Arteriosclerosis, Thrombosis, and Vascular Bi

BASIC SCIENCES

Progression of Established Atherosclerotic Lesions Is Not Inhibited by Endothelial Knockout of Caveolin-1—Brief Report

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BACKGROUND: Eradicating endothelial caveolae by deleting the *Cav1* (caveolin-1) gene reduces LDL (low-density lipoprotein) uptake in arteries and efficiently prevents early atherogenesis, but the role in established atherosclerosis is unknown. Here, to examine CAV1 as a potential therapeutic target, we deleted endothelial *Cav1* in mice after lesion development and analyzed the effect on LDL uptake and lesion progression.

METHODS: To allow timed endothelium-specific *Cav1* deletion, we generated male and female mice with floxed *Cav1* alleles and endothelium-specific inducible Cre recombinase. Atherosclerosis was induced by virus-mediated PCSK9 (proprotein convertase subtilisin/kexin type 9) gene transfer and a high-cholesterol diet. After 16 weeks of lesion development, endothelial *Cav1* deletion was induced by a series of tamoxifen injections, repeated after 4 weeks, and the mice were followed for another 4 weeks. Mice were injected with fluorescently labeled LDL at 1 and 18 hours before euthanasia to study uptake and retention in lesions. Sections of the aortic root were analyzed for lesion size, composition, and LDL accumulation.

RESULTS: Efficient conditional knockout of endothelial *Cav1* was confirmed by CAV1 immunostaining and by the loss of caveolae by electron microscopy. Loss of endothelial *Cav1* for 8 weeks reduced LDL entry into lesions but did not significantly decrease LDL retention, lesion lipid accumulation, fibrous tissue, or lesion size. In males, a reduction in macrophages was seen.

CONCLUSIONS: Targeting CAV1 does not efficiently block LDL entry or reduce lesion progression in established atherosclerosis. These findings open several questions for further research, including alternative LDL entry mechanisms that could circumvent caveolar transport in established atherosclerosis.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ caveolin 1 ■ cholesterol ■ endothelium ■ lipoproteins, LDL

The entry and retention of LDL (low-density lipoprotein) and other small ApoB-containing lipoproteins in the arterial intima is the central mechanism by which atherosclerotic lesions initiate and progress in humans and experimental animal models.[1] Consequently, drugs lowering LDL levels are the main therapy in patients with established atherosclerosis, but they fail to protect a significant proportion of patients against new clinical events.[1] To potentiate the effects of LDL lowering, inroads have been made to understand how LDLs enter and are retained in the arterial wall, with the outlook of potentially blocking these mechanisms.[2] Several interventions have been efficient in mouse models of atherogenesis but have yet to translate into clinical therapies.[2,3]

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Nonstandard Abbreviations and Acronyms

ACTA2	actin alpha 2
CAV1	caveolin 1
CD	cluster of differentiation
LDL	low-density lipoprotein
rAAV8-PCSK9	recombinant adeno-associated virus encoding D377Y gain-of-function proprotein convertase subtilisin/kesin type 9
SMC	smooth muscle cell
VCAM1	vascular cell adhesion molecule 1

What Are the Clinical Implications?

Blocking LDL (low-density lipoprotein) uptake could serve as an adjunct to LDL-lowering therapy to stop disease activity in atherosclerosis. In normal arteries and early-stage atherosclerosis, LDL primarily crosses the endothelium through transcytosis in caveolae. Importantly, knocking out the gene encoding *Cav1*—the gene essential for caveolae formation—efficiently prevents disease initiation and early lesion growth. The present study attempted to translate these encouraging results into a therapy by examining whether endothelial-specific knockout of *Cav1* in established atherosclerosis can prevent further progression. This scenario is clinically relevant because drug therapy, including for primary prevention, is rarely initiated before lesions are well advanced. We found that although endothelial *Cav1* deletion diminished LDL entry into plaques, the reduction was insufficient to alter retained LDL, lipid accumulation, or overall lesion growth. These results indicate that caveolae-mediated transcytosis, while important in early atherogenesis, plays a diminished role in advanced atherosclerosis, possibly due to disruption of endothelial barrier properties. Therapies focusing on reducing endothelial transcytosis may therefore not be sufficient to obtain strong reductions in LDL uptake in advanced lesions.

