Role of Lipid Rafts in Phagocytic Uptake of Polystyrene Latex Microspheres by Macrophages

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Abstract. *Background: Micro- and nanospheres have been used as carrier materials for effective delivery of drugs into macrophages via endocytosis including phagocytosis for the treatment of cancer and infections. Materials and Methods: To analyze the role of lipid rafts in the endocytic uptake of microspheres by macrophages, we studied the incorporation of polystyrene latex microspheres (PSL MS) by treating macrophages with reagents that affect the physicochemical and biochemical functions of their lipid rafts, such as the cholesterol depletor methyl-β-cyclodextrin (MβCD) and the inhibitor of scavenger receptors polyinosinic acid (poly I). For this study, we used 3-μm fluorescent polystyrene latex microspheres (F-PSL MS) and J774 murine macrophage. Results: We found that the endocytosis of J774 cells was remarkably reduced in a concentration-dependent manner by treatment of the cells with MβCD and that endocytosis was suppressed by approximately 50% by treatment with 100 μg/ml poly I. These results suggest that scavenger receptors are associated with phagocytosis of F-PSL MS and that their functions are regulated by lipid rafts. Conclusion: PSL MS are phagocytosed via a raft-dependent pathway.*

To achieve efficient therapy for cancer and infections by delivering drugs to the target cells or organs, various systems including polymers and liposomes have been employed (1). These delivery systems are based on the targeting of cells utilizing their specific biological activities including receptor-ligand binding and incorporation systems such as endocytosis (including phagocytosis). Of these, phagocytosis by macrophages is initiated by the interaction of ligands with specific receptors associated with phagocytosis on the surface of the cells (2). These phagocytic receptors are categorized into two functional groups, namely phagocytic receptors associated with and those without opsonization (3). Fcγ receptors (4) and complement receptor 3 (CR3) are typical phagocytic receptors associated with opsonization (5), and the mannose receptor (6) and scavenger receptors (SRs) (3, 7) are representative members of those without association.

Several pathways of endocytosis toward foreign materials/pathogens by macrophages are known: *e.g.* clathrin-mediated endocytosis, caveolae/raft-dependent endocytosis, and macropinocytosis/phagocytic receptors-mediated endocytosis (8). In particular, significant contributions of caveolae/raft-dependent pathways to the incorporation of certain drugs have been reported. For example, rituximab, a humanized monoclonal antibody directed against CD20 for the treatment of B-cell non-Hodgkin's lymphoma, induces apoptosis of B lymphocytes by activating *src* kinase *via* caveolae/raft-dependent signals (9). Abraxane™ , which is albumin-bound paclitaxel nanoparticles for the treatment of metastatic breast cancer, targets angiogenic endothelial cells in tumors by interacting with the albumin-binding protein GP60 localized in caveolae on the endothelial cell surface (10).

Lipid rafts are the microdomains enriched in glycosphingolipids and cholesterol within the plasma membrane of cells; and caveolae, a subset of lipids rafts, are thought to control various biological events, such as operation of signaling cascades and cell adhesion, depending on the functional organization of the membrane (11-14). Cholesterol plays a critical function, maintaining the rafts in a functional state (12). Alteration of cholesterol status is likely to affect functions associated with rafts, because the structures and functions of rafts are dependent on cholesterol. In addition, lipid-rafts are reported to be associated with the incorporation of pathogens, such as *Brucella suis* (15) and influenza virus (16), by macrophages. However, little is known about the role of lipid raft domains in the uptake of artificial microspheres by macrophages. Recently, we developed rifampicin-loaded poly(lactic-co-glycolic) acid microspheres (PLGA MS) for the effective treatment of *Mycobacterium tuberculosis* (MTB) by targeting them to MTB-

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infected alveolar macrophages (17). PLGA MS loaded with anticancer drugs are also promising for the treatment of cancer (18). However, the pathway of uptake of PLGA MS is unknown. Hence, characterization of the uptake of synthetic microspheres is of importance. In the first step of our present study on the mechanism of uptake of MS, we examined the role of lipid rafts of the macrophage membranes in the uptake of 3-μm fluorescent PSL MS (F-PSL MS), because PSL MS have been used commonly as representative synthetic microspheres in the study of uptake of microspheres by various macrophages (19). In this study, we examined the role of lipid rafts of murine J774 macrophage cells in the uptake of PSL MS, mainly in terms of the effects of the depletion of cholesterol by treatment with methyl-β-cyclodextrin (MβCD) (15, 20, 21). The pathway of the incorporation of PSL MS was subsequently examined with special attention to lipid rafts and SRs, one of the phagocytic receptors not associated with opsonization.

Materials and Methods

Materials and reagents. The materials and reagents used and their sources were as follows: RPMI 1640-medium, from Invitrogen (Carlsbad, USA); thioglycolate broth, from BD Biosciences (San Jose, USA); MβCD, cholesterol and anti-flotillin-1 polyclonal antibody, from Sigma-Aldrich (St. Louis, MO, USA); Triton X-100, sucrose and skim milk, from Wako Pure Chemical Industry (Osaka, Japan); anti-caveolin-1 polyclonal antibody and goat anti-rabbit IgG-HRP, from Santa Cruz Biotechnology (Santa Cruz, USA); anti-SR-A1 monoclonal antibody, from R&D Systems (Minneapolis, USA); peroxidase-conjugated AffiniPure Goat Anti-Rat IgG (H+L), from Jackson ImmunoResearch Laboratories, Inc. (West Grove, USA); primulin, from MP Biomedicals, LLC (Eschwege, Germany); Tween-20, from Nacalai Tesque, Inc. (Kyoto, Japan); Fluoresbrite® YG Microspheres (3.00 μm diameter), from Polysciences Inc. (Warrington, UK); Amersham ECL™ Western Blotting Analysis System, from GE Healthcare UK Ltd. (Buckinghamshire, UK); Micro BCA™ Protein Assay Reagent Kit, from Pierce (Rockford, USA); Clear Blot Membrane-P (PVDF membrane), from ATTO Co., Ltd. (Tokyo, Japan); and HPTLC plates (silica gel 60 F_{254}), from Merck (Darmstadt, Germany).

