Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Lysosomal Cholesterol Hydrolysis Couples Efferocytosis to Anti-Inflammatory Oxysterol Production

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Circ Res. 2018;122:1369-1384; originally published online March 9, 2018;
doi: 10.1161/CIRCRESAHA.117.312333
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Supplement Material

Reagents. Cell culture media and heat-inactivated fetal bovine serum (FBS) were from Invitrogen. Chemical reagents were from Sigma unless specified below. Lalistat was purchased from Enamine ad used as previously described.[1] The antibodies were obtained as follows: $p47^{phox}$ (sc-7660), caspase-1 (sc-1780), LIPA (sc-58374) and hsp90 (sc-13119) from Santa Cruz Biotechnology, Tfeb (A303-673A) from Bethyl Laboratories, LC3-I/II (2775S) from Cell Signaling and Rac1 (610651) from BD Biosciences. Flow cytometry probes were obtained as follows: Bodipy-cholesterol Green and AnnexinV from Life Technologies, FAM-Flica from AbCys Eurobio and Lysosensor Green, LysoTracker^{R} Deep Red, ROS (CM-H2DCFDA), MitoSox and Fluo4-AM from Molecular Probes.

Plasma content analysis. Plasma multi-analyte profiling was performed using a Cobas^{R} clinical chemistry analyzer (Roche Diagnostics).

Plasma HDL preparation. Apo-B containing particles was precipitated from plasma by adding 100µL plasma to 40µL of 20% polyethyleneglycol (PEG, Sigma P-2139 in 200mM glycine, pH 10) solution. This mixture was incubated at room temperature for 15 minutes. After this incubation, the solution was centrifuged at 4,000 rpm for 20 minutes. The supernatant, containing HDL fractions (PEG-HDL), was removed and used for experiments as previously described.[2].

***In vivo* clearance of apoptotic or stressed cells.** To generate apoptotic lymphocytes (ALs), thymi and spleens from 10- to 12- week-old C57BL/6J mice were harvested and mechanistically dissociated, filtered, pelleted and resuspended in DMEM medium supplemented with 10% fetal bovine serum (FBS) (1:1 mixture). Lymphocytes were labelled with Cell-Tracker red (Invitrogen) according to the manufacturer's instructions. Apoptosis was induced by UV radiation at 312nm for 10min and cells were maintained in culture for an additional 2hours. This method results in 70-90% apoptotis.[2] Fluorescent ALs were washed twice with PBS and $6.5x10^{7}$ ALs per mouse were injected intravenously as previously described.[3] A second *in vivo* clearance assay used mice that were injected with stressed red blood cells (sRBCs) as previously described.[4] Briefly, whole blood collected from C57BL/6J mice was pooled, centrifuged at 400*g* for 10min, and the buffy coat was removed. In a second step, RBCs were diluted in PBS and leukoreduced using an adapted Ficoll gradient protocol (Lymphocytes separation medium, AbCys Eurobio). Erythrocytes were labelled with Cell-Tracker red (Invitrogen) according to the manufacturer's instructions. RBCs were then heated for 20min at 48°C under continuous shaking, generating stressed erythrocytes. Fluorescent sRBCs were washed twice with PBS and 400µL of sRBCs adjusted at approximately 16 to 17g/dL of hemoglobin level per mouse were injected intravenously. Mice were sacrificed 16h after injection, and the isolated liver and spleen cells were analyzed by flow cytometry for Cell-Tracker red labeling with up to six mice per group. At this time point, most sRBCs and ALs had disappeared from the circulation. The efferocytic index was determined by flow cyometry and calculated as the number of cells ingested per the total number of macrophages x 100.

***In vivo* flow cytometry analysis.** Leukocytes were collected from spleens, peritoneal cavities, livers and perigonadic adipose tissues. Splenocytes were extracted by pressing spleens through a stainless steel grid. Peritoneal leukocytes were harvested by PBS lavage. Liver and adipose tissue were cut in small piece and digested with 1mg/mL collagenase D (Roche) or collagenase A (Roche) for 30min at 37ºC, respectively. For liver preparation, and additional purification step was performed by Percoll gradient. Single-cell suspension was submitted to red blood cell lysis, filtration and centrifugation for 5min at 1,000rpm. Cell suspensions were stained with the appropriate antibodies for 30min on ice. The following antibodies were used for macrophage flow cytometric analysis: CD45 (30-F11, BD

Bioscience), CD64 (X54-5/7.1, BioLegend), CD11b (M1/70, BioLegend), CD115 (AFS98, BioLegend), CD206 (MR5D3, Bio-Rad) and F4/80 (BM8, BioLegend) using dilution recommended by the manufacturer. Cells were first gated using FSC/SSC characteristics, and doublets were excluded by comparing FSC-height and -area signals. CD45 antibody is used to exclude non-hematopoietic cells. Data were acquired on BD FACS Canto II cytometer and analysed with FlowJo (Tree Star).

Histochemical analysis. Mice were sacrificed and spleen was harvested and fixed in 4% paraformaldehyde. Spleen was embedded in paraffin and 5µm sections were performed using a Microm HM340E microtome (Microm Microtech, Francheville France). Tissue iron distribution was visualized using the Perl's Prussian blue staining method as previously described,[5] and counterstained with eosin.

Tissues and serum iron and ferritin contents. Spleens, livers, perigonadic adipose tissues and serum were analysed for non-heme iron as previously described.[5] Briefly, tissues were weighted and digested in 3M hydrochloric acid/10% trichloroacetic acid, at 65°C for 20h. Iron and ferritin levels were quantified in acid extracts or serum using Cobas^{R} clinical chemistry kits (Roche Diagnostics).

Human THP-1 macrophages and treatments. THP-1 monocytes (TIB-202, ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Cells were treated with 100nM PMA (Phorbol myristate acetate) for 3days to facilitate differentiation into macrophages. Adherent cells consisting of macrophages were used for experiments as described in the figure legend in absence or presence of 10µM lalistat during the course of the experiment. The use and concentration of different inhibitors are described in the figure legends. Where indicated, macrophages were incubated for the indicated period of time with 2.5% plasma, 2.5% PEG-HDL, 50ng/mL lipopolysaccharides (LPS, Escherichia coli 0111:B4, Sigma), 20ng/mL IL-4 or apoptotic Jurkat cells (see below). In some experiments, macrophages were incubated with 5 µM 25-hydroxycholesterol (25OHC), 3 µM LXR agonist (TO901317), 5µM 27-hydroxycholesterol (27OHC), 25 nM CP-4567773, 10 mM Dimethyl Malonate (DMM), 1 µM 3-nitropropionic acid (3-NPA), 1µM rotenone, 150 nM cell membrane-permeant derivative of nictotinic acid adenine dinucleotide phosphate (NAADP-AM), 20 µM Mucolipin TRP channel 1 agonist (ML-SA1), 100 µM 2-aminoethyl diphenylborinate (2-ABP) or 50 µM NSC23766 after incubation of the cells with apoptotic Jurkat cells for 30 minutes (see below). RNA interference to suppress CH25H expression was obtained from GE Dharmacon (On-Targetplus Smartpool siRNA) and deliverd into cells using lipofectamine 2000 as previously described in macrophages.[2] Stable LIPA overexpressing THP-1 macrophages and control cells were generated after electroporation of a neomycin resistant pCDN3.3 vector containing human LIPA gene. One out of 7 clones was selected for further analysis based on LIPA expression and activity.

Isolation of mouse peritoneal macrophages. Peritoneal macrophage cells were harvested from WT mice 3 days after receiving an i.p injection of thioglycollate and plated in 10% FBS in DMEM media. After a 1-hour incubation at 37°C, non-adherent cells were removed and adherent cells consisting of macrophages were used for experiment as described in the figure legend. In some experiments, caspase 1 deficient macrophages were used (B6N.129S2-Casp1^{tm1Flv}/J from Jax).

Lipa and Nlrp3 deficient macrophages. Lipa bone marrow cells and immortalized Nlrp3$^{-/-}$ bone marrow cells were kindly provided by Dr. Kratky (Medical University of Gratz, Austria) and Dr. Stelhik (Feinberg School of Medicine, Northwestern University), respectively. Bone-marrow-derived macrophages were cultured in 10% FBS in DMEM media supplemented with macrophage-colony stimulating factor (M-CSF; 20ng/mL; R&D Systems) for 5-10 days before the experiment.[2]

Gpr78 overexpressing macrophages. GRP78 adenoviruses were kindly provided by Dr. Foufelle (Inserm UMRS 1138, Centre de Recherche des Cordeliers). Adenoviruses were amplified in HEK 293 cells and purified on a cesium chloride gradient. THP-1 macrophages were infected at 10 Multiplicity of Infection 48 hours before the start of the experiment.

***In Vitro* Efferocytosis experiments.** Efferocytosis experiments were performed as previously described.[2] Briefly, apoptosis of Jurkat T cells (TIB-152, ATCC) was induced by UV radiation at 312nm for 10min and maintained in culture for 2hours before incubation with macrophages (ratio 5:1) for 30min. For measurement of uptake of apoptotic Jurkat T cells into macrophages, Jurkat T cells were prelabeled with Cell-Tracker red (invitrogen) according to the manufacturer's instructions. The efferocytic index was determined by flow cytometry and calculated as the number of cells ingested per the total number of macrophages x 100. In some experiments, PI was confirmed by microscopy from at least three separate fields (containing ~1,000 cells) from triplicate wells.