The transport of LDLs across the endothelium is thought to occur mainly by transcytosis through caveolae,[2,4–7] a process inhibited by estrogen, which may contribute to the protective effect against early atherosclerosis in females.[8] Caveolae are flask-shaped nanoinvaginations of the plasma membrane with a distinct protein and lipid composition, which can form on one side of the cell, move through the cytoplasm, and fuse with the contralateral membrane, thereby providing a transcellular route of molecular transport. Caveolae are particularly abundant in endothelial cells and other cells exposed to significant variations in membrane tension, such as smooth muscle cells and adipocytes.[9] Both native and oxidized LDL have been observed in caveolae-like structures in the endothelium by electron microscopy.[4,10,11]

The main structural protein component in caveolae is CAV1 (caveolin-1),[12] and the absence of CAV1 results in the complete loss of caveolae organelles.[13,14] Global deletion also leads to reduced LDL uptake in normal arteries and is one of the most potent interventions described against atherogenesis in mice, with some studies showing ≈7-fold reduction in atherogenesis.[4,7] The strong protective effect is abolished by adding back *Cav1* as an endothelial-expressed transgene, demonstrating that the loss of endothelial CAV1 mediates the protective effect.[15,16] In addition to the effect on LDL transport, endothelial CAV1 and caveolae have multiple other biological functions that are centrally involved in atherogenesis, including mechanotransduction and cellular lipid homeostasis.[9]

To investigate the role of caveolae in atherogenesis, previous research has used constitutive *Cav1* knockouts in which the defect in caveolae formation is present both during the initiation of atherosclerosis and subsequent lesion progression.[4,7] The importance of CAV1 in each of these phases is unknown, but mechanisms of LDL entry may change with disease progression because the endothelium loses part of its intercellular barrier function in established lesions.[17] This is important from a translational perspective because lesion initiation occurs early in life; therefore, lesion progression rather than initiation is the relevant target for antiatherosclerotic therapies, which are typically administered later in life.

In the present study, we established tools to delete *Cav1* in the endothelium of established atherosclerotic lesions and investigated its effect on disease progression. We find that the loss of CAV1 partially blocks LDL uptake in lesions but not sufficiently to reduce LDL retention or significantly alter the course of lesion development.

METHODS

The raw data that support the findings of this study are available from the corresponding authors on reasonable request.

Animals

To generate mice with floxed *Cav1* alleles (*Cav1*$^{fl/fl}$ mice), C57BL/6J oocytes were fertilized in vitro with sperm from C57BL/6N-*A*^{tm1Brd} *Cav1*$^{tm1a(KOMP)Mbp/MbpMmucd}$ mice (MMRRC_046921-UCD, other names: CSD76557), obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis. The *Cav1*$^{fl/fl}$ mice carry *loxP* sites inserted in the introns around the exon III of the *Cav1* gene (Figure S1). Cdh5-CreER^{T2} mice (B6.Cg-Tg[Cdh5-cre/ERT2]1Rha), expressing the tamoxifen-inducible Cre recombinase CreER^{T2} under the endothelial-specific *Cdh5* promoter, were kindly gifted by Ralf Adams to Centro Nacional de Investigaciones Cardiovasculares and subsequently

backcrossed multiple times to the C57BL/6J background. Littermate male and female Cdh5-CreER^{T2} (hemizygous) mice with *Cav1*$^{fl/fl}$ (*Cav1*$^{EC-KO}$) and *Cav1*$^{wt/wt}$ (*Cav1*^{WT}) genotypes were generated by crossing *Cav1*$^{wt/fl}$ mice with either the male or female homozygous for the Cdh5-CreER^{T2} transgene.

Mice were group-housed in a specific pathogen-free facility with a 12-hour/12-hour light-dark cycle and free access to water and feed. Where not noted otherwise, the feed was standard laboratory diet (D184, SAFE, Rosenberg, Germany), containing 70.2% cereals, 25% vegetable and fish protein, and 4.8% vitamins and minerals. All animal procedures were approved by the ethical review boards at Centro Nacional de Investigaciones Cardiovasculares and Universidad Autónoma de Madrid and authorized by the Comunidad de Madrid (PROEX 266/16).

Conditional Cav1 Deletion and Atherosclerosis Induction

To test the efficiency with which endothelial *Cav1* can be deleted in the new strain, 8-week-old *Cav1*$^{EC-KO}$ (n=4 male, n=4 female) and *Cav1*^{WT} (n=5 male, n=5 female) mice were injected intraperitoneally with tamoxifen (2 mg IP dissolved in corn oil) on 5 consecutive days. Four weeks later, mice were anesthetized (pentobarbital 250 mg/kg and lidocaine 20 mg/kg IP), euthanized by exsanguination, and perfusion-fixed with 4% phosphate-buffered formaldehyde. Cross-sections of the aortic root near the commissures of the aortic cusps were stained for CD31 (cluster of differentiation 31) and CAV1 and analyzed by confocal microscopy.

