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Macropinocytosis contributes to the macrophage foam cell formation in RAW264.7 cells

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The key event in the atherosclerosis development is the lipids uptake by macrophage and the formation of foam cell in subendothelial arterial space. Besides the uptake of modified low-density lipoprotein (LDL) by scavenger receptor-mediated endocytosis, macrophages possess constitutive macropinocytosis, which is capable of taking up a large quantity of solute. Macrophage foam cell formation could be induced in RAW264.7 cells by increasing the serum concentration in the culture medium. Foam cell formation induced by serum could be blocked by phosphoinositide 3-kinase inhibitor, LY294002 or wortmannin, which inhibited macropinocytosis but not receptor-mediated endocytosis. Further analysis indicated that macropinocytosis took place at the gangliosides-enriched membrane area. Cholesterol depletion by β-methylcyclodextrin-blocked macropinocytosis without affecting scavenger receptor-mediated endocytosis of modified LDLs. These results suggested that macropinocytosis might be one of the important mechanisms for lipid uptake in macrophage. And it made significant contribution to the lipid accumulation and foam cell formation.

Keywords macrophage; macropinocytosis; foam cell; PI3-kinase; atherosclerosis

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Introduction

Atherosclerosis is a chronic inflammatory process initiated by the accumulation of macrophage foam cell in the subendothelial arterial space [1]. The circulating monocytes are attracted into the arterial intima by modified low-density lipoprotein (LDL) and differentiate into macrophages, which take up the modified LDL to form macrophage foam cell [2]. The macrophage scavenger receptors have been considered as the major cellular surface receptors in macrophage to take up the modified LDL through the receptor-mediated endocytosis [3]. Scavenger receptor class A and CD36 have been considered as the two most important scavenger receptors in the foam cell formation [4]. Foam cell is considered to be the major culprit in atherosclerosis. Mice that lack macrophages are resistant to atherosclerosis [5,6]. However, it has been reported that deletion of both receptors could not avoid atherosclerosis and macrophages could still form foam cells in hyperlipidemic mice [7]. Hence, there should be other mechanisms of lipid uptake in macrophage *in vivo*.

In addition to receptor-mediated endocytosis, macrophages possess high-level macropinocytic activity [8]. Macropinocytosis is an actin-dependent process including membrane ruffling and the formation of membrane vesicles [9]. Unlike receptor-mediated endocytosis that concentrates specific solute by the receptor into clathrin-coated pits for internalization, macropinocytosis takes up the solute by engulfing it with membrane ruffling [10]. The macropinosomes formed by macropinocytosis are heterogeneous in size and much larger than clathrin-coated endocytotic vesicles [11]. At high solute concentration, the internalization of solute via macropinocytosis is much more efficient than micropinocytosis through clathrin-coated vesicles [12].

Because of its high efficiency and non-selective nature in taking up the solute, macropinocytosis could be the

lipid uptake pathway other than the receptor-mediated pathway in macrophage. To investigate the role of macropinocytosis in the formation of macrophage foam cell, we used a mouse macrophage-like cell line RAW264.7 cells. Macropinocytosis requires PI3-kinase (phosphoinositide 3 kinase) activity and the inhibition of PI3-kinase blocks macropinocytosis, but not receptor-mediated endocytosis [13]. In RAW264.7 cells, macropinocytosis and cell formation were inhibited by the PI3-kinase inhibitors, LY294002. Macropinocytosis took place at the membrane where it is relatively enriched with glycosphingolipids. Furthermore, the depletion of membrane cholesterol by β-methylcyclodextrin blocked the macropinocytosis but not the receptor-mediated modified LDL uptake. Our results suggested that macropinocytosis played an important role in the lipid accumulation and foam cell formation in macrophage.

