Electronic Supplemental Information (ESI)

Unique cellular interaction of macrophage-targeted liposomes potentiates anti-inflammatory activity

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Contents

1. **Experimental section**
2. **Size of phosphatidylserine-displaying liposomes (PSLs) (Fig. S1)**
3. **PSL uptake by RAW 264.7 macrophages (Fig. S2)**
4. **Cytotoxicity of PSLs toward RAW 264.7 macrophages (Fig. S3)**
5. **Changes in mouse body weight during PSLs treatment (Fig. S4)**
6. **F4/80 (a macrophage marker)-stained liver section (Fig. S5)**

Experimental section

Preparation of phosphatidylserine-containing liposomes (PSLs): Soybean-derived PS (purity >98%) and egg-derived phosphatidylcholine (PC) (purity >98%) (all Sigma-Aldrich, St. Louis, MO, USA) were used as received. PS and PC were dissolved in chloroform/methanol (90:10). PS and PC were mixed at a molar ratio of 3:7 and the solvent removed under nitrogen gas, followed by drying in a desiccator overnight. PSL was prepared by resuspending the lipid film in Tris-HCl buffer (100 mM, pH 7.5) at a concentration of 10 mg/mL. In the cellular uptake assay, *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Fluorescein DHPE, Thermo Fisher Scientific, Waltham, MA, USA) was mixed (1mol% of total lipids) to prepare fluorescein-labeled PSL. The size of PSLs was controlled by extrusion through polycarbonate membranes of different pore sizes (800, 400, 200, 100, 50, and 30 nm) (Avanti Polar Lipids, Alabaster, AL, USA). The diameter of PSLs was determined by dynamic light scattering technique using a Zetasizer NS (Malvern Instruments, Malvern, UK).

Preparation of bone marrow-derived macrophages (BMMΦ): BMMΦ were obtained by differentiating bone marrow cells from C57BL/6J mice (female, 8 weeks, Japan SLC, Shizuoka, Japan). Briefly, bone marrow cells were isolated from mouse femurs, and cultured in Roswell Park Memorial Institute medium (RPMI 1640; Fujifilm Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT), 1% penicillin-streptomycin (Fujifilm Wako Pure Chemical) and, 40 ng/mL mouse macrophage-colony stimulating factor (M-CSF; Miltenyi Biotec, Gladbach, Germany) for 5 days at 37°C in a humidified atmosphere containing 5% CO_2. On day 3, 5 mL of fresh medium was added to the dish. Only adherent cells (BMMΦ) were used for further experiments.

Cellular PSL uptake: BMMΦ were grown in a 24-well plate at an initial density of 1×10^5/well at 37°C for 24 h. NIH-3T3 mouse fibroblasts (RIKEN BioResource Research Center, Ibaraki, Japan) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Fujifilm Wako Pure Chemical) containing 10% FBS and 1% antibiotics and were grown in a 24-well plate at an initial density of 5×10^4/well at 37°C for 24 h. RAW 264.7 mouse macrophages (RIKEN BioResource Research Center) were maintained in DMEM supplemented with 10% FBS and 1% antibiotics and were grown in 24-well plate at an initial density of 5×10^4/well at 37°C for 24 h. Fluorophore-labeled PSLs (100 μg/mL) were added to the cells. After 1–24 h, the cells were washed three times with Hank's balanced salt solution (HBSS; Fujifilm Wako Pure Chemical) and lysed in 200 μL of lysis buffer M (Fujifilm Wako Pure Chemical; 20 mM Tris-HCl, pH 7.4, 200 mM sodium chloride, 2.5 mM magnesium chloride, 0.05 w/v% NP-40 substitute). Fluorescence intensity was measured using a plate reader (Synergy HT, BIO-TEK Instruments Inc., Winooski, VT, USA) and total protein concentration was determined by the Bradford

method (Coomassie Brilliant Blue G-250 reagent; BIO-RAD Lab., Hercules, CA, USA). Fluorescence intensity was normalized with respect to the total protein in lysates.