To verify that the knockout of *Cav1* reduced caveolae formation in endothelial cells, we treated *Cav1*^{WT} (n=3) and *Cav1*$^{EC-KO}$ (n=4) male mice with tamoxifen (2 mg IP in corn oil) for 5 days at 8 weeks of age and again at 10 weeks of age. A week later, the mice were euthanized as described above, perfusion-fixed with glutaraldehyde-formaldehyde, and the ascending aorta was processed for electron transmission microscopy.

To study endothelial CAV1 during atherogenesis, we stained aortic root sections near the commissures of the aortic valves from a previous study of male C57BL/6J mice with 0, 2, 4, 6, and 12 weeks of atherosclerosis (n=5 per time point), induced by rAAV8-PCSK9 (recombinant adeno-associated virus encoding D377Y gain-of-function proprotein convertase subtilisin/kesin type 9; 10^{11} viral genomes) and feeding a high-cholesterol diet (0.3% cholesterol, 4.2% fat, S9167-E150, Sniff).[18]

To study the effect of endothelial *Cav1* deletion in established atherosclerotic lesions, we injected 6-week-old *Cav1*$^{EC-KO}$ (n=30 female, n=26 male) and *Cav1*^{WT} (n=33 female, n=32 male) mice with rAAV8-PCSK9 (10^{11} viral genomes, produced at the Viral Vector Unit at Centro Nacional de Investigaciones Cardiovasculares) and put them on the high-cholesterol diet (S9167-E150, Sniff). Cre recombinase activity was induced 12 weeks later (18 weeks of age), by 5 days of tamoxifen injections (2 mg IP in corn oil), which was repeated 4 weeks later. After an additional 4 weeks (26 weeks of age), mice were euthanized and processed as described above. Sections collected ≈0, 100, and 200 µm from the aortic valve commissures were used for microscopic analysis.

Rates of LDL entry and retention were measured in subgroups of mice by tail vein injection of 250 µg Atto565- or Atto647-conjugated human LDL at 1 hour (to measure entry) or 18 hours (to measure retention) before euthanasia, as previously described and validated.[18] The small Atto fluorophores bind covalently with the ApoB backbone of LDL, enabling their tracking in tissues. LDL entry was measured in the subendothelial region of the atherosclerotic lesion (up to 30 µm from the lumen, 4 separate experiments), whereas LDL retention was measured in the full lesion (3 separate experiments). Each experiment included mice comprising all genotypes. Steps to quantify LDL entry and retention in plaque sections are shown in Figure S2. Data from individual LDL tracking experiments were combined by normalizing values to the average level in female *Cav1*^{WT} mice.

A separate study with *Cav1*$^{EC-KO}$ (n=14 female, n=9 male) and *Cav1*^{WT} (n=11 female, n=14 male) mice that received 2 series of 5-day tamoxifen injections (2 mg IP in corn oil) at 8 and 10 weeks of age was conducted to study plasma lipids in a nonhypercholesterolemic context.

Statistics

All statistical analyses were performed in Prism 8 (GraphPad Software, San Diego, CA). The experimental unit was the individual mouse. Randomization occurred in each mouse by the random Mendelian assortment of alleles from the heterozygous *Cav1*$^{wt/fl}$ parents. In this way, litters included both *Cav1*$^{wt/wt}$ and *Cav1*$^{fl/fl}$ mice that were raised, group-housed, and treated together, preventing confounding by cage and litter effects. Two-way ANOVA was used to analyze the effects of *Cav1* genotype, sex, and their interaction, after confirming approximate normal data distribution and homoscedasticity of data in sex-genotype categories by the Shapiro-Wilk and Brown-Forsythe tests, respectively. Some data were log-transformed before analysis to meet model assumptions as indicated in figure legends. When appropriate, Holm-Šidak post hoc tests were performed to explore simple effects in each sex. P values <0.05 were considered significant.

Two mice were excluded from the atherosclerosis study (not reported in group numbers) after finding very low cholesterol values, which we attributed to rAAV8-PCSK9 injection failure. All analyses were performed blinded for genotype and sex. The primary end point was lesion size, and the necessary group size to detect a 30% decline in lesion size with 90% certainty at a coefficient of variation of 0.35 was calculated to n=29 per group using published variance estimates.[19] Other end points included LDL entry and retention, necrotic size, changes in inflammation, cap smooth muscle cells, and fibrosis.

Description of mouse breeding, plasma lipid measurements, LDL isolation and conjugation procedures, tissue processing, staining procedures, image analysis, additional statistical analysis, and the Major Resources Table can be found in the Supplemental Material.

