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-rich 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 macropinocytosis 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 macropinocytosis uptake of fluid and LDL. Also, nystatin and filipin, inhibitors of macropinocytosis 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 DiI-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 undegraded 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 macrophage 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 micropinocytotic vesicles (micropinocytosis) or macropinocytotic 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 envelopes 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-

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1 The abbreviations used are: LDL, low density lipoprotein; PMA, phorbol 12-myristate 13-acetate; BSA, bovine serum albumin; DPBS, Dulbecco’s phosphate-buffered saline; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; N.A., numerical aperture.

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phatidylinositol 3-kinase and the Rab family GTPases, 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). Macropino- cytosis can occur in clathrin-associated, caveolin-associated, or non-clathrin-caveolin-associated vesicles (no larger than ~0.1 um). The latter two modes of macropinocytosis usually occur in lipid-raft domains of the plasma membrane (19). Macropinocy- tosis is an actin-dependent endocytic process while micropino- cytosis 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 macropino- cytosis mediated by Rho-GTPase and phatidylinositol 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 mono- cyte-derived macrophage cultures were rinsed three times with RPMI 1640 medium and then incubated at 37 °C for the indicated times with the indicated additions to RPMI 1640 medium with 5% FBS. All sera served macrophages grown in a T-25 flask with a 0.17-mm thick cover-glass bottoms (Electron Microscopy Sciences). Five microwells were incubated with [3H]sucrose in parallel incubations, and gave a background count less than 3% of the [3H]sucrose radioactivity detected in control unstimulated 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 standard electron microscopy of LDL macrophages, we used a 12-μg/ml culture plates. After incubations with PMA and LDL, macrophages were fixed, embedded in LR White resin, and prepared for immunogold labeling as described previously (20) except that 1% dry skim milk instead of 1% BSA was used to block nonspecific staining. LDL was labeled by incubating thin sections with 10 μg/ml affinity-purified rabbit anti-human LDL antibody (cat. DT-905, BTI), and then with a 1:10 dilution of 10 ng/ml of rabbit IgG (Sigma) in 1% dry skim milk. For controls, the rabbit anti-human LDL antibody was substituted with the same concentration of purified rabbit IgG. This control showed no labeling.

For chemical analysis of the fluid-phase tracer horseradish per- oxidase, macrophages were preincubated 30 min with 2 mg/ml mannannan, and then incubated 24 h with 1 μg/ml PMA, 0.5 μg haefolinic acid A1, 1 mg/ml dextran, and 2 μg/ml human mannannan. Macropinosomes have been shown to block receptor-mediated but not fluid-phase uptake of horse- radish peroxidase (25). After incubation, macrophages were rinsed three times with DPBS plus Ca^2+, Mg^2+, and 0.2% BSA, followed by three times with DPBS plus Ca^2+ and Mg^2+ all at 4 °C. Then, macro- phages were fixed 1 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature. Horseradish peroxidase was localized with 512 long-working

Time-lapse Microscopy—Time-lapse confocal fluorescence images were obtained with a >65 planapo objective lens (1.4 N.A.) mounted on an Olympus IX81 inverted microscope equipped with a Yokogawa spinning disk confocal system. Macropinosomes 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 DiI-LDL fluorescence in living macrophages were acquired with 512 × 512 pixel resolution. DiI label was excited with the 588-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 image thickness was about 1 μm. Media Cybernetics QED InVivo software was used for stack preparation and conversion to QuickTime format.

Time-lapse phase-contrast video microscopy was carried out by observing macrophages grown in a T-25 flask with a ×20 long-working distance planfouler objective lens (0.4 N.A.) mounted on an Olympus L70 inverted microscope. 5% CO_2/95% hydrated air and 37 °C were maintained in an enclosed chamber during recording from the macrophages. Analogue video images were recorded using a Dage-MTI CD100 camera and JVC BR9000-U VHS video recorder. Video images were captured

