Materials and Methods

Generation and processing of AKR x DBA/2 F4 mice

Parental male AKR/J and female DBA/2J mice (The Jackson Laboratory) were crossed to generate an F1 generation, fixing the Y chromosome from the AKR strain. Two breeding pairs of F1 mice were bred to generate the F2 mice, and two breeding pairs of F2 mice were used to generate F3 mice. Six breeding pairs of F3 mice were used to generate the 122 F4 mice, which consisted of 70 males and 52 females. Healthy F4 mice were sacrificed at 8-10 weeks of age. Ear tissue was collected from each mouse and digested overnight at 55°C in lysis buffer containing 20mg/mL proteinase K. DNA was ethanol precipitated and stored for eventual genotyping. Femurs were promptly flushed after sacrifice, and resultant bone marrow cells were washed, aliquoted, and cryopreserved. Cells were thawed and differentiated into macrophages at the time of experimentation, as described below.

Mouse genotyping

DNA from AKR x DBA/2 F4 mice was genotyped via the GeneSeek MegaMUGA SNP array. The array consisted of 77,800 markers, with an average of 33kb between adjacent markers, and marker locations based on NCBI Mouse Genome Build 37. Markers were filtered by genotyping the parental and F1 strains and only using markers which were bi-allelic and perfectly called in these test samples, yielding 16,975 informative SNPs that were used for QTL analysis.

Bone marrow-derived macrophages

Bone marrow cells were flushed from femur bones, resuspended, and plated in macrophage growth medium (DMEM, 10% FBS, 20% L-cell conditioned media as a source of MSCF) as previously described.[1, 2] The media was renewed twice per week. Cells were used for experiments 10 to 14 days after plating, when the bone marrow cells were confluent and fully differentiated into bone marrow-derived macrophages (BMDMs).

AcLDL preparation, loading, and cholesterol mass assays

Human LDL ($1.019 < d < 1.063$ g/mL) was prepared by density gradient ultracentrifugation. LDL was acetylated as described previously.[3, 4] AcLDL was dialyzed against PBS with 100μM EDTA and 20μM BHT and its protein concentration was determined using an alkaline Lowry assay. F4 BMDMs were loaded with 50 μg/mL acLDL for 24 hours in 12-well plates. After removing the media and washing cells with PBS, total lipids were extracted in hexane:isopropanol (3:2). The remaining cell protein was dissolved by overnight incubation in 0.2% SDS 0.2N NaOH. For one experiment, GC-MS was used to quantify total and free cholesterol as previously described.[5] Briefly, lipids were extracted in hexane:isopropanol (3:2) for each sample. Half the sample was left untreated to quantify total cholesterol (TC), while the other half was saponified in 0.5 M potassium hydroxide in methanol for 1 hour at 37°C to cleave cholesterol esters and quantify free cholesterol (FC). 50 μL Sylon™ HTP (Sigma; 33038) was added to each sample, and trimethylsilyl (TMS) derivatives were formed via a 1 hour incubation at 90°C. Calibration curves were prepared after TMS derivatization using varying concentrations of cholesterol spiked with 100 ng of stable deuterium-labeled cholesterol (cholesterol-d_6) internal standard (Sigma; 488577). One μL of TMS-derivatized samples or standards were injected onto a 6890/5973 GC-MS equipped with an automatic liquid sampler (Agilent Technologies) using the positive ion chemical ionization mode with methane as the reagent gas. Cholesterol levels were determined via stable isotope dilute analysis after

integrating the GC chromatograms at m/z = 368 for cholesterol and m/z = 374 for deuterated cholesterol. A separate experiment utilized an enzymatic cholesterol mass assay to quantify cholesterol levels, as previously described.[5] For both experiments, cholesterol levels were normalized to cellular protein, as determined by the bicinchoninic acid protein assay (ThermoFisher; 23227).

