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Macrophage Sortilin Promotes LDL Uptake, Foam Cell Formation, and Atherosclerosis

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Abstract

Rationale—Non-coding gene variants at the *SORT1* locus are strongly associated with LDL-C levels as well as with coronary artery disease (CAD). *SORT1* encodes a protein called sortilin, and hepatic sortilin modulates LDL metabolism by targeting apoB-containing lipoproteins to the lysosome. Sortilin is also expressed in macrophages, but its role in macrophage uptake of LDL and in atherosclerosis independent of plasma LDL-C levels is unknown.

Objective—To determine the effect of macrophage sortilin expression on LDL uptake, foam cell formation, and atherosclerosis.

Methods and Results—We crossed *Sort1−/−* mice onto a 'humanized' *Apobec1−/−; hAPOB* Tg background and determined that *Sort1* deficiency on this background had no effect on plasma LDL-C levels but dramatically reduced atherosclerosis in the aorta and aortic root. In order to test whether this effect was a result of macrophage sortilin deficiency, we transplanted *Sort1−/−;LDLR−/−* or *Sort1+/+;LDLR−/−* bone marrow into *Ldlr−/−* mice and observed a similar reduction in atherosclerosis in mice lacking hematopoetic sortilin without an effect on plasma LDL-C levels. In an effort to determine the mechanism by which hematopoetic sortilin deficiency reduced atherosclerosis, we found no effect of sortilin deficiency on macrophage recruitment or LPS-induced cytokine release in vivo. In contrast, sortilin deficient macrophages had significantly reduced uptake of native LDL ex vivo and reduced foam cell formation in vivo, whereas sortilin overexpression in macrophages resulted in increased LDL uptake and foam cell formation.

Conclusions—Macrophage sortilin deficiency protects against atherosclerosis by reducing macrophage uptake of LDL. Sortilin-mediated uptake of native LDL into macrophages may be an important mechanism of foam cell formation and contributor to atherosclerosis development.

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DISCLOSURES

None.

Keywords

Atherosclerosis; low density lipoprotein cholesterol; macrophage; foam cell; receptor-mediated endocytosis

INTRODUCTION

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in the world. A central hallmark of atherosclerosis is the cholesterol-loaded macrophage or 'foam cell.' Despite decades of research, the molecular mechanisms by which arterial macrophages take up cholesterol-rich lipoproteins, such as low density lipoproteins (LDL), leading to the development of foam cells and atherosclerotic lesions remain to be fully elucidated. Kruth and colleagues have shown that macrophages internalize native LDL through a process of macropinocytosis, although LDL uptake cannot be fully accounted for by this process[1]. Gene deletion of known receptors of modified LDL, such as scavenger receptor A (SRA) and CD36, do not reduce foam cell formation or the development of atherosclerosis in mice[2]. Thus, pathways that mediate macrophage uptake of LDL leading to foam cell formation and atherosclerosis remain of substantial interest.

Unbiased genome-wide association studies (GWAS) of coronary artery disease (CAD) have the potential to identify new pathways involved in atherosclerosis. In one of the first GWAS for CAD, non-coding genetic variants at chromosome 1p13 were reported to be significantly associated with myocardial infarction and CAD[3], a finding that has been widely replicated[4, 5]. The same variants have also shown to be significantly associated with plasma levels of LDL cholesterol[6–8]. The *SORT1* gene, encoding the protein sortilin, appears to be the causal gene at the locus regulating LDL cholesterol levels[9–11]. Sortilin is a type I transmembrane trafficking receptor initially characterized by its ability to serve as a receptor for proneurotrophins[9–11] and for its role as a sorting receptor for lysosomal hydrolases[12, 13]. Hepatic sortilin expression modulates VLDL production rates[7, 910]; in addition, hepatic sortilin binds LDL and promotes its cellular uptake and lysosomal degradation[10]. Sortilin is also expressed in macrophages, but little is known about its function in this cell type or its relationship to atherosclerosis.[14, 15] We hypothesized that macrophage sortilin mediates macrophage LDL uptake. Through a combination of in vivo mouse studies and ex vivo macrophage studies utilizing *Sort1−/−* macrophages, we show here that macrophage sortilin promotes macrophage LDL uptake, foam cell formation, and atherosclerosis independent of plasma LDL-C levels.

METHODS

Detailed descriptions of all Methods can be found in the Online Supplement. Following is a summary of the key experimental approaches.