Assay of Cholesterol Esteresterification—[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, mac-rophages were rinsed, lipids were extracted with hexane/isopropyl alco- hol, and macropinosomes were harvested for determination of their protein content. Cholesteryl ester synthesis was determined as described previously by quantifying [14C]oleate incorporation into cholesteryl esters, which were separated by thin layer chromatography (24). Measurement of Fluid-phase Endocytosis—Fluid-phase endocytosis was determined by incubating macrophages with 0.8 mmol/ml H2sucrose (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+, Mg^2+, and 0.2% BSA, and then three times with DPBS plus Ca^2+ and Mg^2+. Macropinosomes were scraped from wells into 1 ml of distilled water. 1 Hr macrophages were washed in 1 ml glass culture wells using a liquid scintillation counter. Macropinosome protein content was de-termined with another aliquot as described above. The amount of fluid accumulated by macropinosomes was calculated by dividing the amount of H2sucrose present in the macrophages following incubations (nmol/ml cell protein) by the concentration of H2sucrose in the medium. Cell-free wells were incubated with H2sucrose in parallel incubations, and gave a background count less than 3% of the H2sucrose radioactivity detected in control unstimulated macrophages.

Cell-Associated [3H]Sucrose Uptake—[3H]Sucrose taken up by macrophages was calculated by dividing the amount of [3H]Sucrose present in the macrophages following incubations (nmol/mg cell protein) by the concentration of [3H]Sucrose in the medium. Cell-free wells were incubated with [3H]Sucrose in parallel incubations, and gave a background count less than 3% of the [3H]Sucrose radioactivity detected in control unstimulated macrophages.

Cell-Associated [14C]Oleate Incorporation into Cholesterol Esters—Macropinosomes were preincubated 30 min with 2 mg/ml mannanan, and then incubated 24 h with 1 μg/ml PMA, 0.5 μg haefolinic acid A1, 1 mg/ml dextran, and 2 μg/ml human mannannan. Macropinosomes have been shown to block receptor-mediated but not fluid-phase uptake of horseradish peroxidase (25). After incubation, macrophages were rinsed three times with DPBS plus Ca^2+, Mg^2+, and 0.2% BSA, followed by three times with DPBS plus Ca^2+ and Mg^2+ all at 4 °C. Then, macro-phages were fixed 1 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature. Horseradish peroxidase was localized with 512 long-working

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and digitized using MGI VideoWave software and converted to Quick-Time format using QuickTime. When viewed at standard rates (i.e. 30 frames/second), the movies prepared from the time-lapse phase-contrast videos are ×120 real time, and the movies prepared from the time-lapse confocal fluorescence images are ×300 real time.

Confocal Microscopic Immunocytochemical Analysis of Endocytic Pathway Markers—Macrophages, cultured on 13-mm diameter glass cover slips, were incubated 24 h with 1 μM PMA, 0.5 μM bafilomycin A1, and a mixture of 0.2 mg/ml DiI-LDL and 2 mg/ml unlabeled LDL. Following incubations, macrophages were rinsed three times with phosphate-buffered saline containing 10% fetal bovine serum (also used for later rinses), and then fixed 10 min with 2% paraformaldehyde all at 4 °C. After 2 rinses, macrophages were permeabilized with 0.2% Triton X-100 for 5 min at room temperature also used for all subsequent staining steps. Next, macrophages were rinsed three times and incubated 1 h with primary mouse monoclonal antibodies (anti-human cathepsin D, clone C5, from Biogenes; anti-human LAMP-1, clone H4A3, from the University of Iowa Developmental Studies Hybridoma Bank; anti-human EEA1, clone 14, from BD Transduction Laboratories; and negative control purified mouse IgG1 from Dako) diluted in the rinsing solution containing 0.2% Triton X-100. For this and subsequent incubations, coverslips were inverted over a drop of antibody labeling solution. After three 5-min rinses, primary mouse monoclonal antibodies were washed for 1 h with Alexa 488-conjugated goat anti-mouse IgG (2.6 μg/ml diluted in rinsing solution/0.1% Tween 20, Molecular Probes). Following three rinses, coverslip cultures were mounted on glass slides with ProLong mounting medium obtained from Molecular Probes.

To assess the localization of DiI-LDL and macrophage subcellular compartment markers, images were obtained with a Leica SP1 confocal system equipped with UV-Vis lasers from Leica Microsystems. Images were acquired sequentially using a 488-nm laser line and emission between 490 and 560 nm for Alexa 488 and a 568-nm laser line and emission between 575–690 nm for DiI. High-resolution (100 nm/pixel) Z-series images were obtained from the macrophages with a ×63 (1.4 N.A.) planapochromat oil-immersion objective and analyzed for colocalization. Overlay images were assembled, zoomed, and cropped using Imaris 4.0 software form Bitplane AG.

