blood. Compared with those in healthy controls, HDL levels were significantly lower, and AI, LDL, TC and TG levels were elevated in AS mice. The levels of these indices were restored after MTZ treatment. Moreover, the HDL level was significantly lower and the levels of AI, LDL, TC and TG were greater in CA1-overexpressing mice than in ApoE [−/−] mice with normal CA1 expression, regardless of whether these mice were induced to develop AS or treated with MTZ. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$.

samples from ordinary ApoE [−/−] mice without genetic modification, regardless of whether these mice had AS and were treated with MTZ (Fig. 6). These observations demonstrated successful establishment of the AS mouse model and significant alleviation of AS with MTZ treatment. The above findings further demonstrated that CA1 overexpression significantly aggravated AS pathogenesis and increased the sensitivity of ApoE [−/−] mice to AS induction.

3.3. Cardiac aorta tissues from ApoE [−/−] mice exhibited extensive CA1 expression and calcification

To determine CA1 expression, mouse aortic tissue sections were examined via immunohistochemistry. CA1 expression was detected in the aortic plaques of the animals with AS. CA1 is also expressed in some VSMCs in aortic tissues. To semiquantify the expression levels, CA1 expression was also measured via immunofluorescent immunohistochemistry. CA1, with red immunofluorescence, was obviously expressed in the adventitia of the cardiac aorta and weakly expressed in intimal plaques. Moreover, CA1 was significantly more highly expressed in the aortic adventitia of CA1-overexpressing Apoe [−/−] mice than in the tissues of ordinary ApoE [−/−] mice, regardless of whether these CA1-overexpressing mice had AS. CA1 exhibited relatively low expression in the aortic adventitia of ordinary Apoe [−/−] mice, although its expression was relatively high in these animals with induced AS (Fig. 7).

Von Kossa staining was performed to detect calcium deposition. The staining revealed extensive calcification signals in the cardiac aorta tissues of CA1-overexpressing AS and AS mice following MTZ treatment. No extensive expression was detected in the aortic tissues of CA1-overexpressing mice without induced AS or in those of AS mice subjected to MTZ-preventive treatment. Little calcification was observed in the aortic tissues of ordinary ApoE [−/−] mice with induced AS, and no calcification was detected in the aortic tissues of ordinary ApoE [−/−] mice without AS or with AS subjected to MTZ treatment (Fig. 8).

3.4. ApoE [−/−] mice with CA1 overexpression presented more M1-type macrophages in cardiac aorta tissues

To understand how CA1 expression affects AS, immunofluorescence immunohistochemistry analysis was performed to determine CD86 and CD163 expression in the aortic tissues of AS model mice. CD86, which represents M1 macrophages, was expressed mainly in the cardiac aortic adventitia. Some M1 macrophages were also found in aortic intima

