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**Abstract**

Induction of cancer-specific cytotoxic T lymphocytes is crucially important to complement therapeutic effects of immune checkpoint inhibitors and to achieve efficient cancer immunotherapy. To induce cancer-specific cytotoxic T lymphocytes, cancer antigen carriers must have multiple functions to deliver cancer antigens to antigen presenting cells, release antigens into cytosol, and promote the maturation of these cells. We earlier achieved cytosolic delivery of antigens and induction of antigen-specific cytotoxic T lymphocytes using carboxylated polyglycidol or polysaccharide derivative-modified liposomes that can induce membrane fusion with endosomes in response to weakly acidic pH. Furthermore, pH-sensitivity and adjuvant properties of these polymers were enhanced strongly by introduction of hydrophobic carboxylated units to dextran. Against our expectations, these polymer-modified liposomes only slightly induce cancer immunity, probably because of the high hydrophobicity of spacer units. This study used a polysaccharide with charged groups (chondroitin sulfate) instead of dextran as a backbone to reduce hydrophobicity. Chondroitin sulfate derivative-modified liposomes showed almost equal pH-sensitivity to that of dextran derivative-modified liposomes and achieved selective delivery to dendritic cells, whereas dextran derivative-modified liposomes were highly taken up by both dendritic cells and fibroblasts. Chondroitin

sulfate derivative-modified liposomes delivered model antigenic proteins into cytosol of dendritic cells and promoted cytokine production from the cells, leading to tumor regression on tumor-bearing mice after subcutaneous administration. Results demonstrate that charged group-having polysaccharide as a backbone can be used in an effective strategy to balance strong hydrophobicity of spacer units with their utilization for immunity-inducing systems.

*Keywords*: chondroitin sulfate / pH-sensitive liposome / dendritic cell / endosome / cancer immunotherapy / hydrophobicity

## Introduction

Recent progress in biology and immunology has revealed the important roles of immune systems on tumor microenvironments. Furthermore, that progress has led to novel therapeutic approaches to fight cancers through artificial control of immune systems. Cancellation of signaling pathways *via* immune checkpoint molecules, such as CTLA-4, PD-1 and PD-L1, using these molecule-specific antibodies has achieved remarkable therapeutic effects in clinical cancer therapy.[1-5] Nevertheless, these therapeutic effects remain limited. In some cases no therapeutic effect was observed, probably because of the lack of cancer-specific cytotoxic T lymphocytes (CTLs) in these cancer patients.[6-11] Therefore, the induction of cancer-specific CTLs is regarded as another important approach to achieve efficient cancer immunotherapy. To date, various induction systems of cancer-specific CTLs, including *ex vivo* and *in vivo* approaches, have been studied.[12-22] CAR-T therapy, a representative example of *ex vivo* approaches, has already been approved for clinical use, although clinical application of *in vivo* approaches to induce cancer-specific CTLs are few. Carriers of cancer antigens have been examined as examples of *in vivo* approaches. These carriers are expected to have multiple functions including encapsulation of cancer antigens, cellular association to antigen presenting cells (APCs), control of intracellular distribution of antigen and, and induction

of APC maturation to activate cancer-specific immune responses.[23]

We earlier reported an *in vivo* induction system of antigen-specific CTLs using antigen-loaded liposomes modified with carboxylated polyglycidols.[22,24,25] Carboxylated polyglycidols show membrane disruptive activity in response to weakly acidic pH and induce membrane fusion with endosomes after internalization to dendritic cells, resulting in the release of antigens into the cytosol of the cells.[22, 25] Cytosolic release of antigens can promote MHC class I-mediated antigen presentation (cross-presentation) in both murine-derived or human-derived dendritic cells.[22,26,27] After subcutaneous administration of these liposomes to mice, antigen-specific CTLs were induced in spleen; these CTLs migrated to tumor tissues, where they exhibited tumor cell-killing effects.[22,24,25] However, for liposomes modified with carboxylated polyglycidols, adjuvant molecules such as monophosphoryl lipid A (MPLA) or CpG-DNA are necessary to induce strong cellular immune responses.[28,29] We further developed carboxylated polysaccharides having not only pH-responsive membrane disruptive activity but also adjuvant function. Actually, liposomes modified with 3-methylglutarylated curdlan achieved the induction of antigen-specific CTLs and strong antitumor effect even in the absence of MPLA.[30] Adjuvant activity of polysaccharide derivatives can be modulated by changing the carboxylated units introduced to polysaccharides. Compared with 3-