Materials and Methods

Materials

OxLDL (oxidized LDL), lipoprotein-deficient serum, and DiO-Ac-LDL (3,3′-dioctadecyloxacarbocyanine perchlorate-labeled acetylated LDL) were from Biomedical Technologies (Stoughton, USA). PIP3 (dipalmitoyl-L-α-phosphatidylinositol-3,4,5-triphosphate), LY294002 and wortmannin were from Merck (San Diego, USA). FITC-labeled transferrin, FITC or Alexa Fluor 546-labeled dextran (10,000 Da, anionic, unfixable or lysine-fixable) and DMEM (Dulbecco's modified Eagle's medium) were from Invitrogen (Carlsbad, USA). Cytochalasin B, FITC-CTB (cholera toxin B subunit), β-methylcyclodextrin, and TRITC-labeled phalloidin were from Sigma (St. Louis, USA).

Cell culture

Mouse RAW264.7 cell was cultured in DMEM with 10% FBS (fetal bovine serum). Foam cell was induced by adding 50 μg/ml oxLDL to the culture medium or by increasing FBS to 40% after RAW264.7 cells reached cell–cell contact. The culture medium was changed every day. Foam cells were visualized by staining the cells with Oil-red-O and the cellular cholesterol was determined by enzymatic measurement [14]. The cellular protein was measured by Lowry method [15]. The lipid contents could be determined by extraction of stained Oil-red-O with isopropanol and measured the OD at 510 nm [16].

Receptor-mediated endocytosis

DiO-Ac-LDL uptake was performed as described by Cao *et al*. [17]. RAW264.7 cells were pretreated with or without the PI3-kinase inhibitor (LY294002 or wortmannin) in DMEM containing 2% lipoprotein deficient serum for 30 min at 37°C. DiO-Ac-LDL was then added at a final concentration of 2 μg/ml. After incubation for another 6 h at 37°C, the cells were washed with PBS and analyzed by FACScan flow cytometer (BD Biosciences, Franklin Lakes, USA).

For transferrin uptake, the RAW264.7 cells were pretreated with or without LY294002 in DMEM containing 1% BSA for 30 min at 37°C. FITC-conjugated transferrin (5 μg/ml in DMEM with 1% BSA) was then added to the cells for another 15 or 30 min. The cells were then washed twice with cold acidic DMEM (pH 3.5) plus 1% BSA and four times with cold PBS, and analyzed by FACScan flow cytometer.

Macropinocytosis

Macropinocytosis analysis was performed according to Duclos *et al*. [18]. For FACScan flow cytometer analysis of dextran uptake, the RAW264.7 cells were pretreated with or without LY294002 in DMEM for 30 min and then incubated with 150 μg/ml FITC-dextran in DMEM for additional 30 min. Cells were then washed, fixed, and prepared for flow cytometry analysis.

For observation of macropinosomes, the RAW264.7 cells cultured on coverslip were pretreated with or without 20 μM LY294002 in DMEM for 30 min and then incubated with 1 mg/ml lysine-fixable FITC-dextran for 30 min. The cells were then washed and fixed for confocal microscopic observation (Leica SP2, Mannheim, Germany).

Time-lapse phase imaging of macropinocytosis

According to Kruth *et al*. [19], RAW264.7 cells grown on coverslip were observed using a Leica AS MDW Multi Dimension Workstation with a 63X/1.3 NA glycerol objective. The images were taken every 10 s.

Immunofluorescence staining

According to Wang *et al*. [20], for observation of actin filaments and glycosphingolipids-enriched membrane, RAW264.7 cells cultured on coverslip were washed with PBS, fixed with 3.7% paraformaldehyde and double stained with phalloidin and FITC-CTB.

For observation of macropinosomes and glycosphingolipids-enriched membrane, RAW264.7 cells were incubated with 1 mg/ml lysine-fixable Alexa Fluor 546-dextran for

1 min. After washed with PBS and fixed with paraformaldehyde, the cells were stained with FITC-CTB.