Efferocyte cholesterol efflux. Jurkat cells were cultured for 24h in 10%FBS in RPMI containing 1μCi/mL [^{3}H]-cholesterol. Apoptosis of [^{3}H]-cholesterol labelled Jurkat cells was next induced and cells were incubated with human THP-1 macrophages as described above. After 3 extensive washes and an equilibration period of 15min, cholesterol efflux was performed for the indicated period of time in 0.2% BSA RPMI containing 25μg/mL apoA-I or 25μg/mL PEG-HDL. The cholesterol efflux was expressed as the percentage of the radioactivity released from the cells in the medium relative to the total radioactivity in cells plus medium.

Intracellular isotopic cholesterol distribution. A sucrose gradient was prepared by layering 1.98mL of 1.1M sucrose, 1.72mL of 0.88M sucrose, and 1.72mL of 0.58M sucrose in a 6.3mL centrifuge tube. Human THP-1 macrophages ($25x10^6$ cells) incubated for 1hour with [^{3}H]-cholesterol labelled apoptotic Jurkat cells (see protocol above) were collected and homogenized in 2.633mL of low-ionic strength buffer (10mM TrisHCl pH 7.5, 0.5mM $MgCl_2$, 1mM Phenyl Methane Sulfonyl Fluorid, 100U/mL aprotinin). The homogenates were then made isotonic by the addition of 0.527mL of low-ionic strength buffer (1.46M sucrose, 0.3M KCl, 6mM β-Mercapto-Ethanol, 49μM $CaCl_2$, 10mM TrisHCl pH 7.5) and then centrifuged at 10,000g for 15 min at 4°C. The supernatant was divided into two equal portions loaded onto two sucrose density gradient tubes, and centrifuged at 100,000g for 2 hours at 4 °C. This procedure resulted in visible bands at each of the four interfaces plus a pellet as previously described.[6] The pellet, which was enriched in endoplasmic reticulum was washed twice and then resuspended in 150 μl of Buffer A (0.25mM sucrose, 0.15M KCl, 3mM β-Mercapto-Ethanol, 20μM $CaCl_2$, 10mM Tris HCl pH 7.5). The radioactivity was quantified in the five fractions collected and expressed as the percentage of the radioactivity ingested.

Thin-Layer Chromatography (TLC)- Total lipid content was extracted according to the method of Bligh and Dyer. The organic phase containing lipids including the [^{3}H]-cholesterol was collected, evaporated under nitrogen flux and resuspended in 50μL isopropranol. The total amount of extracted lipids was dropped on silica gel POLYGRAM precoated TLC sheets (Sigma). Separation of [^{3}H]-free cholesterol (FC) and [^{3}H]-cholesteryl esters (CE) was achieved in hexane/diethylether/formic acid (80:20:1, v/v/v) running buffer. The radioactivity was quantified and expressed as the percentage of the radioactivity ingested.

Impedance reading of protrusion dynamics. Electric cell-substrate impedance sensing (ECIS) analyser (Applied BioPhysics) was used for real time analysis of morphological changes of intact cells and analysis was performed by ECIS platform (Acquire Innovation). Briefly, human THP-1 monocytes were seeded at $5x10^5$ cells/well in ECIS plates containing gold film electrodes before differentiation into macrophages. After incubation with apoptotic Jurkat cells ($2.5x10^6$ cells/mL), the frequency dependent electrical impedance was measured

in cell-covered electrodes subject to an alternate small electric current. Results were expressed as Ohm normalized to baseline immediately after impedance recordings

Cellular respiration Assays. XPF extracellular flux analyzer (Seahorse Biosciences) was used for real time analysis of the Oxygen Consumption Rate (OCR) of intact cells according to manufacturer's instructions. Briefly, macrophages were seeded at $5x10^5$ cells/well in XPF plates. After incubation with apoptotic Jurkat cells ($2.5x10^6$ cells/mL), efferocytes were treated according to manufacturer's instructions to determine mitochondrial respiration. Results were normalized to the actual cell count immediately after OCR recordings

Lipa activity measurement. LIPA activity was performed as previously described.[7] Briefly, cells were homogenized in 10mM Tris pH8, 50 mM NaCl, 1% Triton X100 buffer containing proteases inhibitors. Protein concentration in cell lysates was determined using a BCA dosage kit (Pearce). A mixture of cell lysate (50μg of protein) was incubated for 30 minutes at 37°C with 250μM of 4-MethylUmbelliferyl Oleate (4-MUO, Sigma) reconstituted in 200mM sodium acetate solution pH 5. To stop the reaction, 1M Tris pH 8 (100μL) was added, and fluorescence intensity was measured.

Rac1 activity assay. Rac1 activity was determined by performing a pull-down of Rac1-GTP (PR-962, Jena Bioscience) according to manufacturer's instructions.

Mitochondrial calcium content. We carried out mitochondrial calcium measurements as previously described,[8] with minor modifications. Briefly, THP-1 macrophages were loaded with 5 μM of the calcium probe Fluo4-AM for 30 minutes at 37°C before the start of the efferocytosis experiments. At the end of the experiment, cells were treated for 10 minutes with 2 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler to release mitochondrial calcium and cellular calcium was determined by flow cytometry. Mitochondrial calcium content was calculated as the difference in mean fluorescence intensity between conditions treated with or without CCCP.

Cellular lipid content. Total lipids were extracted with chloroform/methanol from total cell lysates. Cholesterol or triglyceride mass in cells was determined using colorimetric kits (Wako Chemicals).

Directed lipidomic assays. Lipidomic analyses were performed with mass spectrometry by Biocrate Life Sciences using AbsoluteIDQ^R p180-oxysterol assay kit. Briefly, Metabolites from sterol pathway (sterol precursors : lanosterol, 24-dihydrolanosterol, 7-dehydrocholesterol and desmosterol); toxic sterols : alpha triol (cholesterol-3beta, 5alpha, 6beta-triol), alpha epox (cholesterol-5alpha, 6alpha-epoxide), 7alpha-hydroxycholesterol and 7-ketocholesterol; oxysterols : 24S-, 4β-, 25- and 27-hydroxy(OH)cholesterol) were analyzed in human THP-1 efferocytes as indicated in the figure legends. Sterols were extracted according to manufacturer's instructions and analyzed by reversed phase liquid chromatography-mass spectrometry (LC-ECI-MS/MS) to realize liquid chromatography separation, and thus individual quantification of isobaric sterols. The most selective detection was performed in positive MRM detection mode using a SCIEX 4000QTrap tandem mass spectrometry instrument (Applied Biosystems). Data were quantified with Analyst software.

Secretion analysis- Secretion levels of IL1-β, IL-18, Cathepsin D, Cathepsin B and Cathepsin K in human THP-1 macrophages were measured using enzyme-linked immunoabsorbent assay (ELISA) (R&D systems and RayBiotech, Inc.).

***In vitro* flow cytometry assays.** Cells were harvested after trypsin or 5mM EDTA treatment of adherent cells and stained for flow cytometry during 30min at 4°C with labeled antibodies as previously described.[9] Briefly, most of the staining were performed on live cells but for staining performed on fixed cells, one additional step of permeabilization and fixation was

added prior of staining using BD Cytofix/Cytoperm (BD Biosciences) according to manufacturer instructions. Flow cytometry analysis was performed on BD FACS Canto II (BD Biosciences) and analysed with FlowJo (Tree Star). All antibodies were from Biolegend and BD Bioscience and gating strategies are depicted in the Figures. All conditions were performed in triplicates with at least n=3 experiments. Cellular cholesterol content was quantified using the Bodipy-cholesterol probe (Life Technologies), lysosomal content and acidification using the LysoTracker^{R} Deep Red and Lysosensor Green probes (Molecular Probes), Ros production using the CM-H2DCFDA probe (Molecular Probes) and caspase-1 activation was monitored in live cells using the FAM-FLICA assay with the flurosescent FAM-YVAD-FMK probe (Fluorescent Labeled Inhibitor of Caspases) (AbCys Eurobio) according to manufacturer's instructions.

Transmission Electronic Microscopy. Cells were observed with transmission electron microscopy (TEM) for ultrastructural analysis. Cells were fixed in a 1.6 % glutataraldehyde solution in 0.1 M sodium phosphate buffer at room temperature (RT) and stored overnight at 4°C. After three rinsing in 0.1 M cacodylate buffer (15 min each), cells were postfixed in a 1 % osmium tetroxide and 1 % potassium ferrocyanide solution in 0.1 M cacodylate buffer for 1 hour at RT. Cells were subsequently dehydrated in a series of acetone baths (90 %, 100% three times, 15 min each) and progressively embedded in Epon 812 resin (acetone / resin 1:1, 100 % resin two times, 2 hours for each bath). Resin blocs were finally left to harden in a 60 °C oven for 2 days. Ultrathin sections (70 nm) were obtained with a Reichert Ultracut S ultramicrotome equipped with a Drukker International diamond knife and collected on 200 mesh copper grids. Sections were stained with lead citrate and uranyl acetate. TEM observations were performed with a JEOL JEM-1400 transmission electron microscope, equipped with a Morada camera, at a 100 kV acceleration voltage. For analysis of cell adhesive/contact properties, digitized images acquired by TEM were analyzed with ImageJ and data were expressed as percentage of cell contact.