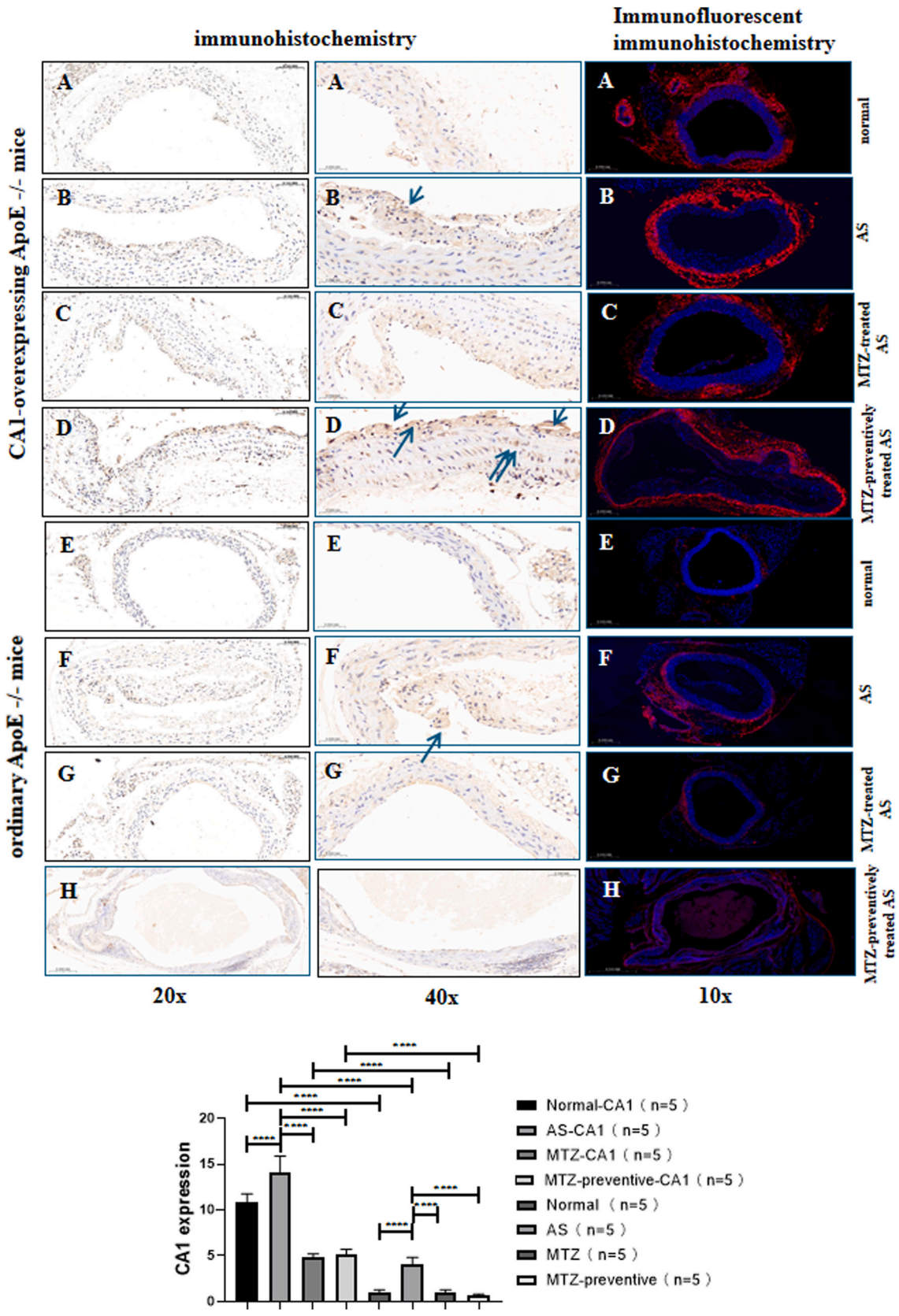

Fig. 7. Immunostaining of CA1 expression in mouse aortic tissues. Immunohistochemistry revealed CA1 expression (brown) in the aortic plaques of the animals with AS (↓). CA1 was also weakly expressed in aortic VSMCs (↓↓). Immunofluorescent immunohistochemistry revealed that CA1 levels were increased in the cardiac aorta tissues of CA1-overexpressing ApoE [−/−] mice, regardless of whether the KI mice had induced AS, compared with those of ordinary ApoE [−/−] mice. The quantified signal is normalized to the total cellularized area. *** indicates $P < 0.001$ and **** indicates $P < 0.0001$.

