Macropinocytosis Is the Endocytic Pathway That Mediates Macrophage Foam Cell Formation with Native Low Density Lipoprotein**

Previously, we reported that fluid-phase endocytosis of native LDL by PMA-activated human monocyte-derived macrophages converted these macrophages into cholesterol-enriched foam cells (Kruth, H. S., Huang, W., Ishii, I., and Zhang, W. Y. (2002) J. Biol. Chem. 277, 34573-34580). Uptake of fluid by cells can occur either by microinocytosis within vesicles (<0.1 μm diameter) or by macropinocytosis within vacuoles (~0.5–5.0 μm) named macropinosomes. The current investigation has identified macropinocytosis as the pathway for fluid-phase LDL endocytosis and determined signaling and cytoskeletal components involved in this LDL endocytosis. The phosphatidylinositol 3-kinase inhibitor, LY294002, which inhibits macropinocytosis but does not inhibit micropinocytosis, completely blocked PMA-activated macrophage uptake of fluid and LDL. Also, nystatin and filipin, inhibitors of micropinocytosis from lipid-raft plasma membrane domains, both failed to inhibit PMA-stimulated macrophage cholesterol accumulation. Time-lapse video phase-contrast microscopy and time-lapse digital confocal-fluorescence microscopy with fluorescent Dil-LDL showed that PMA-activated macrophages took up LDL in the fluid phase by macropinocytosis. Macropinocytosis of LDL depended on Rho GTPase signaling, actin, and microtubules. Bafilomycin A1, the vacuolar H+-ATPase inhibitor, inhibited degradation of LDL and caused accumulation of undergraded LDL within macropinosomes and multivesicular body endosomes. LDL in multivesicular body endosomes was concentrated >40-fold over its concentration in the culture medium consistent with macropinosome shrinkage by maturation into multivesicular body endosomes. Macropinocytosis of LDL taken up in the fluid phase without receptor-mediated binding of LDL is a novel endocytic pathway that generates macrophage foam cells. Macropinocytosis in macrophages and possibly other vascular cells is a new pathway to target for modulating foam cell formation in atherosclerosis.

Macrophage foam cell formation is an important process in atherosclerotic plaque development. Uptake and storage of plasma lipoprotein-derived cholesterol within monocyte-derived macrophages affects macrophage functions in ways that influence plaque development and stability. Whether macrophage storage of cholesterol contributes to cholesterol retention within the plaque or facilitates its removal from plaques remains to be determined (1, 2). In either case, macrophage storage of cholesterol promotes macrophage expression of proteases and tissue factor that contribute to plaque rupture and thrombosis, respectively (3–5).

Previously, macrophage foam cell formation was thought to occur only through uptake of modified forms of low density lipoprotein (LDL) such as oxidized or aggregated LDL (2). Recently, we showed that macrophage foam cell formation can occur through uptake of native LDL in a receptor-independent fashion (6). Activation of human monocyte-derived macrophages with the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) stimulates macropage fluid-phase endocytosis. Fluid-phase endocytosis can mediate substantial macrophage uptake of LDL when LDL levels are high at about 0.5 to 2 mg/ml. While these LDL levels are high relative to levels that saturate receptor-mediated uptake of LDL, this LDL concentration range is comparable to LDL levels that occur in the normal intima of arteries and atherosclerotic plaques (7–9). Thus, consideration of two conditions that occur in atherosclerotic plaques, activated macrophages and high LDL levels, has led to our discovery of a novel mechanism of macrophage foam cell formation mediated by fluid-phase endocytosis.

Fluid-phase endocytosis by cells can occur by two pathways, uptake of fluid within either micropinocytic vesicles (micropinocytosis) or macropinocytic vacuoles (macropinocytosis) (10, 11). With both micropinocytosis and macropinocytosis, solute uptake is directly proportional to the volume of fluid internalized and solute concentration. During macropinocytosis, plasma membrane ruffling occurs that can lead to membrane fusion of the plasma membrane with itself. This membrane fusion envelope extracellular fluid that enters the cell within 0.5–5.0-μm vacuoles formed during the ruffling and membrane fusion process. Ruffling of surface membranes is necessary for macropinocytosis and is mediated by many Ras superfamily GTPases (12, 13). However, ruffling alone is not sufficient for macropinocytosis as other signaling factors including phos-
phatidylinositol 3-kinase and the Rab family GTPase, Rab/Rab34, have been implicated in the closure of ruffles to form macropinosomes (14, 15).