methyl glutarylated dextran (MGlu-Dex), 2-carboxycyclohexane-1-carboxylated dextran (CHex-Dex) markedly promoted cytokine production from dendritic cells, probably because of strong hydrophobicity derived from CHex units.[31] In addition, CHex-Dex-modified liposomes showed markedly higher cellular association than that of MGlu-Dex-modified liposomes and achieved cytosolic release of antigenic protein.[31] Despite this superior antigen delivery performance *in vitro*, CHex-Dex-modified liposomes exhibited only a slight antitumor effect on tumor-bearing mice compared with MGlu-Dex-modified liposomes (Figure S1). After subcutaneous administration, the strong hydrophobicity of CHex-Dex might induce non-specific interaction with skin-resident cells rather than APCs.

These data suggest that modulation of hydrophobicity of CHex unit-introduced polymers is necessary, although the introduction of CHex units is an effective strategy to provide strong pH-sensitivity, cellular association property, and adjuvant function to the liposomes. For this purpose, we conceived the use of polysaccharides with charged groups as a backbone instead of dextran to modulate the hydrophobicity of CHex unit-introduced polymers. Chondroitin sulfate (CS) was selected as a first example to verify our hypothesis because CS has many carboxy groups and sulfo groups in its repeating units. Especially, sulfo group stably possesses a negative charge at the physiological pH

region, which might suppress non-specific binding of CHex unit-introduced polymers *via* strong hydrophobic interaction. Additionally, CS, a main component of the extracellular matrix, is a biocompatible material.[32-34] Polysaccharide having a sulfo group including CS are known to show adjuvant functions.[35-38] Here, MGlu units or CHex units were introduced to CS. These CS derivatives were modified onto antigen-loaded liposomes. Then, their pH-responsive capability, antigen delivery performance, adjuvant property and cancer immunity-inducing function were investigated and compared with those of CHex-Dex-modified liposomes (Figure 1).

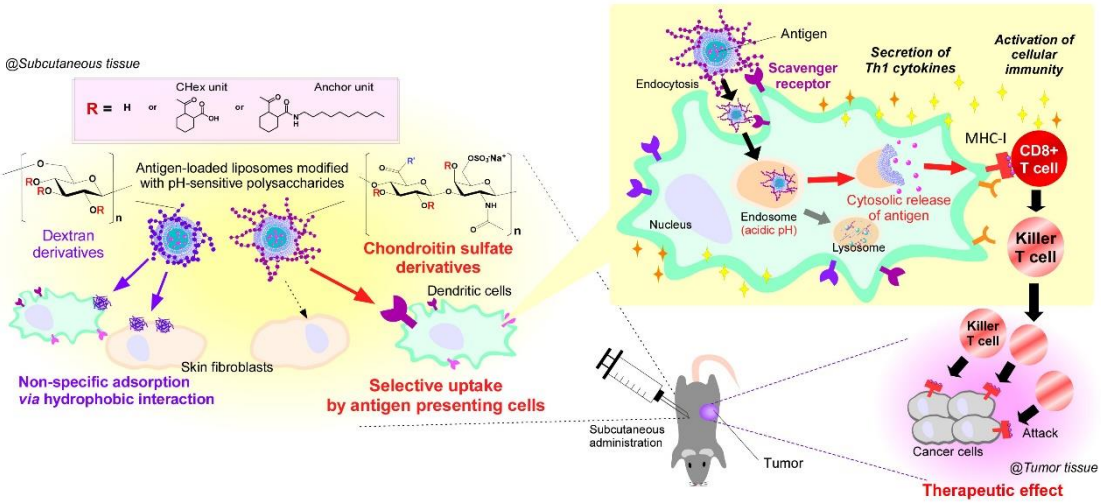


**Figure 1.** Design of chondroitin sulfate derivative-modified liposomes for induction of cancer-specific immunity. Conventional pH-sensitive polysaccharide, dextran derivative-modified liposomes could not induce antitumor immunity probably because of non-specific adsorption to skin-resident cells *via* its hydrophobic nature (Figure S1).