Macrophage cells. J774 cells derived from mouse macrophage-like cells (TIB-67; ATCC, Rockville, USA) were cultured with RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 60 μg/ml ampicillin, and incubated at 37°C under a stream of 5% CO_2. Murine peritoneal macrophages were isolated from C57BL/6 mice by peritoneal lavage using 5 ml of phosphate buffered saline (PBS) 4 days after an intraperitoneal injection of 3 ml sterile 3% thioglycolate broth (Becton Dickinson, Franklin Lakes, USA).

Extraction of lipid raft domains. Lipid raft domains were extracted from macrophages with Triton X-100 and purified by sucrose density gradient ultracentrifugation according to standard protocols. Briefly, the cells (1×10^8 cells) were lysed in 2 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes on ice. The lysate was then mixed with an equal volume

of 80% sucrose, after which the mixture was added to an ultracentrifuge tube. The sample was overlaid with 3 ml of 30% sucrose and 3 ml of 5% sucrose, and centrifuged at 100,000 ×g for 16 hours at 4°C in a preparative Himac CP80MX ultracentrifuge equipped with a P40ST Swing Rotor (Hitachi Koki Co. Ltd., Tokyo, Japan). The detergent-resistant membrane (DRM) fraction at the interface between 5% sucrose and 30% sucrose was collected as the lipid-raft fraction. DRMs were washed 5 times with PBS and then centrifuged at 15,000 ×g for 20 minutes to remove the sucrose. Otherwise, after the ultracentrifugation, 10 fractions (each 1.0 ml) were collected from the top of the tube. Each fraction was subjected to Western blot analysis.

Western blotting. The DRMs were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels followed by transfer of the sample onto a PVDF membrane. Blocking of the membrane was performed by incubating the membrane for 2 hours in PBS containing 5% skim milk and 0.1% Tween 20. The membrane was then incubated for 2 hours at room temperature or overnight at 4°C with the primary antibody in PBS containing 1% skim milk and 0.1% Tween 20. After the membrane had been washed 3 times for 10 minutes each time with PBS containing 0.1% Tween 20, it was incubated with the secondary antibody in the same buffer for 2 hours at room temperature. Following washing 3 times for 10 minutes each time with PBS containing 0.1% Tween 20, the proteins were detected by using an Amersham ECL™ Western Blotting Analysis System (GE Healthcare) according to the manufacturer's protocol. Visualization was carried out with conventional X-Ray films (Amersham), and band intensities were quantified with an ATTO Printgraph image analyzing system AE-6905CF (Tokyo, Japan).

High-performance thin-layer chromatography (HPTLC). Total lipids of DRMs were prepared by extraction with chloroform-methanol (2:1) overnight at room temperature, and they were analyzed by HPTLC using a chloroform-methanol-acetic acid-formic acid-water mixture (35:15:6:2:1, v/v) after developing with a mixture of hexane-isopropylether-acetic acid (65:35:2, v/v). The cholesterol was detected under UV light, after spraying with primulin reagent (0.01% w/v solution in acetone-water [80:40, v/v]) and quantified by using the AE-6905CF ATTO Printgraph image analyzing system.

Quantification of proteins in DRMs. The amount of proteins in DRMs from J774 cells was quantified by use of a BCA assay kit (Micro BCA™ Protein Assay Kit; Pierce), according to the manufacturer's instructions.

Endocytosis of J774 cells. J774 cells (5×10^5 cells/ml) were incubated with 3-μm F-PSL MS (Fluoresbrite® YG Microspheres; Polyscience, Warrington, USA) at microsphere: macrophage ratio of 10 in RPMI-1640 with a reduced concentration of FBS (1%) for different times at 37°C in 96-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lake, USA). The population of J774 cells that had taken up F-PSL MS was determined with a flow cytometer (BD FACSAria; Becton Dickinson). The viability of J774 cells in each well was determined by performing the WST-8 assay (Dojindo, Kumamoto, Japan) (22, 23), in which the optical absorbance at 450 nm due to WST-8-formazan produced by dehydrogenases in live cells is measured in a spectrophotometer, model 680 (Bio-Rad Laboratories, Hercules, USA).

A : Caveolin-1 (C57BL/6)
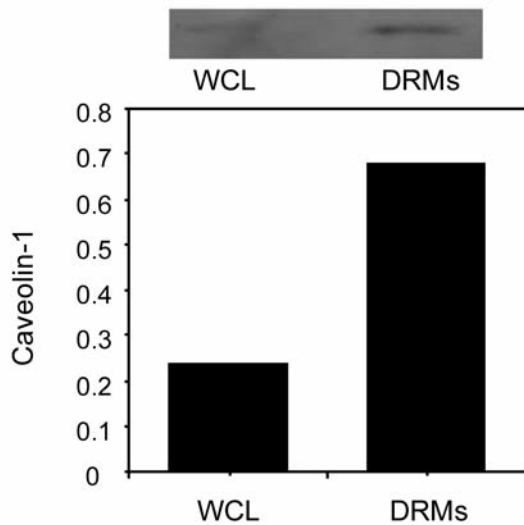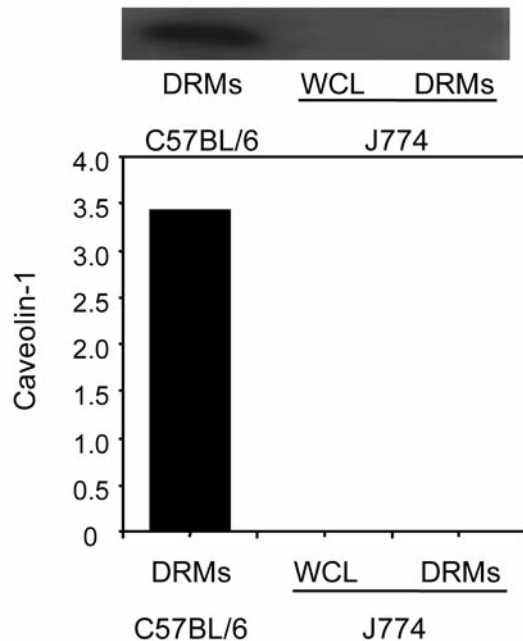