Macropinocytosis occurs in amoeba and mammalian cells such as macrophages, dendritic cells, neutrophils, fibroblasts, and epithelial cells. Macropinocytosis is either constitutive as in dendritic cells or can be induced in different cells by PMA, growth factors, and modified LDLs (10, 11, 16–18). Macropinocytosis can occur in clathrin-associated, caveolin-associated, or non-clathrin-caveolin-associated vesicles (no larger than 0.1 μm). The latter two modes of macropinocytosis usually occur in lipid raft domains of the plasma membrane (19). Macropinocytosis is an actin-dependent endocytic process while micropinocytosis generally is an actin-independent process.

In the current investigation, we have sought to identify the endocytic pathway that mediates PMA-stimulated macrophage uptake of native LDL. Through analysis of cytoskeletal and signaling dependence of LDL uptake, and microscopic analysis of macrophages, we have identified actin-dependent macropinocytosis mediated by Rho-GTPase and phosphatidylinositol 3-kinase signaling as the mechanism of LDL-induced foam cell formation in PMA-activated macrophages.

**EXPERIMENTAL PROCEDURES**

**Culture of Human Monocyte-derived Macrophages**—Human monocytes were purified with counterflow centrifugal elutriation of mononuclear cells obtained by monocytophoresis of normal human donors. The monocytes were cultured in pooled human AB, heat-inactivated serum (Pel-Freez) as described previously (20) except that 0.4 × 10^6 monocytes/cm^2 were initially seeded in 12-well (22-mm diameter) culture plates (Plastek C from MatTek). For experiments, 2-week-old monocyte-derived macrophage cultures were rinsed three times with RPMI 1640 medium and then incubated at 37 °C for the indicated times with the indicated agents or to RPMI 1640 medium with serum.

**Preparation of LDL for Use in Experiments**—Before use, human Dil-LDL (Intracel) and DiI-LDL (Molecular Probes) were dialedyzed against 1 liter of 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 12 h at 4 °C, then against RPMI 1640 medium (2 changes, 1 liter/each change) for 24 h. Human 125I-LDL (Biomedical Technologies) was dialyzed against 0.15 μM sodium chloride and 0.3 mM EDTA (pH 7.4) over 36 h at 4 °C (3 changes, 1 liter/each change). All dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular weight cut-off). After dialysis, lipoproteins were sterilized by passage through a 0.45-μm (pore size) low protein binding filter (Gelman Acrodisc). 125I-LDL specific content was adjusted to 2.25 × 10^−8 μCi/μg by adding unlabeled LDL.

**Measurement of Fluid-phase Endocytosis**—Fluid-phase endocytosis was determined by incubating macrophages with 0.8 nmol/ml [3H]sucrose (specific activity of 12.3 Ci/mmol obtained from American Radiolabeled Chemicals). Following incubations, macrophages were rinsed 3 times with ice-cold DPBS plus Ca^2+ and Mg^2+ and 0.2% BSA, and then three times with DPBS plus Ca^2+ and Mg^2+. Macropinocytosis mediated by Rho-GTPase and phosphatidylinositol 3-kinase signaling as the mechanism of LDL-induced foam cell formation in PMA-activated macrophages.

**Electron Microscopic Analysis of Macrophages**—For standard electron microscopy, macrophages were seeded in 2-well plastic slide chambers (Lab-Tek). After incubations, macrophages were fixed, stained with ruthenium red to distinguish extracellular from intracellular membranes, and further prepared for electron microscopy (20). For cytochemical analysis of the fluid-phase tracer horseradish peroxidase, macrophages were fixed 1 h with 2.5% glutaraldehyde in 0.1M cacodylate buffer at room temperature. Horseradish peroxidase was localized with diaminobenzidine and hexazone as previously described (25, 26). Next, macrophages were preincubated 30 min with 2 mg/ml mannan, label with anti-human LDL antibody (cat. BT-905, BTI), and then with a 1:10 dilution of 10 nm gold rabbit IgG (Intracel). After controls, the rabbit anti-human LDL antibody was substituted with the same concentration of purified rabbit IgG. This control showed no labeling.

**McKee Microscopy**—Time-lapse confocal fluorescence images were obtained with a ×63 planapo objective lens (1.4 N.A.) mounted on an Olympus IX81 inverted microscope equipped with a Yokogawa spinning disk confocal system. Macrophages were cultured in dishes with 0.17-mm thick cover-glass bottoms (Electron Microscopy Sciences). Five % CO_2/95% hydrated air and 37 °C were maintained in an enclosed chamber during recording from the macrophages. 12-bit confocal images of Dil-LDL fluorescence in living macrophages were acquired with 512 × 512 pixel resolution. DiI label was excited with the 586-nm line of a Krypton laser. Specimen fluorescence passed through a DP 570–620 emission filter before images were collected at a rate of one every 5 s and recorded with a Hamamatsu ORCA-ER digital CCD camera. The confocal optical slice thickness was about 1 μm. Media Cybernetics QED InVivo software was used for stack preparation and conversion to QuickTime format.

