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

Kevin M. Patel, Alanna Strong, Junichiro Tohyama, Xueting Jin, Carlos R. Morales, Jeffery Billheimer, John Millar, Howard Kruth, Daniel J. Rader

Rationale: Noncoding gene variants at the SORT1 locus are strongly associated with low-density lipoprotein cholesterol (LDL-C) levels, as well as coronary artery disease. SORT1 encodes a protein called sortilin, and hepatic sortilin modulates LDL metabolism by targeting apolipoprotein B-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 transgenic 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. To test whether this effect was a result of macrophage sortilin deficiency, we transplanted Sort1−/−;LDLR−/− or Sort1−/−;LDLR−/− mouse bone marrow into Ldlr−/− mice and observed a similar reduction in atherosclerosis in mice lacking hematopoietic sortilin without an effect on plasma LDL-C levels. In an effort to determine the mechanism by which hematopoietic sortilin deficiency reduced atherosclerosis, we found no effect of sortilin deficiency on macrophage recruitment or lipopolysaccharide-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. (Circ Res. 2015;116:789-796. DOI: 10.1161/CIRCRESAHA.116.305811.)

Key Words: atherosclerosis • foam cell • low-density lipoprotein cholesterol • macrophage • receptor-mediated endocytosis

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 lipoprotein (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. Gene deletion of known receptors of modified LDL, such as scavenger receptor A and CD36, do not reduce foam cell formation or the development of atherosclerosis in mice. Thus, pathways that mediate macrophage uptake of LDL, leading to foam cell formation and atherosclerosis, remain of substantial interest.

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Unbiased genome-wide association studies of coronary artery disease have the potential to identify new pathways involved in atherosclerosis. In one of the first genome-wide association studies for coronary artery disease, noncoding genetic variants at chromosome 1p13 were reported to be significantly associated with myocardial infarction and coronary artery disease, a finding that has been widely replicated. The same variants have also been shown to be significantly associated with plasma levels of LDL cholesterol.

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Correspondence to Dr Daniel J. Rader, 11–125 Smilow Center for Translational Research, 3400 Civic Center Blvd, Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA 19104. E-mail rader@mail.med.upenn.edu

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SORT1 gene, encoding the protein sortilin, seems to be the causal gene at the locus regulating LDL cholesterol levels.\(^5\)–\(^11\)

Sortilin is a type I transmembrane trafficking receptor initially characterized by its ability to serve as a receptor for proenkephalin\(^9\)–\(^11\) and for its role as a sorting receptor for lysosomal hydrolases.\(^12\),\(^13\)

Hepatic sortilin expression modulates cholate experiment).\(^12\),\(^13\)

and 1-way ANOVA with a Bonferroni correction (for lipopolysaccharide–induced inflammatory response).\(^7\),\(^8\),\(^10\)

in addition, hepatic sortilin binds LDL and promotes its cellular uptake and lysosomal degradation.\(^9\)

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 using 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 Data 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 transgenic mouse, in which the human apolipoprotein B (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.\(^16\)–\(^18\)

These mice were crossed with total body Sort1\(^−/−\) mice and experiments compared Sort1\(^+/−\); hAPOB transgenic mice to Sort1\(^+/−\) transgenic littermates. Mice were fed a western-type diet for 18 weeks and assessed for atherosclerosis in the aortic roots or lipopolysaccharide–induced inflammatory response. (Online Figure I). Mice transplanted with Sort1\(^+/−\); Ldlr\(^−/−\) bone marrow had a 69% reduction in en face aortic lesion area (\(P<0.0001\)) and a 34% reduction in aortic root lesion area (\(P<0.01\)) compared with mice transplanted with Sort1\(^+/−\); Ldlr\(^−/−\) bone marrow (Figure 2A–2D), suggesting that hematopoietic and potentially macrophage sortilin influences the development of atherosclerotic disease.