Immunohistochemistry. Human THP-1 macrophages were cultured on coverslips and incubated with the different preparations described below. Cells were then fixed with 4% paraformaldehyde (Sigma) for 15 min at 4°C. Unspecific staining was avoided with a blocking step in PBS 0.1% Triton X100 1% BSA. The coverslips were mounted using Mowiol (Calbiochem) and visualized with a Axioskop 2 FS MOT upright confocal microscope (Nikon 1AR+). Images were obtained by implementing z-scanning, enhanced for publication purposes and analyzed using the ImageJ software as previously described.[2] 3D reconstruction from confocal Z-stack images was also generated. At least three separate fields from triplicate wells for each treatment condition were randomly analyzed.

- *Bodipy-cholesterol diffusion.* UV-induced apoptotic Jurkat cells were stained with 5μM Bopidy-cholesterol for 30min and incubated with THP-1 macrophages for an additional 30min. Then, cells were stained with LysoTracker^{R} Deep Red during 30 minutes at 37°C, as recommended by the manufacturer (Molecular Probes). Cells were washed several times and fixed in 4% formaldehyde solution. Slides were counterstained with DAPI (Sigma). For the quantification of Bopidy-cholesterol in phagolysosome, the mean gray intensity of Bopidy-cholesterol was divided by the mean gray intensity of LysoTracker^{R} Deep Red.

- *Lysosome acidification*. UV-induced apoptotic Jurkat cells were incubated with THP-1 macrophages for 30min as described above and after several washes, 1μM LysoSensor Green was added to cell medium for an additional 30min at 37°C as recommended by the manufacturer's instructions. Cells were washed several times and fixed in 4% formaldehyde solution. Nuclei were counter-stained by addition of DAPI (Sigma). Quantification of LysoSensor Green intensity was performed using the ImageJ software and expressed as mean LysoSensor intensity.

- *p47^{phox} clustering.* Human THP-1 macrophages were incubated with apoptotic Jurkat cells for 30min as described above. Cells were then fixed with 4% paraformaldehyde for 15 min at 4°C and permeabilized with 0.5% Triton for 5min before overnight immunostaining with p47^{phox} antibody as previously described.[2] For the quantification of p47^{phox} in phagolysosome,

the mean gray intensity of $p47^{phox}$ was divided by the mean gray contrast of apoptotic Jurkat cells.
- *Membrane ruffling.* UV-induced apoptotic Jurkat cells were stained with LysoTracker Deep Red for 30min and incubated with THP-1 macrophages for an additional 30min. Then, cells were kept in culture for an additional 30min and washed several times before staining. THP-1 efferocytes were immunostained overnight with Rac1 antibody (Clone 610651, BD Biosciences) in PBS 0,1% Triton X-100, 1% BSA. F-actin was stained with Texas Red-X Phalloidin as recommended by the manufacturer (ThermoScientific). Slides were counterstained with DAPI (Sigma).

Western Blotting. The expression of caspase-1, Nlrp3, Tfeb, LC3-I/II, Rac-1 and LIPA were measured in human THP-1 macrophages or in the cell and supernatant of bone marrow derived macrophages by Western blot analysis. Briefly, cell extracts were electrophoresed on 4-20% gradient SDS-PAGE gels and transferred to 0.22-μm nitrocellulose membranes. The membrane was blocked in Tris-buffered saline, 0.1% Tween20 containing 5%(w/v) nonfat milk (TBST-nfm) at room temperature (RT) for 1h and then incubated with the primary antibody in TBST-nfm at RT for 4h, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Proteins were detected by ECL chemiluminescence (Pierce). References for primary antibodies are provided in the 'Materials' section. Intensity of each protein strips was quantified using Image J software.

RNA analysis. Total RNA extraction, cDNA synthesis and real-time PCR were performed as described previously.[2] m36B4 RNA expression was used to account for variability in the initial quantities of mRNA.

Statistical analysis
Data are shown as mean ± SEM. Statistical significance was performed using two-tailed parametric student's t test or by one-way analysis of variance (ANOVA, 4-group comparisons) with a Bonferroni multiple comparison post-test according to the dataset (GraphPad software, San Diego, CA). Results were considered as statistically significant when $P<0.05$.

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Supplemental Figure I. Defective lysosomal cholesterol hydrolysis promotes lysosomal cholesterol accumulation during efferocytosis. (A) Lysosomal acid lipase (LIPA) mRNA expression (normalized to m36B4) was determined by real-time polymerase chain reaction (PCR) in human THP-1 macrophages after LIPA knockdown (using lentiviral ShRNA particles or siRNA) or after LIPA inhibition achieved with 10 μM lalistat. (B, upper panel) Immunoblot of LIPA from control and LIPA-overexpressing THP-1 macrophages (LIPA Ovex) generated by lentiviral transfection. LIPA Ovex THP-1 macrophage clone #2 was selected for further analysis. (B, lower panel) LIPA activity from control, lalistat-treated and LIPA-overexpressing (clone #2) THP-1 macrophages. Enzymatic activity (normalized to control DMSO) was quantified at acidic pH at 37°C after the reaction was initiated by adding 250 μM 4MUO substrate and monitored for 30 minutes. (C) WT and Lipa deficient bone marrow-derived macrophages were incubated for the indicated times in the presence or absence of 10 μM lalistat together with Cell-Tracker Deep Red-prelabeled apoptotic Jurkat cells, and the efferocytic index was quantified by flow cytometry. (D) WT and Lipa deficient bone marrow-derived macrophages incubated in the presence or absence of 10 μM lalistat were stimulated with apoptotic Jurkat cells for the indicated times. BODIPY staining was quantified by flow cytometry. (E) Control and LIPA-overexpressing THP-1 efferocytes, cultured for 1 hour after the ingestion of [^{3}H]-cholesterol-prelabeled apoptotic Jurkat cells, were fractionated by sucrose step gradient, and fractions were assayed for [^{3}H]-cholesterol incorporation as described in the Methods section. (F) Quantification of [^{3}H]-free cholesterol (FC) and cholesteryl ester (CE) incorporation by thin-layer chromatography (TLC) in high-density membrane (HDM) containing lysosomes, plasma membrane (PM) and endoplasmic reticulum (ER) of control or lalistat-treated THP-1 efferocytes 1 hour after the ingestion of [^{3}H]-cholesterol-prelabeled apoptotic Jurkat cells. (G) Representative images of transmission electron microscopy of control or lalistat-treated THP-1 macrophages (eight to ten different transmission electron microscopy images from individual cells with over four hundred lysosomes were analyzed per condition) showing higher lysosome numbers with similar structures (left panels). Lysosomal membrane structure including 'onion-ring' or 'whorl-shaped' phospholipid-rich lysosomes is shown in control cells (upper right panels) and cholesterol crystal-containing lysosomes is shown in lalistat-treated cells (lower right panel; 1 identified among more than 400 visualized lysosomes) (scale bar, 5 and 1 μm). (H) Control, lalistat-treated and LIPA Ovex THP-1 macrophages were stimulated with BODIPY green-prelabeled apoptotic Jurkat cells for 30 minutes. The cells were counterstained with LysoTracker^R Deep Red (phagolysosome staining) and DAPI (nuclear staining), and representative confocal images (magnification 80x and 160x) are depicted (eight to ten different confocal images were analyzed per condition from experiments performed in triplicate). The data represent the averages ± SEM of at least three independent experiments. *P<0.05 *vs.* controls.

Supplemental Figure II. Defective lysosomal cholesterol hydrolysis does not initiate phagolysosome dysfunction but promotes lysosomal biogenesis after efferocytosis to prevent cell from apoptosis. (A) Representative immunoblots of LC3I/II and phospho-Tfeb from control or LIPA overexpressing (Ovex) THP-1 macrophages incubated for 30 minutes with apoptotic Jurkat cells and cultured for various times. (B) Visualization of the phagolysosome (gray contrast) surrounding p47^{phox} staining after performing 3D reconstruction from confocal Z-stack images of an ingested apoptotic cell. Quantification of p47^{phox} in phagolysosomes 1 hour post-efferocytosis was performed as described in the Methods section with the use of ImageJ software. (C) Confocal analysis of LysoSensor-stained (green) control or lalistat-treated THP-1 macrophages exposed to apoptotic Jurkat cells for 1 hour. Cells were counterstained with DAPI (nuclear staining). The quantification of LysoSensor fluorescence intensity was performed as described in the Methods section with the use of ImageJ software. **(**D) The lysosomal acidification of control, lalistat-treated or LIPA-Ovex THP-1 efferocytes was analyzed by flow cytometry with the fluorescent LysoSensor probe at the indicated time points and was expressed as the mean fluorescence intensity (MFI). (E) Flow cytometry analysis of fluorescent LysoTracker^R Deep Red at the

indicated time points and expressed as the mean fluorescence intensity (MFI) in control, lalistat-treated or LIPA-Ovex efferocytes. (F). The percentage of apoptosis was determined using the Annexin V probe by flow cytometry in THP-1 macrophages after LIPA knockdown (using lentiviral ShRNA particles or siRNA), LIPA inhibition (10 μM lalistat treatment) or LIPA overexpression (Ovex) and exposure to apoptotic cells for 16 hours. The data are given as the mean ± SEM from three independent experiments. *P<0.05 *vs.* controls.

