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Research Article

Title: A new adjuvant delivery system 'cyclic di-GMP/YSK05 liposome' for cancer immunotherapy

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ABSTRACT

Cyclic dinucleotides are of importance in the field of microbiology and immunology. They function as second messengers and are thought to participate in the signal transduction of cytosolic DNA immune responses. One such dinucleotide, cyclic di-GMP (c-di-GMP), stimulates the immune system. C-di-GMP is thought to be recognized by ATP dependant RNA helicase (DDX41) in the cytosol, forms a complex with the Stimulator of interferon genes protein (STING), triggers the signal via the tank binding kinase 1 - interferon regulatory factor 3 (TBK1-IRF3) pathway and induces the production of type I interferons. Therefore c-di-GMP can be thought of as a new class of adjuvant. However, because c-di-GMP contains two phosphate groups, this prevents its use as the adjuvant because it cannot pass through the cell membrane, even though the target molecule of c-di-GMP is located in the cytoplasm.

Our group have been developing a series of liposomal drug delivery systems and recently investigated YSK05 which is a synthetic lipid with a pH sensitivity and has a high fusogenicity. We utilized this lipid as a carrier to send c-di-GMP into the cytosol to then use c-di-GMP as an adjuvant. Based on screening experiments, YSK05/POPE/Cholesterol = 40/30/30 was found to induce IFN- β in Raw264.7 cells. The induction of IFN- β from c-di-GMP liposomes was inhibited by adding BX795, a TBK1 inhibitor, indicating that the production of IFN- β caused the activation of STING-TBK1 pathway. C-di-GMP liposomes also showed significantly higher levels of expression of CD80, CD86 and MHC class I. The c-di-GMP/YSK05 liposome facilitated antigen specific cytotoxic T cell activity and the inhibition of tumor growth in a mouse model. These findings indicate that c-di-GMP/YSK05 liposomes could be used, not only to transfer c-di-GMP to the cytosol and induce an innate immune system but also as a platform for investigating the mechanism of immune sensing with cyclic dinucleotides *in vitro* and *in vivo*.

INTRODUCTION

Cancer immunotherapy is a frequently used approach in the treatment of cancer [1]. Cancer specific peptides or proteins are used as antigens to induce an immune system and to kill cancer cells. Sipuleucel-T (Provenge) is a cancer vaccine for prostate cancer that has been approved by the FDA and prostatic acid phosphatase is the antigen in this system [2]. In addition, antigen nonspecific cancer immunotherapy in which an adjuvant is involved, is also of current interest, because stimulation of the innate immune system aids in this type of therapy. A tumor-specific immune response starts by recognizing the cancer antigen by antigen presenting cells (eg. dendritic cells). The captured antigen is present on MHC class I and II molecules and stimulated dendritic cells then elicit the formation of anticancer effector T cells with the help of co-stimulatory molecules, such as CD80 and CD86. Antigen educated T cells then kill the cancer cells [3, 4]. Adjuvant can help to induce the expression of MHC class I and II and also CD80 and CD86 [5]. Many efforts have been made in this area using cancer vaccines and the results show that the stimulation of innate immune system with a suitable adjuvant is also important for achieving cancer immunotherapy. An adjuvant can reduce the amount of antigen produced and induce the desired immune responses. Because of this, the development of efficient adjuvants is an important issue in the field of cancer immunotherapy.

Cyclic dinucleotides function as second messengers in microbes and animals. For a long time, the key molecule involved in the innate immune response stimulated by cytosolic dsDNA was unknown. In 2013, however, it was revealed that cytosolic dsDNA binds to cGAMP synthase (cGAS) and produces cyclic G(2'-5')pA(3'-5')p as the second messenger and cyclic G(2'-5')pA(3'-5')p binds to stimulator of interferon genes protein (STING) and induce the production of type I interferons. Therefore cGAS is a DNA sensor and cyclic G(2'-5')pA(3'-5')p is a cytosolic DNA sensor [6-8]. Cyclic di-GMP (c-di-GMP) is also a cyclic dinucleotide and is a cyclic guanosine dimer that contains two (3'-5') phosphate linkages and functions as a co-factor of cellulose synthase in *Gluconabactor xylinum* [9, 10]. C-di-GMP also controls biofilm formation, exopolysaccharide production, toxin production and related outcomes, therefore c-di-GMP can be considered to be a second messenger in bacteria [11-13]. In addition, our group recently reported that c-di-GMP can stimulate the innate immune system [14] and, given the fact that c-di-GMP binds to DDX41 in the cytosol, can form a complex with STING, send the signal via the TBK1-IRF3 pathway and activate type I interferons [15, 16]. Therefore the receptor for c-di-GMP is DDX41 in the cytosol is different from known adjuvants such as polyIC (toll like receptor 3 on the cell surface) and CpG oligonucleotides (toll like receptor on endosome). It has recently been reported that type I interferons help to stimulate CD8 positive T cells to present the tumor associate antigen [17]. This is because c-di-GMP is considered to be a new class of useful adjuvants and some researchers have attempted to use this compound as a vaccine.

Recently, several groups have attempted to use c-di-GMP to prevent bacterial infections [14, 18-23], problems were encountered when c-di-GMP was used as a drug. C-di-GMP contains two phosphate groups and this prevents it from passing through the cell membrane, even though the target molecule of c-di-GMP is located in the cytoplasm [24]. Clearly, a suitable drug delivery system (DDS) is needed to permit c-di-GMP to penetrate the cell membrane and pass into the cytoplasmic region, if it is to be used as an adjuvant.

Liposomes are lipid bilayer nanoparticles encapsulating small molecules, such as DNAs and RNAs. Liposomes are readily taken up by cells and pharmacological findings indicate that

they are relatively safe for use, both *in vitro* and *in vivo* also for these kinds of small molecules. Our group has been investigating functional liposomes that are referred to as MENDs for the delivery of plasmid DNA, siRNA and related molecules [25, 26]. Liposomes were already used as the vesicle for adjuvants [27], employed for clinical trials such as AS01 and showed good results [28]. We recently developed a synthetic lipid named YSK05. YSK05 was designed to have optimal functionality at pH = 6.4 and to possess a high fusogenic activity, with a high endosomal escape ability which is well known promote better gene silencing [27]. Therefore we hypothesize that a c-di-GMP/YSK05 liposome system could be used to enhance the immunostimulating activity of c-di-GMP and related compounds. C-di-GMP/YSK05 liposome can be uptaken well to cells and large amount of c-di-GMP can be released to the cytosolic region by using the high fusogenic property of YSK05 liposome. The released high level of c-di-GMP will recognize DDX41 well, activate TBK1-IRF3 pathway and produce large amount of type I interferons which can work as an effective adjuvant. In this report, we have reported on attempts to prepare YSK05 liposomes that contain encapsulated c-di-GMP, for use as an efficient adjuvant system for cancer immunotherapy (Fig. 1).

