의학석사 학위논문

# Development and characterization of zoledronic acid encapsulated liposome for macrophage polarization in a tumor model

# 종양 모델에서 대식세포 분극화를 위한 졸레드론산 봉입 리포좀의 개발 및 특성 분석

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A thesis of the Master's Degree

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by

Eunjin Cho

A thesis submitted to the Department of Biomedical Science in partial fulfillment of the requirements for the Degree of Master in Master of Science in Medicine at Seoul National University College of Medicine

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논문 제목: Development and characterization of zoledronic acid encapsulated liposome for macrophage polarization in a tumor model

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# Abstract

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**Introduction:** Zoledronic acid (ZA) is an FDA-approved bisphosphonate drug that facilitates polarization from M2 to M1 macrophages in the tumor microenvironment (TME). Accordingly, various ways have been suggested to deliver ZA to the TME for anti-cancer therapeutic applications. Among them, liposomes are attractive vehicles for drug delivery because their surface can be easily modified to achieve various functions. Mannose was used as a targeting ligand to actively target the mannose receptor (CD206) on the surface of M2 macrophages, including tumor-associated macrophages (TAMs). This study aimed to explore the use of liposomes as nanocarriers to deliver ZA to the TME, especially TAMs, which are similar to M2 macrophages. This study also investigated the contribution of ZA encapsulated in liposomes in mouse tumor

models to the polarization of TAMs.

Methods: Liposomes were prepared with a 5.7:3.8:0.5 molar ratio of HSPC, cholesterol, and PEG2k-PE or a 5.7:3.8:0.25:0.25 molar ratio of HSPC, cholesterol, PEG2k-PE, and Man-PEG2k-PE. The liposome size, stability, and properties were evaluated by dynamic light scattering and nanoparticle tracking analysis (NTA). To investigate whether liposomes specifically target M2 macrophages when they were mannosylated, liposomes were labeled with FNR648 dye, and cellular uptake was assessed in M0 and M2 macrophages using confocal microscopy. A liposome-mediated macrophage polarization experiment was performed to assess the effect of ZA-encapsulated liposomes on macrophage polarization *in vitro* in RAW264.7 cells treated with M2-inducing cytokines, tumor-conditioned media (TCM) or none. For *in vivo* study, 4T1-luc2 cells were injected subcutaneously into both thighs of mice to generate mouse tumor models. After intratumoral injection of PBS, ZA, Lipo, Lipo/ZA, M-Lipo, and M-Lipo/ZA, the bioluminescence signals were detected with IVISspcetrum. To examine the effect of liposomal ZA on macrophage polarization in vivo, flow cytometry analysis and immunohistochemical staining were performed using iNOS (M1

macrophage marker) and CD206 (M2 macrophage marker) on tumor tissue obtained from 4T1-luc2 tumor-bearing mouse models.

**Results:** The hydrodynamic sizes of the liposomes were about 100 nm, which was cross-checked by showing the sizes of about 112 nm using NTA. The PDI values of all liposomes were determined to be reliably low, less than 0.1. The size and PDI values were stable for up to 8 weeks at 4 ℃. Zeta potentials tended to decrease over time. The encapsulation efficiencies of ZA were 17.63% and 18.12% for Lipo/ZA and M-Lipo/ZA, respectively. Unlike FNR648-Lipo in M2 macrophages, FNR648-M-Lipo showed 2.6-fold higher uptake at 1 hour and 2.5-fold higher at 4 hours after liposomes treatment, and there was no significant difference in cellular uptake in M0 macrophages. Lipo/ZA and M-Lipo/ZA increased CD80 expression and decreased CD206 expression in differently stimulated RAW264.7 cells into M2 macrophages, TCM-treated macrophages, and M0 macrophages compared to Lipo and M-Lipo. Treatment with ZA in 4T1-luc2 tumor models significantly increased tumor volume. Liposome treatment containing ZA also tended to increase tumor volume, but this was not statistically significant. As a result of flow cytometry

analysis of 4T1 tumors with liposome treatment, the M-Lipo/ZA group showed the lowest M2 marker expression compared to the other groups. Immunohistochemistry results showed M-Lipo/ZA also increased iNOS marker expression and decreased CD206 expression 7 days after tumor inoculation. At 15 days after tumor inoculation, the CD206 expressions in the ZA, Lipo/ZA, and M-Lipo/ZA groups were decreased compared to the control group.

**Conclusion:** Liposomes were successfully fabricated. When mannose was labeled on the liposome surface, it was more effectively taken up by M2 macrophages. Also, ZA-encapsulated liposomes decreased CD206 expression and increased CD80 expression in macrophages *in vitro* and *in vivo*. It was observed that M1 polarization of TAMs in the tumor microenvironment was induced when M-Lipo/ZA was treated every other day.

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**Keywords:** zoledronic acid, liposome, tumor microenvironment, tumor-associated macrophages, macrophage polarization, drug delivery, flow cytometry, immunohistochemistry

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# LIST OF ABBREVIATIONS

**BMDM,** Bone marrow-derived macrophage

**Cryo-TEM,** Cryogenic transmission electron microscopy

**DBCO,** dibenzocyclooctyne

**DLS,** Dynamic Light Scattering

**DMEM,** Dulbecco's Modified Eagle's Medium

**DPBS,** Dulbecco's Phosphate Buffered Saline

**FACS,** Fluorescence Activated Cell Sorting

**FBS,** Fetal Bovine Serum

**IL,** Interleukin

**IVIS,** *In Vivo* Imaging System

**LPS,** Lipopolysaccharide

**NTA,** Nanoparticle Tracking Analysis

**PDI,** polydispersity index

**TAMs,** Tumor-associated macrophages

**TME,** Tumor microenvironment

**ZA,** Zoledronic acid

# Introduction

A tumor microenvironment (TME) is defined as the complex cellular environment around a tumor, including immune cells, blood vessels, stromal cells, the extracellular matrix (ECM), and other secreted molecules (1, 2). The TME serves critical biological roles in solid tumors' subsequent evolution, such as metabolic support, angiogenesis, metastasis, and immune regulation (2, 3).

The macrophage is one of the most abundant immune cell types in the TME (3). The phenotype of macrophages is primarily divided into M1 and M2 types. Both are closely related to inflammatory responses. M1 macrophages participate in the pro-inflammatory responses against pathogens and produce pro-inflammatory cytokines such as IL-6, IL-12, and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (4). In contrast, M2 macrophages participate in anti-inflammatory responses and tissue repair (4).

Depending on the progression of cancer, their TME can be characterized into two types; tumor-suppressive or tumor-supporting (1, 5). Tumor-suppressive TME contains a high infiltration of $CD8^+$ T cells and M1 macrophages and a low

infiltration of myeloid-derived suppressor cells (MDSCs) (6). However, in tumor-supporting TME, $CD8^+$ T cells are absent. They have a large population of regulatory T cells (Tregs), M2 macrophages, and MDSCs (6). These immune cells promote the development of tumors, which are non-inflammatory tumors, and are correlated with poor response to immunotherapy (6, 7). Therefore, changing TME from a tumor-supporting state to a tumor-suppressive state would be an essential breakthrough in cancer therapy.

Tumor-associated macrophages (TAMs) are generally thought to be more similar to M2 macrophages and play essential roles in the progression of tumors by secreting tumor-promoting cytokines and accelerating tumor growth, angiogenesis, and immune suppression in TME (8-11). They also promote Tregs induction, inactivation of T cells, tumor invasion, and metastasis (8-11). TAMs are currently recognized as potential therapeutic targets for cancer and biomarkers associated with diagnosis and prognosis in various solid tumors. Therefore, the repolarization of M2 macrophages to M1 macrophages can be the foothold for changing the properties of TME to be tumor-suppressive and increasing the efficacy of cancer therapy.

CD206, a mannose receptor, is mainly expressed on the surface of M2 macrophages. It acts as a pattern recognition receptor (PPR) for pathogens like bacteria and plays a role in innate and adaptive immunity (12, 13). Many pathogenic microbes are coated with structures containing mannose, and CD206 on M2 macrophages can bind to those pathogens with high affinity (13). TAMs are M2-like macrophages that express CD206 and exhibit similar functional properties (8-11). Therefore, CD206 is a promising biomarker targeting M2 macrophages and TAMs.

Clodronic acid is one of the best-known bisphosphonates that prevent bone metastases in patients with breast cancer (13). In addition, clodronic acid depletes mature macrophages, inhibiting tumor progression in various malignant tumors, including melanoma and ovarian cancer (15, 16). However, various drugs using clodronic acids, such as bonefos and clodron, have been developed, but the U.S. FDA currently approves no drugs due to many side effects.

Zoledronic acid (ZA) is an FDA-approved nitrogen-containing bisphosphonate drug soluble in water and is used to treat many bone diseases, such as osteoporosis and bone metastases (17). Recently, ZA has been found to facilitate

macrophage polarization in the TME (18-21). An increase in TLR-4 and an increase in M1 polarization of macrophages were detected after ZA administration both in vitro and in vivo in mice. (18). TEP-1 macrophage-like cells were M1 polarized by ZA. (19). However, bisphosphonates containing ZA tend to accumulate a lot in bones due to their poor membrane permeability and strong binding ability with calcium ions (22, 23). Therefore, a nanocarrier is required to deliver ZA effectively to the TAMs in TME.

Liposomes are easy to change their composition, and different compositions provide physical properties, functions, and uses that have not existed before. Liposome properties depend primarily on lipid composition, surface charge, size, and preparation method (24). Representative advantages of liposomes are biocompatibility, biodegradability, and excellent stability (24, 25). Also, phospholipids have hydrophobic tails and hydrophilic heads in one molecule (24, 25). Therefore, liposomes have an aqueous core and a bilayer interface, so it is possible to partition and solubilize both hydrophilic and hydrophobic materials (24, 25).

Various ways have been suggested to deliver ZA to the TME for anti-cancer therapeutic applications. In this study, we

explored the use of liposomes as nanocarriers for the delivery of ZA to TAMs in TME. This study also aimed to investigate the contribution of liposomal ZA to macrophage polarization and changes in the tumor microenvironment in mouse tumor models.

# MATERIAL AND METHODS

## 1. Preparation of the liposomes

### Extrusion and purification of liposomes

L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (18:0 PEG2000-PE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzocyclooctyl(polyethylene glycol)-2000] (DSPE-PEG2000-DBCO) were acquired from Avanti Polar Lipids (Alabaster, AL, USA). DSPE-PEG-Man was purchased from Biopharma PEG (Watertown, MA, USA). Zoledronic acid monohydrate was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Lipo, Lipo/ZA, M-Lipo, and M-Lipo/ZA were prepared by the thin-film hydration-extrusion method. HSPC, cholesterol, and $PEG_{2000}$-PE in a molar ratio of 5.7:3.8:0.5 were dissolved in chloroform: methanol (2:1 %v/v), and then the solvent was removed under vacuum to form thin lipid films. The resulting lipid films were hydrated with distilled water for blank liposomes or ZA solution (6.89415 mM) for ZA-loaded liposomes at 65℃. The resulting suspension was subjected to

extrusion 21 times using an Avanti Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL USA) via 100-nm PC membrane with a temperature of 65℃, close to the $T_c$ of HSPC (55℃) to obtain liposomes without mannose (Lipo and Lipo/ZA). Unencapsulated ZA was removed with the PD-10 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and determined whether it was completely removed using the NanoDrop OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Mannose-labeled liposomes (M-Lipo and M-Lipo/ZA) were composed of HSPC, cholesterol, PEG2000-PE, and DSPE-PEG-Man in a ratio of 5.8:3.8:0.25:0.25. M-Lipo and M-Lipo/ZA were prepared by the thin-film hydration-extrusion method as previously described.

For fluorescently labeled FNR648, liposomes (FNR648-Lipo and FNR648-Lipo/ZA) were prepared with HSPC, cholesterol, and $PEG_{2000}$-PE or HSPC, cholesterol, $PEG_{2000}$-PE, and DSPE-PEG-Man in a molar ratio of 5.7:3.8:0.5 or 5.7:3.8:0.25:0.25 in addition to 5 mol% of DSPE-$PEG_{2000}$-DBCO. To verify *in vitro* uptake of liposomes in RAW264.7, $N_3$-FNR648 and DBCO-Liposome were reacted for 30 minutes at 25℃.