Supplemental Figure III. Defective lysosomal cholesterol hydrolysis activates the NLRP3 inflammasome and promotes Rac1-dependent phagocytic cup disassembly. (A) Cathepsin K secretion levels from control or lalistat-treated THP-1 efferocytes cultured for the indicated times after the ingestion of apoptotic cells were measured and expressed in pmol/L. (B) IL-1β secretion levels (expressed in pg/mL) from control or lalistat-treated murine thyoglycollate-elicited peritoneal macrophages cultured for 16 hours after the ingestion of apoptotic cells. (C) IL-1β secretion levels from control or LIPA overexpressing (Ovex) THP-1 efferocytes cultured for the indicated times after the ingestion of apoptotic cells were measured and expressed in pg/mL. (D) Caspase-1 activity determined by flow cytometry with the FAM-FLICA assay kit in control and lalistat-treated THP-1 efferocytes cultured for 16 hours after the ingestion of apoptotic cells in the presence or absence of 25 nM of the Nlrp3 inflammasome inhibitor (CP-456773). The data are expressed as the mean fluorescence intensity (MFI). (E) IL-1β secretion levels from control and immortalized Nlrp3$^{-/-}$ bone marrow-derived macrophages incubated in the presence or absence of 10 μM lalistat and cultured for an additional 16 hours after the ingestion of apoptotic Jurkat cells. (F) Treatment of control and Nlrp3$^{-/-}$ bone marrow-derived efferocytes in the presence or absence of 10 μM lalistat and cultured for 16 hours. Caspase-1 cleavage was quantified by Western blot. (G) Control and lalistat-treated thyoglycollate-elicited peritoneal macrophages isolated from WT or caspase 1 deficient mice were incubated in the presence or absence of 25 nM Nlrp3 inflammasome inhibitor (CP456773) together with Cell-Tracker Red-prelabeled apoptotic Jurkat cells, and the efferocytic index was quantified by flow cytometry 16 hours later. (H) Representative transmission electron microscopy images of control or lalistat-treated THP-1 efferocytes showing membrane structure (scale bar, 20 μm). (I) THP-1 macrophages incubated in the presence or absence of 10 μM lalistat were stimulated with cell tracker Deep Red-prelabeled apoptotic Jurkat cells for 30 minutes. After an additional culture period, the cells were counterstained with Rac1 (green), F-actin (red) and DAPI (nuclear staining); representative confocal images are depicted (eight to ten different confocal images were analyzed per condition from experiments performed in triplicate). The results are expressed as the mean ± SEM of at least two experiments performed in triplicate. *P<0.05 *vs.* controls.

Supplemental Figure IV. Impaired mitochondrial metabolic repurposing but not lysosomal calcium release is the culprit of the inflammasome activation after LIPA inhibition. (A) Oxygen consumption rate (OCR) recordings of control or lalistat-treated THP-1 macrophages incubated with apoptotic Jurkat cells for the indicated times. The OCR of apoptotic Jurkat cells was also recorded. (B) OCR of control or lalistat-treated thyoglycollate-elicited murine peritoneal macrophages in presence or absence of apoptotic Jurkat cells and in Lipa deficient efferocytes. (C-D) mRNA expression of *Ucp-2* and *Hmox1* in control or lalistat-treated THP-1 efferocytes at the indicated time points. The expression of mRNA was normalized to m36B4 and expressed in arbitrary units (a.u.). (E) Caspase-1 activity determined by flow cytometry with the FAM-FLICA assay kit in control and lalistat-treated THP-1 efferocytes cultured for 3 hours after ingestion of apoptotic cells in the presence or absence of 150 nM of the cell membrane-permeant derivative of nictotinic acid adenine dinucleotide phosphate (NAADP-AM), 20 μM of the lysosomal Mucolipin TRP channel 1 agonist (ML-SA1) that release calcium from the acidic compartment or 10 μM of 2-aminoethyl diphenylborinate (2-APB), a membrane permeable IP3 receptor modulator that stimulates store-operated calcium release at 10 μM concentration. Data are expressed as mean fluorescence intensity (MFI). (F) IL-1β secretion levels in these cells at the end of the

incubation period. The results are expressed as the mean ± SEM of at least two independent experiments performed in triplicate. *P<0.05 *vs.* controls.

Supplemental Figure V. LIPA favors the generation of 25-OHC during efferocytosis to down-regulate the expression of cholesterol biosynthesis genes and prevent activation of the inflammasome. Effect of LIPA inhibition on (A) sterol precursors (lanosterol, 24-dihydrolanosterol, 7-dehydrocholesterol and desmosterol) and (B) toxic sterols (alpha-triol (cholesterane-3beta,5alpha,6beta-triol), alpha-epox (cholesterol-5alpha, 6alpha-epoxide), 7alpha-hydroxycholesterol and 7-ketocholesterol) determined by liquid chromatography-mass spectrometry (LC-MS) in THP-1 macrophages 3 hours post-efferocytosis. (B) mRNA expression of oxysterol-producing enzymes in THP-1 macrophages 3 hours post-efferocytosis. (D-E) Transcript levels of endoplasmic cholesterol biosynthesis genes (*Srebf2* and *Hmgcr*) in control or lalistat-treated THP-1 efferocytes at the indicated time points. Quantified transcript levels (normalized to m36B4) are expressed in arbitrary units (a.u.). (F) WT and Lipa deficient murine efferocytes were incubated in the presence or absence of 5 μM 25-hydroxycholesterol (25-OHC) and the efferocytic index was quantified by flow cytometry 16 hours later. (G) LIPA-overexpressing (Ovex) THP-1 macrophages treated with scrambled or *Ch25h* SiRNA were incubated for 30 minutes with apoptotic Jurkat cells, extensively washed, and cultured for an additional 16 hours. Ch25h mRNA expression, normalized to m36B4, was expressed in arbitrary units (a.u.) and IL-1β secretion levels were expressed in pg/mL. The results are expressed as the mean ± SEM of two to five independent experiments performed in triplicate. *P<0.05 *vs.* controls. #P<0.05 treatment effect.

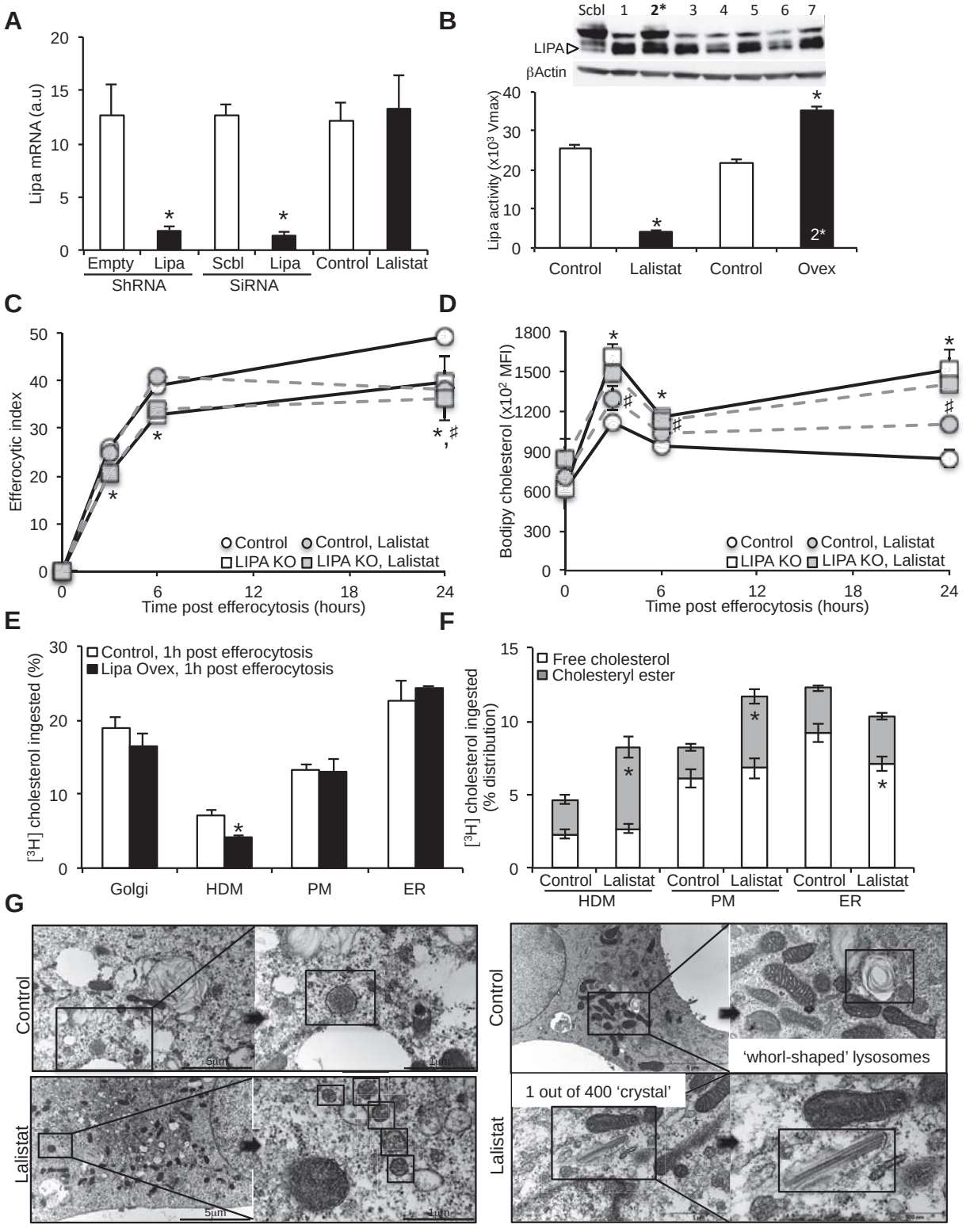
Supplemental Figure VI. LIPA induces liver X receptor (LXR) activation during efferocytosis. (A-B) THP-1 macrophages were incubated for the indicated times in the presence or absence of 10 μM lalistat together with apoptotic Jurkat cells, and the transcript levels of *Abca1* and *Abcg1* (normalized to m36B4) were determined by real-time polymerase chain reaction (PCR; n=3). (C-D) THP-1 macrophages were incubated for 30 minutes in the presence or absence of 10 μM lalistat together with [^{3}H]-cholesterol-prelabeled apoptotic Jurkat cells. Then, apoA-I or polyethylene glycol (PEG)-HDL was added as an acceptor and incubated for 6 or 24 hours before the media and cells were collected for isotopic cholesterol efflux analysis. (E) Control and LIPA Ovex THP-1 macrophages were incubated in the presence or absence of apoptotic Jurkat cells for 30 minutes, extensively washed, and cultured for an additional 24 hours. At the end of the incubation, *Abca1*, *Abcg1* and *MertK* transcript levels (normalized to m36B4) were quantified and expressed in arbitrary units (a.u). (F) Control and LIPA Ovex THP-1 macrophages were incubated for 30 minutes with [^{3}H]-cholesterol-prelabeled apoptotic Jurkat cells. After an equilibration period, polyethylene glycol (PEG)-HDL was added as an acceptor and incubated for 6 or 24 hours before the media and cells were collected for isotopic cholesterol efflux analysis. (G) mRNA expression of LXR target genes (*Abca1* and *Abcg1*) in control or lalistat-treated THP-1 macrophages 24 hours after efferocytosis in the presence or absence of 3 μM LXR agonist (TO901317). The expression of mRNA was normalized to m36B4 and expressed in arbitrary units (a.u). (H) THP-1 macrophages were incubated for 30 minutes in the presence or absence of 10 μM lalistat or 3 μM LXR agonist (TO901317) together with [^{3}H]-cholesterol-prelabeled apoptotic Jurkat cells. Then, polyethylene glycol (PEG)-HDL was added as an acceptor and incubated for 24 hours before the media and cells were collected for isotopic cholesterol efflux analysis. The data are given as the mean ± SEM from three independent experiments. *P<0.05 *vs.* controls. #P<0.05 treatment effect.

