SUPPLEMENTAL MATERIAL

Detailed methods:

Materials – Macrophage serum-free medium was from Gibco and recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) from Invitrogen. Other cell culture media were from Sigma-Aldrich. The anti-NPC-1 antibody was from Novus Biologicals, anti-LAMP-1 (H4A3) from Developmental Studies Hybridoma Bank, anti-ABCA1 from Abcam, anti-CHOP (B-3) from Santa Cruz Biotechnology and secondary antibodies from Invitrogen. LDL was provided by Dr. Matti Jauhiainen (National Public Health Institute, Helsinki, Finland) and lipid-free apoA-I by Dr. Peter Lerch (Swiss Red Cross, Bern, Switzerland). DiI-acLDL was from Invitrogen, [^{3}H]oleic acid, [^{3}H]cholesterol oleate, [^{14}C]cholesterol oleate and [^{14}C]oleoyl coenzymeA were from Amersham. BODIPY-FTY720 was synthesized as in [1].

Quantitative PCR – Total RNA from monocyte derived human macrophages was isolated with RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. 1µg of total RNA was treated with DNase I (Promega, Madison, WI) in the presence of RNase Inhibitor (Promega) and reverse-transcribed by using Superscript II (Invitrogen, Carlsbad, CA) and random hexamer priming (Random hexamers from Applied Biosystems, Foster City, CA). Each sample was amplified in quadruplicate for ABCA1 and human RPII, which was used as a reference gene. The samples were run on a CFX96 detection system (BioRad, Hercules, CA) using iQ SYBR Green Supermix (BioRad). The data was analyzed as described previously [2]. The primers used were: ABCA1; forward: 5'-GCACTGAGGAAGATGCTGAAA-3', reverse: 5'-AGTTCCTGGAAGGTCTTGT TC-3'. RPII; forward: 5´-GCACCACGTCCAATGACA-3', reverse: 5'-GTGCGGCTGCTTCCATAA -3'.

Cell culture and viability – Human monocytes from healthy control subjects were isolated from buffy coat cells (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Monocytes were allowed to adhere to the flask for 1 h in RPMI medium, washed once with PBS and differentiated into macrophages for one week in macrophage serum-free medium + 10 ng/ml GM-CSF + 100 U/mL penicillin and 100 µg/mL streptomycin. CHO cells were cultured in a 1:1 solution of Ham's F12 and D-MEM supplemented with 5% FBS + 100 U/mL penicillin and 100 µg/mL streptomycin. FTY720 was added to the cells from DMSO and control samples received DMSO only. The DMSO concentration did not exceed 0.1%. Cell viability was measured using the CellTiter 96®Aq$_{ueous}$ assay (Promega).

RNA Interference in Primary Human Macrophages - Differentiated macrophages were collected by trypsinization and were electroporated with control, NPC1, or S1P1-specific siRNA using Human macrophage nucleofection kit (Amaxa) according to the manufacturers instructions. After electroporation, cells were incubated in M-SFM +5% FBS for 18 hours, after which the medium was changed to M-SFM. The siRNA sequences (sense strand) used were GL2 (control); 5'-CGUACGCGGAAUACUUCGA-3', NPC1; 5'-CCAGGTTCT-TGACTTACAA-3', S1P1 siRNA was an ON-target plus SMARTpool from Dharmacon.

Sterol determinations – Cells were washed three times with PBS and harvested in 1% NP-40 in PBS + protease inhibitors (chymostatin, leupeptin, antipain and Pepstatin A at 25 µg/mL). Protein concentrations were determined using the BioRad protein assay kit. Cholesterol and cholesteryl ester concentrations were determined using the Amplex Red cholesterol assay kit (Invitrogen) from amounts corresponding to 10µg of protein. Radiolabeling was carried out by incubating acLDL (1.5 mg/mL) with 1:10 volume of [^{3}H]cholesterol in DMSO (40 µCi [^{3}H]cholesterol/mg acLDL). The solution was incubated for 2h at 40^{o}C and dialyzed against 150 mmol/L NaCl + 1 mmol/L EDTA, pH 7.4. Oxysterol determinations were performed as in [3].