Chondroitin sulfate derivative-modified liposomes would be selectively taken up by antigen presenting cells at skin and promote both the cytokine production from the cells and endosomal escape of antigenic protein *via* pH-responsive membrane destabilization property, leading to induction of antigen-specific cellular immunity.

## Results and Discussion

**Characterization of Chondroitin Sulfate Derivatives.** Chondroitin sulfate (CS) derivatives with different contents of MGlu groups or CHex groups as pH-sensitive moiety were synthesized by reacting CS with various amounts of 3-methylglutaric anhydride or 1,2-cyclohexanedicarboxylic anhydride (Figure 2 and Table 1). Decyl amidated groups were further introduced to CS derivatives by reaction of decylamine with carboxy groups in CS derivatives for fixation of these polymers onto liposome membrane (Figure 2 and Table 2). As a control, carboxy groups of CS were reacted directly with decylamine to obtain anchor group-having CS (CS-A7). The obtained CS derivatives were characterized using $^{1}$H NMR. Comparison to spectra for CS (Figure S2A) revealed the existence of new peaks corresponding to MGlu/CHex groups and decyl groups in Figures S2B–F, which confirmed the introduction of MGlu/CHex groups and decyl amidated groups to CS. From the integration ratio of peaks of MGlu/CHex residues

or decyl residues to those of sugar backbone (2 ppm, 3.3–4.8 ppm), the contents of MGlu/CHex residues and decyl residues were calculated and calculated. They are shown in Tables 1 and 2 as the number per 100 sugar units.

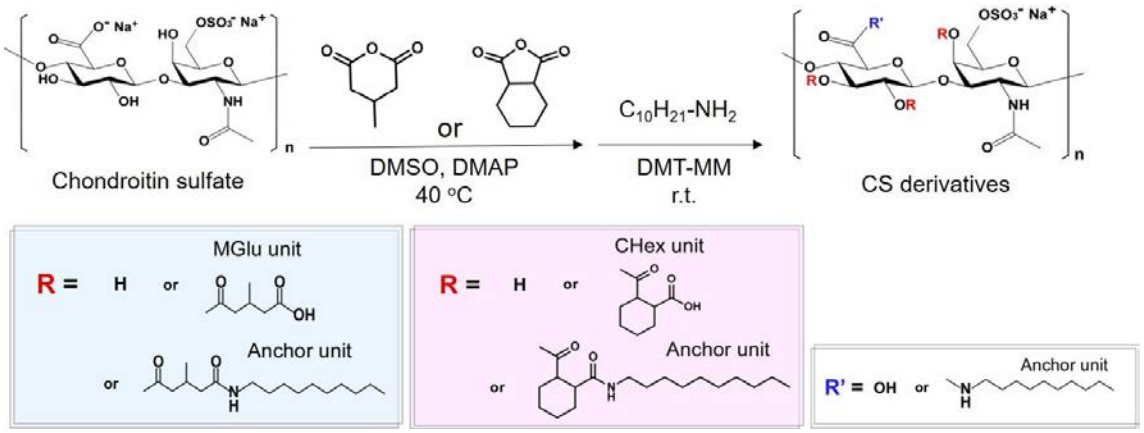


**Figure 2.** Synthetic route for chondroitin sulfate derivatives having carboxy groups and alkyl chains as anchor units to liposomal membrane.

**Table 1. Synthesis of Chondroitin Sulfate Derivatives**

| Polymer | CS [g] | MGluAn or CHexAn [g] | DMSO [mL] | DMAP [g] | Time [h] | Yield [g] | MGlu or CHex contents/100 sugar units* |
|---|---|---|---|---|---|---|---|
| MGlu24-CS | 0.494 | 0.417 | 30 | 0.372 | 24 | 0.498 | 24 |
| MGlu45-CS | 0.568 | 1.794 | 50 | 0.281 | 144 | 0.690 | 45 |
| MGlu71-CS | 0.462 | 1.594 | 30 | 0.268 | 96 | 0.564 | 71 |
| MGlu72-CS | 0.490 | 0.792 | 27 | 0.749 | 74 | 0.650 | 72 |
| CHex42-CS | 0.870 | 0.930 | 25 | 0.464 | 24 | 0.917 | 42 |
| CHex43-CS | 0.204 | 0.448 | 28 | 0.124 | 164 | 0.206 | 43 |
| CHex79-CS | 1.281 | 1.966 | 25 | 0.571 | 24 | 1.451 | 79 |
| CHex131-CS | 0.455 | 0.921 | 25 | 0.232 | 111 | 0.599 | 131 |
| CHex139-CS | 0.868 | 1.927 | 25 | 0.464 | 24 | 1.204 | 139 |

*Determined by $^1$H NMR.