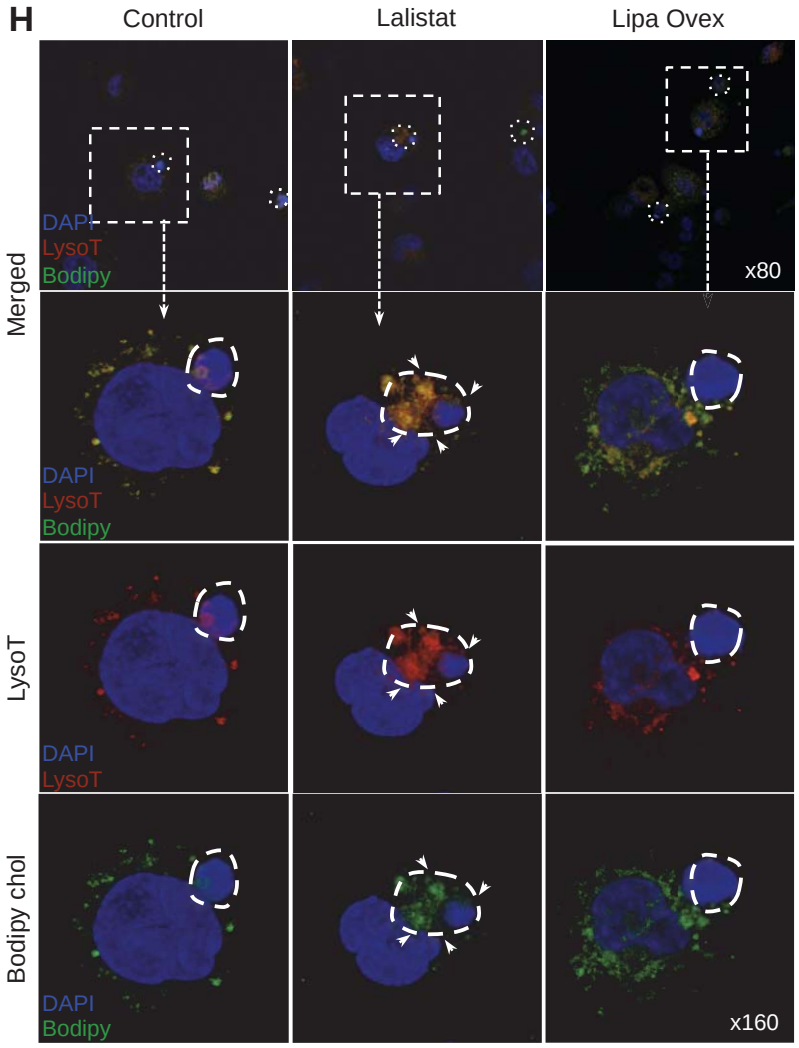
Supplemental Figure VII. Targeted gene expression profiling of LIPA and related lysosomal and efferocytic functions in tissue-resident immune cells and effects of LIPA inhibition on the efferocytosis of stressed erythrocytes and apoptotic lymphocytes. (A) Heat map of LIPA, lysosomal and efferocytic functions, LXR target genes,

heme/iron metabolism and inflammasome markers in various hematopoietic, lymphoid and myeloid cell populations generated from a publicly available Immgen dataset. (B) Representative dot plots showing the uptake of CellTracker^{+} stressed erythrocytes (sRBCs, red) or apoptotic lymphocytes (UV-T, green) in the liver (top) and spleen (bottom) 16 hours after *i.v.* injection. (C-D) Quantification of the percentage of efferocytic cells of each cell type in the liver (myeloid, T-mac and KC) or the spleen (myeloid and RPM). The results are expressed as the mean ± SEM of 4 to 6 animals per group. *P<0.05 *vs.* saline-injected control mice.

Supplemental Figure VIII. Inhibition of lysosomal lipid hydrolysis promotes pathogenic tissue-resident macrophage expansion and lysosomal dysfunction. (A) Representative dot plots of tissue-resident macrophages (F4/80^{+}CD64^{+} splenic RPMs, liver KCs and adipose tissue ATMs and F4/80^{+}CD115^{+} peritoneal PCMs) by flow cytometry from 12-week high-fat-fed WT or *Ldlr*$^{-/-}$ mice treated for the last 2 weeks with subcutaneous injections of either saline or 20 mg/kg lalistat every two days. (B) Quantification of tissue-resident macrophages expressed as a percentage of whole tissue. (C) Representative histograms and quantification of CD206 cell surface expression in splenic RPMs by flow cytometry and expressed as the mean fluorescence intensity (MFI). (D) Lysosomal content and acidification in tissue-resident macrophages quantified by flow cytometry using LysoTracker^{R} Deep Red and LysoSensor Green, respectively, and expressed as the mean fluorescence intensity (MFI). The results expressed as are the mean ± SEM from 4 to 6 animals per group. *P<0.05 *vs.* saline injected *Ldlr*$^{-/-}$ mice.

Supplemental Figure I.

