Monocyte-Derived Dendritic Cells Upregulate Extracellular Catabolism of Aggregated Low-Density Lipoprotein on Maturation, Leading to Foam Cell Formation

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Objective—Although dendritic cells are known to play a role in atherosclerosis, few studies have examined the contribution of the wide variety of dendritic cell subsets. Accordingly, their roles in atherogenesis remain largely unknown. We investigated the ability of different dendritic cell subsets to become foam cells after contact with aggregated low-density lipoprotein (LDL; the predominant form of LDL found in atherosclerotic plaques).

Approach and Results—We demonstrate that both murine and human monocyte-derived dendritic cells use exophagy to degrade aggregated LDL, leading to foam cell formation, whereas monocyte-independent dendritic cells are unable to clear LDL aggregates by this mechanism. Exophagy is a catabolic process in which objects that cannot be internalized by phagocytosis (because of their size or association with extracellular structures) are initially digested in an extracellular acidic lytic compartment. Surprisingly, we found that monocyte-derived dendritic cells upregulate exophagy on maturation. This contrasts various forms of endocytic internalization in dendritic cells, which decrease on maturation. Finally, we show that our in vitro results are consistent with dendritic cell lipid accumulation in plaques of an ApoE−/− mouse model of atherosclerosis.

Conclusions—Our results show that monocyte-derived dendritic cells use exophagy to degrade aggregated LDL and become foam cells, whereas monocyte-independent dendritic cells are unable to clear LDL deposits. Furthermore, we find that exophagy is upregulated on dendritic cell maturation. Thus, exophagy-mediated foam cell formation in monocyte-derived dendritic cells could play a significant role in atherogenesis. (Arterioscler Thromb Vasc Biol. 2015;35:2092-2103. DOI: 10.1161/ATVBAHA.115.305843.)

Key Words: atherosclerosis ■ dendritic cells ■ foam cells ■ lipoproteins, LDL ■ phagocytosis

Atherosclerosis is a lipid-related inflammatory disease in which the interaction of immune cells with subendothelial lipoproteins and ensuing foam cell formation play a pivotal role. Although macrophages are the predominant immune cells associated with atherosclerosis, almost 20 years ago, Bobryshev and Lord used electron microscopy to demonstrate that dendritic cells (DCs) are also present in human arteries.1–3 Since then, several studies have shown the accumulation of DCs in human and murine atherosclerotic lesions using a variety of markers.4–14 In addition to regulation of the inflammatory response,11,15,16 DCs also play a direct role in the regulation and processing of lipid.17,18 Conditional depletion of CD11c+ cells in low-density lipoprotein receptor knockout (Ldlr−/−) mice significantly reduced the intimal lipid area, and few foam cells were found. This suggests that DCs may be responsible for foam cell formation at the earliest stages of plaque development.18 Although this study indicated a proatherogenic role of DCs, it did not differentiate between the wide variety of DC subsets, and accordingly their roles in atherogenesis remain largely unknown.

Both classical DCs, formed by the cytokine fms-like tyrosine kinase 3 ligand (flt-3) acting on monocyte-independent precursors,19–21 and nonclassical (monocyte-derived) DCs22 are present in atherosclerotic plaques.11 Recently, the Steinman laboratory examined the role of classical flt-3–derived DCs in atherosclerosis and, surprisingly, found that they seem to be atheroprotective.11 Other studies have used various manipulations to perturb nonclassical monocyte-derived DC function in mouse models of atherosclerosis, such as restricted monocyte entry using CX3CR1-deficient mice23 or inhibited monocyte differentiation into DCs using granulocyte macrophage colony-stimulating factor (GM-CSF)-deficient mice.24,25 Although
atherosclerosis was decreased when monocyte plaque entry was restricted, suggesting a proatherogenic role for nonclassical monocyte-derived DCs, loss of CX3CR1 concomitantly reduces macrophage plaque accumulation, leaving the relative contribution of these 2 cell types to atherogenesis unresolved. Furthermore, studies using GM-CSF–deficient mice report conflicting phenotypes, so the role of nonclassical monocyte-derived DCs in atherosclerosis remains elusive.

In the arterial intima, lipid accumulation can be found within vascular CD11c⁺ DCs in Ldlr⁻/⁻ mice after only a few days of hypercholesterolemia, where they adopt a foam cell–like appearance that may constitute the earliest stages of plaque formation. Historically, the macrophage has been the predominant lesional cell type studied to assess foam cell formation during atherogenesis. Although macrophage foam cell formation is generally considered atherogenic, there are several notable differences between macrophages and DCs that are relevant for the biological consequences of lipid loading. For instance, molecules involved in handling cholesterol, such as ATP-binding cassette (ABC) A1 and ABCG1, are differentially expressed by macrophages and DCs (http://www.imgen.org). Also, macrophage migration and ensuing emigration from tissues are much less efficient than that of DCs, although evidence suggests that hypercholesterolemia affects DC mobilization. Thus, it is possible that the consequences of foam cell formation by these 2 cell types may be disparate. Although macrophage foam cell formation has received much attention, the mechanism(s) by which DCs internalize lipoproteins to become foam cells and the functional significance of DC lipid accumulation remains to be determined.

