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Receptor-independent fluid-phase pinocytosis mechanisms for induction of foam cell formation with native LDL particles

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Abstract

Purpose of review—Because early findings indicated that native low density lipoprotein (LDL) did not substantially increase macrophage cholesterol content during in vitro incubations, investigators presumed that LDL must be modified in some way to trigger its uptake by the macrophage. The purpose of this review is to discuss recent findings showing that native unmodified LDL can induce massive macrophage cholesterol accumulation mimicking macrophage foam cell formation that occurs within atherosclerotic plaques.

Recent findings—Macrophages that show high rates of fluid-phase pinocytosis also show similar high rates of uptake of native unmodified LDL through non-receptor mediated uptake within both macropinosomes and micropinosomes. Non-saturable fluid-phase uptake of LDL by macrophages converts the macrophages into foam cells. Different macrophage phenotypes demonstrate either constitutive fluid-phase pinocytosis or inducible fluid-phase pinocytosis. Fluid-phase pinocytosis has been demonstrated by macrophages within mouse atherosclerotic plaques indicating that this pathway contributes to plaque macrophage cholesterol accumulation.

Summary—Contrary to what has been believed previously, macrophages can take up large amounts of native unmodified LDL by receptor-independent, fluid-phase pinocytosis converting these macrophages into foam cells. Thus, targeting macrophage fluid-phase pinocytosis should be considered when investigating strategies to limit macrophage cholesterol accumulation in atherosclerotic plaques.

Keywords

LDL; macrophages; fluid-phase pinocytosis; cholesterol; macropinocytosis

Introduction

Macrophage accumulation of cholesterol that transforms the macrophages into so called foam cells within atherosclerotic plaques is considered to be a key pathologic event in the development of plaques. Macrophages may take up cholesterol that has accumulated in various physical forms within the extracellular spaces of plaques [1]. This cholesterol burden is comprised in part by lipoproteins such as LDL that enter the vessel wall from the

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blood and remain unbound. Unbound LDL levels in the intima, the innermost layer of the vessel wall where atherosclerotic plaques form, are twice that of plasma LDL levels [2, 3], a finding that is not widely appreciated, but which has important implications for studies of how macrophages accumulate cholesterol. Macrophage uptake of extracellular bound cholesterol may be necessary to initiate reverse cholesterol transport of this cholesterol out of the vessel wall. On the other hand, macrophage uptake of extracellular unbound LDL may directly contribute to vessel wall cholesterol accumulation by preventing the LDL from exiting the vessel wall through transport either back into the blood or into the vessel lymphatics. Although it is not clear to what extent macrophage uptake of cholesterol contributes to vessel wall cholesterol accumulation, macrophage cholesterol accumulation has pathologic consequences. Macrophage accumulation of cholesterol promotes macrophage release of metalloproteinases and expression of tissue factor, processes that can promote plaque rupture and subsequent plaque thrombosis, respectively [4–6].

Because macrophage foam cell formation could not be achieved in earlier studies through incubation of cultured macrophages with even high levels of native LDL, investigators concluded that LDL must undergo some type of modification that would promote macrophage binding and uptake of the modified LDL [7]. In this regard, the LDL receptor cannot explain macrophage foam cell formation because patients and animals that lack the LDL receptor nevertheless show foam cells in their atherosclerotic plaques, and macrophage LDL receptors appear to be down regulated in atherosclerotic plaques reflecting LDL receptor down-regulation in the presence of excess cholesterol [8]. Various enzymatic and non-enzymatic modifications to LDL or complexing of LDL with other potential macrophage ligands were shown to promote macrophage uptake of LDL producing various levels of macrophage cholesterol accumulation [9]. The most extensively studied foam cell formation hypothesis is that oxidative modification of LDL facilitates its recognition by macrophage scavenger receptors that mediate macrophage uptake of the oxidized LDL. However, certain findings challenge this hypothesis. LDL isolated from human aorta is not oxidized sufficiently to trigger macrophage uptake through scavenger receptors [10]; macrophage foam cells form in mice with genetic deletion of those scavenger receptors that mediate mouse macrophage uptake of oxidized LDL [11••]; and macrophage uptake of oxidized LDL produces mostly lysosomal rather than mostly lipid droplet accumulation of cholesterol [12–14], the latter a characteristic of most plaque macrophage foam cells.