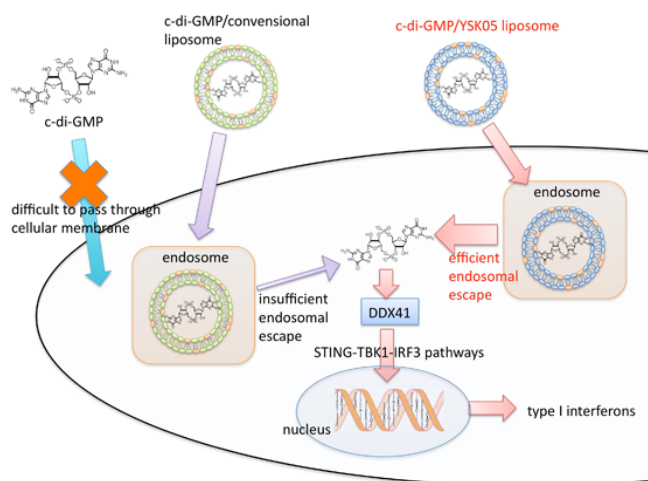


Figure 1. Schematic representation of this research.

Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (SOPE), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride (DOTAP) and Cholesterol (Chol) were purchased from Avanti polar lipids Inc. (Alabaster, AL). Egg phosphatidylcholine (EPC) was purchased from NOF corp. (Tokyo, Japan). Lipofectamine 2000 and Carboxyfluorescein diacetate succinimidyl ester (CFSE) were purchased from Life Technologies corp. (Carlsbad, CA). YSK05 was synthesized by a previously reported method [23]. C-di-GMP was obtained from the Yamasa corporation (Chiba, Japan). BX795, an inhibitor of TBK1 was purchased from InvivoGen (San Diego, CA). Anti-mouse CD80, anti-mouse CD86, anti-mouse H-2Db and anti-mouse I-Ab antibodies and their

isotype controls were purchased from BioLegend (San Diego, CA). Mouse IFN- β ELISA kit was purchased from PBL Interferon Source (Piscataway, NJ). Ovalbumin (OVA) was purchased from Sigma-Aldrich (St. Louis, MO) and OVA257-264 peptide was purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

Mice and cell cultures

Raw 264.7 macrophage cells and E.G7-OVA cells, generated by transducing the chicken OVA gene into the murine lymphoma cell line EL4 were obtained from the American Type Culture Collection (Manassas, VA). Raw 264.7 cell was grown in Roswell Park Memorial Institute (RPMI) 1640 (Sigma Aldrich, MO) supplemented with 10% fetal calf serum and 100 Units/mL of penicillin/streptomycin. E.G7-OVA cell was grown in Roswell Park Memorial Institute (RPMI) 1640 (Sigma Aldrich, MO) supplemented with 50 μ M of β -mercaptoethanol, 10% fetal calf serum, 10 mM HEPES, 1 mM sodium pyruvate and 100 Units/mL of penicillin/streptomycin. Cell were cultured at 37°C in 5% CO₂ incubator. C57BL/6J (H-2b) female mice (6 to 8 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). All animal experiments were approved by the Hokkaido University, Pharmaceutical Science Animal Committee.

Preparation of c-di-GMP encapsulated YSK05 liposome

All liposomes were prepared with cationic lipids, phospholipids, cholesterol and PEG₂₀₀₀-DMG using the t-BuOH dilution procedure. 2 mM of YSK05, POPE and cholesterol in 90% t-BuOH were prepared and mixed to result in a whole lipid amount of 400 nmol. This solution and 0.5 mM DMG-PEG₂₀₀₀ (8 μ L) were dissolved in 92 μ L of 90% t-BuOH solution. 5 μ g of c-di-GMP was dissolved in 1 mM citric buffer (pH = 4.0) up to 150 μ L. This c-di-GMP solution was added dropwise to the lipid solution with vortexing. This c-di-GMP/lipid mixture was added to 1.6 mL of 1 mM citrate buffer using a 25G syringe with vortexing and diluted with 4 mL of 1X PBS. This solution was concentrated to 100 μ L with Amicon Ultra (MWCO 100,000, 1000 rpm, 25 °C, 15 min) and washed with another 4 mL of 1X PBS. The average diameter and zeta-potential of liposomes were determined with a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, UK).

Quantification of encapsulated c-di-GMP amount

C-di-GMP encapsulated liposomes were lysed with 5% SDS (12 μ L) and diluted with 1X PBS up to 600 μ L. The lysate was applied to a manual reverse phase chromatography column (Wakogel 50C₁₈, Wako, Japan) and the fraction eluted with 20% methanol was collected. The collected solution was concentrated and measured the UV-Vis spectroscopy. The recovery amount of c-di-GMP was estimated with the absorbance at 252 nm (ϵ = 24,700) [9].

Quantification of IFN- β via ELISA

Raw 264.7 cells were inoculated in 24 well plates (8×10^4 cells/well) and incubated 24 hours at 37 °C in 5% CO₂. A suitable amount of c-di-GMP/YSK05 liposomes were diluted with RPMI-1640 medium (FBS -, S/P -) up to 500 μ L. Cells were washed with 1X PBS prior to use

and added to c-di-GMP/YSK05 liposome solution (250 μ L/well) and incubated at 37 °C in 5% CO₂ for 3 hours. After incubation, RPMI-1640 medium (FBS+, P/S+) was added and incubated for 24 hours. All medium were collected and applied for ELISA kit followed with their protocols. For the TBK1 inhibitory experiment, cells were treated with 1 μ M BX795 (200 μ L/wells) diluted with RPMI-1640 (FBS -, P/S -) 1 hour prior to the administration of the c-di-GMP/YSK05 liposome or unencapsulated c-di-GMP plus Lipofectamine2000. Cells were incubated 3 hours at 37 °C in 5% CO₂ and applied for ELISA described previously.

Quantification of maturation markers via Flow cytometry

Prior to the administration, Raw 264.7 cells were inoculated in 6 well plates (4 x 10⁵ cells/well). After washing with 1X PBS (1 mL/well), c-di-GMP/YSK05 liposomes were added to cells followed by incubation at 37 °C in 5% CO₂ for 3 hours. RPMI-1640 (FBS+, P/S+) was then added, followed by a 24 h period of incubation. Cells were collected by pipetting and washed with FACS buffer via centrifugation (500 xg, 4 °C, 5 minutes). Cells were treated with 5 μ g/mL of IgG1k (50 μ L) and incubated at 4 °C for 30 minutes, then washed with FACS buffer (1 mL) twice. 5 μ g/mL of a suitable fluorescent tagged antibody (50 μ L) was added and the solution was then incubated at 4 °C for 30 minutes. Cells were washed with FACS buffer (500 μ L) twice, filtered via nylon filter and applied for FACS analysis (FACSCalibur, BD Biosciences, CA, USA).