In atherosclerotic plaques, >90% of the low-density lipoprotein (LDL) that interacts with immune cells is aggregated and avidly bound to the subendothelial matrix. This aggregated LDL (agLDL) can be modified by oxidation and exposure to lipases. Mouse models in which the subendothelial retention of LDL is blocked develop significantly less atherosclerosis than their wild-type counterparts, with lesion area reduced by as much as 86%. Thus, mechanisms of foam cell formation based on catabolism of agLDL likely have significant physiological relevance. However, most studies of foam cell formation use modified monomeric LDL, such as oxidized, acetylated, or minimally modified LDL. The contributions of these different pathways to foam cell formation in atherosclerosis are not known. In a cell culture model of the interaction of macrophages with agLDL, we showed that the macrophages create deeply invaginated structures that are actively acidified in which extracellular agLDL is digested by exocytosed lysosomal enzymes. We describe this method of catabolism as exophagy and the compartment used for degradation as a lysosomal synapse.

In light of research indicating that DCs play an important role in atherosclerotic lesion development, we investigated the ability of both monocyte-independent (flt-3 derived, classical) and monocyte-derived (GM-CSF derived, nonclassical) DCs to perform exophagy-mediated catabolism of agLDL. We found that, similar to macrophages (which also derive from monocytes), GM-CSF bone marrow–derived DCs use exophagy to degrade agLDL and that this interaction leads to foam cell formation. Conversely, flt-3 bone marrow–derived DCs did not perform exophagy, did not become foam cells, and were unable to degrade the aggregate. Next, we compared the ability of immature and mature monocyte-derived DCs to perform exophagy because it is well known that several types of endocytosis are dampened during nonclassical DC maturation. Surprisingly, we found that mature DCs (mDC) upregulate exophagy and consequently take up more cholesterol from the aggregates than immature DCs (iDC). Finally, we show that consistent with our in vitro cell culture results, mDC contain more neutral lipid than iDC in an ApoE⁻/⁻ mouse model of atherosclerosis, whereas monocyte-independent DC contained scant amounts of lipid. These results show that monocyte-derived DCs can use exophagy to degrade agLDL that is not taken up by standard phagocytic mechanisms and that in contrast to other forms of endocytosis, exophagy is upregulated on DC maturation. We suggest that exophagy-mediated foam cell formation in monocyte-derived DCs could play a significant role in atherogenesis.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

GM-CSF Bone Marrow–Derived DCs Form Specialized Extracellular Compartments at Sites of Contact With agLDL

To characterize the topological organization of compartments formed for exophagy, we labeled the plasma membrane with fluorescent cholera subunit B (CtB). In this experiment, the cells are not permeabilized, so the macromolecular CtB can only label glycolipids on the plasma membrane. We use this technique to examine whether plasma membrane surrounds the aggregate, thereby establishing the surface connectivity of the compartment. We note lipid molecules and even glycosylphosphatidylinositol-anchored proteins can redistribute on the plasma membrane after fixation with 3% paraformaldehyde, so we would expect the GM₁ ganglioside, which binds CtB, to remain mobile after fixation. Both flt-3 bone marrow–derived and GM-CSF bone marrow–derived primary murine DCs were incubated with AlexaFluor-546 (Alexa546)-agLDL for 60 minutes, labeled with Alexa488-CtB on ice for 3 minutes, and fixed. Sites of contact between GM-CSF bone marrow–derived DCs and agLDL were labeled by CtB (arrows,
Figure 1A and 1B), indicating that the aggregate is contained in a compartment that is connected to the cell surface. An axial slice through the confocal stack at the position of the red line shows that although the aggregate resembles a separate vesicle in the xy plane (Figure 1B), it is contained in a compartment contiguous with the extracellular space (Figure 1B). In contrast, flt-3 bone marrow–derived DCs do not create deeply invaginated structures at sites of contact with the aggregate (arrows, Figure 1C and 1D), indicating that they do not form specialized extracellular compartments in response to contact with agLDL.

We note that the flt-3 bone marrow–derived DC culture contains a mix of 2 distinct CD24(high) and CD11b(high) DCs, equivalent to splenic CD8+ and CD8− DCs, as well as CD45RA high plasmacytoid DCs.41 Sufficient agLDL was added to the culture system so that >95% of cells were in contact with aggregate. None of the cells in our mixed flt-3 bone marrow–derived DC culture formed extracellular compartment in response to contact with agLDL, indicating that both types of flt-3 bone marrow–derived DCs were unresponsive to the agLDL.

Compartments used for exophagy are formed via F-actin–driven membrane protrusions surrounding the aggregate.39 We examined F-actin near sites of contact with agLDL in both flt-3 bone marrow–derived and GM-CSF bone marrow–derived primary murine DCs. Cells were incubated with Alexa546-agLDL followed by fixation and labeling of F-actin by Alexa488-phalloidin (Figure 1E–1H). After a 60-minute agLDL incubation, an enrichment of F-actin was detected near the sites of contact with agLDL in GM-CSF bone marrow–derived DCs (arrows, Figure 1E and 1F) but not appreciably in flt-3 bone marrow–derived DCs (Figure 1G and 1H). Again, no cells in our mixed flt-3 bone marrow–derived DC culture formed local F-actin structures in response to contact with agLDL. We also examined primary flt-3–derived DCs isolated from spleens of mice treated with flt-3 ligand. Figure I in the online-only Data Supplement shows roughly a 20-fold expansion of splenic flt-3–derived DCs on treatment with flt-3 ligand. No increase in local F-actin was seen at points of contact between primary flt-3–derived DCs and agLDL (data not shown). Actin polymerization, typical of a lysosomal synapse, was confirmed in human monocyte-derived DCs (huMDDCs).