**Table 2. Synthesis of Chondroitin Sulfate Derivatives Having Anchor Moieties**

| Polymer | MGlu-CS or CHex-CS [g] | Decyl amine [g] | DMT-MM [g] | Time [h] | Yield [mg] | Anchor contents/100 sugar units* | MGlu or CHex contents/100 sugar units* |
|---|---|---|---|---|---|---|---|
| MGlu43-CS-A16 | 0.099 | 0.022 | 0.048 | 21 | 0.099 | 16 | 43 |
| MGlu55-CS-A27 | 0.312 | 0.053 | 0.117 | 7 | 0.308 | 27 | 55 |
| MGlu73-CS-A10 | 0.118 | 0.029 | 0.070 | 48 | 0.139 | 10 | 73 |
| CHex27-CS-A9 | 0.452 | 0.027 | 0.013 | 17.5 | 0.429 | 9 | 27 |
| CHex34-CS-A15 | 0.621 | 0.053 | 0.032 | 27.5 | 0.572 | 15 | 34 |
| CHex75-CS-A21 | 0.100 | 0.010 | 0.027 | 7 | 0.095 | 21 | 75 |
| CHex77-CS-A9 | 0.353 | 0.020 | 0.054 | 8.5 | 0.348 | 8 | 77 |
| CS-A7 | 1.948 | 0.199 | 0.434 | 18 | 1.066 | 7.2 | 0 |

*Determined by $^1$H NMR.

**Preparation of Chondroitin Sulfate Derivative-Modified Liposomes.** CS derivative-modified liposomes were prepared by film hydration method using a mixed thin film of egg yolk phosphatidylcholine (EYPC) and CS derivatives. The liposome suspension was extruded further through a polycarbonate 100 nm pore size membrane. The size and zeta potential of liposomes were evaluated (Table 3). Most liposomes were found to have a narrow size distribution and average sizes were 130–200 nm. CS derivative-modified liposomes showed more negative zeta potentials than those of

unmodified liposomes. The zeta potentials decreased concomitantly with the increase of polymer (CHex77-CS-A8) contents in the lipid/polymer mixture, indicating the modification of CS derivatives having carboxy groups and sulfo groups onto the liposome surface. Considering the condensation reaction by DMT-MM, both carboxy groups in MGlu/CHex units and glucronic acid units of CS can react with decylamine to form anchor moiety. The anchor modification position might affect the amounts of the polymers modified onto the liposomes. Therefore, polymer contents in the liposomes after purification were ascertained by quantification of sugar contents using phenol-sulfuric acid assay.[39] Table S1 shows that the recovery rates of CS-A7 and CHex77-CS-A8 in each liposome were almost equal (37% and 30%, respectively), irrespective of differences in possible modification sites of decylamines. Moreover, the recovery rate of MGlu55-CS-A27 in MGlu55-CS-A27-modified liposomes was quite high (75%). These results suggest that the number (not introduced position) of the anchor group affects the amounts of CS derivatives modified onto the liposomes. These liposomes can encapsulate the model antigenic proteins (ovalbumin, OVA) in range to 50-100 g/mol lipids, which is consistent with results reported earlier.[29] To ascertain the pH-responsive properties of CS derivatives, liposomes containing a fluorescence dye (pyranine) and its quencher (DPX) were prepared. The leakage of pyranine from liposomes was monitored at various pH