C: Flotillin-1 (J774)
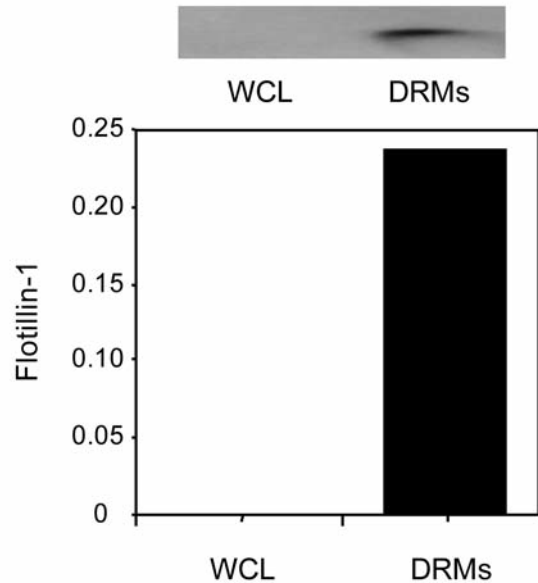
B : Caveolin-1 (J774)

Figure 1. *Identification of detergent-resistant membranes (DRMs) from macrophage cells. DRMs were prepared from thioglycollate-elicited peritoneal macrophage cells of C57BL/6 mice and J774 murine macrophage cells by treatment with lysis buffer containing 1% Triton X-100, followed by sucrose density gradient ultracentrifugation. Proteins in DRMs and whole cell lysate (WCL) were analyzed by Western blotting with murine anti-caveolin-1 antibody or murine anti-flotillin-1 antibody. Electrophoretic protein profiles of both were visualized on X ray films by using the Amersham ECLTM blotting system and the amounts of proteins in DRMs and WCL, quantified by an ATTO Printgraph image analyzing system, are shown for caveolin-1 from C57BL/6 (A) and J774 (B), and for flotillin-1 from J774 cells (C). In the quantification analysis of proteins in WCL and DRMs, total protein concentrations were adjusted to be the same, and the band intensity of caveolin-1 or flotillin-1 is shown.*
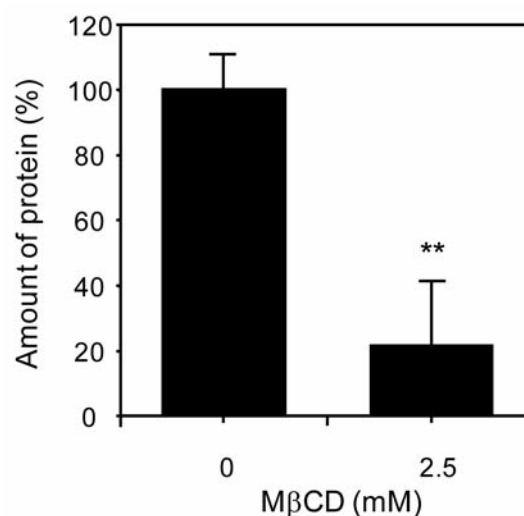
Results

Detection of lipid-raft markers in the DRMs from macrophage cells. For a biochemical approach to the study of the role of lipid rafts in the endocytosis of PSL MS, DRM fractions were collected by treatment of J774 cells and thioglycollate-elicited peritoneal macrophage cells of C57BL/6 mice, with 1% Triton X-100 solution for 30 minutes, followed by sucrose density-gradient ultracentrifugation. Specific protein markers in lipid rafts on the DRMs were analyzed for by Western blotting with specific antibodies. Caveolin-1, known as a prominent protein marker for the subset of lipid rafts known as caveolae (24), was detected in DRMs from C57BL/6 mice to a level approximately 3 times higher than that in the whole-cell lysate (WCL), showing that caveolin-1 was highly enriched in DRMs derived from C57BL/6 mice (Figure 1A). However, it was not detected in DRMs derived from J774 cells (Figure 1B), in accordance with results reported previously (25). DRMs from J774 cells were highly enriched in flotillin-1, one of the ubiquitous markers of lipid rafts (26) (Figure 1C). These results show that lipid-raft domains in the macrophage membranes were definitely isolated as DRMs, and, hence, that DRMs would be available to analyze lipid raft domains by biochemical approaches. In J774 cells, DRMs can be regarded as non-caveola-type lipid rafts.
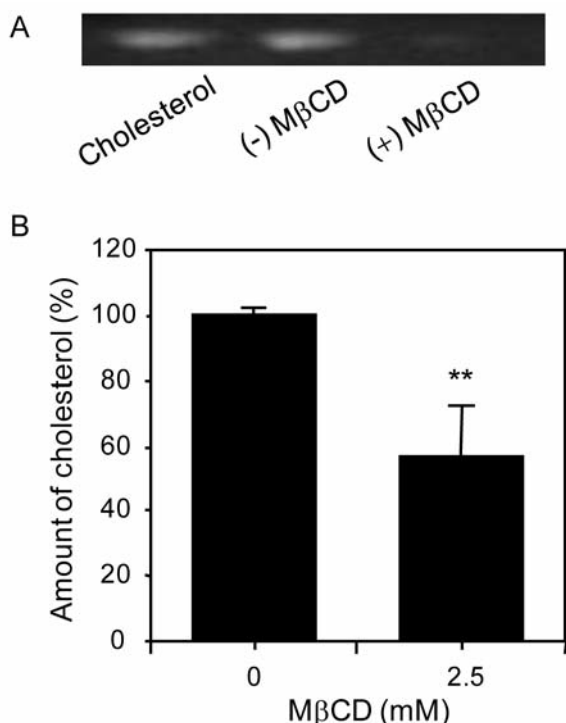
Figure 2. *Cholesterol depletion of macrophage lipid rafts by MβCD. A total lipid extract of DRMs from J774 cells treated with 2.5 mM MβCD for 2.5 hours was analyzed by HPTLC. Cholesterol bands were visualized by illumination from ultraviolet light, after spraying with primulin reagent (A), and they were quantified by an ATTO Printgraph image analyzing system in terms of the value (%) relative to that without MβCD treatment (B). Total lipid extract of DRMs from cells without MβCD treatment was prepared as a control. Data were expressed as the mean±SD in 3 separate experiments. The results of HPTLC for a commercial sample of cholesterol (3 μg), DRMs from J774 cells, and DRMs from J774 cells after treatment with MβCD are shown. The asterisks indicate a statistically significant difference (p<0.01) from the control, as assessed by Student's t-test.*

