Loss of ACAT1 Attenuates Atherosclerosis Aggravated by Loss of NCEH1 in Bone Marrow-Derived Cells

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**Aim:** Acyl-CoA cholesterol acyltransferase 1 (ACAT1) esterifies free cholesterol to cholesteryl esters (CE), which are subsequently hydrolyzed by neutral cholesterol ester hydrolase 1 (NCEH1). The elimination of ACAT1 in vitro reduces the amounts of CE accumulated in Nceh1-deficient macrophages. The present study aimed at examining whether the loss of ACAT1 attenuates atherosclerosis which is aggravated by the loss of NCEH1 in vivo.

**Methods:** Low density lipoprotein receptor (Ldlr)-deficient mice were transplanted with bone marrow from wild-type mice and mice lacking ACAT1, NCEH1, or both. The four types of mice were fed a high-cholesterol diet and, then, were examined for atherosclerosis.

**Results:** The cross-sectional lesion size of the recipients of Nceh1-deficient bone marrow was 1.6-fold larger than that of the wild-type bone marrow. The lesions of the recipients of Nceh1-deficient bone marrow were enriched with MOMA2-positive macrophages compared with the lesions of the recipients of the wild-type bone marrow. The size and the macrophage content of the lesions of the recipients of bone marrow lacking both ACAT1 and NCEH1 were significantly smaller than the recipients of the Nceh1-deficient bone marrow, indicating that the loss of ACAT1 decreases the excess CE in the Nceh1-deficient lesions. The collagen-rich and/or mucin-rich areas and en face lesion size were enlarged in the recipients of the Acat1−/− bone marrow compared with those of the recipients of the WT bone marrow.

**Conclusion:** The loss of ACAT1 in bone marrow-derived cells attenuates atherosclerosis, which is aggravated by the loss of NCEH1, corroborating the in vitro functions of ACAT1 (formation of CE) and NCEH1 (hydrolysis of CE).

**Key words:** Cholesterol, Atherosclerosis, Macrophage, Inflammation, Foam cells
isoforms have been identified in mammals: ACAT1 and ACAT2. ACAT1 is ubiquitously expressed, especially in steroidogenic organs and macrophages, while ACAT2 is expressed exclusively in intestinal epithelial cells and hepatocytes where it is involved in lipoprotein assembly and secretion.

The hydrolysis of intracellular CE is the initial step of reverse cholesterol transport⁵. The enzymes hydrolyzing CE at neutral pH have been collectively called neutral CE hydrolases (NCEHs)⁶. Under certain circumstances, lysosomal acid lipase may be involved in reverse cholesterol transport, because of the potential involvement of autophagy⁵. We have shown previously that neutral cholesterol ester hydrolase 1 (NCEH1)⁶,⁷, also known as KIAA1363 or arylacetamide deacetylase-like 1, is responsible for most of the activity in human monocyte-derived macrophages⁸ and half of the activity in mouse peritoneal macrophages, where the rest of the activity is mediated by hormone-sensitive lipase (HSL, LIPE)⁹-¹¹). The overexpression of NCEH1 inhibited the accumulation of CE in THP-1 macrophages⁹. Conversely, the pharmacological and genetic inhibition of NCEH1 increased the accumulation of CE in peritoneal macrophages treated with acetylated low density lipoproteins (acLDL)⁸,¹⁰. In an experiment in which mice deficient in LDL receptor (Ldlr⁻/⁻) were transplanted with bone marrow from wild-type (WT) and Nceh1⁻/⁻ mice and, then, fed a high-cholesterol diet⁹, the recipients of the Nceh1⁻/⁻ bone marrow developed larger atherosclerotic lesions than the recipients of the WT bone marrow⁹.

### Aim

To test whether the elimination of ACAT1 in the macrophages mitigates the worsening of atherosclerosis in Ldlr⁻/⁻ mice whose bone marrow is transplanted from Nceh1⁻/⁻ mice, we transplanted bone marrow from WT and mice which lack NCEH1, ACAT1 or both to Ldlr⁻/⁻ mice and we compared the degree of atherosclerosis after feeding them a high-cholesterol diet.

