Objective—Recent genome-wide association studies revealed that a genetic variant in the loci corresponding to histone deacetylase 9 (HDAC9) is associated with large vessel stroke. HDAC9 expression was upregulated in human atherosclerotic plaques in different arteries. The molecular mechanisms how HDAC9 might increase atherosclerosis is not clear.

Approach and Results—In this study, we show that systemic and bone marrow cell deletion of HDAC9 decreased atherosclerosis in LDLr−/− (low density lipoprotein receptor) mice with minimal effect on plasma lipid concentrations. HDAC9 deletion resulted upregulation of lipid homeostatic genes, downregulation of inflammatory genes, and polarization toward an M2 phenotype via increased accumulation of total acetylated H3 and H3K9 at the promoters of ABCA1 (ATP-binding cassette transporter), ABCG1, and PPAR-γ (peroxisome proliferator-activated receptor) in macrophages.

Conclusions—We conclude that macrophage HDAC9 upregulation is atherogenic via suppression of cholesterol efflux and generation of alternatively activated macrophages in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2014;34:1871-1879.)

Key Words: atherosclerosis ■ epigenomics ■ histones ■ histone deacetylases ■ macrophages

Recent genome-wide association studies revealed that a genetic variant in the loci corresponding to histone deacetylase 9 (HDAC9) is associated with large vessel stroke.1 Moreover, HDAC9 is also a common genetic variant that share genetic susceptibility to ischemic stroke and coronary artery disease.2 HDAC9 expression was upregulated in human atherosclerotic plaques in different arteries.3 The molecular mechanisms how HDAC9 region might increase the risk of atherosclerosis is unknown.

There are 18 mammalian HDACs, which fall into 4 classes on the basis of their structural and biochemical characteristics.4 HDAC9 is expressed in heart, pancreatic islets, neuron, spinal cord, teeth, smooth and skeletal muscles, T lymphocytes, endothelium, and adipose tissues.4 One of the best characterized mechanisms of action of HDAC9 is its ability to partner with MEF-2 (myocyte enhancer-binding factor) and repress p53-inducible genes.5 Macrophages consist of ≥2 subgroups M1 and M2. Whereas M1 macrophages are proinflammatory and have a central role in atherosclerosis development and plaque rupture, M2 macrophages are associated with response to anti-inflammatory reactions, tissue remodeling, fibrosis, and atherosclerosis regression.6 Recent studies demonstrate that among HDACs, HDAC3 and HDAC7 have been identified to play a key role in inflammatory gene expression program and alternative activation of macrophages.7,8 However, the role of HDAC9 in these processes in macrophages is unknown. Understanding whether HDAC9 plays a role in macrophage development and the pathogenesis of atherosclerosis will provide the rationale for the development of selective HDAC9 inhibitors that target macrophages for the treatment and prevention of atherosclerosis because of its significance with GWAS (Genome-Wide Association Study) study.

In the present study, we report that HDAC9 is expressed in macrophages and its role in cholesterol homeostasis and inflammation in macrophages and on atherosclerosis development. Our experiments revealed that systemic and bone marrow (BM) cell HDAC9 deficiency in LDLr−/− (low density lipoprotein receptor) mice reduced atherosclerosis. HDAC9 deletion resulted upregulation of lipid homeostatic genes, downregulation of inflammatory genes, and polarization of macrophages toward M2-like phenotype via increased accumulation of acetylated H3 at the promoters of ABCA1 (ATP-binding cassette transporter), ABCG1, and PPAR-γ (peroxisome proliferator-activated receptor) in macrophages. We conclude that macrophage HDAC9 upregulation is...
We determined the effect of HDAC9 deletion in genes expression levels in lesions that play key roles in atherogenesis in vivo. Real-time polymerase chain reaction analysis was done in pooled samples of aortic roots from the SKO and DKO mice. Aortic roots of DKO mice had increased mRNA expression of ABCA1, ABCG1, and arginase-1, decreased expression of interleukin-1β and MCP-1 (monocyte chemoattractant protein), but no change in expression of CD36, scavenger receptor class A (SRA), tumor necrosis factor-α, and inducible nitric oxide synthase (iNOS) compared with SKO mice (Figure IV in the online-only Data Supplement).