in vivo CTL assay with c-di-GMP/YSK05 liposome

C57BL/6J mice were anesthetized with diethyl ether and c-di-GMP/MEND was injected subcutaneously using a 26G syringe. Spleen tissues were harvested from naive mice, and the resulting dissociated cells were passed through a cell strainer (40 μ m pore, BD Falcon, CA). The recovered cells were spun down (500 xg, 4 °C, 5 minutes) to remove the supernatant, resuspended in ACK lysing buffer (3 mL, Lonza, MD) and incubated for 5 minutes at room temperature. These treated cells were washed with RPMI medium by spinning (500 xg, 4 °C, 5 minutes) twice and were then passed through a cell strainer. The concentration of cells was adjusted to 1 x 10⁷ cells/mL and the resulting cells were separated to 2 groups. Half of the cells were treated with the OVA₂₅₇₋₂₆₄ peptide (5 μ M final conc.) and incubated 37 °C for 1 hour. These cells were washed with RPMI medium (10 mL) and 1X PBS (10 mL) and suspended with CFSE^{high} (5 μ M solution) or CFSE^{low} (0.5 μ M) solutions at the cell concentration of 3 x 10⁷ cells/mL. The resulting reaction mixtures were incubated at 37 °C for 10 minutes and washed twice with medium (10 mL) and twice with 1X PBS (10 mL), respectively. These cells were resuspended with 1X PBS (5 x 10⁷ cells/mL) and mixed CFSE^{high} and CFSE^{low} with each volume. An aliquot containing 1 x 10⁷ cells/200 μ L were administered to mice that had been immunized one week previously via the tail vein injection. After 20 hours, spleen tissues were harvested, washed and incubated in ACK lysing buffer (1 mL) at room temperature for 5 minutes. Cells were added with 9 mL of FACS buffer and spun down at 500 xg under 4 °C for 3 minutes and washed again with FACS buffer (10 mL). These cells were washed with 5 mL of FACS buffer and applied for flow cytometry. The number of CFSE^{low} cells was set at 7500 and the intensities of the CFSE^{high} cells measured.

Preventive antitumor effect of c-di-GMP/YSK05 liposome on mice

C57BL6/J mice (6-9 weeks old) were anesthetized with diethyl ether. PBS control, 40 µg of OVA only, 40 µg of OVA and 0.3 µg of c-di-GMP, 0.3 µg of c-di-GMP/YSK05 liposomes or the same lipid amount of empty YSK05 liposomes were injected subcutaneously. One week later, E.G7 OVA cells (8.0×10^6 cells/50 µL/mouse) were inoculated subcutaneously in the right flank. Tumor volumes were calculated at 3 day intervals by the following method (tumor volume = major axis x minor axis² x 0.52).

Statistical analysis

Comparisons between multiple treatments were made by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test. For preventive antitumor experiment, a two-way repeated analysis of variance was used, followed by the Scheffe's *F* test or Dunnett test. A *P* value of < 0.05 was considered to be a significant difference.

Results and Discussion

Optimization of c-di-GMP encapsulating liposome

We evaluated the induction of IFN-β by the stimulation with the c-di-GMP/YSK05 liposome system compared to other liposomes such as cationic liposomes, anionic liposomes, neutral liposomes and Lipofectamine2000 which is a conventional transfection reagent. The induction of IFN-β was quantified by ELISA and none of the liposomes showed IFN-β production except for the c-di-GMP/YSK05 liposomes (data not shown). The YSK05 lipid is a synthetic lipid developed in our laboratory and has properties that permit it to be protonated at weakly acidic conditions (*pK*_a = 6.4) [27] and also has a high fusogenic property, because of its dilinoleyl lipid tail structure (Fig. 2). C-di-GMP/YSK05 liposome was prepared with alcohol dilution method which is also used for the encapsulation of siRNA to YSK05 liposome [23]. The encapsulation ratio of c-di-GMP was quantified with UV/Vis spectroscopy after purifying c-di-GMP with C18 short column chromatography and 2% of c-di-GMP was incorporated in YSK05 liposome by this method (4 mM lipid concentration, 1.4 µM c-di-GMP). This c-di-GMP/YSK05 liposome was used for further experiments. Next, helper phospholipids such as POPE, SOPE and DOPE were investigated and POPE was found to be the optimal material for the use in c-di-GMP/YSK05 liposomes (Fig. 3a). Next, the lipid compositions of POPE and cholesterol (Chol) were optimized and a ratio of YSK05/POPE/Chol/DMG-PEG₂₀₀₀ = 40/25/35/1 was determined to be the optimal ratio for inducing the production of IFN-β in the c-di-GMP/YSK05 liposome system (Fig. 3b, c). Physical properties of optimized c-di-GMP/YSK05 liposome and empty YSK05 liposome was cited on Table 1. This liposome was compared with a conventional cationic liposome (DOTAP/DOPE/Chol = 30/40/30) and Lipofectamine2000 and showed a 7 times higher IFN-β production than Lipofectamine2000 (Fig. 3d). As a result, we used this system for further experiments.

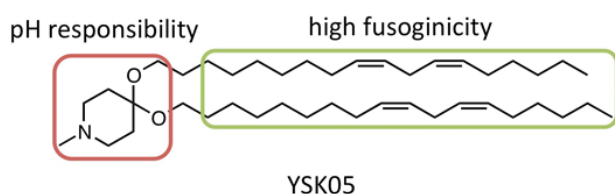


Figure 2. Structure and proposed property of YSK05

Table 1. Physical properties of c-di-GMP/YSK05 liposome.

The size, polydispersity index (PdI) and zeta-potential of c-di-GMP encapsulated YSK05 liposomes and empty YSK05 liposomes were measured (n = 3).

	size (nm)	PdI	zeta-potential (mV)
c-di-GMP/YSK05 liposome	172.0 ± 24.8	0.14 ± 0.03	- 4.30 ± 4.45
YSK05 liposome	143.3 ± 31.8	0.12 ± 0.03	1.65 ± 2.07

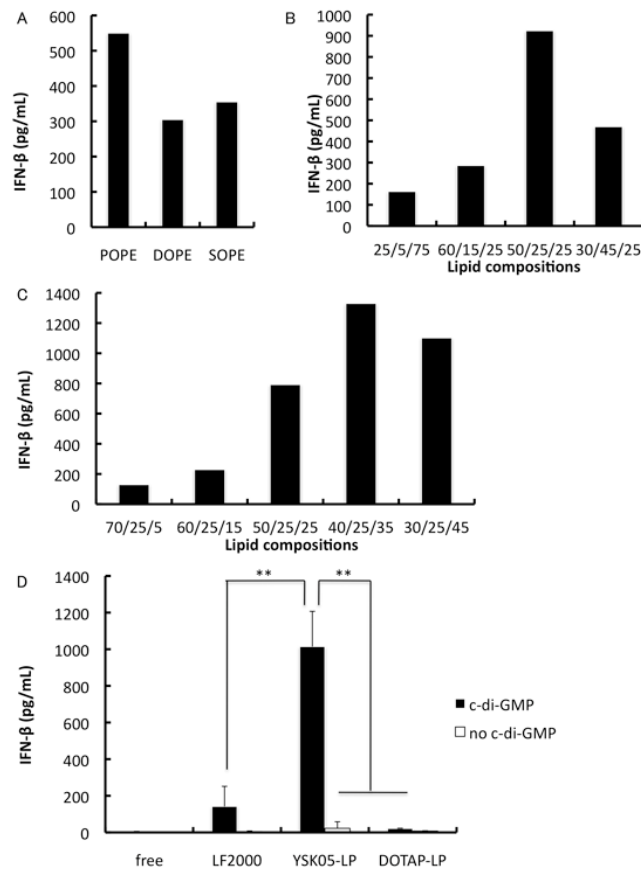


Figure 3. Optimization of lipids for c-di-GMP liposome

Amounts of IFN-β were estimated by ELISA. (a) POPE, SOPE, DOPE were investigated as a helper lipid. Liposomes were prepared with the ratio of YSK05/helper lipid/Chol = 50/25/25 and compared with Lipofectamine2000. (b) The amount of POPE was investigated. (c) The amount of cholesterol was investigated. (d) IFN-β induction of Lipofectamine2000, c-di-GMP encapsulated cationic liposome (DOTAP/DOPE/Chol = 30/40/30) and optimized c-di-GMP-liposome (YSK05/POPE/Chol = 40/25/35) were compared. Values are the mean ± SD of at least three different experiments (n = 3, *P<0.05, **P<0.01).