**Assay of Cholesterol Esterification**—[14C]Oleate-albumin complex was added at the beginning of each incubation and was prepared as described by Goldstein et al. (24) except that the final [14C]oleate concentration was increased to 800 μM to prevent the supply of [14C]oleate from becoming rate limiting. Following incubations, macrophages were rinsed, lipids were extracted with hexane/isopropyl alcohol, and macrophages were harvested for determination of their protein content. Cholesterol ester synthesis was determined as described previously by quantifying [14C]oleate incorporation into cholesterol esters, which were separated by thin layer chromatography (24).
Inhibition of cholesterol accumulation was secondary to...

tion only at later times, this would suggest that nocodazole...macrophages incubated with LDL. If nocodazole inhibited cholesterol accumulation by PMA-activated macrophages incubated with LDL, this would be decreased, but this was not the case. While nocodazole was not due to nocodazole inhibition of endosome to lysosome transport. If nocodazole inhibited degradation of the 125I-LDL inter-

The effect of nocodazole on 125I-LDL degradation showed additional evidence that nocodazole inhibition of LDL uptake was not due to nocodazole inhibition of endosome to lysosome transport. If nocodazole inhibited this transport, an expected buildup of LDL in endosomes and not due to a primary inhibition of fluid endocytosis of LDL. Nocodazole showed similar inhibition of cholesterol accumulation at both early and later times during incubation of PMA-activated macrophages with LDL suggesting that the initial steps of fluid-phase LDL uptake were microtubule-dependent (Fig. 2B).

The effect of nocodazole on 125I-LDL degradation showed additional evidence that nocodazole inhibition of LDL uptake was not due to nocodazole inhibition of endosome to lysosome transport. If nocodazole inhibited this transport, an expected result would be that macrophage degradation of 125I-LDL would be decreased, but this was not the case. While nocodazole inhibited total uptake of 125I-LDL as discussed above (Fig. 1), nocodazole did not inhibit degradation of the 125I-LDL internalized by PMA-activated macrophages, even over a 2-day incubation: 83 ± 7% was degraded without nocodazole, and 80 ± 4% was degraded with nocodazole. Thus, these results with nocodazole inhibition show evidence for both microtubule-de-
DEPENDENT AND INDEPENDENT FLUID-PHASE PATHWAYS FOR UPTAKE OF LDL.

Cytochalasin D, an inhibitor of actin function, decreased by more than 82% macrophage accumulation of [3H]sucrose, 125I-LDL, and cholesterol showing that both the microtubule-dependent and microtubule-independent fluid-phase endocytic LDL uptake pathways were actin-dependent (Table I). Myosins are the motor proteins that function together with actin to generate movement including membrane ruffling. ML-9, an inhibitor of myosin light chain kinase that phosphorylates myosin II, inhibited PMA-stimulated macrophage cholesterol accumulation no more than 40% in three separate experiments (data not shown). This showed that myosin II and another myosin besides myosin II, must function in fluid-phase endocytosis of LDL. ML-9 and nocodazole were synergistic in their inhibition of cholesterol accumulation indicating that they inhibited different actin-dependent fluid-phase uptake pathways for LDL (Table II).

Macropinocytosis-mediated Fluid-phase Endocytic Uptake of LDL—PMA activation of macrophages has been reported to stimulate macropinocytosis in mouse macrophages (29). Therefore, we examined our cultured human monocyte-derived macrophages for PMA stimulation of macropinocytosis using time-lapse phase-contrast video microscopy. Addition of PMA to macrophage cultures stimulated extensive surface membrane ruffling and formation of phase-bright macropinosomes (~0.5–5.0 μm diameter) by 10–15 min (Fig. 3F). Macropinosomes formed in the cell periphery and on the dorsal cell surface of some macrophages. The macropinosomes often fused with other macropinosomes and moved toward the center of the macrophage, decreasing in size to a point where they were no longer visible by phase microscopy. PMA-stimulated macropinocytosis was sustained over a 24-h period of observation compared with DiI-LDL. Prolonged observation of DiI-LDL in macrophages incubated with DiI-LDL showed that macropinocytosis was limited by photobleaching of the DiI.

Uptake of LDL in the fluid-phase content of macropinosomes was confirmed with time-lapse confocal fluorescence microscopy of PMA-activated macrophages incubated with fluorescent 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-LDL (Fig. 4 and Supplemental Movie 3). Macropinosomes showed homogeneous fluorescence consistent with fluid-phase uptake and receptor-mediated uptake of DiI-LDL. No fluorescence was visible when macrophages were incubated without DiI-LDL. Prolonged incubation of DiI-LDL in macrophages incubated without DiI-LDL was limited by photobleaching of the DiI.