For studies of the effect of total body sortilin deficiency on atherosclerosis, we used the *Apobec1−/−; hAPOB* Tg mouse, in which the human apoB transgene is overexpressed in the liver and, in contrast to the wild-type mouse, is not edited, thus producing only the apoB-100 protein.[21–23] These mice were crossed with total body *Sort1−/−* mice and

experiments compared *Sort1−/−;Apobec1−/−; hAPOB* Tg mice to *Sort1+/+;Apobec1−/−; hAPOB* Tg littermates. Mice were fed a western-type diet for 18 weeks and assessed for atherosclerosis in the aortic roots and the entire aorta by en face quantitation. A detailed description of the atherosclerosis methods can be found in the Online Supplement.

For studies of hematopoietic sortilin deficiency on atherosclerosis, we transplanted donor *Sort1−/−;Ldlr −/−* and *Sort1+/+;Ldlr −/−* bone marrow into irradiated recipient *Ldlr−/−* mice. Six weeks post bone marrow transplantation the mice were placed on a western diet for 18 weeks and then assessed for atherosclerosis.

For studies of macrophage LDL uptake, both thioglycollate-elicited peritoneal macrophages and bone-marrow derived macrophages were used. The macrophages were incubated with ^{125}I-LDL for five hours and uptake and degradation were assessed.

Statistical analyses were done using 2-tailed paired student's t test and 1 way ANOVA with a Bonferroni correction (for LPS experiment).

RESULTS

Sortilin deficiency in hematopoetic cells protects against atherosclerosis

Total body *Sort1* deficiency on an *Ldlr−/−* background is associated with reduced plasma cholesterol levels, confounding attempts to address its role in atherosclerosis independent of LDL-C levels. We crossed *Sort1−/−* mice onto the background of an atherosclerosis-prone *Apobec1−/−; hAPOB* Tg mouse model, which has a human-like lipoprotein profile, and fed the mice a western type diet for 18 weeks. On this genetic background, total and LDL cholesterol levels were not different in *Sort1−/−* mice compared with *Sort1+/+* mice (Figure 1a,b). After 18 weeks on diet, *Sort1−/−* mice had a 68% reduction in en face aorta lesion area ($P < 0.0001$ Figure 1c,d) and an 87% reduction in aortic root lesion area ($P < 0.0001$ Figure 1e,f) compared with *Sort1+/+* mice, demonstrating a major effect of sortilin deficiency in reducing atherosclerosis despite no effect on plasma cholesterol in this model.

Macrophages express sortilin and we hypothesized that macrophage sortilin deficiency might account specifically for the reduced atherosclerosis. In order to test this hypothesis, irradiated *Ldlr−/−* mice were transplanted with bone marrow from *Sort1−/−;Ldlr−/−* mice or *Sort1+/+;Ldlr−/−* mice and 6 weeks after transplantation were started on a western type diet and fed for 18 weeks. Bone marrow engraftment was 74% (Online Figure Ia). Body, liver and spleen weights, plasma cholesterol, peripheral blood counts, and hepatic *Sort1* expression were similar between groups (Online Figure I). Mice transplanted with *Sort1−/−;Ldlr−/−* bone marrow had a 69% reduction in en face aortic lesion area ($P<0.00001$) and a 34% reduction in aortic root lesion area ($P < 0.01$) compared to mice transplanted with *Sort1+/+;Ldlr−/−* bone marrow (Figure 2a–d), suggesting that hematopoietic, and potentially macrophage sortilin influences the development of atherosclerotic disease.

Sortilin deficiency has no effect on thioglycollate-elicited monocyte recruitment or LPS-induced inflammatory response in vivo

Monocyte recruitment is a key determinant of the macrophage content of atherosclerotic lesions. To determine if *Sort1* deficiency affects macrophage recruitment, *Sort1*−/− and *Sort1*+/+ mice were injected i.p. with thioglycollate to elicit an inflammatory response. Three days after injection, peritoneal macrophages were harvested and counted. There was no difference in macrophage counts between *Sort1*+/+ and *Sort* −/− mice (Online Figure IIa). Monocyte recruitment and atherosclerosis development is strongly influenced by inflammation and cytokine production. To determine if *Sort1*−/− mice have reduced cytokine levels, cytokine multiplexing assays were performed on *Sort1*−/− and *Sort1*+/+ mice injected with lipopolysaccharide (LPS). Cytokine levels post LPS injection were found to be similar between *Sort1*−/− and *Sort1* +/+ (Online Figure IIb,c).