Local F-actin–rich structures were detected near the sites of contact with agLDL in huMDDCs (arrows, Figure 1I and 1J). Both the local F-actin–rich structures and CtB staining indicate that GM-CSF bone marrow–derived DCs form deeply invaginated surface compartments in response to incubation with agLDL but flt-3 bone marrow–derived DCs do not.

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**Figure 1.** Granulocyte macrophage colony-stimulating factor (GM-CSF) bone marrow (BM)–derived dendritic cells (DCs) form extracellular compartments at sites of contact with aggregated low-density lipoprotein (agLDL) but fms-like tyrosine kinase 3 ligand (flt-3) BM–derived DCs do not. A–J, GM-CSF BM (A, B, E, F, I, and J) and flt-3 BM (C, D, G, and H) DCs were incubated with Alexa546-agLDL (red) for 60 minutes and the interface between the aggregate and cells examined. A–D, The plasma membrane was labeled via incubation with Alexa488-CtB (green) on ice for 3 minutes. Cells were then washed and fixed. Sites of contact between mature GM-CSF BM DCs and agLDL were labeled by cholera toxin subunit B (CtB; arrows, A and B). An axial slice through the confocal stack at the position of the red line shows that although the aggregate resembles a separate vesicle in the xy plane (B), it is contained in a compartment contiguous with the extracellular space (arrow, B’). flt-3 BM DCs do not surround the aggregate with CtB positive membrane at sites of contact (arrows, C and D). E–J, Cells were stained with Alexa488-phalloidin to show F-actin (green). An enrichment of F-actin was detected near the sites of contact with agLDL in GM-CSF BM (arrows, E and F) but not flt-3 BM (arrows, G and H) DCs. I and J, Actin polymerization, at sites of contact with agLDL, was also seen in mature human monocyte-derived DCs (arrows, I and J).
GM-CSF Bone Marrow–Derived DCs Form a Lysosomal Synapse to Degrade agLDL but flt-3 Bone Marrow–Derived DCs Are Unable to Clear agLDL

We have previously reported that compartments used for extracellular catabolism, described as a lysosomal synapse, function as an extracellular hydrolytic organelle because of targeted exocytosis of lysosomes and compartment acidification by V-ATPase on the plasma membrane. We tested whether lysosomal contents, which include lysosomal acid lipase, a lysosomal hydrolase essential for the intracellular degradation of cholesteryl esters, were delivered to points of contact with the aggregate in both flt-3 and GM-CSF bone marrow–derived DCs. Biotin–fluorescein–dextran was incubated with DCs overnight, leading to endocytosis of the dextran and delivery to lysosomes. Cells were then exposed to streptavidin-Alexa546-labeled agLDL for 90 minutes followed by a 30-s treatment with biotin-Alexa633 to label extracellular aggregates. Next, excess biotin was applied to block any remaining free streptavidin on the aggregates. Cells were then fixed and permeabilized to remove the bright lysosomal fluorescence and facilitate identification of the exocytosed fluorescein signal.

When GM-CSF bone marrow–derived DCs were in contact with agLDL for 90 minutes (Figure 2A–2D), there was significant deposition of biotin–fluorescein–dextran onto the aggregate in regions in contact with cells (arrows, Figure 2A).
Consistent with previous studies, the biotin-Alexa633 staining (Figure 2D) clearly indicated that the aggregate was largely extracellular after the 90-minute incubation.\(^4\) We have previously shown that the extracellular compartments formed by macrophages are dynamic, allowing catabolic products and lysosomal enzymes to be released into the extracellular space as well as the entry of large molecules, such as biotinylated fluorophores.\(^3\) Although we display a compartment that is positive for Alexa633, we note that many regions of exocytosis excluded biotin-Alexa633 (data not shown). Colocalization of the fluorescein and Alexa633 signals demonstrated that lysosomal contents were exocytosed and delivered to an extracellular compartment in GM-CSF bone marrow–derived DCs. Consistent with our findings about compartment formation, no lysosomal exocytosis was seen at points of contact between the aggregate and any of the flt-3 bone marrow–derived DCs in the mixed culture (Figure 2E–2G). We also observed no lysosome exocytosis in primary flt-3–derived DCs isolated from mouse spleen (data not shown). Lysosome exocytosis to an extracellular agLDL-containing compartment was also seen in huMDDCs (arrows, Figure 2H–2J). These data show that lysosomal contents were delivered to an extracellular compartment, and the previously internalized biotin–fluorescein–dextran became associated with the agLDL contained in these compartments in GM-CSF bone marrow–derived but not flt-3 bone marrow–derived DCs.