Mechanism of PSL uptake by BMMΦ: To examine whether inhibition of energy-dependent endocytosis at low temperature changed BMMΦ PSL uptake of by, BMMΦ were incubated at 4°C for 1 h, after which 100 μg/mL fluorescein-labeled PSLs were added, and the cells were further incubated at 4°C for 1 h. In addition, to investigate the role of CD300a receptor in PSL uptake of BMMΦ, the cells were pre-treated with 10 μg/mL of anti-mouse CD300a antibody (clone 172224, R&D Systems, Minneapolis, MN, USA) for 30 min, mixed with 100 μg/mL of fluorescein-labeled PSLs and 10 μg/mL of anti-CD300a antibody, and further incubated for 1 h. Levels of PSL uptake were then determined as described above.

Anti-inflammatory activity of PSLs: BMMΦ were grown in 24-well plates at an initial density of 1×10^5/well at 37°C for 24 h. THP-1 human monocytes (RIKEN BioResource Research Center) were cultured in 24-well plates at an initial density 1×10^5/well and were differentiated into macrophages in RPMI-1640 containing 10% FBS, 1% antibiotics, and 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) at 37°C for 48 h. After that, a mixture of 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich) and 20 ng/mL interferon-γ (IFNγ; Miltenyi Biotec), and 30 μg/mL of PSLs was added to the cells. After 6 h, the media were collected in cryotubes and frozen at –80°C until analysis. Concentrations of cytokines and chemokines were measured using Ready-SET-Go ELISA kits (Affymetrix Inc., CA, USA) according to the manufacturer's instructions.

To investigate the long-lasting inflammatory activity of the PSLs, we added PSLs into the cultured BMMΦ and incubated them at 4°C for 1 h. Unbound PSLs were removed by

washing thrice with PBS, after which LPS (100 ng/mL) and IFNγ (20 ng/mL) were added into the media. After 2 and 24 h incubation, the media were collected in cryotubes, and cytokines and chemokines analyzed following the above-mentioned protocol. For IL-1β, cells were further treated with 500 μL of medium containing 3 mM adenosine 5′-triphosphate (ATP; purity>99%, Sigma-Aldrich). At 45 min, media were collected in cryotubes and were frozen at –80°C until analysis.

CDAHFD-induced murine nonalcoholic fatty liver disease (NAFLD) model: All animal studies were performed in accordance with the Guidelines for Animal Experiments established by the Ministry of Health, Labour and Welfare of Japan and by the National Institute of Advanced Industrial Science and Technology (#A2018-0313, A2019-0313). The C57BL/6J mice (male, 5 weeks-old, Japan SLC Inc., Shizuoka, Japan) were maintained in a temperature-controlled room (22˚C) with a 12-h light-dark cycle. The animals were also provided a standard diet (MF, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* for a week. Once the mice were 6 weeks old, the standard diet was changed to CDAHFD (Research Diets, New Brunswick, NJ, USA), while still providing tap water *ad libitum*. After 1 week of initiating CDAHFD diet, the mice were intraperitoneally injected with 200 μL of PSLs (1 mg/shot) twice a week.

Histopathology and immunohistochemistry: After 5 weeks of PSL injection, the mice were sacrificed, and their livers were collected. The livers were fixed in 4% neutral paraformaldehyde, and embedded in paraffin. Liver sections were then stained with hematoxylin and eosin (HE) or processed for immunostaining. Immunostaining was conducted using rabbit anti-mouse CD11c antibody (clone D1V9Y; Cell Signaling Technology, Danvers, MA, USA) and colors were developed by 3,3’-diaminobenzidine.

Pictures of histological sections were acquired and analyzed using a light microscope (BX43, Olympus, Tokyo, Japan). Four high power fields (original magnification ×200) per HE-stained section were counted and averaged.

Statistical analysis: Data are presented as mean ± standard deviation. Statistical analyses were performed using two-tailed Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison test. Variations with a p-value < 0.05 were considered statistically significant.