Macrophage sortilin deficiency reduces LDL uptake and foam cell formation

To determine if macrophage sortilin deficiency reduces foam cell formation, primary bone marrow macrophages were isolated from *Sort1*−/−; *Ldlr*−/− and control *Sort1*+/+; *Ldlr*−/− mice, cells were differentiated with M-CSF for 7 days, incubated with 1 mg/mL LDL for 5 hours, and Oil Red O staining was performed. *Sort1*−/−;*Ldlr*−/− macrophages had a clear and consistent reduction in Oil Red O staining (Figure 3a). *Sort1* deficient macrophages had a significant 28% reduction in total cellular cholesterol, a 25% reduction in free cholesterol, and a 32% reduction in cholesteryl ester ($P<0.05$; Figure 3b–d). In vivo foam cell formation assays were performed by isolating thioglycollate-elicited peritoneal macrophages from *Sort1*+/+;*Apobec*−/−;*hApob Tg* and *Sort1*−/−;*Apobec*−/−;*hApob Tg* mice fed a western type diet for 18 weeks. Consistent with the in vitro loading experiments, macrophages isolated from *Sort1*−/− mice had reduced Oil Red O staining and a significant 33% reduction in cellular cholesterol content compared to macrophages isolated from *Sort1*+/+ mice ($P<0.05$; Figure 3e–f). These studies indicated that sortilin-deficient macrophages have reduced capacity to form foam cells when exposed to high levels of LDL.

As sortilin can act as a receptor for LDL in hepatocytes, we hypothesized that sortilin promotes the internalization of LDL by macrophages. To test the response of sortilin expression to increasing cholesterol concentration in macrophages, thioglycollate-elicited peritoneal macrophages were isolated from wild-type mice and incubated for 24 hours in lipoprotein-deficient serum, lipoprotein-deficient serum supplemented with 25-hydroxycholesterol to reduce intracellular cholesterol content, or with lipoprotein deficient serum supplemented with high concentrations of LDL. In contrast to the LDL receptor, whose expression was reduced by co-incubation with LDL, *Sort1* mRNA abundance increased over 400-fold with LDL incubation ($P<0.05$; Figure 4a) and sortilin protein also increased significantly with LDL incubation (Figure 4b).

To test the hypothesis that sortilin is able to promote the uptake of LDL into macrophages, ^{125}I-LDL uptake studies were performed in thioglycollate-elicited and bone marrow derived macrophages from *Sort1*+/+ and *Sort1*−/− mice. *Sort1* deficiency was associated with a 48% and 33% percent reduction in LDL uptake, respectively ($P<0.05$ for both; Figure 4c–d). We next tested if this effect on LDL uptake was independent of the LDL

receptor. Bone marrow derived macrophages were isolated from *Sort1+/+;Ldlr−/−* and *Sort1−/−;Ldlr−/−* mice and ^{125}I-LDL uptake studies were performed. *Sort1* deficiency was associated with a 39% percent reduction in LDL uptake in the absence of the LDLR ($P<0.05$; Figure 4f).

To further confirm that sortilin deficiency confers atheroprotection by eliminating a receptor dependent pathway for LDL uptake and not by modulating macropinocytosis, LDL uptake studies were performed in bone marrow derived macrophages in the presence of cytochalasin D, a potent inhibitor of actin polymerization and macropinocytosis. Under these conditions, while LDL uptake is reduced, substantial residual LDL uptake still takes place.[1] *Sort1* deficiency was associated with a 38% reduction in LDL uptake in the presence of cytochalasin D ($P< 0.05$; Figure 4f). These studies indicate that macrophage sortilin deficiency reduces macrophage uptake of LDL and formation of foam cells and this effect is independent of the LDL receptor and of macropinocytosis.

Finally, to determine whether increased macrophage sortilin results in increased LDL uptake, J774 cells were transduced with lentivirus encoding GFP or *Sort1* and LDL uptake studies were performed. *Sort1* overexpression in macrophages led to a 29% increase in LDL uptake ($P< 0.05$; Figure 4e).

DISCUSSION

Genetic variation at the 1p13 *SORT1* locus is strongly associated both with CAD, as well as with plasma LDL-C levels. We have previously shown that sortilin is a cell surface receptor for LDL on hepatocytes and its elevated expression in liver reduces LDL-C at least in part by facilitating LDL clearance from blood. Sortilin is expressed in macrophages, which actively take up LDL, leading us to investigate the role of macrophage sortilin in LDL uptake, foam cell formation, and atherosclerosis. After a series of studies of atherosclerosis in mice and LDL uptake in macrophages, we conclude that macrophage sortilin promotes LDL uptake, foam cell formation, and atherosclerosis and that deficiency is protective against atherosclerosis at least in part by reducing LDL uptake.