Statistical analysis

Data were presented as mean ± SD. Differences were analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Induction of foam cell formation in cultured RAW264.7 cells

The uptake of modified LDL by macrophage induces foam cell formation and promotes the development of atherosclerosis [21]. In cultured RAW264.7 cells, the addition of oxLDL to the culture medium induced the foam cell formation as the cytoplasmic lipid droplets accumulation was visibly increased [**Fig. 1(A)**]. FBS supplemented in the culture medium is also a rich source of cholesterol and lipids. In fact, after the cultured RAW264.7 cells reach cell–cell contact, the prolonged culture in 10% FBS medium could induce a low level foam cell formation without the addition of external oxLDL [**Fig. 1(A)**]. By switching to medium containing 40% FBS after RAW264.7 cells reached cell–cell contact, the cells were exposed to a condition of high lipids concentration and more foam cells were induced [**Fig. 1(A)**]. In 40% FBS medium, the induction of foam cell was gradually increased [**Fig. 1(B)**]. During this process, both the lipid droplets accumulation and the cellular cholesterol level were markedly increased [**Fig. 1(B,C)**]. The cell number, however, was decreased as more foam cells were induced and became less adherent [**Fig. 1(C)**].

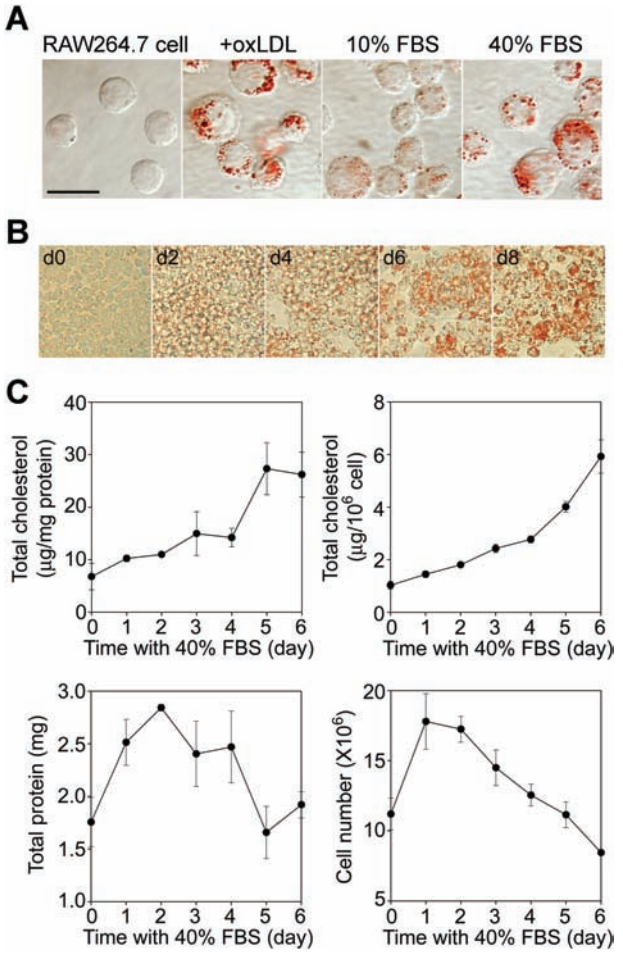

Figure 1 The induction of foam cell formation in RAW264.7 cells (A) RAW264.7 cells, after reaching cell–cell contact, were induced for 6 days in indicated medium, replated for 2 h and stained with Oil-red-O. RAW264.7 cell, cells before induction; +oxLDL, cells induced with 10% FBS medium plus 50 μg/ml oxLDL; 10% FBS, cells induced with 10% FBS medium; 40% FBS, cells induced with 40% FBS medium. Bar = 10 μm. (B) After induction with 40% FBS medium for 0, 2, 4, 6, 8 days (d0, d2, d4, d6, and d8), cells were stained with Oil-Red-O. Magnification, 40×. (C) Cells induced with 40% FBS medium were harvested at indicated time for measuring cholesterol and protein. Cell numbers were counted every day.