Ironically, while Brown and Goldstein first suggested the modified LDL hypothesis of macrophage foam cell formation that led to subsequent decades of related research, these investigators also described non-receptor mediated, fluid-phase uptake of LDL by mutant fibroblasts that lacked the LDL receptor [15, 16]. Fluid-phase uptake by cells occurs when cells engulf extracellular fluid and any material such as a lipoprotein contained in that fluid. Thus, uptake of lipoprotein by fluid-phase pinocytosis (also called bulk-phase endocytosis) does not involve cell binding of the lipoprotein. Uptake of the lipoprotein is linearly related to its concentration and does not show saturation of uptake that is characteristic of receptor-mediated uptake processes (Figure 1). Also, in contrast to receptor-mediated uptake processes, fluid-phase mediated uptake of labeled lipoprotein is not competed by excess unlabeled lipoprotein. These characteristics are summarized in Table 1.

Fluid-phase pinocytosis can occur by either micropinocytosis in small micropinosome vesicles less than 0.1 μm, or macropinocytosis in large macropinosome vacuoles typically greater than 0.2 μm (Figure 2). Because of the large size of the forming macropinosomes, this uptake mechanism can be observed by phase-contrast light microscopy (see supplemental online material, Figure S1). Macropinocytosis is an actin-dependent endocytic pathway carried out by some cell types including macrophages in which ruffling plasma membranes fuse to enclose fluid and contained solute within the macropinosome vacuoles. Macropinocytosis is thus different from actin-dependent phagocytosis triggered by plasma membrane binding of large particles that are then engulfed by cells within phagocytic vacuoles. Phagocytic vacuoles are relatively free of fluid because of the tight apposition of the engulfed particle with the cell's plasma membrane. The characteristics of micropinocytosis and macropinocytosis are summarized in Table 2.

Brown and Goldstein showed that human skin fibroblasts take up LDL both through the LDL receptor and through fluid-phase pinocytosis [15, 16]. In fibroblasts, LDL taken up through the LDL receptor led to cellular cholesterol accumulation, while LDL taken up by fluid-phase pinocytosis was likewise degraded by lysosomes, but its cholesterol was excreted rather than being retained by the fibroblast. Other early studies showed non-saturable uptake of LDL that did not stimulate cholesteryl ester accumulation in unactivated mouse peritoneal macrophages and unactivated human LDL receptor-negative, monocyte-derived macrophages differentiated with human serum [19, 20]. While most of the non-saturable LDL uptake could be attributed to fluid-phase pinocytosis [15, 21], it is likely that LDL uptake in these studies was mediated by fluid-phase micropinocytosis rather than macropinocytosis because macropinocytosis does not occur in these cells without some form of activation. J774 mouse macrophages show cholesterol esterification induced by native LDL taken up through both LDL receptor and non-LDL receptor-mediated mechanisms. Some of the LDL uptake possibly occurred by fluid-phase pinocytosis in this macrophage cell line [22].

Because fluid-phase pinocytosis of LDL in these early studies did not result in substantial cellular cholesterol accumulation, for decades there was little subsequent attention paid to fluid-phase pinocytosis as a mechanism for macrophage foam cell formation. This review will discuss recent studies that show fluid-phase pinocytosis is an important mechanism for cellular cholesterol accumulation, especially for macrophages, a cell type that can show extremely high levels of fluid-phase pinocytosis of LDL converting these cells into foam cells.

Macrophages take up large quantities of native LDL by fluid-phase pinocytosis

The long delay in the recognition of fluid-phase pinocytosis as an important mechanism for macrophage cholesterol accumulation also can be explained by the fact that most studies of macrophage LDL uptake have been carried out with rather low concentrations of LDL, typically 50 ug protein/ml or less, reflecting the concentrations that saturate receptors for uptake of native or modified LDL. As mentioned above, much higher levels of LDL that can exceed 2 mg/ml exist in the vessel intima [2, 3]. Fluid-phase pinocytosis shows substantial

levels of LDL uptake compared to receptor-mediated uptake only at these higher LDL levels. Secondly, cultured macrophages show phenotype heterogeneity depending on the macrophage source including tissue of origin (e.g., blood monocytes, bone marrow, peritoneum, and spleen), species of origin, and culture conditions especially with respect to differentiation factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF)[23, 24]. Not all macrophages necessarily show constitutive high levels of fluid-phase pinocytosis, but activation of this process can occur in these macrophages similar to the activation of macropinocytosis in some non-macrophage cells following stimulation with various growth factors [25, 26].