Figure 3. *Effect of MβCD treatment on proteins in lipid-raft domains. The amount of proteins in DRMs from J774 cells treated with 2.5 mM MβCD for 2.5 hours was determined by using the BCA assay, according to the manufacturer's instructions. The protein amount in DRMs is shown as the value relative to that of cells not treated with MβCD. Data are expressed as the mean±SD from 3 separate experiments. The asterisks indicate a statistically significant difference (p<0.01) from the amount for the control, as assessed by Student's t-test.*

Cholesterol depletion and lipid-raft disruption in macrophage cells by MβCD. Cholesterol depletion of macrophage cell membranes by MβCD was confirmed by HPTLC. The quantity of cholesterol in DRMs from J774 cells after MβCD treatment was reduced to approximately one-half that without treatment, indicating that MβCD efficiently depleted cholesterol in the lipid-raft domains of the J774 cell membrane (Figure 2). The disruption of lipid rafts concomitant with cholesterol depletion by MβCD treatment was verified by quantifying total protein in DRMs with the BCA assay after treating the cells with MβCD. The amount of proteins in DRMs of J774 cells treated with 2.5 mM MβCD for 2.5 hours was decreased to approximately 20% of the normal amount by treatment with MβCD (Figure 3).

Effect of MβCD on incorporation of F-PSL MS by J774 cells. To analyze the role of lipid rafts in the incorporation of MS by macrophage cells, we examined the effect of MβCD treatment on the uptake of 3-μm F-PSL MS by J774 cells. As shown in Figure 4A, the endocytic activity of J774 cells toward F-PSL MS for 2 hours (2.5 hours for total treatment period with MβCD) decreased with an increase in the MβCD concentration used for depletion of cholesterol. In contrast, the viability of the cells was not affected by treatment with MβCD up to 2.5 mM, but was decreased considerably by treatment with 5.0 mM MβCD (Figure 4B). Next, the time-dependency of the effect of MβCD treatment on the uptake of PSL MS and viability of J774 cells was examined. As shown in Figure 5A, the endocytic activity of J774 cells increased with increasing time of incubation, both with and without MβCD treatment. However, the degree of incorporation of F-PSL MS into macrophage cells treated with MβCD compared to that in those without MβCD treatment was independent of the incubation period. Namely, endocytic activities of J774 cells after incubation for 1, 2 and 4 hours with F-PSL MS (total MβCD incubation period of 1.5, 2.5 and 4.5 hours, respectively) were approximately 63%, 45%, and 48%, respectively, of those without MβCD treatment. Hence, the effect of MβCD treatment seemed to be completed after incubation for just a few hours, such as for 2.5 hours.
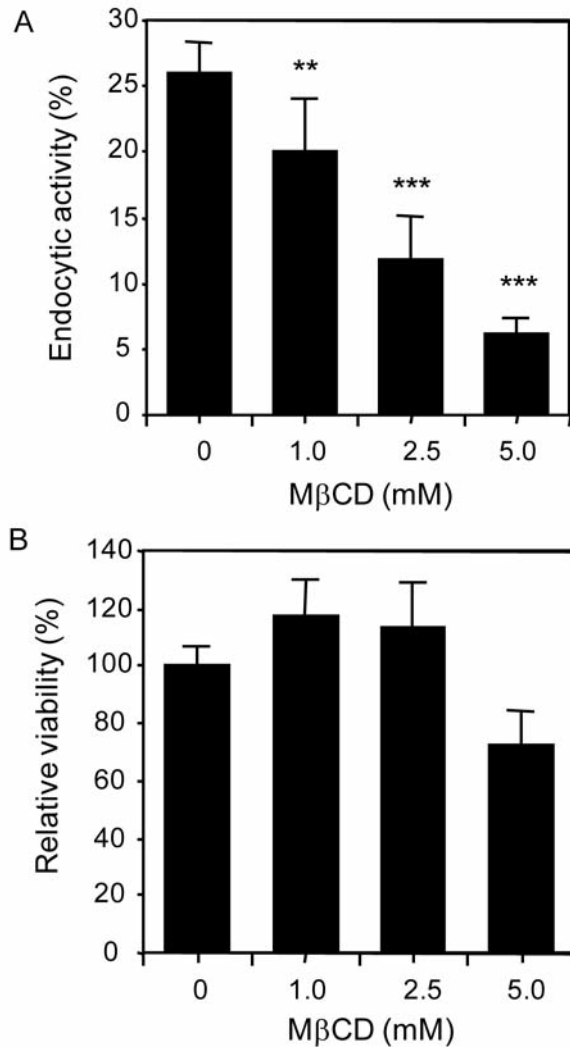
Figure 4. *Concentration-dependent effects of MβCD treatment on the endocytic activity and viability of J774 cells. J774 cells (5×10^5 cells/ml) were incubated with different concentrations of MβCD for 30 min, and then F-PSL MS were added to give a microsphere:macrophage ratio of 10, followed by incubation for 2 hours (total incubation with MβCD for 2.5 hours). The population of J774 cells that had taken up the MS (endocytic macrophages) was determined by counting 10,000 cells with a flow cytometer (A). The viability of J774 cells was determined by performing the WST-8 assay, and is shown as the value relative to that of Mφ cells without treatment with MβCD (B). Results are expressed as the mean±SD (n=9) from at 3 separate experiments. Statistically significant differences at the level of **p<0.01 and ***p<0.001, from the control (untreated cells), as assessed by Student's t-test, are shown.*