Finally, we tested whether portions of extracellular compartments formed at points of contact with the aggregate were acidic in flt-3 and GM-CSF bone marrow–derived DCs. In order for lysosomal acid lipase or other secreted lysosomal acid hydrolases to function optimally, an acidic environment is required. To test whether portions of the compartment are acidic, we labeled LDL with CypHer 5E Mono N-hydroxysuccinimide ester, a pH sensitive fluorophore, and Alexa488, a pH insensitive fluorophore.\(^4\) DCs were incubated with agLDL, and the pH surrounding the aggregate was determined from the ratio of CypHer 5E Mono N-hydroxysuccinimide ester/Alexa488 fluorescence when compared with values obtained in pH calibration buffers. When GM-CSF bone marrow–derived DCs interacted with the dual-labeled agLDL, regions of low pH could be seen at the contact sites (arrow, Figure 2K). Consistent with our data about compartment formation and lysosome exocytosis, no acidification was observed in aggregates in contact with any flt-3 bone marrow–derived DCs in the mixed culture (Figure 2L). Regions of low pH were also seen in contact sites between huMDDCs and agLARD (arrow, Figure 2M).

To determine whether cholesteryl ester hydrolysis occurs in extracellular compartments formed for exophagy, we used filipin, a fluorescent sterol-binding polyeone that can be used for detection of free cholesterol.\(^4\) flt-3 and GM-CSF bone marrow–derived DCs were incubated with Alexa546-agLDL for 1 hour, fixed, and labeled with filipin (Figure 2N and 2O). As expected, GM-CSF bone marrow–derived DCs exhibited intense filipin staining (green) in the region of contact with the agLDL (arrows, Figure 2N), whereas flt-3 bone marrow–derived DCs did not exhibit filipin staining near the aggregate (Figure 2O). The bright filipin labeling seen in GM-CSF bone marrow–derived DCs indicates that there is an increase in free cholesterol at sites of contact between agLDL and the cell.

**GM-CSF Bone Marrow–Derived DCs Use Exophagy to Degrade agLDL and Become Foam Cells**

Next, we investigated whether incubation of flt-3 and GM-CSF bone marrow–derived DCs with agLDL leads to exophagy-mediated foam cell formation. flt-3 and GM-CSF bone marrow–derived DCs were incubated with Alexa546-agLDL for 0, 2, 4, 6, and 24 hours. At each time point, the cells were fixed and labeled with LipidTOX-Green to detect neutral lipid droplets, indicative of foam cell formation (arrows, Figure 3A and 3B). The percentage of LipidTOX-positive cells was quantified for each time point. Consistent with previous results, we did not observe foam cell formation when flt-3 bone marrow–derived DCs were incubated with agLDL (Figure 3C–3E). The number of GM-CSF bone marrow–derived DCs in contact with aggregates that were LipidTOX-positive increased as a function of agLDL incubation time indicating increased levels of intracellular neutral lipid (Figure 3E). This shows that in vitro, GM-CSF bone marrow–derived DCs use exophagy to degrade agLDL and become foam cells.

Foam cell formation is accompanied by increases in cellular free cholesterol. To assess the effects of increasing cellular cholesterol on exophagy, we raised cellular free cholesterol levels and examined local actin polymerization and lysosome exocytosis to the compartment. To increase cellular free cholesterol, GM-CSF bone marrow–derived DCs were treated with 5 mmol/L cholesterol:methyl-β-cyclodextrin for 15 minutes. Treated and resting DCs were then incubated with agLDL in the presence of an acyl-coenzyme A cholesterol acyltransferase inhibitor to maintain cellular free cholesterol levels (ie, prevent esterification of cholesterol) for the duration of the experiment. Under the conditions used in these experiments, the cellular free cholesterol level in treated cells was increased 1.7-fold over resting cells as determined by analysis of cellular lipid extracts using gas chromatography–mass spectrometry. The cellular free cholesterol was visualized using filipin staining, which revealed an increase in free cholesterol in cholesterol-methyl-β-cyclodextrin–treated DCs (Figure 3F and 3G) compared with resting DCs (Figure 3H and 3I). Both compartment formation, quantified by local F-actin–rich structures (Figure 3J), and lysosome exocytosis (Figure 3K) were unaffected by a substantial increase in cellular free cholesterol levels.

**DC Maturation Results in an Upregulation of Exophagy**

It is well established that downregulation of endocytosis, including macropinocytosis, phagocytosis, and effrocytosis, occurs with DC maturation.\(^7,8\) Thus, we wondered whether exophagy would also be downregulated on DC maturation. To this end, the ability of GM-CSF bone marrow–derived iDCs and mDCs to form a lysosomal synapse and perform exophagy-mediated catabolism of agLDL was compared. mDCs were generated on day 10 of GM-CSF culture by overnight treatment with lipopolysaccharide and examined on day 11. First, the formation of F-actin in iDCs and
mDCs near sites of contact with agLDL was examined. Both iDCs and mDCs were incubated with Alexa546-agLDL for 60 minutes followed by fixation and labeling of F-actin with Alexa488-phalloidin. After 60 minutes, an enrichment of F-actin was detected near the sites of contact with agLDL in both iDCs (Figure 4A and 4B) and mDCs (Figure 4C and 4D). Surprisingly, the local F-actin in mDCs was significantly more intense than that observed in iDCs. The iDC culture represents a mixed population of cells. The main contaminants are macrophages and B cells. However, macrophages adhere strongly to the culture dish and are not resuspended with the DCs when they are plated for experiments, whereas B cells are nonadherent and do not stick to our microscopy dishes.