Table 1. Various formulations of liposomes

| Sample | Composition | Molar ratio |
|---|---|---|
| Lipo<br>Lipo/ZA | HSPC/Chol/PE-$PEG_{2k}$ | 5.7/3.8/0.5 |
| Lipo-DBCO<br>(for click chemistry) | HSPC/Chol/PE-$PEG_{2k}$/DSPE-$PEG_{2k}$-DBCO | 5.7/3.8/0.5/0.5 |
| M-Lipo<br>M-Lipo/ZA | HSPC/Chol/PE-$PEG_{2k}$/DSPE-$PEG_{2k}$-Mannose | 5.7/3.8/0.25/0.25 |
| M-Lipo-DBCO<br>(for click chemistry) | HSPC/Chol/PE-$PEG_{2k}$/DSPE-$PEG_{2k}$-Mannose/DSPE-$PEG_{2k}$-DBCO | 5.7/3.8/0.25/0.25/0.5 |

Lipo: liposome, M-Lipo: mannose-labeled liposome, Chol: Cholesterol

### Characterization of the liposomes

Hydrodynamic diameter, size distributions, and surface charge of the liposomes were determined with dynamic light scattering (DLS) using the Malvern Zetasizer Nano ZS90 system (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) and with nanoparticle tracking analysis (NTA) using NanoSight NS300 system (Malvern Panalytical Ltd.). For DLS measurement, the liposomes solution was diluted 10 times in distilled water and mixed for 10 seconds by sonication. The liposomes solution was diluted $10^4$ times in distilled water for NTA measurement and mixed by the vortexer. The analysis was performed using Zetasizer v8.01.4906 software (Malvern Panalytical Ltd.). The morphologies of liposomes were observed by cryogenic transmission electron microscopy (cryo-TEM, JEM-2100Plus, JEOL Ltd., Akishima, Tokyo, Japan). Specimens for cryo-TEM were prepared with 200 mesh Cu holey carbon grids using an FEI Mark IV Vitrobot (FEI Company, Hillsboro, OR, USA). TEM images were taken using JEOL TEM with an acceleration voltage of 200 kV. To obtain encapsulated efficiency (EE) of ZA-loaded liposomes (Lipo/ZA and M-Lipo/ZA), 100 μL of liposomes were added to 100 μL of

Chloroform and mixed for 15 minutes by sonication. Then, density gradient centrifugation was performed to obtain a supernatant containing ZA. ZA in the supernatant was measured using NanoDrop OneC Microvolume UV-Vis Spectrophotometer at a wavelength of 210 nm. EE was determined using the following equation.

$$\text{EE}\ (\%) = \frac{Amount\ of\ ZA\ in\ liposomes}{Total\ amount\ of\ ZA\ used\ for\ loading} \times 100$$

## 2. *In vitro* experiments of liposomes for macrophage polarization

### Cell culture

The murine macrophage cell line, RAW264.7 was grown in DMEM media (Welgene, Daegu, South Korea) containing 10% (v/v) fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% antibiotics containing penicillin/streptomycin (GenDEPOT, Baker, TX, USA). The murine breast cancer cell line, 4T1-luc2, was grown in RPMI media (Welgene). Cells were incubated in a 37℃ humidified incubator with a 5% $CO_2$ atmosphere.

4T1, known to form a tumor-supporting TME, was used to

achieve the research goal of converting the TME from a tumor-supporting state to a tumor-suppressive state using ZA (26). Especially firefly luciferase-expressing 4T1 cells (4T1-luc2) were finally selected for visualizing the tumor cells *in vivo*.

### Induction of macrophage polarization

RAW264.7 cells were seeded in 6-well plates at $2\times10^5$ cells per well. After 24 hours of incubation, INF-$\gamma$ (20 ng/ml) and LPS (50 ng/ml) were incubated with RAW264.7 cells to induce polarization into M1 macrophages. IL-4 (20 ng/ml) and IL-10 (20 ng/ml) were incubated with RAW264.7 cells for M2 polarization.

For tumor-conditioned media (TCM) treated macrophages, TCM was generated from the culture supernatant of 4T1 tumor cells. 4T1 cells($1\times10^7$ cells/dish) were cultured in RPMI media for 24 hours. the culture supernatant was filtered with 0.22 μm syringe filter (Millipore, Billerica, MA, USA) after centrifugation for 10 minutes at 1500 rpm. Macrophages were incubated for 24 hours with cytokines or TCM before flow cytometry analysis.

### Confocal microscopy

For the study of the cellular uptake of Lipo and M-Lipo, FNR648-labeled liposomes were used. $1\times10^5$ cells (RAW264.7) were seeded in each well of the 12-well plate (Nalgene NUNC International, Naperville, IL, USA) since the day before. Macrophages were induced into M0 or M2 macrophages, respectively. FNR648 labeled liposomes were added to the plates to a total lipid concentration of 0.4 mmol/L and incubated at 37℃ for 0.5, 1, 4, 8, and 24 hours. Cells were washed with DPBS and fixed using 4% paraformaldehyde at 37℃ for 10 minutes. Finally, the slides were mounted with the prolonged gold reagent. Fluorescence images were taken by confocal laser scanning microscope (Olympus LX83, Olympus Corp., Shinjuku, Tokyo, Japan).

### *In vitro* flow cytometry analysis

The macrophages stained with FACS antibody were detected by flow cytometry using FACS Canto II (BD Biosciences, San Jose, CA, USA). The analysis was performed using FlowJo v10.7.1 software (Tree Star Inc., Ashland, OR, USA).

For *in vitro* macrophage polarization study, macrophages were plated on 6-well plates, cultured for 24 h, and then induced into M1 or M2 macrophages using M1-induced cytokines and M2-induced cytokines, respectively. Various samples (DW, free ZA, Lipo, and Lipo/ZA with a ZA concentration of 1.1 mM) were treated for 24 hours, and cells were separated using a cell scraper. Cells were counted and transferred to a 5 ml test tube. $1X10^6$ cells were suspended in 100 μl of cold FACS buffer and stained with FACS antibodies for 30 minutes at 4℃. And they were labeled with CD206-PECy7 and CD80-PE for flow cytometry. CD206-PECy7 was used to label M2 macrophages, and CD80-PE was used to label M1 macrophages. All antibodies were diluted 1:100 in FACS buffer. Cells were washed with 1 ml of FACS buffer and resuspended in 100 μl of FACS buffer before analysis.

### 3. *In vivo* experiments of liposomes for macrophage polarization

#### Animal model

Female BALB/C mice were selected for tumor models because 4T1 is a breast cancer cell line from the mammary gland tissue of BALB/C mice and closely mimics human breast

cancer in tumor progression when injected into them. Six-week-old female BALB/C mice were obtained from Orient Bio, Inc. (Seongnam, Korea). To establish the mouse tumor models, 4T1-luc2 cell lines ($5x10^5$) were injected subcutaneously into both thigh legs. The tumor was formed in the thigh leg, and tumor size was measured by a caliper every 2 days. Non-invasive bioluminescence imaging was used for monitoring tumor growth.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

### Liposome injection

ZA (10 $\mu$g/site) was injected intratumorally into 4T1-luc2 tumor-bearing mice every 2 days after tumor inoculation. For Lipo/ZA and M-Lipo/ZA, the dose was calculated such that ZA encapsulated in liposomes could be 10 $\mu$g/site, and the total injection volume was 25 $\mu$l per tumor. Mice were sacrificed 7 or 15 days after tumor inoculation, and tumors were excised.

### Bioluminescence optical imaging

An *in vivo* IVISspectrum imaging system (Perkin Elmer,

Waltham, MA, USA) was used to monitor luciferase-expressing tumor cells. D-luciferin (3 mg/mouse, Promega, Madison, WI, USA) was injected intraperitoneally into 4T1-luc2 tumor-bearing mice 10 minutes before optical imaging. Bioluminescence optical imaging was performed for about 0 to 30 minutes to obtain the maximum radiance. The bioluminescent signal was acquired and analyzed by Living Image v2.50.1 software (Xenogen, Alameda, CA, USA)

*In vivo* flow cytometry analysis

The macrophages stained with FACS antibody were detected and analyzed as above.

For *in vivo* macrophage polarization study, tumors from 4T1-luc2 tumor-bearing mice were ground into single cells using a homogenizer. Ground tumors were filtered through a cell strainer (40 $\mu$m nylon, Falcon, Oneonta, NY, USA) and washed with 1 ml of FACS buffer (DPBS with 5% FBS). As a positive control, the spleen was isolated. The ground spleen was centrifuged (1500 rpm, 5 minutes, 4℃), and Red blood cell lysis buffer (1 ml/spleen, Sigma-Aldrich) was added to the cell pellet. The mixture was incubated at RT for 2 minutes to remove red blood cells, and a double volume of RPMI medium

was added to the cells. After repeating 2 or 3 times, cells were filtered through a cell strainer. Cells were counted and transferred to a 5 ml test tube. $1X10^6$ cells were suspended in 100 μl of cold FACS buffer and stained with FACS antibodies for 30 minutes at 4℃. The following FACS antibodies were used; CD45R-APC, CD11b-V450, F4/80-PE, CD206-PECy7, and iNOS-FITC. All antibodies were diluted 1:100 in FACS buffer except F4/80-PE (1:400). Cells were washed with 1 ml of FACS buffer and resuspended in 500 μl of 4% PFA for 30 minutes at 4℃. After fixation, cells were washed with 1 ml of FACS buffer and resuspended in 100 μl of FACS buffer before analysis.

### Immunohistochemistry

4T1-luc2 tumor tissues from 4T1-luc2 tumor-bearing mice were embedded in paraffin and sectioned to a thickness of 4 μm segments. Heat-induced method for antigen epitope retrieval was used. Tumor tissue was blocked with 3% normal horse serum in PBS for 30 minutes, and the primary antibodies were incubated overnight at 4℃. The following primary antibodies were used; Anti-CD31, Anti-CD45, Anti-CD68, anti-CD206, and Ki-67 (Abcam, Cambridge, MA, USA), anti-iNOS (Santa

Cruz Biotechnology, Dallas, TX, USA). The tumor tissues were washed with PBS three times and then incubated with biotinylated secondary antibodies (anti-rabbit IgG for CD31, CD45, CD68, iNOS, CD206, and Ki-67; secondary antibody from Vector Laboratories (Burlingame, CA, USA). Avidin-biotin solution (Vector Laboratories) was incubated for 1 hour at RT. DAB (3,3-diaminobenzidine) substrate (Vector Laboratories, Burlingame, CA, USA) was used for developing the antigenic signals, according to the manufacturer's instructions. The tumor tissues were then counterstained with hematoxylin and mounted with Permount Mounting solution (ThermoFisher Scientific, Fair Lawn, NJ, USA).

### 4. Statistical analysis

All results were calculated as means standard (SD). Statistically significant differences were analyzed by a paired 2-sample Student t-test. Statistical significance was considered $p<0.05$. All statistical analysis was measured using GraphPad Prism 9 software (San Diego, CA, USA).

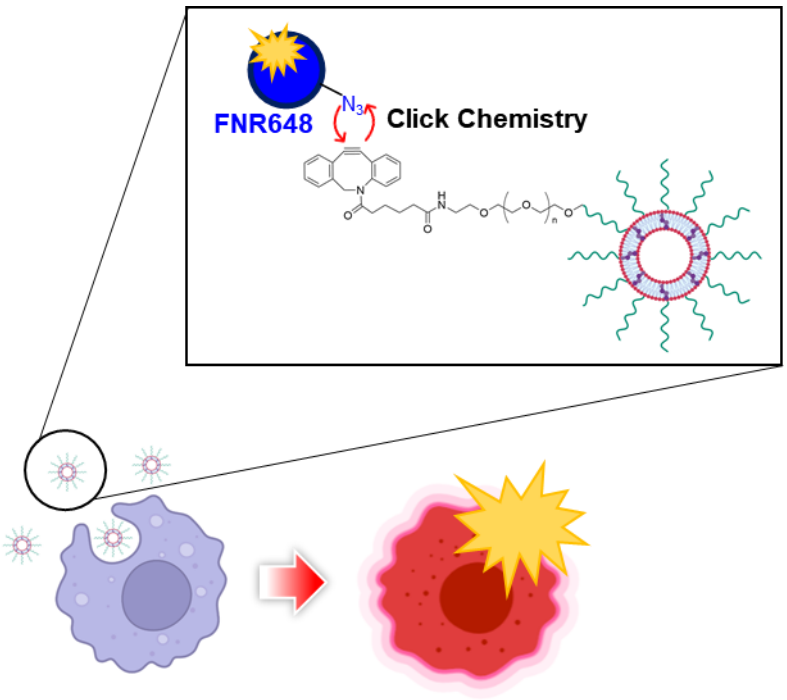



Figure 1. Click chemistry design for FNR648 labeled liposomes

Click reaction was used to conjugate liposomes and FNR648 dye for 30 minutes at RT. The DBCO group from liposomes and the $N_3$ group from FNR648 dye formed a covalent bond in a click reaction.

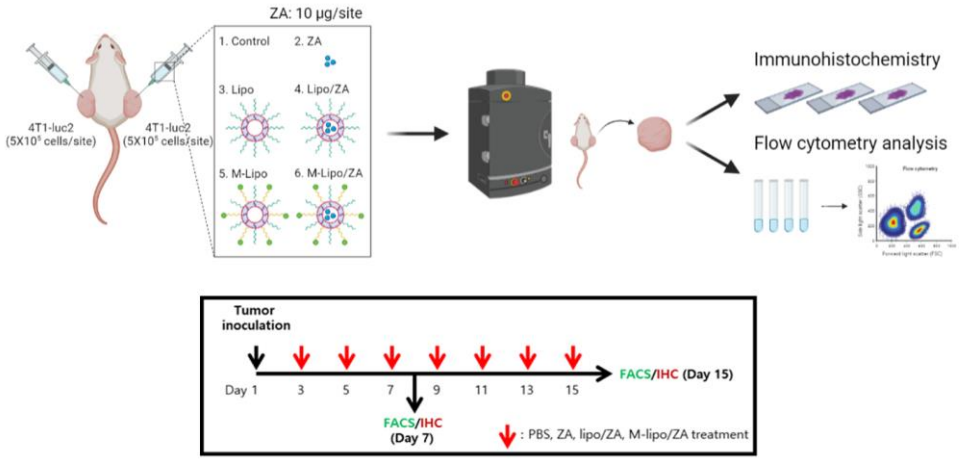



Figure 2. *In vivo* experimental scheme

4T1 cells ($5 \times 10^5$) expressing firefly luciferase were transplanted subcutaneously into the left and right thighs. Intratumoral injections of 10 μg/site ZA (free ZA, Lipo/ZA, and M-Lipo/ZA), Lipo, M-Lipo, or control (PBS) every 2 days with a total injection volume of 25 μL were performed. Tumor growth was followed by IVIS imaging. Before injection, IVIS images were obtained. Tumors were prepared for flow cytometry and immunohistochemistry 7 or 15 days after tumor inoculation.