Micropinocytotic uptake of culture fluid containing LDL did not contribute significantly to macrophage cholesterol accumulation. Macrophage macropinocytosis but not micropinocytosis depends on phosphatidylinositol 3-kinase (14). We found that the phophatidylinositol 3-kinase inhibitor, LY294002, completely blocked the fluid and 125I-LDL macropinocytosis uptake that was stimulated by PMA (Table III). Also, two inhibitors of micropinocytosis that originates from lipid-raft plasma membrane domains, nystatin (50 μg/ml) and filipin (1 μg/ml, the highest concentration that was not toxic), both failed to inhibit
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Panels A–D follow the same field of macrophages during LDL incubation at 4 min (A), 15 min (B), 5.5 h (C), and 17 h (D). LDL initially caused a slight contraction of the peripheral cytoplasm. Retraction fibers (black arrows and shown at higher magnification in the inset) were present at 15 min. Over the entire 18 h of LDL incubation, very few macropinosomes were seen (arrow in D). After 18 h of LDL incubation, 1 μg/ml PMA was added to the macrophage culture. Panels E–H are a new field of macrophages incubated with PMA for 1 min (E), 15 min (F), 30 min (G), and 60 min (H). Phase-bright macropinosomes (white arrows) were seen forming in most macrophages by 10–15 min after PMA addition, and continued forming over the ensuing 2.5 h that the macrophages were observed in this experiment. See Movies 1 and 2 from which the figure is extracted. The entire movie field width is 250 μm.

Fig. 3. Time-lapse phase-contrast video microscopy of macrophage macropinocytosis. Macrophages were first observed for 2 h in serum-free medium to determine the basal level of macropinocytosis (data not shown), which was the same low level as for macrophages incubated with LDL below. Then, the macrophages were rinsed and incubated for 18 h with 2 mg/ml LDL in serum-free medium. Panels A–D follow the same field of macrophages during LDL incubation at 4 min (A), 15 min (B), 5.5 h (C), and 17 h (D). LDL initially caused a slight contraction of the peripheral cytoplasm. Retraction fibers (black arrows and shown at higher magnification in the inset) were present at 15 min. Over the entire 18 h of LDL incubation, very few macropinosomes were seen (arrow in D). After 18 h of LDL incubation, 1 μg/ml PMA was added to the macrophage culture. Panels E–H are a new field of macrophages incubated with PMA for 1 min (E), 15 min (F), 30 min (G), and 60 min (H). Phase-bright macropinosomes (white arrows) were seen forming in most macrophages by 10–15 min after PMA addition, and continued forming over the ensuing 2.5 h that the macrophages were observed in this experiment. See Movies 1 and 2 from which the figure is extracted. The entire movie field width is 250 μm.

Fig. 4. Time-lapse confocal fluorescence microscopy of macrophage macropinocytosis of fluorescent Dil-LDL. Macrophages were incubated with a mixture of 2 mg/ml LDL, 200 μg/ml Dil-LDL and 1 μg/ml PMA in serum-free medium. The elapsed time after addition of the LDL and PMA is indicated on each frame. The time course of formation of three different macropinosomes containing fluorescent Dil-LDL is indicated. Notice the decrease in size of the macropinosomes with time. See Movie 3 from which the figure is extracted. The entire movie field width is 55 μm.

TABLE III
Effect of phosphatidylinositol 3-kinase inhibition on LDL and fluid uptake

| Parameter measured | Macrophase total uptake |
|--------------------|-------------------------|
| Experiment 1       |                         |
| 125I-LDL           | 2.7 ± 0.2               |
| 125I-LDL+PMA (1 μg/ml) | 15.7 ± 1.1             |
| 125I-LDL+PMA+LY294002 (50 μM) | 2.4 ± 0.1 |
| Experiment 2       |                         |
| [3H]Sucrose        | 4.9 ± 0.6               |
| [3H]Sucrose+PMA (1 μg/ml) | 25.1 ± 2.1            |
| [3H]Sucrose+PMA+LY294002 (50 μM) | 3.2 ± 0.2 |

cholesterol accumulation when added to PMA-activated macrophages incubated 1 day with 2 mg/ml LDL. Lastly, the Rho GTPase inhibitor, toxin B, inhibited LDL-induced cholesterol accumulation in PMA-activated macrophages (Table II). This is consistent with the known required functioning of Rho family GTPases for membrane ruffling and macropinocytosis to occur (12, 13, 15, 30–32). In contrast, Rho family GTPases inhibit clathrin-associated micropinocytosis (33).