Zbtb46 classical DCs are not only a minor contaminant in this culture system, but they are also nonadherent. To ensure that contaminant cells were not affecting our F-actin measurements, we isolated CD11c+ iDCs from the culture using flow cytometry. No quantitative difference was seen in the amount of local F-actin polymerization between the unsorted culture and the isolated CD11c+ iDC cells (Figure IIA in the online-only Data Supplement), indicating that the cells used for microscopy experiments represent a homogenous population of CD11c+ iDC.

Quantification of the average local F-actin intensity per cell revealed a 60% increase in mature murine DCs compared with immature murine DCs (Figure 4E). Furthermore, we...
examined the formation of the lysosomal synapse in immature and mature huMDDCs and found a similar increase in local F-actin–rich structures used for compartment formation in mDCs compared with iDCs (Figure 4E). We also examined the formation of local F-actin–rich structures in flt-3 bone marrow–derived DCs treated with lipopolysaccharide to induce maturation. No compartment formation at sites of contact with the aggregate was observed in these mature flt-3 bone marrow–derived DCs (data not shown).

It has been shown that mDC contain higher basal levels of F-actin than iDC,47 which might partially account for the increased F-actin at sites of contact with agLDL. Thus, we next quantified another aspect of exophagy, the amount of lysosome exocytosis to the lysosomal synapse, in iDCs and mDCs. Biotin–fluorescein–dextran was delivered to DC lysosomes via overnight incubation.42 Cells were then exposed to streptavidin-Alexa546-labeled agLDL for 90 minutes, fixed, and permeabilized. Lysosome exocytosis was observed to aggregate-containing compartments in both GM-CSF BM iDCs (F–H) and mDCs (I–K). L, Quantification of the amount of biotin–fluorescein–dextran exocytosed in both murine and human monocyte-derived iDC and mDC. M, GM-CSF BM iDCs, mDCs, and J774 macrophage-like cells were incubated with CypHer 5E, a pH sensitive fluorophore, and Alexa488, a pH insensitive fluorophore, dual-labeled agLDL, and the pH surrounding the aggregate was measured. Quantification of the lowest pH achieved in the compartment at a single time point in iDCs, mDCs, and J774 macrophage-like cells. The central mark on each box is the median, whereas the edges of the box represent the 25th and 75th percentiles. Error bars (E and L) represent the SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 Student t test. Data are pooled from 3 independent experiments.
unsorted and CD11c+ cells (Figure IIB in the online-only Data Supplement).

We confirmed that our DCs behaved as expected in regard to the activity of other maturation and endocytic mechanisms. Consistent with previous studies, major histocompatibility complex II (MHC II) and CD86 were upregulated, and phagocytosis and macrophagocytosis were downregulated on DC maturation (Figure III in the online-only Data Supplement). Thus, despite the fact that macrophagocytosis delivered significantly more biotin–fluorescein–dextran to the lysosomes of iDCs, less exocytosed fluorescein was detected during exophagy.

We also examined the amount of lysosome exocytosis in immature and mature huMDDCs and again found that mDCs exhibited more lysosome exocytosis to agLDL-containing compartments than iDCs (Figure 4L). These data indicate a significant upregulation of exophagy, presumably indicative of greater agLDL catabolism, with DC maturation. Consistent with previous results, no lysosome exocytosis was observed in areas of contact with the aggregate in mature flt-3 bone marrow–derived DCs (data not shown).

We expected that greater actin polymerization and lysosome exocytosis might result in enhanced acidification of the lysosomal synapse in mDCs. We made live cell pH measurements of lysosomal synapses in iDCs and mDCs containing agLDL labeled with pH-sensitive and pH-insensitive fluorophores to determine the lowest pH achieved in the compartment after a 30-minute agLDL incubation. We observed no significant differences in pH values obtained from lysosomal synapses in iDCs and mDCs (Figure 4M). Furthermore, compartments used for exophagy by DCs were less acidic than those used by J774 macrophage-like cells (Figure 4M). This observation parallels previous work which found that DC phagosomes are less acidic than those of macrophages.48 In spite of this, we did observe pH values in the lysosomal synapse as low as 5.5 in DCs (Figure 4M), indicating that sufficient acidification takes place to allow lysosomal enzymes, such as lysosomal acid lipase, to be active.49

**Figure 5.** Mature dendritic cell (DC) accumulates more free cholesterol than immature DC on incubation with aggregated low-density lipoprotein. Immature and mature DCs were incubated with agLDL for 0, 30, or 90 minutes. Lipids were extracted, and free cholesterol was determined by gas chromatography–mass spectrometry (GC-MS) analysis. Values were normalized for extraction using β-sitosterol and for protein content. No significant difference was seen in the free cholesterol content of immature DC (iDC) and mature DC (mDC) at 30 minutes (data not shown). Error bars represent the SEM. *P ≤ 0.05 Student t-test. Data pooled from 3 independent experiments.