Macropinocytosis and receptor-mediated endocytosis in RAW264.7 cells

The receptor-mediated uptake of modified LDL in macrophage is mostly mediated by scavenger receptors (class A and CD36) [4]. The modified LDL is bound by scavenger receptors on the cell surface and internalized through receptor-mediated endocytosis [22]. Fluorescence-labeled modified LDL, such as DiO-Ac-LDL, can be used to measure the scavenger receptor-mediated modified LDL uptake [23]. Macropinocytosis, on the other hand, is independent from cell surface receptors and can be measured by fluorescence-labeled dextran [24,25]. In murine bone marrow-derived macrophages, PI3-kinase inhibitor (LY294002 or wortmannin) blocks the cellular macropinocytosis without affecting scavenger receptor-mediated modified LDL uptake [13]. In RAW264.7 cells, the uptake of DiO-Ac-LDL was insensitive to the treatment of LY294002 or wortmannin, whereas the uptake of FITC-dextran was inhibited [**Fig. 2(A,B)**]. The uptake of transferrin, which is a standard control for receptor-mediated endocytosis [26], was not affected by LY294002 [**Fig. 2(C)**]. Further analysis showed that the uptake of DiO-Ac-LDL was insensitive to LY294002 even in prolonged incubation time or at higher DiO-Ac-LDL concentration [**Fig. 2(D,E)**].