Importance of Vacuolar H⁺-ATPase for Fluid-phase Processing of LDL—PMA not only stimulates fluid-phase-mediated uptake of LDL, it also stimulated esterification of cholesterol derived from this LDL (Fig. 5). This implies that LDL-derived cholesteryl esters are hydrolyzed and are then available for cholesterol re-esterification that we previously showed was mediated by acylCoA:acyl cholesterol transferase (6). To determine whether LDL degradation was dependent on vacuolar H⁺-ATPase, whose activity is necessary for protein degrada-

tion within endosomal/lysosomal compartments, we examined the effect of bafilomycin A1, an inhibitor of this enzyme, on LDL degradation. Without bafilomycin A1, PMA-activated macrophages degraded 83% of the 125I-LDL that they took up during a 1-day incubation with 125I-LDL. On the other hand, with bafilomycin A1, these macrophages degraded only 22% of the 125I-LDL (Table IV). As a result of bafilomycin A1 treatment, a substantial amount of degraded 125I-LDL accumulated within the bafilomycin A1-treated macrophages.

Electron microscopic examination of bafilomycin A1-treated macrophages showed the accumulation of many vacuoles filled with LDL-like particles (~20-nm diameter) (Fig. 6A). The vacuoles, varied in size with some as small as 0.1 μm (Fig. 6A, inset), but most were similar to the size range of macropinosomes observed by phase microscopy (0.5 um-5 μm). The vacuoles and their contained LDL-like particles did not label with ruthenium red confirming that there was no connection of the vacuoles with the extracellular space. Immunoelectron microscopy showed that the LDL-like particles within the bafilomycin A1-induced vacuoles labeled with an anti-LDL antibody (Fig. 7A). Most of the smaller vacuoles also contained small vesicles (~50–70-nm in diameter) within the vacuoles showing that these vacuoles were multivesicular body endosomes (Fig. 6A).

The multivesicular body endosomes were made to accumulate fluorescent LDL by incubating macrophages with Dil-LDL, PMA, and bafilomycin A1 for 1 day. The multivesicular body endosomes with Dil-LDL showed immunofluorescent labeling.
with the late endosome/lysosome marker, LAMP-1, but lacked the early endosome marker, EEA1, and the lysosome marker, cathepsin D. LAMP-1 staining occurred as a ring enclosing the fluorescent DiI-LDL, a pattern consistent with the membrane location of LAMP-1 protein. LAMP-1 also labeled spherical structures (presumably lysosomes) that did not contain DiI-LDL (Fig. 8).

Because LDL enters macrophages by fluid-phase endocytosis, we tested whether bafilomycin A1 also caused the fluid-phase tracer, horseradish peroxidase, to accumulate within multivesicular body endosomes. This was the case as shown in Fig. 6B where the horseradish peroxidase electron dense reaction product can be seen to surround the vesicles contained within the multivesicular body endosomes.

Most lipid droplets rather than mostly LDL-containing vacuoles accumulated in control macrophages incubated with LDL and PMA without bafilomycin A1 (Fig. 7B). The few vacuoles in these macrophages also contained vesicles but showed much less anti-LDL labeling (data not shown). LDL-containing vacuoles did not occur in macrophages incubated with LDL alone.

Ketoconazole inhibits trafficking of unesterified cholesterol from lysosomes to the plasma membrane and the endoplasmic reticulum where unesterified cholesterol undergoes ACAT-mediated esterification (34, 35). As would be expected for an agent that inhibits cholesterol trafficking from lysosomes, ketoconazole increased the unesterified cholesterol content and decreased the percentage of esterified cholesterol in PMA-activated macrophages incubated with LDL (Table V).

**DISCUSSION**

Macropinocytosis mediated foam cell formation has been thought to occur only by receptor-mediated uptake of modified LDL. We recently showed that activated macrophages take up native LDL through non-receptor-mediated fluid-phase endocytosis and form foam cells (6). In this report we have identified macropinocytosis as the endocytic pathway that functions in sustained fluid-phase uptake of LDL by PMA-activated macrophages. Macropinocytosis is an actin-dependent endocytic pathway in which ruffling plasma membranes fuse to enclose surrounding fluid within vacuoles that are then internalized by the cell. Macropinocytosis is thus different from actin-dependent phagocytosis that is triggered by plasma membrane binding of large particles that are then engulfed by the cell within phagocytic vacuoles. Phagocytic vacuoles are relatively free of fluid because of the tight apposition of the engulfed particle with the cellular plasma membrane.

Macropinocytosis accounted for nearly all of the fluid-phase uptake of LDL because cytochalasin D and the phosphatidylinositol 3-kinase inhibitor, LY294002, drugs that inhibit macrophage macropinocytosis but not micropinocytosis inhibited greater than 80% of LDL uptake (14, 36). Also, two inhibitors of micropinocytosis that originate from lipid-raft plasma membrane domains, nystatin, and filipin, both failed to inhibit cholesterol accumulation when added to PMA-activated macrophages.