**AgLDL Incubation Causes Mature GM-CSF Bone Marrow–Derived DCs to Accumulate More Cellular Free Cholesterol Than Immature GM-CSF Bone Marrow–Derived DCs**

We examined the consequences of more robust lysosomal synapse formation on DC cholesterol internalization. Immature and mature nonclassical DCs were incubated with agLDL for 0, 30, and 90 minutes in the presence of an acyl-Coenzyme A cholesterol acyltransferase inhibitor to prevent reesterification of internalized cholesterol. At the indicated time points, cellular lipids were extracted, and amounts of free and total cholesterol were determined by gas chromatography–mass spectrometry. After a 90-minute incubation with agLDL, mDCs internalized more free cholesterol than iDCs (Figure 5). No significant difference was seen in the free cholesterol content of iDC and mDC after 30 minutes. These data are consistent with the results presented in Figure 4, indicating that mDCs form larger F-actin–dependent extracellular compartments and exhibit increased lysosome exocytosis to these compartments than iDCs. Taken together, these data suggest that exophagy is upregulated in mDCs compared with iDCs. This upregulation contrasts with various forms of endosomal internalization in DCs, such as macrophagocytosis and phagocytosis, which are dampened with maturation.37,38

**Monocyte-Derived mDCs Isolated From Murine Atherosclerotic Plaques Accumulate More Lipid Than iDCs**

To examine the capacity of different DC subsets to accumulate lipid and form foam cells in vivo, we isolated DCs from the aorta of 5 ApoE−/− mice on a high-fat diet for 12 weeks. Cell suspensions were prepared from the aortic root (valves and aortic sinus) and both ascending and descending aorta of the mice. The aorta was dissociated with an enzyme mixture and then stained with monoclonal antibodies, for dissecting DC subsets, and LipidTOX-Red, for quantifying neutral lipid content. The Steinman laboratory has previously used a similar approach to examine aortic leukocyte populations.11 Although CD11c is commonly accepted as a pan-DC marker, a clear identification of DCs is still limited by the lack of unambiguous surface markers. In particular, the discrimination of DCs from macrophages that share many markers and functions remains challenging. The findings by the Steinman laboratory and others indicate that some macrophage foam cells can express CD11c.11,55 Thus, to avoid including macrophages in our analysis, cells that were CD11b+ were excluded whether they were CD11c+ or CD11c−. However, we note that the CD11b expression is somewhat variable on both DCs and macrophages, and perfect discrimination of these cell types is difficult.
Consistent with the studies of the Steinman laboratory, we observed a minor fraction of CD11c+ and MHC II hi cells in the aorta with low to intermediate levels of CD11b. These cells have previously been shown to possess immune-stimulating and cross-presentation capacities similar to DCs from lymphoid tissues.51 CD11c+ and MHC II hi cells were further subdivided into mDCs and iDCs based on quantitative analysis of MHC II expression. Representative MHC II hi CD11c+ CD11b low, predominantly monocyte-derived mDC, and MHC II int CD11c+ CD11b low, predominantly monocyte-derived iDC, are shown in Figure 6A–6E. The mDC, highlighted with an arrow in Figure 6E, is positive for neutral lipid staining indicative of foam cell formation. A CD11b hi cell, possibly a macrophage, that was excluded from analysis can also be seen (Figure 6E).

We also examined the neutral lipid content of monocyte-independent DCs isolated from the plaque of ApoE−/− mice on a high-fat diet for 12 weeks. Monocyte-independent DCs were defined by their expression of CD103, a ligand for E-cadherin expressed by most epithelial cells and also a marker for CD11b− DCs in many tissues.52–56 Most CD11b F4/80− DCs express CD103 in normal murine aorta, and this molecule has not been found on any other population of aortic leukocytes.11 Examples of CD103+ Cd11c+ cells are shown in Figure 6F–6I.

Consistent with the studies of the Steinman laboratory, we observed a minor fraction of CD11c+ and MHC II hi cells in the aorta with low to intermediate levels of CD11b. These cells have previously been shown to possess immune-stimulating and cross-presentation capacities similar to DCs from lymphoid tissues.51 CD11c+ and MHC II hi cells were further subdivided into mDCs and iDCs based on quantitative analysis of MHC II expression. Representative MHC II hi CD11c+ CD11b low, predominantly monocyte-derived mDC, and MHC II int CD11c+ CD11b low, predominantly monocyte-derived iDC, are shown in Figure 6A–6E. The mDC, highlighted with an arrow in Figure 6E, is positive for neutral lipid staining indicative of foam cell formation. A CD11b hi cell, possibly a macrophage, that was excluded from analysis can also be seen (Figure 6E).

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The LipidTOX signal per cell was quantified for each DC subset (Figure 6J). MHC II hi CD11c+ CD11b low DCs contained more neutral lipid, indicative of foam cell formation, than MHC II int CD11c+ CD11b low DCs. CD103+ CD11c− monocyte-independent DCs contained scant amounts of neutral lipid compared with monocyte-derived DCs. These results demonstrate that our cell culture findings examining exophagy-mediated degradation of agLDL mirror in vivo patterns of DC subset neutral lipid accumulation.