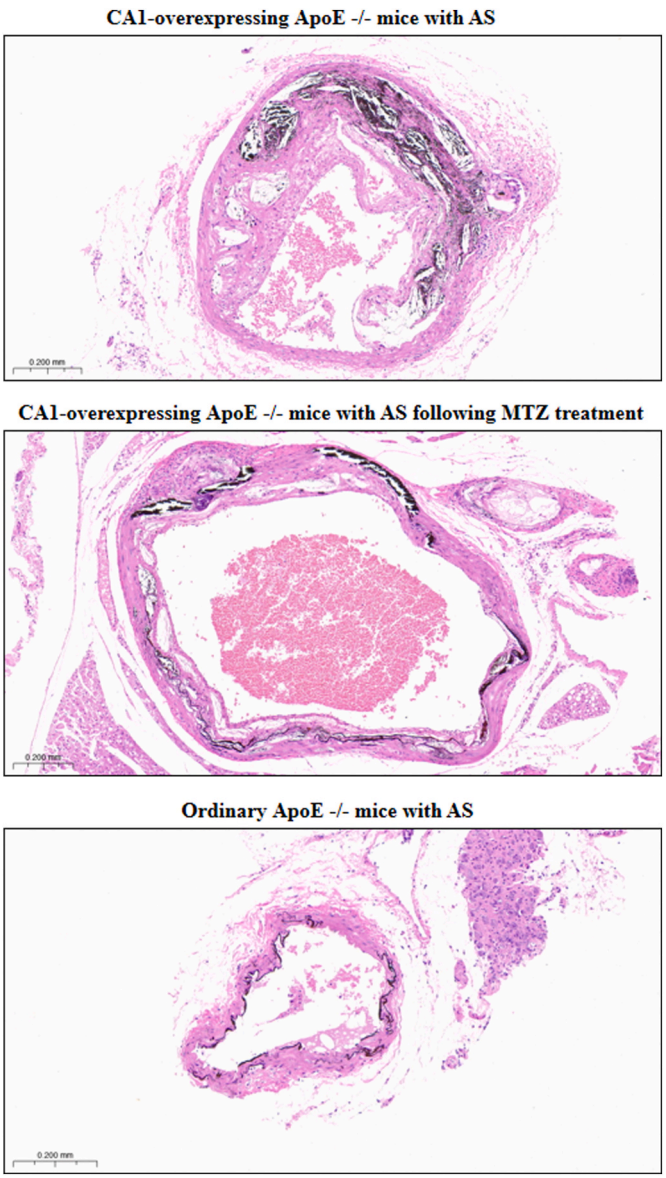

Fig. 8. Calcification of mouse cardiac aorta tissues via Von Kossa staining. Extensive calcium deposition was detected in cardiac aorta tissues from CA1-overexpressing AS and AS mice following MTZ treatment. Little calcification was observed in the aortic tissues of ordinary ApoE [−/−] mice with induced AS.

plaques. Importantly, CD86 was extensively expressed and loosely distributed in the aortic tissues of CA1-overexpressing mice, regardless of whether these mice were normal or in AS. Increased expression of CD86 was also detected in ordinary ApoE [−/−] mice with AS, but CD86 expression level was very low in normal ApoE [−/−] mice without CA1 overexpression. CD86 was distributed tightly surrounding the aortic intima of ApoE [−/−] mice without CA1 overexpression. This surface of M1 macrophages was decreased in the aortic tissues of animals subjected to MTZ-preventive treatment. CD163, which is expressed mainly by M2 macrophages, is expressed at relatively low levels and is expressed mainly in the inner layer of the cardiac aorta. Semiquantitative analysis of CD86 expression was performed on the basis of the signal density. The analysis revealed that CA1-overexpressing mice presented more M1-type macrophages in aortic tissues than did normal ApoE [−/−] mice when they were under normal conditions without AS induction, indicating that CA1-overexpressing mice had more M1-type macrophages in their aortic tissues than did ordinary ApoE [−/−] mice. Additionally, increased CD86 expression was detected in the aortic tissues of both CA1-overexpressing mice and ordinary ApoE [−/−] mice when they were induced to AS, and the expression decreased when these AS animals were treated with MTZ, indicating the important role of M1 macrophages in the AS process (Fig. 9).