Cellular uptake of c-di-GMP liposome

To elucidate why the c-di-GMP/YSK05 liposomes showed a higher immune stimulation compared to conventional liposomes, the relationship between cellular uptake efficiency and

IFN- β induction activity of each liposome was investigated. Cationic liposomes (DOTAP/DOPE/Chol = 30/40/30) was chosen as the control to compare because they had good efficacy when the transfection of plasmid DNA [28]. Raw264.7 cells were treated with each type of fluorescently labeled liposomes. We then measured the fluorescence intensity by flow cytometry. The relationship between uptake efficacy (Fig. 4) and IFN- β production (Fig. 3d.) was investigated between YSK05 and cationic liposomes. Cationic liposomes showed a higher uptake but the production of IFN- β was low. This suggests that the high IFN- β production for the c-di-GMP YSK05 liposomes is likely due to the high fusogenic and endosomal escaping properties of YSK05 [27].

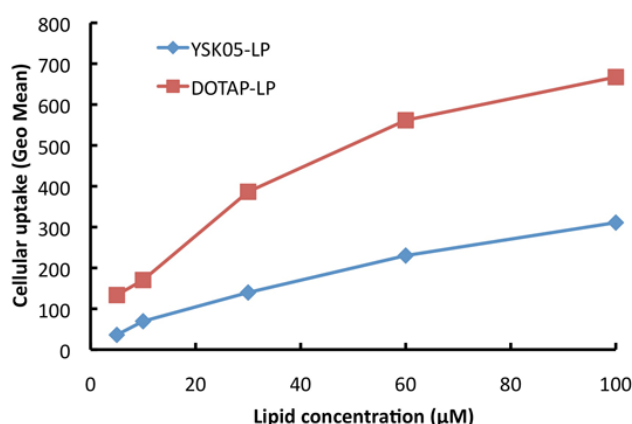


Figure 4. Uptake efficiency of c-di-GMP encapsulated YSK05 and cationic liposomes. The lipid composition of the YSK05 liposomes and the cationic liposomes were YSK05/POPE/Chol = 50/25/25 and DOTAP/DOPE/Chol = 30/40/30 with 1% of DiD-DOPE respectively. The physico-chemical properties of cationic liposome was size: 162 nm, PDI: 0.181, zeta-potential: 25.6 mV. Cells were applied for flow cytometry.

Inhibition of IFN- β induction with c-di-GMP liposomes using a TBK1 inhibitor

The c-di-GMP/YSK05 liposome system showed a high IFN- β induction, while empty YSK05 liposomes did not. This result suggests that the encapsulated c-di-GMP was, in fact, transported to the cytoplasmic region, where it then binds DDX41, triggering the production of type I interferons via the TBK1-IRF3 pathway. To verify that IFN- β induction was the result of an interaction between c-di-GMP and DDX41-STING, we examined the inhibition of IFN- β production with BX795, a TBK1 inhibitor. Raw264.7 cells were treated with 1 μ M BX795 for 1 h prior to treatment with c-di-GMP/YSK05 liposomes or free c-di-GMP and Lipofectamine2000. The inhibitory effect of IFN- β production was quantified by means of ELISA. In both cases, the induction of IFN- β was reduced by around 90%. This result suggests that the immunostimulation by c-di-GMP/YSK05 liposomes was actually induced via the TBK1 pathway (Fig. 5).

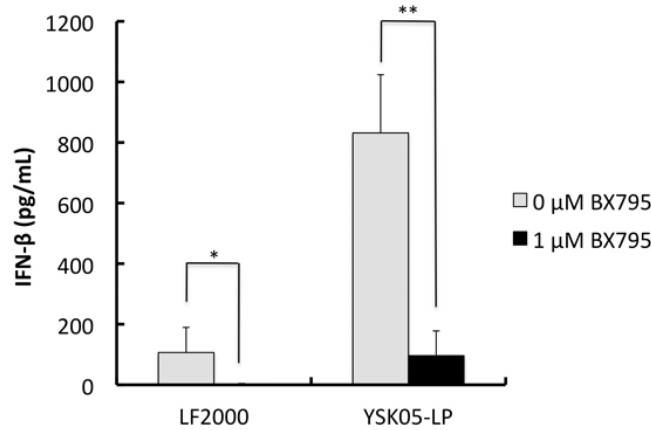


Figure 5. Inhibition of IFN- β induction with BX795

0 or 1 μ M of BX795 that is known as the inhibitor of TBK1 was treated with Raw264.7 cells 1 hour before and c-di-GMP was administrated with Lipofectamine2000 (left column) or YSK05-liposome (right column). Amounts of IFN- β were quantified with ELISA. Values are the mean $\pm$ SD of at least three separate experiments (n = 3, *P<0.05, **P<0.01).

The expression of costimulatory and MHC molecules by treatment with c-di-GMP/YSK05 liposomes

The above findings prove that the availability of c-di-GMP/YSK05 liposomes and the IFN- β production of this system resulted in the binding of c-di-GMP to the receptor protein, followed by induction via the TBK1-IRF3 pathway. We then evaluated the expression of costimulatory molecules (CD80, CD86) and MHC molecules (MHC class I, II) to analyze the effect of the c-di-GMP/YSK05 liposomes. Costimulatory and MHC molecules are important in terms of the activation of B and T lymphocytes for antigen presentation and certain adjuvants such as CpG DNA are able to induce the expression of these costimulatory molecules [29]. Raw264.7 cells were treated with c-di-GMP liposomes and the production of MHC and costimulatory molecules were measured by flow cytometry. The expression of CD80, CD86 and MHC class I molecules was up-regulated (Fig. 6), suggesting that the c-di-GMP/YSK05 liposome system can activate the maturation of antigen presenting cells (APCs), indicating that this system can be used as an adjuvant system.

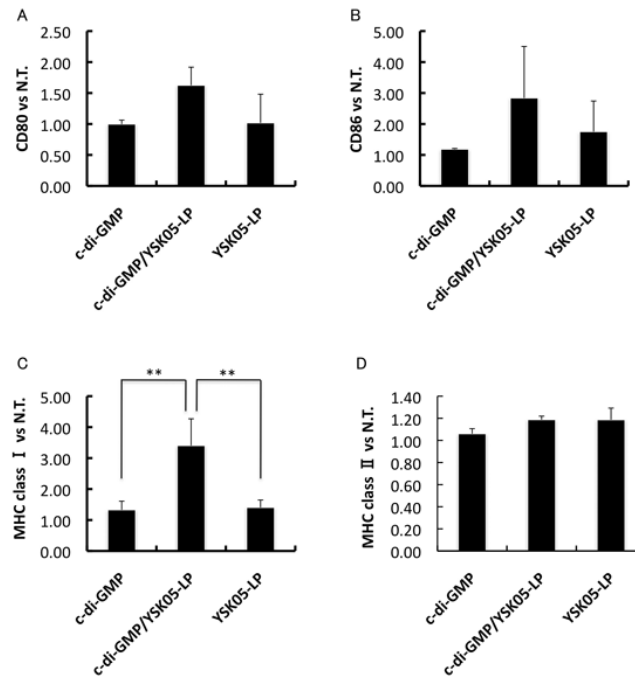


Figure 6. Expression of costimulatory and MHC molecules

Raw264.7 cells were treated with c-di-GMP only, c-di-GMP/YSK05 liposomes or empty YSK05 liposomes. After 24 hours, the cells were treated with (A) CD80, (B) CD86, (C) MHC class I or (D) MHC class II antibodies and applied for flow cytometry. Values are the mean±SD of at least three different experiments (n = 3-4, *P<0.05, **P<0.01).