Macrophage uptake of modified LDL can be mediated by scavenger receptors such as SRA and CD36. However, deletion of SRA or CD36 does not reduce macrophage uptake of native LDL[1], nor does it ameliorate atherosclerosis in hypercholesterolemic mice.[17] Even CD36−/−; SRA −/− mice still contain abundant lipid laden macrophages in vessel wall and develop atherosclerosis.[18] Kruth has shown that macrophages can take up native LDL through fluid-phase macropinocytosis, but there remains substantial LDL uptake even when this pathway is inhibited.[1] Our data establish macrophage sortilin as the first receptor-mediated pathway of uptake of native LDL leading to foam cell formation and promoting atherosclerosis development. We also found that increasing concentrations of extracellular LDL causes an upregulation of macrophage *Sort1* mRNA and protein. Because a function of macrophages is to phagocytose LDL, it is very possible that increased exposure of macrophages to LDL triggers the transcriptional upregulation of sortilin, which then mediates increased LDL uptake. The mechanisms of this upregulation of macrophage *Sort1* by LDL require further exploration.

While this manuscript was under preparation, Mortensen et al. reported that sortilin deficiency reduced atherosclerosis in the ApoE −/− mouse model.[19] While the fundamental observation is consistent with our data, these authors suggested a different mechanism, namely that decreased proinflammatory cytokines may have been responsible for the reduced atherosclerosis. We performed our cytokine assays prior to initiation of atherosclerotic disease, while Mortensen et al measured the cytokine profile after disease was present. In addition, these authors did not see a reduction in LDL uptake by sortilin deficient macrophages, although they used an ATTO dye conjugated to the LDL that may have influenced the interaction with sortilin. We also used a different mouse model, the *Apobec1−/−; hAPOB* Tg mouse, in which human apoB-100 containing LDL is the dominant lipoprotein,in a human-like lipoprotein profile, while Mortensen et al used the *Apoe*−/− mouse, which is characterized primarily by mouse apoB-48 containing remnant lipoproteins. Overall, the top-line results of the two studies, which used very different mouse atherosclerosis models, are highly comparable, whereas the mechanisms responsible for the reduced atherosclerosis may be complex and multifactorial.

In summary, our findings indicate that *SORT1* deficiency in macrophages reduces LDL uptake and macrophage cholesterol loading independent of the LDL receptor or macropinocytosis, and protects against the development of atherosclerosis. The macrophage sortilin pathway is a novel pathway of macrophage cholesterol loading that quantitatively contributes to atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

LDL	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
Tg	transgenic
ApoB	Apolipoprotein B
CAD	coronary artery disease
GFP	green fluorescent protein

GWAS genome wide association studies

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Novelty and Significance

What Is Known?

- Genetic variants at the *SORT1* locus are associated with plasma low density lipoprotein cholesterol (LDL-C) levels as well as myocardial infarction and coronary artery disease (CAD).
- The gene *SORT1* encodes a protein called sortilin, which in hepatocytes can mediate the uptake and degradation of LDL.

What New Information Does This Article Contribute?

- Deletion of sortilin in macrophages reduces foam cell formation and atherosclerosis without influencing plasma LDL-C levels.
- Macrophage sortilin is a new receptor-mediated pathway promoting uptake of native LDL by macrophages promoting foam cell formation and atherosclerosis.

The mechanisms linking genetic variation at the SORT1 locus with CAD have not been fully elucidated. Using atherosclerosis-prone mice with a human-like lipoprotein profile, we found that deletion of sortilin in the whole body or specifically in macrophages substantially reduced atherosclerosis without affecting plasma cholesterol. We found that macrophage sortilin mediates the uptake of native (unmodified) LDL leading to foam cell formation, a novel receptor-mediated process for macrophage uptake of LDL. This pathway could potentially be targeted as an approach to reducing risk of atherosclerosis.

