MATERIALS AND METHODS

Reagents

Chemicals were obtained from Sigma unless otherwise noted. Human lipoproteins (acetylated LDL) were obtained from Biomedical Technologies Inc (Stoughton, MA) and human ApoAI was obtained from Meridian Life Sciences. Anti-LC3 was obtained from MBL international (PD014 for western blotting and PM036 for immunostaining). Anti-SQSTM1 (p62) was obtained from Abnova (Cat#H00008878-M01). Anti-adipophilin was obtained from Fitzgerald (#20R-AP002). Anti-Lysosomal-associated membrane protein 1 (LAMP-1) was obtained from Abcam (Ab25245). Anti-Tubulin (Cat#T6074) was obtained from Sigma-Aldrich. Anti-HSP90 (BDB610419) was from Fisher Scientific. Anti-CD68 (MCA1957) was purchased from AbD Serotec. The lysosomal acid lipase inhibitor Lalistat1 was provided by Drs. Fred Maxfield and Anton Rosenbaum (Weill Cornell Medical College).

Cell Culture

THP-1 and Jurkat cells were obtained from American Type Tissue Collection. THP-1 cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% P/S. THP1 differentiation into macrophages was induced using 100nM phorbol-12-myristate acetate (PMA) for 72h. Jurkat cells were maintained in RPMI 1640 media supplemented with 10% FBS, 1% P/S, and 1% Glutamax. Peritoneal macrophages from adult C57BL/6J mice were harvested by peritoneal lavage four days after intraperitoneal (i.p.) injection of thioglycollate, as previously described[1]. The cells were maintained in culture as adherent monolayer in medium containing DMEM, 10% FBS, and 20% L929-conditioned medium. Bone marrow derived-macrophages (BMDMs): bone marrow cells were flushed from the tibia and femurs of 6-8 week old C57BL/6 mice, or from $Atg5^{fl/fl}Lysmcre^{+/-}$ or control $Atg5^{fl/fl}$ mice, and differentiated into macrophages by incubation in DMEM media supplemented with 10% FBS, 1% P/S, and 15% L929-conditioned media for 7 days. Peritoneal macrophages from $Atg5^{fl/fl}Lysmcre^{+/-}$ or $Atg5^{fl/fl}$ mice were harvested 4 days after i.p. injection of methyl-BSA in mice previously immunized with this antigen, as previously described[2].

Animal Studies

The Institutional Animal Care Use Committee of New York University Medical Center approved all animal experiments. Six-week old C57BL/6 and *Ldlr*$^{-/-}$ mice were obtained from Jackson Laboratory. For anti-miR-33-induced regression of atherosclerosis, *Ldlr*$^{-/-}$ mice were placed on a Western diet (21% [wt/wt] fat, 0.3% cholesterol; Research Diets) for 14 weeks, at which point mice were either sacrificed (baseline) or switched to chow diet for 4 weeks, as previously described[3]. Mice received 2 subcutaneous injections of 10mg/kg 2′F/MOE control anti-miR (TTATCGCCATGTCCAATGAGGCT) oligonucleotide, or 2′F/MOE anti-miR-33 (TGCAATGCAACTACAATGCAC) oligonucleotide (Regulus Therapeutics) the first week, spaced 2 days apart, and weekly injections of 10mg/kg anti-miR (or PBS) thereafter for 4 weeks[4]. At sacrifice, mice were anaesthetized with isoflurane and exsanguinated by cardiac puncture, perfused with PBS, followed by 10% sucrose in PBS. Aortic roots were embedded in OCT medium and frozen immediately for subsequent sectioning and staining. For *in vivo* macrophage cholesterol loading, *Apoe*$^{-/-}$ mice 8 months of age were placed on a Western diet (Harlan-Teklad) for 2 weeks after which peritoneal macrophages were harvested 5 days after thioglycolate injection. For *in vivo* anti-miR-33 treatment, mice were injected i.p. with 10mg/kg of 2′-F/MOE-modified, phosphorothioate-backbone-modified antisense miR-33 (TGCAATGCAACTACAATGCAC) and mismatch control (TCCAATCCAACTTCAATCATC) anti-miRNA (mismatched bases underlined) (Regulus Therapeutics) 3 days prior to macrophage

isolation.

miR-33 and anti-miR-33 Transfections
Mouse peritoneal macrophages, differentiated THP-1 macrophages, or MEFs were transfected with 80-120nM miRIDIAN miRNA mimics (miR-33) or with 80-120nM miRIDIAN miRNA inhibitors (anti-miR-33) (Dharmacon) utilizing Lipofectamine RNAiMax Transfection Reagent (Invitrogen). All experiment control samples were treated with an equal concentration of a non-targeting control mimics sequence (Ctrl miR) or inhibitor negative control sequence (Ctrl Inh), for use as controls for non-sequence-specific effects in miRNA experiments.