Figure 5. *Time-dependent effect of MβCD treatment on endocytic activity and viability of J774 cells. J774 cells (5x105 cells/ml) were incubated with 2.5 mM MβCD for 30 min, and then F-PSL MS were added to give a microsphere: macrophage ratio of 10, followed by incubation for different periods of time. The endocytic activity and viability of J774 cells were determined as described in the Materials and Methods. Note that the incubation period in the shown is that for F-PSL MS, and hence the total incubation period with MβCD is 30 minutes longer than that shown in the figures. A: Endocytic activity of F-PSL MS by J774 cells. B: Viability of J774 cells relative to the value after incubation for 1 hour without treatment with MβCD. In the case of treatment without MβCD, J774 cells were first incubated in the incubation medium for 30 minutes, and then F-PSL MS were added. Results are expressed as the mean±SD (n=9). Statistically significant differences at the level of **p<0.01 and ***p<0.001, from the control (untreated cells), as assessed by Student's t-test, are shown.*

As the viability of J774 cells was not affected essentially by treatment with 2.5 mM MβCD for up to 4.5 hours of incubation (Figure 5B), the decrease in the endocytic activity by treatment with MβCD was not due to the loss of the viability of the J774 cell population, but due to disruption of their lipid rafts. A typical microscopic image of a J774 cell
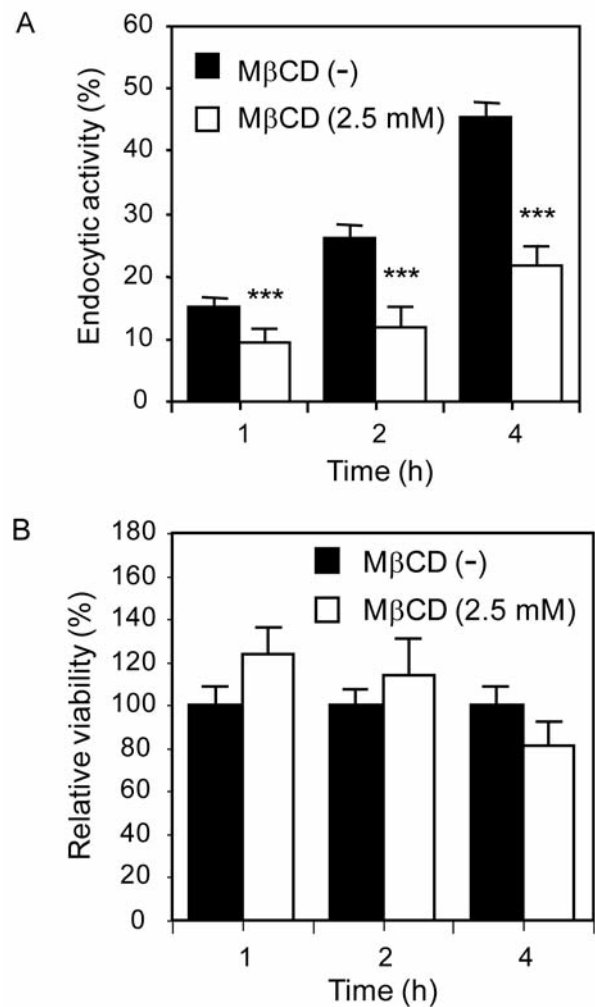
that had taken up F-PSL MS is shown in Figure 6. These results show that incubation with MβCD up to 5.0 mM did not essentially affect the macrophage viability, that it decreased the viability at 5.0 mM MβCD and higher, and that the depletion of the membrane cholesterol was
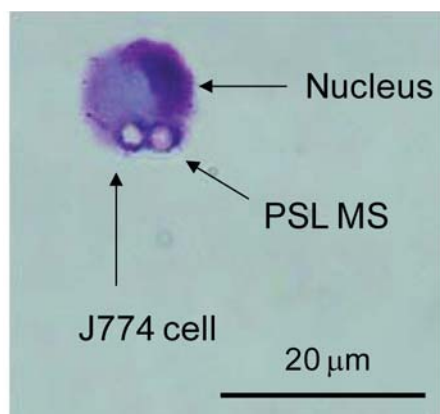
Figure 6. *Typical microscopic image of a J774 cell that had taken up F-PSL MS. Optical microscopic appearance of a J774 cell incubated with 3-μm F-PSL MS at a microsphere:macrophage ratio of 10 for 2 hours is shown.*
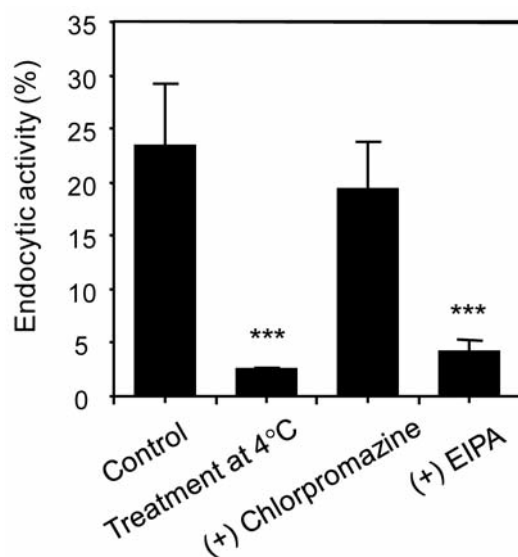

