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).[20] 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 500ug/ml of LDL in 10% LPDS overnight. The next day the LDL uptake assay was performed with 25ug/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 ug/ml I-125 LDL. For lentiviral expression of *SORT1* in J774 macrophages, lentivirus encoding human was generated as previously described.[10] 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 ug/ml I-125 LDL.

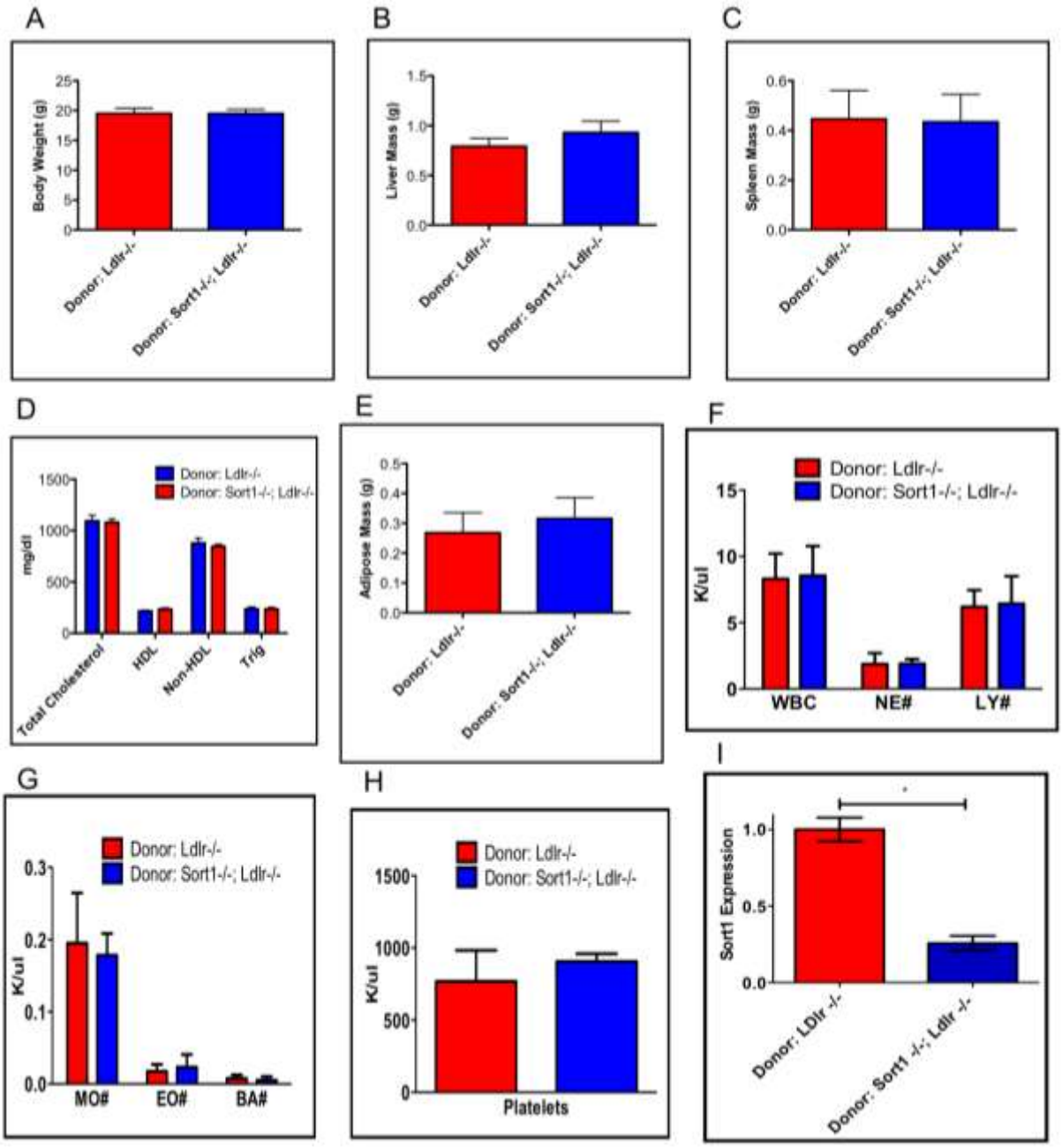
LDL uptake assay

The macrophages (thioglycollate-elicited peritoneal or bone marrow derived) were incubated with ^{125}I-LDL for five hours. In some experiments, cytochalasin D (4ug/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 bicinchoninic 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