The Ras superfamily of GTPases is comprised of four subfamilies, Ras, Rho, Rab, and ARF. Members of each subfamily (Ha-ras and K-ras, Rac and Cdc42, Rab34, and ARF6, respectively) have been implicated in mediating macropinocytosis in other cell systems (12, 13, 15, 30–32). These GTPases regulate the organization of the actin cytoskeleton and plasma membrane ruffling, two processes involved in macropinosome formation. Macropinocytosis in human monocyte-derived macrophages in our study was dependent on Rac activity. In HeLa human epithelial cells, ARF6 mediates plasma membrane ruffling independent of Rac and each of these GTPases mediates a different pattern of membrane ruffles (31). In contrast, in RAW mouse macrophages, ARF6 is required for Rac-mediated membrane ruffling (37). Targeting of K-Ras and Ha-Ras to different plasma membrane domains has been proposed as a possible explanation for the two different patterns of membrane ruffling and macropinosomes (different in size and number) formed when REF-52 rat embryo fibroblasts are transiently transfected with these Ras family GTPases (38). It is possible that different GTPases mediated both types of macropinocytosis we observed in human monocyte-derived macrophages (i.e. microtubule-dependent and microtubule-independent macropinocytosis discussed below). Examination of which GTPases are specifically involved in human monocyte-derived macrophages will have to await development of techniques to successfully transfect dominant-negative GTPases into this primary cell type macrophage. In any case, because we have stimulated macropinocytosis through protein kinase C activation with PMA, our results suggest that protein kinase C somehow signals to GTPases in the activation of macrophage macropinocytosis.

PMA-activated human monocyte-derived macrophages showed both microtubule-dependent and microtubule-independent macropinocytotic uptake of LDL. Previously, nocodazole was shown to partially inhibit macropinocytosis in mouse bone marrow-derived macrophages, with some macropinosomes still forming in the presence of nocodazole (39, 40). Some plasma membrane ruffling of cells is dependent on microtubules, while other plasma membrane ruffling is independent of microtubules (41). Microtubule function is necessary for plasma membrane targeting of two GTPases, K-Ras and Rac, that mediate macropinocytosis (42, 43). Thus, microtubule dependence of macropinocytosis could be due to microtubule-dependent transport of signaling molecules such as GTPases to specific plasma membrane domains where they can be activated, rather than due to microtubule generation of motor forces in order for plasma membrane ruffling to occur.

Myosin function is necessary for the contractile force that closes plasma membrane ruffles to form macropinosomes (44). In our study, the myosin light chain kinase inhibitor, ML-9, only blocked macrophage cholesterol accumulation that did not depend on microtubule function. A related myosin light chain
kinase inhibitor, ML-7, partially inhibits plasma membrane ruffling activity and macropinocytosis in bone marrow-derived mouse macrophages (44). Myosin-II is the myosin type that is inhibited by ML-9 and ML-7. These findings suggest that myosin-II, which localizes to plasma membrane ruffles during macropinocytosis in epithelial cells and macrophages (44, 45), functioned in the microtubule-independent macropinocytotic LDL uptake pathway. Some other myosin type, possibly myosin-I that has been implicated in amoeba macropinocytosis (46, 47), must have functioned in the microtubule-dependent macropinocytotic LDL uptake pathway.

The fate of macropinosomes varies in different cell types. In epidermal growth factor-stimulated A431 human epithelial cells, a major portion of macropinosomes is recycled out of the cells, and is not delivered to lysosomes (48). In contrast, macropinosomes formed in M-CSF-stimulated mouse bone marrow-derived macrophages shrink and acidify as they move toward the center of the macrophage (49). Then, these mature macropinosomes merge with pre-existing lysosomes. Our results show that macropinosome degradation of LDL depended on vacuolar H⁺-ATPase implicating this proton pump in the acidification process that activates endosomal/lysosomal degradative enzymes. Bafilomycin A1 inhibition of the acidification process blocks transport of endocytosed materials at different steps in the endocytic process depending on the cell type. In some cell types, bafilomycin A1 blocks transport from early endosomes to late endosomes (50). In other cell types, transport is blocked between late endosomes and mature lysosomes (51, 52). In the presence of the vacuolar H⁺-ATPase inhibitor, bafilomycin A1, undegraded LDL and the fluid-phase tracer horseradish peroxidase accumulated within macropino-