**Deletion of HDAC9 in BM Cells Inhibits the Formation of Atherosclerosis**

HDAC9 is expressed in multiple tissues including endothelial cells, T lymphocytes, and adipose tissues. To test the hypothesis whether HDAC9 expressed on hematopoietic cells (eg, monocytes/MΦ, lymphocytes, and platelets) versus endothelial cells or all the cell types those promote the development of atherosclerosis lesions, we transplanted male SKO and DKO BM into female LDLr−/− recipient mice (Figure V in the online-only Data Supplement). Five weeks after transplantation, the recipient mice were fed an atherogenic diet for 16 weeks and euthanized. Plasma total cholesterol, free cholesterol, and cholesterol ester levels were similar, but triglyceride concentrations were reduced in LDLr−/− mice reconstituted with DKO versus SKO BM (Figure 2A). Very-low-density lipoprotein cholesterol was reduced, whereas HDL was increased in mice receiving DKO BM (Figure 2B and 2C). LDLr−/− mice transplanted with DKO versus SKO BM had decreased visible atherosclerotic plaques in the aortic arch (Figure 2D), aortic surface lesion area (Figure 2E and 2F), and aorta root intimal area and lipid staining (Figure 2G–2I). Although BM transplantation experiments suggested that the possible deletion of macrophage HDAC9 was sufficient to decrease atherosclerosis, a beneficial role for other BM cell types in this process cannot be excluded.

**HDAC9 Expression Is Increased During Macrophage Differentiation**

To determine the role of HDAC9 biological and pathological functions in macrophages, we performed following experiments. We first determined HDAC9 expression in human THP-1 (acute monocytic leukemia cell) monocytes during in vitro differentiation into macrophages and mouse BM-derived macrophages. HDAC9 mRNA was most highly expressed in differentiated macrophages (Figure 3A and 3B). HDAC9 is alternatively spliced to generate different proteins. Among these, 2 major isoforms (HDAC9—a form containing HDAC domain and a truncated form HDRP (HDAC-related protein)/MITR (MEF-2 interacting transcription repressor protein) without HDAC domain but acquire deacetylase activity by recruitment of HDAC1 or HDAC3) expressed in tissue-specific manner. These major isoforms were expressed in mouse and human macrophages (Figure 3C and 3D). HDAC9 mRNA expression is further increased in response to oxidized low-density lipoprotein, acetylated low-density lipoprotein, and toll-like receptor (TLR) (LTA [lipoteichoic acid], LPS [lipopolysaccharide], flagellin but not CpG [cytosine–phosphate–guanine] DNA) signals and thioglycollate-elicited peritoneal macrophages (TEPMs; Figure VI in the online-only Data Supplement).
HDAC9 Deficiency in Macrophages
Results Increased Cholesterol Eflux via Increased Expression of ABCA1 and ABCG1 via Accumulation of Acetylated Histone at H3 and H4 at Promoters
Cholesterol efflux capacity from macrophages via ABCA1 and ABCG1 has a strong inverse relationship with atherosclerosis development. To determine the role of macrophages HDAC9 in cholesterol efflux pathway, we performed following experiments. HDAC9-deficient macrophages demonstrated increased expression of the cholesterol efflux genes ABCA1 and ABCG1 with acetylated low-density lipoprotein and liver X receptor (LXR) ligand stimulation compared with wild-type mice (both HDAC9-deficient and wild-type mice were on C57/
Bl6 back ground fed on chow diet) in vitro (Figure VIIA and VIIB in the online-only Data Supplement). Apolipoprotein A1– and HDL-mediated cholesterol efflux was increased in HDAC9-deficient versus wild-type macrophages (Figure VIIC and VIID in the online-only Data Supplement). ACAT (acyl-coenzyme A:cholesterol acyltransferase) inhibition further increased apolipoprotein A1– but not HDL-mediated cholesterol efflux in HDAC9-deficient (Figure VIIIE and VIIF in the online-only Data Supplement). To determine whether cholesterol efflux genes were upregulated in macrophages...
from atherogenic diet-fed mice, TEPMs were isolated from SKO and DKO mice fed an atherogenic diet for 16 weeks. TEPMs from DKO mice had significantly less cholesterol ester compared with foam cells from SKO mice (Figure 4A), consistent with the finding of decreased atherosclerosis in DKO mice. Expression of ABCA1 and ABCG1 in DKO macrophages was increased compared with SKO macrophages (Figure 4B–4D). HDAC9 regulates gene expression via multiple mechanisms, including protein–protein interaction and histone and nonhistone protein acetylation, but these pathways are not necessarily mutually exclusive.5,16,17 Western blot analyses revealed no global changes in histone acetylation in HDAC9-deficient macrophages (Figure VIII in the online-only Data Supplement). Control of inducible gene expression is dependent on signal-induced transcriptional elongation. This process is facilitated by deposition of histone acetylation mark associated with transcriptional activation at the promoters.18 Using stable isotope labeling in combination with mass spectrometry analysis in splenocytes from knockout and MRL (Murphy Roths Large)/LprHDAC9+/+ (wild-type) mice, we had previously demonstrated that lysine residues H3K9 and H3K18 were hyperacetylated (≈5-fold) in splenocytes from HDAC9-deficient mice.17 To investigate the consequences of HDAC9 deletion in macrophages and these histone acetylation marks, we performed chromatin immunoprecipitation (ChIP) assay using total H3, H4, site specific H3K9ac, and H3K18 ab. Quantitative ChIP assays demonstrated increased levels of total H3, H4, and H3K9 acetylation but not H3K18 acetylation at the promoters of ABCA1 and ABCG1 in HDAC9-deficient macrophages compared with controls fed on atherogenic diet (Figure 4E and 4F). We also performed ChIP assays in BM-derived macrophages from these 2 groups of mice loaded with acetylated low-density lipoprotein in vitro. Similar results were observed in ChIP assay as above (Figure IXB and IXC in the online-only Data Supplement). There was no increase in deposition of these acetylation marks in SRA promoter where transcription was unchanged between HDAC9 versus wild-type macrophages (Figure IXD in the online-only Data Supplement).
arginase-1, and interleukin-10) in the basal states compared with wild-type mice fed on atherogenic diet (Figure 5C). Compared with wild-type mice macrophages, the macrophages from HDAC9-deficient mice fed on chow diet had a significant increase in basal, interleukin-4, and PPAR-γ agonists (rosiglitazone) stimulated expression of CD206, MGL-1, MGL-2, arginase-1, Chi3l3, and interleukin-10 mRNA (Figure X in the online-only Data Supplement). The upregulation of M2 and downregulation of M1 genes in HDAC9-deficient macrophages are probably through PPAR-γ pathway.19 Expression of PPAR-γ in DKO macrophages was increased compared with SKO macrophages (Figure 5D). Quantitative ChIP assays demonstrated increased levels of total H3, H4, H3K9 but not H3K18 acetylation at the promoters of PPAR-γ (Figure 5E) but not at the arginase-1 promoter (Figure XI in the online-only Data Supplement).