# RESULTS

## 1. Preparation of liposomes

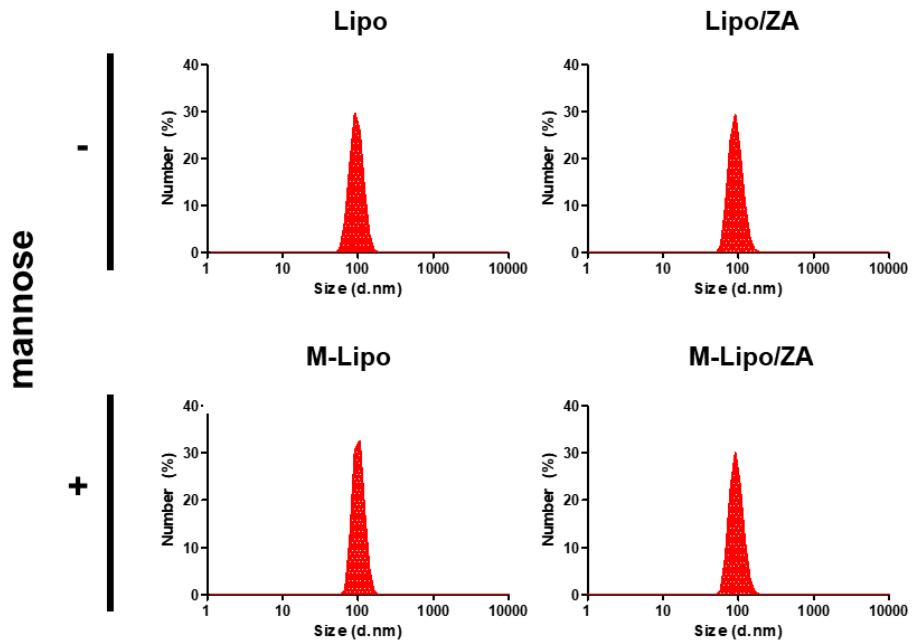
### Preparation and characterization of liposomes

Liposomes were prepared using the thin-film hydration method. The sizes and zeta potentials of liposomes were measured using DLS and NTA.

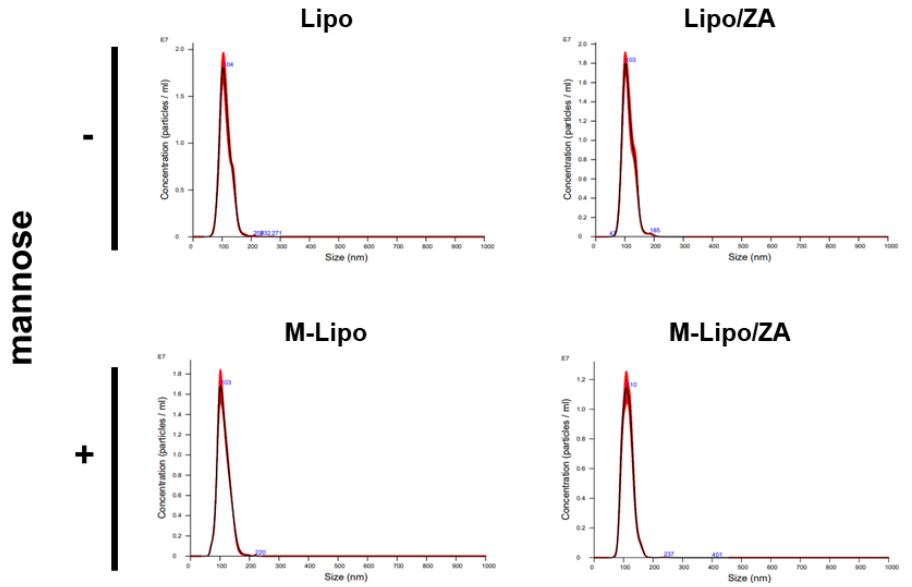
The hydrodynamic diameters of all liposomes were similar by about 100 nm, irrespective of mannosylation and drug-loading (Figure 3A and Table 2). As shown in Table 2, the polydispersity index (PDI) values of all liposomes were below 0.1, which indicates monodispersity. The NTA also reported single peaks of liposomes with an average size of approximately 112.9 nm (Figure 3B and Table 2). Liposomes showed an average zeta potential of −24.4 mV (Figure 3C and Table 2). The zeta potentials tended to decrease over time, and in particular, Lipo/ZA and M-Lipo/ZA showed slightly lower values than other liposomes (Table 2). The morphologies of liposomes were examined via cryo-TEM. The cryo-TEM images showed mainly spherical structures with uniform diameters ranging from 95 to 120 nm, consistent with the results of DLS and NTA (Figure 3C). In addition, liposomes

were well dispersed. The EE of Lipo/ZA was 17.63%, which decreased to 13.79% over 8 weeks (Table 2). During the same period, the EE of M-Lipo/ZA decreased from 18.12% to 17.13% (Table 2). Liposomes showed good stability for 8 weeks when they were stored at 4℃ (Table 2).

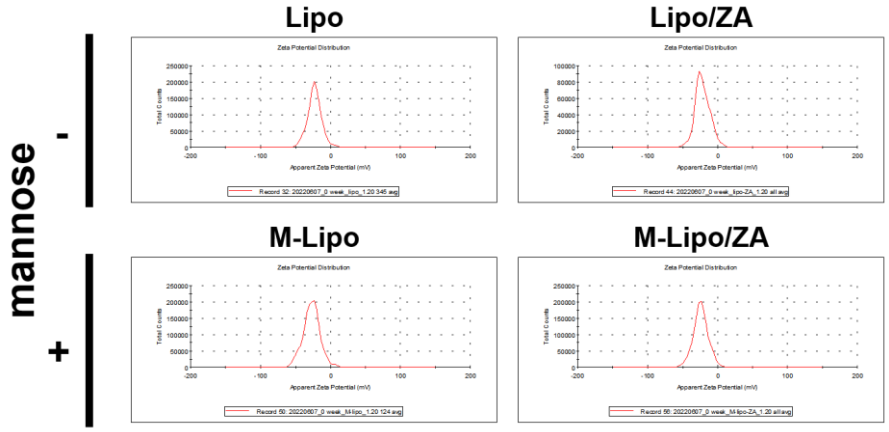
(A)


Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA
mannose
-
+
Number (%)
Size (d.nm)
0
10
20
30
40
1
10
100
1000
10000

(B)


Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA
mannose
-
+
Concentration (particles / ml)
Size (nm)
E7
0
100
200
300
400
500
600
700
800
900
1000

(C)


Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA
mannose
-
+

(D)

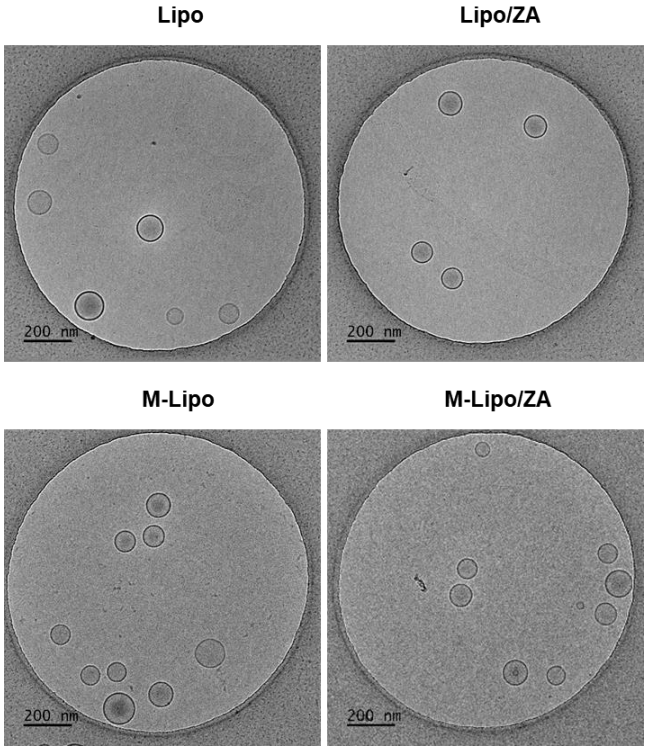

Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA
200 nm
200 nm
200 nm
200 nm

Figure 3. Characterization of liposomes

(A) Size distributions of all liposomes (Lipo, Lipo/ZA, M-Lipo, and M-Lipo/ZA) measured by DLS. (B) Sizes of liposomes measured by NTA were similar to the hydrodynamic diameters from DLS. (C) The zeta potential graph showed one peak at about -20 mV. (D) Cryo-TEM images of liposomes. The scale bar indicates 200 nm.

Table 2. Physiochemical characterization of liposomes and stability at 4℃

| Formulation | Time (week) | Size (nm) | | PDI | Zeta potential (mV) | EE (%) |
|---|---|---|---|---|---|---|
| | | DLS | NTA | | | |
| Lipo | 0 | 97.03 ± 18.96 | 112.6 ± 21.0 | 0.028 | -23.0 ± 10.1 | - |
| | 1 | 103.2 ± 17.69 | 119.5 ± 18.3 | 0.019 | -31.7 ± 12.3 | - |
| | 2 | 99.63 ± 18.50 | 117.3 ± 17.9 | 0.026 | -33.7 ± 10.7 | - |
| | 4 | 102.4 ± 16.98 | 113.3 ± 17.9 | 0.056 | -33.1 ± 10.6 | - |
| | 8 | 99.84 ± 18.20 | 116.1 ± 19.9 | 0.034 | -34.6 ± 12.3 | - |
| Lipo/ZA | 0 | 93.91 ± 19.25 | 112.4 ± 20.4 | 0.065 | -23.6 ± 11.6 | 17.63 |
| | 1 | 95.70 ± 19.44 | 117.3 ± 20.5 | 0.033 | -31.7 ± 11.1 | 16.03 |
| | 2 | 96.64 ± 19.34 | 113.1 ± 17.0 | 0.057 | -34.2 ± 11.1 | 16.35 |
| | 4 | 94.26 ± 19.22 | 111.0 ± 16.7 | 0.078 | -34.8 ± 7.75 | 8.64 |
| | 8 | 94.04 ± 20.16 | 110.8 ± 17.3 | 0.064 | -38.5 ± 7.87 | 13.79 |
| M-Lipo | 0 | 102.9 ± 17.34 | 112.6 ± 20.3 | 0.019 | -27.1 ± 12.1 | - |
| | 1 | 102.2 ± 18.44 | 116.3 ± 17.9 | 0.023 | -29.7 ± 11.0 | - |
| | 2 | 99.48 ± 18.96 | 114.9 ± 17.6 | 0.052 | -33.3 ± 10.6 | - |
| | 4 | 96.46 ± 18.74 | 112.8 ± 17.5 | 0.046 | -31.8 ± 11.2 | - |
| | 8 | 100.9 ± 19.37 | 114.1 ± 17.7 | 0.042 | -31.6 ± 10.8 | - |
| M-Lipo/ZA | 0 | 95.34 ± 19.05 | 114.0 ± 18.6 | 0.062 | -24.0 ± 10.7 | 18.12 |
| | 1 | 94.20 ± 19.61 | 113.9 ± 19.2 | 0.051 | -30.3 ± 10.8 | 16.15 |
| | 2 | 94.60 ± 20.57 | 110.5 ± 19.9 | 0.039 | -34.8 ± 12.0 | 14.80 |
| | 4 | 94.44 ± 20.39 | 111.0 ± 17.5 | 0.045 | -34.0 ± 11.7 | 12.37 |
| | 8 | 95.03 ± 19.87 | 108.6 ± 19.2 | 0.059 | -42.5 ± 12.1 | 17.13 |

PDI: polydispersity index

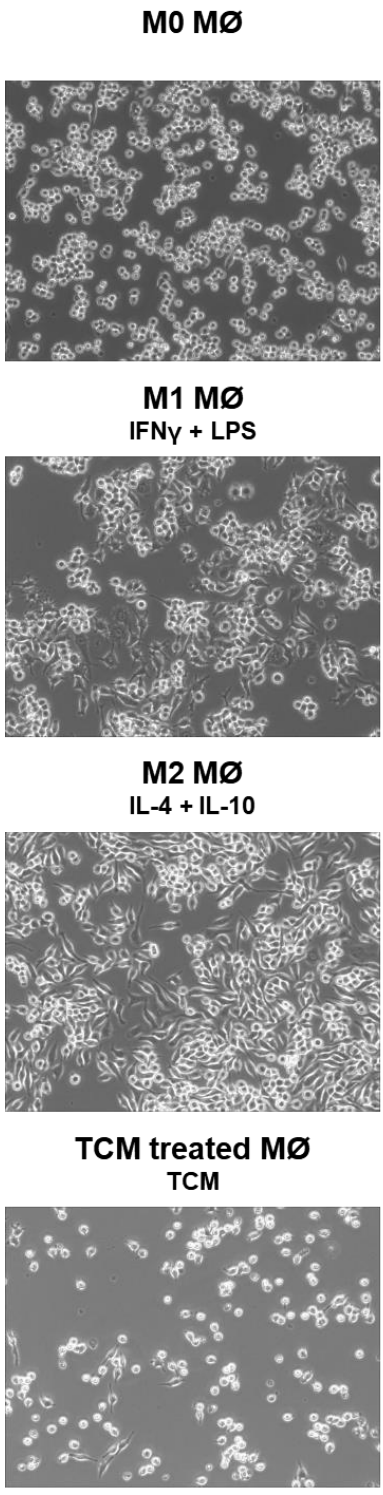
## 2. *In vitro* experiments of liposomes for macrophage polarization