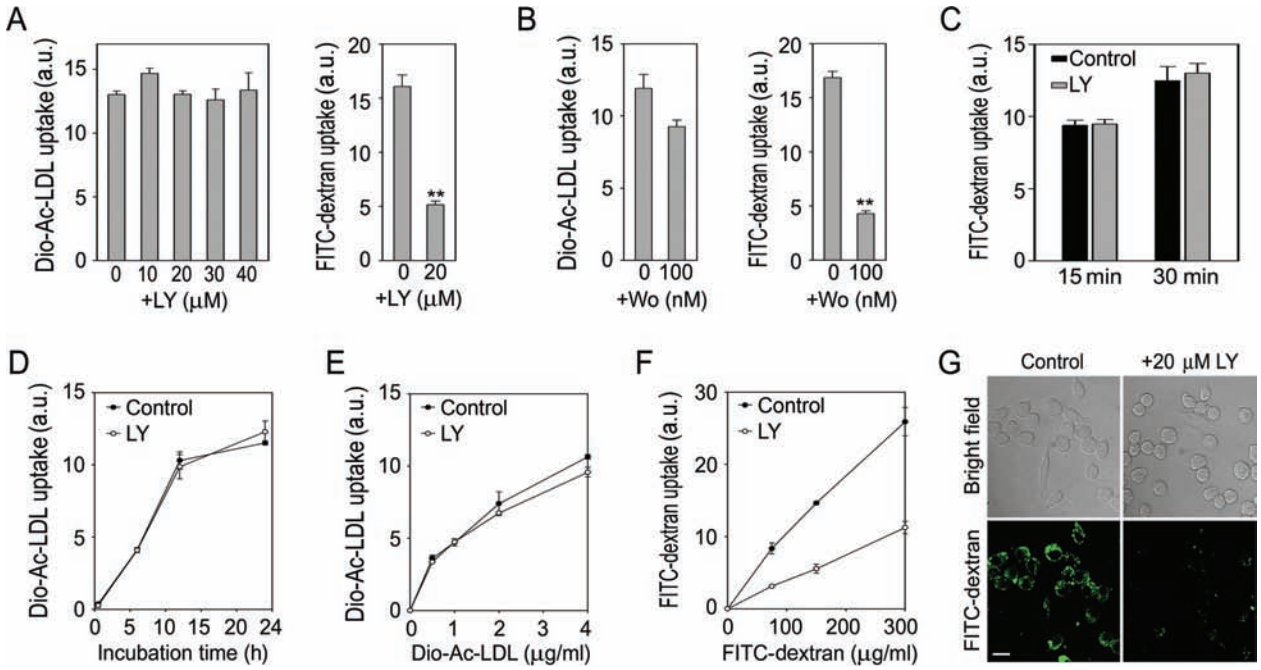

Figure 2 The inhibition of macropinocytosis but not receptor-mediated endocytosis by PI3-kinase inhibitor in RAW264.7 cells (A) The inhibition of FITC-dextran uptake, but not DiO-Ac-LDL uptake by LY294002. FITC-dextran and DiO-Ac-LDL uptake were measured by fluorescence intensity in RAW264.7 cells. $^{**}P < 0.01$. (B) The inhibition of FITC-dextran uptake, but not DiO-Ac-LDL uptake by wortmannin. $^{**}P < 0.01$. (C) Transferrin uptake in the presence (LY) or absence (control) of 20 μM LY294002. (D) Time course of DiO-Ac-LDL uptake in the presence or absence of 20 μM LY294002. RAW264.7 cells were incubated with 2 μg/ml DiO-Ac-LDL for indicated times. (E) RAW264.7 cells were incubated with DiO-Ac-LDL at indicated concentration for 6 h in the presence or absence of 20 μM LY294002. (F) RAW264.7 cells were incubated with FITC-dextran at indicated concentration for 30 min in the presence or absence of 20 μM LY294002. (G) RAW264.7 cells were pretreated with LY294002 (+20 μMLY) or without (control) for 30 min and lysine-fixable FITC-dextran was added for additional 30 min. FITC-dextran fluorescence was visualized by confocal microscope. Bar = 10 μm. a.u., arbitrary unit.

As no cell surface receptor is involved in macropinocytosis, the uptake of FITC-dextran by RAW264.7 cells was in linear proportion with FITC-dextran concentration [**Fig. 2(F)**]. The macropinosomes formed in RAW264.7 cells were clearly visible by confocal microscope [**Fig. 2(G)**]. With LY294002 treatment, number of FITC-dextran containing macropinosomes was greatly reduced [**Fig. 2(G)**]. These findings in RAW264.7 cells were consistent with the previous study in murine bone marrow-derived macrophages [13].

Inhibition of macrophage foam cell formation by LY294002

To assess the effect of macropinocytosis on the macrophage foam cell formation, RAW264.7 cells were induced to form foam cells by high serum concentration medium in the presence of LY294002. As shown in **Fig. 3(A)**, foam cell formation was blocked by LY294002, but not by MEK inhibitor, PD98059 or U0126. One of the most important functions of PI3-kinase is to catalyze the production of 3-phosphorylated phosphatidylinositol (PIP3) [27]. LY294002 inhibits PI3-kinase activity and blocks the production of PIP3. Indeed, the supplement of PIP3 to LY294002 treated RAW264.7 cells could reverse LY294002 blocked foam cell formation [**Fig. 3(A)**].

The inhibition of foam cell formation by LY294002 was dose-dependent [**Fig. 3(B)**]. Quantitative analysis of lipids storage in foam cell was measured by Oil-red-O absorption [**Fig. 3(C)**]. The cholesterol content in RAW264.7 cells was also determined. LY294002 markedly reduced the amount of cholesterol accumulated in RAW264.7 cells [**Fig. 3(D)**].

F-actin is involved in the process of macropinocytosis. Cytochalasin B, which depolymerizes the actin filaments, can inhibit macropinocytosis [28]. Similar to LY294002, the treatment of cytochalasin B in RAW264.7 cells could also inhibit foam cell formation and the lipids accumulation [**Fig. 3(B,E)**]. The inhibition of foam cell formation in RAW264.7 cell by LY294002 and cytochalasin B suggested that macropinocytosis provided significant contribution for cholesterol accumulation during high serum concentration-induced foam cell formation.