Fluorescence microscopy studies

Lipid droplet visualization was performed on ESDMs plated on either 4-chamber slides or 12-well plates. Cells were either loaded with 50 µg/mL acLDL for 24 hours or left untreated as a control. Cells were fixed at room temperature for 20 minutes in 10% buffered formalin phosphate (Fisher scientific), lipid droplets were stained with 100 ng/mL Nile Red (Sigma; N3013) at room temperature for 10 minutes, and nuclei were stained with 300nM DAPI (Fisher; D1306) for 5 minutes, as described by the manufacturer's protocol. Images were captured using either the Cytation 3 Cell Imaging Multi-Mode Reader (BioTek) or the IX51 microscope (Olympus).

QTL mapping analysis

QTL mapping of cholesterol metabolism phenotypes (*Mcmm*s) from 122 AKR x DBA/2 F4 BMDMs was performed using R/qtl software.[6] The "scanone" function was utilized using Haley-Knott regression by specifying the "method" argument as "hk". False discovery rates (FDRs) were estimated via permutation analysis, using 10,000 permutations by specifying the "n.perm" argument in the "scanone" function. QTL credible intervals were determined using the Bayesian credible interval ("bayesint") function in R/qtl, with the "prob" argument set at 0.95 unless stated otherwise. QTL mapping for *Mcmm2-Mcmm10* was performed using the genotype from the most strongly associated *Mcmm1* marker as an additive covariate ("addcovar") in the "scanone" function of R/qtl. The *Mcmm1* corrected data were subjected to 10,000 permutation analyses to determine FDRs. Loci that reached significance were designated *Mcmm2-Mcmm10* based on their occurrence scanning left to right across the genome. To aid in prioritizing candidate genes, a custom R function termed "flank_LOD" was written (http://www.github.com/BrianRitchey/qtl) "flank_LOD" utilizes the "find.flanking" function in R/qtl and returns the LOD score of the nearest flanking marker for a given candidate gene position based on "scanone" output data.

Bioinformatic analysis

Genes in QTL intervals were determined by custom written R functions ("QTL_gene" and "QTL_summary") which utilized publicly available BioMart data from Mouse Genome Build 37. A custom written R function ("pubmed_count") which utilized the rentrez package in R was used to determine the number of PubMed hits for Boolean searches of gene name and terms of interest. Custom written R functions ("sanger_AKRvDBA_missense_genes" and "missense_for_provean") were used to determine the number of missense (non-synonymous) mutations between AKR/J and DBA/2J in QTLs, as documented by the Wellcome Trust Sanger Institute's Query SNP webpage for NCBIm37 (https://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1211). Custom written VBA subroutines ("Provean_IDs" and "Navigate_to_PROVEAN") were used to automate PROVEAN software (http://provean.jcvi.org/seq_submit.php) queries for functional effects of missense mutations in each QTL, with rentrez functions utilized to retrieve dbSNP and protein sequence data. Ultimately, custom R code was used to generate output tables. Deleterious mutations were designated as defined by PROVEAN parameters.[7] All custom code can be found at http://www.github.com/BrianRitchey/qtl.

