Lysosomal acid lipase promotes reverse cholesterol transport *in vitro* and *in vivo*

Data Supplement

Supplemental Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (SH3024301) and RPMI 1640 media (SH3002701) were from HyClone (Logan, Utah). Fetal bovine serum (12483020) was from Invitrogen. Complete Mini Protease Inhibitor tablets (1836153) were from Roche Applied Science. Nitrocellulose membranes (162-0115) and SDS-PAGE supplies, were purchased from Bio-Rad. Pre-stained protein molecular mass markers (P77032) were from New England Biolabs. Antibodies against ABCA1 (NB400-105), ABCG5 (NBP1-59803), and ABCG8 (NB400-117) were from Novus Biologicals. The primary antibody against protein disulfide isomerase (PDI) (SPA-890F) was from Enzo Life Sciences. The β-actin primary antibody (AB8227) was from Abcam. Super Signal West Femto chemiluminescence substrate (PI-34096) was from Pierce. PerfeCTa SYBR Supermix (CA101414-148) was from Quanta, VWR. GelRed (41003-BT) nucleic acid stain was from Biotium. The anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (A0545) was from Sigma. [Cholesteryl –1,2-^{3}H (N)] cholesteryl oleate (NET746L) was from Perkin Elmer (Waltham, Massachusetts). Whatman pre-SIL G plastic-backed flexible plates (05-713-161) used for thin layer chromatography analysis were from Fisher. Purified recombinant human LAL enzyme (rhLAL) was by donated by Shire (Lexington, MA).

LAL-deficient mice

Mice were group housed in micro-isolation under a 12-hour light cycle and fed a chow diet *ad libitum* with free access to water. Mice of ages between 8 and 13 weeks were shipped from Cincinnati Children's Hospital, (Cincinnati, OH) to the University of British Columbia (Vancouver, BC) and allowed to acclimatize for 1 week prior to the beginning of experiments. Animals were cared for in accordance with the guidelines of the US National Institutes of Health and the Canadian Council on Animal Care and all procedures were carried out under protocols approved by the UBC Animal Care Committee. A total of 46 wild type mice (n= 20 males, n=26 females) and 30 LAL KO mice (n=16 males, n=14 females) were used for the study.

Cell Culture

Immortalized $lal^{+/+}$ and $lal^{-/-}$ mouse peritoneal macrophages were grown in monolayer in continuous culture at 33°C and 8% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics and 5 units/ml IFN-γ (growth medium) for the first 10 passages only.

Primary dermal fibroblasts isolated from skin sections from $lal^{+/+}$ and $lal^{-/-}$ mice were grown in DMEM medium supplemented with 20% FBS, antibiotics and anti-fungal

reagents during clonal cell growth. The individual colonies of cells isolated were then propagated in DMEM with 10% FBS and antibiotic, incubated at 37°C and 5% CO_2. Cells were used for experiments between passages 3 and 10.

Tissue homogenization and Western blotting

Mouse tissues were collected immediately following euthanasia, flash-frozen in liquid nitrogen and stored until use. Tissues were crushed to a frozen powder using a mortar and pestle cooled in liquid nitrogen and 30-50mg of powdered tissue was homogenized on ice in 0.5 ml lysis buffer containing 20 mM Tris, 5 mM EDTA, 5 mM EGTA, 0.5% maltoside and 1X protease inhibitor. Macrophages were harvested on ice in 0.25 ml lysis buffer and then homogenized using a glass mortar and Teflon pestle. Cell proteins were separated by 7.5% (top half) and 12% (bottom half) SDS-PAGE and transferred to nitrocellulose overnight at 35 V. Immunoblotting was performed using polyclonal antibodies against ABCA1 (2 µg/mL), ABCG5 (0.5 µg/mL), ABCG8 (1 µg/mL), protein disulfide isomerase (PDI) (1 µg/mL) or β-actin (1 µg/mL) and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (0.4 µg/mL), detected using chemiluminescence substrate.