4. Discussion

In the present study, ApoE [−/−] mice with CA1 overexpression were generated via CRISPR/Cas-mediated genome engineering. PCR assays demonstrated the successful establishment of CA1 [KI/KI] ApoE [−/−] mice. Mice with CA1 overexpression generally have greater body weights than do those without CA1 overexpression. Immunohistochemistry revealed increased CA1 expression in the cardiac aorta tissues of CA1-overexpressing mice, especially in the tissues of the AS animals. Sudan IV, HE and Oil Red O staining revealed more disrupted tissue structures, thicker aortic walls, more plaques, and more fat deposits in the aorta intima of AS mice with CA1 overexpression than in those of the AS ApoE [−/−] mice without CA1 overexpression. Additionally, extensive calcification was observed in the aortic tissues of CA1-overexpressing ApoE [−/−] mice with AS or AS following MTZ treatment, but little calcification was detected in ordinary ApoE [−/−] mice with AS. Consistent with the above observations, higher serum LDL, AI, TC and TG levels were detected in CA1-overexpressing mice than in ordinary ApoE [−/−] mice, and HDL levels were always lower, regardless of whether these two mouse strains were in AS conditions. LDL stimulates AS, whereas HDL reduces vascular calcification in AS [17]. HDL has antiatherogenic functions as a scavenger for atherosclerotic therapy [18]. The above observations indicate that CA1-overexpressing ApoE [−/−] mice are more susceptible to AS induction than ordinary ApoE [−/−] mice and demonstrate that CA1 expression can aggravate AS progression. The measurements also revealed that LDL, AI, TC and TG levels were declined, and HDL levels were elevated in the AS animals following MTZ treatment; moreover, the weights of the AS model mice were generally greater than those of the control mice and were reduced after MTZ treatment. The AS mice that received MTZ treatment or MTZ-preventive treatment presented an aortic structure with less plaque formation and thickening of the heart aorta intima, which was very similar to the observations of our previous study [10]. These results again verified the therapeutic effect of MTZ on AS progression. Additionally, the present study revealed obvious calcium deposition in the aortic tissues of both AS CA1-overexpressing mice and AS CA1-overexpressing mice treated with MTZ. These observations suggest that MTZ treatment can suppress CA1 activity and calcification, a process of calcium deposition, but the drug does not affect the formed calcium deposits, although MTZ can decrease LDL, AI, TC and TG levels and partially restore aortic structure.

The present study revealed more M1-type macrophages in the aortic tissue of CA1-overexpressing mice. These findings indicate that CA1 overexpression increased the number of M1-type macrophages in aortic tissues. Proinflammatory M1-type macrophages in coronary arteries are induced and accumulate in response to proatherogenic stimuli. M1-type macrophages prompt chronic inflammation in cardiac vessels in AS [19]. M1-type macrophages also predominantly promote initial calcium deposition within the necrotic core of lesions, called microcalcification, through vesicle-mediated mineralization resulting from the apoptosis of macrophages and VSMCs [25]. Cholesterol metabolism is integral to macrophage activation [21]. Cholesterol crystals can drive metabolic reprogramming and M1-type macrophage polarization in primary human macrophages [22]. On the other hand, CA1 can regulate acid–base balance by mediating carbon dioxide production [5,6]. Acid accumulation is associated with metabolic alterations such as energy expenditure, fat formation and protein intake [20]. Fat is an important raw material for cholesterol formation. AS is a chronic inflammatory disease characterized by the accumulation of lipids in the vessel wall, leading to the formation of an atheroma [23,24]. These