Statistical Analysis—All data are presented as the mean ± S.E. The means were determined from three culture wells for each data point. The standard error is not shown for line graphs when its value was less than the symbol height.

RESULTS

Actin and Microtubule Dependence of LDL Uptake and Fluid Endocytosis—PMA activation of human monocyte-derived macrophages stimulated sustained fluid endocytosis in these cells as measured by the fluid-phase tracer [3H]sucrose (Fig. 1). Previously, we showed that fluid endocytosis mediated the LDL uptake and cholesterol accumulation in these PMA-activated macrophages (6). To learn whether fluid-phase endocytosis of LDL depended on actin microfilaments and tubulin microtubules, we examined the effects of CytoD and nocodazole inhibitors on this process. Inhibition of microtubule function with nocodazole decreased [3H]sucrose, 125I-LDL, uptake, and macrophage cholesterol accumulation by about 50% (Table I). This result suggests that there were both microtubule-dependent and microtubule-independent pathways that functioned in fluid-phase uptake of LDL. This result was not due to an insufficient amount of nocodazole inhibitor as shown by the dose response effect of increasing nocodazole concentration producing a maximum 50% inhibition of macrophage cholesterol accumulation (Fig. 2A).

Microtubules have been implicated in transport from endosomes to lysosomes in non-macrophage cell types (27, 28). Thus, it is possible that the inhibition of fluid-phase LDL uptake represented inhibition of transport of LDL to lysosomes. Blocking LDL transport to lysosomes could secondarily impede LDL from entering macrophages. To test this possibility, we examined the time course of nocodazole inhibition of cholesterol accumulation by PMA-activated macrophages incubated with LDL. If nocodazole inhibited cholesterol accumulation only at later times, this would suggest that nocodazole inhibition of cholesterol accumulation was secondary to 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-depe...
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 \( ^{3}H \) sucrose, \( ^{125}I \)-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 (\( \sim 0.5-5.0 \, \mu m \) 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 consistent with sustained fluid-phase endocytosis as discussed above in Fig. 1. Nocodazole inhibited macropinocytosis in some but not all macrophages consistent with chemical data above showing that microtubule-dependent and microtubule-independent macropinocytosis of LDL occurred. Incubation with LDL alone (2 mg/ml) did not stimulate membrane ruffling and macropinosome formation (Fig. 3, A–D, and Supplemental Movie 1). When PMA was added to the macrophages with or without LDL, regardless of the order of addition, membrane ruffling and macropinosome formation were stimulated (Fig. 3, E–H, and Supplemental Movie 2). However, with inclusion of LDL, the PMA-activated macrophages accumulated phase-reactive lipid (data not shown) as we showed previously (6). This did not occur in PMA-activated macrophages incubated without LDL.

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 rather than membrane-bound uptake of DiI-LDL. No fluorescence was visible when macrophages were incubated without DiI-LDL. Prolonged observation of DiI-LDL in macrophages was limited by photobleaching of the DiI.

**Micropinocytic 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 phosphatidylinositol 3-kinase inhibitor, LY294002, completely blocked the fluid and \( ^{125}I \)-LDL macrophage uptake that was stimulated by PMA (Table III). Also, two inhibitors of macropinocytosis that originate from lipid-raft plasma membrane domains, nystatin (50 \( \mu g/ml \)) and filipin (1 \( \mu g/ml \), the highest concentration that was not toxic), both failed to inhibit...
Macrophages were incubated 1 day with either 500 μg/ml 125I-LDL in experiment 1 or 0.8 nmol/ml [3H]sucrose in experiment 2 and the indicated additions. Following incubations, media were collected, and then macrophages were rinsed and harvested to measure their protein and 125I-LDL or [3H]sucrose content depending on the experiment. Fluid uptake was determined from [3H]sucrose content as described under “Experimental Procedures.” Total 125I-LDL uptake is shown and was calculated as the sum of the cell-associated and degraded 125I-LDL (i.e., trichloroacetic acid-soluble 125I-LDL in the media).