CRISPR/Cas9 Soat1 editing of DBA/2 embryonic stem cells

DBA/2J AC173/GrsrJ mES, hereafter referred to as DBA/2 embryonic stem (ES) cells, were purchased from The Jackson Laboratory (catalog #:000671C02). Puromycin-resistant mouse embryonic fibroblast (MEF) feeder cells (Cell Biolabs; CBA-312), which had been mitotically inactivated by 10 μg/ml mitomycin C were cultured on 0.1% gelatin coated 6-well plates overnight. DBA/2 ES cells were cultured on the feeder cells in ES culture medium (DMEM high glucose with 15% fetal bovine serum, 1% PenStrep, 1% MEM Non-Essential Amino Acids, 0.1 mM 2-Mercaptoethanol, 103 unit/ml leukemia inhibitory factor (LIF), 1 uM PD0325901 and 3 uM CHIR99021). DBA/2 ES cells were used or passaged with trypsin detachment before they reached 80% confluency. $8x10^5$ DBA/2 ES cells were transfected with 5μg Cas9 expression plasmid pSpCas9(BB)-2A-Puro (gift from Feng Zhang, Addgene; PX459)[8], using a Lonza Amaxa nucleofector II device with program A-24 and mouse ES cell nucleofector kit (Amaxa; VAPH-1001). Transfected cells were plated in 2 mg/ml puromycin in ES culture medium on the puromycin resistant MEFs in P100 tissue culture dishes. One week later, the medium was replaced with ES medium without puromycin medium. 3 to 7 days later individual colonies were picked and plated on MEFs in a 96-well plate and expanded. 5 colonies were screened for Cas9 expression by western blot using a Cas9 antibody (Diagenode; C15200203), 3 of which were strongly positive, and the highest expressing line was used subsequently. Cas9 expression in this line was also confirmed by immunohistochemistry with the same Cas9 antibody (Figure IV).

Two sgRNAs were designed at positions proximal and distal to the AKR deletion endpoints (Figure 5B) to generate an ~ 6.6kb deletion of *Soat1*, making them almost identical to the AKR allele of *Soat1*, with exon 2 deleted. sgRNA1 ds oligos (FWD: TAGGGCGAGATTCCCCAGCCAT and REV: AAACATGGCTGGGGAATCTCGC, designed in CCTop[9]), and sgRNA2 ds oligos (FWD: TAGGGACAGCTATTTCACAATG and REV: AAACCATTGTGAAATAGCTGTC, designed in CCTop) were annealed and inserted into Bsa1 digested pDR274 (gift from Keith Joung, Addgene; 42250).[10] After linearization with HindIII, these sgRNA plasmids were purified by phenol-chloroform extraction and ethanol precipitation. 2-4 μg of linear DNA was in vitro transcribed using RiboMAX™ Large Scale RNA Production System-T7 according to manufacturer's protocol (Promega; P1300). The RNA product was purified by phenol-chloroform and ethanol precipitation and verified by running on 6% TBE Urea gels (Invitrogen, USA). $8x10^5$ Cas9 stably transfected DBA/2 ES cells were transfected by nucleofection with 0.75 μg each of sgRNA1 and sgRNA2 as describe above. Individual colonies were grown and expanded as described above.

Genomic DNA was extracted from a sgRNA1/sgRNA2 transfected cell pool and from individual transfected colonies after Histopaque centrifugation to separate ES cells from feeder cells.[11] Genomic DNA was genotyped for the presence or absence of the 6.6kb deletion using primers that spanned the deletion or included the deletion. PCR primer pair 1 (P1) was designed to flank the AKR deletion. The length of this PCR product was 340bp for AKR cells, 540bp for DBA/2 cells with the deletion, and no band was observed for non-deleted DBA/2 cells. PCR primer pair 2 (P2) was designed to detect the non-deleted allele by spanning the intron 1 deletion end point. The length of PCR product was 703bp on non-deleted DBA/2 DNA, and no band was observed using either AKR DNA or DBA/2 DNA after deletion.

P1 FWD: TCATCAGGATTCCACAAAACATTA

P1 REV: GCTCAGGCCTATTATCCCAGTA

P2 FWD: GGGGGATCTAAATAAATAAAAG

P2 REV: GGCAGCCCTGGAAAGAA

After PCR, the products were analyzed by 0.8% agarose gel electrophoresis. The P1 PCR product from the DBA/2 ES line homozygous for the 6.6 kb *Soat1* deletion was sequenced to ascertain the exact deletion endpoints. Subsequent studies were performed on cells expanded from one non-deleted DBA/2 ES line, one heterozygous deleted line, and one homozygous deleted line (referred to as DBA/DBA, DBA/Δ, and Δ/Δ, respectively).