Supplemental data

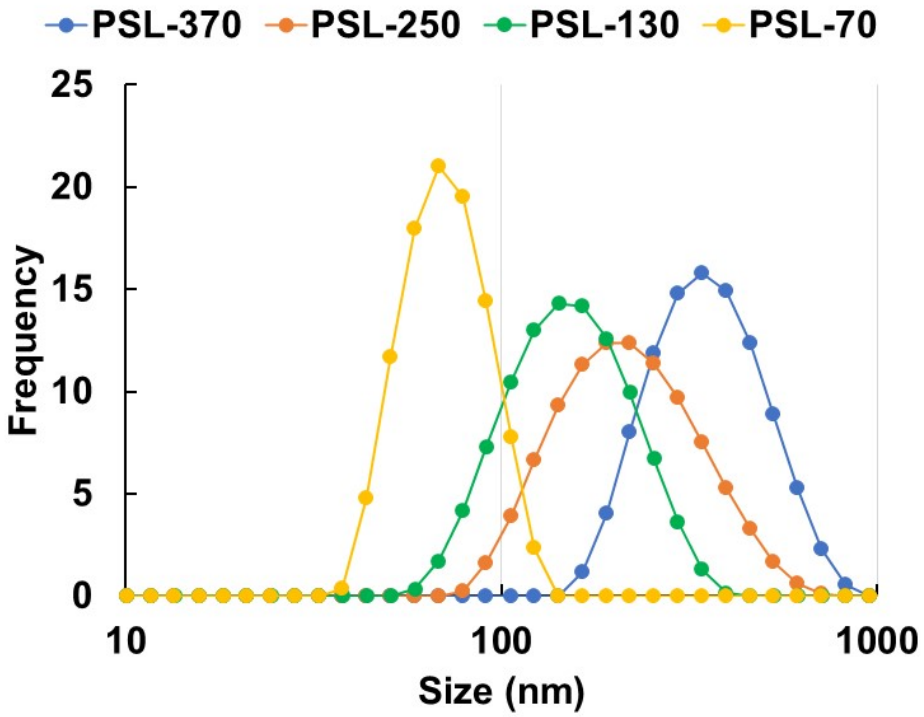

Fig. S1 Size distribution of phosphatidylserine-displaying liposomes determined by dynamic light scattering measurement.

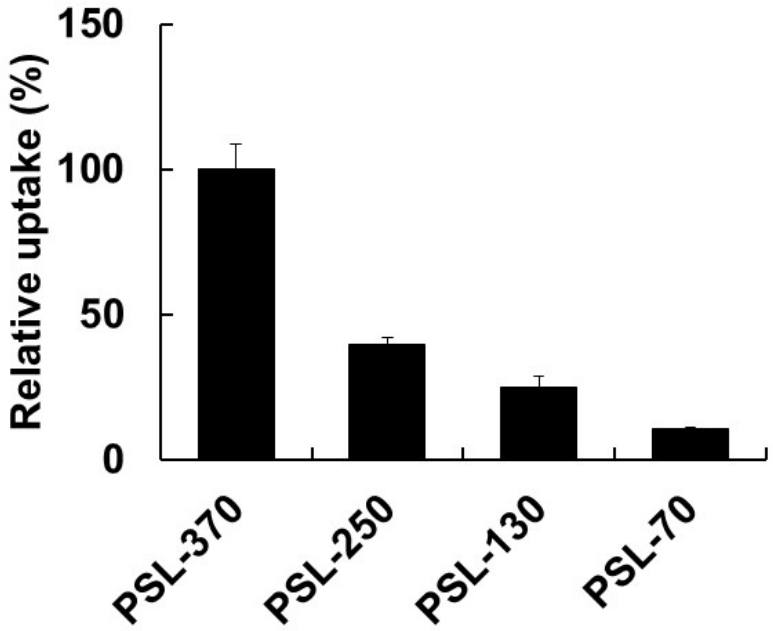

Fig. S2 Effect of phosphatidylserine-displaying liposome (PSL) size on its uptake by RAW 264.7 murine macrophages (n=4). Data are mean ± standard deviation.