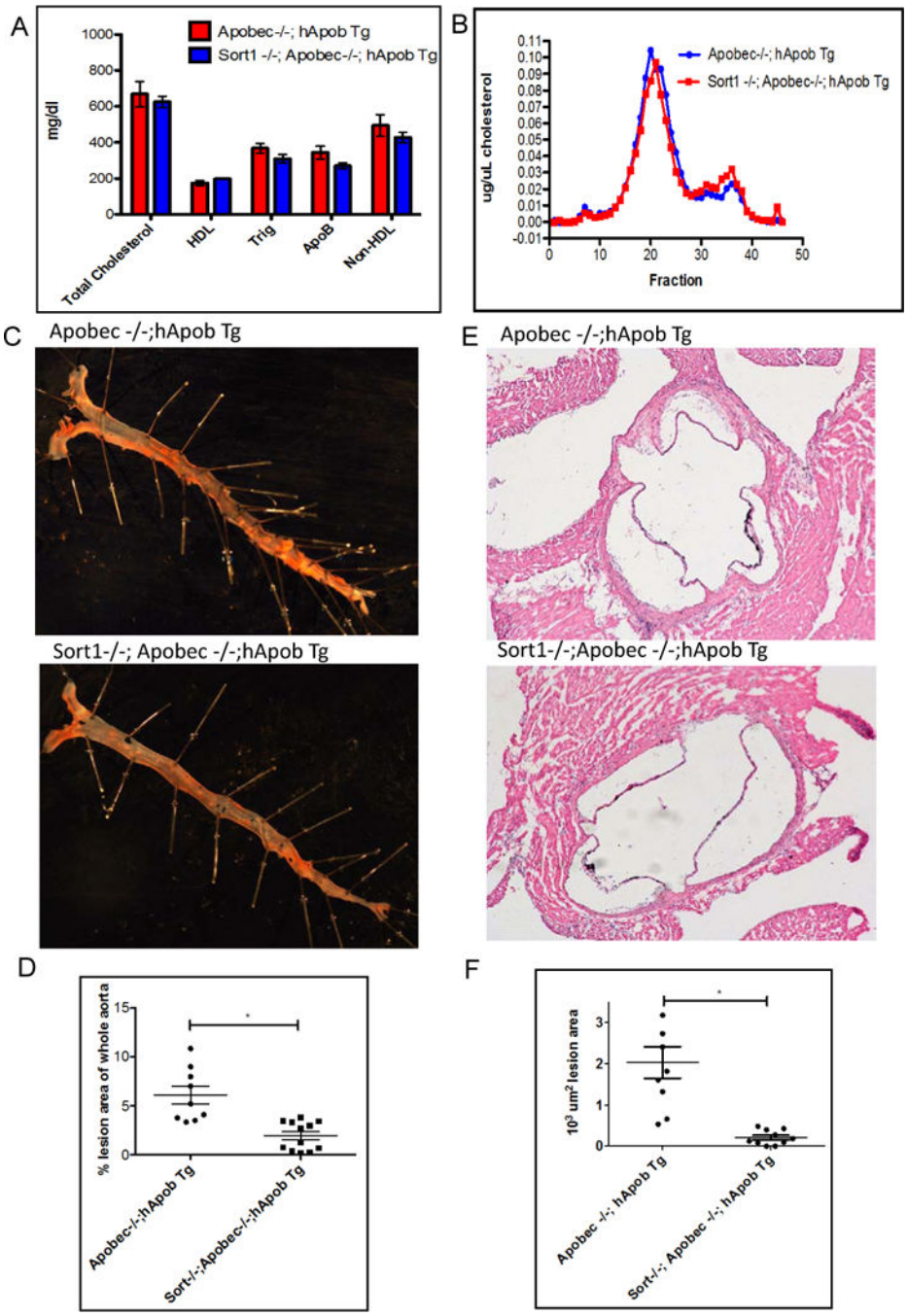

Figure 1. Whole body *Sort1* deficiency reduces atherosclerosis independent of plasma lipid level

A. Eight week old Apobec −/−; hApob Tg and Sort −/−;Apobec −/−; hApobTg mice (n=10 per group) placed on a western diet for 18 weeks and retroorbital bleeds were performed after a 4 hr fast. Plasma was isolated by centrifugation and samples were run individually on a Cobas Mira Autoanalyzer (Roche Diagnostic Systems). **B.** Samples were pooled and run on FPLC. **C.** Whole aortas were dissected and tissues harvested. Aortas were stained with Oil Red O. **D.** Quantification of lesions on aorta **E.** Aortic roots were sectioned & stained

with hematoxylin eosin (H&E) **F.** Quantification of atherosclerotic lesion area at aortic root P value <0.01