RESULTS

Validation of Endothelial Cav1 Deletion

To validate the Cre-mediated deletion of floxed *Cav1* alleles in *Cav1*$^{EC-KO}$ mice, we stained aortic root sections of *Cav1*$^{EC-KO}$ (n=4 female, n=4 male) and *Cav1*^{WT} (n=5 female, n=5 male) male mice 1 week after a series of tamoxifen injections. CAV1 was abundantly

expressed in the CD31+ endothelium of *Cav1*^{WT} mice but was significantly reduced in *Cav1*$^{EC-KO}$ mice in both females and males (Figure S3A through S3D). As expected, the reduced CAV1 level was specific for endothelium, with no changes observed in arterial media (Figure S3A through S3D). Tamoxifen alone did not significantly alter endothelial CAV1 levels (Figure S3E through S3G).

To confirm the consequences of *Cav1* deletion on caveolae formation in endothelial cells, we examined ascending aortas from an independent batch of *Cav1*$^{EC-KO}$ (n=4) and *Cav1*^{WT} (n=3) male mice by transmission electron microscopy. Endothelial caveolae content was significantly decreased by 82% in *Cav1*$^{EC-KO}$ compared with *Cav1*^{WT} mice (Figure S4).

Deletion of Endothelial Cav1 After Induction of Hypercholesterolemia and Atherosclerosis

Immunostaining of aortic root sections from rAAV-PCSK9–injected wild-type mice with 0, 2, 4, 6, and 12 weeks of disease development (n=5 per group) showed a significant decrease in average endothelial CAV1 levels during lesion development compared with healthy arteries, but CAV1 remained clearly detectable in established lesions after 12 weeks (Figure S5). To study the effect of endothelial *Cav1* deletion from this time point onwards, lesions were first induced in *Cav1*$^{EC-KO}$ (n=30 females, n=26 males) and *Cav1*^{WT} (n=33 females, n=32 males) mice by injection of rAAV-PCSK9 and 12 weeks of high-cholesterol diet feeding. Mice were then injected with tamoxifen, which was repeated after 4 weeks, and the effects were analyzed after another 4 weeks (Figure 1A). The 8-week intervention period was deemed sufficient to detect a potential therapeutic effect of reducing plaque LDL uptake, as previous studies have shown that 2 to 4 weeks of LDL lowering induces regression of many features of murine plaques, including inflammation and lipid content.[20,21] The repeated tamoxifen injections were performed to reinforce endothelial CAV1 deficiency and minimize the risk that residual CAV1-competent cells could expand and outcompete CAV1-deficient cells. Immunostaining of the aortic root confirmed an efficient, ≈90% loss of CAV1 in the CD31-stained plaque endothelium in male and female *Cav1*$^{EC-KO}$ mice at the study end point (Figure 1B and 1C).

Plasma Lipids

Plasma total and LDL cholesterol levels before endothelial *Cav1* deletion were not influenced by genotype but were more severe in females than males (Figure 1D and 1E). In both genotypes, there was a marked drop after the first tamoxifen injections, which corresponds to a known Cre-independent effect of tamoxifen in the rAAV-PCSK9 model, presumably caused by tamoxifen-induced reductions in hepatic very low-density lipoprotein output.[22] The direct effect of tamoxifen is persistent over months,[22] which helps explain the stability of the lowered plasma cholesterol levels and why no drop was seen with the second set of tamoxifen injections. Notably, after tamoxifen-induced endothelial *Cav1* deletion, total and LDL cholesterol showed small but significant increases in *Cav1*$^{EC-KO}$ compared with *Cav1*^{WT} mice (Figure 1D and 1E). No significant differences in high-density lipoprotein cholesterol levels or body weight were found (Figure S6). In short-term studies conducted in nonhypercholesterolemic mice, similar changes were observed, including a significant increase in total and LDL cholesterol caused by endothelial *Cav1* deletion (Figure S7).

Endothelial Cav1 Deletion Reduces LDL Entry

To study the impact on the rates of LDL entry and retention into atherosclerotic lesions, a subset of the mice was injected with Atto647- or Atto565-labeled LDL at 18 hours and 1 hour before the end point to measure LDL retention and entry, respectively. Figure 1F shows confocal microscopy images of Atto-labeled LDLs in aortic root lesions. Entry of Atto-labeled LDL was significantly reduced by endothelial *Cav1* deletion with no interaction with sex (Figure 1G), whereas no significant differences in the rate of LDL retention were observed (Figure 1H).