These in vitro and ex vivo experiments in macrophages support the concept that increased HDAC9 expression in macrophages is atherogenic via suppression of cholesterol efflux and generation of alternatively activated macrophages via decreased accumulation of histone acetylation at ABCA1, ABCG1, and PPAR γ promoters. The consequences of HDAC9 deletion in macrophages on a genome-wide scale in response to different toll-like receptor agonists or infections need further investigations.

**Discussion**

In this study, we report several new findings. Among class IIa HDACs, HDAC9 is most abundantly expressed during macrophage differentiation. Systemic and macrophage HDAC9 deficiency reduces atherosclerosis development in different sites in LDLr−/− mice fed an atherogenic diet. The molecular mechanisms behind the decreased atherosclerosis in HDAC9-deficient mice are likely multifactorial, including increased macrophage cholesterol efflux by ABCA1 and ABCG1 and phenotypic switching of macrophages from a proinflammatory M1 to a less inflammatory M2 state via PPAR-γ.

We have not observed cardiac hypertrophy or polydactyly in DKO mice in contrast to previously reported studies in HDAC9 knockout mice.20,21 The difference between the phenotypes observed between ours and others are most likely because of difference in background (C57Bl6 versus mixed 129 and C57Bl6) and age of the mice studied. Interestingly, in contrast to Chatterjee et al study, the double knockout (LDLr−/−HDAC9−/−) mice have increased body, liver, and adipose tissue weight compared with single knockout (LDLr−/−) mice on the atherogenic diet; these phenotypes were neither observed in chow-fed mice nor during atherogenic diet feeding of LDLr−/− mice transplanted with HDAC9 knockout versus wild-type BM (data not shown). The difference between the phenotypes observed between theirs and ours are most likely because of difference in background (C57Bl6 versus LDLr) and diet. The mechanisms behind the phenotypes observed with systemic deficiency of HDAC9 in atherogenic diet-fed LDLr−/− mice are currently unknown.

Recent genome-wide association studies revealed that a genetic variant in the loci corresponding HDAC9 is associated with large vessel stroke.1 Moreover, HDAC9 is also a common genetic variant that share genetic susceptibility to ischemic stroke and coronary artery disease. HDAC9 expression was upregulated in human atherosclerotic plaques in different arteries.3 HDAC9 knockout mice are protected from adipose tissue dysfunction and systemic metabolic disease during high fat feeding.12
Moreover, HDAC9 deficiency increases regulatory T cells and decreases effector T cells. Finally, HDAC9 promotes angiogenesis by targeting the antiangiogenic microRNA-17 to -92 cluster in endothelial cells. These studies consistent with our results to support the concept of inhibition of HDAC9 may have beneficial effect in atherosclerosis development.