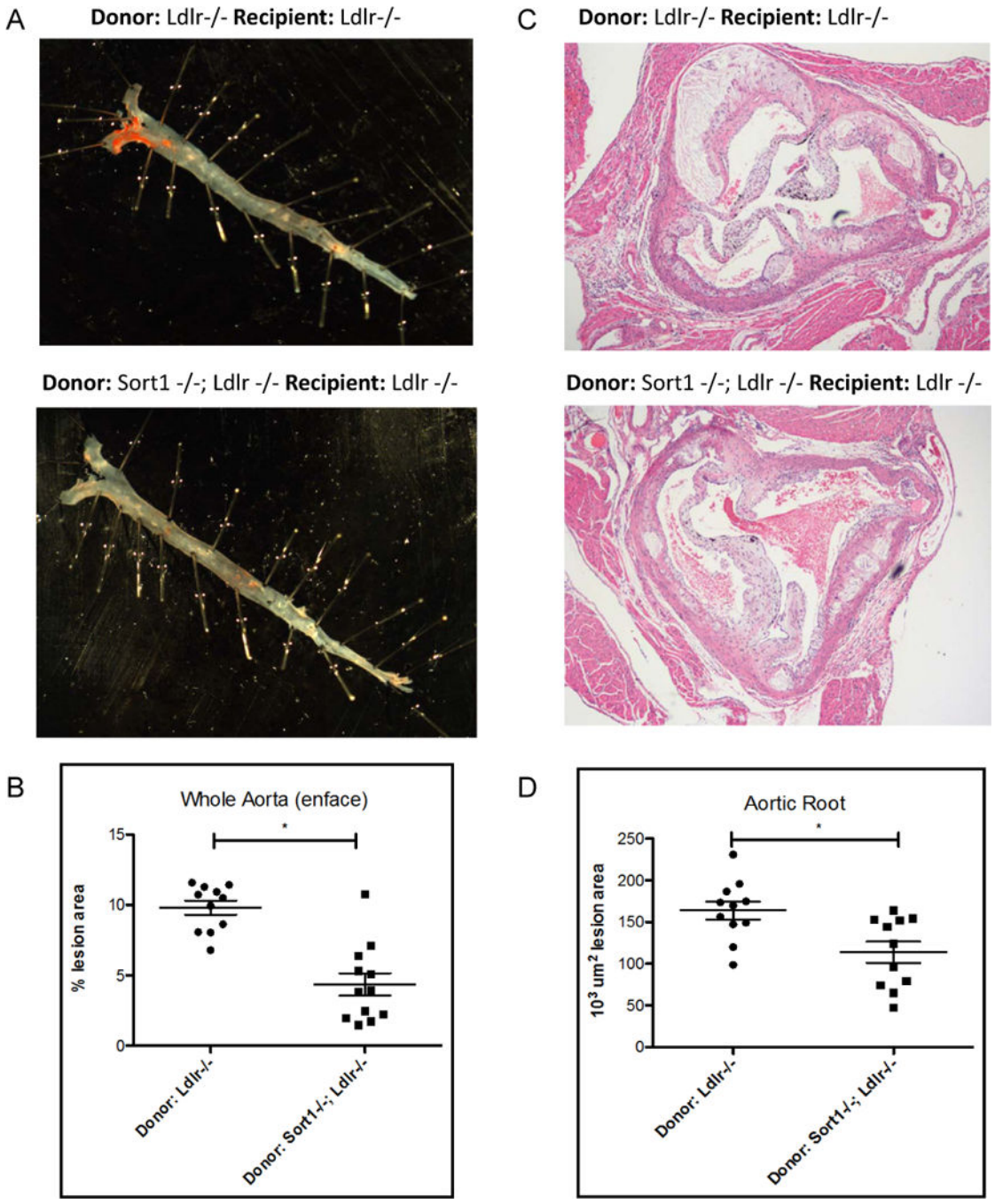

Figure 2. Sort1 hematopoietic deficiency reduces atherosclerosis

A. After 18 weeks on western diet recipient *Ldlr−/−* mice carrying donor *Ldlr−/−* or *Sort1−/−;Ldlr−/−* (n=11 per group) bone marrow transplanted mice aortas were dissected and stained with Oil Red O **B.** Quantification of lesions on aorta P<0.00001 **C.** Representative aortic roots stained with H&E **D.** Quantification of aortic roots P value <0.01