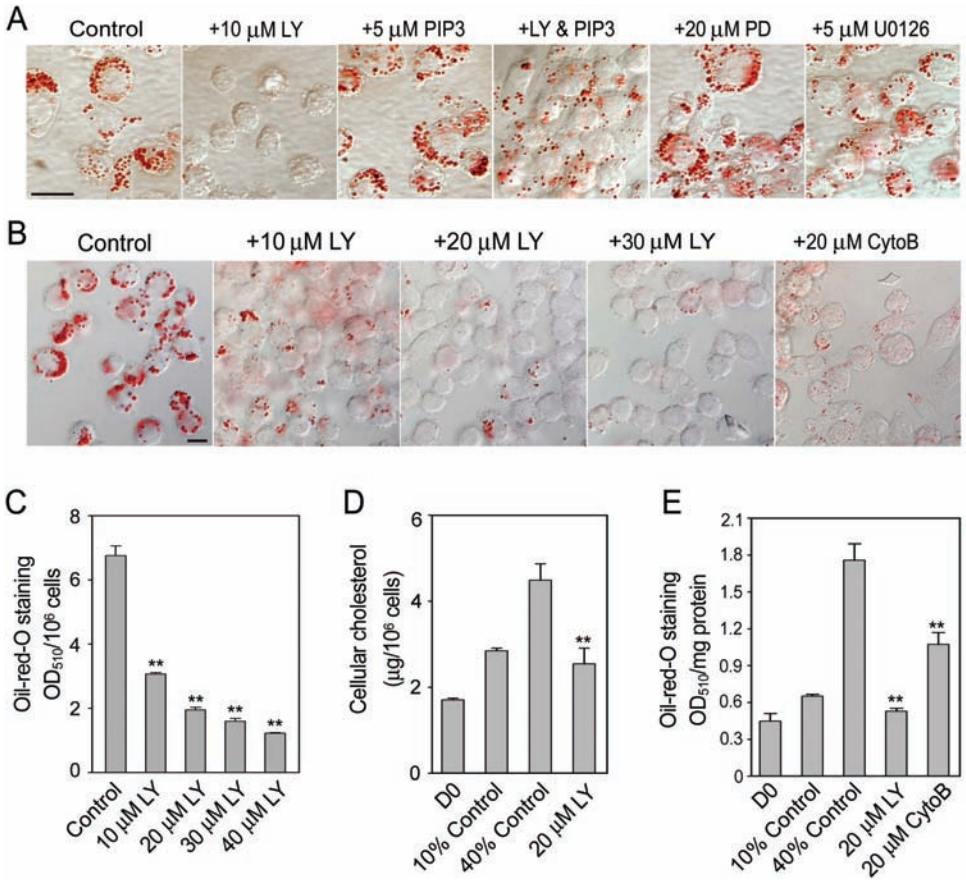

Figure 3 The inhibition of foam cell formation by LY294002 in RAW264.7 cells (A) RAW264.7 cells, after reaching cell–cell contact, were induced with 40% FBS medium for 4 days in the presence of LY294002 (+10 μM LY), PIP3 (+5 μM PIP3), both LY294002 and PIP3 (+LY & PIP3), PD98059 (+20 μM PD), U0126 (+5 μM U0126) or without (control). Cells were replated for 2 h and stained with Oil-red-O. Bar = 10 μm. (B) Dose-effect of LY294002 on foam cell formation. Cells were induced with 40% FBS medium in the presence of LY294002 at indicated concentration or 20 μM cytochalasin B and stained with Oil-red-O. Magnification, 100×. (C) RAW264.7 cells were induced and stained with Oil-red-O as in panel B. The Oil-red-O was extracted and OD_{510} was measured. $^{**}P < 0.01$. (D) Cellular cholesterol. D0, RAW264.7 cells at cell–cell contact; 10% control, 10% FBS-induced cells; 40% control, 40% FBS-induced cells; 20 μM LY, 40% FBS-induced cells in the presence of 20 μM LY294002. $^{**}P < 0.01$. (E) OD_{510} of Oil-red-O stained cells. D0, RAW264.7 cells at cell–cell contact; 10% control, 10% FBS-induced cells; 40% control, 40% FBS-induced cells; 20 μM LY, 40% FBS-induced cells in the presence of 20 μM LY294002. 20 μM CytoB, 40% FBS-induced cells in the presence of 20 μM cytochalasin B. $^{**}P < 0.01$.

Involvement of lipid rafts in the macropinocytosis of RAW264.7 cells

Lipid rafts, liquid-ordered plasma membrane microdomains enriched with cholesterol and sphingolipids [29], have been implicated to be involved in the macropinocytosis in A431 cells [30]. The process of macropinocytosis that begins with membrane ruffling and is followed by the formation of macropinosomes could be observed with live-cell imaging microscope [**Fig. 4(A)**]. Gangliosides (GM1), a kind glycosphingolipids, can be used as a lipid marker for lipid rafts and they can be bound by CTB [31]. Double staining for F-actin and lipid rafts showed that the serried F-actin membrane ruffling areas were also enriched with lipid rafts [**Fig. 4(B)**]. The macropinosomes, which were labeled by internalized Alexa Fluor 546-dextran, were surrounded by the membrane enriched with gangliosides [**Fig. 4(C)**].