Embryonic stem cell differentiation into macrophages

The macrophage differentiation protocol was adapted from two published protocols.[12, 13] DBA/DBA, DBA/Δ, and Δ/Δ ES cell lines were cultured on inactivated MEFs as described above. In order to separate the ES cells from MEF cells after trypsin treatment, cells were bound to gelatin coated tissue culture plates in ES culture medium at 37°C for 15 minutes, so that the MEF cells were bound to the plate, while the ES cells remained in the supernatant.[11] This step was repeated one additional time. $6x10^5$ ES cells were resuspended in macrophage differentiation medium (MDM) in low-adherence petri dishes for 8 days. MDM consists of DMEM high glucose, 15% FBS, 1% PenStrep, 1% MEM Non-Essential Amino Acids, 0.1 mM 2-Mercaptoethanol, 1 ng/ml IL-3 and 20% L-cell conditioned medium. On day 8, newly forming floating embryoid bodies were transferred to gelatin coated P-100 tissue culture plates in MDM. On the specified day, macrophage progenitor cells floating in the media were harvested and filtered through a 30 μm sterile filter (Sysmex, Lincolnshire, USA) to remove any embryoid bodies, and plated on tissue culture plates. This harvest of macrophage progenitors was repeated every other day. In order to determine the efficacy of differentiation into macrophages, we performed a DiI-acLDL uptake time course assay from 3-13 days after plating the macrophage progenitors. Cells were incubated with DiI-labeled acLDL for 30 minutes at 37°C and uptake was quantified by flow cytometry [14] (Online Supplemental Table II). The DiI-acLDL uptake assay was also used to compare differentiation of ESDM from DBA/DBA, DBA/Δ, and Δ/Δ cells 14 days after plating the macrophage progenitors (Online Supplemental Figure VI).

Characterization of DBA/DBA, DBA/Δ, and Δ/Δ ES derived macrophages

Cell lysates were made using a 1% SDS containing RIPA buffer. Cell protein concentration was determined by the BCA assay (Pierce). Proteins were separated on 4-20% Tris-Glycine gels (Invitrogen, Carlsbad, USA) and transferred to PVDF membrane. The membrane was blocked with Blocker Casein in TBS (Thermo Scientific, Waltham, USA) overnight and incubated with five different ACAT1 antibodies (Novus Biologicals NB400-141; Abcam ab72229; Santa Cruz Biotechnology sc-21029; Aviva Systems Biology ARP45419; ABclonal A6311) and subsequently incubated with the appropriate HRP-labeled secondary antibody. The antigen-antibody complex was detected using chemiluminescent HRP reagents (Denville Scientific Inc.). RNA was extracted and purified from ESDMs using the miRNeasy Mini Kit (Qiagen; 217004) with on-column DNA digestion performed according to manufacturer's instructions. cDNA was generated using the IScript cDNA Synthesis Kit (BioRad; 1708891). mRNA levels were determined via TaqMan RT-qPCR assays for mouse *Soat1* (ThermoFisher; 4331182), with *Actb* (ThermoFisher; 4352341E) serving as an internal control. Samples were run for 40 cycles on an Applied Biosystems StepOnePlus Real-Time PCR System using the comparative Ct method. Resultant data were analyzed using the 2^-ΔΔCt method relative to the average DBA/DBA ΔCt. PCR to detect *Soat1* cDNA

was performed using primers in exons 1 and 4 (FWD: CCGCGGGCTGGGAGGTT, REV: CCAGCGATGCGGACTTTTCAATG). Cholesterol mass assays and lipid staining after acLDL loading were performed as described above.

Statistics

Normality tests were performed on cholesterol mass data. The CE/FC ratio did not pass, so a square root transformed was performed to achieve a normal distribution. Comparisons of two conditions were performed by two-tailed student t-test, and comparisons of multiple conditions were performed by ANOVA with Newman-Keuls posttest. Evaluation of F4 BMDM cholesterol levels separated by their *Mcmm1* genotype were performed using Kruskal-Wallis non-parametric ANOVA with Dunn's posttest. Statistics were performed using GraphPad Prism (v.6) software.

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