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Fluid-Phase Pinocytosis of LDL by Macrophages: A Novel Target to Reduce Macrophage Cholesterol Accumulation in Atherosclerotic Lesions

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Abstract

Circulating low-density lipoprotein (LDL) that enters the blood vessel wall is the main source of cholesterol that accumulates within atherosclerotic plaques. Much of the deposited cholesterol accumulates within plaque macrophages converting these macrophages into cholesterol-rich foamy looking cells. Cholesterol accumulation in macrophages contributes to cholesterol retention within the vessel wall, and promotes vessel wall inflammation and thrombogenicity. Thus, how macrophages accumulate cholesterol and become foam cells has been the subject of intense investigation. It is generally believed that macrophages accumulate cholesterol only through scavenger receptor-mediated uptake of modified LDL. However, an alternative mechanism for macrophage foam cell formation that does not depend on LDL modification or macrophage receptors has been elucidated. By this alternative mechanism, macrophages show receptor-independent uptake of unmodified native LDL that is mediated by fluid-phase pinocytosis. In receptor-independent, fluid-phase pinocytosis, macrophages take up LDL as part of the fluid that they ingest during micropinocytosis within small vesicles called micropinosomes, and by macropinocytosis within larger vacuoles called macropinosomes. This produces cholesterol accumulation in macrophages to levels characteristic of macrophage foam cells in atherosclerotic plaques. Fluid-phase pinocytosis of LDL is a plausible mechanism that can explain how macrophages accumulate cholesterol and become disease-causing foam cells. Fluid-phase pinocytosis of LDL is a relevant pathway to target for modulating macrophage cholesterol accumulation in atherosclerosis. Recent studies show that phosphoinositide 3-kinase (PI3K), liver X receptors (LXRs), the macrophage colony-stimulating factor (M-CSF) receptor, and protein kinase C (PKC) mediate macrophage macropinocytosis of LDL, and thus, these may be relevant targets to inhibit macrophage cholesterol accumulation in atherosclerosis.

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CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

Keywords

Atherosclerosis; macrophages; LDL; cholesterol; fluid-phase pinocytosis; macropinocytosis; LXR; phosphoinositide 3-kinase; M-CSF; GM-CSF

INTRODUCTION

Engorgement of macrophages with cholesterol is the defining pathological characteristic of atherosclerotic plaques, the cause of most heart attacks and strokes. Cholesterol accumulation in macrophages not only contributes to cholesterol retention within the vessel wall, but also alters macrophage biology. Cholesterol-loaded macrophages secrete plaque disrupting matrix metalloproteinases, and produce tissue factor that promotes thrombosis when plaques rupture [1, 2]. Thus, how macrophages accumulate cholesterol and become so called foam cells has been the subject of intense investigation.

LDL, the main carrier of plasma cholesterol, enters the vessel wall, and then enters macrophages by some mechanism. LDL concentration within the vessel wall is about twice ($\cong 2$ mg/ml) the blood concentration of LDL [3–5], considerably higher than the LDL concentrations investigators typically use when simulating macrophage foam cell formation *in vitro*. Previously, investigators could not show native LDL to cause foam cell formation, because the cellular receptor (i.e., LDL receptor) that binds LDL is poorly expressed on differentiated macrophages, and this receptor downregulates during cholesterol uptake limiting total cholesterol accumulation [6–9]. Moreover, the LDL receptor is not expressed in human atherosclerotic plaques [10]. Thus, most previous studies of macrophage foam cell formation have focused on modifying LDL in some way that increases its binding and subsequent uptake by macrophages.