**Sortilin Deficiency Has no Effect on Thioglycollate-Elicited Monocyte Recruitment or Lipopolysaccharide-Induced Inflammatory Response In Vivo**

Monocyte recruitment is a key determinant of the macrophage content of atherosclerotic lesions. To determine whether Sort1 deficiency affects macrophage recruitment, Sort1\(^+/−\) and Sort1\(^+/−\) mice were injected IP with thioglycollate to elicit an inflammatory response. (Figure 1A).

Three days after injection, peritoneal macrophages were harvested and counted. There was no difference in macrophage counts between Sort1\(^+/−\) and Sort1\(^+/−\) mice (Online Figure IIA). Monocyte recruitment and atherosclerosis development is strongly influenced by inflammation and cytokine production. To determine whether Sort1\(^−/−\) mice have reduced cytokine levels, cytokine multiplexing assays were performed on Sort1\(^−/−\) and Sort1\(^+/−\) mice injected with lipopolysaccharide. Cytokine levels post lipopolysaccharide injection were found to be similar between Sort1\(^−/−\) and Sort1\(^+/−\) (Online Figure IIB and IIC).

**Macrophage Sortilin Deficiency Reduces LDL Uptake and Foam Cell Formation**

To determine whether 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 (macrophage colony stimulating factor) 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–3D). In vivo foam cell formation assays were performed by isolating thioglycollate-elicited peritoneal macrophages from Sort1+/--;Apoec−/−;hApop transgenic and Sort1+/--;Apoec−/−;hApop transgenic 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 with macrophages.
isolated from Sort1+/+ mice (P<0.05; Figure 3E and 3F). 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 coinubcation 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, 125I-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% reduction in LDL uptake, respectively (P<0.05 for both; Figure 4C and 4D). We next tested whether 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 incubated with LDL. Sort1−/− mice showed a 48% reduction in LDL uptake compared to Sort1+/+ mice (P<0.05; Figure 4D). These results suggest that sortilin promotes the internalization of LDL into macrophages, independent of the LDL receptor.

**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<0.0001. C, Representative aortic roots stained with H&E. D, Quantification of aortic roots; P value <0.01.
Ldlr−/− mice, and 125I-LDL uptake studies were performed. Sort1 deficiency was associated with a 39% 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, although LDL uptake is reduced,
substantial residual LDL uptake still takes place.\textsuperscript{1} \textit{Sort1} deficiency was associated with a 38\% reduction in LDL uptake in the presence of cytochalasin D (\textit{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 green fluorescent protein or \textit{Sort1}, and LDL uptake studies were performed. \textit{Sort1} overexpression in macrophages led to a 29\% increase in LDL uptake (\textit{P}<0.05; Figure 4E).

### Discussion

Genetic variation at the 1p13 \textit{SORT1} locus is strongly associated both with coronary artery disease, 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 scavenger receptor A and CD36. However, deletion of scavenger receptor A or CD36 does not reduce macrophage uptake of native LDL,\textsuperscript{4} nor does it ameliorate atherosclerosis in hypercholesterolemic mice.\textsuperscript{19} Even CD36\textsuperscript{−/−}; SRA\textsuperscript{−/−} mice still contain abundant lipid laden macrophages in vessel wall and develop atherosclerosis.\textsuperscript{20} 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.\textsuperscript{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 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.

Although this article was under preparation, Mortensen et al reported that sortilin deficiency reduced atherosclerosis in the ApoE−/− mouse model. 21 Although 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 before initiation of atherosclerotic disease, whereas 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 transgenic mouse, in which human apoB-100 may be complex and multifactorial.

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References


What Is Known?

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

What New Information Does This Article Contribute?

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

Novelty and Significance

The mechanisms linking genetic variation at the SORT1 locus with coronary artery disease 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) low-density lipoprotein, leading to foam cell formation, a novel receptor-mediated process for macrophage uptake of low-density lipoprotein. This pathway could potentially be targeted as an approach to reducing risk of atherosclerosis.
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SUPPLEMENTAL MATERIAL

METHODS

Animals

Sort1-/- mice were obtained from Dr. Carlos Morales at McGill University and crossed onto the Apobec1-/-; hAPOB Tg background. Ldlr-/- mice for the bone marrow transplant studies. The University of Pennsylvania IACUC approved all animal protocols.