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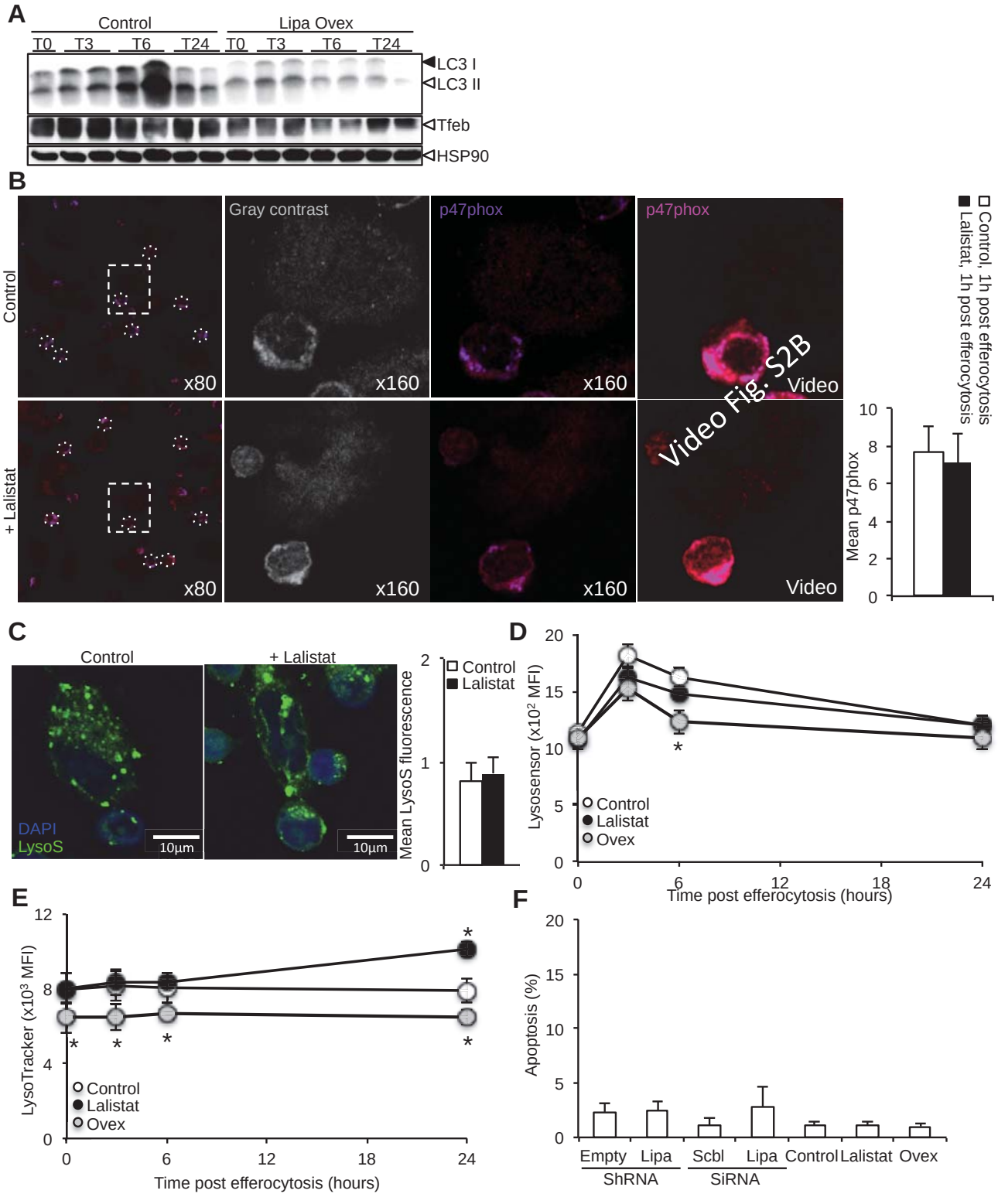

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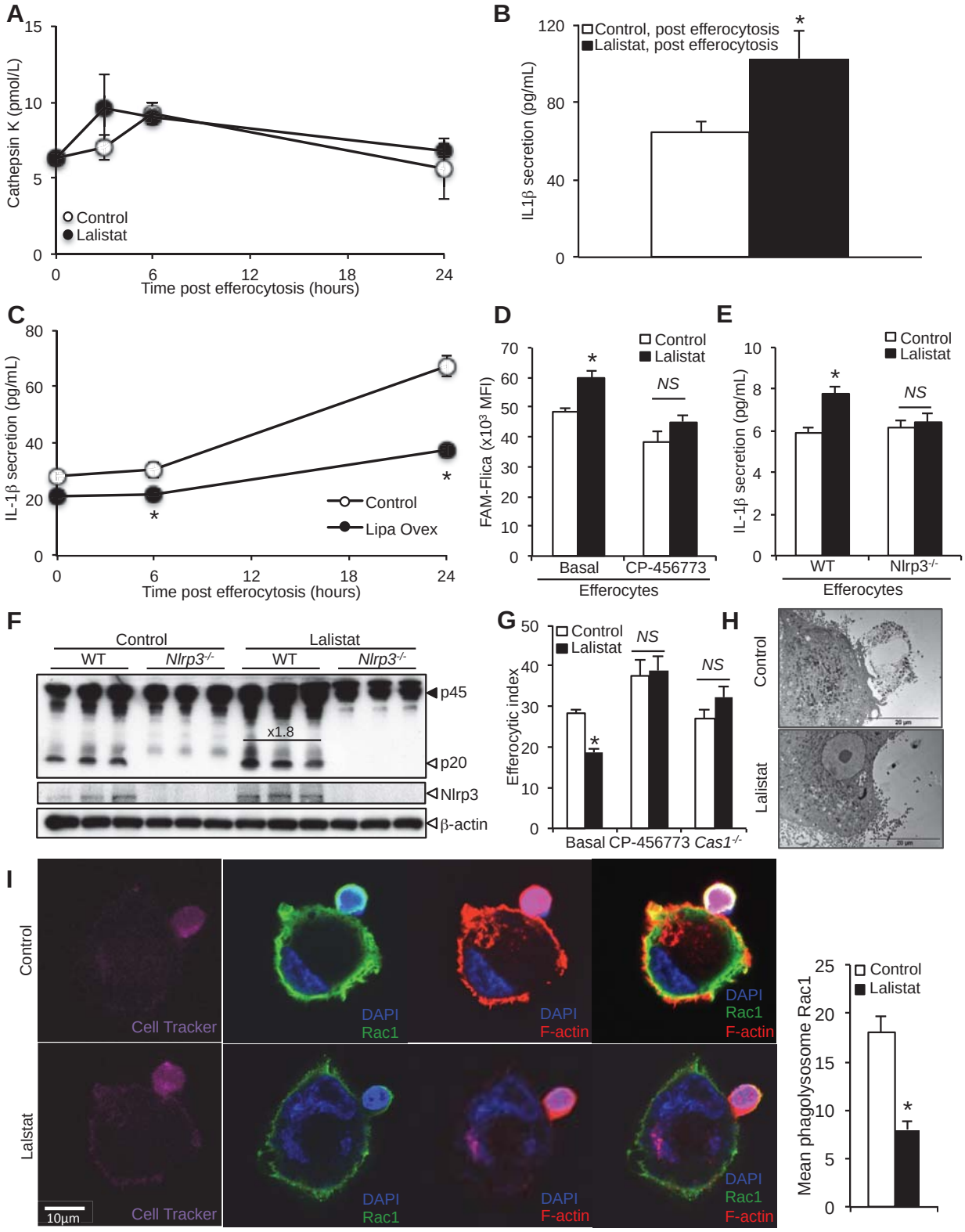

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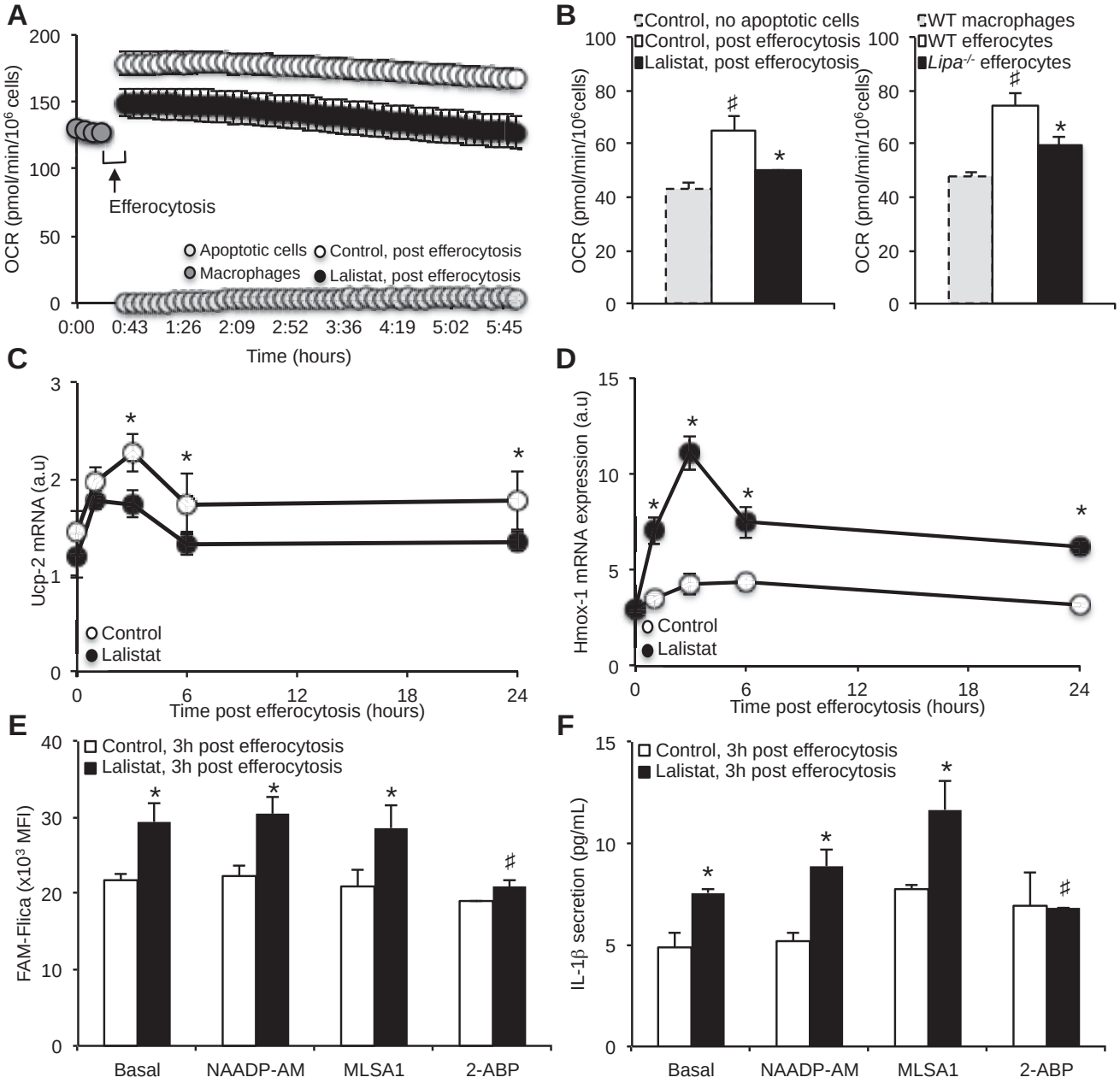