A widely accepted hypothesis for foam cell formation involves LDL oxidation. LDL oxidation promotes macrophage LDL uptake that is mediated by various macrophage scavenger receptors [11]. While oxidation of LDL has important biological effects that could influence atherosclerotic plaque development [12], oxidation of LDL does not readily explain foam cell formation. LDL isolated from human atherosclerotic lesions is not sufficiently oxidized for macrophage scavenger receptors to bind and take up the LDL [13]. Also, incubation of monocyte-derived human macrophages with LDL even strongly oxidized by artificial chemical means produces little cholesterol accumulation in human macrophages [14]. Any oxidized LDL (OxLDL) taken up by macrophages is poorly metabolized within lysosomes because OxLDL partially inactivates the lysosomal enzymes that degrade LDL [15–17]. This limits the capacity of OxLDL to induce acyl-CoA:cholesterol acyltransferase (ACAT)-mediated cholesterol esterification, and cholesteryl ester lipid droplet formation, the hallmark of macrophage foam cell formation. Lastly, macrophage foam cell formation occurs in mouse models of atherosclerosis even when both receptors mediating mouse macrophage uptake of OxLDL are genetically deleted [18 19].

Recently, it has been shown that macrophages can take up and degrade large amounts of native LDL under certain conditions of macrophage differentiation and activation [20–22]. LDL uptake does not depend on macrophage oxidation of LDL. In fact, LDL uptake does

not depend on macrophage binding of LDL. Rather, macrophages show high levels of pinocytosis of fluid and unbound LDL contained within the fluid, transforming the macrophages into lipid droplet-enriched foam cells. This novel mechanism of fluidphase mediated macrophage cholesterol accumulation shows that oxidative modification of LDL is not necessary for foam cell formation to occur. Thus, the 30-year-old belief that macrophage foam cell formation cannot occur with native LDL, a belief that has driven much past research in the atherosclerosis field, turns out to be untrue. The existing paradigm of macrophage foam cell formation must be broadened to include fluid-phase pinocytosis of LDL (discussed below) as a mechanism mediating macrophage foam cell formation.

TRANSFORMATION OF MACROPHAGES INTO FOAM CELLS THROUGH FLUID-PHASE PINOCYTOSIS OF LDL

Incubation of bone marrow-derived mouse and monocyte-derived human macrophages with increasing concentrations of unmodified native LDL similar to concentrations found in the vessel wall [3–5] results in progressive macrophage accumulation of cholesterol (Fig. 1) [20, 21, 23, 24]. Total cholesterol levels can increase greater than 400 nmol/mg protein (4-times control levels) with the majority of cholesterol accumulating as ACAT-generated esterified cholesterol [20]. These macrophages show progressive uptake of LDL over a 48-hour incubation (Fig. 2) [21], reflecting that the LDL uptake pathway does not readily down-regulate. Macrophage uptake of native LDL causes lipid droplet formation (Fig. 3) and cholesterol accumulation in the macrophages to levels characteristic of macrophage foam cells in atherosclerotic plaques [20, 23–25].

Cholesterol accumulation in wild-type and LDL receptor-deficient mice is similar indicating that cholesterol accumulation does not depend on LDL receptor function [23, 24]. Scavenger receptor family members SR-A and CD36 each mediate a portion of oxLDL uptake by mouse macrophages [26]. However, uptake of native LDL by SR-A or CD36 knockout M-CSF differentiated mouse macrophages is similar to the LDL uptake by wild-type macrophages showing that these scavenger receptors do not mediate uptake of LDL [24]. Consistent with the LDL uptake occurring through fluid-phase rather than a binding receptor mechanism, LDL uptake is non-saturable and linearly related to LDL concentration (Fig. 4) [20, 21, 23, 24, 27], uptake of labeled LDL cannot be competed by excess unlabeled LDL [20, 21, 23, 24], and LDL uptake can be completely accounted for by the level of fluid-phase pinocytosis determined with a fluid-phase tracer [20, 28].