Lipid rafts can be disrupted by cholesterol depletion with cholesterol-binding β-methylcyclodextrin [29]. By treating RAW264.7 cells with β-methylcyclodextrin, macropinocytosis, measured by the uptake of FITC-dextran, was greatly reduced [**Fig. 4(D)**]. In contrast, the uptake of DiO-Ac-LDL was not significantly inhibited by the treatment of β-methylcyclodextrin [**Fig. 4(E)**]. The DiO-Ac-LDL uptake in 20 mM β-methylcyclodextrin treated RAW264.7 cells was slightly decreased due to the cytotoxicity of the high β-methylcyclodextrin

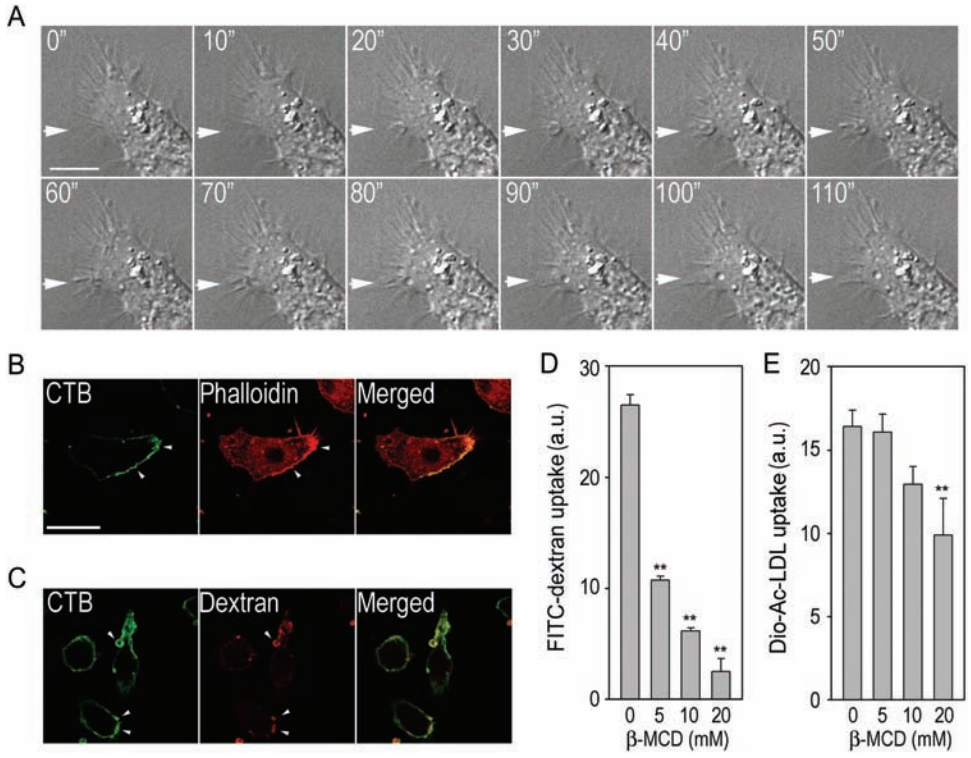

Figure 4 The involvement of gangliosides-enriched membrane in macropinocytosis (A) Live-cell images of macropinocytosis. Arrow head indicates a macropinosome formation process in RAW264.7 cell. RAW264.7 cells grown on coverslip were observed using a Multi Dimension Workstation and images were taken every 10 s. Bar = 10 μm. (B) Enrichment of F-actin at gangliosides-enriched membrane. RAW264.7 cell was stained with FITC-CTB and TRITC-phalloidin (phalloidin). Bar = 10 μm. (C) Macropinosome formation at gangliosides-enriched membrane. The scale is the same as in panel B. RAW264.7 cells were pulsed with Alexa Fluor 546-dextran for 1 min and then fixed for staining with FITC-CTB. Dextran, the fluorescence of Alexa Fluor 546-dextran. Magnification, 63×. (D) The inhibition of macropinocytosis by cholesterol depletion. β-MCD (mM), cells pretreated with β-methylcyclodextrin at indicated concentration for 30 min and 150 μg/ml FITC-dextran was added for additional 1 h. Fluorescence intensities of the probe were measured by FACScan flow cytometer. $^{**}P < 0.01$. (E) DiO-Ac-LDL uptake is insensitive to cholesterol depletion. Cells pretreated with β-methylcyclodextrin at indicated concentration for 30 min and 10 μg/ml DiO-Ac-LDL was added for additional 1 h. a.u., arbitrary units.

concentration. These results suggested that lipid raft might also be involved in the macropinocytosis of RAW264.7 cells.

Discussion

Macropinocytosis is often operated constitutively in macrophages and many tumor cells [32]. In phorbol-myristate acetate or macrophage-colony-stimulating factor stimulated human monocyte-derived macrophages, the activated macropinocytosis is the endocytic pathway for LDL uptake and the generation of foam cells [19,24]. These findings suggest that other than the scavenger receptor-mediated modified LDL uptake, the uptake of native LDL by macropinocytosis is also an important endocytotic pathway for cholesterol accumulation and macrophage foam cell formation. The cultured mouse macrophage-like cell line RAW264.7 exhibits both scavenger receptor-mediated endocytosis and constitutive macropinocytosis (**Fig. 2**). It can be induced to form foam cell by the addition of oxidized LDL [**Fig. 