### Methods

#### Animals

Acat1⁻/⁻¹²) and Nceh1⁻/⁻ mice⁹ were generated as described previously. Mice lacking both ACAT1 and NCEH1 (Acat1⁻/⁻;Nceh1⁻/⁻) were generated by mating the mutant genes. In brief, the heart was perfused with saline containing EDTA for the separation of plasma. Kits (Determiner TC II, Kyowa Medex and L-Type TG M, Wako) were used to determine the plasma levels of total cholesterol (TC) and triglyceride (TG) enzymatically. High performance liquid chromatography (HPLC) analyses of plasma were performed, as described previously¹⁴).

#### Plasma Lipids and Lipoproteins

After a 16-h fast, blood was collected into tubes containing EDTA for the separation of plasma. Kits (Determiner TC II, Kyowa Medex and L-Type TG M, Wako) were used to determine the plasma levels of total cholesterol (TC) and triglyceride (TG) enzymatically. High performance liquid chromatography (HPLC) analyses of plasma were performed, as described previously¹⁴).

### Quantification of Atherosclerotic Lesions

After feeding with HCD, the mice were euthanized and their aortas and hearts were isolated for the evaluation of cross-sectional lesion area at the aortic roots and en face atherosclerotic lesion area of the aorta. The cross-sectional lesion area was evaluated according to the method of Paigen et al.¹⁵) with slight modifications. In brief, the heart was perfused with saline containing 4% (w/v) formalin, and was fixed for more than 48 h in the same solution. The basal half of the heart was embedded in Tissue-Tek OCT compound (Sakura Finetek), and serial sections were cut using

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cryostat (6 µm thick), as described previously. Four sections, each separated by 60 µm, were used to evaluate the lesions; two at the end of the aortic sinus and two at the junctional site of sinus and ascending. The sections were stained with Oil red O (ORO) and counterstained with hematoxylin to quantify the atherosclerotic plaque area and the neutral lipid positive area. The en face lesion area of the aorta was evaluated as described previously. In brief, the aorta was opened from the aortic root to the iliac bifurcation, and was pinned out flat on a wax surface. Atheromatous plaques in the aorta were visualized by staining with Sudan IV, and the luminal side of the stained aorta was photographed. Adobe Photoshop 6 image analysis software was used to perform the image capture and analysis. The extent of atherosclerosis was expressed as the percent of surface area of the entire aorta covered by lesions.

**Histology**

The sections were stained with hematoxylin and eosin (HE), Movat’s pentachrome, or Masson trichrome. HE staining was used to determine the size of the necrotic core. Immunostaining was performed as described previously. In brief, the sections were incubated with the primary antibody against mouse MOMA-2 (1:600; Bio-Rad) or α-SMA (1:100; abcam) overnight at 4°C. After washing, the sections were incubated with biotinylated anti-rat antibody (1:200; Vector Labs) or anti-rabbit antibody (1:200; Vector Labs) for 2 h at 37°C, and then with avidin-biotin peroxidase complex (Vector Labs) for 30 min. Last, the sections were developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma), and counterstained with hematoxylin.

**Preparation of Peritoneal Macrophages**

Peritoneal macrophages were obtained 3 days after the intraperitoneal injection of 2 ml of 5% thioglycolate broth. The macrophages were plated to each well of 12-well plates, and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics for 3 h. Thereafter, the non-adherent cells were washed with PBS.

**Preparation of Lipoproteins**

Blood was drawn from healthy volunteers and LDL (d=1.019–1.063 g/ml) and lipoprotein deficient serum (LPDS; d>1.21) were isolated by ultracentrifugation. LDL was acetylated with acetic anhydrate as described previously. Protein concentrations were determined by the BCA kit (Pierce).

**CE Formation Assay**

Following a 24-h incubation period in medium containing 5 mg/ml of LPDS, mouse peritoneal macrophages were incubated with 100 µg/ml of acLDL, 5 mg/ml of BSA, and 0.1 mM [1-14C]oleate-albumin complex at 37°C for 24 h. CE formation was determined as described previously. Protein concentrations were determined by the BCA kit (Pierce).

**Cellular Neutral Lipids**

The macrophages were incubated with 100 µg/ml of acLDL in DMEM containing 5 mg/ml of BSA for 24 h in a Lab-Tek II Chamber slide system (Thermo Scientific). After having been fixed with 4% paraformaldehyde, the macrophages were stained with Oil red O.