Two distinct macrophage phenotypes can be differentiated *in vitro* from human monocytes or mouse bone marrow cells using fetal bovine serum (FBS) containing either M-CSF or granulocyte macrophage colony-stimulating factor (GM-CSF) [29]. Human monocytes differentiated with human serum are similar in phenotype to human monocytes differentiated with FBS and GM-CSF [20, 30]. Both macrophage phenotypes are present within human atherosclerotic lesions [30]. Importantly, both macrophage phenotypes show fluid-phase pinocytosis of LDL, which is either mostly constitutive for GM-CSF differentiated bone marrow-derived mouse macrophages and M-CSF differentiated monocyte-derived human macrophages [21, 23], partly constitutive for M-CSF differentiated mouse macrophages

[24], or completely dependent on PKC activation in the case of GM-CSF differentiated monocyte-derived human macrophages, [20, 30].

MACROPHAGE FLUID-PHASE PINOCYTOSIS OCCURS IN ATHEROSCLEROTIC LESIONS

In order to show that fluid-phase pinocytosis of lipoproteins such as LDL can occur within atherosclerotic lesions *in vivo*, it is necessary to have a tracer that does not bind cells and is similar in size to LDL. Two different fluorescent nanoparticles, designed not to bind cells, can be employed as tracers for qualitative and quantitative analysis of fluid-phase pinocytosis. AngioSPARK nanoparticles are highly near-infrared fluorescent nanoparticles specifically designed for *in vivo* imaging [31]. These nanoparticles are pegylated to minimize their interaction with cells, and remain localized in the vasculature for extended periods, showing a half-life of 14 hours in blood of mice. Qtracker non-targeted Quantum Dots are also stable, bright fluorophores that have high quantum yields, and narrow fluorescence emission bands [32]. Qtracker non-targeted Quantum Dots are also pegylated and exhibit a circulating half-life of 18.5 hours. Both nanoparticles are similar in size (20–50 nm for AngioSPARK and 10–20 nm for Quantum Dots) to LDL (22 nm). Like LDL, cultured macrophages take up these fluorescent nanoparticles by fluid-phase pinocytosis [27]. Macrophage uptake of Quantum Dots and AngioSPARK is linearly related to the concentration of these nanoparticles in the medium, showing no saturation and therefore not mediated by cell binding, also confirmed by fluorescence microscopy [27]. AngioSPARK or Quantum Dots nanoparticles injected into apolipoprotein E (ApoE) knockout mice accumulate within macrophages of the atherosclerotic lesions that develop in these mice [27]. Fluid-phase pinocytosis persists even in lipid-filled macrophage foam cells because macrophages that show extensive lipid deposits also accumulate substantial amounts of nanoparticles [27]. Thus, macrophage lipid accumulation does not downregulate fluid-phase pinocytosis consistent with *in vitro* findings that LDL-induced macrophage cholesterol accumulation does not downregulate macrophage pinocytosis [21, 28].

Two findings make it unlikely that monocytes take up the fluorescent nanoparticles in the circulation and carry them into the vessel wall. Rare monocytes attached to the luminal surface of the vessel wall do not show accumulation of nanoparticles. Moreover, atherosclerotic aortas removed from ApoE knockout mice and exposed to nanoparticles *in vitro* rather than *in vivo*, also show accumulation of nanoparticles within atherosclerotic lesion macrophages [27]. This shows that the nanoparticles can enter the vessel wall, and that macrophages already present within atherosclerotic lesions can take up the nanoparticles.

Substantial uptake of the fluid-phase tracer nanoparticles in atherosclerotic lesion macrophages shows that fluid-phase pinocytosis functions at high levels in these cells. Thus, macrophage fluidphase pinocytosis of LDL is not only a mechanism for foam cell formation with cultured macrophages [20, 21, 23, 24, 33, 34], but also is a plausible mechanism to explain foam cell formation with atherosclerotic plaque macrophages.

