Materials and Methods

Animals. Mice were housed in a pathogen-free environment at Weill Cornell Medical College or The Rockefeller University and used in accordance with protocols approved by the Institutional Animal Care and Utilization Committees. C57BL/6 mice, purchased from Taconic, were used to prepare bone marrow and spleen derived DCs. For the examination of the lipid content of DC subsets in atherosclerotic plaque, 5 $ApoE^{-/-}$ female mice were purchased from Jackson laboratories. They were transitioned at 5 weeks of age to a high fat diet (21% milk fat, 0.15% cholesterol; Harlan Teklad) and maintained on the diet for 12 weeks. Mice were then euthanized via carbon dioxide inhalation.

Cells and Cell Culture. The principal method for generating non-classical DCs was adapted from a pervious method[1] by Lutz *et al.*[2]. On day 0, mice were euthanized and bone marrow cells flushed from ethanol sterilized femurs and tibias of mice using serum-free Roswell Park Memorial Institute (RPMI) and a 26-gauge needle. Cells were centrifuged at 300 g for 5 min and resuspended in 10 ml of DC media (RPMI, 10% endotoxin free and heat-inactivated fetal bovine serum (FBS), 58 U/ml penicillin, 58 U/ml streptomycin, 1X non-essential amino acids, 1 mM sodium pyruvate, 43 μM β-mercaptoethanol, 20 ng/ml recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF)). Cells were plated in non-tissue culture treated 10 cm petri dishes at a density of 0.25 x 10^6/ml and cultured at 37°C in a 5% CO_2 incubator. On day 3, 10 ml of fresh DC media containing 40 ng/ml recombinant murine GM-CSF was added to dishes. On days 6 and 8, 10 ml of the DC suspension was taken and centrifuged at 300 g for 5 min. Cells were resuspended in 10 ml of fresh DC media containing 40 ng/ml GM-CSF and added back to the culture dish. On day 6, $Cd11c^+$ cells (AlexaFluor647-Cd11c purchased from AbD Serotech, Raleigh, NC) were selected by fluorescence-activated cell sorting and used as immature cells. No difference in our lysosome exocytosis experiments was observed between Cd11c sorted and unsorted immature DCs. Thus, for all future experiments, unsorted DCs were used. On day 10, 1 μg/ml lipopolysaccharide (LPS) was added to the cell suspension to induce maturation. On day 11 mature cells, which represent a highly homogeneous population[2], were used without sorting.

Human monocyte derived dendritic cells were prepared as described previously[3] with minor modifications. Buffy coat cells drawn from healthy donors were subjected to hypertonic lysis by resuspension at a 1:4 ratio in 0.168 M NH_4Cl followed by washing in phosphate buffered saline (PBS). The remaining leukocytes were resuspended at a concentration of 1 x 10^6 cells/ml in RPMI containing 10% low endotoxin and heat-inactivated FBS and 50 ng/ml recombinant murine GM-CSF and cultured at 37°C in a 5% CO_2 incubator.

Flt-3 DCs were prepared as described previously[4] with minor modifications. Bone marrow was isolated as described above and red blood cells lysed by resuspension in 0.168 M NH_4Cl, followed by washing with PBS. Cells were cultured at 37°C in a 5% CO_2 incubator at a density of 1.5 x 10^6 cells/ml in non-tissue culture treated 10 cm tri-partition dishes in DC media containing 300 ng/ml recombinant murine flt-3 ligand. Cells were used on day 11 of culture. Flt-3 classical DC were enriched by flt-3L treatment *in vivo* by 10-20 fold[5] and isolated from the spleens of mice treated with recombinant sterile human flt-3L (Celldex, Hampton, NJ) at 10 μg/mouse, intraperitoneally for 10-12 consecutive days as described[6, 7]. Splenic single-cell suspensions were prepared by incubation with 400 U/ml collagenase D at 37°C for 30 min. The cell suspensions were passed through a 40 μm cell strainer and flt-3 DCs isolated using a CD11c isolation kit (Miltenyi, San Diego, CA) according to the manufacturer's instructions. Classical DC viability was confirmed with propidium iodide staining (data not shown).