RNA Isolation and Quantitative Real Time-PCR
Total RNA was isolated using TRIzol reagent (Invitrogen) and and Direct-zol columns (Zymo Research), according to the manufacturer's protocol. For mRNA quantification, cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed in triplicate using KAPA SYBR FAST Universal 2X qPCR Master Mix (Kapa Biosystems) on the Mastercycler Realplex (Eppendorf). The following primer sequences were used (m=murine):
mGAPDH_F TGTGAGGGAGATGCTCAGTG; mGAPDH_R TGTTCCTACCCCCAATGTGT;
mABCA1_F AAAACCGCAGACATCCTTCAG; mABCA1_R CATACCGAAACTCGTTCACCC;
mAtg5_F AAAGATGTGCTTCGAGATGTGT; mAtg5_R CACTTTGTCAGTTACCAACGTCA;
mLamp1_F CCCCACTGTATCCAAGTACAATG; mLamp1_R
TGTTGTCCTTTTTCAGGTAGGTG;
mPrkaa1_F GTCAAAGCCGACCCAATGATA; mPrkaa1_CGTACACGCAAATAATAGGGGTT;
mFoxo3_F GGGGAACCTGTCCTATGCC; mFoxo3_R TCATTCTGAACGCGCATGAAG;
mAtg4b_F CATGAATTTCTGGGGCACTT; mAtg4b_F AGCCTTGGGATTCTCTCCAT;
mAtg12_F TGGCCTCGGAACAGTTGTTTA; mAtg12_R GGGCAAAGGACTGATTCACAT;
mMap1lc3b_F CGCTTGCAGCTCAATGCTAAC; mMap1lc3b_R TGCCCATTCACCAGGAGGA;
mTcfeb_F GCAGCCACCTGAACGTGTA; mTcfeb_R TGTTAGCTCTCGCTTCTGAGT;
mCtsb-F TTGCGTTCGGTGAGGACATAG; mCtsb-R GCAGGAGCCCTGGTCTCTA;
mLipa_F TGTTCGTTTTCACCATTGGGA; mLipa_R CGCATGATTATCTCGGTCACA;
mUvrag mUvrag-F ACATCGCTGCTCGGAACATT; mUvrag-R
CTCCACGTCGGATTCAAGGAA;
mHprt_F TCAGTCAACGGGGGACATAAA; mHprt_R GGGGCTGTACTGCTTAACCAG;
mCd68_F TGTCTGATCTTGCTAGGACCG; mCd68_R GAGAGTAACGGCCTTTTTGTGA
Fold change in mRNA expression was calculated using the comparative cycle method ($2^{-\Delta\Delta Ct}$).

Efferocytosis Assays
Jurkat cells were labeled with Cell Tracker Orange CMRA and rendered apoptotic by cycloheximide treatment (100µg/mL) for 6hrs. Apoptotic Jurkat cells were added onto macrophages plated in a LabTek Chamber Slide system, at a 5:1 ratio of apoptotic cells:macrophages, and incubated for 75min. Media was removed, and cells were washed 4X with ice cold 1X PBS to remove uninternalized apoptotic cells. Macrophages were fixed and stained with Phalloidin Green and Dapi for microscopy. For efferocytosis engulfment quantification, macrophages having internalized apoptotic bodies were scored from 6 images / condition (10X objective, with an average of 450cells / image) and results are expressed as % efferocytosis (# macrophages with engulfed apoptotic cells / total # macrophages x 100). Necrotic core analysis was performed on H&E-stained sections, as previously described[5]. Acellular areas lacking hematoxylin stained nuclei were selected and quantified using Image J analysis software. Quantification was conducted in 6 mice per group under blinded conditions.