**Statistics**

The data were presented as means ± S.D. The one way analysis of variance (ANOVA) was used for multiple comparisons. When the ANOVA results were statistically significant (i.e., p<0.05), then, individual comparisons were made with the Tukey post-hoc test.
Results

To assess the role of ACAT1 in the worsening of atherosclerosis in the \textit{Ldlr}^{-/-} mice whose bone marrow was transplanted with that from \textit{Nceh1}^{-/-} mice, we transplanted the bone marrow obtained from male WT, \textit{Acat1}^{+/+}, \textit{Nceh1}^{+/+}, \textit{Acat1}^{-/-};\textit{Nceh1}^{-/-} mice into female \textit{Ldlr}^{-/-} mice which were irradiated to eliminate the endogenous bone marrow. Four weeks after the transplantation, the recipient mice were fed HCD for 12 additional weeks.

PCR-assisted amplification of the \textit{Sry} gene (a male marker) and \textit{Acat1} or \textit{Nceh1} mutant gene was used to verify the successful reconstitution of recipients with cells of donor origin after the bone marrow transplantation (Supplemental Fig. 1).

Bone marrow specific inactivation of the \textit{Acat1} and/or \textit{Nceh1} gene did not significantly affect the body weight, plasma lipids and lipoprotein fractions in \textit{Ldlr}^{-/-} mice before and after feeding with HCD (Table 1 and Supplemental Fig. 2). All the types of mice developed severe hypercholesterolemia.

After feeding with HCD for 12 weeks, atherosclerosis was evaluated by cross-sectional analysis of aortic roots and the \textit{en face} surface lesion area of the aorta. Regarding the size of the cross-sectional lesions (Fig. 1), there was no difference between the recipients of the WT bone marrow and those of the \textit{Acat1}^{-/-} bone marrow. The recipients of \textit{Nceh1}^{-/-} bone marrow showed a 1.6-fold increase in the lesions compared with those of the WT bone marrow. The recipients of the \textit{Acat1}^{-/-};\textit{Nceh1}^{-/-} bone marrow showed a significant decrease of the lesions to a level indistinguishable from the recipients of the WT and \textit{Acat1}^{-/-} bone marrow.

Regarding the size of \textit{en face} lesion area (Supplemental Fig. 3), the recipients of the \textit{Acat1}^{-/-} bone marrow showed a 2.2-fold increase compared with those of the WT bone marrow. The recipients of the \textit{Nceh1}^{-/-} bone marrow showed a 2.1-fold increase in the lesions compared with those from the WT bone marrow (\(p < 0.01\)). There was no difference between the recipients of the \textit{Acat1}^{-/-} and \textit{Nceh1}^{-/-} bone marrows. The lesion size of the recipients of the \textit{Acat1}^{-/-};\textit{Nceh1}^{-/-} bone marrow was not significantly different from either that of the \textit{Acat1}^{-/-} bone marrow or the \textit{Nceh1}^{-/-} bone marrow.

To determine the characteristics of the lesions, we employed several different ways to stain the cross-sectional sections (Figs. 2 and 3). ORO staining was performed to visualize neutral lipids, mostly CE, accumulated in the lesions. The percentage of the ORO-positive area of the recipients of the \textit{Acat1}^{-/-} bone marrow was significantly smaller than that of the WT bone marrow by 44\% (\(p < 0.01\)) (Fig. 3A). The percentage of the ORO-positive area of the recipients of \textit{Nceh1}^{-/-} bone marrow was larger than that of the WT bone marrow by 34\% (\(p < 0.05\)). The percentage of the ORO-posi-
The percentages of the area filled with mucins of the recipients of $Acat1^{-/-}$ and $Acat1^{-/-};Nceh1^{-/-}$ bone marrow were 2.2-fold and 1.9-fold larger than that of the WT bone marrow, respectively. There was no difference in the percentage between the recipients of the $Nceh1^{-/-}$ bone marrow and those of the WT bone marrow.