Lund et al demonstrated that epigenetic changes, such as DNA methylation polymorphisms, preceded any histological sign of atherosclerosis in apolipoprotein E–deficient mice and human THP-1 cells cultured with mixtures of different proportions of lipoproteins (very-low-density lipoprotein, low-density lipoprotein, and HDL) decreased histone H4 acetylation. Studies in endothelial cells have demonstrated opposing roles of HDACs in endothelial function. HDAC3 expression protected endothelial integrity, and HDAC3 knockdown increased atherosclerosis in aortic isografts of apolipoprotein E mice. Laminar blood flow inhibited HDAC5 expression, resulting in decreased binding of monocytes to endothelial cells. HDAC2 and HDAC5 played an important role in smooth muscle cell differentiation. Moreover, short interfering RNA-mediated knockdown of HDAC1, 2, or 3, and pharmacological inhibition of HDAC by scriptaid prevented smooth muscle cell differentiation.
proliferation and neointima formation. HDAC3 and HDAC7 promote TLR-4 dependent proinflammatory gene expression, whereas HDAC3 promotes macrophage alternate activation. Finally, pan-HDAC inhibitors have both anti- and proinflammatory effects on macrophages and prevent smooth muscle cells migration in vitro. Importantly, endogenous HDAC inhibitor β-hydroxybutyrate protects against oxidative stress by upregulating oxidative stress resistance genes Foxo3a and MT2. Based on these opposing roles of HDACs in different cell types that are involved in atherosclerosis development, it is not surprising that trichostatin A, which inhibits both class I and class II HDACs, increased atherosclerosis in LDLr−/− mice. It is also possible that the high concentration of dietary cholesterol (1.25%) overwhelms a beneficial effect of trichostatin A.

The nuclear receptors (PPARs, LXR, and RXRs [retinoid X receptors]) not only influence lipid metabolism at the systemic level but also regulate lipid homeostasis and inflammation in macrophages. There is considerable interest in the development of selective PPAR and LXR agonists for the treatment of atherosclerosis. Active repression of LXR target genes, such as ABCA1 and ABCG1, is mediated, in part, by interaction with corepressors nuclear receptor corepressor (NCoR) and SMART (silencing mediator of retinoic acid and thyroid hormone receptor) to repress basal expression of target promoters. Intron function, and atherosclerosis.

The nuclear deacetylase HDAC3 for the inflammatory gene expression program in macrophages. Activation of the genes in macrophages decreased foam cell formation and inflammation via increased cholesterol efflux and phenotypic switching of macrophages from a proinflammatory M1 to anti-inflammatory M2 phenotype, ultimately promoting oxidative stress resistance genes Foxo3a and MT2. Based on these opposing roles of HDACs in different cell types that are involved in atherosclerosis development, it is not surprising that trichostatin A, which inhibits both class I and class II HDACs, increased atherosclerosis in LDLr−/− mice. It is also possible that the high concentration of dietary cholesterol (1.25%) overwhelms a beneficial effect of trichostatin A.

Although, it is not clear from our study whether enzyme activity of HDAC9 or protein itself is required for atherogenesis, it is tempting to speculate that HDAC9 inhibition in macrophages may provide a therapeutic benefit in atherosclerosis in humans. HDAC9 is recently identified a common genetic variant that share genetic susceptibility to large artery stroke and coronary artery disease. Moreover, HDAC9 expression was upregulated in human atherosclerotic plaques in different arteries. Our study provides a mechanistic clue how HDAC9 gene variant may cause atherosclerosis. We envision that this study may provide the foundation for development of HDAC9 isoform–specific inhibitors that can selectively be delivered to macrophages via chemical conjugates technology may provide therapeutic benefit in individuals with coronary artery disease and stroke with genetically defined HDAC9 population.

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Disclosures
None.

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11. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.


Histone Deacetylase 9 Represses Cholesterol Efflux and Alternatively Activated Macrophages in Atherosclerosis Development

Qiang Cao, Shunxing Rong, Joyce J. Repa, Richard St. Clair, John S. Parks and Nilamadhab Mishra

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Supplementary Fig. I. Class IIa HDACs mRNA levels, body weight and plasma lipid profiles in LDLr<sup>−/−</sup> (SKO) and LDLr<sup>−/−</sup> HDAC9<sup>−/−</sup> (DKO) mice fed on chow diet

(A) Real-time PCR analysis of Class IIa HDACs mRNA in liver (A) and peritoneal macrophages isolated from SKO and DKO mice. Data were normalized using the housekeeping gene GAPDH. mRNA expression was calculated relative to SKO mice (control). Data are mean ± SD.

(B) Body weight of male and female SKO and DKO mice fed on chow diet.