For example, human monocytes differentiated in fetal bovine serum with added GM-CSF or with human serum without added GM-CSF generate macrophages with a rounded “fried-egg” appearing morphology (i.e., GM-CSF phenotype), and show low levels of fluid-pinocytosis unless activated with phorbol 12-myristate 13-acetate (PMA) or other activators of protein kinase C [27••, 28•]. On the other hand, mouse bone marrow-derived macrophages differentiated with GM-CSF show high levels of fluid-phase pinocytosis of LDL that is constitutive not requiring macrophage activation [29]. PMA-activation of human of macrophages having the GM-CSF phenotype increases fluid-phase pinocytosis to levels about 5 times that shown by unactivated macrophages [27••, 28•]. Incubation of these PMA-activated macrophages with native LDL results in a non-saturable, concentration-dependent uptake and lysosomal degradation of LDL that transforms the macrophages into foam cells because of the massive amount of cholesterol that accumulates due to the stimulated LDL uptake [24, 27••, 28•]. Protein kinase C isoforms delta and beta each mediate about half the cholesterol accumulation suggesting that macropinocytosis may function through multiple signaling pathways [30].

Human monocytes and mouse bone marrow-derived macrophages differentiated with fetal bovine serum and added M-CSF generate elongated macrophages (i.e., M-CSF phenotype) that show high levels of constitutive fluid-phase pinocytosis. As a result, these macrophages show constitutive fluid-phase uptake of LDL and cholesterol accumulation transforming these macrophages into foam cells [31••](Barthwal, M, Anzinger, JJ, Kruth, HS, unpublished data). Consistent with the LDL uptake occurring through fluid-phase rather than a binding mechanism in M-CSF and GM-CSF macrophage phenotypes, ^{125}I- LDL uptake shows a non-saturable concentration-dependent uptake, ^{125}I- LDL uptake cannot be competed by excess unlabeled LDL, and ^{125}I- LDL uptake can be completely accounted for by the level of fluid-phase pinocytosis determined with a fluid-phase tracer [27••, 31••]. In the case of the M-CSF macrophage phenotype, micropinocytosis and macropinocytosis each contribute to about one-half the LDL uptake and cholesterol accumulation [18•]. This could be shown with the use of bafilomycin and SU6656 that inhibit respectively, micropinocytosis and macropinocytosis in these cells. In contrast, in the case of the GM-CSF macrophage phenotype, macropinocytosis mediates most of the fluid-phase pinocytosis [28•, 29].

Interestingly, how cholesterol is delivered to macrophages, either through receptor-mediated compared with fluid-phase pinocytosis, has been shown to influence the mechanism of efflux of cholesterol from macrophages [32••]. When cholesterol is delivered to mouse

bone-marrow derived macrophages by scavenger receptor-mediated uptake of acetylated LDL, subsequent cholesterol efflux occurs predominantly through an apo (apolipoprotein)A-I/ ATP-binding cassette transporter A1(ABCA1) mediated efflux mechanism. In contrast, macrophage uptake of LDL cholesterol by fluid-phase pinocytosis results in cholesterol efflux that is predominantly independent of apoA-I/ABCA1 and likely due to diffusional cholesterol efflux mechanisms.