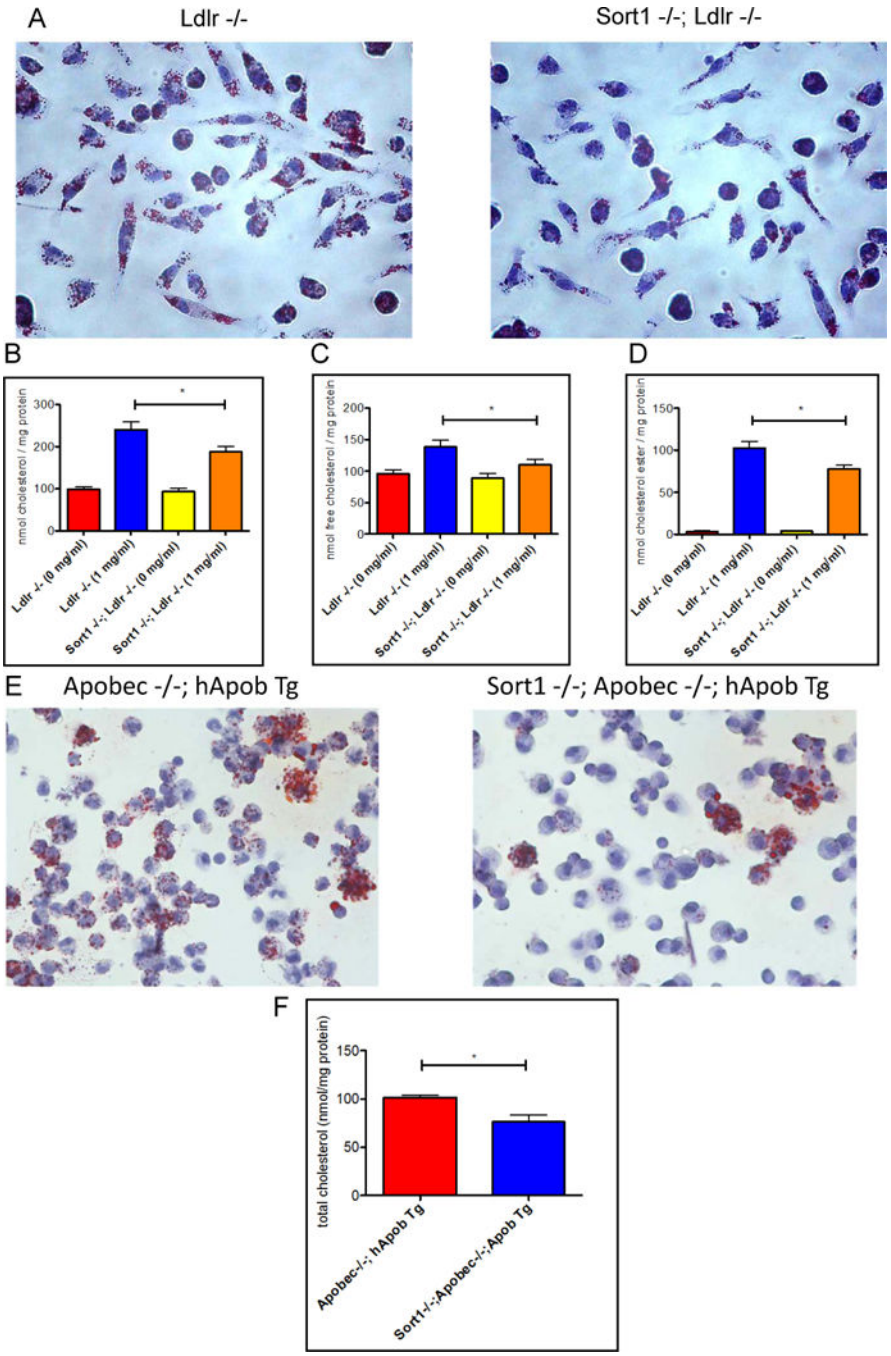

Figure 3. Sort1 deficiency reduces macrophage cellular cholesterol levels and Oil Red O staining in vivo and in vitro

A. Bone marrow-derived macrophages were stained with Oil Red O (n=3) **B.** Cellular total cholesterol **C.** Free cholesterol **D.** Cholesterol ester measured by gas chromatography (GC/MS). **E.** Peritoneal macrophages from mice fed a western diet for 18 weeks were isolated and stained with Oil Red O **F.** Cellular total cholesterol of peritoneal macrophages measured by GC/MS. All experiments were replicated.