TABLE IV

Effect of bafilomycin A1, a vacuolar H⁺-ATPase inhibitor, on 125I-LDL processing by PMA-activated macrophages

| Condition                  | Cell-associated 125I-LDL | Degraded 125I-LDL | Total 125I-LDL |
|----------------------------|--------------------------|------------------|--------------|
| 125I-LDL + PMA             | 2.7 ± 0.2                | 13.1 ± 1.2       | 15.7 ± 1.1   |
| 125I-LDL + PMA + bafilomycin A1 (0.5 μM) | 7.1 ± 0.6                | 2.0 ± 0.2        | 9.1 ± 0.7   |

![Image](317x511 to 563x624)

**FIG. 6.** Accumulation of LDL-like particles in vacuoles of bafilomycin A1-treated macrophages. Macrophages were incubated 1 day with 2 mg/mL LDL plus 1 μg/mL PMA and with 0.5 μM bafilomycin A1 (shown in A), a vacuolar H⁺-ATPase inhibitor. Other control macrophages were incubated with bafilomycin A1 without and with LDL. Then, the culture media were fixed in glutaraldehyde, treated with ruthenium red to label any cellular structures with a physical connection to the extracellular space, and prepared for electron microscopic analysis as previously described (20). Besides their content of ∼20-nm LDL-like particles, the vacuoles contained small peripheral vesicles (∼50–70 nm indicated with arrows) identifying the vacuoles as multivesicular body endosomes. The inset shows an example of a smaller multivesicular body endosome, which usually contained more vesicles. The vacuoles and their content did not stain with ruthenium red showing that the vacuoles were not connected to the extracellular space. B shows macrophages that were incubated 1 day with the fluid-phase tracer, 1 mg/mL horseradish peroxidase, rather than LDL in the presence of 0.5 μM bafilomycin A1, 1 μg/mL PMA, and 2 mg/mL mannan to block receptor-mediated uptake of the horseradish peroxidase. Multivesicular body endosomes show electron dense horseradish peroxidase reaction complex surrounding the multivesicular body electron lucent vesicles.

![Image](380x245 to 500x377)

**FIG. 7.** Immunogold labeling of LDL contained in bafilomycin A1-induced vacuoles. Macrophages were incubated 1 day with 2 mg/mL LDL plus 1 μg/mL PMA with (A) or without (B) 0.5 μM bafilomycin A1. Both sets of macrophages were rinsed, fixed, and embedded in plastic for thin sectioning and immunocytochemistry as described previously (20). LDL was immunogold-labeled with rabbit anti-human LDL antiserum and goat F(ab)₂ anti-rabbit IgG conjugated to 10-nm gold particles. LDL was localized to the bafilomycin A1-induced vacuoles (arrows) confirming that the LDL-like particles they contained were LDL. Immunogold labeling of LDL in the vacuole indicated by the different arrow in A is shown at higher magnification in the inset. Lipid droplets (arrowheads) rather than LDL-containing vacuoles accumulated in control macrophages incubated with LDL and PMA without bafilomycin A1 (B).

![Image](382x483)

**FIG. 8.** Confocal fluorescence microscopy of Dil-LDL and endocytic compartment markers. Macrophages were incubated 1 day with 1 μg/mL PMA, 0.5 μM bafilomycin A1, and a mixture of 0.2 mg DI-LDL and 2 mg/mL LDL. Then, macrophages were rinsed, fixed, and labeled with either mouse monoclonal anti-human cathepsin D, anti-human LAMP-1, anti-human EEA1, or purified mouse IgG1 serving as a negative control. Mouse antibodies were then labeled with Alexa 488-conjugated goat anti-mouse IgG. LAMP-1 (a membrane protein) showed staining (green) in a ring-like pattern at the periphery of most DI-LDL-positive (red) structures (A). The early-endosome marker EEA1 (green) (B) and lysosome marker cathepsin D (green) (C) exhibit ring-like or filled-round patterns distinct from DI-LDL-positive structures (red). The bar is 5.0 μm.
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TABLE V
Effect of ketoconazole on cholesterol accumulation by PMA-activated macrophages

| Condition                                | Cholesterol |
|------------------------------------------|-------------|
|                                          | Total       | Unesterified | Esterified | cholesterol |
|                                          | nmol/mg cell protein | %          |           |            |
| No addition                              | 78 ± 2      | 75 ± 2       | 3 ± 1      | 4 ± 1       |
| LDL                                      | 120 ± 3     | 98 ± 2       | 22 ± 3     | 18 ± 2      |
| LDL + PMA                                | 472 ± 12    | 148 ± 2      | 324 ± 9    | 69 ± 0      |
| LDL + PMA + ketoconazole                 | 542 ± 13    | 325 ± 1      | 217 ± 12   | 40 ± 1      |

Macrophages were incubated 2 days with RPMI 1640 and the indicated additions of 2 mg/ml LDL, 1 μg/ml PMA, and 20 μM ketoconazole. Following incubations, macrophages were rinsed, harvested, and analyzed for their protein and cholesterol contents.