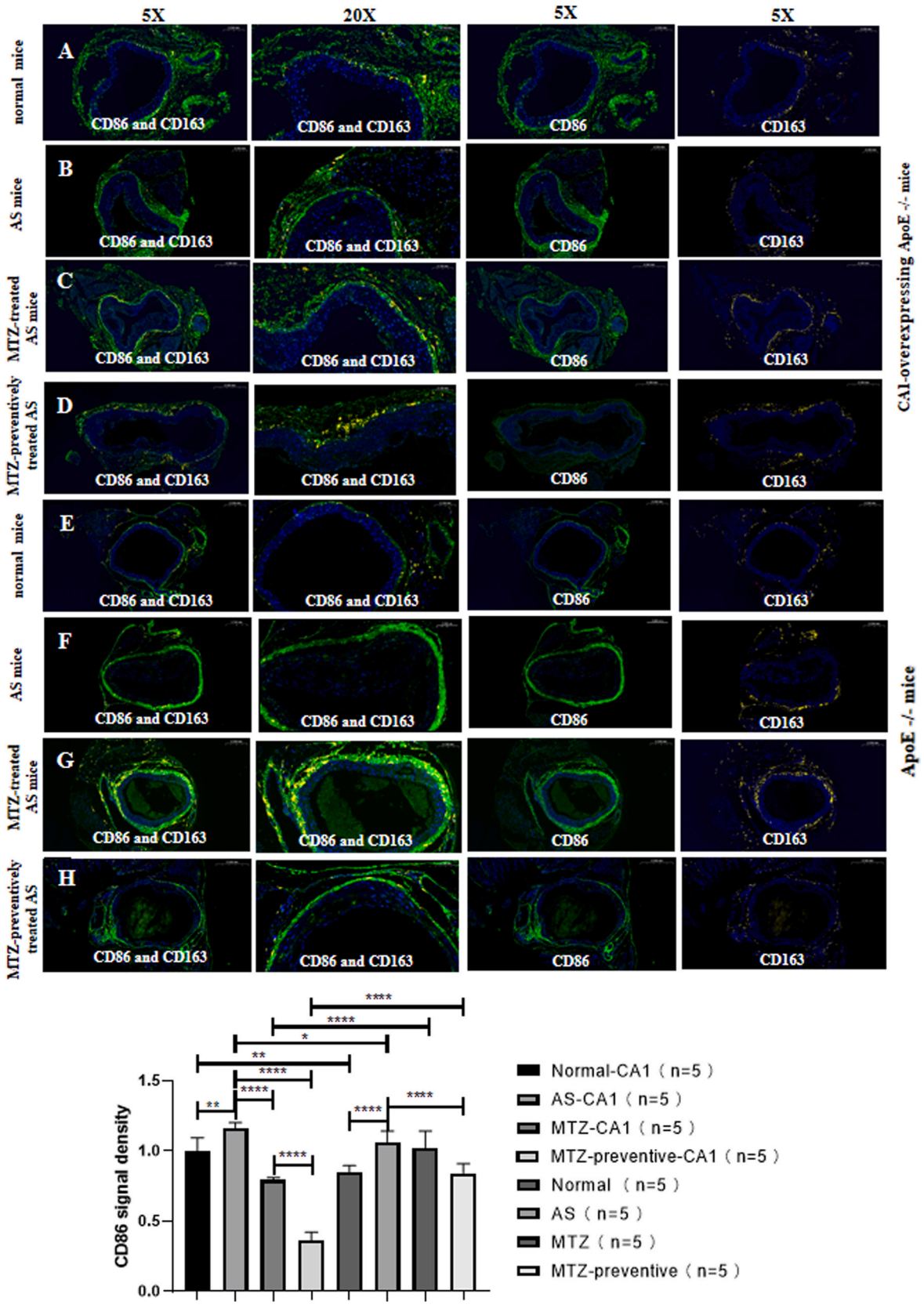

Fig. 9. Immunofluorescence analysis of macrophages in mouse cardiac aorta tissues. The aortic tissues were stained with DAPI (blue). CD86 (green) represents the M1 macrophage subtype, and CD163 (yellow) represents the M2 macrophage subtype. The expression levels of CD86 were semiquantitatively analyzed. Higher CD86 expression was detected in the aortic tissues of CA1-overexpressing mice than in those of ordinary Apoe [−/−] mice. Increased CD86 expression was detected in the aortic tissues of both CA1-overexpressing mice and ordinary ApoE [−/−] mice when they were induced to AS. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$.

proinflammatory M1-type macrophages are incapable of digesting lipids, thus resulting in foam cell formation in atherosclerotic plaques [13,14,24]. On the basis of our findings and those of other studies, we hypothesize that CA1 expression increases the proportion of M1-type macrophages in atherosclerotic aortic tissues by mediating acid base balance to stimulate aortic lipid deposits, inflammation and calcification in AS. A high proportion of M1-type macrophages thus contributes to the high severity of AS in CA1-overexpressing mice.