### Macrophage polarization

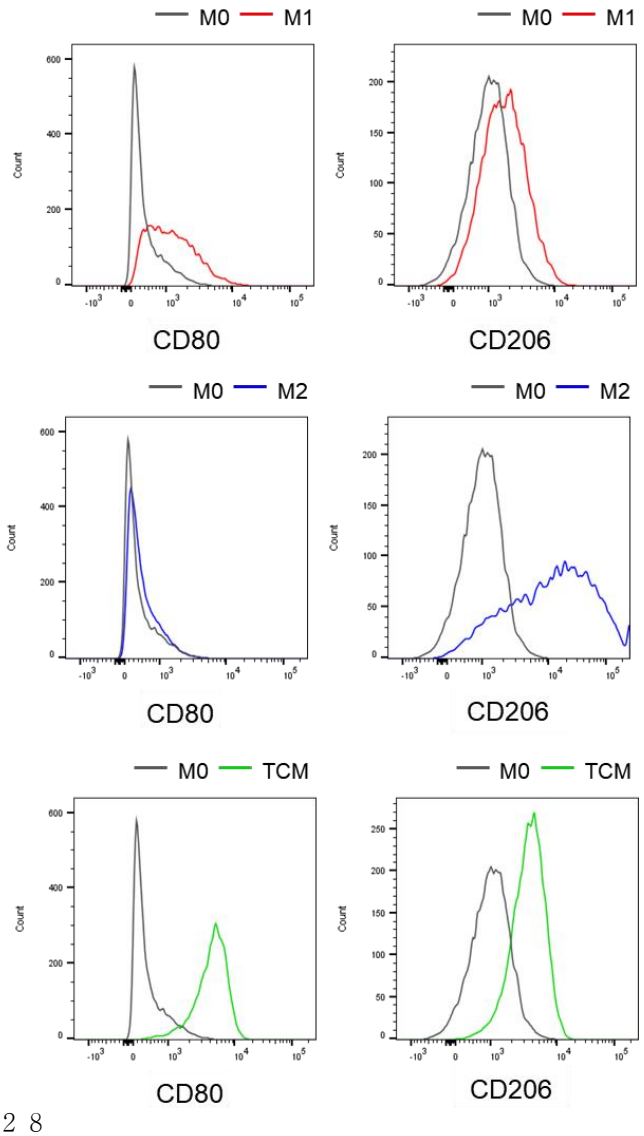
To test the induced M1/M2 polarization, M1-inducing cytokines (LPS and INF-$\gamma$) and M2-inducing cytokines (IL-4 and IL-10) were treated in RAW264.7 cells. Morphological changes, such as spreading (M1 macrophages) and elongation (M2 macrophages) of the cells, were observed (Figure 4A). Surface marker expressions of iNOS and CD206 in M1 and M2 macrophages were analyzed using flow cytometry (Figure 4B). M1-inducing cytokines increased iNOS expression in RAW264.7 cells (Figure 4B). However, CD206 expression was also slightly increased. M2-inducing cytokines highly increase CD206 expression in RAW264.7 cells, apart from no significant difference in iNOS expression. In tumor-conditioned media (TCM) treated macrophages, both iNOS and CD206 expression were increased compared to the control group. Interestingly, the size of polarized RAW264.7 cells varied. Upon activation, the size of RAW264.7 cells increased, as evidenced by the shift of FSA-A of M1, M2 macrophages, or TCM-treated macrophages compared to M0 macrophages (Figure 4C). Cell size was smallest for M0 macrophages and largest when M1

polarization was induced.

(A)


M0 MØ
M1 MØ
IFNγ + LPS
M2 MØ
IL-4 + IL-10
TCM treated MØ
TCM

(B)


M0
M1
M2
TCM
Count
CD80
CD206

(C)

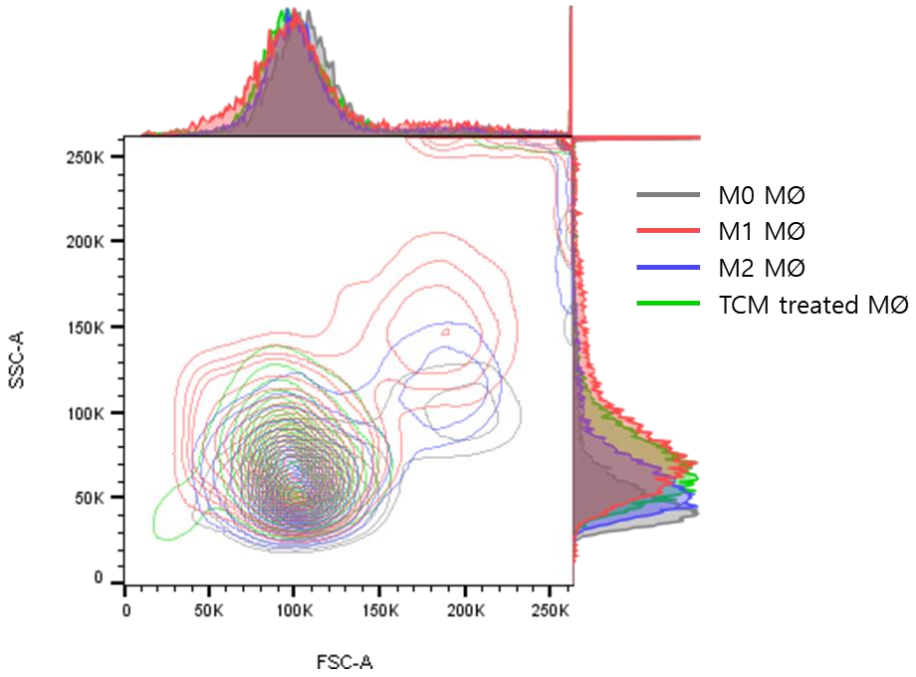



Figure 4. Macrophage activation analysis by flow cytometry

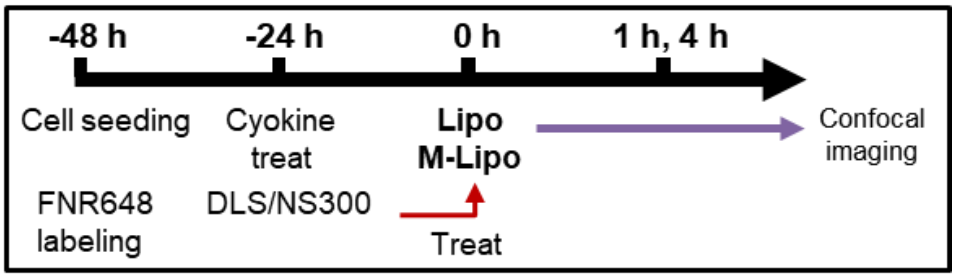
(A) RAW264,7 cells were stimulated with INF-γ (20 ng/ml) and LPS (50 ng/ml) for inducing M1 macrophages and with IL-4 and IL-10 (20 ng/ml) for inducing M2 macrophages. TCM-treated macrophages were incubated with TCM of 2 ml. (B) The activation-related surface markers were analyzed by flow cytometry after M1/M2 polarization or TCM treatment. (C) Depending on stimulation with M1/M2-inducing cytokines or TCM, the size of RAW264.7 cells increased in M1, M2, or TCM-treated macrophages compared to M0 macrophages.

*In vitro* cellular uptake of liposomes in RAW264.7 cells

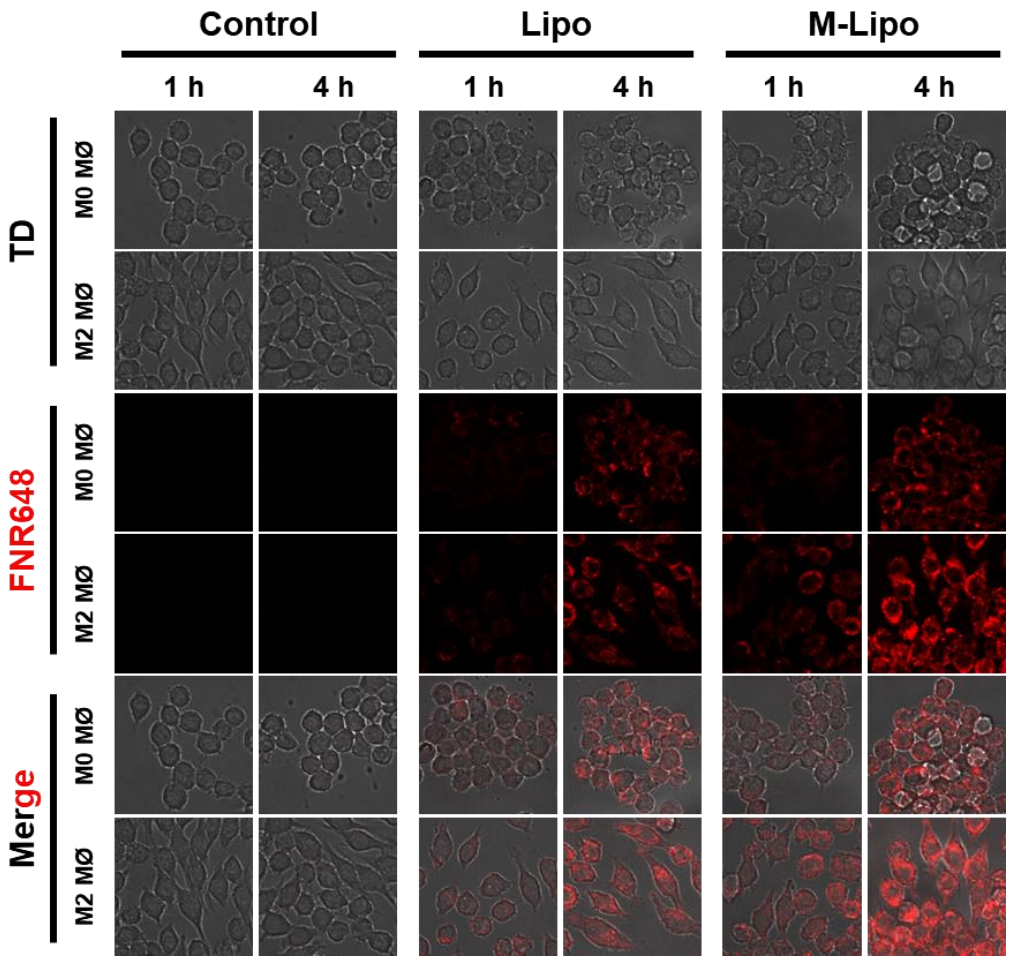
To compare the cellular uptake of Lipo and M-Lipo in RAW264.7 cells, those two liposomes were conjugated with fluorescence, FNR648, and visualized using confocal microscopy. (Figure 5A). Two phenotypes of RAW264.7 cells were used; non-activated (M0 macrophages) and anti-inflammatory (M2 macrophages).

It was observed that M0 macrophages had a round morphology, and the M2 macrophages had a pointed morphology (Figure 5B). Both liposomes labeled with FNR648 were accumulated in the cytoplasm. When RAW264.7 cells were treated with M-Lipo, liposome uptake increased more in M2 macrophages than in M0 macrophages. When incubated with M-Lipo for 1 hour, M2 macrophages showed a 2.6-fold increase in median fluorescence intensity (MFI) compared to M0 macrophages (Figure 5C). MFI increased 2.5-fold in M2 macrophages than in M0 macrophages after 4 hours of incubation with M-Lipo. Cellular uptake of Lipo was not significantly different between M0 and M2 macrophages (Figure 5C). Results indicate that M-Lipo accumulated more effectively in M2 macrophages than in M0 macrophages.

(A)


-48 h
-24 h
0 h
1 h, 4 h
Cell seeding
Cyokine treat
Lipo
M-Lipo
Confocal imaging
FNR648 labeling
DLS/NS300
Treat

(B)


Control
Lipo
M-Lipo
1 h
4 h
1 h
4 h
1 h
4 h
TD
FNR648
Merge
M0 MØ
M2 MØ

(C)

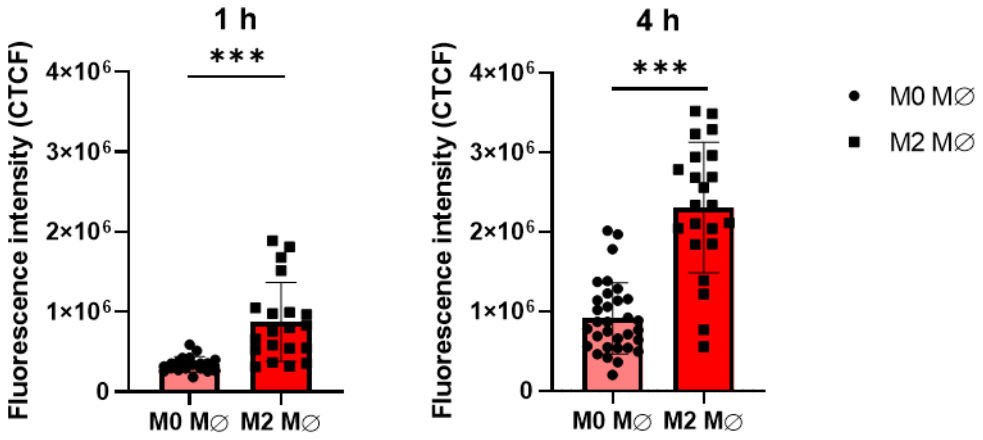



Figure 5. Cellular uptake of FNR648 labeled Lipo or M-Lipo

(A) Experimental scheme for treatment of cytokines and liposomes to induce M2 macrophages and to observe cellular uptake. (B) Representative confocal laser scanning microscopy (CLSM) images were obtained using FNR648-Lipo and FNR648-Lipo/ZA (red signal) in M0 or M2 macrophages. The scale indicates 63.640 mm. M-Lipo was better in uptake by cells than Lipo when RAW264.7 cells were polarized into M2 macrophages. (C) Images of M-Lipo were captured, and fluorescence intensity was quantitated using Image J software and displayed in corrected total cell fluorescence (CTCF).