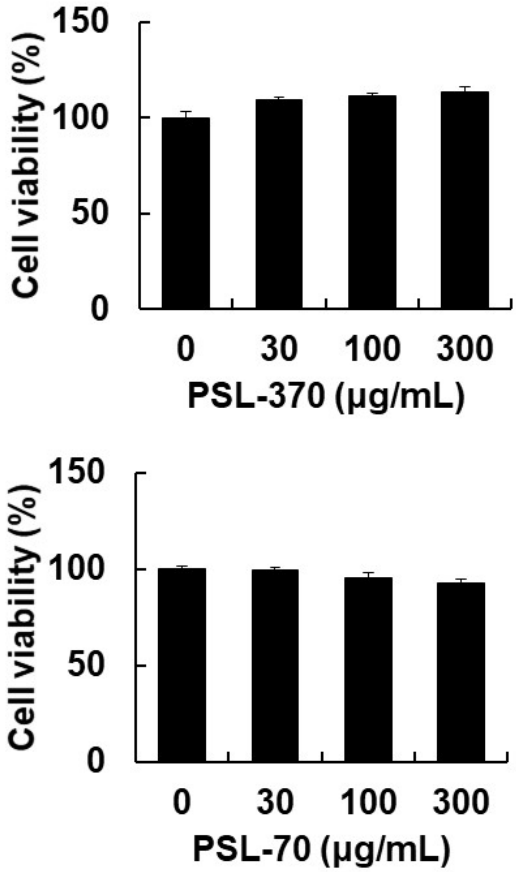

Fig. S3 Cytotoxicity of phosphatidylserine displaying liposomes toward RAW 264.7 murine macrophages (n=5). Both PSL-370 and PSL-70 do not show cytotoxicity at a concentration below 300 μg/mL. Data are mean ± standard deviation.

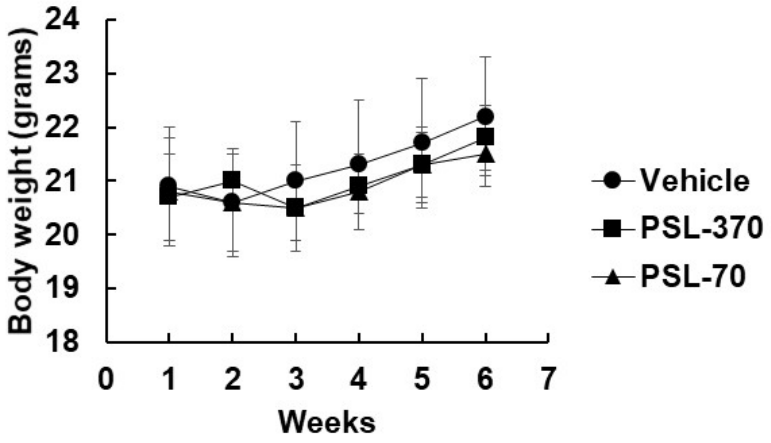

Fig. S4 Changes in the body weight of choline-deficient, methionine-defined high-fat diet (CDAHFD)-fed C57BL/6 mice during treatment (n=6). Data are mean ± standard deviation.

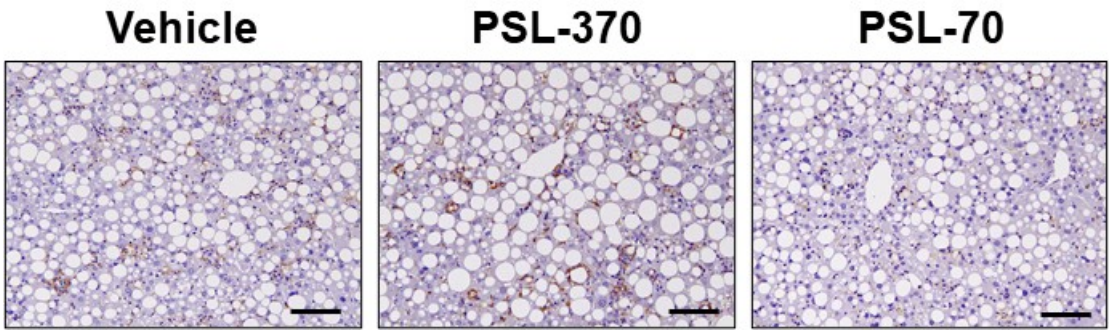

Fig. S5 F4/80 (a macrophage marker)-stained liver sections (original magnification ×200). F4/80-positive cells were stained in brown color. Scale bars are 100 μm.