Cholesterol esterification – Cells were labeled with [^{3}H]-oleic acid in serum-free medium containing 2% fatty acid free BSA for the indicated times. The cells were then washed three times with cold PBS and scraped into a Kimax tube in 800 µL 2% NaCl. Samples of cell suspensions were taken for protein determinations. ^{14}C-cholesteryl oleate was added as an internal standard and 3 mL MeOH:$CHCl_3$ (2:1) was added. The samples were vortexed and 1/10 was removed for calculating extraction losses. 2 mL $CHCl_3$:H_2O (1:1) was added to the samples for phase separation. Following vortex and centrifugation, the lower phase was collected and dried. The samples were spotted on a TLC plate and run with hexane : diethyl ether : acetic acid (80:20:1) as solvent. The spots corresponding to cholesteryl ester were scraped and scintillation counted.

ACAT activity – Cells were washed with ice-cold PBS, scraped and the pellet was resuspended in Buffer A (20 mmol/L Tris-HCl, 1 mmol/L EDTA; pH 7.7). The suspension was passed through a 25 gauge needle 10 times for homogenization. Aliquots of the homogenate were put aside for protein determination. A 40 µL aliquot was mixed with 10 µL Buffer A + 2 mg/mL fatty acid free BSA + 250 µg/mL cholesterol. The solution was incubated at 37^{o}C for 2 min and the reaction started by adding 50µl Buffer A + 2 mg/mL fatty acid free BSA + [^{14}C]oleoyl CoA. The solution was vortexed briefly, incubated for 10 min at 37^{o}C and the reaction stopped by the addition of methanol (1 mL) and of $CHCl_3$ (1 mL). [^{3}H]Cholesteryl oleate was added as an internal standard and cholesteryl esters measured as described above.

Electron microscopy – For electron microscopy macrophages were cultured on glass coverslips, fixed and osmificated and a plastic capsule filled with embedding resin was inverted over the coverslip. After polymerization the coverslip was removed and the cells sectioned horizontally. Multilamellar bodies/cell profile were counted in all cells in a section of ± 1 µmol/L FTY720 treated, ac-LDL loaded cells from the same donor. For quantification of mitochondrial-late endosomal/lysosomal contacts every fourth cell was systematically photographed at 15000X magnification and the number of mitochondria making contact with late endosomes/lysosomes versus the total number of mitochondria/cell profile were counted in ± 1µmol/L FTY720 treated cells from the same donor. Late endosomes/lysosomes were identified by the

morphological criteria in [4]. Contact was defined as a proximity of $\leq$ 50 nm between membranes.

Western blotting – Proteins were separated by SDS-PAGE (15 µg/lane; 6-15% gels depending on protein size), transferred to nitrocellulose membrane and incubated with primary antibodies at 4°C overnight and secondary antibodies for 45 min at room temperature. After extensive washing with 0.1% Tween in TBS, the blots were developed using the Enhanced Chemiluminiscense kit (Amersham). Western blot quantifications were performed using ImageJ (NIH, Bethesda, Maryland, USA) after scanning of the films.

Surface Biotinylation – The cells were transferred to +4°C and were washed twice with ice cold PBS^+. The cells were the incubated at for 30 minutes +4°C with 1 mg/mL EZ-Link Sulfo-NHS-SS-Biotin in PBS^+. The cells were rinsed with cold PBS^+ and incubated for two times five minutes with 0.1 mol/L gycine + 0.3% BSA in PBS^+ at +4°C. Cells were lysed in 0.2% SDS – 2% NP-40 in PBS on ice. The supernatant was then transferred to eppendorf tubes. The tubes were centrifugated for 10 min at 13000 rpm and the supernatant was transferred to new tubes. One-tenth of the volume was removed to represent the total sample and 9/10 was precipitated with Streptavidin-agarose (Pierce) overnight at 4°C, followed by washes (once with lysis buffer, 3 times with 1% NP40 in PBS, twice in 0.1% NP40, 0.5 mol/L NaCl in PBS, twice with 50 mmol/L Tris–HCl, pH 7.5), and Western blotting. The fraction of ABCA1 on the cell surface was calculated by comparing the total versus biotinylated ABCA1 for each donor.