**Discussion**

It has long been recognized that osteoclasts create extracellular lysosome-like compartments that play an essential role in bone remodeling.57,58 We have recently shown that a similar extracellular lysosomal hydrolysis process, termed exophagy, can be used by macrophages to clear large aggregates of LDL.36,39 Thus, we wondered whether DCs could also perform exophagy. Based on emerging evidence that DCs contribute to atherosclerotic
foam cell formation, we investigated whether DCs, both flt-3 bone marrow–derived (monocyte-independent, classical) and GM-CSF bone marrow–derived (monocyte-derived, nonclassical), could perform exophagy of aggregated lipoproteins.

Our studies elucidate the mechanism of a novel pathway for catabolism of agLDL by DCs and demonstrate that, in addition to macrophages and osteoclasts, monocyte-derived DCs are capable of exophagic degradation. When GM-CSF bone marrow–derived DCs were incubated with agLDL, the formation of extracellular aggregate-containing compartments was observed. Lysosomal contents were delivered to these compartments, and they were acidified, thereby enabling activation of lysosomal hydrolases. Together, these results demonstrate that during monocyte-derived DC engagement of agLDL, exophagic cholesteryl ester hydrolysis occurs as an initial step in foam cell formation. Conversely, flt-3 bone marrow–derived DCs did not exhibit exophagic degradation of agLDL and consequently were unable to internalize the aggregate and did not become foam cells.

While revising our article, heterogeneity in the GM-CSF mouse bone marrow culture system used in this study was reported. Although this culture system has been widely used to generate CD11c+MHCII+ cells, previously considered a highly pure population of DCs, it was recently demonstrated that on day 6 of culture it is comprised of a small number of bona fide DCs, in addition to monocyte-derived macrophages and uncharacterized cells. These distinctions were made on the basis of ontogenetic, morphological, and gene expression criteria. Culture conditions used to generate the mDC in this study were not examined using these criteria. Although future studies will be needed to evaluate exophagy in the DC population defined in this manner, many of the cells in the current study exhibit morphological features (eg, dendritic processes) characteristic of DCs (see Figures 1A and 2K and 2M), thus supporting the fact that monocyte-derived DCs can perform exophagy of agLDL.

We also investigated the efficiency of exophagy in iDC and mDC internalization of agLDL. In most tissues, DCs are present in an immature state. The forms of endocytosis, such as phagocytosis, macropinocytosis, and efferocytosis, are efficient in iDCs and serve as a means of antigen capture. In response to inflammatory stimuli, DCs trigger the process of maturation, a terminal differentiation program required to initiate T-cell responses. In maturation, DCs exhibit reduced antigen capture capacity (downregulation of phagocytosis and macropinocytosis) and increased surface expression of MHC II and costimulatory molecules. Thus, we were surprised to find that exophagy of agLDL was substantially more efficient in mDCs than iDCs. This finding may be particularly significant for atherogenesis as both human and animal studies have demonstrated the accumulation of mDC in atherosclerotic plaques with lesion progression. Our results indicate that mDC will take up more lipid than iDC and will rapidly become foam cells. This may exacerbate plaque progression as DCs under conditions of hyperlipidemia exhibit a reduced migratory ability and emigration with their lipid load may be impaired. However, the role of DC emigration during atherogenesis remains to be defined. Furthermore, mDCs are located mainly in rupture-prone areas of the plaque. Thus, exophagy-mediated catabolism of agLDL by mDC may directly contribute to plaque instability through the release of lysosomal enzymes that could destabilize the collagen cap. Indeed, significantly higher numbers of DCs reside in carotid plaques with characteristics of vulnerable lesions than in stable lesions. Thus, exophagic degradation of agLDL by DCs could play a role in plaque rupture and ensuing cardiac events.

mDCs serve 2 dominant functions, antigen presentation and migration to lymph nodes. It is possible that exophagy is increased in mDCs because it is related to the formation of the immunologic synapse or, as has recently been suggested, is necessary for matrix degradation to facilitate cell migration. Alternatively, it has been shown that during maturation, DCs use tubular lysosomes to deliver MHCII to the plasma membrane. Thus, it is possible that lysosome exocytosis occurs more readily in mDCs thereby enhancing exophagy. Our finding that exophagy is upregulated in mDC, taken together with recent data indicating that receptor-mediated endocytosis remains an efficient means of antigen internalization in mDC, indicating that the biology of DC maturation with regard to endocytosis is more complex than originally appreciated. Interestingly, several therapeutic interventions for atherosclerosis, such as aspirin and statins, have shown to maintain DCs in an immature state. Our results suggest that in addition to quelling the inflammatory cascade associated with atherosclerosis, these therapies may also directly modulate the efficacy of DC foam cell formation.

Examination of foam cell formation in plaque DC subsets isolated from a standard mouse model of atherosclerosis showed that in vivo monocyte-derived mDCs (MHC II+CD11c+CD11b+ nonclassical DCs) contain more neutral lipid than monocyte-derived iDCs (MHC II+CD11c+CD11b+), whereas monocyte-independent DCs (CD103+CD11c+) contained scant amounts of lipid. These findings mirror our cell culture results. Based on our findings, we propose that rapid cholesteryl ester hydrolysis and the transfer of free cholesterol directly from LDL aggregates to nonclassical DCs and macrophages may occur when these cells encounter subendothelial lipoproteins in developing atherosclerotic lesions. Furthermore, the data expand the types of monocyte-derived cells known to perform exophagy.