*In vitro* flow cytometry analysis of macrophage polarization induced by liposomes

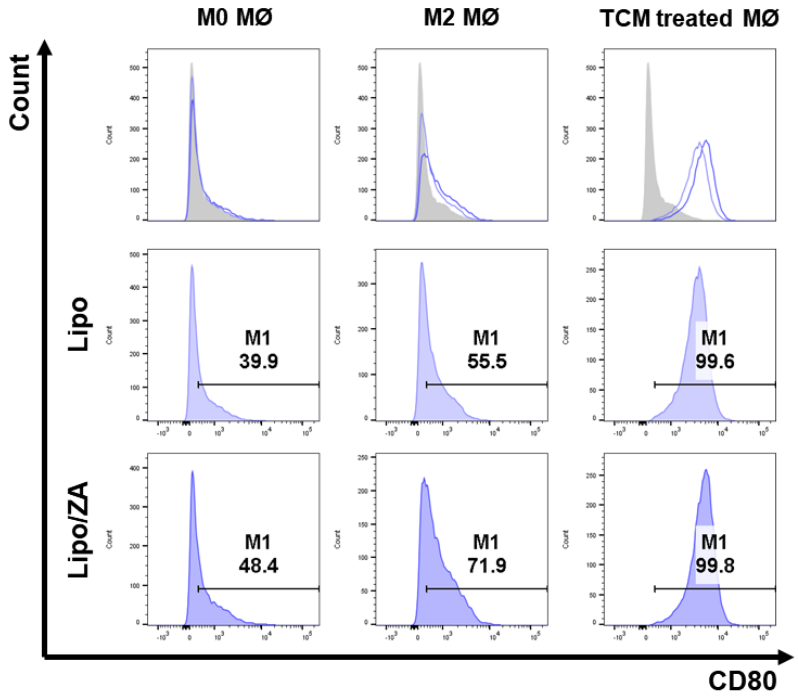
For the liposome-mediated macrophage polarization study, M1 macrophage markers (CD80) and M2 macrophage markers (CD206) on RAW264.7 were analyzed using flow cytometry.

When M0 macrophage was treated with Lipo/ZA, it was observed that the expression of CD80, an M1 marker, increased slightly from 39.9% to 48.4% compared to when Lipo was treated (Figure 6A). In M2 macrophage, CD80 expression in the Lipo/ZA group increased about 1.3-fold from 55.5% to 71.9% compared to the Lipo group, clarifying this difference. In TCM-treated macrophages, the peak seemed to increase slightly towards CD80 positive according to Lipo/ZA treatment, but there was no significant difference in % population. The expression of CD206, an M2 marker, did not decrease in M0 macrophages by Lipo/ZA treatment but decreased in M2 and TCM-treated macrophages (Figure 6B). CD206 expression in M2 macrophages was 73.6% in the Lipo group and 48.6% in the Lipo/ZA group, significantly lower in the Lipo/ZA group. This is the same in TCM-treated macrophage, which decreased by 37.5% from 49.7% to 10.4%.

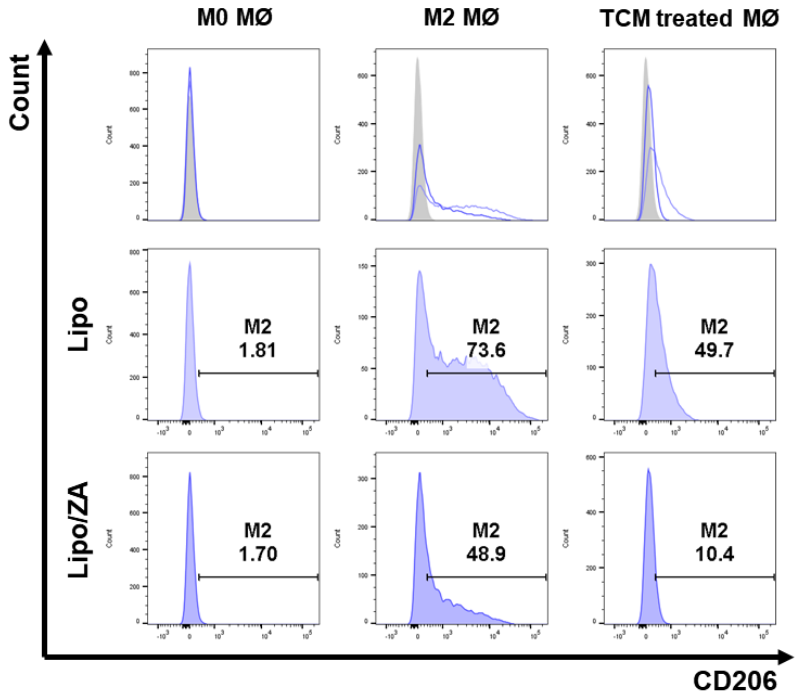
Mannosylated liposomes also showed similar results to

liposomes. M0 macrophages treated with M-Lipo/ZA showed a slight increase in CD80 expression from 43.0% to 53.9% compared to those treated with M-Lipo (Figure 6C). In addition, the CD80 expression on M2 macrophages in the M-Lipo/ZA group was 67.0%, 1.3-fold higher than that of 51.3% of the M-Lipo group. There was no significant difference between the two groups in TCM-treated macrophages. When M0 macrophages were treated with M-Lipo/ZA, the expression of CD206 remained at 1.47%, decreasing by half compared to that when treated with M-Lipo (Figure 6D). In the case of M2 macrophages, CD206 expression was decreased by 19.4% in M-Lipo/ZA compared to the M-Lipo group. The same trend was observed in TCM-treated macrophages, which decreased from 29.9% to 8.29%. These results indicate that ZA encapsulated in liposomes increases the proportion of M1 macrophages and decreases the proportion of M2 macrophages.

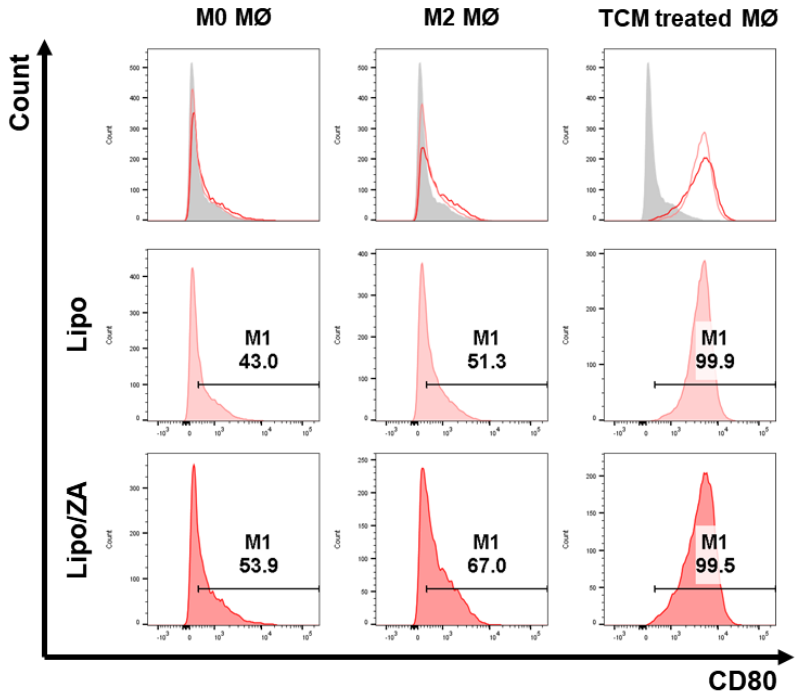
(A)


M0 MØ
M2 MØ
TCM treated MØ
Count
Lipo
Lipo/ZA
M1
39.9
M1
55.5
M1
99.6
M1
48.4
M1
71.9
M1
99.8
CD80

(B)


M0 MØ
M2 MØ
TCM treated MØ
Count
Lipo
Lipo/ZA
M2
1.81
M2
73.6
M2
49.7
M2
1.70
M2
48.9
M2
10.4
CD206

(C)


M0 MØ
M2 MØ
TCM treated MØ
Count
Lipo
Lipo/ZA
M1
43.0
M1
51.3
M1
99.9
M1
53.9
M1
67.0
M1
99.5
CD80

(D)

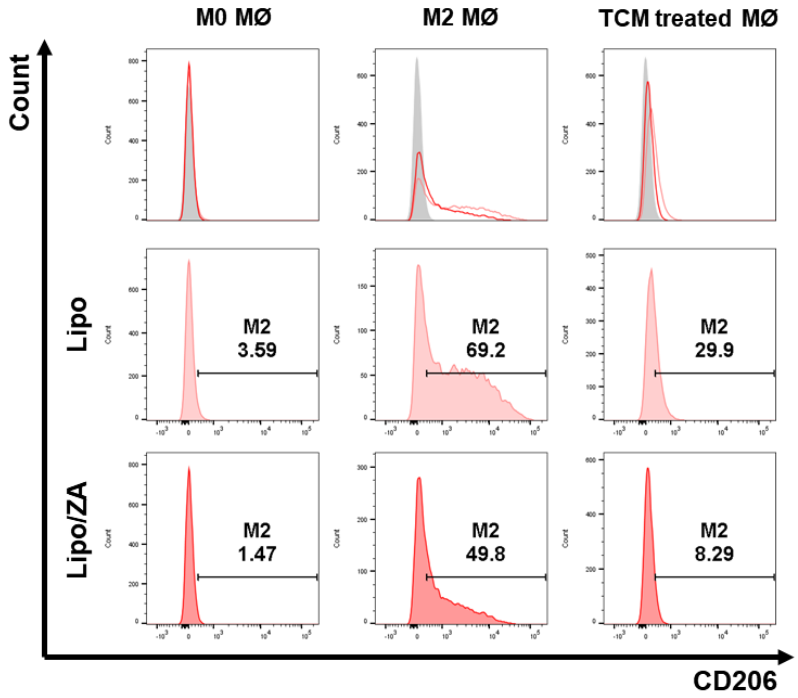

M0 MØ
M2 MØ
TCM treated MØ
Count
Lipo
Lipo/ZA
M2
3.59
M2
69.2
M2
29.9
M2
1.47
M2
49.8
M2
8.29
CD206

Figure 6. Liposome-mediated macrophage polarization

Representative flow cytometry histogram of CD80 (M1 macrophage marker) and CD206 (M2 macrophage marker) expression in differently stimulated RAW264.7 cells into M0, M2, and TCM-treated macrophages. (A) CD80 and (B) CD206 expression in the Lipo and Lipo/ZA groups. (C) CD80 and (D) CD206 expression in the M-Lipo and M-Lipo/ZA groups. Macrophages were incubated with normal, M2-inducing media and TCM for 24 hours and then treated with Lipo, Lipo/ZA, M-Lipo, and M-Lipo/ZA for another 24 hours before flow cytometry analysis.

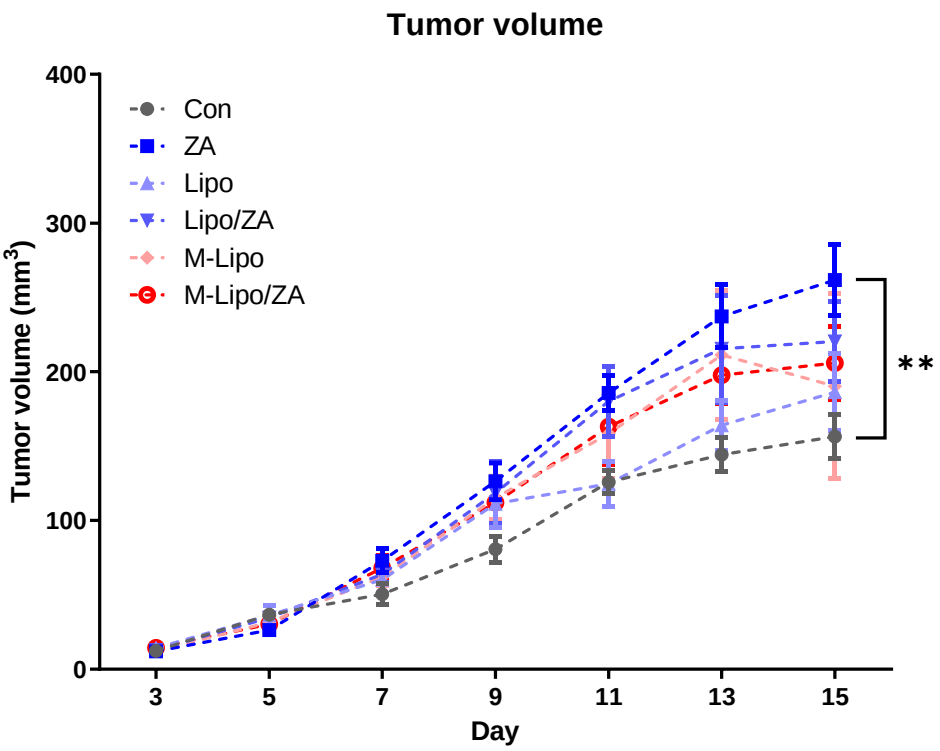
### 3. *In vivo* experiments of liposomes for macrophage polarization

#### *In vivo* bioluminescence imaging of liposomes treated 4T1-luc2 tumor

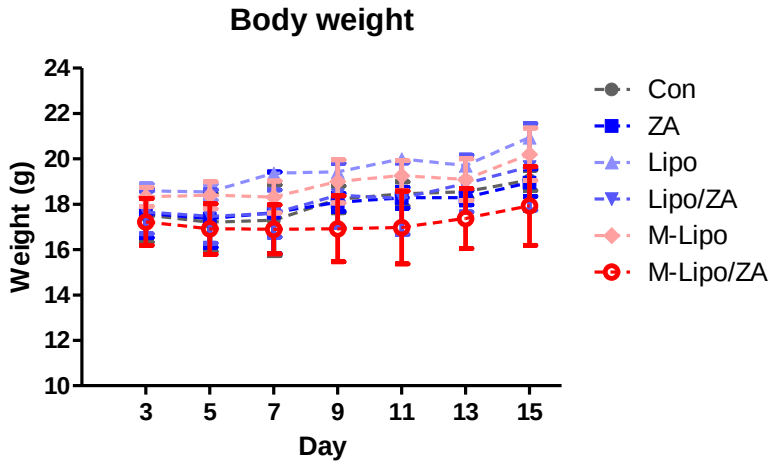
IVIS images were obtained every other day for 15 days after 4T1 tumor inoculation to monitor tumor growth.