Immunocytochemistry- The macrophages were washed two times with PBS and were fixe with 4% paraformaldehyde for 30 minutes at RT. Following 3 washes with PBS the cells were incubated at RT for 10 minutes with 50mmol/L NH_4Cl in PBS. The cells were washed with PBS three times and incubated for 30 minutes with 0.5 mg/mL + 10 % FBS in PBS at 37°C to stain free cholesterol and permeabilize the cells. Following a wash with PBS, the cells were incubated with primary antibody diluted in 10% FBS in PBS for 1 h at 37°C. The macrophages were washed with PBS, and were incubated with secondary antibody in 10% FBS/PBS for 1 h at 37°C. The

coverslips were washed three times with PBS, rinsed briefly in MQ-H_2O and were mounted on microscope slides using Mowiol.

Annexin V staining was carried out on macrophages on coverslips using the Annexin V FITC staining kit from Invitrogen. The cells were washed three times in PBS and were then incubated for 15 minutes with Annexin V FITC in 1 x binding buffer for 15 minutes. The cells were then washed three times with binding buffer and were imaged by fluorescence microscopy.

Analysis of BODIPY-FTY720 by thin layer chromatography – Native and phosphorylated BODIPY-FTY720 were extracted from cells resuspended in 800 µL 2% NaCl or directly from 800 µL cell culture medium. 5 mL $CHCl_3$: MeOH : H_2O (2 : 2 : 1) was then added to the suspension with 10 µL HCl. The samples were vigorously vortexed and centrifuged at 2500 rpm for 10 minutes. The lower phase was transferred to new vials, dried and dissolved in $CHCl_3$:MeOH (9:1). The labelled lipids were separated on SilicaGel 60 TLC plates with 1-butanol/acetic acid/water (3 : 1 : 1) and were visualized on a UV-table.

Statistics – Results are expressed as mean ± SEM from a minimum of three independent experiments. Statistical analysis was made using Student's *t* test for paired observations. When three or more means were tested, one way ANOVA was performed followed by Dunnett's test for multiple comparisons against a single control. Statistical significance ($p<0.05$) is denoted with *.

Supplemental table I

Cholesterol handling in macrophages treated with FTY720 or other membrane active amphiphils.

	Control	FTY720	U18666A[5]	1-oct[6]	Oleic acid[6]	Sphingosine[6]
Cholesterol esterification	100	44±3 ↓	4±3 ↓	84±11	130±4 ↑	99±19
Cholesterol efflux	100	127±8 ↑	83±4 ↓	119±8 ↑	101±4	117±3 ↑
ABCA1 expression	100	247±43 ↑	95±13	94±17	148±6 ↑	76±10

Cholesterol esterification was carried out as [^{3}H] oleic acid incorporation into [^{3}H]cholesterol oleate on load with acLDL for 4 h. Cholesterol efflux was measured as 3-h efflux to apoA-I following loading with [^{3}H]cholesterol labelled acLDL for 4 h. ABCA1 expression was measured by western blotting. All pre-treatments were for 24 h. The concentrations used were 1 µmol/L FTY720, 5 µg/mL U18666A, 0.2 mmol/L 1-octanol, 1µmol/L oleic acid and 1µmol/L sphingosine. Results are given as percent of control ± SEM. Statistical significance is shown as an arrow in the direction the effect was regulated.

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