Quantitative real time PCR analysis of mRNA

Total RNA was extracted from cell monolayers or frozen powdered frozen liver tissue (see above) using 1 ml TRIzol extraction reagent (Invitrogen), and cDNA libraries were constructed by reverse transcription. DNA amplification was performed following initial denaturation at 95°C for 3 min, 40 cycles [denaturation at 95°C for 20 seconds, annealing for 20 seconds, extension at 72°C for 40 seconds] with SYBR Green to detect PCR products in real-time using a Realplex^2 Mastercycler thermocycler (Eppendorf). The annealing temperatures and sequences of primers used were: m-cyclophilin, 58°C, 5'-ACCCAAAGGGAACTGCAGCGAGAGC-3' (forward) and 5'-CCGCGTCTCCTTTGAG CTGTTTGCAG-3' (reverse); ABCA1, 58°C, 5'-GACATCCT GAAGCCAATCCTG-3' (forward), and 5'-CCTTGTGGCTCCACTGTCAGGT-3' (reverse); ABCG1, 60.9°C, 5'-CACCTCGCACATTGGGATCG-3' (forward), and 5'-GCC AGGTAGTAGGCCTTCAG-3' (reverse); ACBG5, 54°C, 5'-ACTGGACTGCATGACTGCA A-3' (forward), and 5'-CAAG CATCTCCTCTGGGGTG-3' (reverse); ABCG8, 54°C, 5'-CAAGACTCCTGTGAGCTGG G-3' (forward), and 5'-ACGTCGAGTAGTGAGGCTCT-3' (reverse)**;** ApoE, 56.2°C, 5'-CAGCAGATACGCCTGCAGG-3' (forward), and 5'-ACTGG CGCTGCATGTCTTCC-3' (reverse)**;** SR-B1, 54°C, 5'-AACAGGGAAGATCGAGCCAG-3' (forward) and 5'-CTCAG AGTAGGCCTGAATGGC-3' (reverse). The mRNA levels for genes listed were the mean cycle times, C_T, corrected relative to the housekeeping gene m-cyclophilin (m-cyc) and expressed as a ratio relative to untreated controls.

Cholesterol efflux assay

Macrophage cell monolayers were grown to confluence and incubated with 50 µg/ml ^{3}H-CE – acLDL for 24 hours followed by incubation with 10 µg/ml apoA-I in RPMI 1640 medium for a further 24 hours. For some samples 20 µg/ml rhLAL was added 1 hour

prior to and during acLDL loading. Medium was removed and centrifuged for 10 minutes at 3000 rpm to precipitate cell debris and ^{3}H-sterol in medium was quantified by liquid scintillation counting (LSC). Cell monolayers were washed extensively with cold PBS, 1 mg/ml BSA and PBS on ice and cellular lipids were isolated by Folch extraction.

ApoE ELISA

Lal$^{+/+}$ and lal$^{-/-}$ macrophages were grown to confluence and loaded with 50 µg/ml acLDL for 24 hrs followed by incubated medium alone for 24 hrs. Medium was then collected, centrifuged at 2000 rpm for 10 min and frozen at -80 ^{o}C until analysis. Total apoE secreted into medium was measured using an ELISA kit (AB215086, Abcam) as per the manufactures protocol and normalized to cell proteins measured using reagents from Bio Rad.

Mevalonolactone incorporation assay

Macrophage cell monolayers were grown to confluence in 12-well plates and then incubated with RPMI 1640 medium containing 0.5 µCi/ml ^{3}H-mevalonolactone ± 50 µg/ml acLDL for 24 hours. Lal$^{-/-}$ macrophages were also pretreated with 10 µg/mL rhLAL1 hour prior to and during acLDL loading. Cell monolayers were then washed extensively with cold PBS containing 1 mg/ml BSA, followed by PBS. Lipids were then extracted and separated by thin layer chromatography. Radioactivity of new cholesterol synthesized was quantified by liquid scintillation counting as previously described and normalized to cell proteins (mg) by the method of Lowry.

Measurement of 27-hydroxycholesterol production

Macrophage cell monolayers were grown to confluence in 60 mm dishes and then incubated with RPMI 1640 medium containing 50 µg/ml acLDL for 24 hours. Lal$^{-/-}$ macrophages were also pretreated with 10 µg/ml rhLAL1 hour prior to and during acLDL loading. Cell monolayers were then extensively washed and equilibrated for 24 hours in medium containing 1 mg/ml fatty acid free albumin. Cell lysates and media were combined and 50 µl of 50 ng/ml 27-hydroxycholesterol-d6 (Avanti Polar Lipids, Inc.; Alabaster, Alabama) added as an internal standard. Lipids were extracted with hexanes. The organic extracts were then evaporated under a stream of nitrogen gas and the residues reconstituted in 100 µl of methanol. The mass of 27-hydroxycholesterol (ng) was determined by HPLC tandem mass spectrometry, and normalized to cell proteins (mg) as determined using reagents from Bio Rad.