Enhancement of antigen-specific CTL activity by c-di-GMP/YSK05 liposome

Stimulation of antigen-specific CTL is an important factor in killing cancer cells [30]. To determine whether c-di-GMP/YSK05 liposomes can activate the CTL response or not, a CTL assay was carried out with OVA as the antigen. C57BL/6J mice were treated with the c-di-GMP/YSK05 liposomes and the OVA (40 µg) 7 days prior to harvesting mouse spleen cells. Half of the recovered spleen cells were treated with the fragment of the OVA peptide and the remainder was treated with 1X PBS, respectively. These cells were labeled with high and low concentrations of CFSE dye and injected into mice that had been immunized with c-di-GMP/YSK05 liposomes or empty YSK05 liposomes. After 20 hours, spleen cells were collected and counted by flow cytometry and the activities of cytotoxic T lymphocytes determined. The findings clearly show that immunization with c-di-GMP/YSK05 liposomes resulted in a significant increase in cytotoxic T lymphocytes (95.7%) compared to empty YSK05 liposomes (40.2%) (Fig. 7). It was surprising for us to show that the empty YSK05 liposome showed 40% cytotoxic effect. The OVA itself didn't show the cytotoxic effect therefore this cytotoxicity came from empty YSK05 liposome. It was known that some cationic liposomes containing synthetic lipid such as DOTAP, DiC14-amidine could work as an adjuvant. For example, Tanaka et al reported that liposome containing DiC14-amidine was recognized by Toll like receptor 4, induced the activation of NF-κB and stimulated myeloid dendritic cells [31]. Yan *et al.* reported that DOTAP containing liposome induced the production of reactive oxygen species (ROS) and recruited the activation of ERK and immune stimulation [32]. YSK05 is pH-sensitive and can be said a kind of cationic lipid therefore YSK05 empty liposome could work as the adjuvant and

showed 40% cytotoxicity. Even this effect, c-di-GMP/YSK05 liposome showed higher cytotoxic effect compared to empty YSK05 liposome with significant difference. This result suggests that the c-di-GMP/YSK05 liposomes stimulated the innate immune system and recruited costimulatory molecules and mature cytotoxic T lymphocytes.

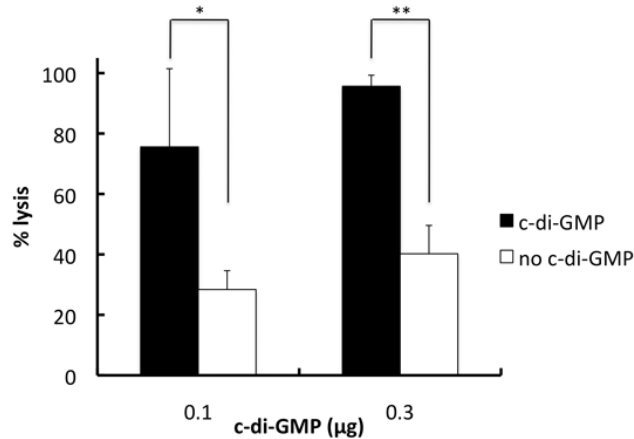


Figure 7. Enhancement of in vivo cytotoxic T cell activities with c-di-GMP liposome

CTL activities with or without c-di-GMP encapsulated liposome were challenged on mouse model. c-di-GMP/YSK05 liposome or empty liposome were administrated at 0.1 and 0.3 µg of c-di-GMP dose subcutaneously. CTL activities were estimated by flow cytometry. Values are the mean±SD of at least three different experiments (n = 3, *P<0.05, **P<0.01).

Enhancement of preventive anticancer effects as the result of a c-di-GMP/YSK05 liposome vaccination

Finally, we investigated the antitumor activities of the system using a mouse model to verify that this c-di-GMP YSK05 liposome system can reduce the size of cancer cells by the stimulation of type I interferons. 5 mice were immunized with c-di-GMP, free OVA, empty YSK05 liposomes and c-di-GMP/YSK05 liposomes 7 days prior to inoculation with cancer cells. The immunized mice were inoculated with E.G7-OVA cells and the size of tumor tissue was measured after 3 days. When mice were immunized with unencapsulated c-di-GMP, free OVA or empty YSK05 liposomes, no difference was found compared to PBS controls, whereas tumor tissue did not grow in mice that had been treated with c-di-GMP/YSK05 liposomes. From these results, it is clear that vaccination with c-di-GMP/YSK05 liposomes resulted in a significant reduction in the growth of cancer tissue (Fig. 8).

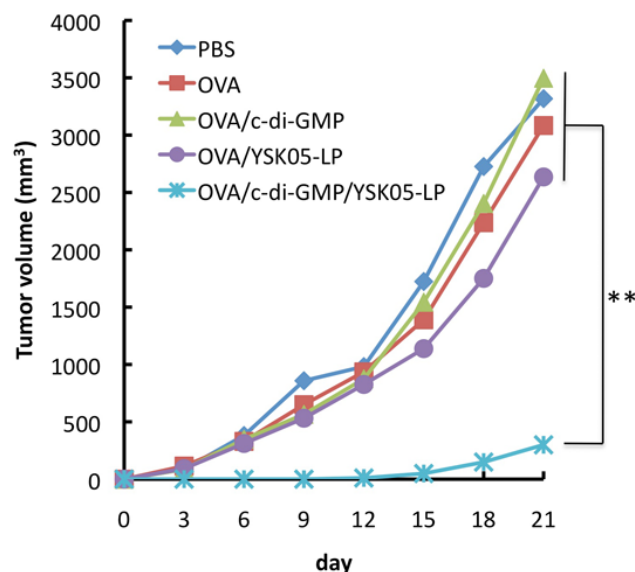


Figure 8. Antitumor activities of c-di-GMP liposome

C-di-GMP/YSK05 liposome, empty YSK05 liposome, c-di-GMP were subcutaneously administrated as the adjuvant one week before the inoculation of E.G7-OVA cancer cells. Tumor volume was measured after 3 days and the results subjected to statistical analysis. Values are the mean $\pm$ SD of at least three different experiments (n = 5, *P<0.05, **P<0.01).