Figure 8. *Effects of poly I and poly C on macrophage uptake of F-PSL MS. After pretreatment of J774 cells (5×10^5 cells/ml) with various concentrations of poly I or poly C for 30 minutes, the cells were incubated with F-PSL MS at a microsphere: macrophage ratio of 10 for 4 hours. The uptake of F-PSL MS by 10,000 cells was then determined with a flow cytometer. Results are expressed as the mean±SD (n=9). The asterisks indicate a statistically significant difference (p<0.001) from the control (cells treated with poly C at the same concentration as poly I), as assessed by Student's t-test.*

Figure 7. *Effects of pathway inhibitors on uptake of PSL MS by J774 cells. After treatment of J774 cells (5x105 cells/ml) with 5 μM chlorpromazine or 50 μM EIPA for 30 min, the J774 cells were incubated with F-PSL MS at a microsphere:macrophage ratio of 10 for 2 hours at 37˚C. In addition, the effect of incubation temperature on the F-PSL MS uptake was examined by incubating J774 cells with F-PSL MS at 4˚C. The endocytic activity in terms of the proportion of macrophage cells that had taken up F-PSL MS to the total number of macrophage cells (%) was determined with a flow cytometer by counting 10,000 cells. Results are expressed as the mean±SD (n=9). The asterisks indicate a statistically significant difference (p<0.001) from the control (untreated cells), as assessed by Student's t-test.*

Incorporation pathway of F-PSL MS. Several pathways of endocytosis to take up foreign materials/pathogens by macrophage cells are known, *i.e.*, clathrin-mediated endocytosis, caveolae/raft-dependent endocytosis, macropino-cytosis, and phagocytosis (phagocytic receptor-mediated endocytosis) (8). However, the mechanism by which PSL MS are incorporated by macrophage cells is not clear at present. As shown in Figure 7, a significant decrease in the incorporation of F-PSL MS into J774 cells by incubation at 4˚C confirmed that F-PSL MS were incorporated into these cells by endocytosis. To determine the incorporation pathway of F-PSL MS, we examined the uptake of F-PSL MS by J774 cells after incubation at a microsphere:macrophage ratio of 10 for 2 hours at 37˚C, for changes after treatment with different pathway inhibitors. Endocytic activity by treatment with chlorpromazine, a clathrin-mediated endocytosis inhibitor (27), did not show significant change, whereas that with EIPA, a macropinocytosis and phagocytosis inhibitor (28), remarkably decreased the uptake of PSL MS (Figure 7). These results suggest that the F-PSL MS were incorporated by macropinocytosis and/or phagocytosis (phagocytic receptor-mediated endocytosis).

Association of SRs with uptake of F-PSL MS. To determine whether SRs in macrophages were associated with the uptake of F-PSL MS, we examined the effect of the SR inhibitor

terminated after a short period of incubation, such as 1.5 hours. As the depletion of lipid-raft domains caused significant loss of the endocytic activity, the endocytosis of F-PSL MS would be expected to be mediated by a specific receptor in the lipid rafts of the macrophage cells.
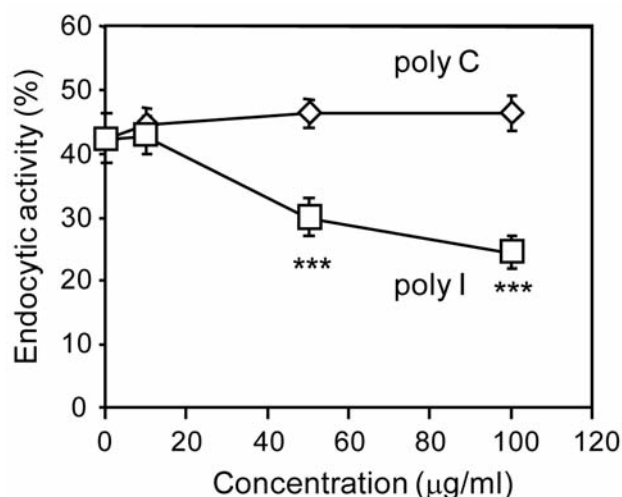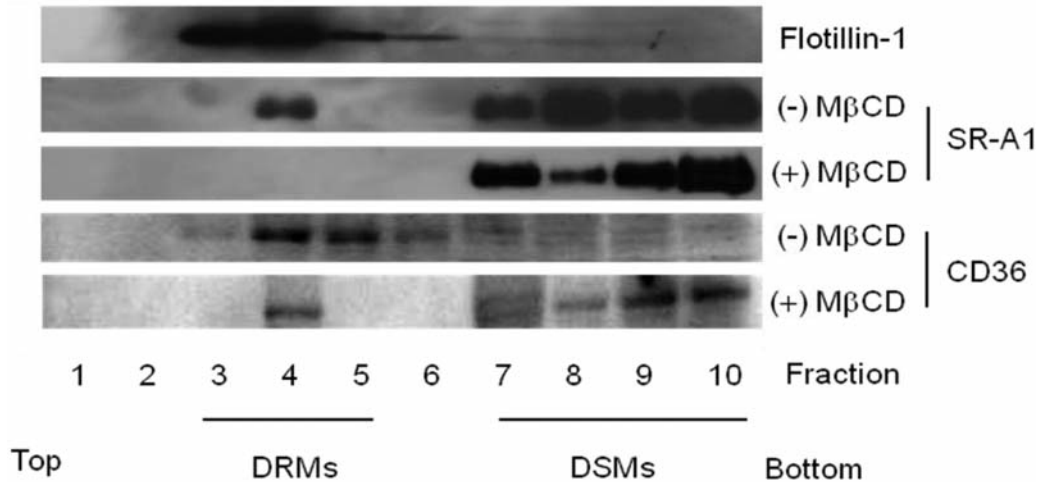
Figure 9. *Association of scavenger receptor A1 and scavenger receptor B/CD36 with lipid rafts. J774 cells (5×10⁵ cells/ml) were treated with 2.5 mM MβCD for 2.5 hours, then lysed with 1% Triton X-100, after which sucrose gradient ultracentrifugation was performed for fractionation. After centrifugation, 10 fractions of 1 ml each were collected from the top of the tube and analyzed by Western blotting with anti-scavenger receptor A1 antibody (SR-A1), and anti-CD36 (scavenger receptor B) antibody. The presence of lipid rafts in the solubilized fraction was judged from blotting with flotillin-1 antibody. DSM: Detergent-soluble membrane.*