Macrophage reverse cholesterol transport

Mice were housed individually in cages with wire mesh floor inserts and were fed *ad libitum* with free access to water for the duration of the experiment. Three days prior to injection, lal$^{+/+}$ and lal$^{-/-}$ peritoneal macrophages were seeded in 100 mm dishes at 3x10^{6} cells/dish in growth medium and incubated for 2 days. Cells were then incubated

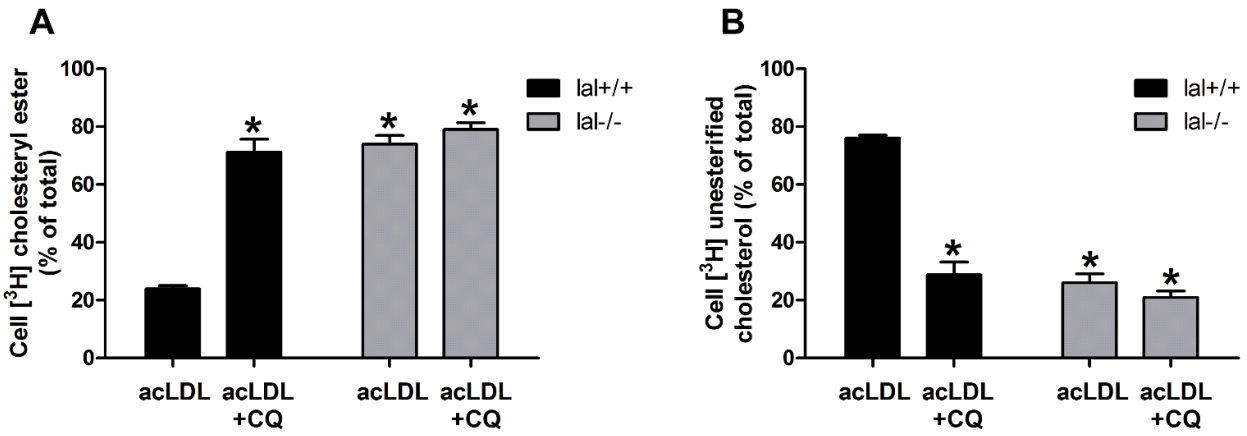
in 6 ml/well RMPI 1640 medium containing 50 µg/ml ^{3}H-CE – acLDL for 24 hours. Prior to injection, cells were gently lifted from dishes using 0.05% trypsin, suspended cells were washed several times in warm PBS. Cells were counted using a hemocytometer, resuspended in warm PBS to 8-12x10^{6} cells/ml and a small aliquot (~50 µl) of cells was removed to quantify the specific activity of ^{3}H by liquid scintillation counting. Cells were drawn into individual 1 ml syringes (500 µl cells/syringe) and injected intraperitoneally into mice using 26 gauge needles within 1 hour after collection. Typically, 6x10^{6} cells containing 7-10x10^{5} counts per minute (CPM) in 0.5ml PBS per mouse was injected.

A blood sample was collected after 24 hours via the facial vein into tubes containing EDTA and placed immediately on ice. Blood was centrifuged at 1500 x g for 30 min at 4°C and the plasma layer removed. Plasma volumes were estimated (~50-100 µl/mouse) and ^{3}H- radioactivity was quantified by LSC. At 44 hours post-injection mice were fasted for 4 hours. Mice were then deeply anesthetized by inhaled isofluorane gas and blood was drawn by cardiac puncture. Plasma was collected as above and an aliquot of 200 µl was counted directly by LSC.

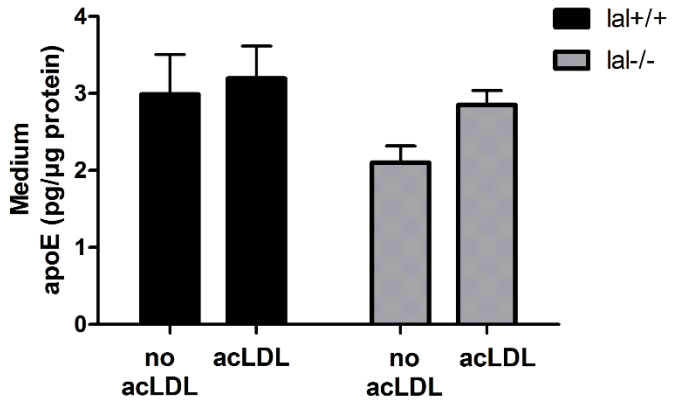
Following anesthesia and removal of blood by cardiac puncture, the mouse vasculature was perfused with 20 ml of ice-cold saline via the left ventricle of the heart and tissues were dissected, frozen in liquid nitrogen and stored at -80°C. Frozen liver sections were crushed to a powder using a mortar and pestle frozen under liquid nitrogen, 30-50 mg of powder was weighed and transferred to a new tube. The frozen powder was homogenized in 1 ml of distilled water and the radioactivity of 500 µl aliquots was quantified by LSC.

Feces were continuously collected from cages over 48 hours and stored at -20°C until assayed. Feces were weighed and soaked at 4°C overnight in distilled water at a ratio of 1 ml per 100 mg feces. The following day, an equal volume of ethanol was added and feces were homogenized by vortexing vigorously. Triplicate aliquots of 200 µl of feces homogenate were quantified for ^{3}H-sterol radioactivity by LSC.