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Macrophage total uptake</th>
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<tbody>
<tr>
<td>125I-LDL</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>125I-LDL + PMA (1 μg/ml)</td>
<td>15.7 ± 1.1</td>
</tr>
<tr>
<td>125I-LDL + PMA + LY294002 (50 μM)</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>[3H]Sucrose</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>[3H]Sucrose + PMA (1 μg/ml)</td>
<td>25.1 ± 2.1</td>
</tr>
<tr>
<td>[3H]Sucrose + PMA + LY294002 (50 μM)</td>
<td>3.2 ± 0.2</td>
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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 degradation 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 DiI-LDL, PMA, and bafilomycin A1 for 1 day. The multivesicular body endosomes with DiI-LDL showed immunofluorescent labeling

![Fig. 3. Time-lapse phase-contrast video microscopy of macrophage macropinocytosis.](image)

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.](image)

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.
Macropinocytosis is thus different from actin-dependent phagocytosis as the endocytic pathway that functions in sustained fluid-phase uptake of LDL by PMA-activated macrophages.

In this report we have identified macropinocytosis as the endocytic pathway that functions in sustained fluid-phase uptake of LDL by PMA-activated macrophages. We recently showed that activated macrophages take up native LDL only by receptor-mediated uptake of modified LDL. We recently showed that activated macrophages take up native LDL only by receptor-mediated uptake of modified LDL. We recently showed that activated macrophages take up native LDL only by receptor-mediated uptake of modified LDL. We recently showed that activated macrophages take up native LDL only by receptor-mediated uptake of modified LDL.

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).

Mycophenolate, 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 cytchalasin 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 originates 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, Rh, 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 Rh-family GTPase 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 the two 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 macropinocytic formation.

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 be 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 inhibitor, ML-9, only blocked macrophage cholesterol accumulation that did not depend on microtubule function.
were collected, and then macrophages were rinsed and harvested to measure their protein, cell-associated 125I-LDL, and the 125I-LDL degraded by the macrophages (i.e., trichloroacetic acid-soluble 125I-LDL in the media). Total 125I-LDL uptake was calculated as the sum of the cell-associated and degraded 125I-LDL.

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell-associated 125I-LDL</th>
<th>Degraded 125I-LDL</th>
<th>Total 125I-LDL mg protein/10^6 cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-LDL + PMA</td>
<td>2.7 ± 0.2</td>
<td>13.1 ± 1.2</td>
<td>15.7 ± 1.1</td>
</tr>
<tr>
<td>125I-LDL + PMA + bafilomycin A1 (0.5 μM)</td>
<td>7.1 ± 0.6</td>
<td>2.0 ± 0.2</td>
<td>9.1 ± 0.7</td>
</tr>
</tbody>
</table>

Macrophinocytosis Mediates LDL-induced Foam Cell Formation

**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 without 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 macrophages 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.

**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 (arrow) 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).

**Fig. 8.** Confocal fluorescence microscopy of DiI-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 DiI-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 DiI-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 DiI-LDL-positive structures (red). The bar is 5.0 μm.

The fate of macropinosomes varies in different cell types. In epidermal growth factor-stimulated A431 human epithelial cells, a major portion of macropinocytosed marker appears to be 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, degraded LDL and the fluid-phase tracer horseradish peroxidase accumulated within macropino-
Macropinocytosis Mediates LDL-induced Foam Cell Formation

Effect of ketoconazole on cholesterol accumulation by PMA-activated macrophages

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cholesterol</th>
<th>Total</th>
<th>Unesterified</th>
<th>Esterified</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/mg cell protein</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>78 ± 2</td>
<td>75 ± 2</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>120 ± 3</td>
<td>98 ± 2</td>
<td>22 ± 3</td>
<td>18 ± 2</td>
<td></td>
</tr>
<tr>
<td>LDL+PMA</td>
<td>472 ± 12</td>
<td>148 ± 2</td>
<td>324 ± 9</td>
<td>69 ± 0</td>
<td></td>
</tr>
<tr>
<td>LDL+PMA+ketoconazole</td>
<td>542 ± 13</td>
<td>225 ± 1</td>
<td>217 ± 13</td>
<td>40 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Following incubations, macrophages were rinsed, harvested, and analyzed for their protein and cholesterol contents.

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).

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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REFERENCES


2 B. Zhao and H. Kruth, unpublished observations.