MACROPINOCYTOSIS AND MICROPINOCYTOSIS MEDIATE FLUID-PHASE PINOCYTOSIS OF LDL BY M-CSF DIFFERENTIATED MONOCYTE-DERIVED HUMAN MACROPHAGES

Fluid-phase pinocytosis can occur by either micropinocytosis of fluid within small vesicles less than $<0.2\ \mu\text{m}$, or by macropinocytosis of fluid within large vacuoles typically greater than $>0.5\ \mu\text{m}$ (Fig. S1) [35]. Macropinocytosis is an actin-dependent endocytic pathway in which ruffling plasma membranes fuse to enclose culture fluid within vacuoles that the macrophage then internalizes. Macropinocytosis is thus different from actin-dependent phagocytosis triggered by plasma membrane binding of large particles that the macrophage then engulfs 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.

Light and electron microscopy have demonstrated what pinocytotic pathways mediate M-CSF differentiated macrophage uptake of fluid-phase solute. These pathways were determined by incubating macrophage cultures with the fluid-phase pinocytosis tracer, horseradish peroxidase (HRP), together with either bafilomycin A1 or SU6656 [34]. Pinocytotic small vesicles (i.e., micropinosomes) and large vacuoles (i.e., macropinosomes) both show uptake of HRP. SU6656-treated macrophages show HRP uptake within micropinosomes, but not within macropinosomes [34]. This finding is consistent with time-lapse phase-microscopy showing that SU6656 inhibits macropinocytosis and formation of macropinosomes [34]. In contrast, bafilomycin A1-treated macrophages show HRP uptake within macropinosomes, but not within micropinosomes [34]. Thus, bafilomycin A1 and SU6656 inhibit micropinocytosis and macropinocytosis, respectively, and pinocytosis of solute in M-CSF differentiated macrophages occurs through both micropinocytosis and macropinocytosis.

Consistent with the above, SU6656 and another agent that blocks macropinocytosis, the Rho GTPase inhibitor, toxin B, decrease LDL uptake and cholesterol accumulation by approximately 40% [34]. Bafilomycin A1, which inhibits micropinocytosis, inhibits LDL uptake also by about 40% [34]. However, these two drugs additively inhibit macrophage uptake of LDL confirming that they target different pathways, micro- and macropinocytosis that each contribute about half of the fluid-phase mediated LDL uptake by M-CSF differentiated macrophages [34].

The relative contributions of macropinocytosis and micropinocytosis to fluid-phase uptake of LDL by GM-CSF phenotype monocyte-derived human macrophages remain to be determined.

IDENTIFICATION OF POTENTIAL DRUG TARGETS FOR INHIBITING MACROPHAGE MACROPINOCYTOSIS OF LDL (TABLE 1)

As discussed above, macropinocytosis substantially contributes to macrophage fluid-phase uptake of LDL. Thus, inhibition of macrophage macropinocytosis should be a potential means of limiting plaque macrophage uptake of LDL and cholesterol accumulation. Below

is a discussion of drug targets that mediate macrophage macropinocytosis. Note that the targets can be both macrophage phenotype and species specific.

LXR Agonists Decrease M-CSF Differentiated Monocyte-derived Human Macrophage Macropinocytosis of LDL

Several studies have indicated LXR transcription factors are involved in the control of lipid metabolism and inflammation (reviewed in [36]). LXR transcription factors inhibit atherosclerosis in experimental mouse studies. Treatment of atherosclerosis-prone LDL receptor-and apoE-deficient mice with synthetic LXR agonists reduces the development of atherosclerosis [37, 38]. Other studies, using macrophage-selective LXR-deficient mice created by bone marrow transplantation, further establish that this reduction in atherosclerosis is dependent on LXR activity in macrophages [39, 40]. One possible mechanism by which LXR transcription factors inhibit atherosclerosis is by promoting macrophage cholesterol efflux via induction of the genes encoding apoE, ATP binding cassette transporter A1 (ABCA1), and ATP binding cassette transporter G1 (ABCG1) in macrophages [41].