When followed up to day 15, tumor size was larger in the groups with ZA (ZA, Lipo/ZA, M-Lipo/ZA) than in those without ZA. The difference was largest when free ZA was treated and decreased when ZA was encapsulated in liposomes (Figure 7A). Compared to other groups, the body weight of the M-Lipo/ZA group was relatively low. However, ZA or liposomes treatment did not significantly reduce body weight (Fig. 7B). Serial bioluminescence imaging of 4T1-luc2 tumor-bearing mice revealed that bioluminescence signals were gradually increased until 9 ~ 11 days and then slightly decreased at 15 days after tumor inoculation (Figure 7C).

(A)


Tumor volume
Con
ZA
Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA
Tumor volume (mm3)
400
300
200
100
0
3
5
7
9
11
13
15
Day
**

(B)


Body weight
Weight (g)
24
22
20
18
16
14
12
10
3
5
7
9
11
13
15
Day
Con
ZA
Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA

(C)

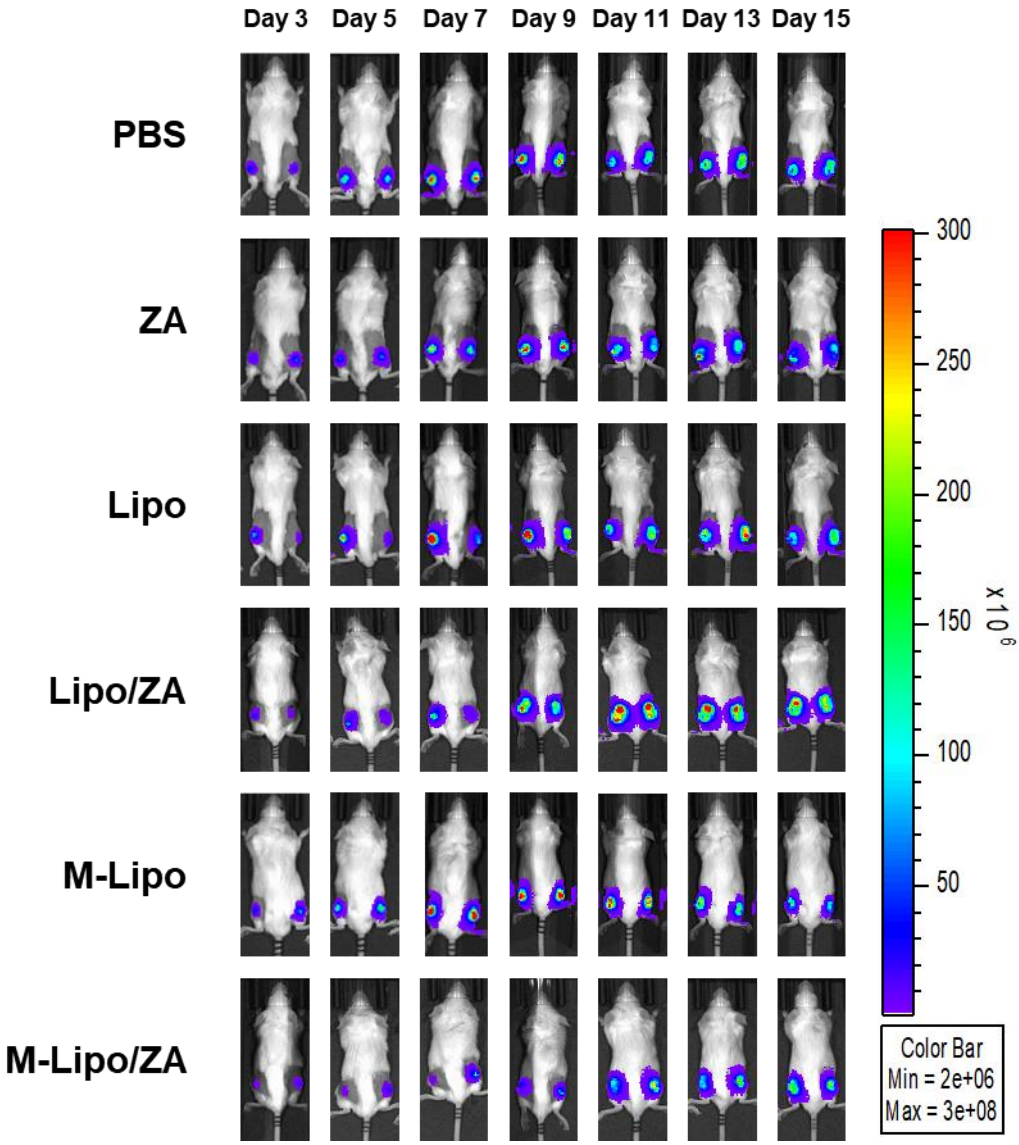

Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15
PBS
ZA
Lipo
Lipo/ZA
M-Lipo
M-Lipo/ZA
300
250
200
150
100
50
x 10 6
Color Bar
Min = 2e+06
Max = 3e+08

Figure 7. Monitoring of 4T1-luc2 tumor growth

The tumor growth curve of the 4T1 tumor with treatments of (A) PBS, ZA, Lipo, Lipo/ZA, M-Lipo, and M-Lipo/ZA was calculated every other day. (B) The effect of liposomes on body weight showed a minor increase compared to day 1. (C) IVIS images were obtained every 2 days for 15 days after 4T1 tumor inoculation. PBS, ZA, Lipo/ZA, and M-Lipo/ZA were treated by intratumoral injection every other day at the same ZA concentration of 10 μg/site until 15 days. Lipo and M-Lipo were treated at the same lipid concentration of Lipo/ZA and M-Lipo/ZA.

Flow cytometry analysis of macrophage polarization in the 4T1-luc2 tumor at 7 days after tumor inoculation

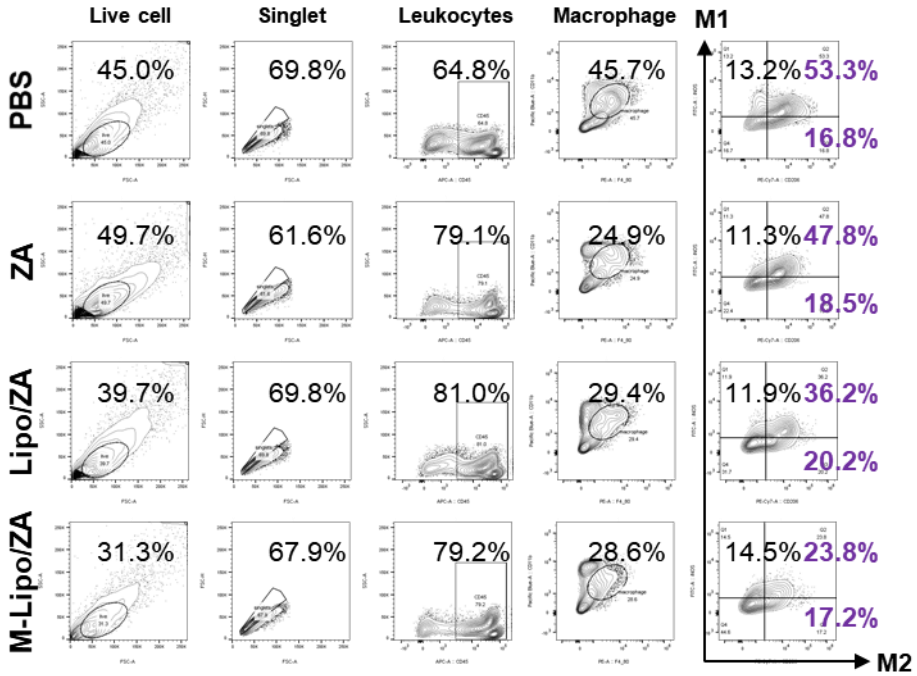
For flow cytometry analysis, 4T1-luc2 tumors grown for 7 days were collected, leukocytes were classified through CD45 antibody, and macrophage was identified using F4/80 and CD11b antibodies. iNOS was used as an M1 macrophage marker, and CD206 was used as an M2 macrophage marker.

$CD45^+$ cells in the control group accounted for 64.8%, whereas $CD45^+$ cells in the ZA, Lipo/ZA, and M-Lipo/ZA groups had higher populations, 79.1%, 81.0%, and 79.2%, respectively (Figure. 8A). On the other hand, $CD11b^+/F4/80^+$ cells classified as macrophages were the most common in the control group at 45.7%. ZA, Lipo/ZA, and M-Lipo/ZA showed a lower population distribution at 24.9%, 29.4%, and 28.6%, respectively. As a result of analyzing macrophage polarization, the population expressing the M1 marker and M2 marker simultaneously was the main. The percentage of the co-positive population was the highest in the control group at 53.3% and the lowest in M-Lipo/ZA group at 23.8%. iNOS and CD206 expressions were represented as histograms (Figure 8B). Differences between all groups were minor in iNOS expression but more evident in CD206 expression. $CD206^+$

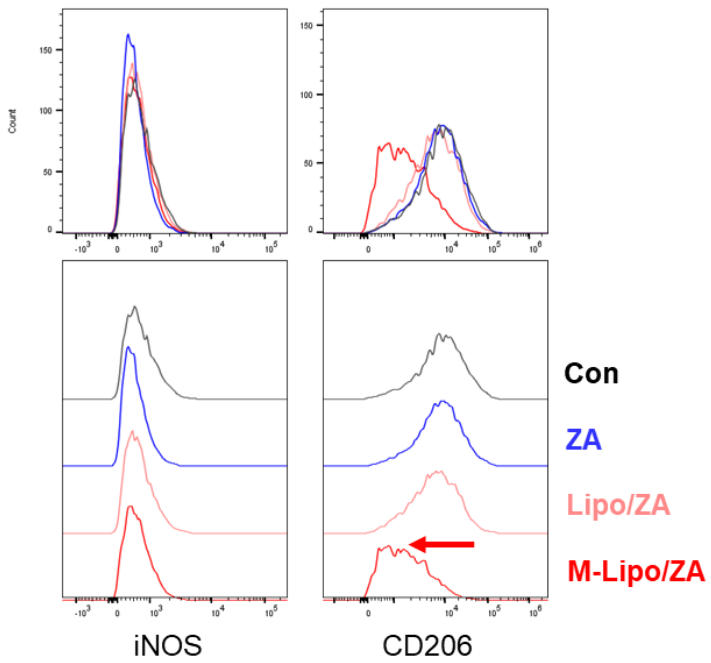
cells decreased compared to other groups upon M-lipo/ZA treatment.

Average iNOS and CD206 expression levels were visualized as measured by median fluorescence intensity (MFI). Compared to the control group, the expression of iNOS showed no significant difference between each group (Figure 8C). The MFI of CD206 expression was lower in the Lipo/ZA and M-Lipo/ZA compared to the other groups, but this was not statistically significant (Fig. 8D).

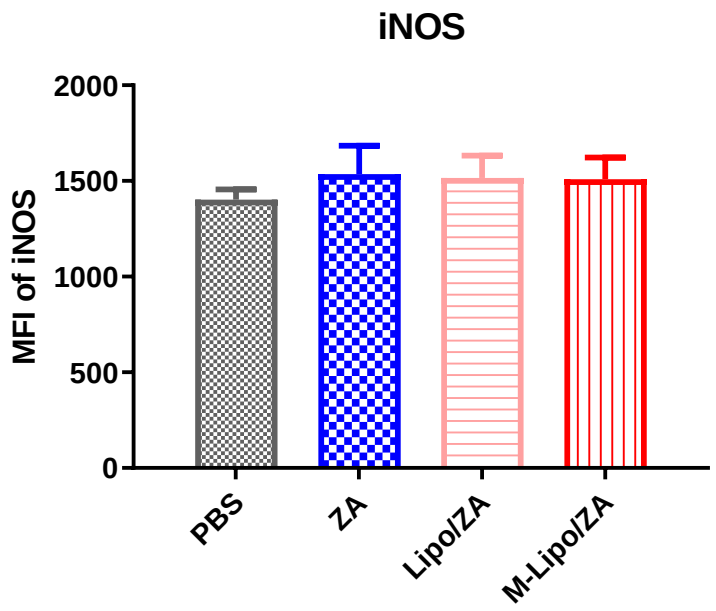
(A)


Live cell
Singlet
Leukocytes
Macrophage
M1
M2
PBS
ZA
Lipo/ZA
M-Lipo/ZA
45.0%
69.8%
64.8%
45.7%
13.2%
53.3%
16.8%
49.7%
61.6%
79.1%
24.9%
11.3%
47.8%
18.5%
39.7%
69.8%
81.0%
29.4%
11.9%
36.2%
20.2%
31.3%
67.9%
79.2%
28.6%
14.5%
23.8%
17.2%

(B)


Count
Con
ZA
Lipo/ZA
M-Lipo/ZA
iNOS
CD206

(C)


iNOS
MFI of iNOS
2000
1500
1000
500
0
PBS
ZA
LipoZA
M-LipoZA

(D)

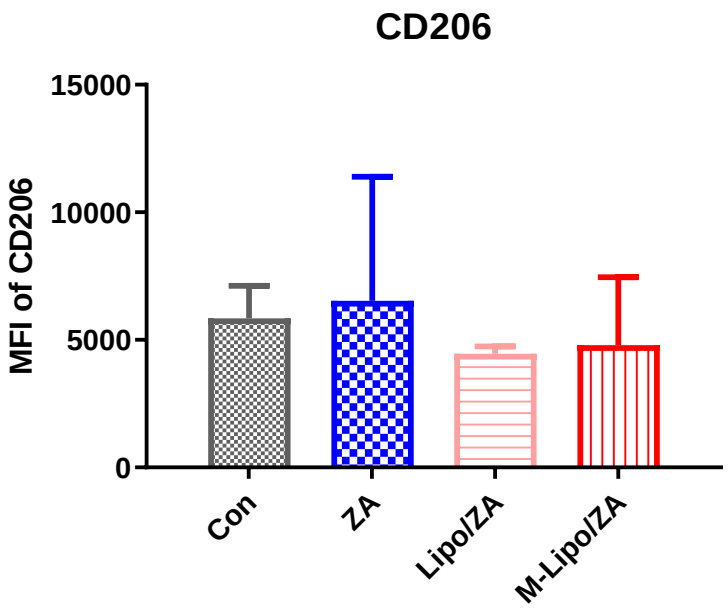

CD206
MFI of CD206
15000
10000
5000
0
Con
ZA
LipoZA
M-LipoZA

Figure 8. Flow cytometry analysis of macrophage polarization at 7 days after tumor inoculation

(A) Contour plots from flow cytometry analysis representing immune cells in 4T1 tumor. Leukocyte (anti-CD45), macrophage (anti-F4/80), monocyte (anti-CD11b), M1 macrophage (anti-iNOS), and M2 macrophage (anti-CD206) markers were used. (B) Histograms of iNOS and CD206 expression on macrophage from TME. Quantification of (C) iNOS and (D) CD206 expression measured using flow cytometry after treatment of PBS, ZA, Lipo/ZA, and M-Lipo/ZA.