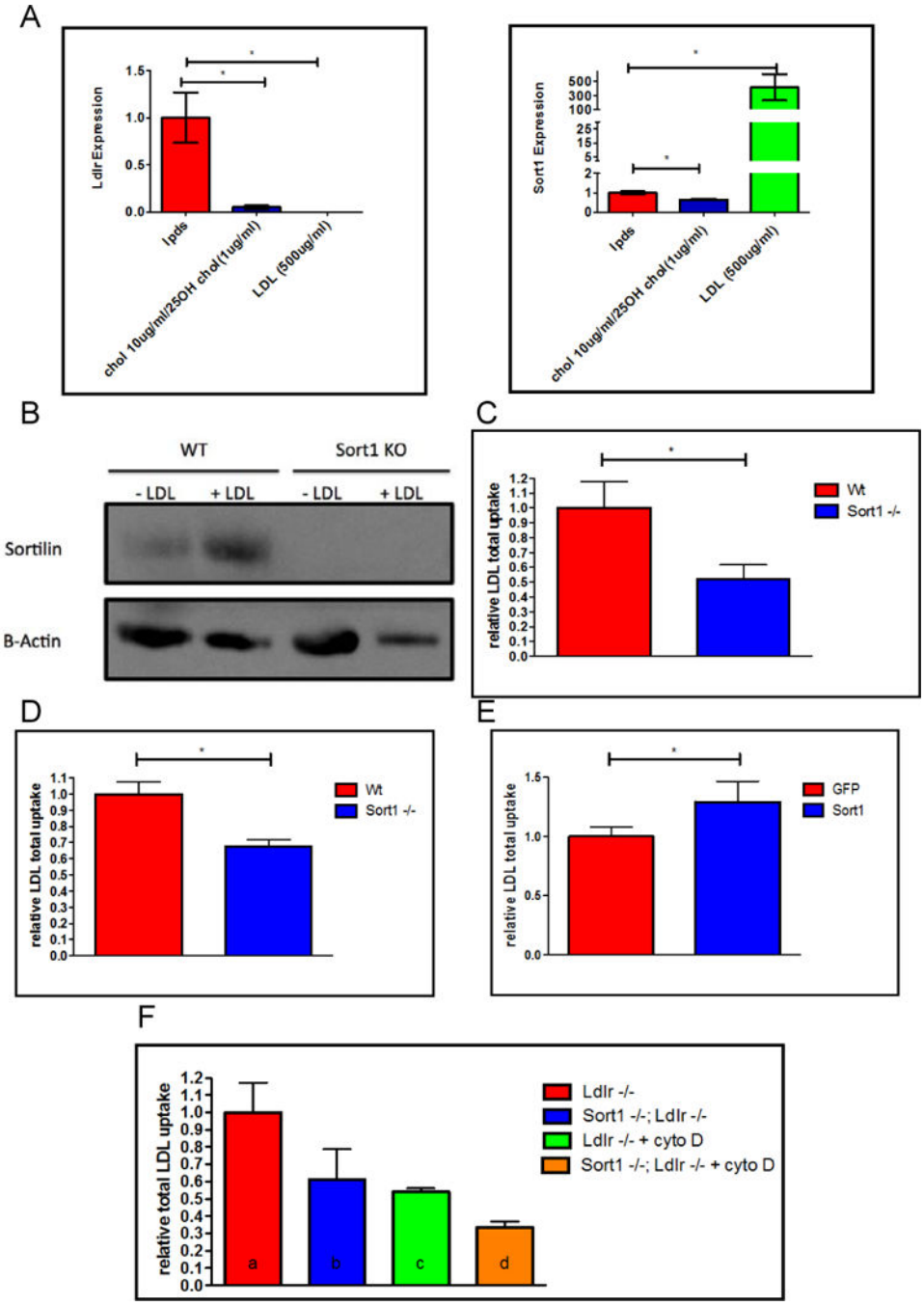

Figure 4. LDL loading increases sortilin in macrophages, where it mediates uptake of LDL

A. Thio-elicited peritoneal macrophages were incubated in conditions listed above. RNA expression and **B.** Westerns **C.** I-125 LDL uptake study from thioglycollate-elicited macrophages from *Sort1* +/+ and *Sort1* −/− mice (n=6) **D.** I-125 LDL uptake study from bone marrow derived macrophages from *Sort1*+/+ and *Sort1*−/− mice (n=6)". and **F.** M-CSF differentiated macrophages from *Ldlr* −/− and *Sort1*−/−*; Ldlr* −/− mice. 250ug/ml LDL was incubated and 4ug/ml cytochalasin D (as indicated) for 5 hours (n=6).