Loss of Endothelial CAV1 Does Not Alter Lesion Size or Necrotic Areas

Female mice developed larger lesions than male mice, as expected,[23] but no differences were found in aortic root lesion size between *Cav1*$^{EC-KO}$ and *Cav1*^{WT} mice in either sex (Figure 2A). Indeed, the estimated genotype effect in the 2-way ANOVA was close to zero with a narrow 95% CI (−0.052 to 0.039×10^5 µm), indicating that the experiment had sufficient power to avoid overlooking large effects by chance.

To understand if the reduced LDL entry after *Cav1* deletion resulted in a significantly reduced accumulation of lipids in lesions, we analyzed Oil Red O–stained aortic root sections (Figure 2B) and longitudinally opened Oil Red O–stained aortic arches (Figure S8) but found no significant differences between genotypes. Furthermore, necrotic core areas in trichrome-stained sections were found unchanged by the intervention (Figure S9).

To investigate whether the small between-group differences in total and LDL cholesterol during the intervention period could explain the lack of effect on lesion size and necrotic area, we performed a linear regression analysis. However, no significant correlations were detected, indicating that the plasma lipid changes were unlikely to have masked important effects of local endothelial *Cav1* deletion on lesion progression (Figure S10).

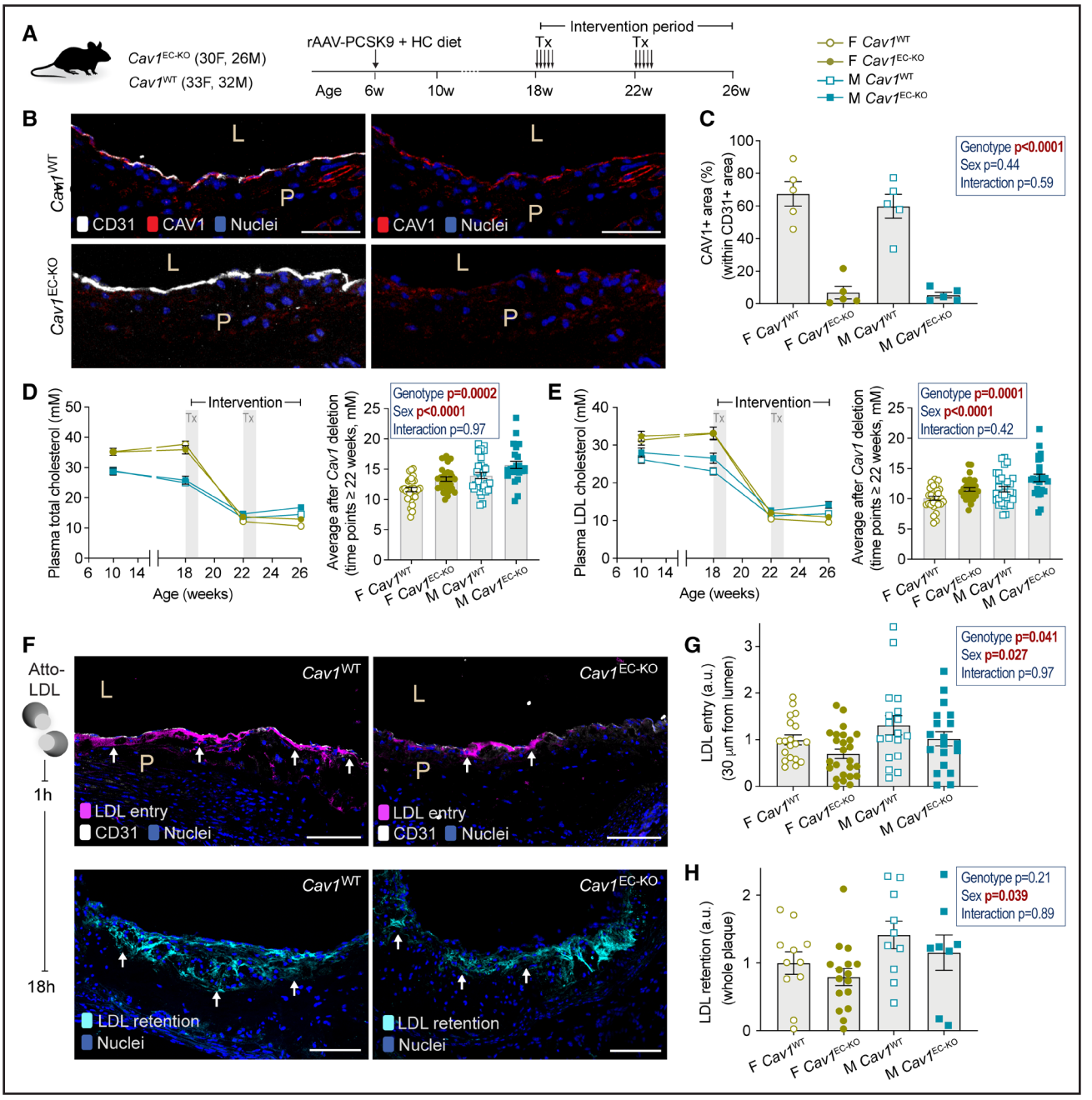

Figure 1. Impact of endothelial *Cav1* (caveolin-1) deletion on LDL (low-density lipoprotein) entry and retention in atherosclerotic plaque.