Total body sortilin deficient mouse atherosclerosis studies

Female Sort1-/-;Apobec1-/-; hAPOB Tg mice (n=10) and Sort1+/-;Apobec1-/-; hAPOB Tg littermates (n=10) at 8 weeks of age were started on a western-type diet. Mice placed on a western diet (21% fat, 50% carbohydrate, 20% protein Research Diets D12079B), which was continued for 18 weeks. Mice were bled at weeks 0, 2, and 9 and after 18 weeks on the diet were sacrificed and assessed for atherosclerosis in the aortic roots and the entire aorta by en face quantitation (see below).

Hematopoietic sortilin deficient mouse atherosclerosis studies

Donor bone marrow was isolated from male Sort1-/-;Ldlr-/- and Ldlr-/- mice by flushing femurs and tibias with sterile PBS. Female Ldlr-/- recipient mice (8-10 weeks old) were irradiated with 900 rads from a cesium g source prior to transplantation. Each irradiated mouse was then injected with 4 E6 donor bone marrow cells via tail vein injection. The recipient Ldlr-/- mice were given water with sulfamethoxazole and trimethoprim for 2 weeks post bone marrow transplantation. For bone marrow engraftment, Sort1 mRNA was quantified in spleen using quantitative PCR. Six weeks post transplantation mice were placed on a western diet for 18 weeks. Mice were bled at weeks 0, 4, and 14, and after 18 weeks on the diet were sacrificed and assessed for atherosclerosis in the aortic roots and the entire aorta by en face quantitation (see below).

Atherosclerosis quantitation and assessment

Mice placed on a western diet (21% fat, 50% carbohydrate, 20% protein Research Diets D12079B) for 18 weeks were anesthetized with isoflurane followed by a cervical dislocation after a four-hour fast. Aortas were collected from the base of ascending aorta and to the iliac bifurcation, whereas aortic roots with heart were harvested and both are fixed in 4% paraformaldehyde. Aortas for en face were stained with Oil Red O. Aortic roots were dehydrated and paraffin embedded and used for lesion area quantification. Images were captured with Leica MZ12 microscope at a 40x magnification and quantification was performed with Image Pro Plus Software. For en face atherosclerotic lesion area was quantified in reference to the total surface area of the aorta as previously described. For aortic root
atherosclerosis, lesion area was measured over the hematoxylin and eosin stained sections prepared from paraffin embedded hearts. In aortic roots atherosclerosis in either Sort1-/- or Sort+/- on Apobec1-/-; hAPOB Tg model, we quantified representative section where captures 3 aortic valve leaflet equally observed in the section slice (same section as described below). In the quantification of aortic roots atherosclerosis in irradiated / bone marrow transplanted experiment; we quantified lesion area of 5 serial sections (80um between sections) in each mice. For choosing 5 sections, we first defied a “zero-point” where meets all 3 leaflet of the aortic valve moving from aortic vessel towards internal lumen of the vessel. Then 2 serial sections towards aortic arch and another 2 distal serial sections to the zero point into the ventricle chamber were quantified. For each mouse, the data was represented as average lesion area (average area across the 5 points).

Monocyte recruitment assay

Sort1+/+ and Sort1 -/- (n=10 per group) were injected i.p. with thioglycollate (3%) and three days later macrophages were isolated and counted by hemocytometer.

Assessment of cytokine response to LPS injection

Sort1+/+ and Sort1 -/- (n=10 per group) were injected i.p. with LPS (3mg/kg). Mice were bled retroorbitally 2 and 5 hours post injection. Serum was isolated and run on the Bioplex Pro Mouse Cytokine 23-plex Immunoassay (#M60-009RDPD).