Masson trichrome staining was performed to estimate the amounts of collagen (Figs. 2 and 3D). The percentages of the area filled with mucins of the recipients of $Acat1^{-/-}$ and $Acat1^{-/-};Nceh1^{-/-}$ bone marrow were 2.2-fold and 1.9-fold larger than that of the WT bone marrow, respectively. There was no difference in the percentage between the recipients of the $Nceh1^{-/-}$ bone marrow and those of the WT bone marrow.

Movat’s pentachrome staining was performed to estimate the area filled with mucins (Figs. 2 and 3C). The percentages of the area filled with mucins of the recipients of $Acat1^{-/-}$ and $Acat1^{-/-};Nceh1^{-/-}$ bone marrow were 2.2-fold and 1.9-fold larger than that of the WT bone marrow, respectively. There was no difference in the percentage between the recipients of the $Nceh1^{-/-}$ bone marrow and those of the WT bone marrow.

**Fig. 2.** Morphological comparison of atherosclerotic lesions in the recipients of WT, $Acat1^{-/-}$, $Nceh1^{-/-}$, $Acat1^{-/-};Nceh1^{-/-}$ bone marrow.

Representative images of aortic root stained with HE, Movat’s pentachrome, Masson trichrome, antibodies against macrophages (MOMA-2) or vascular smooth muscle cells ($\alpha$-SMA). The Movat’s pentachrome stains nuclei and elastic fibers black, collagen yellow, mucins blue to green, muscle red, and fibrin intense red.
Fig. 3. Quantification of ORO-positive area (A), necrotic core area (B), mucin-positive area (C), collagen-positive area (D), macrophage-dominated area (E) and vascular smooth muscle cell-dominated area (F) in the atherosclerotic lesions in the recipients of WT, Acat1−/−, Nceh1−/−, Acat1−/−;Nceh1−/− bone marrow.

(A) Quantification of ORO-positive area as a percentage of whole plaque area (n=13−18 animals per group). (B) Quantification of necrotic core from HE staining as a percentage of whole plaque area (n=6 animals per group). (C) Quantification of mucin-positive area from Movat's pentachrome (n=6 animals per group). (D) Quantification of collagen-positive area from Masson trichrome staining as a percentage of whole plaque area (n=5−6 animals per group). (E) Quantification of MOMA-2 positive area as a percentage of whole plaque area (n=5−6 animals per group). (F) Quantification of α-SMA positive area as a percentage of whole plaque area (n=5−6 animals per group). Values are expressed as means ± S.D. *p<0.05, **p<0.01, ***p<0.001.
estimate macrophage contents (Figs. 2 and 3E). The percentage of macrophage content in the recipients of the \textit{Nceh1^{-/-}} bone marrow tended to be larger than that of the WT bone marrow; however, the difference was not statistically significant.

Immunostaining for \(\alpha\)-SMA was performed to estimate the distribution and amounts of vascular smooth
muscle cells (VSMCs) (Figs. 2 and 3F). α-SMA-positive cells were primarily distributed in the subendothelial areas and media. There were no differences in the amounts of the α-SMA-positive cells among the four types of mice.

Next, we compared the ORO staining (Fig. 4A), the amounts of CE formed from oleate (Fig. 4B) in peritoneal macrophages cultured in the presence of 100 μg/ml acLDL in the medium for 24 h among the four types of mice. Numerous ORO-positive lipid droplets were observed in WT and Nceh1-deficient macrophages, while they were barely detectable in Acat1-/- and Acat1-/-;Nceh1-/- macrophages (Fig. 4A). The CE formation was drastically reduced to almost undetectable level in both Acat1-/- and Acat1-/-;Nceh1-/- macrophages (Fig. 4B). The CE formation in Nceh1-/- macrophages was 1.4-fold higher than that in the WT macrophages (p < 0.05).

Discussion

In the present study, we show that loss of both ACAT1 and NCEH1 in the bone marrow-derived cells reversed the size of the cross-sectional atherosclerotic lesion area which was aggravated by the loss of NCEH1 in the bone marrow-derived cells in Ldlr-/- after feeding with HCD. In parallel, the loss of both ACAT1 and NCEH1 decreased the ORO-positive area, macrophage content and necrotic area, which were worsened by the loss of NCEH1. These results provide in vivo proof of our premise that NCEH1 mediates the hydrolysis of CE, which is counteracted by the action of ACAT1, cholesterol esterification, in macrophages. The loss of ACAT1 may abrogate the pro-atherogenic effects of loss of NCEH1 by suppressing the supply of its substrate, FC. Since the differences in the ORO-positive area among the different genotypes of mice (Fig. 3A) were largely proportional to the differences in CE contents of the macrophages in culture (Fig. 4A and 4B), the ORO-positive area may faithfully reflect the degree of CE accumulation in each macrophage in this model.