Regulation of fluid-phase pinocytosis

Recent findings show that liver X receptors (LXRs), which bind oxysterols, regulate fluid-phase uptake of LDL by human M-CSF differentiated monocyte-derived macrophages [33•]. When monocytes are differentiated into macrophages in the presence of the LXR agonists T0901317 or 22(R)-hydroxycholesterol, both fluid-phase uptake of LDL and cholesterol accumulation by the M-CSF differentiated macrophages are decreased by greater than 50%. The LXR-agonist decrease in the uptake of LDL is completely accounted for by a similar decrease in the uptake of the fluid-phase tracer albumin. The LXR-agonist treatment effect is specific for fluid-phase pinocytosis because the treatment has no effect on macrophage receptor-mediated uptake of acetylated LDL. Although it was not quantitatively determined to what degree LXR-agonist treatment downregulated fluid-phase micropinocytosis compared with fluid-phase macropinocytosis, LXR-agonist treatment substantially decreases macropinocytosis assessed by time-lapse phase microscopy (Anzinger JJ, Kruth HS, unpublished data).

While it remains to be determined by what mechanism LXRs regulate fluid-phase pinocytosis in macrophages, the LXR up-regulated gene, ABCA1, has been shown to affect fluid-phase pinocytosis in other cell types. Fluid-phase pinocytosis is increased in cultured Tangier disease fibroblasts [34], which are genetically deficient in ABCA1, a protein best known for its function in cellular cholesterol export. In contrast, increased expression of ABCA1 in cultured MDCK2 epithelial cells causes reduced fluid-phase pinocytosis [35]. Thus, the anti-atherogenic properties of LXR agonists may be not only due to their well known stimulation of macrophage cholesterol efflux, but also be contributed to by an LXR agonist decrease in macrophage fluid-phase uptake of LDL. In contrast to LXRs that decrease fluid-phase pinocytosis, overexpression of another oxysterol-binding protein, oxysterol-binding protein-related protein 2 (ORP2), in HeLa epithelial cells causes an increase in fluid-phase pinocytosis [36]. The increased fluid-phase pinocytosis may mediate some of the increased LDL uptake observed in ORP2 overexpressing HeLa cells.

While fluid-phase pinocytosis can be constitutive in M-CSF phenotype macrophages and dendritic cells [31••, 37], fluid-phase pinocytosis can be induced in other macrophage phenotypes. Besides PMA activation of macropinocytosis in certain types of macrophages, interestingly, modified forms of LDL such as acetylated LDL and oxidized LDL have been shown to trigger macropinocytosis and fluid-phase pinocytosis in pigeon monocyte-derived macrophages, THP-1 human macrophages, resident mouse peritoneal macrophages, and J774 mouse macrophages [38••, 39••]. Induction of fluid-phase pinocytosis in mouse peritoneal macrophages by minimally oxidized 15-lipoxygenase-treated LDL is mediated by toll-like receptor 4 and spleen tyrosine kinase [39••, 40], similar to toll-like receptor ligand-

induced fluid-phase pinocytosis in mouse bone marrow- and spleen-derived dendritic cells [41]. Fluid-phase pinocytosis can be stimulated by 15-lipoxygenase treated cholesteryl arachidonate suggesting that cholesteryl ester hydroperoxides likely mediate the minimally oxidized LDL triggering of fluid-phase pinocytosis. However, some caution must be taken in the interpretation of the signaling studies reported in [39••–41] because FITC-dextran was used to monitor fluid-phase uptake, and as discussed below, dextran also can be internalized through receptors. Acetylated LDL and minimally oxidized LDL both stimulate macrophage uptake of co-incubated native LDL, and it was suggested but not demonstrated that fluid-phase pinocytosis rather than LDL bound to macrophages mediated the native LDL uptake [39••, 42•]. Lastly, many viruses have been shown to trigger macropinocytosis (reviewed in [43]), and thus, viruses could be another trigger for fluid-phase pinocytosis of LDL and foam cell formation that should be examined in future research.

Fluid-phase pinocytosis due to macropinocytosis is phosphoinositide (PI) 3-kinase dependent [44]. We have recently shown that PI3-kinase gamma mediates fluid-phase macropinocytosis of LDL by murine bone marrow-derived macrophages differentiated with GM-CSF [29]. This finding helps explain why genetic deletion or pharmacologic inhibition of PI3-kinase gamma substantially decreases development of atherosclerotic plaques in mice [45].