(Figure 3). For the cases of unmodified liposomes and CS-A7-modified liposomes, no change in pyranine release was observed under any examined pH conditions (Figure 3A). MGlu-CS-A-modified liposomes retained their contents at neutral pH but they induced content release below at pH 5 (Figure 3A), suggesting that MGlu-CS chains on the liposomes possessed hydrophobic nature after protonation of carboxy groups in MGlu residues, leading to destabilization of the liposomal membrane *via* hydrophobic interaction. Compared with MGlu55-CS-A27-modified liposomes, MGlu73-CS-A10-modified liposomes showed less content release, perhaps because of the higher modification amounts of MGlu55-CS-A27, which has many decyl chains, than the amounts of MGlu73-CS-A10. Actually, MGlu73-CS-A10-modified liposomes prepared in high polymer content (50 wt%) exhibited almost identical pH-responsive behavior to that found for MGlu55-CS-A27-modified liposomes. Figure 3B presents effects of the amounts of CHex77-CS-A8 on pH-responsiveness. Actually, CHex77-CS-A8-modified liposomes exhibited release of contents at pH below 6.0, which is higher pH than that of MGlu-CS-A-modified liposomes, indicating that CHex residues possess more hydrophobic properties than MGlu residues have.[40,41] The extent of content release increased concomitantly with increase of the feed ratio of CHex77-CS-A8 per lipid. By contrast, liposomes modified with CHex27-CS-A9, CHex34-CS-A15 and CHex77-CS-

A8 showed almost identical pH-responsive behaviors (Figure 3C). In our earlier results for MGlu/CHex unit-introduced polymers, the pH region in which content release is induced increased with MGlu/CHex unit contents because of the p*K*a shift of carboxy groups and increased hydrophobic interactions.[40,41] For the case of CS derivatives, the increase of hydrophobicity by MGlu/CHex group introduction might be suppressed by the existence of sulfo groups in the backbone, resulting in no dependence in MGlu/CHex residue contents on pH-responsiveness of the liposomes (Figures 3A and 3C). When CHex group-introduced dextran derivative (CHex81-Dex-A18) was used for liposome preparation, remarkable aggregation was observed for polymer content more than 3 wt%, probably because of the quite strong hydrophobic nature of CHex81-Dex-A18 (data not shown). Therefore, liposomes modified with 1 wt% of CHex81-Dex-A18 were prepared as a comparison of CHex-CS-A-modified liposomes. Even with a quite low feed ratio of polymer, CHex81-Dex-A18-modified liposomes showed almost equal content release behavior with those of CHex-CS-A-modified liposomes (Figure 3C).

**Table 3. Characteristics of Liposomes Modified with or without CS Derivatives**

| Liposome | Polymer content per lipid | Size [nm] | PDI | ζ-potential [mV] | OVA/lipid [g/mol] |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| Unmodified | – | 156 ± 9.8 | 0.090 | −10.1 ± 2.0 | 51.9 ± 9.7 |
| CS-A7 | 50 wt% | 151 ± 22 | 0.14 | −36.4 ± 3.4 | 73.0 ± 33 |
| MGlu55-CS-A27 | 30 wt% | 149 ± 8.4 | 0.13 | −52.3 ± 5.9 | 73.8 ± 17 |
| CHex34-CS-A15 | 30 wt% | 158 ± 31 | 0.15 | −19.4 ± 6.2 | 48.4 ± 11 |
| CHex75-CS-A21 | 30 wt% | 143 ± 23 | 0.37 | −42.2 ± 0.78 | 65.3 ± 15 |
| CHex77-CS-A8 | 30 wt% | 130 ± 11 | 0.14 | −17.6 ± 3.2 | 64.5 ± 25 |
| CHex77-CS-A8 | 50 wt% | 118 ± 5.2 | 0.19 | −29.3 ± 6.7 | 77.4 ± 19 |
| CHex81-Dex-A18 | 1 wt% | 138 ± 50 | 0.23 | −26.0 ± 4.3 | 105 ± 19 |