Another possible mechanism by which LXR transcription factors may affect atherosclerosis is by decreasing macrophage receptor-independent, fluid-phase pinocytosis of LDL. Differentiating M-CSF monocyte-derived human macrophages with either T0901317, or the natural LXR agonist, 22(R)-hydroxycholesterol reduces macropinosome formation [28], and subsequently macropinocytosis of LDL. T0901317 and 22(R)-hydroxycholesterol reduce macrophage LDL uptake and cholesterol accumulation by about 50%. The inhibition of fluid uptake can account for the decrease in LDL uptake, because LXR agonist treatment reduces bovine serum albumin uptake (a measure of fluid-phase pinocytosis) and LDL uptake to the same degree [28].

When T0901317 is added to differentiating monocytes at different time points following initiation of the culture, macrophage total uptake of LDL is progressively reduced as the time of T0901317 treatment is increased [28]. Incubation of macrophage for 7 days with T0901317 that is added beginning on day 7 of culture produces much less inhibition of ¹²⁵I-LDL uptake compared with a 7-day incubation with T0901317 added from the beginning of culture. This finding indicates that for maximal effectiveness of LXR agonist inhibition of LDL uptake, the LXR agonist must be added during early differentiation of the M-CSF macrophages. The finding also explains why with even 2 days of cholesterol accumulation, 7-day differentiated macrophages do not downregulate macropinocytosis of LDL [21] despite the probable generation of endogenous LXR activators such as oxysterols and desmosterol [42].

Acetylated LDL is taken up by macrophage scavenger receptors [43]. However, while LXR agonist inhibits receptor-independent, fluid-phase uptake of LDL, it does not inhibit receptor-mediated acetylated LDL uptake. This shows that LXR agonist specifically inhibits fluid-phase pinocytosis of lipoproteins, and does not affect receptor-mediated endocytosis of lipoproteins. Although peroxisome proliferator-activated receptors (PPARs) affect macrophage lipoprotein and cholesterol trafficking through their modulation of LXRs [44,

45, 46,], PPAR agonists do not affect LXR inhibition of macrophage macropinocytosis of LDL.

PI3K Gamma Signaling Mediates Macropinocytosis of LDL by GM-CSF Differentiated Bone Marrow-derived Mouse Macrophages

Previous studies show an important role for PI3K signaling in macropinosome formation [47–49]. Recently, PI3Kgamma has been identified as a mediator of macropinocytosis in GM-CSF differentiated bone marrow-derived mouse macrophages as LDL uptake and cholesterol accumulation were inhibited about 50% by the PI3Kgamma inhibitor, AS605240 [23]. This finding was confirmed with PI3Kgamma-null and PI3Kgamma kinase-dead, knock-in macrophages that also show about a 50% decrease in LDL uptake and a corresponding decrease in cholesterol accumulation compared with wild-type macrophages [23]. Because electron microscopy shows that macropinocytosis mediates all of the fluid-phase pinocytosis of LDL, and AS605240 inhibits macropinocytosis of LDL by only ~50%, at least one additional fluid-phase macropinocytosis pathway that is independent of PI3Kgamma exists in the GM-CSF differentiated macrophages.

Interestingly, inhibition of PI3Kgamma signaling by AS605240 treatment or genetic deletion of PI3Kgamma inhibits by about 50% the development of atherosclerosis in ApoE and LDL receptor-deficient mice [50]. Inhibition of macrophage fluid-phase uptake of LDL may contribute to the reduction of atherosclerosis caused by AS605240 treatment or genetic deletion of PI3Kgamma.