Fluid-phase pinocytosis occurs within atherosclerotic plaques and other tissues

LDL receptor-independent uptake of LDL by tissues occurs in all species examined and ranges between 22–28% of total LDL uptake in animals, but is higher in humans reaching 42% of total tissue uptake of LDL [46]. Approximately 70% of this LDL receptor-independent LDL uptake occurs in extra-hepatic tissues. The observation that LDL receptor-independent uptake of LDL in vivo shows linear dependence on plasma LDL concentration is consistent with this process being mediated by fluid-phase pinocytosis. In this regard, it has been shown that the levels of fluid-phase pinocytosis in mouse spleen and liver account for the levels of LDL receptor-independent uptake of LDL in these organs [47••]. In mutant NPC1 mice, which show a defect in cholesterol trafficking that causes lysosomal unesterified cholesterol accumulation, fluid-phase pinocytosis contributes to increased cholesterol accumulation in extrahepatic organs. These organs show infiltration with macrophage foam cells suggesting that macrophages fluid-phase pinocytosis contributes to the increased fluid-phase pinocytosis of LDL in the organs [47••]. Interestingly, enrichment of mouse peritoneal macrophages with unesterified cholesterol stimulates increased M-CSF secretion [48••], an inducer of macropinocytosis in some mouse macrophage phenotypes. Thus, a possible amplification mechanism may exist for macrophage foam cells to induce other macrophages to macropinocytose lipoproteins.

M-CSF administered in vivo decreases plasma cholesterol levels in non-human primates, humans, and rabbits including WHHL rabbits that lack the LDL receptor [49–51]. On the other hand, both hypercholesterolemic apoE- and LDL receptor-deficient mice show a further increase in plasma cholesterol when these mice are additionally made genetically deficient in M-CSF [52, 53]. Studies show that the cholesterol lowering effect of M-CSF

treatment is due in part to M-CSF stimulation of LDL clearance by an LDL receptor-independent mechanism [49, 50]. Thus, M-CSF stimulation of macrophage number and macrophage fluid-phase pinocytosis of LDL could be how M-CSF regulates plasma cholesterol levels.

Recently, it has been shown that macrophages in mouse atherosclerotic lesions carry out fluid-phase pinocytosis [17••]. This was demonstrated with the use of fluorescent pegylated nanoparticles (similar in size to LDL) that do not bind to cells, but are taken up by cultured macrophages through fluid-phase pinocytosis. Thus, the pegylated nanoparticles are useful in vivo tracers of LDL fluid-phase pinocytosis. The pegylated nanoparticles injected into apoE-deficient mice accumulated rather selectively within the macrophage foam cells of atherosclerotic lesions. This demonstrates that foam cells within atherosclerotic lesions show active fluid-phase pinocytosis and this can be a mechanism for uptake of lipoproteins such as LDL within plaques.

Considerations when measuring fluid-phase pinocytosis

The ideal probe for quantifying fluid-phase pinocytosis should reflect uptake exclusively in the fluid-phase and thus, the chosen probe should not bind to cells or extracellular matrix. If it is desired to measure total fluid-phase pinocytosis, the probe should be of small enough size to enter cells by both micropinocytosis and macropinocytosis. Also, once taken up by the cell, the probe should not be excreted by the cell during the period of probe uptake. Of course it must be possible to quantify the cell accumulated probe. Unfortunately, few probes satisfy all of these requirements. Commonly used probes such as radiolabeled sucrose or polyvinylpyrrolidone, the fluorescent dye lucifer yellow, and fluorescently labeled dextrans typically underestimate fluid-phase pinocytosis due to their concurrent cellular excretion during cellular uptake [54]. ^{125}I-labeled albumin is an especially useful fluid-phase tracer for studies of LDL uptake because, similar to studies of ^{125}I- LDL uptake, cellular metabolism of albumin provides a means to account for all cellular uptake including retained albumin and excreted albumin degradation products [15, 31••, 33•].

Another commonly used probe type, fluorescently labeled dextrans, potentially bind to pattern recognition receptors and this should be checked before their use as fluid-phase probes [25, 37, 55–57]. Thus, it is imperative to check concentration-dependent linearity of probe uptake before determining fluid-phase pinocytosis in the cell type under investigation. Another problem with the application of fluorescently labeled dextrans relates to the assumption by many investigators that all sized-dextrans selectively measure uptake by macropinocytosis rather than micropinocytosis. 150 kilodalton dextran has been shown to selectively label macropinosomes in mouse bone-marrow derived macrophages, while smaller sized-dextrans label micropinosomes in addition to macropinosomes [44, 58].