A, Experimental design. **B**, Representative images of CD31 (cluster of differentiation 31; white) and CAV1 (red) staining in aortic root plaques of *Cav1*^{WT} and *Cav1*$^{EC\text{-}KO}$ mice. Scale bar, 50 μm. **C**, CAV1 signal within CD31-stained endothelium was reduced ≈90% in *Cav1*$^{EC\text{-}KO}$ mice of both sexes. **D** and **E**, Plasma total (P-Total) and LDL (P-LDL) cholesterol levels during the study (**left**). A direct cholesterol-lowering effect of tamoxifen was observed, independent of genotype. Average total and LDL cholesterol levels were significantly increased in *Cav1*$^{EC\text{-}KO}$ compared with *Cav1*^{WT} mice after endothelial *Cav1* deletion (average of 22 and 26 week time points, **right**). **F**, Representative images of Atto-LDL injected at 1 hour (magenta) and 18 hours (cyan) before analysis to measure LDL entry and retention rates, respectively. Arrows point to tracked LDL. Scale bar 100 μm. **G**, LDL entry, measured as Atto signal in the luminal 30 μm of plaque at 1 hour after injection, was significantly reduced with no significant interaction with sex. **H,** LDL retention, measured in whole plaque at 18 hours after injection, was not significantly altered. Values in G and H were normalized to the level in *Cav1*^{WT} females (F). *P* values in **C**, **D**, **E**, **G**, and **H** were calculated by 2-way ANOVA. Error bars indicate mean±SEM. HC indicates high cholesterol; L, lumen; M, male; P, plaque; rAAV8-PCSK9, recombinant adeno-associated virus encoding D377Y gain-of-function proprotein convertase subtilisin/kesin type 9; and Tx, tamoxifen.