1(A)**]. However, by switching to medium-containing high-level serum, foam cell formation can also be induced in RAW264.7 cell (**Fig. 1**). Serum is the rich source of lipoproteins, cytokines, and growth factors. The cytokines and growth factors in serum might further stimulate the spontaneous macropinocytosis in RAW264.7 cells [33]. And the lipoproteins provide the cholesterol, which is taken up through macropinocytosis and accumulated in the cell. All these findings suggest that the macropinocytosis in macrophage is an important endocytotic pathway for cholesterol accumulation and foam cell formation. Thus, the factors that activate macropinocytosis in macrophage have the possibility to promote foam cell formation and can be the risk factors of atherosclerosis. And the activation of macropinocytosis should be a particular risk

in the case of hyperlipidemia because of its non-selective uptake of solutes. We described spontaneous constitutive macropinocytosis played an important role in the foam cell formation. It was previously reported that inductive macropinocytosis stimulated by growth factors or phorbol esters contributed greatly to foam cell formation [19]. In contrast to the macropinocytosis induced by stimuli, the spontaneous constitutive macropinocytosis is much milder and spontaneously occurs *in vivo*. Our data here provide new insight into the mechanism of foam cell formation *in vivo*.

F-actin plays important roles in macropinocytosis [**Fig. 3(B,E)**] [9]. The membrane ruffling, which is the initial stage of macropinocytosis, is actin-dependent [9]. Coincidently, actin-enriched membrane ruffling area is gangliosides-enriched membrane [**Fig. 4(B)**]. And the macropinocytic vesicle membrane is also gangliosides enriched [**Fig. 4(C)**]. When membrane lipid microdomains are disrupted by cholesterol-binding β-methylcyclodextrin, macropinocytosis but not receptor-mediated endocytosis is blocked [**Fig. 4(D,E)**]. These observations indicate that membrane lipid microdomains are involved in macropinocytosis. It is possible that the diversified oligosaccharide chains on gangliosides facilitate the absorption of solute onto membrane.

All plasma proteins can enter and retain in the intima of blood vessel. Larger molecule will be retained longer in intima [34]. LDL concentrations in arterial intima have been shown to range from about 0.7 to 2.7 mg/ml [34–36]. The modified LDL in arterial intima may be taken up by the macrophage through scavenger receptor-mediated endocytosis. However, spontaneous or activated macropinocytosis may also take up the LDL directly. It might be another important pathway for macrophage foam cell formation in human intima in the presence of high concentration of LDL.

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