polyinosinic acid (poly I) (29-31). The endocytosis was significantly suppressed by poly I in a concentration-dependent manner. The proportion of endocytic cells, being referred to as endocytic activity, was decreased from the control value of 46% to 24%, corresponding to an approx. 50% decrease, by treatment of J774 cells with 100 μg/ml poly I. In contrast, the endocytic activity was not affected at all by treatment with poly C up to 100 μg/ml (Figure 8). These results suggest that SRs were associated, at least in part, with the uptake of F-PSL MS.

J774 cells treated with 2.5 mM MβCD for 30 minutes were lysed and subjected to sucrose density-gradient ultracentrifugation. Subsequently, 10 fractions of 1 ml each were collected from the top of the tube, and were analyzed by Western blotting, as shown in Figure 9. Flotillin-1, a marker of lipid rafts, was detected in fractions 3, 4, and 5, showing that these fractions contained DRMs. Scavenger receptor A1 (SR-A1) was detected in fraction 4 (containing DRMs), whereas most of the SR-A1 was detected in fractions 7 to 10, which contained the detergent-soluble membrane (DSM) without lipid rafts. On the other hand, the vast majority of CD36 (also known as SR-B) was detected in fractions 4 and 5, which contained DRMs. In addition, SR-A1 was not detected in the fractions corresponding to DRMs after disruption of lipid rafts by MβCD treatment; and the vast majority of CD36 in DRMs was shifted to DSMs, supporting the probability that SR-A1 and CD36 in the lipid rafts were shifted to a membrane region other than the lipid raft domain by the treatment with MβCD.
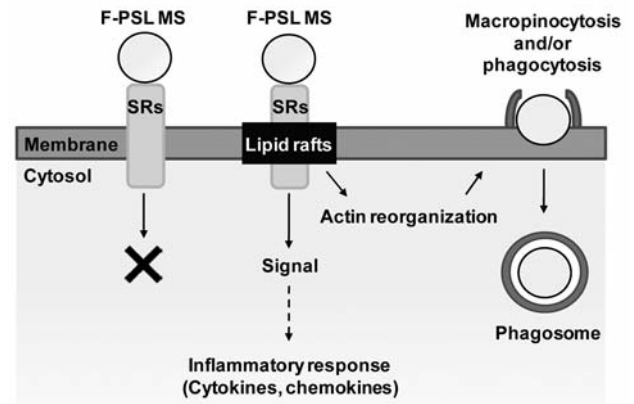

Figure 10. *Possible mechanism of incorporation of F-PSL MS by macrophage. F-PSL MS interact with scavenger receptors (SRs) located in lipid rafts, and this interaction induces rearrangement of the structure of the actin cytoskeleton, leading to phagocytosis and/or macropinocytosis. Accordingly, F-PSL MS are incorporated into the cytosol in the form of phagosomes.*

Discussion

Lipid rafts have been suggested as key cellular apparatus in various biological events, such as signaling cascades (32), cell adhesion and migration (33), entry of viruses (34), bacteria, and toxins (35) and immune responses (36). Lipid rafts have also been implicated in macrophage functions such as endocytosis (37), cholesterol export (38), major histocompatibility complex (MHC) class II antigen

presentation (39), and cytokine production (40). In addition to these biochemical events, they are expected to play a prominent role in the therapeutic effect of drugs administered by use of the drug delivery systems such as those in forms of antibodies, liposomes, microspheres, and nanoparticles. Recently, we reported that the uptake of the poly(lactic-co-glycolic) acid (PLGA) microsphere loaded with the antitubercular agent rifampicin is effective for the treatment of *Mycobacterium tuberculosis* (MTB), because rifampicin-loaded PLGA MS were effectively incorporated by alveolar macrophage cells infected with MTB, resulting in a significant bactericidal effect (17). However, the mechanism of endocytosis of PLGA MS is not clear, although the contribution of caveolae/raft-dependent pathway in the therapeutic effect has been suggested (41, 42). For understanding the pathway of the endocytosis of synthetic microspheres, we analyzed the endocytosis of the commonly used F-PSL MS by J774 cells.