(C) Plasma lipid profiles of male and female SKO and DKO mice fed on chow diet.
Supplementary Fig. II. HDAC9 deficiency reduces atherosclerosis in male LDLr−/− mice

Plasma lipid and atherosclerosis measurements were made after 16 weeks of atherogenic diet consumption by SKO (LDLr−/−) and DKO (LDLr−/− HDAC9−/−) male mice. FPLC analysis of lipoproteins was done using pooled plasma from the terminal bleeds after 15 hours of fasting.

(A) Plasma lipid concentrations.
(B-C) Cholesterol distribution among VLDL, LDL and HDL fractions by FPLC.
(D) Representative examples of light microscopic images taken of gross dissected aortas in situ. Atherosclerotic lesions are visualized as white areas, denoted by arrows, inside the arteries.
(E-F) Representative en face image (E) and surface lesion quantification (F) in aortic samples.
(G-H) Representative cross-sectional image of aortic root stained with Oil Red O (G), and quantification of aortic intimal area and Oil Red O positive area (H).

Data are shown as the mean ± SD. * p<0.05
Supplementary Fig. III. Content of SMC and collagen in atherosclerotic plaques in SKO and DKO mice
(A-D) Representative cross-sectional image of aortic root stained with SMC (A) and collagen (C). Quantification of area stained positive for SMC (B) and collagen (D) in aortic lesions of SKO and DKO mice.
Supplementary Fig. IV. Gene expression in atherosclerotic lesions determined by real-time PCR in aortic root of SKO and DKO mice

RNA was pooled from SKO and DKO mice. Real time PCR was performed in triplicates from these pooled samples. Data were normalized using the housekeeping genes GAPDH. mRNA expression was calculated relative to SKO mice (control).

(A-B) Lipid homeostatic genes (A), inflammatory macrophage M1 and anti-inflammatory M2 genes (B) in aortic root of SKO and DKO mice fed an atherogenic diet for 16 weeks.

Data are presented as mean ± SD.)
Supplementary Fig. V. Confirmation of donor bone marrow cell reconstitution in recipient mice

(A) Successful bone marrow reconstitution from the male donor mice to female recipient mice was verified by PCR amplification of the *Sry* gene expression in blood leukocytes. Male and female genomic DNA was used as positive and negative controls for the *Sry* gene, respectively. For the *HDAC9* gene, SKO and DKO mice genomic DNA was used as positive controls for WT and mutant genes, respectively.
Supplementary Fig. VI. HDAC9 mRNA expression is increased in response to OXLDL, acLDL and TLR agonists in TEPMs.

(A) HDAC9 mRNA level is increased in TEPMs cultured with modified LDL. TEPMs were isolated from C57BL/6J mice and cultured with ac-LDL (50 μg/mL) and ox-LDL (50 μg/mL) for 24 hours. Data were normalized using the housekeeping gene GAPDH. Relative mRNA expression was calculated relative to DMSO-treated cells (control).

(B) Real-time PCR analysis of HDAC9 mRNA levels in macrophages stimulated with different TLR agonists. TPEMs were isolated from C57BL/6J mice and treated with LTA (5 μg/mL), LPS (100 ng/mL), flagellin (50 ng/mL), and CpG (3μM) for 24 hours. Data were normalized using the housekeeping gene GAPDH. mRNA expression was calculated relative to vehicle (DMSO)-treated cells (control).
Supplementary Fig. VII. Cholesterol efflux is increased in HDAC9 deficient macrophages.

(A-B) Real-time PCR analysis of ABCA1 and ABCG1 mRNA in BMDMs macrophages isolated from SKO and DKO mice and stimulated with acLDL and LXR agonists.

(C-D) apoA1- and HDL-mediated cholesterol efflux is increased in HDAC9 deficient macrophages.

(E-F) ACAT inhibition further increases apo-A1 mediated cholesterol efflux but, not HDL mediated cholesterol efflux in HDAC9 deficient macrophages.
Supplementary Fig. VIII. Global or site-specific acetylation status of histone 3 and histone 4 are not affected by HDAC9 deficiency in macrophages.

(A-B) Histones were isolated from SKO and DKO bone marrow-derived macrophages and subjected to Western blot analysis using antibodies against anti-Ac-H3, Ac-H4, Ac-H3K9, Ac-H3K14, Ac-H3K18, Ac-H3K23, Ac-H4K5, Ac-H4K8, Ac-H4K12, and Ac-H4K16. Coomassie blue staining demonstrated equal protein loading.
Supplementary Fig. IX. Differential accumulation of acetylated histones at ABCA1, ABCG1 and SRA promoter in DKO BMDMs compared to SKO macrophages.
BMDMs from DKO and SKO mice are treated with acLDL for 18hrs.
(A) Real-time PCR analysis of ABCA1, ABCG1 and SRA mRNA in BMDMs
(B-D) Quantitative ChIP assays were performed on the promoter regions of ABCA1, ABCG1 and SRA using anti-Ac-H3, Ac-H3K9, Ac-H3K18, Ac-H4 or IgG (control) for immunoprecipitation. Non-immunoprecipitated sample served as an input control.
Supplementary Fig. X. Increased expression of certain M2 genes in HDAC9 deficient BMDMs in response to IL-4 and rosiglitazone.