Plaque calcification develops via inflammation-dependent mechanisms involved in AS [21]. TNF-α antagonists may have a beneficial effect on preventing the progression of subclinical atherosclerosis and arterial stiffness [26]. We previously reported that M1-type macrophages secrete TNF-α to stimulate CA-1 expression and calcification in VSMCs *in vitro* [12]. In this study, calcification was strongly increased in VSMCs treated with β-glycerophosphate (β-GP), a chemical inducer of cellular calcification, following incubation with M1-type macrophages or their culture supernatants. M0 and M2 macrophages or their supernatants did not significantly stimulate calcification in VSMCs because we found that TNF-α levels were significantly increased in the culture medium of M1-type macrophages. Following transfection with anti-CA1 or CA2 siRNAs, β-GP-induced VSMCs showed decreased calcification, but the calcification level was partially increased when those VSMCs were incubated with the supernatants of M1-type macrophages. When VSMCs were treated with TNF-α without β-GP induction, calcification and CA1 and CA2 expression were also significantly increased. On the basis of the findings of the present study and previous studies of ours, we suggest that the increased number of M1-type macrophages produces a large amount of TNF-α, which prompts calcification in the VSMCs of the cardiac aorta and inflammation to aggravate AS progression. The possible regulatory effects of CA1 on AS progression are summarized in Supplementary Fig. 1.

Although some studies have clearly demonstrated a novel immunomodulatory role for CA1 in macrophage function [27], how CA1 increases the number of M1 macrophages is still unknown. The direction of macrophage polarization can be regulated by a series of factors, including interferons, lipopolysaccharides, interleukins, and noncoding RNAs. An imbalance in M1/M2 polarization is found in many autoimmune diseases [28]. Most likely, CA1 overexpression prompts autoimmunity to affect macrophage polarization, as Ando et al. reported [7].

We recently investigated the aortic tissues of ordinary ApoE [−/−] mice via single-cell sequencing and found atheroprotective B1/MZB B cells and $CD8^{+}CD122$ Treg-like cells as well as atherogenic Spp1+ macrophages in the tissues [29]. The present study established CA1-overexpressing ApoE [−/−] mice and compared these knock-in mice with ordinary ApoE [−/−] mice to identify the functional mechanism of CA1 in AS progression. The present study not only verified the stimulatory role of CA1 expression in AS progression by establishing CA1-overexpressing ApoE [−/−] mice but also revealed that CA1 plays an essential role by increasing the number of M1 macrophages in aortic tissues. Therefore, the present study obtained more in-depth results by comparing two strains of experimental animals.

There is a limitation of the present study. In the present study, CA1 was globally overexpressed in ApoE [−/−] mice. On the other hand, we detected increased CA1 expression in the cardiac aortas of CA1-expressing mice via immunohistochemistry, regardless of whether these mice were normal or had induced AS, and CA1 expression was further increased in the mice with AS. To date, many studies have investigated CA1 expression in different tissues, but no studies have reported which factors affect CA1 expression and tissue distribution. The human CA1 gene has differential promoters in erythroid and non-erythroid tissues [30], indicating a complicated regulatory mechanism in CA1 expression. Additionally, we do not know whether CA1 overexpression elevates body weight primarily by increasing body fat or body water/fluid. We have no data on body composition to clarify this question. Most likely, CA1 overexpression elevated body fat, as we detected increased oil deposition in the cardiac aortas of CA1-overexpressing mice.

5. Conclusion

In summary, this study confirmed that CA1-overexpressing knock-in ApoE [−/−] mice are more sensitive to AS induction and that CA1 can aggravate AS by increasing M1-type macrophages. Additionally, the current study again revealed that MTZ may be a potential drug for AS therapy.

Availability of data and materials

The data and materials that support the study are available from the corresponding author on reasonable request.

Ethics approval

All animals were housed under specific pathogen-free conditions and handled in accordance with the Helsinki Convention on Animal Protection and EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental design was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (QYFYWZLL27438).

Author contributions

Jinbao Zong: Investigation, Methodology, Data curation, Funding acquisition and data analysis and interpretation. Changyuan Wang, Hongji Zhou and Yu Song: Investigation, Methodology and Data curation. Kehua Fang: Data curation, Validation, Formal analysis, Resources, Writing-review & editing. Xiaotian Chang: Funding acquisition, Project administration, Supervision, Writing-review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors agree to take responsibility for all aspects of the research.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2025.03.003.

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