Conclusion

The findings reported herein show that c-di-GMP/YSK05 liposomes were taken up by antigen presenting cells, that the encapsulated c-di-GMP was released to the cytosol, and this process resulted in the stimulation in the induction of IFN- β via the STING-TBK1-IRF3 pathway. The large induction of IFN- β can mainly be attributed to the high fusogenic properties of the YSK05 lipid. The activity of the vaccinated c-di-GMP/YSK05 liposomes was inhibited by treatment with the TBK1 inhibitor, in other words the immune stimulation of c-di-GMP/YSK05 liposome proceeded through the TBK1-IRF3 pathway. C-di-GMP/YSK05 liposome vaccination also increased the expression of CD80, CD86 and MHC class I and showed higher CTL activities, indicating that the c-di-GMP/YSK05 liposome system also functions as an adjuvant *in vivo* and also c-di-GMP/YSK05 liposomes prevented the growth of tumor tissue. These findings establish c-di-GMP/YSK05 liposomes have promise for use as an adjuvant system, both *in vitro* and *in vivo*. This system can be used, not only for the *in vivo* vaccination of a adjuvant but also for the investigation mechanistic aspects of the innate immune response stimulated by cyclic dinucleotides *in vitro* and *in vivo*.

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Figure Legends

Figure 1. Schematic representation of this research

Figure 2. The chemical structure of YSK05

Figure 3. Optimization of lipids for c-di-GMP liposome

Amounts of IFN- β were estimated by ELISA. (a) POPE, SOPE, DOPE were investigated as a helper lipid. Liposomes were prepared with the ratio of YSK05/helper lipid/Chol = 50/25/25 and compared with Lipofectamine2000. (b) The amount of POPE was investigated. (c) The amount of cholesterol was investigated. (d) IFN- β induction of Lipofectamine2000, c-di-GMP encapsulated cationic liposome (DOTAP/DOPE/Chol = 30/40/30) and optimized c-di-GMP liposome (YSK05/POPE/Chol = 40/25/35) were compared. Values are the mean $\pm$ SD of at least three different experiments (n = 3, *P<0.05, **P<0.01).

Figure 4. Uptake efficiency of c-di-GMP encapsulated YSK05 and cationic liposomes.

The lipid composition of the YSK05 liposomes and the cationic liposomes were YSK05/POPE/Chol = 50/25/25 and DOTAP/DOPE/Chol = 30/40/30 with 1% of DiD-DOPE respectively. Cells were applied for flow cytometry. (a) Histograms of flow cytometry were shown. Red, blue and black lines represented YSK05-liposome, cationic liposome and non treated respectively. (b) Scattered graph of cellular uptake on each liposomes were prepared. Geo mean values were calculated and plotted to each lipid concentrations.

Figure 5. Inhibition of IFN- β induction with BX795

0 or 1 μ M of BX795 that is known as the inhibitor of TBK1 was treated with Raw264.7 cells 1 hour before and c-di-GMP was administrated with Lipofectamine2000 (left column) or YSK05-liposome (right column). Amounts of IFN- β were quantified with ELISA. Values are the mean $\pm$ SD of at least three separate experiments (n = 3, *P<0.05, **P<0.01).

Figure 6. Expression of co-stimulatory molecules

Raw264.7 cells were treated with c-di-GMP only, c-di-GMP/YSK05 liposomes or empty YSK05 liposomes. After 24 hours, the cells were treated with CD80, CD86, MHC class I or MHC class II antibodies and applied for flow cytometry. (a) Histograms were shown for each experiment. Relative expression of (b) CD80, (c) CD86, (d) MHC class I and (e) MHC class II were summarized by bar graphs. Values are the mean $\pm$ SD of at least three different experiments (n = 3-4, *P<0.05, **P<0.01).

Figure 7. Enhancement of in vivo cytotoxic T cell activities with c-di-GMP liposome

CTL activities with or without c-di-GMP encapsulated liposome were challenged on mouse model. C-di-GMP/YSK05 liposome or empty liposome were administrated at 0.1 and 0.3 μ g of c-di-GMP dose subcutaneously. CTL activities were estimated by flow cytometry. Values are the mean $\pm$ SD of at least three different experiments (n = 3, *P<0.05, **P<0.01).

Figure 8. Antitumor activities of c-di-GMP liposome

C-di-GMP/YSK05 liposome, empty YSK05 liposome, c-di-GMP were subcutaneously administrated as the adjuvant one week before the inoculation of E.G7-OVA cancer cells. Tumor

volume was measured after 3 days and the results subjected to statistical analysis. Values are the mean $\pm$ SD of at least three different experiments (n = 5, *P<0.05, **P<0.01).

Table 1. Physical properties of c-di-GMP/YSK05 liposome.

The size, polydispersity index (PdI) and zeta-potential of c-di-GMP encapsulated YSK05 liposomes and empty YSK05 liposomes were measured.

Figure 1.

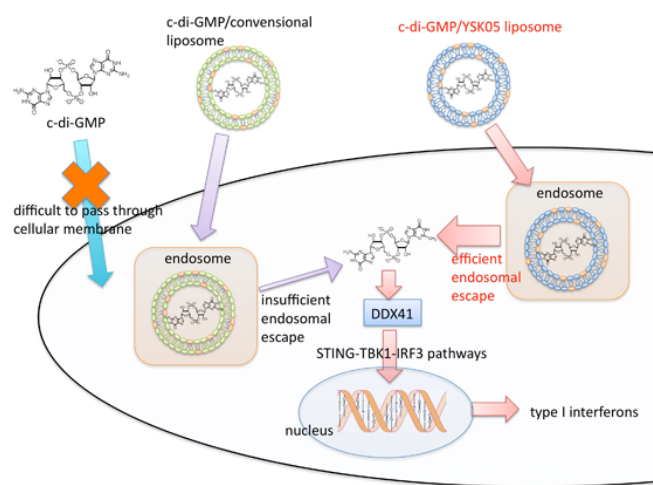


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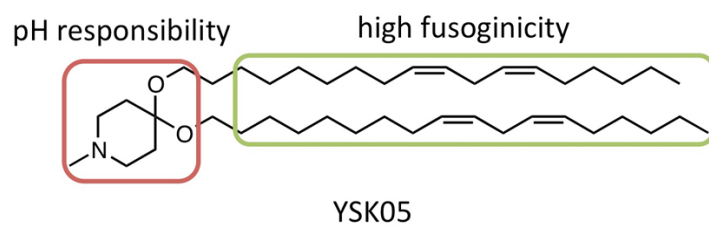