Studies of macrophage foam cell formation

For in vitro studies, M-CSF differentiated bone marrow macrophages from Sort1-/-;Ldlr -/- and Ldlr -/- mice (n=3 per group; for procedure see below) on day 7 were incubated with 1 mg/ml LDL for 5 hours. The cells were fixed with paraformaldehyde and stained with Oil Red O and hematoxylin. For in vivo foam cell formation studies, Sort1-/-;Apobec1-/-; hAPOB Tg mice and Sort1+/+;Apobec1-/-; hAPOB Tg littermates (n=3 per group) were placed on a western diet for 18 weeks. Thioglycollate (3%) was injected i.p. and 3 days later cells were peritoneal macrophages were isolated, plated, and stained with Oil Red O and hematoxylin.

Studies of macrophage LDL uptake

For studies of macrophage LDL uptake, both thioglycollate-elicited peritoneal macrophages and bone marrow derived macrophages were used. For isolation of thioglycollate-elicited peritoneal macrophages, Sort1+/+ and Sort1 -/- (n=6 per group) were injected i.p. with thioglycollate (3%) and three days later macrophages were isolated. Thioglycollate-elicited peritoneal macrophages after plating were incubated
with 500μg/ml of LDL in 10% LPDS overnight. The next day the LDL uptake assay was performed with 25μg/ml I-125 LDL. For isolation of bone marrow derived macrophages, bone marrow was isolated from mice femurs from Sort1+/+ and Sort1 -/- (n=2 per group). Monocytes were differentiated for 7 days in M-CSF media into macrophages and on day 7 an LDL uptake study was performed with 250 μg/ml I-125 LDL. For lentiviral expression of SORT1 in J774 macrophages, lentivirus encoding human was generated as previously described. J774 cells were transduced with viral supernatant (Control: GFP + rtTA, Experimental SORT1 + rtTA). The cells were incubated at 37°C overnight, viral supernatant was removed, and cells were grown in RPMI medium. LDL uptake assay was performed with 25 μg/ml I-125 LDL.

**LDL uptake assay**

The macrophages (thioglycollate-elicited peritoneal or bone marrow derived) were incubated with125I-LDL for five hours. In some experiments, cytochalasin D (4μg/ml) was added to bone marrow derived macrophages as indicated right before LDL incubation. Cells were dissolved in 0.2M NaOH. The values were standardized to protein content of the dissolved cells in NaOH by bicinechonic acid (BCA) assay (Thermo). After incubation the media was removed. Trichloroacetic acid was added to precipitate out unreacted LDL, followed by chloroform extraction of free iodine. Total Uptake values represent the sum of LDL associated and degraded.

**Statistics**

Statistical analyses were done using 2-tailed paired student’s t test for total body knockout atherosclerosis, bone marrow transplant atherosclerosis, LDL uptake, macrophage recruitment, cellular cholesterol experiments. A 1 way ANOVA with a Bonferroni correction was done for the LPS experiment.
Online Figure I: Characterization of recipient Ldlr-/- mice carrying donor Ldlr-/- or Sort1-/-;Ldlr-/- bone marrow. N=11. A. Sort1 deficiency in bone marrow has no effect on body weight. B. Sort1 deficiency in bone marrow has no effect on Liver, C. Spleen, or D. Adipose Mass. E. Sortilin deficiency in bone marrow has no effect on plasma lipids. F. Sort1 deficiency in bone marrow has no effect on white blood cells (WBC), neutrophils (NE#), lymphocytes (LY#), G. monocytes (MO#), eosinophils (EO#), basophils (BA#). H. Sort1 deficiency in bone marrow has no effect on platelet number. I. Bone marrow engraftment was 74% as measured by Sort1 expression in spleen. N=11 P value <0.01
Online Figure II

Online Figure II: Sortilin deficiency has no effect on macrophage recruitment or modulation of inflammation. A. Thioglycollate (3% w/v) was injected into peritoneal cavity and after 3 days peritoneal macrophages were counted from Sort1 +/+ and Sort -/- mice (n=10). B. Serum cytokine levels were measured 2 hours and C. 5 hours post lipopolysaccharide injection in Sort1 +/+ and Sort -/- mice (n=10).