Non-classical PI3K Signaling Mediates Macropinocytosis of LDL by M-CSF Differentiated Bone Marrow-derived Mouse Macrophages

LY294002 and wortmannin, two inhibitors of all PI3K isoforms, inhibit macropinocytosis in M-CSF differentiated bone marrow-derived mouse macrophages as assessed by phase-contrast microscopy [24, 47]. LY294002 also inhibits serum-induced lipid accumulation that may be mediated by macropinocytosis in RAW264.7 macrophages [51]. Consistent with these observations that macropinocytosis is dependent on PI3K, LY294002 and wortmannin inhibit both LDL uptake and cholesterol accumulation almost completely in PMA-activated human serum differentiated monocyte-derived human macrophages, and by about 50% in M-CSF differentiated bone marrow-derived mouse and human macrophages [24](and unpublished data). However, there was no decrease in cholesterol accumulation with PI3K kinase-dead, knock-in class I PI3K isoforms beta, delta, or gamma in M-CSF differentiated bone marrow-derived mouse macrophages. Also, wild-type macrophages treated with class I PI3K isoform-specific small molecule inhibitors did not show any decrease in LDL uptake or cholesterol accumulation compared with controls [24]. These results suggest that in contrast to GM-CSF differentiated bone marrow-derived mouse macrophages discussed above, a PI3K isoform other than PI3Kgamma and other class I PI3K isoforms mediates M-CSF differentiated mouse macrophage macropinocytosis of LDL.

M-CSF (i.e., colony-stimulating Factor 1 (CSF-1)) Receptor Kinase Mediates Macropinocytosis of LDL in M-CSF Differentiated Bone Marrow-derived Mouse Macrophages

M-CSF receptor kinase signaling is necessary for macropinocytosis by M-CSF differentiated bone marrow-derived mouse macrophages as indicated by the complete loss of macropinosomes when macrophages are incubated without M-CSF or with M-CSF in the presence of the M-CSF receptor kinase inhibitor, GW2580 [24, 52, 53]. GW2580 inhibition of macropinocytosis decreases by about 50% cholesterol accumulation in these mouse macrophages, thus implicating macropinocytosis in mediating about one-half of macrophage cholesterol accumulation, similar to the contribution of macropinocytosis in M-CSF differentiated monocyte-derived human macrophages [34]. Recently, it was shown that GW2580 inhibits the development of mouse atherosclerosis [54]. Because M-CSF receptor signaling regulates multiple macrophage functions, GW2580 may be anti-atherogenic through multiple mechanisms, but inhibition of macrophage macropinocytosis of LDL could be one of those mechanisms.

Macropinocytosis in M-CSF differentiated monocyte-derived human macrophages is less dependent on M-CSF or M-CSF receptor signaling as removal of M-CSF or addition of GW2580 only slightly decreases macropinocytosis of LDL. The reason for this apparent species difference remains to be determined.

PKC Isoforms Mediating LDL Uptake by Phorbol 12-myristate 13-acetate (PMA)-activated Human Serum Differentiated Monocyte-Derived Human Macrophages

Early work showed that PMA stimulates macropinocytosis by M-CSF differentiated bone marrow-derived mouse macrophages and peritoneal-derived mouse macrophages [55, 56]. Although PMA activation stimulates macropinocytosis in human serum or GM-CSF+FBS differentiated monocyte-derived human macrophages, M-CSF does not [30, 33 and unpublished data]. With PMA stimulation of macropinocytosis in this macrophage phenotype, macropinocytosis mediates LDL uptake and cholesterol accumulation transforming these macrophages into foam cells [20, 33]. Beta and delta PKC isoenzymes mediate the PMA-stimulated cholesterol accumulation by human serum differentiated monocyte-derived human macrophages [57]. These two PKC isoenzymes may function independently to promote PMA-stimulated cholesterol accumulation. Inhibition of PKC beta with either Go6976 (a PKC classical group inhibitor that includes alpha, beta, and gamma PKC isoforms) or LY333531 (PKC beta isoform specific inhibitor) decreases PMA-stimulated cholesterol accumulation by about 50%, while inhibition of both PKC beta and delta with Go6850 and Go6983, pan PKC inhibitors, decrease PMA-stimulated cholesterol accumulation by >90%. These findings suggest that PKC beta and delta function independently and additively to stimulate cholesterol accumulation. It remains to be determined whether both PKC isoenzymes mediate macropinocytosis of LDL or whether macropinocytosis and some other pathway such as micropinocytosis also functions in LDL uptake by these macrophages.