(A) Real-time PCR analysis of M2 genes in BMDMs isolated from SKO and DKO mice and stimulated with IL-4 and rosiglitazone.
Supplementary Fig. XI. Accumulation of acetylated histones at the arginase 1 promoter are not affected by HDAC9 deficiency in macrophages

(A) Quantitative ChIP assays were performed on the promoter regions of Arginase-1 using anti-Ac-H3, Ac-H3K9, Ac-H3K18, Ac-H4 or IgG (control) for immunoprecipitation. Non-immunoprecipitated sample served as an input control.
### Table I. Composition of atherogenic diet

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Supporting Online Materials and Methods

HDAC9 represses cholesterol efflux and generation of alternatively activated macrophages in atherosclerosis development

Qiang Cao, Shunxing Rong, Joyce J. Repa, Richard St. Clair, John S. Parks, Nilamadhab Mishra

Materials and methods

Mice
HDAC9−/− mice were kindly provided by Dr. Eric N. Olson 1 (UT Southwestern Medical Center, Dallas, TX) and backcrossed to C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) for 12 generations. Backcrossed HDAC9−/− mice were bred with C57BL/6J LDLr−/− mice (SKO; Jackson Laboratory, Bar Harbor, ME) to obtain LDLr−/−HDAC9−/− double knockout mice (DKO). All mice were housed at Wake Forest School of Medicine (WFSM) in a specific pathogen-free animal facility, with a daylight cycle from 6AM to 6PM. Mice were fed with standard chow diet (Prolab RMH 3000) and water ad libitum after weaning. At eight weeks of age, mice were fed an atherogenic diet containing 12% calories from palm oil and 0.1% (w/w) cholesterol (Table 1) prepared in animal kitchen at Wake Forest University 2. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at WFSM. After 16 weeks of atherogenic diet consumption, mice were fasted for 15 hours and then sacrificed.

Plasma lipid and lipoprotein analysis
After a 15-hour fast, mice were anesthetized and then euthanized via cardiac puncture. The concentrations of plasma triglyceride (TG) (461-08992, Wako), total cholesterol (TC) (CHOLESTEROL, Genchem), free cholesterol (FC) (435-35801, Wako) and phospholipids (PL) (433-36201, Wako) were measured by enzymatic assay following the manufacturer’s protocol. Esterified cholesterol (EC) was calculated by subtracting free cholesterol from total cholesterol, measured after saponification. The esterified cholesterol value is then multiplied by 1.67 to correct for the loss of fatty acid during saponification 3. PlasmaVLDL, LDL and HDL cholesterol concentrations were measured on lipoproteins separated using Superose 6 (1x30 cm) and Superose 12 (1x30cm) FPLC columns in series as previously described 4.

Quantification of atherosclerosis
Mouse hearts were perfused in situ with PBS. The top 1/3 of the heart and aortic arch were cut, embedded in O.C.T. compound (Sakura Tissue-Tek, Torrance, CA), and frozen at -80°C. Frozen tissue blocks were serially cut at 8μm intervals from the aortic sinus and aortic arch using a Leica tissue-processing machine and mounted on slides as described previously 5. The sections were fixed with 10% neutral buffered formalin for 10 minutes, dehydrated with propylene glycol (Fisher Scientific) for 2 minutes, and stained with 0.5% Oil Red O (Sigma) solution (dissolved in propylene glycol) for 25 minutes at 60°C. The sections were counterstained with hematoxylin (Sigma). Atherosclerotic intimal area and Oil Red O stained area were analyzed with Image-Pro Plus software. During computer
analysis, the pictures were zoomed in to compute averages per group maximal atherosclerotic lesion areas of the total aortic root wall, medial, and intimal area as well as the maximal thickness of the total aortic wall (media + intima) of each mouse. Positive oil red O area was determined by computer-assisted image quantification (Image pro). Oil red O area was utilized to determine the total lipid contents in the atherosclerosis lesion area. Aortic en face surface lesion area was measured as described previously.

**Immunohistochemistry**

For immunohistochemical analysis, serial cryostat sections of the aortic arch and aortic root were collected, and sections were stained with SMC antibody (1:250, Epitomics). Total lesion areas and antibody-positive areas were quantified with Image-Pro Plus software.