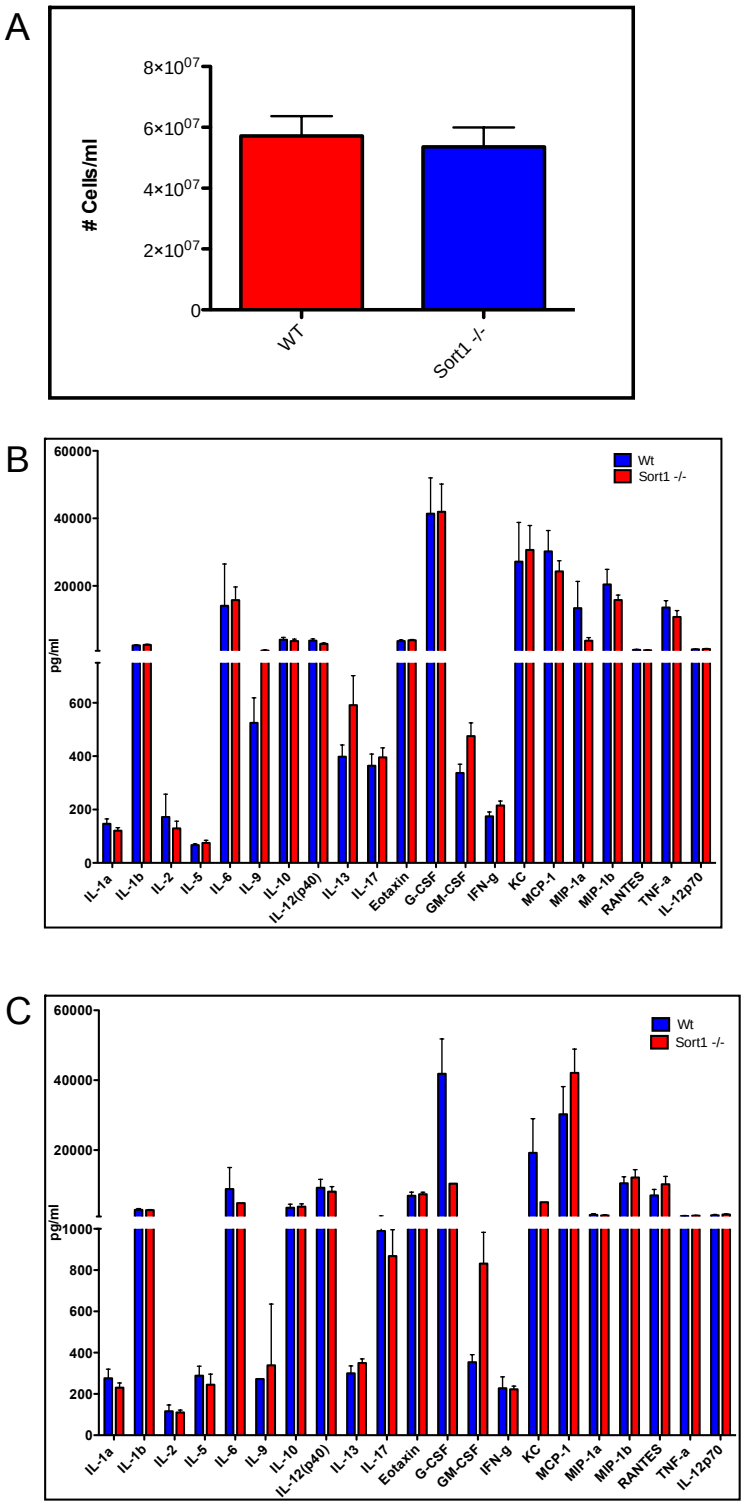
Statistical analyses were done using 2-tailed paired student's t test for total body knockout atherosclerosis, bone marrow transplant atherosclerosis, LDL uptake, macrophage recruitment, cellular cholesterol experiments. A 1 way ANOVA with a Bonferroni correction was done for the LPS experiment.

Online Figure I



<u>Online Figure I:</u> 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)