Effect of Src Family Kinase Inhibitors on M-CSF Differentiated Monocyte-derived Human Macrophage Macropinocytosis of LDL

As discussed above, the kinase inhibitor SU6656 inhibits M-CSF differentiated macrophage macropinocytosis, and consequently, LDL uptake and cholesterol accumulation [34].

SU6656 is considered a specific small-molecule inhibitor of Src family kinases [58]

However, other Src family kinase inhibitors, PP1 or PP2, do not inhibit LDL uptake by M-CSF differentiated monocyte-derived human macrophages suggesting that SU6656 inhibits macropinocytosis mediated by a non-Src family kinase that remains to be determined [34].

Spleen Tyrosine Kinase (Syk)-dependent Macropinocytosis by Resident Peritoneal-derived Mouse Macrophages

LDL modified by either acetylation or minimal oxidization with 15-lipoxygenase treatment induces macropinocytosis in monocyte-derived pigeon macrophages and resident peritoneal-derived mouse macrophages, respectively [59, 60]. The induced macropinocytosis mediates fluid-phase uptake of co-incubated native LDL [59, 60]. The minimally oxidized LDL binds CD14 and triggers toll-like receptor-4 and Syk-dependent fluid-phase macropinocytosis in the resident peritoneal-derived mouse macrophages [59]. Whether tolllike receptor-4 stimulated macropinocytosis is sustained sufficiently to induce macrophage foam cell formation remains to be determined. Demonstrating the specificity of signaling in different macrophage phenotypes, Syk does not mediate macropinocytosis in M-CSF differentiated monocyte-derived human macrophages [34].

CONCLUSION

Macrophages show fluid-phase mediated uptake and degradation of native LDL, a novel mechanism to explain macrophage foam cell formation. LDL uptake does not depend on modification of LDL or macrophage binding of LDL. Rather, macrophages take up LDL by macropinocytosis and micropinocytosis. In this receptor-independent uptake process, macrophages take up LDL as part of the fluid that they ingest by these pinocytosis pathways. This produces cholesterol accumulation in macrophages to levels characteristic of macrophage foam cells in atherosclerotic plaques. Thus, the existing paradigm that macrophage cholesterol accumulation leading to foam cell formation can only occur when LDL is modified and taken up by macrophage scavenger receptors is not correct.

Macrophages in mouse atherosclerotic lesions demonstrate fluid-phase uptake of fluorescent LDL-like surrogate nanoparticles [27]. Thus, fluid-phase pinocytosis of LDL in atherosclerotic plaques is a plausible mechanism that can explain how macrophages accumulate cholesterol and become disease-causing foam cells. The findings indicate that macrophage fluid-phase pinocytosis is a relevant pathway to target for modulating macrophage cholesterol accumulation in atherosclerosis.

Macropinocytosis is induced by transfection of Ras, Rac, Pak1, and certain Src-family kinases signaling molecules in nonmacrophage cell types [61–69], and some of these factors have been implicated in the signaling that mediates macropinocytosis induced by platelet-derived growth factor and epidermal growth factor in non-macrophage cells [62, 63].