Lipoproteins and Reagents. LDL was isolated from fresh human plasma by preparative ultracentrifugation as previously described[8]. LDL was labeled using succinimidyl esters of AlexaFluor546 (Alexa546) and Alexa488 (Invitrogen, Carlsbad, CA), biotin (Sigma-Aldrich, St. Louis, MO) or CypHer 5E Mono N-hydroxysuccinimide ester (CypHer5E) (GE Healthcare, Chalfont St. Giles, U.K.). LDL was vortex aggregated for 10 sec[9]. Alexa488-phalloidin, Alexa488-Cholera toxin subunit B (CtB), LipidTOX, biotin-fluorescein-dextran (10,000 MW) and non-essential amino acids were purchased from Invitrogen. All solvents (isopropanol, hexane, methanol, potassium hydroxide, NH_4Cl), as well as biotin, streptavidin, 4-(2-hydroxyethyl)-1-pipierazine ethane sulphonic acid (HEPES), PFA, saponin, acyl-coenzyme A cholesterol acyltransferase (ACAT) inhibitor 58035, hyaluronidase, Collagenase I/XI, Collagenase D, beta-sitosterol, beta-mercaptoethanol, LPS, filipin, methyl-β-cyclodextrin, cholesterol and Triton were purchased from Sigma Chemicals. DNase was purchased from New England BioLabs (Ipswich, MA). Flt-3 and GM-CSF were purchased from Peprotech (Rocky Hill, NJ). Sodium pyruvate was purchased from Corning (Corning, NY).

CtB Plasma Membrane Labeling. For surface labeling with fluorescent CtB cells were incubated on ice for 3 min in the presence of 5μg/ml Alexa488-CtB in experimental medium, rinsed with ice-cold medium and fixed with 3.3% PFA. Images were taken with a 63x 1.4 numeric aperture (NA) objective on a Zeiss LSM 510 laser scanning confocal microscope (axial resolution 0.8 μm).

Actin Measurements. To visualize F-actin, DCs were incubated with Alexa546-agLDL for 60 min, washed with PBS and fixed for 15 min with 3.3% PFA. Cells were subsequently washed with PBS, and incubated with 2 U/ml of fluorescent phalloidin in 0.05% (w/v) saponin in PBS for 60 min at room temperature. Images were acquired on the confocal microscope described above with a 40x 0.8 NA objective. For image quantification, MetaMorph software, Universal Imaging/Molecular Devices Corporation (Sunnyvale, CA) was used. All images subjected to comparative quantification were acquired on the same day using the same microscope settings. Each experiment was repeated at least three times and >100 cells were examined per condition in each experiment. The procedures used to quantify the amount of F-actin in the vicinity of agLDL has been described in detail previously[10]. Briefly, we obtained stacks of confocal images for each field at wavelengths appropriate for Alexa546-agLDL (red) and Alexa488-phalloidin (green). We determined a threshold in the red channel (agLDL) that would include nearly all of the observable agLDL in the images. We then measured the total Alexa488-phalloidin fluorescence intensity within the thresholded area for each field and normalize by the number of cells. By this procedure the total phalloidin fluorescence intensity within the thresholded regions per cell touching agLDL was measured.

Delivery of Lysosomal Contents. Lysosome labeling of DCs plated on Poly-D-lysine coated glass-coverslip bottom dishes was accomplished via an 18 hr pulse with 1 mg/ml biotin-fluorescein-dextran. Cells were chased for 2 hrs in RPMI and subsequently incubated with streptavidin-Alexa546-agLDL for 90 min. Next, cells were incubated with 200 μM biotin for 10 min in order to bind any unoccupied streptavidin sites prior to cell permeabilization. Cells were then fixed with 1% PFA for 15 min, washed, and permeabilized with 1% Triton for 10 min. Images were acquired with the confocal microscope described above using a 40x 0.8 NA objective. For image quantification, MetaMorph software was used. All images subjected to comparative quantification were acquired on the same day using the same microscope settings. Each experiment was repeated at least three times. For every experiment >30 randomly chosen fields with a total >200 cells per condition were imaged and subjected to quantification. To quantify the amount of lysosome exocytosis, we obtained a single plane for each field at wavelengths appropriate for streptavidin-Alexa546-agLDL (red) and biotin-fluorescein-agLDL (green). We determined a threshold in the red channel (agLDL) that would include nearly all of the observable agLDL in the images. We then

measured the total fluorescein fluorescence intensity within the thresholded area for each field. We used the same threshold level for each image within an experimental data set. By this procedure the total fluorescein signal intensity within the thresholded regions per field was measured. Data was normalized by the amount of biotin-fluorescein-dextran delivered to lysosomes as determined by confocal imaging of non-permeabilized cells.

pH Measurements. DCs were plated on Poly-D-lysine coated glass-coverslip bottom dishes. The cells were incubated for 30 min with CypHer 5E, a pH sensitive fluorophore, and Alexa488, a pH insensitive fluorophore, dual labeled agLDL. The pH values within each pixel were assessed quantitatively by comparison with ratio images obtained in calibration buffers of varying pH. Live cells were imaged on the confocal microscope described above using a 63x 1.4 NA objective. Cell temperature was maintained at 37°C with a heated stage and objective heater. For all live cell imaging experiments, media was changed to RPMI containing 25 mM HEPES without phenol red or sodium bicarbonate. Data were analyzed with MetaMorph image analysis software. A binary mask was created using the Alexa488 signal intensity and applied to both channels to remove background noise. Images were convolved with a 7x7 pixel Gaussian filter, and ratio images were generated.