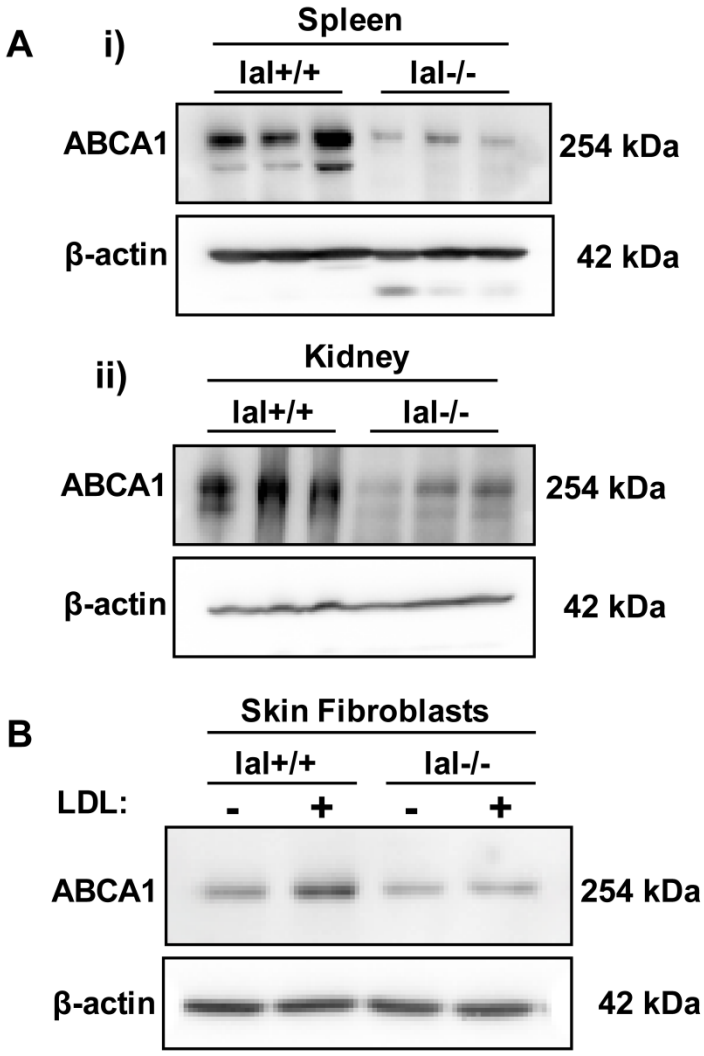
Supplemental Figures



Supplemental Figure I. Inhibition of lal$^{+/+}$ macrophage lysosomal function with chloroquine recapitulates cholesterol distribution of lal$^{-/-}$ macrophages. Lal$^{+/+}$ and lal$^{-/-}$ macrophages were grown to confluence in RPMI and 10% FBS. Cells were then treated for 1 hour with or without 100 µM chloroquine followed by incubation with 50 µg/ml [^{3}H]-CE-labeled acLDL with 2 mg/ml fatty acid-free albumin for 24 hr also with or without chloroquine. After a 6 hr equilibration period in medium alone with or without chloroquine cell lipids were extracted with hexane/isopropanol and (**A**) [^{3}H]-CE and (**B**) [^{3}H]-FC were then separated by thin-layer chromatography and the corresponding radioactivity measured. Data are n=6 replicates from 2 experiments **(**avg±SEM**,** *p<0.05, Kruskal-Wallis test with Dunn's comparisons, significantly different compared to lal$^{+/+}$ with acLDL).



Supplemental Figure II. ApoE is secreted into medium by lal$^{+/+}$ and lal$^{-/-}$ macrophages. Lal$^{+/+}$ and lal$^{-/-}$ macrophages were grown to confluence and loaded with 50 µg/ml acLDL for 24 hrs followed by incubation medium alone for 24 hrs. Medium was then collected, centrifuged at 2000 rpm for 10 min and frozen at -80 ^{o}C until analysis. Total apoE secreted into medium was measured using an ELISA kit (AB215086, Abcam) and normalized to cell proteins. Data are from 3 experiments (avg±SEM, One-way ANOVA with Bonferroni comparisons, no statistical difference between treatment groups).



Supplemental Figure III. Tissue-specific ABCA1 protein expression in wild-type and LAL knockout mice. **A.** Mouse spleen and kidney were dissected and homogenized. **B.** Primary dermal fibroblasts were isolated from mouse skin and grown in culture and then treated with or without 50 µg/ml LDL for 24 hours. Membrane and soluble proteins were extracted and resolved by SDS-PAGE and probed by Western blot using antibodies against ABCA1 and β-actin loading control. Results (**A**) are representative of samples taken from 6 $lal^{+/+}$ and 6 $lal^{-/-}$ mice or (**B**) 3 separate experiments showing similar results.