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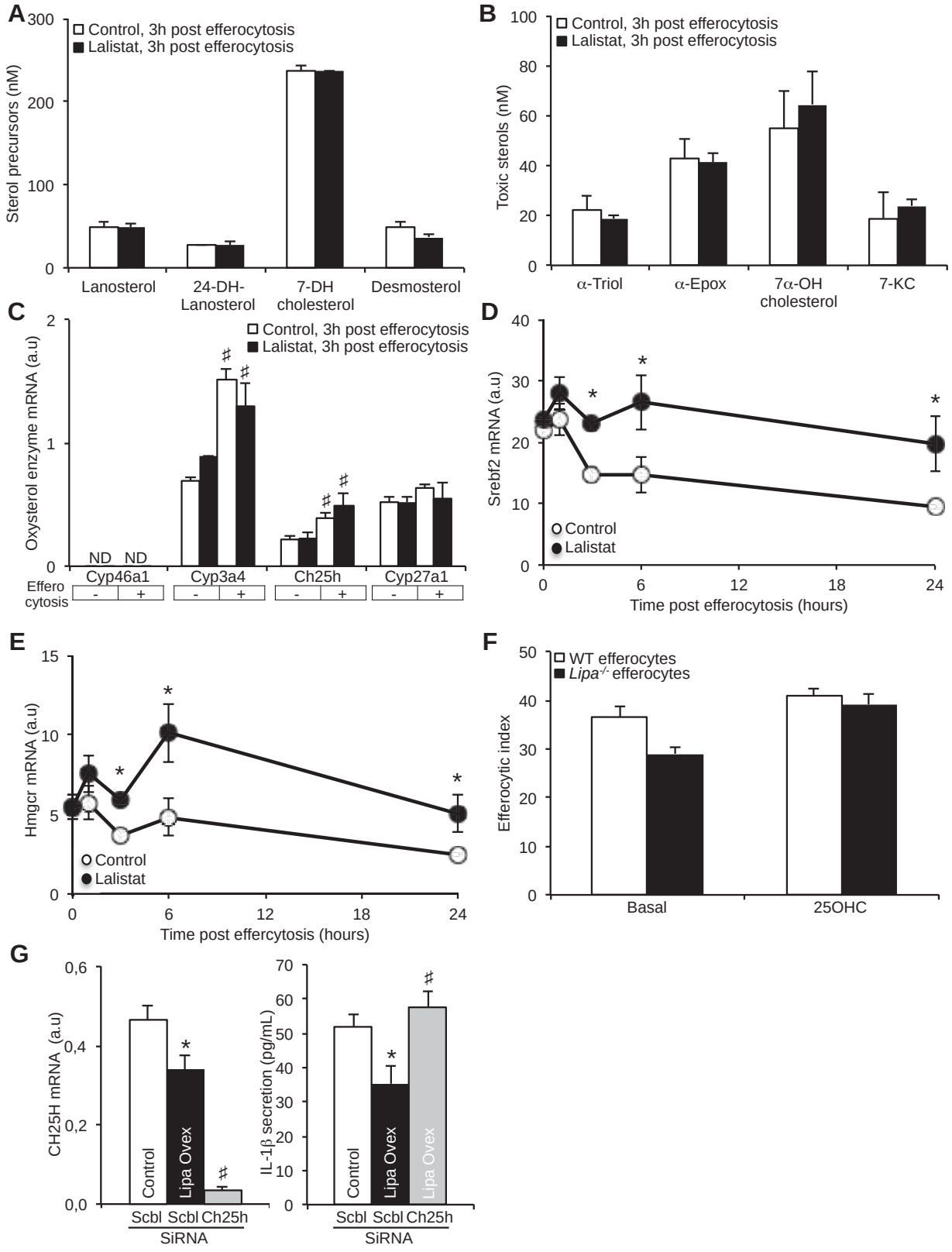

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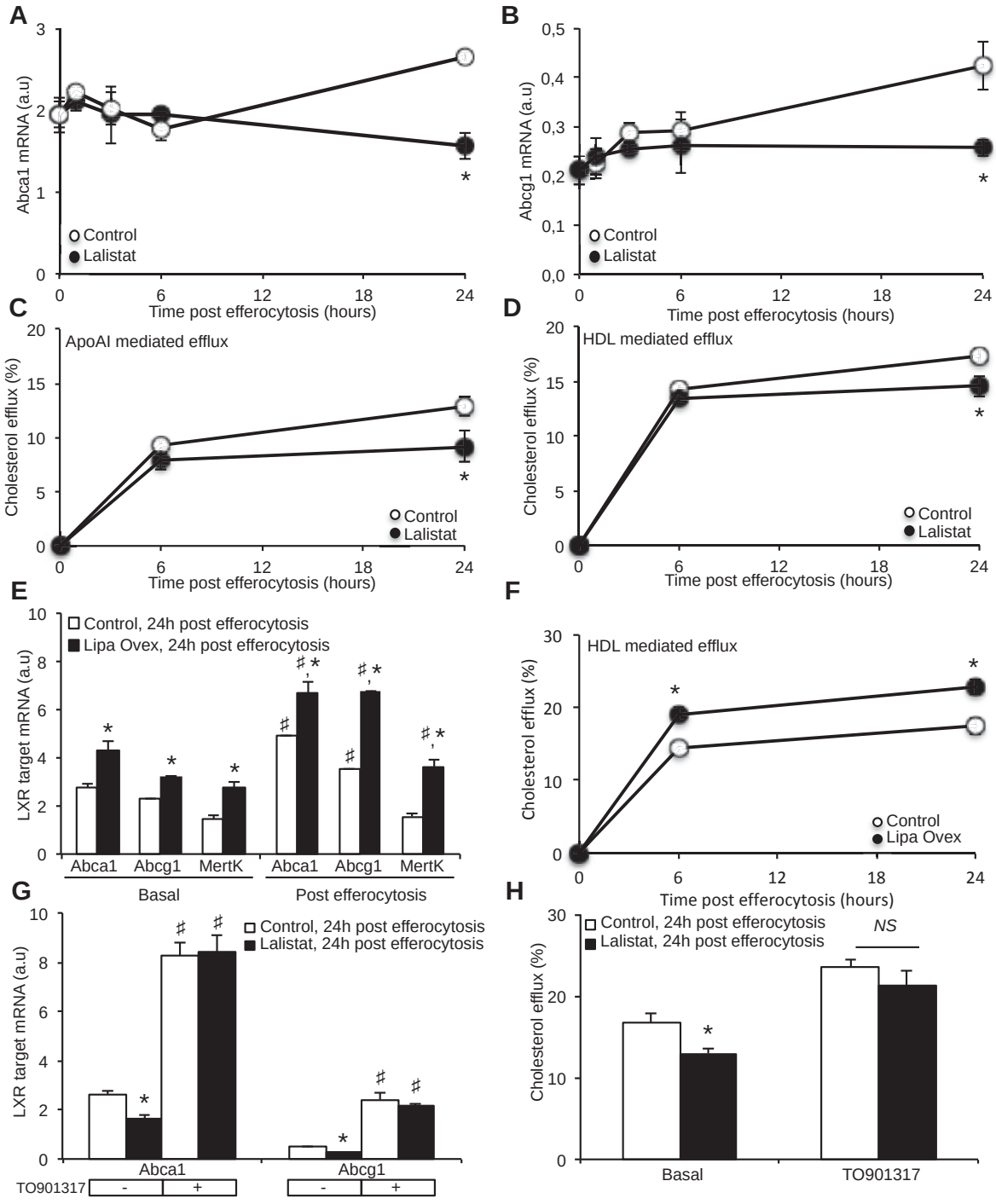