Filipin Labeling. DCs were plated on Poly-D-lysine coated glass-coverslip bottom dishes and incubated with Alexa546-agLDL for 1 hr. Cells were fixed with 3% PFA for 20 min, washed and stained with 50 μg/ml filipin for 45 min. Filipin images were taken with a Leica DMIRB epi-fluorescence microscope equipped with an Andor iXon^{EM} Blue EMCCD camera driven by MetaMorph Imaging System software. Images were acquired with a 63x, 1.32-0.6 NA plan Apochromat oil objective with an A4 filter cube (Chroma).

Foam Cell Formation. Immature monocyte derived DCs and classical DCs were plated onto poly-D-lysine coated glass-coverslip bottom dishes overnight and incubated with Alexa546-agLDL for the indicated times at 37°C and 5% CO_2. Cells were fixed with 3% PFA for 20 min and washed with PBS. They were subsequently stained with LipidTOX-green for 30 min at room temperature and washed with PBS. Cells were analyzed by wide-field fluorescence microscopy using the Leica DMIRB microscope described above. Images were acquired using a 40x 1.25 NA oil-immersion objective with standard FITC/TRITC filter cubes (Chroma). The percentage of LipidTOX positive cells was quantified.

Cholesterol Loading. DCs were plated on Poly-D-lysine coated glass-coverslip bottom dishes and incubated with 5mM cholesterol:methyl-β-cyclodextrin in medium 2 for 15 min. Cells were then incubated with agLDL in the presence of 2 μM ACAT inhibitor (to prevent esterification of cholesterol) and assays for actin and delivery of lysosomal contents were carried out as described above.

Determination of DC Free and Total Cholesterol by GC-MS. DC were differentiated and matured as indicated and grown on 6-well plates (in triplicate for each condition) until approximately 60% confluent. Cells were treated with 2 μM ACAT inhibitor (to prevent re-esterification of hydrolyzed cholesteryl ester)[11] for 20 min prior to addition of agLDL. After addition of 0.5 μg agLDL per well for 30 min, cells were washed once with RPMI and incubated at 37^{o}C for indicated times (0, 30 and 90 min) in presence of ACAT inhibitor. The cells were washed twice with PBS and lipids were extracted using hexane:isopropanol (3:2 v/v) containing β-sitosterol as an internal standard such that the final concentration of β-sitosterol was 5 μg per sample. Lipid extracts were transferred to borosilicate tubes and dried under nitrogen gas and used to determine free cholesterol levels. All samples were resuspended in hexane and lipids separated using a Varian Factor Four capillary column (VF-1 ms 30 m×

0.25 mm ID DF 0.25) using Varian 4000 GC/MS/MS system. Free cholesterol measurements were normalized for extraction using β-sitosterol measurements and for total protein content.

Lipid Analysis of DC Subsets Isolated from Murine Atherosclerotic Plaque. Aortic single cells were prepared in accordance with a previous method[12] with minor modifications. In brief, after removal of the perivascular fat and cardiac muscle tissues, single-cell suspensions from aortic segments, including aortic sinus, aortic arch and thoracic aorta were prepared by incubation with an enzyme mixture containing 675 U/ml collagenase I, 187.5 U/ml collagenase XI, 90 U/ml hyaluronidase, and 90 U/ml DNase in Hank's balanced salt solution with calcium and magnesium for 75 min at 37°C with gentle shaking. Cell suspensions were cytospun onto glass slides at 1000 rpm for 5 min and left to dry for 5 min prior to fixation in 3% PFA for 15 min. After blocking with 10% goat serum for 1 hr, DC subsets were distinguished using combinations of the following monoclonal antibodies: For analysis of non-classical mature and immature DC, Cd11b at 1:300 dilution overnight at 4°C (anti-mouse Cd11b, clone M1-70; BD Biosciences, San Jose, CA) and Alexa350 anti-rat secondary at 1:200 dilution for 2 hr at 37°C (Invitrogen). Followed by Cd11c at 1:100 dilution overnight at 4°C (Alexa647 anti-mouse Cd11c, clone 30-F11; BioLegend, San Diego, CA) and major histocompatability complex II at 1:100 dilution overnight at 4°C (Alexa488 anti-mouse MHC II; BioLegend). For analysis of classical DC, CD103 antibody was used at 1:100 dilution overnight at 4°C (anti-mouse CD103, Ebioscience, San Diego, CA) and Alexa488 anti-hamster secondary at 1:200 dilution for 2 hr at 37°C. Followed by Cd11c at 1:100 dilution overnight at 4°C (Alexa647 anti-mouse Cd11c, clone 30-F11; BioLegend). All antibody labeling was carried out in PBS containing 3% goat serum. Last, all samples were stained with LipidTOX-red 1:1000 in PBS for 20 min. Samples were imaged on the widefield microscope described above. Images were acquired using a 40x 1.25 NA oil-immersion objective with A4, FITC, TRITC, and Cy5 filter cubes (Chroma). All images subjected to comparative quantification were acquired on the same day using the same microscope settings.