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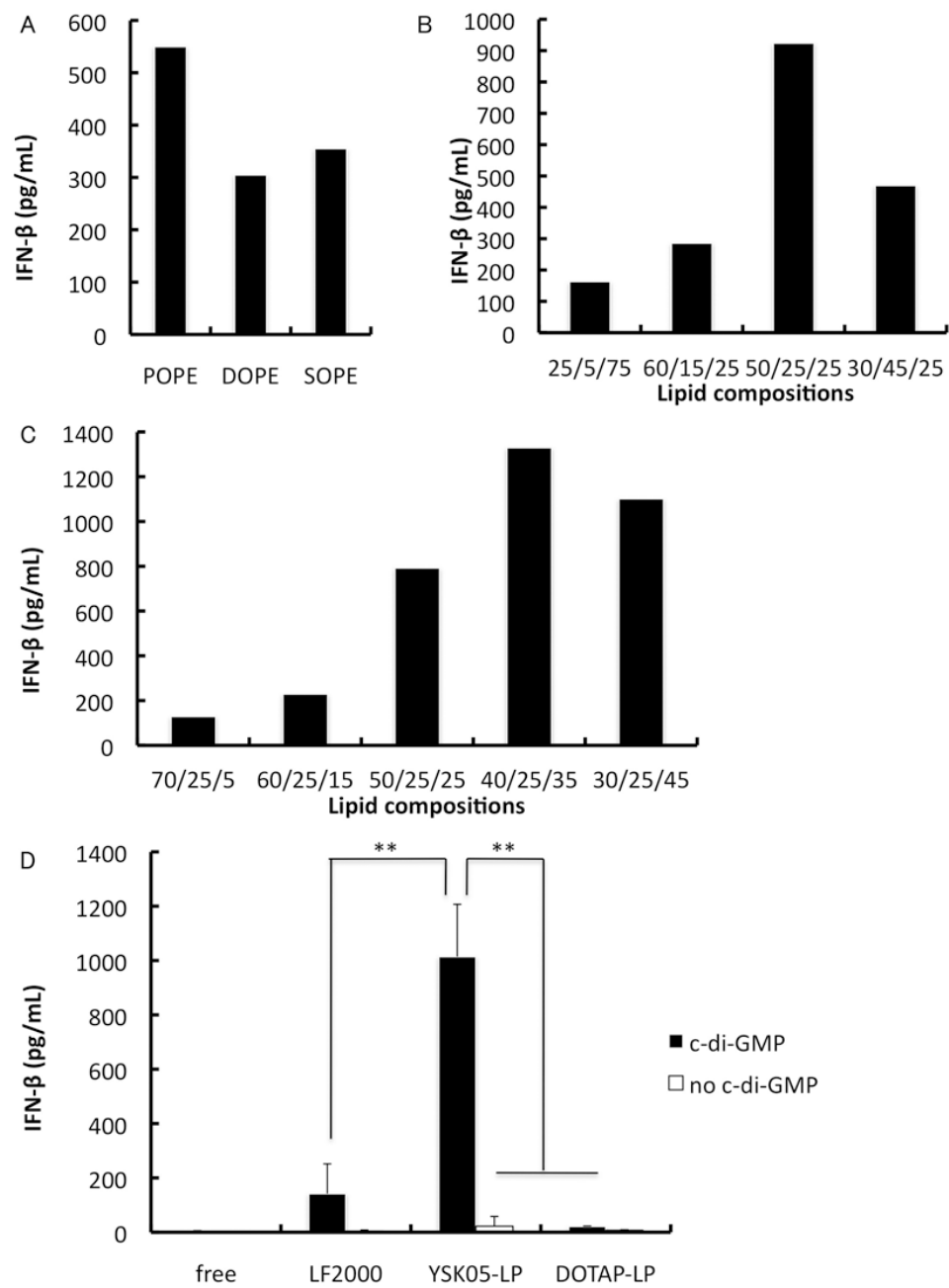


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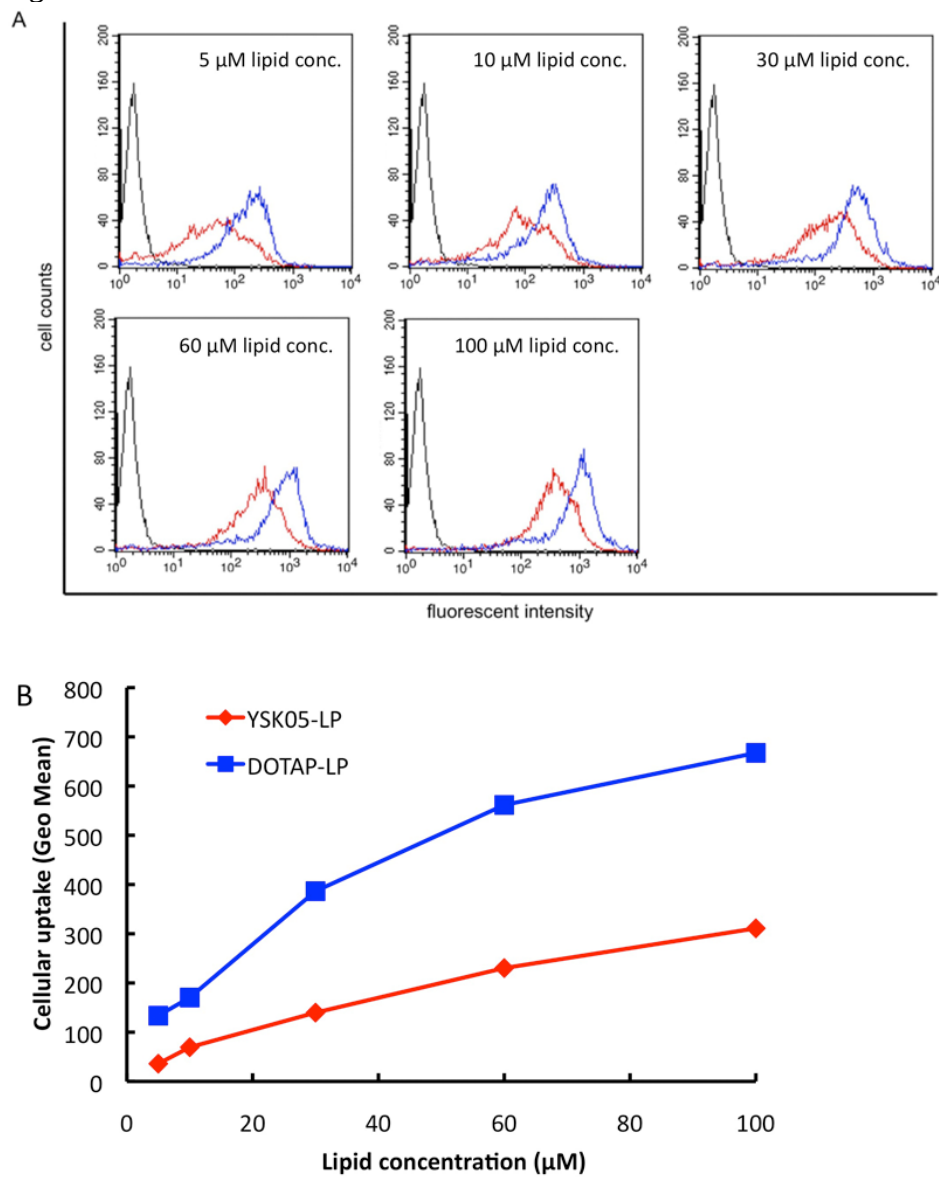


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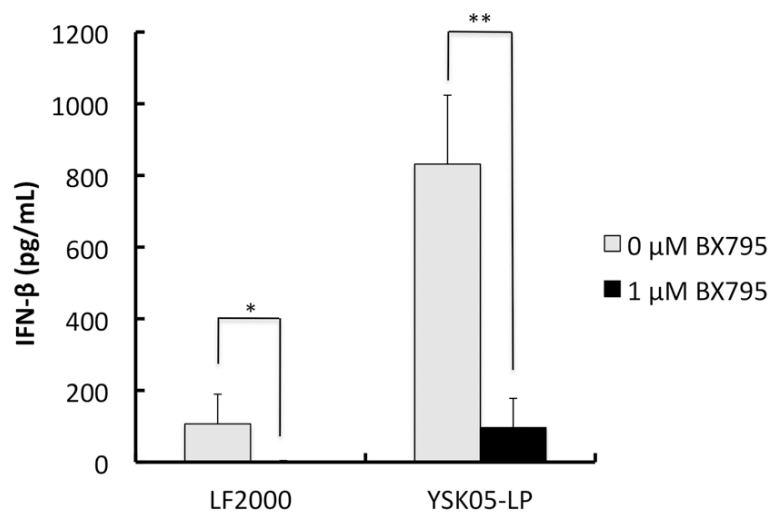