In this study, we prepared DRMs from macrophage cells by cell lysis followed by sucrose density-gradient ultracentrifugation (43). Characterization of DRMs as lipid rafts was performed by Western blotting with antibodies of raft-specific marker proteins. Caveolin-1, the caveola-specific marker protein, was not at all detected in either WCL or DRMs of J774 cells, in contrast to its major presence in DRMs from C57BL/6 murine peritoneal macrophage cells. In addition, the DRMs from J774 macrophage cells were enriched in flotilin-1, a lipid raft-specific marker protein. These results suggest that DRMs collected by this method reflected lipid-raft domains of J774 cells but that they did not contain caveolae, this being consistent with previous results (25).

Cholesterol-depleting agents, such as MβCD, and the cholesterol-binding reagents filipin and nystatin are useful in studying the function of lipid rafts (15,20,21). In this study, we performed cholesterol depletion associated with raft disruption by treating J774 cells with MβCD and found that approximately 50% of the cholesterol in DRMs from these cells was depleted by the treatment with 2.5 mM MβCD for 2.5 hours, showing that cholesterol in the plasma membrane had been depleted successfully by this treatment. It is noteworthy that depletion of cholesterol in lipid rafts did not affect the viability of these macrophage cells under such mild conditions as described above, this being consistent with the results of cholesterol depletion from the plasma membrane of the rat epithelial clone 9 cell line (44). Similarly, a reduction in the amount of proteins in DRMs from J774 cells by MβCD treatment was observed: the amount of proteins in DRMs from J774 cells treated with MβCD was approximately 20% of that of DRMs collected from J774 cells without the treatment.

Lipid rafts are dynamic biological functional machinery composed of sphingolipids, cholesterol, and certain proteins in a well-organized structure. However, our results suggest that J774 cells retained the dynamic cellular compartmentation indispensable for maintaining the viability of macrophage cells even on depletion of membrane cholesterol by up to 50% and proteins by up to 80%, because depletion of such amounts of cholesterol and proteins did not affect the viability of the cells. It is noteworthy that the endocytic uptake of F-PSL MS was significantly dependent on the depletion of cholesterol and proteins.

Uptake of F-PSL MS by J774 cells was inhibited by the inhibitor of phagocytosis/macropinocytosis EIPA (28), but not by the clathrin-mediated endocytosis inhibitor chlorpromazine (27), showing that F-PSL MS were taken up by J774 cells by phagocytosis, but not by clathrin-mediated endocytosis. Phagocytosis of F-PSL MS was suppressed by MβCD treatment in a concentration-dependent manner, and treatment with 2.5 mM MβCD for 2.5 hours suppressed phagocytosis to one-half that without MβCD pretreatment. These results suggest that the lipid rafts of J774 cells were associated with phagocytosis of F-PSL MS.

It is likely that the F-PSL MS were incorporated into the macrophage cells by either caveola-mediated endocytosis or raft-mediated phagocytosis or both. The unique physical features of caveolae includes a distinctive membrane coat composed of caveolin-1 and cholesterol, which is concentrated around the rim of the domain, and it is associated with flask-shaped, invaginated morphology during internalization. The lower size limit appears to be the diameter of flask-shaped caveola, the size of which is in a range of 50 to 80 nm. The cytoplasmic coat occupies an area up to ~150 nm in diameter (46). However, J774 cells do not express caveolin and appear to lack typical flask-shaped membrane invaginations (47); and hence, PSL MS have been suggested to be incorporated *via* lipid raft-dependent phagocytosis. Therefore, lipid rafts are likely to function as sites for signal transduction *via* phagocytic receptors such as SRs to interact with PSL MS. Flotillin-1, which we detected here in DRMs from J774 cells, was reported to be involved in phagosomes, as determined by a proteomics approach (48). Stomatin, a protein associated with lipid-raft domains (49), is also found in phagosomes (48). These results support the possibility that F-PSL MS are incorporated by lipid raft-dependent phagocytosis in J774 cells.

Phagocytosis of particles by macrophage cells is affected by their size and surface charge (45), and several receptors of macrophage cells are known to be associated with such phagocytosis (50). It is possible that SRs, which recognize a wide range of negatively charged macromolecules (3, 7, 29, 30), are also responsible for phagocytosis of F-PSL MS, because the surface of F-PSL MS is negatively charged (51). To examine this possibility, we analyzed the phagocytosis of F-PSL MS by J774 cells by using poly I, a general inhibitor of SRs. The phagocytosis was markedly suppressed by poly I in a concentration-dependent manner, but not by poly C. In

addition, the SR-A1 and CD36, associated with lipid rafts, were dissociated by disruption of the lipid rafts by the treatment of the J774 cells with MβCD, and this was accompanied by a decrease in phagocytosis. Hence, SRs can be regarded as one of the phagocytic receptors for PSL MS; and phagocytic receptors are functional when associating with lipid rafts. It is likely that the formation of the complex of PSL MS with phagocytic receptors in the lipid rafts initiates various events in the signaling-cascades, inducing rearrangement of the actin cytoskeleton, leading to phagocytosis (Figure 10).

In conclusion, we found in this study that i) SR-A1 and CD36 existed in lipid rafts of J774 cells; ii) F-PSL MS were incorporated by phagocytosis and/or macropinocytosis, but not by clathrin-mediated endocytosis; iii) phagocytosis and macropinocytosis of PSL MS were mediated by SRs in lipid rafts; and iv) F-PSL MS were incorporated by a lipid raft-dependent pathway, but not by a caveola-dependent one. Thus, F-PSL MS were found to be incorporated into macrophage cells *via* lipid raft-dependent phagocytosis.

Acknowledgements

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