However, these signaling molecules do not mediate macropinocytosis in M-CSF differentiated bone marrow-derived mouse macrophages [24]. The specificity of signaling in different cell types and different macrophage phenotypes as reviewed here suggests the therapeutic possibility of selectively modulating macropinocytosis of LDL by atherosclerotic plaque macrophages. It will be necessary to learn what fluid-phase pathways, macropinocytosis or micropinocytosis, and what signaling intermediates function in macrophage LDL uptake in atherosclerotic plaques in order to target effectively this process. On the other hand, learning how to stimulate fluid-phase pinocytosis of LDL by macrophages in liver and spleen could be a useful way to lower blood LDL levels as another anti-atherosclerosis treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ABCA1	ATP binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
ACAT	Acyl-CoA:cholesterol acyltransferase
ApoE	Apolipoprotein E
CSF-1	Colony-stimulating factor 1
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage colony-stimulating factor
HRP	Horseradish peroxidase
LDL	Low-density lipoprotein
LXRs	Liver X receptors
M-CSF	Macrophage colony-stimulating factor
OxLDL	Oxidized LDL
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PPARs	Peroxisome proliferator-activated receptors

Syk Spleen tyrosine kinase

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Fig. (1).

Effect of LDL concentration on cholesterol accumulation by M-CSF differentiated monocyte-derived human macrophages. Monocytes were differentiated 7 days in FBS containing M-CSF. Then, the differentiated macrophages were incubated 24 hours in serum-free medium with M-CSF and the indicated concentrations of LDL. Following incubations, macrophage cholesterol content was determined. Shown is the total cholesterol and in parentheses, the percentage of cholesterol that was esterified. Reproduced from *J. Biol. Chem.* 281(23): 15757–62 2006 [21].

Fig. (2).

Time-course of ^{125}I -LDL uptake by M-CSF differentiated monocyte-derived human macrophages. Monocytes were differentiated 7 days in FBS containing M-CSF. Then, the differentiated macrophages were incubated 24 hours in serum-free medium with M-CSF and 500 $\mu\text{g/ml}$ ^{125}I -LDL for the indicated times. ^{125}I -LDL uptake was determined as the sum of cell-associated and degraded ^{125}I -LDL. Reproduced from J. Biol. Chem. 281(23): 15757–62 2006 [21].

Fig. (3). Oil red O-stained GM-CSF differentiated, LDL receptor-null, bone marrow-derived mouse macrophage foam cells. Bone marrow cells were differentiated 3 weeks in FBS containing GM-CSF. Then, the differentiated macrophages were incubated 24 hours in serum-free medium containing GM-CSF without (a) or with 4 mg/ml LDL (b). After fixation, macrophages were stained with oil red O to demonstrate macrophage lipid droplets. The bars = 40 μ m. Reproduced from *J Lipid Res.* 53(1): 34–42 2012 [23].

Fig. (4).

Effect of ^{125}I -LDL concentration on ^{125}I -LDL uptake by M-CSF differentiated monocyte-derived human macrophages. Monocytes were differentiated 7 days in FBS containing M-CSF. Then, the differentiated macrophages were incubated 24 hours in serum-free medium with M-CSF and the indicated concentrations of ^{125}I -LDL. Total uptake of the ^{125}I -LDL was determined as the sum of cell-associated and degraded ^{125}I -LDL. Reproduced from J. Biol. Chem. 281(23): 15757–62 2006 [21].

Table 1.

Signaling Targets Mediating Fluid-Phase Macropinocytosis of LDL

Macrophage Type	Species	Macropinocytosis Stimulus	Target	References
M-CSF+FBS differentiated blood monocytes	Human	constitutive	PI3-kinase LXR	[21] / [28]
GM-CSF+FBS differentiated blood monocytes	Human	PMA	PKC	[30]
Human serum differentiated blood monocytes	Human	PMA	PKC (beta and delta isoforms) PI3-kinase	[20, 57] [33]
M-CSF+FBS differentiated bone marrow cells	Mouse	M-CSF	PI3-kinase (non-classical isoform) M-CSF (i.e., CSF-1) receptor	[24, 47] [24]
GM-CSF+FBS differentiated bone marrow cells	Mouse	constitutive	PI3-kinase (gamma isoform)	[23]
FBS cultured resident peritoneal macrophages	Mouse	oxidized LDL	Syk	[59]

/ and unpublished data