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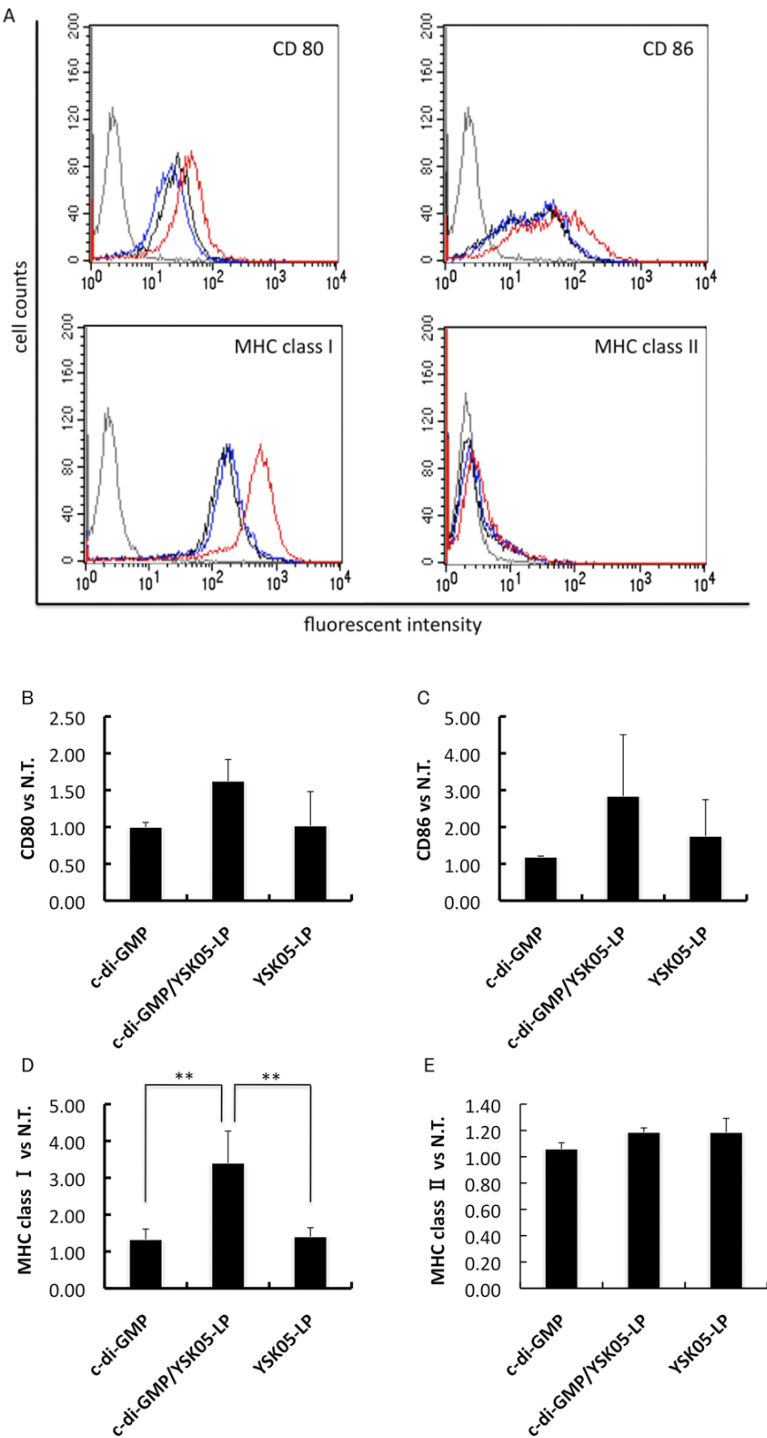


Figure 7.

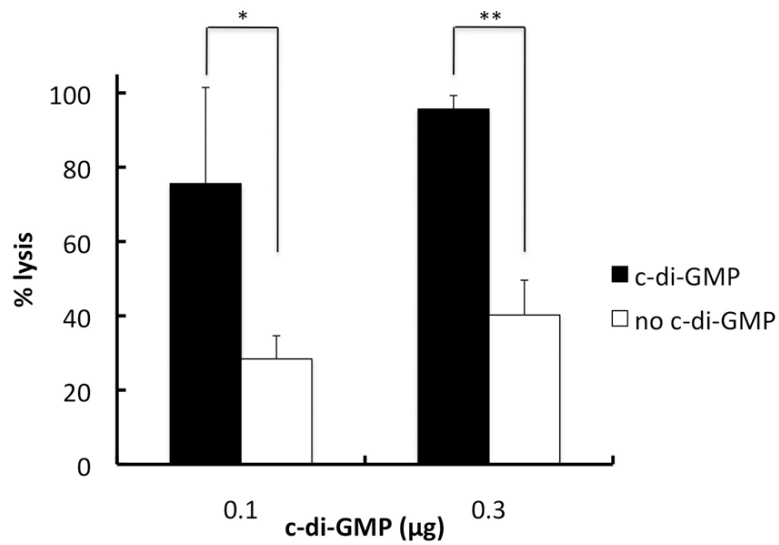


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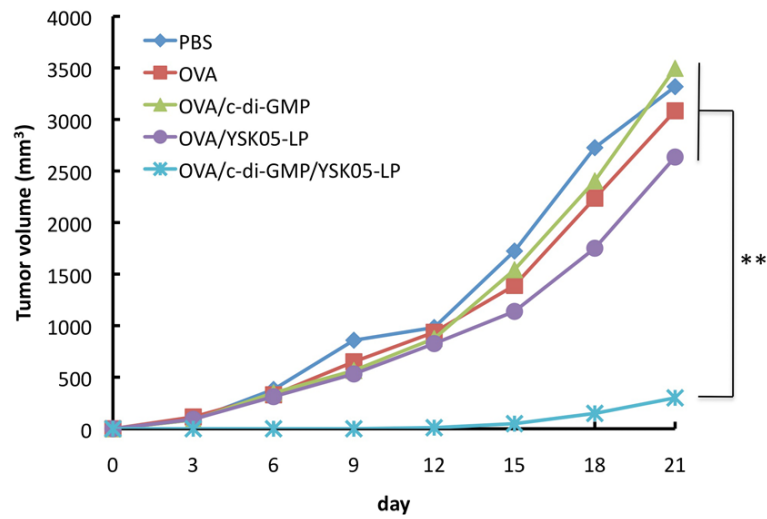


Table 1.

	size (nm)	PdI	zeta-potential (mV)
c-di-GMP/YSK05 liposome	172.0 ± 24.8	0.14 ± 0.03	- 4.30 ± 4.45
YSK05 liposome	143.3 ± 31.8	0.12 ± 0.03	1.65 ± 2.07