**Staining of collagens with Sirius Red**

Collagens type I and III was stained with 0.1% Sirius Red (Polysciences, Inc., 09400) in saturated picric acid (VWR Scientific, RC586032) as previously described. Briefly, frozen sections were fixed with 10% formalin for 10 minutes, stained with 0.1% Sirius Red for 4.5 hours, rinsed twice with 0.01M HCl, and dehydrated with gradient ethanol (70% ethanol for 45 seconds, 95% ethanol for 5 minutes, and 100% ethanol for 5 minutes). Sirius Red staining was analyzed with polarization microscopy and quantified using Image-Pro Plus software.

**Bone Marrow Transplantation (BMT)**

BMT was conducted as previously described with minor modifications. Briefly, recipient mice (female LDLr\(^{-/-}\) mice, age 6 weeks) were given acidified water (pH 2.6) with 100mg/L neomycin and 10mg/L polymyxin B sulfate 3 days before and 2 weeks after BMT. Recipient mice were fasted overnight, irradiated (900 rad), and transplanted with BM 4 hours later. Donor bone marrow cells were collected from male SKO and DKO mouse femurs and tibias, and 5x10\(^6\) (150\(\mu\)L) bone marrow cells were injected into the retro-orbital venous plexus of anesthetized recipient mice. Four weeks after BMT, genomic DNA was isolated from white blood cells (Wizard genomic DNA purification kit, Promega) to check donor bone marrow reconstitution by detecting sequences for Sry and HDAC9 genes. Five weeks after BMT, mice were fed an atherogenic diet containing 12% calories from palm oil and 0.1% (w/w) cholesterol for 16 weeks before termination for atherosclerosis evaluation.

**Peritoneal macrophage isolation and culture**

Mice were injected with 1mL of 10% thioglycollate intraperitoneally and macrophages were isolated after 4 days. For some studies, SKO and DKO mice were fed an atherogenic diet for 16 weeks before peritoneal macrophages were isolated. Five million cells were plated onto 60 mm plates in RPMI 1640 medium with 10% fetal bovine serum (FBS) and allowed to adhere for 2 hours. The cells were washed and cultured for an additional 24 hours before stimulation or incubation with different compounds. Cells and supernatants were harvested for RNA and cytokine measurement, respectively.
Isolation and culture of Bone Marrow-Derived Macrophages (BMDMs)

BMDMs were obtained as described previously with minor modifications \(^{11}\). Briefly, the femurs and tibias of mice were isolated, and bone marrow cells were collected into 1.5mL Eppendorf tubes by centrifugation. Bone marrow-derived cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) with 30% L929 cell-conditioned medium, 20% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 100 units/mL penicillin and 100μg/mL streptomycin. At 1, 3, 5, and 7 days of culture, cells were harvested for RNA analysis.

Differentiation of THP-1 cells

THP-1 cells were cultured with RPMI-1640 medium supplemented with or without 12-O-tetradecanoylphorbol-13-acetate (PMA) (20 ng/mL) for 4 days \(^{12}\). THP-1 cells or macrophages differentiated from THP-1 cells were harvested for RNA analysis.

Cholesterol efflux

Thiglycollate-elicited peritoneal macrophages were plated at a density of 1x10^6 cells in 24-well plates. After 2 days of culture in DMEM with 10% lipoprotein-deficient (LPDS) serum, cells were washed with PBS and treated with vehicle (DMSO), LXR agonist T0901317 (1μM), ACAT inhibitor CP-113 (1.25 μg/mL) (Sigma) in 0.2% BSA for 24 hours in the presence of 50μg ac-LDL and 1μCi/mL of \[^3\text{H}\]-cholesterol (Perkin Elmer Life Sciences). Cells were washed with PBS and equilibrated with media containing 0.2% BSA and drugs for 1 hour, and then the medium was replaced with fresh medium containing human apoA-I (10 μg/mL) or HDL (100 μg protein/mL) with the drugs. The cells were incubated at 37°C and 200 μL of medium was removed at 4 hours for apoA-I-mediated cholesterol efflux and at 24 hours for HDL-mediated cholesterol efflux. The culture medium was centrifuged to remove cell debris and \[^3\text{H}\] radioactivity was determined. The macrophages were washed with PBS and then lysed with 0.2N NaOH. The cell lysate was used to determine \[^3\text{H}\] radioactivity and protein content. The results were expressed as the percentage of \[^3\text{H}\] cholesterol in the medium divided by the total \[^3\text{H}\] cholesterol in the medium and cells \(^{11}\).