Statistical Analysis. Statistical analysis was performed using Excel. For comparisons of two groups, student's t test was used. For comparisons of more than two groups, the Kruskal-Wallis test was used followed by the Wilcoxon rank-sum test for pairwise comparisons.

References

1. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *Journal of Experimental Medicine.* 1992;176:1693-1702.
2. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999;223:77-92.
3. Sanarico N, Ciaramella A, Sacchi A, Bernasconi D, Bossu P, Mariani F, Colizzi V, Vendetti S. Human monocyte-derived dendritic cells differentiated in the presence of IL-2 produce proinflammatory cytokines and prime Th1 immune response. *Journal of Leukocyte Biology.* 2006;80:555-562.
4. Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, Lahoud MH, O'Keeffe M, Shao QX, Chen WF, Villadangos JA, Shortman K, Wu L. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *Journal of Immunology.* 2005;174:6592-6597.
5. Bozzacco L, Trumpfheller C, Huang Y, Longhi MP, Shimeliovich I, Schauer JD, Park CG, Steinman RM. HIV gag protein is efficiently cross-presented when targeted with an antibody towards the DEC-205 receptor in Flt3 ligand-mobilized murine DC. *Eur J Immunol.* 2010;40:36-46.
6. Mollah SA, Dobrin JS, Feder RE, Tse SW, Matos IG, Cheong C, Steinman RM, Anandasabapathy N. Flt3L dependence helps define an uncharacterized subset of murine cutaneous dendritic cells. *J Invest Dermatol.* 2014;134:1265-1275.
7. Anandasabapathy N, Feder R, Mollah S, Tse SW, Longhi MP, Mehandru S, Matos I, Cheong C, Ruane D, Brane L, Teixeira A, Dobrin J, Mizenina O, Park CG, Meredith M, Clausen BE, Nussenzweig MC, Steinman RM. Classical Flt3L-dependent dendritic cells control immunity to protein vaccine. *Journal of Experimental Medicine.* 2014;211:1875-1891.
8. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest.* 1955;34:1345-1353.
9. Buton X, Mamdouh Z, Ghosh R, Du H, Kuriakose G, Beatini N, Grabowski GA, Maxfield FR, Tabas I. Unique cellular events occurring during the initial interaction of macrophages with matrix-retained or methylated aggregated low density lipoprotein (LDL). Prolonged cell-surface contact during which ldl-cholesteryl ester hydrolysis exceeds ldl protein degradation. *Journal of Biological Chemistry.* 1999;274:32112-32121.
10. Grosheva I, Haka AS, Qin C, Pierini LM, Maxfield FR. Aggregated LDL in contact with macrophages induces local increases in free cholesterol levels that regulate local actin polymerization. *Arteriosclerosis, Thrombosis & Vascular Biology.* 2009;29:1615-1621.
11. Tabas I, Weiland DA, Tall AR. Inhibition of acyl coenzyme A:cholesterol acyl transferase in J774 macrophages enhances down-regulation of the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase and prevents low density lipoprotein-induced cholesterol accumulation. *Journal of Biological Chemistry.* 1986;261:3147-3155.
12. Choi JH, Do Y, Cheong C, Koh H, Boscardin SB, Oh YS, Bozzacco L, Trumpfheller C, Park CG, Steinman RM. Identification of antigen-presenting dendritic cells in mouse aorta and cardiac valves. *Journal of Experimental Medicine.* 2009;206:497-505.