- Macrophage uptake of LDL by macropinocytosis also is relevant to immune functioning of macrophages and dendritic cells within the atherosclerotic plaque. We have observed that like monocyte-derived macrophages, monocyte-derived dendritic cells show PMA-stimulated uptake of LDL. Macropinocytosis is one mechanism by which macrophages and dendritic cells internalize extracellular antigens for processing and presentation of the processed antigens to T cells in complex with both class I and class II MHC molecules (69–71).
- Thus, as we have shown that macrophages internalize large amounts of LDL by macropinocytosis, it is possible that these same macrophages process and present LDL-derived peptide and lipid antigens to immune cells contributing to the immune responses occurring within atherosclerotic plaques (72).

Macropinosomes and multivesicular bodies can play an organelle considered to be a late endosome. The LDL-containing multivesicular bodies showed the late endosome/lysosome marker, LAMP-1, but not the early endosome marker, EEA1, or the lysosome marker, cathepsin D. Thus, in our study, bafilomycin A1 blocked transport of LDL from late endosomal multivesicular bodies to lysosomes.

Ketoconazole, an agent that blocks transport of lipoprotein-derived cholesterol out of lysosomes (34, 35), increased the unesterified cholesterol content of PMA-activated macrophages during macropinocytosis of LDL. This was similar to what we reported previously for scavenger-receptor mediated uptake of acetylated LDL by inactivated macrophages (35). This finding suggests that processing of cholesterol derived from either recepto- or fluid-phase-mediated lipoprotein uptake enters a common cholesterol trafficking pathway that is susceptible to ketoconazole inhibition.

LDL was maximally concentrated (i.e. LDL particles were packed side by side) within the smaller vacuoles of bafilomycin A1-treated PMA-activated macrophages. Thus, we were able to calculate that within the vacuoles, LDL had concentrated 43-fold over the 2 mg/ml LDL concentration originally added to the culture medium. Macropinocytotic vacuoles decreased in size over time. A 3.5-μm diameter macropinocytotic vacuole would have to shrink only to 1 μm to produce this degree of LDL concentration. Reduction in macropinocytotic vacuolar size would require reduction of both the limiting membrane of the macropinocytotic vacuole and the vacuolar water content. The mechanism of water removal from shrinking vacuoles remains to be determined. However, our findings suggest that macropinocytotic vacuoles may reduce their limiting membrane by transforming into multivesicular bodies. The internal vesicles of multivesicular bodies are formed from infolding of the limiting multivesicular body membrane (53). Less than 10% of the volume of a 1-μm multivesicular body would be occupied by vesicles of the size we observed in the LDL-containing multivesicular bodies in order to accommodate the amount of internalized membrane necessary to reduce a macropinocytotic vacuole diameter from 3.5 μm to 1 μm. Thus, transformation of macropinocytotic vacuoles into multivesicular bodies is a mechanism that can account for both the size reduction of macropinocytotic vacuoles and the concentration of LDL within multivesicular bodies.

Identification of macropinocytosis as an endocytic pathway that mediates macrophage foam cell formation has many implications for the study of the pathogenesis of atherosclerosis. Macrophage macropinocytosis of LDL is relevant to how atherosclerosis (54–59). Viral and bacterial infectious agents can stimulate macropinocytosis, in some cases by secreting toxins that activate cellular GTPases (60–66). M-CSF stimulates macropinocytosis in mouse macrophages (40). Furthermore, modified LDLs, acetylated LDL and oxidized LDL, have been shown to stimulate macropinocytosis and be internalized bound to forming macropinosomes in pigeon monocyte-derived macrophages (16). When native LDL is co-incubated at moderately high concentrations (50–100 μg/ml) with macrophages and macropinocytosis is stimulated by acetylated LDL, the native LDL is internalized via macropinosomes, with some of the LDL possibly entering in the fluid-phase (67).

In conclusion, we have identified macropinocytosis as the endocytic pathway that functions to generate foam cells when activated human monocyte-derived macrophages are incubated with native LDL. Uptake of LDL through this fluid-phase pathway does not depend on modification of LDL or the binding of LDL to receptors for its internalization. We have shown that this macropinocytosis mechanism of foam cell formation depends on actin, microtubules in part, myosin, protein kinase C, Rho-family GTPase, and phosphatidylinositol 3-kinase. Because macropinocytosis is not limited to macrophages, it will be of interest to learn whether other vascular wall cells such as smooth muscle and endothelial cells can also macropinocytose LDL.

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