### Identification of infiltrated macrophage polarization at 7 days after tumor inoculation

Immunohistochemical staining was performed to identify the types of macrophage phenotypes in 4T1-luc2 tumors 7 days after tumor inoculation (Figure 9).

In the iNOS marker, $iNOS^+$ cells were not observed in the control and Lipo/ZA groups but were identified in the ZA group. (arrow). Especially, iNOS was remarkably positive in the M-Lipo/ZA group compared to other groups, indicating that M1 macrophages were increased. In the CD206 marker, $CD206^+$ cells were slightly less in the ZA group and significantly decreased in M-Lipo/ZA compared to the control and Lipo/ZA groups.

Day 7
iNOS
CD206
Con
ZA
Lipo/ZA
M-Lipo/ZA

Figure 9. Immunohistochemical staining of 4T1-luc2 tumor at 7 days after tumor inoculation

PBS, ZA, Lipo/ZA, and M-Lipo/ZA were treated by intratumoral injection every 2 days at 10 $\mu$g/site of ZA concentration until 7 days after tumor inoculation. M1 macrophage (anti-iNOS) and M2 macrophage (anti-CD206) were stained.

Flow cytometry analysis of macrophage polarization in the 4T1-luc2 tumor at 15 days after tumor inoculation

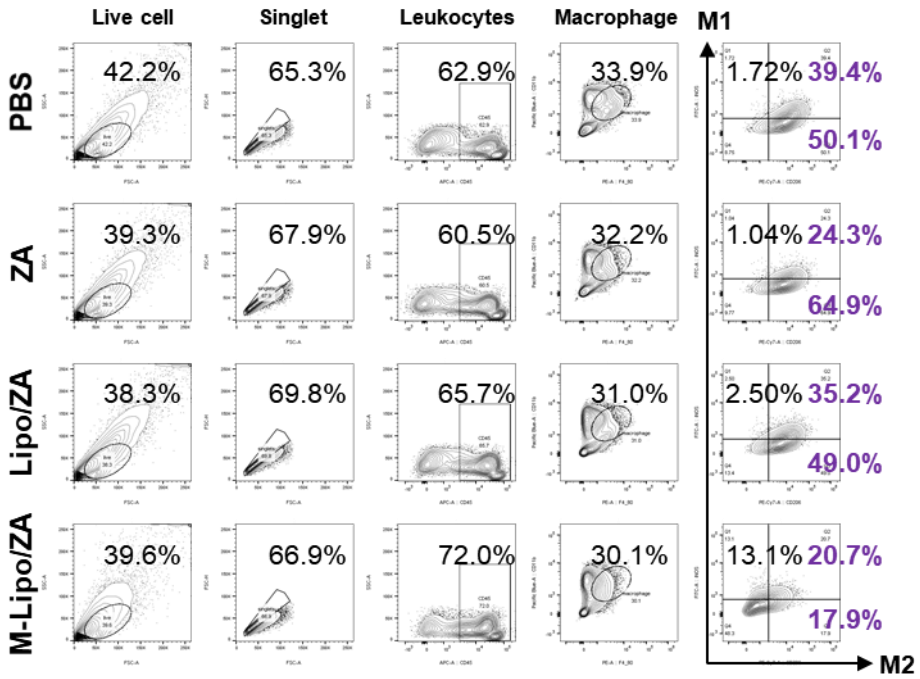
For flow cytometry analysis, 4T1-luc2 tumors grown for 15 days were collected. The gating strategy was the same as above.

In the M-Lipo/ZA group, $CD45^+$ cells were 72.0%. In comparison, the other groups showed about 10% lower percentages, 62.9%, 60.5%, and 65.7 (Fig. 10A). While there was a significant difference in the early time point, $CD11b^+/F4/80^+$ cells were about 30% in all groups at 15 days after tumor inoculation, showing no significant difference (Figure 10A). Compared to 7 days after tumor inoculation, the overall macrophage population ($CD11b^+/F4/80^+$) shifted toward $iNOS^-/CD206^+$ (Figure 8A, Figure 10A). The percentages of the co-positive population in the control, ZA, and Lipo/ZA groups were 39.4%, 24.3%, and 35.2%. Especially the co-positive population is the lowest at 20.7% in the case of the M-Lipo/ZA group. The population of $iNOS^-/CD206^+$ cells classified as M2 macrophages in the M-Lipo/ZA group was 17.9%, significantly lower than 50.1%, 64.9%, and 49.0% of control ZA and Lipo/ZA, respectively. In addition, the population of $iNOS^+/CD206^-$ cells classified as M1 macrophages in the M-

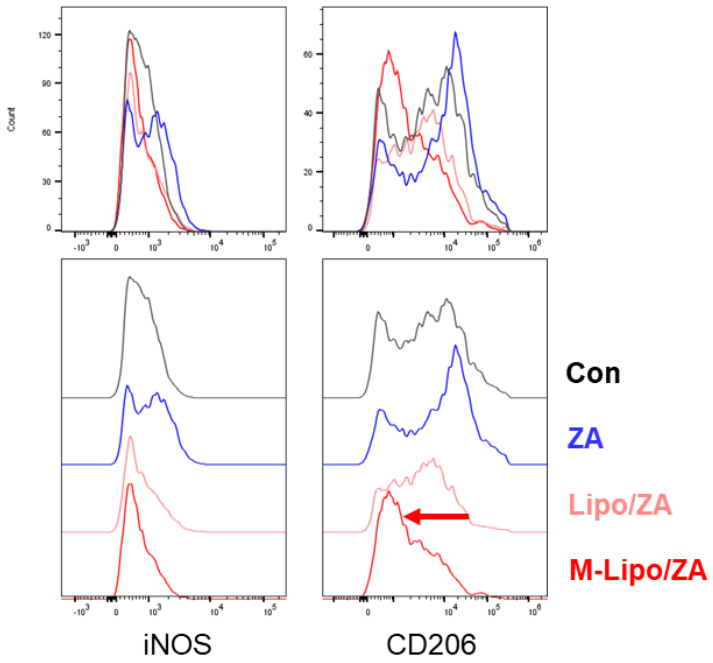
Lipo/ZA group was 13.1%, which was significantly increased compared to other groups. iNOS and CD206 expressions were represented as histograms (Figure 10B). There was a peak that appeared to be $iNOS^+$ cells in the ZA group. In $CD206^+$ cells, a decrease in M-Lipo/ZA was observed compared to other groups.

There is still no significant difference between other groups of iNOS (Figure 8C, 10C). The MFI of CD206 expression was the lowest in the M-Lipo/ZA group and the second lowest in the Lipo/ZA group (Figure 10D).

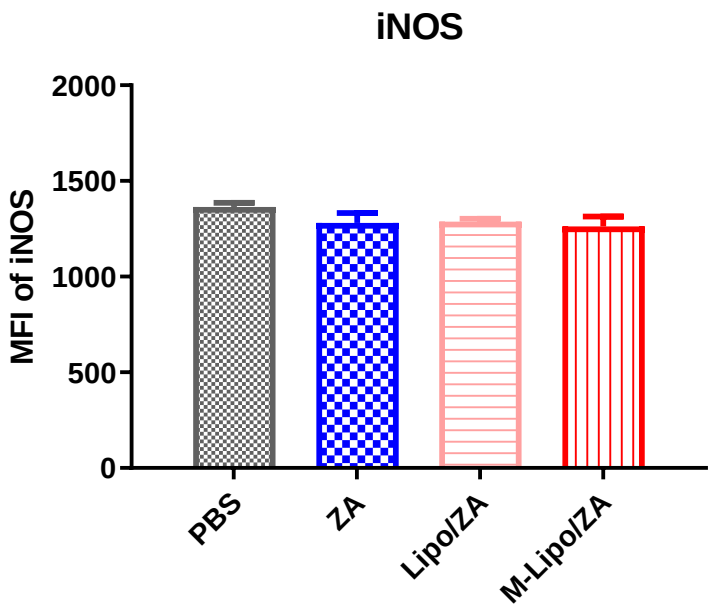
(A)


Live cell
Singlet
Leukocytes
Macrophage
M1
M2
PBS
ZA
Lipo/ZA
M-Lipo/ZA
42.2%
65.3%
62.9%
33.9%
1.72%
39.4%
50.1%
39.3%
67.9%
60.5%
32.2%
1.04%
24.3%
64.9%
38.3%
69.8%
65.7%
31.0%
2.50%
35.2%
49.0%
39.6%
66.9%
72.0%
30.1%
13.1%
20.7%
17.9%

(B)


Count
Con
ZA
Lipo/ZA
M-Lipo/ZA
iNOS
CD206

(C)


iNOS
MFI of iNOS
2000
1500
1000
500
0
PBS
ZA
LipoZA
M-LipoZA

(D)

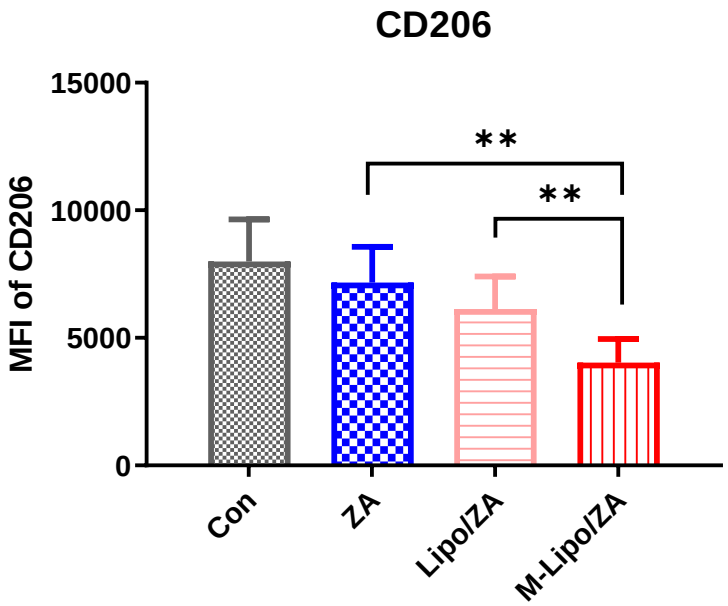

CD206
MFI of CD206
15000
10000
5000
0
**
**
Con
ZA
LipoZA
M-LipoZA

Figure 10. Flow cytometry analysis of macrophage polarization at 15 days after tumor inoculation

(A) Contour plots from flow cytometry analysis representing immune cells in 4T1 tumor. Leukocyte (anti-CD45), macrophage (anti-F4/80), monocyte (anti-CD11b), M1 macrophage (anti-iNOS), and M2 macrophage (anti-CD206) markers were used. (B) Histograms of iNOS and CD206 expression on macrophage from TME. Quantification of (C) iNOS and (D) CD206 expression measured using flow cytometry after treatment of PBS, ZA, Lipo/ZA, and M-Lipo/ZA. There was a significant decrease in CD206 expression according to drug-loading and mannosylation on liposomes.

### Identification of infiltrated macrophage polarization at 15 days after tumor inoculation

Immunohistochemical staining was performed to identify the types of macrophage phenotypes in 4T1-luc2 tumors 15 days after tumor inoculation (Figure 11).

$iNOS^+$ cells were not observed in the control group. $iNOS^+$ cells were identified in the ZA and Lipo/ZA groups and stained more positively in the M-Lipo/ZA group. Positive staining of CD206 markers was decreased in the ZA, Lipo/ZA, and M-Lipo/ZA groups than in the control group. This result indicates that M2 macrophages were reduced.

Day 15
iNOS
CD206
Con
ZA
Lipo/ZA
M-Lipo/ZA

Figure 11. Immunohistochemical staining of 4T1-luc2 tumor at 15 days after tumor inoculation

PBS, ZA, Lipo/ZA, and M-Lipo/ZA were treated by intratumoral injection every 2 days at 10 μg/site of ZA concentration until 15 days after tumor inoculation. M1 macrophage (anti-iNOS) and M2 macrophage (anti-CD206) were stained.

# DISCUSSION

M1 polarization of TAMs plays an important role in altering the tumor-supportive TME to a tumor-suppressive TME and improving the response to immunotherapy by activating immunity (1, 2). ZA has been proposed as a drug for this purpose and encapsulated in liposomes for efficient delivery to M2-like TAMs (18-21). It was verified that the liposomes were highly stable in size and PDI values when the stability test was performed for up to 8 weeks (Figure 3A, 3B, Table 2). This was the same when the surface of the liposome was modified with mannose (Figure 3), and in this case, it was demonstrated that M-Lipo targeted CD206-expressing M2 macrophages (Figure 5). In flow cytometry analysis, ZA delivered by liposomes induced a shift in macrophage polarization both *in vitro* and *in vivo* (Figure 6, 8, 10). This indicates that ZA encapsulated in mannosylated liposomes can change the polarization of TAMs in TME.