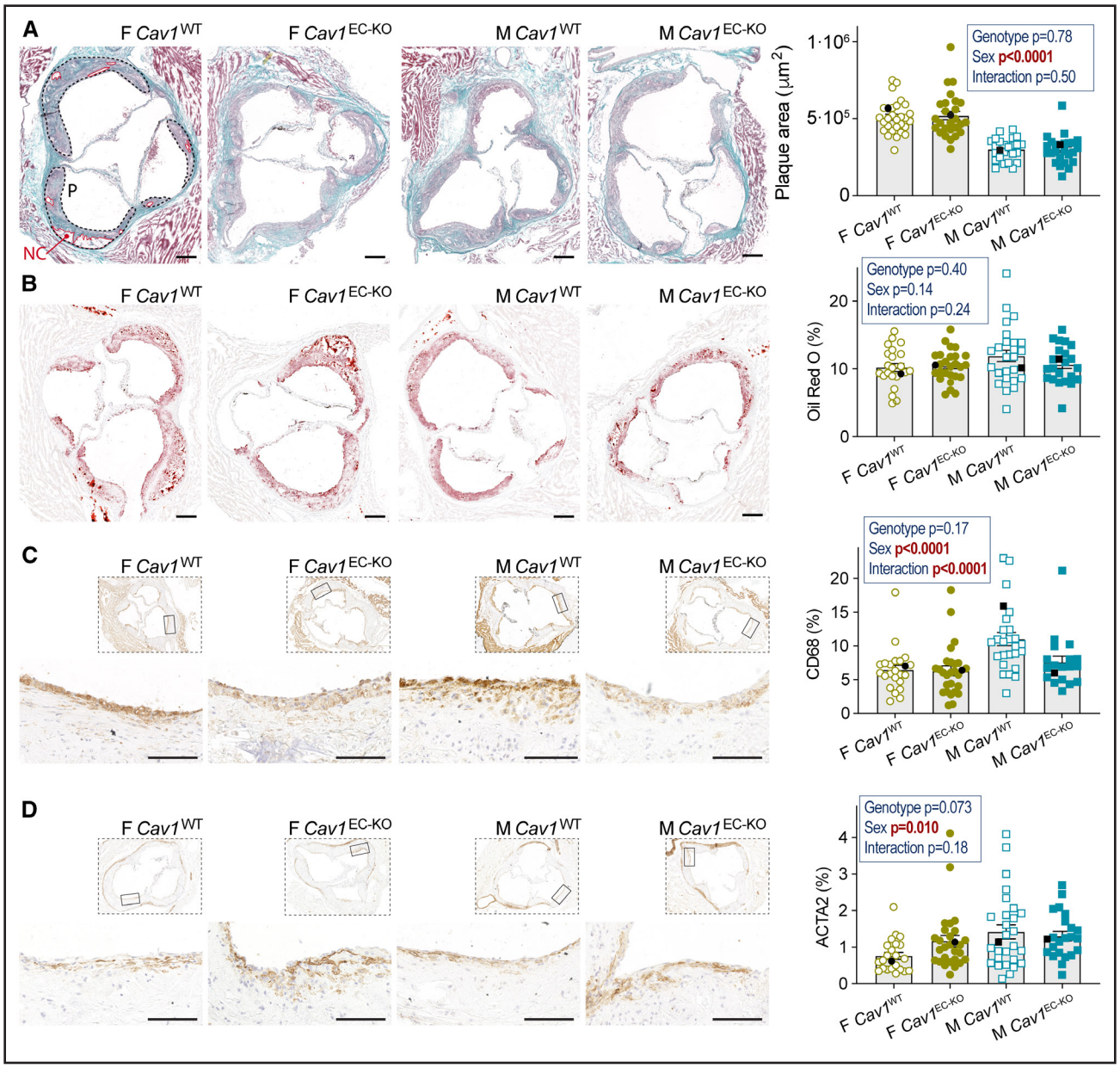

Figure 2. *Cav1* (caveolin-1) deletion in established plaque endothelium does not significantly impact progression.
A, Representative examples of trichrome stains and plaque area quantification. The dashed lines in the leftmost image illustrate how plaque (P) and necrotic core (NC) areas were delineated. Scale bar 200 μm. **B**, Representative examples of Oil Red O–stained sections and the percentage of Oil Red O–staining area within plaques. Scale bar 200 μm. **C**, Representative CD68 (cluster of differentiation 68) staining and quantification of plaque macrophages, showing a significant decline in *Cav1*$^{EC-KO}$ vs *Cav1*^{WT} mice, which was, however, restricted to male (M) mice (Holm-Šidak post hoc test P=0.037 for M, P=0.58 for female [F]). Scale bars 100 μm. **D**, Representative ACTA2 (actin alpha 2) staining and quantification of plaque cap smooth muscle cells. Scale bars 100 μm. The black symbols in the graphs in **A** through **D** indicate data points corresponding to the shown staining examples. *P* values in **A** through **D** were calculated by 2-way ANOVA (using log-transformed data in **A**, **C**, and **D**). Error bars indicate mean±SEM.

Analysis of Changes in Inflammation, Cap Smooth Muscle Cells, and Fibrosis

Loss of CAV1 dampens disturbed flow-induced inflammatory signaling and VCAM1 (vascular cell adhesion molecule 1) expression in cultured ECs,[4] and it reduces VCAM1 expression and macrophage accumulation in early atherosclerosis.[4,15,16] The reduced endothelial activation may be facilitated by decreases in fibronectin.[24,25] To analyze whether *Cav1* deletion can ameliorate endothelial activation in established atherosclerotic lesions, we quantified fibronectin, endothelial VCAM1, and the macrophage marker CD68 in lesions by immunostaining. CD68+ macrophages were reduced in *Cav1*$^{EC-KO}$ male but not female mice (Figure 2C), whereas fibronectin and endothelial VCAM1 signal were similar in all experimental groups (Figure S11).

To investigate if smooth muscle cell–derived lesion components were altered, we stained for ACTA2 (actin alpha 2), which marks the contractile cells of the fibrous cap, and found no significant changes between genotypes (Figure 2D). Furthermore, we used polarization microscopy of Sirius Red–stained sections to test whether lesional collagen, a product of smooth muscle cell–derived cells from lesions, was altered by the loss of endothelial CAV1. No differences were found between genotypes in yellow-red (collagen fibers) or green (smaller fibers) polarized light signals (Figure S12).