It is worth discussing the data which were apparently different from those reported by previous reports, including ours. In the present study, first, the cross-sectional lesion area of the recipients of the Acat1-/- bone marrow was not different from those of the WT bone marrow (Fig. 1). This was apparently inconsistent with the results of en face lesion size (Supplemental Fig. 3) and those reported by Fazio et al. in which the lesions of the recipients of the Acat1-/- bone marrow were larger than those of the WT bone marrow22, 23. Although we and Fazio et al. used the same diet (1.25% cholesterol), we fed the mice longer than Fazio et al. did (12 w vs 10 w). Potential reasons for the inconsistency may be the difference in the duration of the feeding with HCD and other unknown factors. However, the histological analyses showed that the mucin-positive and collagen-positive area were enlarged in the lesions of the recipients of the Acat1-/- bone marrow compared with those of the WT bone marrow (Figs. 2 and 3CD), indicating that increased production of mucin, complex mixtures of glycosaminoglycans and proteoglycans secreted from VSMCs,24 and increased fibrosis. Similar stimulation of fibrosis was reported in apoE-null mice treated with K-604, ACAT1 selective inhibitor.25

It is also puzzling to note that the results of the recipients of the Acat1-/- bone marrow were inconsistent between the cross sectional and en face analyses (Fig. 1 and Supplemental Fig. 3). Site-specific discrepancy has been reported in several studies examining the effects of drugs on the lesions. For example, pravastatin inhibited the lesions at brachiocephalic arteries and aortic roots, but not on the surface of the aorta.26 Although the precise reasons are unknown, we speculate that the stage of the lesions which are dependent on age or the duration of feeding with HCD is a potential contributing factor. Our preliminary study showed that the lesions of the aortic roots of the recipients of Acat1-/- bone marrow were larger than those of the recipients of the WT bone marrow at 2 months of feeding, which are apparently similar to the results of the surface lesions at 3 months of feeding in the current study (Supplemental Fig. 3). It is well known that the lesions develop at aortic roots first and progress distally to thoracic, abdominal aorta, and iliac arteries.27 Therefore, it is reasonable to speculate that the stage of the lesions of the aortic roots were more advanced than that of the aortic surface. Since the lesions of the recipients of the Acat1-/- or Acat1-/-;Nceh1-/- bone marrow were much more fibrotic than the recipients of the WT or Nceh1-/- bone marrow, the expansion of the lesions of the recipients of the Acat1-/- or Acat1-/-;Nceh1-/- bone marrow might be halted by fibrosis at a certain stage between 2 and 3 months of feeding, whereas the expansion of the lesions of the recipients of the WT or Nceh1-/- bone marrow might not be restricted by fibrosis. Although we did not examine the pathology of the lesion of the aortic surface, they might reflect the earlier stage than those at the aortic root, thus, sparing the restrictive effects of extensive fibrosis at later stages.

Theoretically, the cells positive for ORO staining should not be derived from bone marrow cells which lack ACAT1. Therefore, the ORO-positive cells observed in the recipients of the Acat1-/- bone marrow might be originated from the VSMCs of the recipients, because the VSMCs can be transformed to cells with macro-
phage-like property. The loss of ACAT1 in macrophages of the donors might stimulate transformation of VSMCs of the recipients into macrophage-like cells, thereby, causing apparent enlargement of ORO-positive area. Staining for α-SMA showed that only the subendothelial areas contained VSMCs and the intensity of ORO staining was not different between the VSMC-dominant areas and the macrophage-dominant areas (Figs. 1 and 2). Therefore, it is more likely that lipoproteins deposited in extra-cellular matrix (ECM) are positive for ORO staining in the recipients of the Acat1−/− or Acat1−/−;Nceh1−/− bone marrow. More studies are needed to determine the identity of the ORO-positive cells/or ECM in the recipients of the Acat1−/− bone marrow.