Histone isolation and immunoblotting

Histones were isolated as described previously \(^{13}\). Briefly, 5x10^6 cells were suspended with 1mL ice-cold lysis buffer (10mM Tris·HCl [pH 6.5], 50mM sodium bisulfite, 1% Triton X-100, 10mM MgCl\(_2\), 8.6% sucrose) and incubated on ice for 10 minutes. After centrifugation, the pellet was washed once with 1mL lysis buffer. Then, the pellet was resuspended with ice-cold Tris·EDTA solution (10mM Tris·HCl [pH 7.4], 13mM EDTA) and washed once with the same buffer. Nuclei were pelleted, resuspended in 1mL ice-cold H\(_2\)O, sonicated, and acidified to 0.2M sulfuric acid. After 1 hour incubation on ice, the pellet was obtained by centrifuging at 10,000g for 10 minutes at 4°C. Then, the supernatant was transferred into 9mL cold acetone and incubated on ice overnight at 4°C. Histones were collected by centrifugation at 4000 rpm for 20 minutes at 4°C, air-dried, and suspended in 100μL H\(_2\)O. The concentration of histones was quantified by BCA kit (BCA protein assay kit, Pierce). Two μg proteins were loaded on an SDS-PAGE gel to detect different acetyl sites of histones. The following primary antibodies were used: total

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed using a ChIP assay kit (Millipore, 17-295) based on the manufacturer’s instructions and as previously described \(^ {14} \). Briefly, 2x10^6 cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C, and the cross-linking was stopped by adding glycine (0.125M final concentration). Cells were lysed with SDS lysis buffer for 10 minutes on ice, and sonicated to shear DNA to lengths between 200 and 1000 base pairs. The lysates were centrifuged, 10% of supernatants containing proteins and chromatin complex were saved as input control, and the remainder was immunoprecipitated with ChIP grade antibodies of IgG (Millipore, PP64), total Ac-H3 (Millipore, 06-599), total Ac-H4(Millipore, 06-866), Ac-H3K9(Millipore, 07-352) and Ac-H3K18Abcam: ab1191)The complex of histones and chromatin was pulled down by protein A agarose, eluted from agarose by elution buffer (1% SDS and 0.1M NaHCO3), the cross-links reversed with 5M NaCl, and proteins digested with proteinase K. DNA was then isolated using standard methods of phenol/chloroform and ethanol precipitation, and quantitated by SYBR Green quantitative real-time PCR (Applied Biosystems). The primers utilized for ABCA1 promoter-#1, and PPARγ1 promoter-#1 were described previously \(^ {15-17} \). Percent of input was calculated by 100 X 2^(Adjusted input - Ct (IP)). The starting input fraction was 10%, therefore, a dilution factor (DF) of 10 or 3.322 (i.e., log2 of 10) was subtracted from the Ct value of diluted input.

**Cholesterol mass in peritoneal macrophages**

Thioglycollate-elicited peritoneal macrophages (1x10^6) isolated from SKO and DKO mice fed an atherogenic diet for 16 weeks were cultured in 6-well plates for 2 hours with serum-free RPMI 1640 medium. After three washes with cold PBS, peritoneal macrophage cholesterol was extracted with isopropyl alcohol and analyzed by gas-liquid chromatography as previously described \(^ 4 \).

**RNA isolation from aortic valves and aortic arch**

RNA from the upper one-third of heart and aortic arch (from the root to 0.5cm under the left subclavian artery) was isolated using TRIzol as previously described \(^ 3 \).

**Quantitative real-time PCR**

Quantitative real-time PCR was performed using SYBR Green Master Mix (Applied Biosystem) or TaqMan Universal Master Mix (Applied Biosystems) and an ABI Prism 7000 or 7500 PCR instrument (Applied Biosystems). The relative mRNA expression was normalized to either 36B4 (when SYBR Green Master Mix was used) or GAPDH (when Taqman primers and Taqman Universal Master Mix were used). Taqman primers were purchased from Applied Biosystems. Primers used for SYBR green real-time PCR assays were synthesized by IDT Inc. The primer sequences are available upon request.
Western blot analysis
Total cell protein was harvested using cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1mM EDTA) containing protease inhibitors (Sigma). Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). An ECL detection system (PerkinElmer) was used to detect proteins and chemiluminescence was captured with an LAS-3000 imaging system (Fujifilm Life Science).

ELISA
The cell culture supernatants were quantified by ELISA according to the manufacturer’s instructions for each BD OptEIA™ ELISA kit, assayed in triplicate, and read on a FLUOstar Omega-Multidetection Microplate Reader. Standard curves and individual well concentrations were determined using MARS Data Analysis Software (BMG LABTECH).

Statistical analysis
The data are presented as mean ± the standard deviation(SD). Statistical differences between groups were determined using the two-tailed Student’s t test and Mann-Whitney test (Graph Pad Prism software). Statistical significance was considered at a value of $p < 0.05$. 
References