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Disclosures
None.

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**Significance**

We show that monocyte-derived dendritic cells can degrade aggregated low-density lipoprotein (the predominant form of low-density lipoprotein found in atherosclerotic plaques), whereas monocyte-independent dendritic cells cannot. Monocyte-derived dendritic cells use a specialized form of catabolism to clear the low-density lipoprotein aggregates in which the cells create an extracellular, acidic compartment into which they secrete lysosomal enzymes. The interaction results in foam cell formation and, surprisingly, is upregulated on dendritic cell maturation. This contrasts with various forms of endocytic internalization in dendritic cells, which decrease on maturation. This finding may be significant for atherogenesis as studies have shown accumulation of mature dendritic cells in rupture-prone areas of the plaque. Thus, extracellular catabolism of aggregated low-density lipoprotein by mature dendritic cells may contribute to plaque instability through the release of lysosomal enzymes that could destabilize the collagen cap.
Monocyte-Derived Dendritic Cells Upregulate Extracellular Catabolism of Aggregated Low-Density Lipoprotein on Maturation, Leading to Foam Cell Formation
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Flt-3L treated mice

WT mice

Figure I. Splenic cells from mice treated with flt-3 ligand show enrichment in flt-3 derived DCs. Flow cytometry analysis of surface expression of CD11c and IA/IE (MHCII) in (A) Mice treated with flt-3 ligand or (B) WT mice. CD11c^+ IA/IE^+ cells were gated and further analyzed for surface expression of CD11b and CD8 (C, D). Numbers given are percentages of cells contained within each gate.
Figure II. Cells used in microscopy experiments represent a homogenous population of CD11c⁺ iDCs. iDC generated via GM-CSF represent a heterogeneous population of cells. However, the main contaminating cells, macrophages and B cells, are not plated for microscopy experiments as macrophage adhere strongly to the culture dish and B cells do not adhere to the microscopy dishes. To confirm that a homogenous population of iDC were used for microscopy experiments, we isolated iDC with flow cytometry using Alexa647-CD11c. (A) Unsorted and CD11c⁺ cells were incubated with Alexa546-agLDL for 60 min, fixed and stained with Alexa488-phalloidin to show F-actin. No quantitative difference in the amount of local F-actin polymerization at sites of contact with the aggregate was seen between the unsorted culture and the isolated CD11c⁺ iDC cells. (B) Unsorted and CD11c⁺ cell lysosomes were loaded with biotin-fluorescein-dextran via overnight incubation. Cells were subsequently incubated with streptavidin and Alexa546 dual-labeled agLDL for 90 min, fixed and permeabilized. Colocalization of dextran with the aggregate indicates areas of exocytosis. No quantitative difference was seen in the amount of exocytosis to areas of contact with the aggregate between the unsorted culture and the isolated CD11c⁺ iDC cells.
Figure III. DCs upregulate MHC II and CD86 and down regulate phagocytosis and pinocytosis upon maturation. (A) Surface expression of MHC II and CD86, markers of DC maturation, were examined in iDC and mDC using flow cytometry. (B) Quantification of the phagocytosis of Fluoresbrite YG latex beads after 60 min in iDC and mDC at 37°C or 4°C to assess non-internalized surface bound beads. (C) The amount of biotin-fluorescein-dextran delivered to lysosomes via pinocytosis was quantified using confocal imaging in iDC and mDC. a.u. arbitrary units. MFI – Mean Fluorescence Intensity.
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<sup>-/-</sup> 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 previous method<sup>1</sup> by Lutz et al.<sup>2</sup>. 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, 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<sub>2</sub> 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<sup>+</sup> 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<sup>2</sup>, were used without sorting.

Human monocyte derived dendritic cells were prepared as described previously<sup>3</sup> 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<sub>4</sub>Cl 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<sub>2</sub> incubator.

Ft-3 DCs were prepared as described previously<sup>4</sup> with minor modifications. Bone marrow was isolated as described above and red blood cells lysed by resuspension in 0.168 M NH<sub>4</sub>Cl, followed by washing with PBS. Cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator at a density of 1.5 x 10<sup>6</sup> 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. Ft-3 classical DC were enriched by flt-3L treatment in vivo by 10-20 fold<sup>5</sup> 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<sup>6,7</sup>. 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 ft-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. LDL was labeled using succinimidyl esters of Alexa Fluor 546 (Alexa546) and Alexa 488 (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. Alexa 488-phalloidin, Alexa 488-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, NH4Cl), as well as biotin, streptavidin, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES), PFA, saponin, acyl-coenzyme A cholesterol acyltransferase (ACAT) inhibitor 58035, hyaluronidase, Collagenase I/XI, Collagenase D, betasitosterol, 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. 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™ 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% CO₂. 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) 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°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×
Lipid Analysis of DC Subsets Isolated from Murine Atherosclerotic Plaque. Aortic single cells were prepared in accordance with a previous method\textsuperscript{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 cytopun 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 histocompatibility 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