Conflicting results have been reported regarding the effects of the inhibition of ACAT1 on the atherosclerosis. In contrast to the mouse model of bone marrow transplantation mentioned above, models of systemic and myeloid cell-specific deletion of ACAT1 and most of pharmacological studies using non-selective and selective ACAT1 inhibitors have shown the protection against atherosclerosis. Although, currently, precise reasons are unknown, we would speculate that the type of non-macrophage cells whose ACAT1 is inhibited is a crucial determinant. According to Rong et al., for example, the inhibition of ACAT1 is nontoxic in VSMCs and, thus, protects against atherosclerosis. Based on the premise, if the contribution of VSMCs to the atherosclerosis dominates over that of the bone marrow-derived cells, global knockout, and pharmacological inhibition of ACAT1 may be anti-atherogenic. According to Yang et al., the inhibition of ACAT1 potentiates effector function and proliferation of pro-atherogenic CD8+ T cells. If this is the case, the pro-atherogenic effects of ACAT1 inactivation in the bone marrow-derive cells can be attributable to the phenotypes of this lymphoid lineage. Together, it is plausible that the role of ACAT1 inhibition in atherogenesis might be dependent on the cell types. Further studies are warranted to define the role ACAT1 in each type of cell in the development of atherosclerosis.

Moreover, the current results suggest that the roles of ACAT1 inhibition in atherogenesis are facet-specific. The loss of ACAT1 in the bone marrow-derived cells seems anti-atherogenic judged by the reduced number of foam cells (Fig. 3A) and increased deposition of collagen which is thought to stabilize plaques (Fig. 3D). Under such a condition as NCEH1-deficiency where foam cell formation is dominant, ACAT1 inhibition is likely to be anti-atherogenic. On the other hand, ACAT1 inhibition can be pro-atherogenic judged by the enlargement of mucin-positive area (Fig. 3C). Cell death can be both pro-atherogenic and anti-atherogenic depending on the contexts. If the surrounding cells have sufficient capacity to clear the dead cells, cell death will lead to involution of the plaque, thus, anti-atherogenic; if not, the presence of dead cells elicits inflammation, thereby, recruiting more inflammatory cells and transforming VSMCs to macrophage-like cells, thus, pro-atherogenic. Probably, all of these are the reasons why clinical trials have not been successful to inhibit the plaque volume in the coronary arteries.

**Conclusion**

Loss of ACAT1 in bone marrow-derived cells attenuates atherosclerosis, which is aggravated by loss of NCEH1. These results are in agreement with the in vitro functions of ACAT1 (formation of CE) and NCEH1 (hydrolysis of CE).

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**Conflicts of Interests**

There is nothing to disclose with regard to this topic.

**Footnotes Added in the Proof**

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**Supplemental Fig. 1.** Successful reconstitution of the transplanted bone marrow

Bone marrow from mice with the indicated genotypes or tails (lane A and B) were used for isolation of genomic DNA. Tail DNA from a female WT mouse was used as a negative control for lane A of all the 4 gels. DNA isolated from the tails of $\text{Acat1}^{--}$, $\text{Nceh1}^{+/--}$, $\text{Ldlr}^{+/--}$ or male WT mouse was used as templates for PCR amplification of the mutant genes of $\text{Acat1}$, $\text{Nceh1}$, $\text{Ldlr}$ or $\text{Sry}$, respectively. Respective PCR products were loaded to lane B of each gel.
Supplemental Fig. 2. Lipoprotein profiles analyzed by HPLC.
Mice (n=13–18) were fed a HCD diet for 12 weeks and plasma lipoprotein profiles were analyzed by HPLC. After a 16-h fast, blood samples were collected, pooled and subjected to HPLC.
Supplemental Fig. 3. En face surface lesion areas of aorta in Ldlr⁻/⁻ mice transplanted with WT, Acat1⁻/⁻, Nceh1⁻/⁻, Acat1⁻/⁻;Nceh1⁻/⁻ bone marrow.

The aorta of mice used for the experiment shown in Figs. 1-3 and Table 1 were stained with Sudan IV. (A) Representative macroscopic images. (B) Quantified lesion areas. The data was calculated as the percentage of surface lesion area of entire aorta. Values are expressed as means±SD. *p<0.05, **p<0.01.