DISCUSSION

In the present study, we demonstrated that targeted endothelial knockout of *Cav1* in established murine atherosclerotic lesions reduced the rate of LDL entry, but at an insufficient level to significantly reduce LDL retention, lipid accumulation, and lesion progression after 8 weeks of intervention. The findings stand in contrast to the potent anti-atherosclerotic effects reported for constitutive global *Cav1* knockouts in *Ldlr*$^{-/-}$, *ApoE*$^{-/-}$, and rAAV-PCSK9–injected mice,[4,5,7] with some studies showing nearly complete eradication of early atherosclerosis despite an increase in plasma cholesterol.[4] Importantly, prior atherosclerosis studies have examined CAV1 function only in contexts where CAV1 was absent during lesion initiation, a phase of the disease that may operate by different mechanisms than the progression of established atherosclerosis in patients amenable to therapy. Importantly, the initiation phases of atherosclerosis, which occur early in life in most regions of the arterial tree, are not an obvious target for drug therapy, and it is, therefore, relevant to reexamine promising targets in models of lesion progression.[26]

Our experiment was made possible by creating a floxed *Cav1* mouse line, enabling the loss of *Cav1* in the endothelium after atherosclerotic lesions had already formed. The new line was validated by immunostaining, demonstrating efficient reductions in CAV1 in the endothelium of both normal and atherosclerotic aorta. Additionally, electron microscopy showed a marked reduction in caveolae structures in endothelial cells after targeted knockout, consistent with previous studies of global knockout models and underscoring the indispensable role of CAV1 in caveolae formation.[4,13,14] The contrasting findings between our study of endothelium-specific *Cav1* deletion and previous global knockout studies may suggest the significance of CAV1 expressed in nonendothelial cell types in atherosclerosis. While this possibility warrants further exploration in future studies, it should be stressed that the importance of endothelial CAV1 has been thoroughly established through alternative techniques. Several studies have shown that reintroducing CAV1 expression in endothelial cells via an endothelium-expressed transgene nullified the effect of global *Cav1* knockout on lesion formation.[15,16]

An alternative, and arguably more plausible, explanation for our findings is that CAV1-related mechanisms lose importance as atherosclerosis progresses. In normal arteries and early lesions, transcytosis through endothelial caveolae may represent the primary route of LDL entry, as recently reviewed,[2] but this pathway may become less dominant in established disease. Murine atherosclerotic lesions exhibit a high capacity for LDL entry and retention that is not saturated even at high endogenous LDL levels,[18] and increased leakiness—potentially resulting from increased endothelial turnover and endothelial-to-mesenchymal transitioning—may be involved. Super-resolution techniques have revealed the intercellular transport of nanoparticles larger than LDL through leaky endothelial junctions in murine atherosclerosis,[17] suggesting that LDL may also enter via this route. At the same time, we found that the level of endothelial CAV1 decreases with plaque development. The partial bypassing of caveolar transcytosis in established atherosclerosis suggested by these findings may explain why *Cav1* deletion in the present study reduced LDL entry less than reported previously for early atherosclerosis.[4,16] That said, it cannot be excluded that a more efficient reduction in endothelial CAV1, beyond the ≈90% achieved here, could unveil a stronger effect, more in line with observations in constitutive *Cav1* knockout mice.

Similarly, the central role of CAV1 in linking disturbed flow forces to inflammatory signaling, evident in cultured endothelial cells,[4] may be overridden in advanced lesions by proinflammatory mediators released during ongoing inflammation. Notably, if endothelial barrier disruption and high inflammatory activity diminish the role of CAV1 in progressive atherosclerosis, some of its importance could be regained during LDL lowering and atherosclerosis regression, which were not studied here.

Despite the lack of effect on overall lesion size, we did detect differences in macrophages, which were reduced by *Cav1* deletion in males, consistent with previous reports.[4,16,24] Although only significant in 1 sex, they are not necessarily sex-dependent effects; rather, they could be stage-dependent because atherosclerosis in males was less advanced than in females, as is often the case for murine models of atherosclerosis.[23]

Limitations

We studied an experimental murine atherosclerosis model and cannot extrapolate directly to human disease. First, LDLs may enter human plaques through neovessels, which are not present in mouse lesions. Second, the high-LDL-driven disease activity and the use of experimental tools, for example, Cre recombinase expression, tamoxifen, corn oil, and high PCSK9 expression, which may interact with disease processes,

distinguish the experimental lesions from typical human lesions.

Conclusions

Targeting endothelial CAV1 in established murine atherosclerosis cannot stop or reverse lesion progression. This contrasts with the strong atherosclerosis protection provided by constitutive CAV1 loss and underscores the context-dependency of gene knockouts in atherosclerosis studies and the relevance of reinvestigating promising targets in models of lesion progression.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Supplemental Methods
Figures S1–S12
Major Resources Table
ARRIVE Checklist

REFERENCES

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