Liposomes have been extensively studied over the past 60 years since Bangham et al. first developed them in the 1960s (27, 28). They are considered ideal drug carriers because they have similar in shape to cell membranes and can incorporate a

variety of substances. There are several reports on the development of liposomes that target and repolarize TAMs. It has been reported that labeling the liposome surface with aCD47 blocked the binding of CD47 to tumor cells and SIPR$\alpha$ to macrophages (29). It also promoted the polarization of TAMs to M1 macrophages. The surface of exosomes obtained from M1 macrophages was modified with IL4RPep-1 to target the IR4R of M2 macrophages and encapsulated NF-kB p50 siRNA to induce M1 polarization (30). It has been also reported study targeting TAMs by modifying liposomes with folate to eliminate M2 macrophages by delivery of doxorubicin (31). Various strategies for inducing polarization and targeting TAMs have been proposed. This study aimed to target CD206 using mannose as a targeting ligand among various TAMs targeting ligands and evaluate its effect on M2 macrophages and TAMs polarization in vitro and in vivo.

RAW264.7 cells, the most widely used murine macrophage cell line, were selected for in vitro experiments. Tumor models for this study should form a tumor supporting TME, not being in an immune-activated state, but should contain enough macrophages within the TME to analyze changes in TAMs. 4T1

cells, a murine breast cancer cell line, are known to form partially inflamed tumors and have a high macrophage ratio in the TME, so they were selected as a cancer cell line for in vivo evaluation. In addition, since TAMs, the target cells of this study, are particularly closely related to poor prognosis of breast cancer patients (32), 4T1 cells mimicking human breast cancer are suitable as tumor models (33).

All liposomes were successfully fabricated and demonstrated stability for up to 8 weeks when stored at 4℃ (Table 2). In this process, it was observed that the zeta potential slightly decreased with time. The decrease was huge in the liposome group containing ZA, which is thought to be because ZA, which is strongly acidic, affected the pH of the liposome solution. The morphology of macrophages changed according to M1/M2 polarization (Figure 4A), and it was showed that macrophages expressed iNOS and CD206 differently depending on the state (Figure 4B). In the TCM-treated macrophages, RAW264.7 cells were somewhat killed, and M1 and M2 marker expressions were significantly increased (Figure 4A, 4B). It was found that the cell size varied depending on the polarized type (Figure 4C) (34).

M2 macrophages are known to overexpress CD206. Liposomes without mannose on the liposome surface showed a similar fluorescence intensity in both M0 and M2 macrophages because they had no targeting ability (Figure 5B). M-Lipo showed stronger fluorescence intensity in M2 macrophages overexpressed with CD206 (Figure 5C). This result indicates that surface modification of liposomes using mannose induces cellular uptake more effectively in M2 macrophages than in M0 macrophages.

Liposome-mediated macrophage polarization experiments showed that CD80 expression increased and CD206 expression decreased in macrophages under various conditions following Lipo/ZA and M-Lipo/ZA treatment (Figure 6). This tendency was more prominent in M2 macrophages than in M0 macrophages, indicating that liposomal ZA successfully induced M1 polarization of macrophages. Interestingly, it was expected that TAMs would be induced when macrophages were treated with TCM, and CD206 expression increased. However, it was demonstrated that CD80, an M1 macrophage marker, also increased by a large proportion (Figure 6). This suggests that M1/M2 macrophages do not fully dichotomize (35). Future

studies need to analyze using more diverse macrophage markers.

The tumor volume increased over time (Figure 7A). In the ZA, Lipo/ZA, and M-Lipo/ZA groups, tumor volume increased more rapidly than in the groups without ZA, such as the control, Lipo, M-Lipo, and M-Lipo. When tumors were extracted, ZA-exposed tumors tended to be less rigid than non-ZA-exposed tumors. ZA acts on TLR-4 (18), and NF-kB is activated by the TLR-4 pathway, secreting pro-inflammatory cytokines such as IL-1$\beta$ and inducing an inflammatory response (36). The increase in tumor volume may be because ZA is delivered to TAMs infiltrated into infiltrating TME, increasing M1 macrophages and causing inflammatory reactions (37).

When liposomes were treated every two days until 7 days after tumor inoculation, the control group showed 45.7% of the $CD11b^{+}/F4/80^{+}$ cells, and the other group showed about 28% (Figure 8A). This tendency shows the same in the $iNOS^{+}/CD206^{+}$ cell population. In the early stages of tumor growth, the number of immune cells infiltrating TME is small (38). They are exposed to relatively high concentrations of ZA, and depletion of macrophages may occur rather than

polarization. In fact, according to the gating strategy, the number of $CD11b^+/F4/80^+$ events in the control group was 4,885, more than 3,806 in the M-Lipo/ZA group. Immunohistochemical staining exhibited the presence of M1 macrophages, especially in the M-lipo/ZA group (Figure 9). The histogram of CD206 shows a significant reduction in CD206 expression. This is consistent with the flow cytometry data. It was demonstrated that the entire population of macrophages shifted to $iNOS^+/CD206^+$ cells 15 days after tumor inoculation (Figure 10A). This seems to be due to the increase in macrophages infiltrated into TME as the tumor volume increased (38). It also suggests that the population was not dichotomized into M1/M2 macrophages (35). Differences in each group could not be identified only by the population of $iNOS^+/CD206^+$ cells. Therefore, a decrease in CD206 expression was identified in the M-Lipo/ZA group when considering the histogram together (Figure 10B). It was challenging to know the difference in iNOS expression because $iNOS^+/CD206^-$cell was nearly 11% higher in the M-lipo/ZA group than in other groups, but there seemed to be no difference due to the decrease in $iNOS^+/CD206^+$ cells. These results can also be seen in immunohistochemical staining

(Figure 11). Positive staining of iNOS expression was observed in the M-lipo/ZA group.

In this study, only CD80 and iNOS were used as markers for M1 macrophages, and CD206 was used as a marker for M2 macrophages. However, each macrophage's surface and functional markers are very diverse (9). When examined more closely using other macrophage markers, it is expected that changes in macrophages caused by liposomal ZA will be observed more clearly. In addition, the RAW264.7 cell line was used for in vitro experiments, but the primary macrophage is more sensitive than the established cell line. Therefore, when the *in vitro* experiments were reproduced using bone marrow-derived macrophage (BMDM), the primary macrophage most similar in function and phenotype to RAW264.7 cells (39), it is expected that the interpretation of the results of the in vivo experiment will be broader.

To apply the liposome developed in this study to a preclinical or clinical stage, it is necessary to verify whether the developed liposome has potentially toxic effects on major organs and its $IC_{50}$ value through toxicity studies. In addition, the enhanced targeting effect on M2 macrophages or TAMs by mannose labeling needs to be demonstrated in vivo.

Basic research necessary for M-Lipo/ZA to advance to preclinical and clinical stages was conducted in this study. ZA promotes the TLR-4 pathway to activate NF-kB and secrete pro-inflammatory cytokines (18). As a result, TAMs receiving ZA could undergo M1 polarization and change the TME to an immunoreactive state. Therefore, it is expected that a more synergistic effect will be obtained when combined with various anticancer drugs, such as other immune anti-antagonist drugs or immune checkpoint inhibitors, than when used alone. This can help treat non-inflamed tumors or partially inflamed tumors with poor prognosis.

In conclusion, mannosylated liposome, including ZA, was prepared in this study. The liposomes had a size of 100 nm with and without ZA and mannosylation and were stable at 4℃ for 8 weeks. Importantly, In vitro, mannose-labeled liposomes were taken up by CD206-expressing M2 macrophages more than normal liposomes. Lipo/ZA and M-Lipo/ZA increased the M1 marker (CD80) expression and decreased the M2 marker expression in RAW264.7 cells. In 4T1 tumor models, when ZA-encapsulated liposomes were periodically injected intratumorally, the M1 marker (iNOS) increased, and the M2

marker (CD206) decreased in the M-Lipo/ZA group. These promising results demonstrate that liposomal ZA was delivered to M2 macrophages and TAMs and induced M1 polarization. Therefore, M-Lipo/ZA is expected to have a synergistic effect with other anticancer drugs by altering the TME and increasing pro-inflammatory macrophages.

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# 국문 초록

**서론:** 졸레드론산(ZA)은 미국 식품의약국이 승인한 비스포스포네이트 계열 의약품으로, 종양 미세 환경(TME)에서 M2에서 M1 대식세포로의 분극을 촉진하는 것으로 밝혀졌다. 이에 따라, 항암 치료 적용을 위해 ZA를 TME에 전달하기 위한 다양한 방법이 제안되었다. 그 중 리포좀은 표면을 쉽게 변형하여 다양한 기능을 수행할 수 있기 때문에 약물 전달을 위한 매력적인 전달체로 여겨진다. 만노스는 종양 관련 대식세포(TAMs)를 포함한 M2 대식세포 표면의 만노스 수용체(CD206)를 능동적으로 표적화하기 위한 표적 리간드로 사용되었다. 본 연구는 TME, 특히 M2 대식세포와 유사하다고 알려진 TAMs에 ZA를 전달하기 위한 나노전달체로서 리포좀의 활용을 모색하고자 하였다. 또한 이 연구는 마우스 종양 모델에서 리포좀에 봉입된 ZA가 TAMs의 분극화에 미치는 영향을 조사했다.

**방법:** 리포좀은 5.7:3.8:0.5 몰비의 HSPC, 콜레스테롤 및 $PEG_{2k}$-PE 또는 5.7:3.8:0.25:0.25 몰비의 HSPC, 콜레스테롤, $PEG_{2k}$-PE 및 Man-$PEG_{2k}$-PE로 제조하였다. 리포좀 크기, 안정성 및 특성은 동적 광산란 및 나노입자 추적 분석(NTA)에 의해 평가되었다. 리포좀이 만노실화되었을 때 M2 대식세포를 특이적으로 표적으로 하는지 여부를 조사하기 위해 리포좀을 FNR648로 표지하고 공초점 현미경을 사용하여 M0 대식세포와 M2 대식세포에서 리포좀 세포 흡수를 평가했다. M2-유도 사이토카인, 종양 조절 배지(TCM) 처

리되거나 아무것도 처리되지 않은 RAW264.7 세포에서 대식세포 분극화에 대하여 ZA 봉입 리포좀의 효과를 평가하기 위해 리포솜-매개 대식세포 분극화 실험을 수행하였다. 생체 내 연구를 위해 4T1-luc2 세포를 마우스 양쪽 허벅지에 피하 주사하여 마우스 종양 모델을 생성했다. PBS, ZA, Lipo, Lipo/ZA, M-Lipo 및 M-Lipo/ZA를 종양 내 주사한 후 IVISspsetrum으로 생물발광 신호를 검출했다. 생체 내에서 리포좀 ZA가 대식세포 분극화에 미치는 영향을 알아보기 위해 4T1-luc2 종양 모델에서 얻은 종양 조직에 대해 iNOS(M1 대식세포 마커) 및 CD206(M2 대식세포 마커)을 사용하여 유세포 분석 및 면역조직화학 염색을 수행했다.

**결과:** 리포좀의 유체역학적 크기는 약 100 nm였으며, 이는 NTA에서 약 112 nm 크기를 보임으로써 교차 확인되었다. 모든 리포좀의 다분산지수(PDI) 값은 0.1 미만으로 안정적으로 낮게 측정되었다. 4℃에서 두달까지 크기 및 PDI 값에 대해 안정적이다. 제타 전위는 시간이 흐를 수록 감소하는 경향을 보였다. ZA의 캡슐화 효율은 Lipo/ZA와 M-Lipo/ZA에서 각각 17.63%, 그리고 18.12%를 나타낸다. M2로 분극화된 대식세포에서 FNR648-Lipo와는 다르게 FNR648-M-Lipo는 1시간에서 2.6배, 4시간에서 2.5배 높은 섭취 정도를 보였으며, 분극화되지 않은 세포에서는 세포 흡수에 유의한 차이가 없었다. Lipo/ZA와 M-Lipo/ZA는 Lipo와 M-Lipo와 비교하여 M2 대식세포, TCM 처리된 대식세포 및 M0 대식세포로 다르게 자극된 RAW264.7 세포에서 CD80 발현을 증가시키고 CD206

발현을 감소시킨다. 4T1-luc2 종양 모델에서 ZA의 처리는 종양 부피를 유의하게 증가시켰다. ZA 봉입 리포좀 처리도 그렇지 않은 때보다 종양 부피를 증가시키는 경향을 보였으나 통계적으로 유의하지는 않았다. 리포좀을 처리한 4T1 종양의 유세포 분석 결과, M-Lipo/ZA를 처리한 그룹이 다른 그룹과 비교하여 M2 마커가 가장 낮게 확인되었다. 또한, M-Lipo/ZA는 종양 접종 후 7일차 시점에서 iNOS 마커 발현을 증가시켰으며 CD206 발현을 감소시켰다. 종양 접종 후 15일차가 되면 ZA, Lipo/ZA, M-Lipo/ZA의 CD206 발현이 대조군과 비교하여 모두 감소하였다.

**결론:** 리포좀은 성공적으로 제작되었다. 만노스가 리포좀 표면에 표지되었을 때 M2 대식세포에 의해 보다 효과적으로 세포내 흡수되었다. 또한, ZA 봉입 리포좀은 대식세포에서 CD206발현을 감소시키고 CD80 발현을 증가시켰다. M-Lipo/ZA를 격일로 처리할 때 TME에 존재하는 TAMs의 M1 분극화를 유도하는 것을 확인하였다.

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**주요어: 졸레드론산, 리포좀, 종양 미세 환경, 종양 관련 대식세포, 대식세포 분극화, 약물 전달, 유세포 분석, 면역화학염색**

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