Conclusion

Fluid-phase pinocytosis is a mechanism that can contribute to receptor-independent uptake of LDL leading to macrophage foam cell formation. Macrophage LDL uptake by fluid-phase pinocytosis is linearly related to LDL concentration consistent with the risk of developing

cardiovascular disease also being linearly related to LDL levels. To the extent that it is desirable to inhibit macrophage accumulation of cholesterol in atherosclerotic plaques, it will be necessary to pharmacologically target fluid-phase pinocytosis in plaques. Macrophage fluid-phase macropinocytosis of LDL depends on Rho GTPase and PI3-kinase function [18•, 28•, 29, 59] suggesting that these are useful signaling molecules to target for inhibition. On the other hand, stimulating fluid-phase pinocytosis of LDL in organs such as the spleen and liver potentially provides a new approach to lowering plasma cholesterol levels. In any case, recent research has shown clearly that fluid-phase pinocytosis of LDL should be considered in any attempt to further investigate the decades-old research problem of macrophage foam cell formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LDL	low density lipoprotein
GM-CSF	granulocyte macrophage-colony stimulating factor
M-CSF	macrophage-colony stimulating factor
PMA	phorbol 12-myristate 13-acetate
apo	apolipoprotein
ABCA1	ATP-binding cassette transporter A1
LXR	liver X receptors
ORP2	oxysterol-binding protein-related protein 2
PI	phosphoinositide

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Key Points

- Uptake of LDL by receptor-independent, fluid-phase pinocytosis produces massive storage of LDL-derived cholesterol in macrophages.
- Fluid-phase pinocytosis occurs within atherosclerotic lesions.
- PI-3 kinase, RhoGTPase, and LXR transcription factor regulate fluid-phase pinocytosis of LDL.
- Fluid-phase pinocytosis of LDL is a novel mechanism of macrophage cholesterol accumulation demonstrating that neither modification of LDL nor receptors are necessary for macrophage foam cell formation.
- Macrophage fluid-phase pinocytosis of LDL is a relevant pathway to target for modulating macrophage cholesterol accumulation in atherosclerosis.

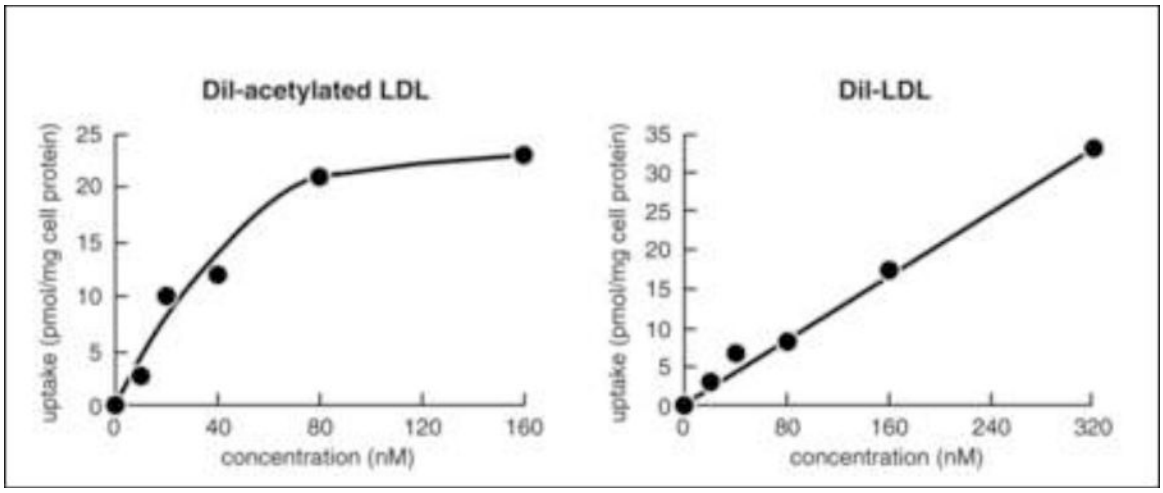

Figure 1. Comparison of receptor-mediated uptake of acetylated LDL and fluid-phase-mediated uptake of LDL