Fluorescence Microscopy

Cells were fixed in 4% PFA and neutral lipids were stained using Bodipy 493/503 (10μg/mL) or Nile Red (50ng/mL), as previously described[6]. For immunofluorescence, cells were fixed in 4% PFA, blocked/permeabilized in 2.5% BSA/0.1% Triton X-100, and stained with the indicated primary antibodies for 1 hr at 37°C or at 4°C overnight. Fluorophore-conjugated secondary antibodies were incubated in the presence of a neutral lipid stain where applicable. Confocal images of lipid droplets were obtained using a 100 3 NA 1.4 objective on an Olympus IX80 FV1000 confocal microscope with appropriate lasers. Fluorescence microscopy of macrophage CD68 and its co-localization with either p62 or LC3 in frozen atherosclerotic sections was observed using a Nikon Eclipse microscope. The mean fluorescence intensity (MFI) for LC3 and p62 on stained sections of aortic roots was measured using Image J software, and measurements were conducted in 7 mice per group under blinded conditions. Macrophage lipid content was quantified using coherent anti-Stokes Raman scattering (CARS) microscopy, as previously described[7]. For lipid droplet quantification using BODIPY, fluorescence images from five different fields per condition were quantified by averaging the mean fluorescence intensity (MFI, obtained using image J) from an average of 80 cells selected randomly from each field. To quantify the % Neutral lipid following incubation with apoA-I, the final MFI / cell for each condition after 4h incubation with apoA-I was divided by the initial MFI / cell and is expressed as a percentage. Quantification of LAMP-1 fluorescence intensity in BMDMs transfected with control anti-miR or anti-miR33 was performed by averaging the MFI within 5 fields per condition, using the same threshold setting for each image, and measurements were conducted in Image J analysis software, and results are an average of 3 independent experiments.

Cholesterol efflux assays

Peritoneal macrophages, BMDMs, or differentiated THP-1 macrophages plated in 24-well plates were transfected with 80-120nM of either miRNA mimics or inhibitors (Dharmacon) in Opti-MEM (Gibco). 24h later, 2X FBS media containing AcLDL, where indicated, at a final concentration of 50µg/mL and ^{3}H-cholesterol at a final concentration of 5µCi/mL and incubated with miRNA mimics or inhibitiors for an additional 30h. The cells were then washed twice with PBS and incubated with 2mg/ml fatty-acid free BSA (FAFA, Sigma) in media for 18hrs hour prior to addition of 50µg/ml human apoA-I in FAFA-media with or without the indicated treatments. Effluxes were carried out for 24h with the indicated reagents (paraoxon 100µM, chloroquine 30µM, Lalistat 1 10µM) for 24h. Because macrophage cholesteryl ester undergoes a continual cycle of hydrolysis and re-esterification[8], we included an Acyl-CoA : cholesterol acyltransferase (ACAT) inhibitor (Sandoz 58-035, 5µg/mL), to prevent re-esterification of newly hydrolyzed cholesterol during efflux. The supernatant was then removed and briefly centrifuged to remove non-adherent cells, and the remaining cells were dissolved in 0.5N NaOH. The radioactivity within aliquots of supernatants and dissolved cells was measured by scintillation counting. Cholesterol efflux is expressed as a percentage of ^{3}H-cholesterol in medium/(^{3}H-cholesterol in medium+^{3}H-cholesterol in cells)x100%. Efflux to apoA-I was calculated by subtracting effluxes of the wells without apoA-I from those containing apoA-I.

Western blotting

Cells were washed twice with ice-cold PBS, scrapped in Rippa buffer with protease and phosphatase inhibitors, or directly in 2X Laemmli sample buffer (Bio-Rad) for LC3 western blots. For aorta lysate preparation, mice were perfused with 1X PBS, followed with 10% sucrose in PBS. Aortas were carefully cleaned to remove all adventitial fat, and dissolved in 100-150µL of lysis buffer, using a mixture of 1.0mm and 2.0mm zirconium silicate beads in the bullet blender, 2x 5min at maximum speed. Total protein samples (25-30μg/well; 60µg/well for phosphorylated

proteins) were electrophoresed on 18% SDS-polyacrylamide gels and transferred to nitrocellulose or PVDF membranes at 125V for 2h. Membranes were incubated overnight with the appropriate antibodies, and proteins were visualized using appropriate secondary antibodies conjugated to IR-dyes (Rockland) and scanned using the Odyssey Imaging System (Licor) as described previously[1].

Statistics

Data are presented as mean ± the standard error of the mean (SEM). Data were tested for normality of distribution by the Kolmogorov Smirnov and Shapiro Wilk tests, and the significance of differences were evaluated using either the Student's t test or in the case of multiple group comparisons, two-way analysis of variance (ANOVA) tests with Tukey post testing. Data in figure 5 were not normally distributed and thus differences were evaluated using the Mann-Whitney test. Significance was accepted at the level of $P \leq 0.05$.

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