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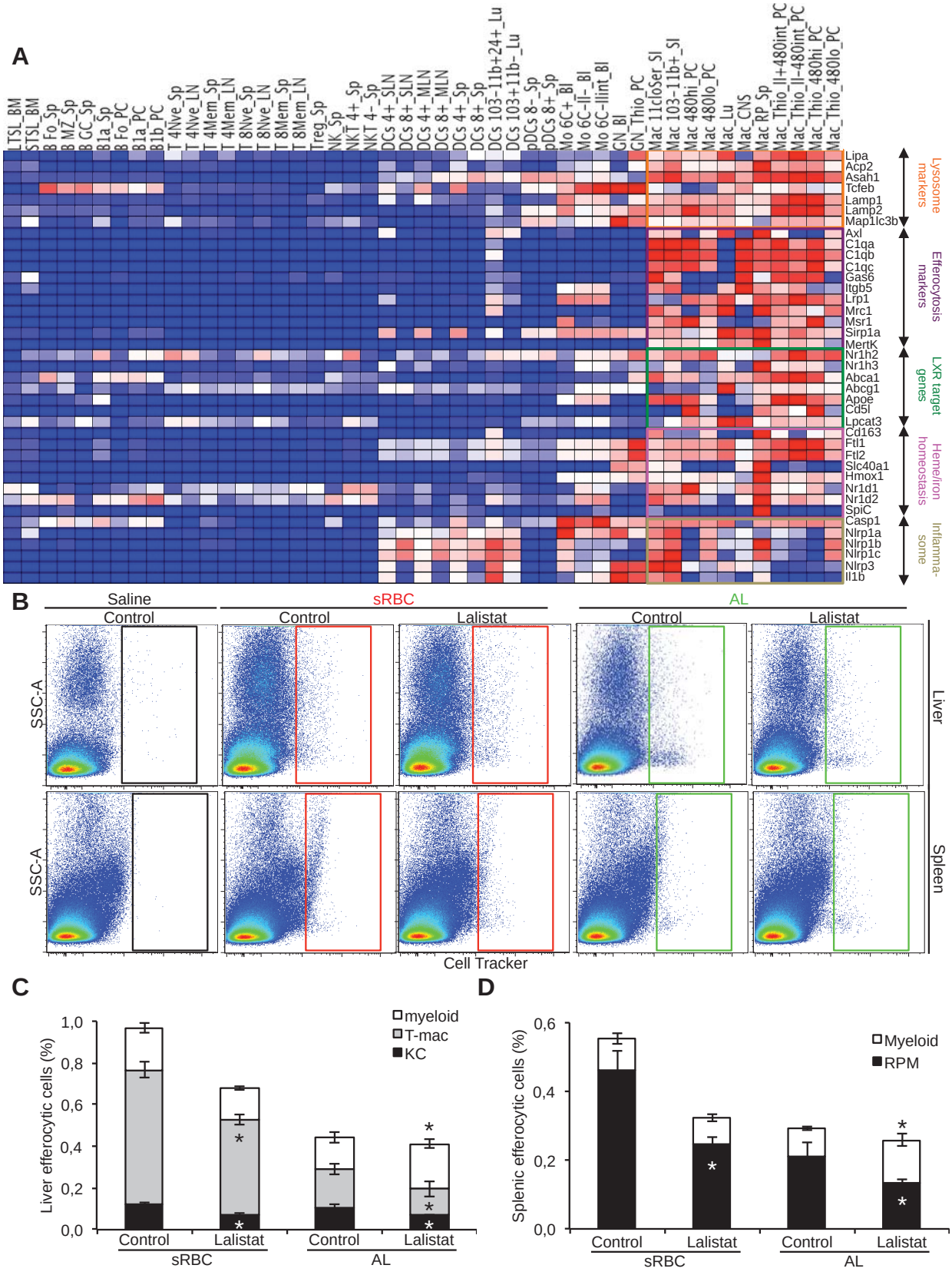

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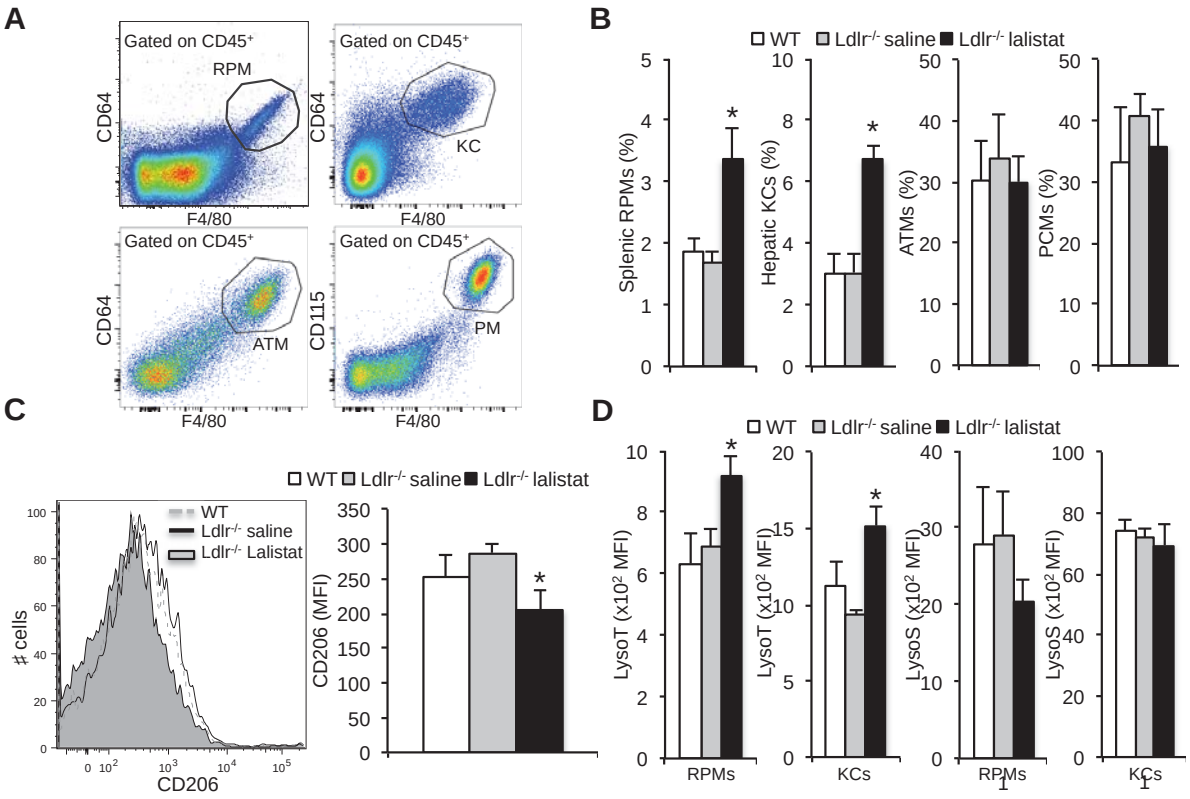

*** Long In Vivo Checklist**

Circulation Research - Preclinical Animal Testing: A detailed checklist has been developed as a prerequisite for every publication involving preclinical studies in animal models. ***Checklist items must be clearly presented in the manuscript, and if an item is not adhered to, an explanation should be provided.*** *If this information (checklist items and/or explanations) cannot be included in the main manuscript because of space limitations, please include it in an online supplement. If the manuscript is accepted, this checklist will be published as an online supplement. See the explanatory editorial for further information.*

This study involves use of animal models:

Yes

Study Design

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.	Yes
An overall study timeline is provided.	Yes
The protocol was prospectively written	Yes
The primary and secondary endpoints are specified	Yes
For primary endpoints, a description is provided as to how the type I error multiplicity issue was addressed (e.g., correction for multiple comparisons was or was not used and why). (Note: correction for multiple comparisons is not necessary if the study was exploratory or hypothesis-generating in nature).	Yes
A description of the control group is provided including whether it matched the treated groups.	Yes

Inclusion and Exclusion criteria

Inclusion and exclusion criteria for enrollment into the study were defined and are reported in the manuscript.	N/A
These criteria were set *a priori* (before commencing the study).	N/A

Randomization

Animals were randomly assigned to the experimental groups. If random assignment was not used, adequate explanation has been provided.	Yes
Type and methods of randomization have been described.	Yes
Allocation concealment was used.	N/A
Methods used for allocation concealment have been reported.	N/A

Blinding

Blinding procedures with regard to masking of group/treatment assignment from the experimenter were used and are described. The rationale for nonblinding of the experimenter has been provided, if such was not performed.	Yes
Blinding procedures with regard to masking of group assignment during outcome assessment were used and are described.	Yes
If blinding was not performed, the rationale for nonblinding of the person(s) analyzing outcome has been provided.	N/A

Sample size and power calculations

Formal sample size and power calculations were conducted before commencing the study based on *a priori* determined outcome(s) and treatment effect(s), and the data are reported.	Yes
If formal sample size and power calculation was not conducted, a rationale has been provided.	N/A

Data Reporting

Baseline characteristics (species, sex, age, strain, chow, bedding, and source) of animals are reported.	Yes
The number of animals in each group that were randomized, tested, and excluded and that died is reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided is provided for all experimental groups.	Yes
Baseline data on assessed outcome(s) for all experimental groups are reported.	N/A
Details on important adverse events and death of animals during the course of the experiment are reported for all experimental groups.	N/A
Numeric data on outcomes are provided in the text or in a tabular format in the main article or as supplementary tables, in addition to the figures.	Yes
To the extent possible, data are reported as dot plots as opposed to bar graphs, especially for small sample size groups.	N/A
In the online Supplemental Material, methods are described in sufficient detail to enable full replication of the study.	Yes

Statistical methods

The statistical methods used for each data set are described.	Yes
For each statistical test, the effect size with its standard error and *P* value is presented. Authors are encouraged to provide 95% confidence intervals for important comparisons.	Yes
Central tendency and dispersion of the data are examined, particularly for small data sets.	N/A
Nonparametric tests are used for data that are not normally distributed.	Yes
Two-sided *P* values are used.	N/A
In studies that are not exploratory or hypothesis-generating in nature, corrections for multiple hypotheses testing and multiple comparisons are performed.	Yes
In "negative" studies or null findings, the probability of a type II error is reported.	N/A

Experimental details, ethics, and funding statements

Details on experimentation including formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring are described.	Yes
Both male and female animals have been used. If not, the reason/justification is provided.	Yes
Statements on approval by ethics boards and ethical conduct of studies are provided.	Yes
Statements on funding and conflicts of interests are provided.	Yes

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