M-CSF-differentiated human monocyte-derived macrophages were incubated with increasing concentrations of either fluorescent DiI-labeled acetylated LDL, which binds the scavenger receptor, or DiI-LDL for 5 hours. Then, macrophage uptake of the fluorescent lipoproteins was determined. DiI-acetylated LDL uptake shows saturation at a lipoprotein concentration of 80 nM (i.e., 44 ug/ml) consistent with receptor-mediated endocytosis, while DiI-LDL uptake does not show saturation, consistent with fluid-phase pinocytosis. Data adapted from [17••].

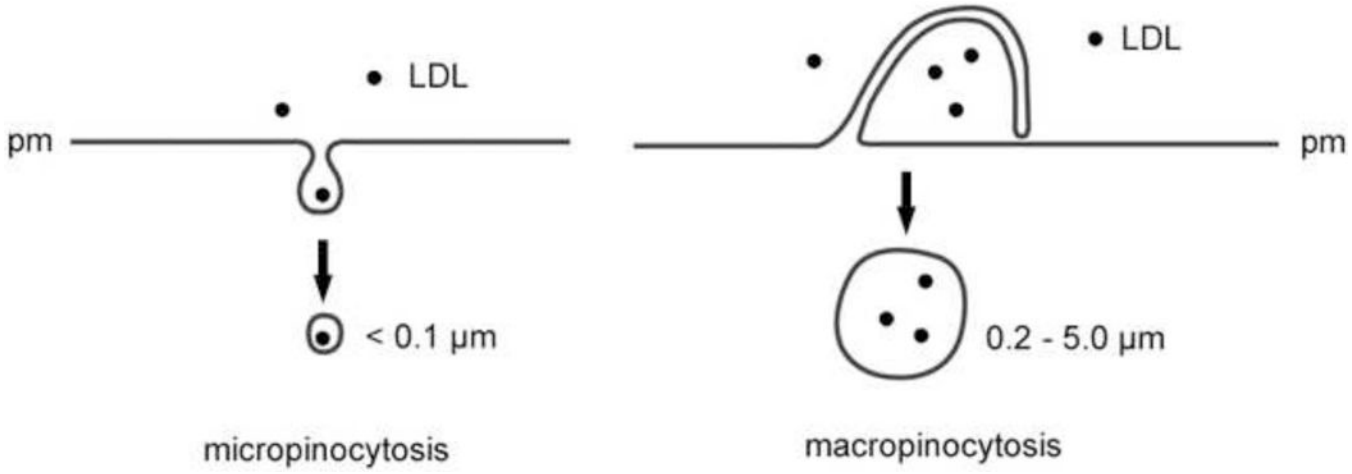

Figure 2. Micropinocytosis and macropinocytosis mediate fluid-phase pinocytosis of LDL

Micropinocytosis includes uptake of fluid into small vesicles by clathrin-mediated, caveolae-mediated, and clathrin and caveolae-independent pinocytosis. Micropinocytosis may be actin-dependent or independent. Macropinocytosis is an actin-dependent pinocytic pathway by which macrophages can engulf droplets of extracellular fluid within large vacuoles formed by fusion of a plasma membrane extension with non-extended plasma membrane (pm) as shown.

Table 1

Comparison of fluid-phase micropinocytosis and macropinocytosis

Characteristic	Micropinocytosis	Macropinocytosis
Size of pinosome	< 0.1 μm	0.2 – 5.0 μm
Multiple pathways	yes	yes
Actin-dependent	[1]some pathways	all pathways
Constitutive	usually	in some cell types
Signaling mediators	vary with cell type	vary with cell type

[1] usually at a very low level in most cells and at a high level in the M-CSF phenotype macrophages

Table 2

Comparison of fluid-phase and receptor-mediated endocytosis of LDL

Characteristic	Fluid-phase	Receptor-mediated
LDL binds to cell	no	yes
LDL uptake shows saturation	no	yes
Uptake of ^{125}I-LDL can be competed by unlabeled LDL	no	yes
Uptake of ^{125}I-LDL and ^{125}I-albumin are similar[1]	yes	no

[1] when added at the same protein concentration