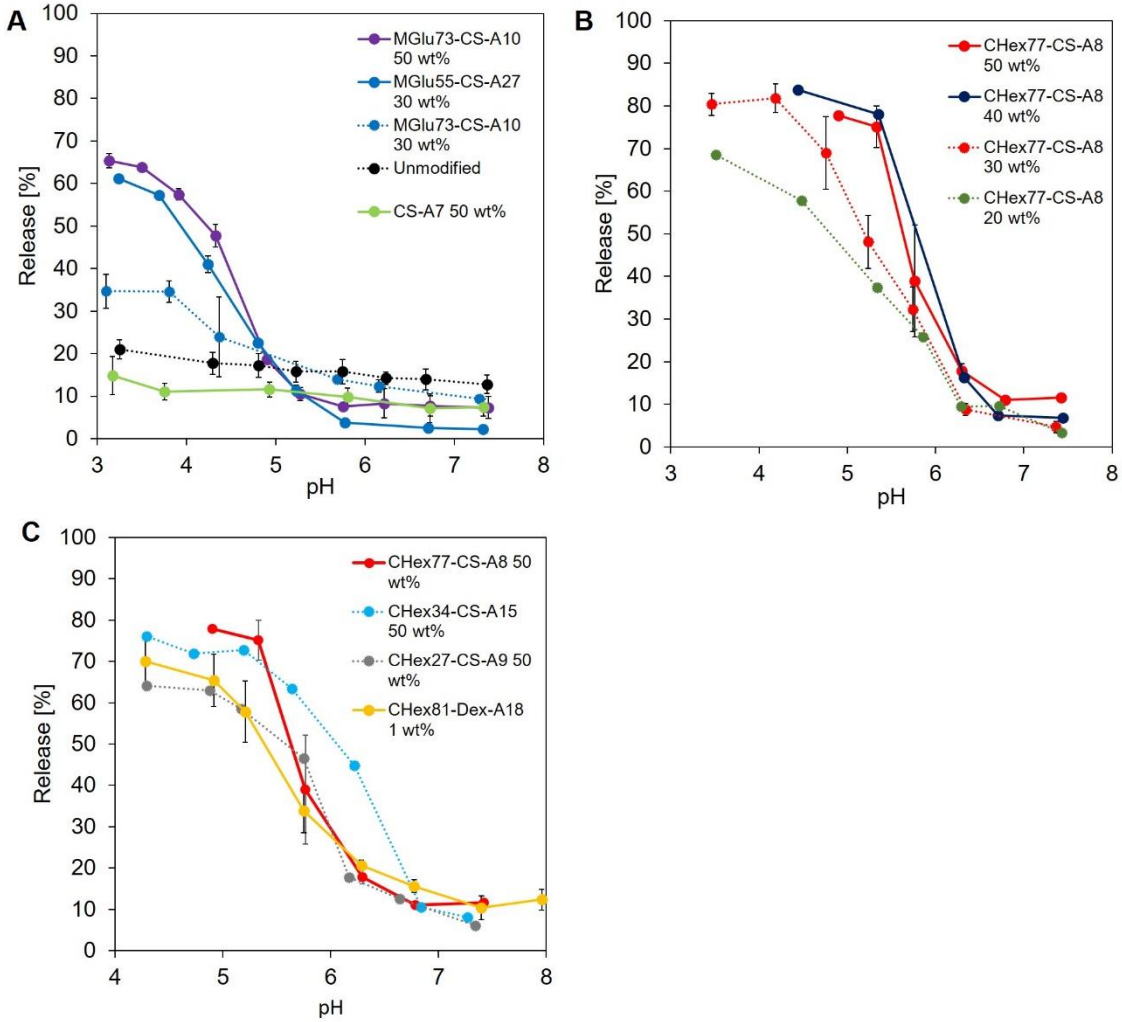


**Figure 3.** pH-Dependence after 30 min-incubation of pyranine release from EYPC

liposomes modified with or without MGlu-CS derivatives (A), with various contents of CHex77-CS-A8 (B) and CHex-CS/CHex-Dex derivatives (C) were shown. Lipid concentrations were $2.0 \times 10^{-5}$ M. Each point is the mean $\pm$ SD ($n = 3$).

**Interaction of Chondroitin Sulfate Derivative-Modified Liposomes with Cells.** Next, interaction of CS derivative-modified liposomes with dendritic cells was examined (Figure 4). The cellular fluorescence intensity of DC2.4 cells treated with DiI-labeled liposomes modified with or without CS derivatives was measured using a flow cytometer. CS-A7-modified liposomes showed almost identical cellular association with unmodified EYPC liposomes, indicating that modification of CS chains on the liposome surface only slightly affected association to dendritic cells (Figure 4A). By contrast, MGlu-CS-A-modified or CHex-CS-A-modified liposomes showed higher cellular association than that of unmodified liposomes (Figure 4A). Especially, liposomes modified with CS derivatives containing high contents of MGlu or CHex units showed markedly high cellular association (Figure 4A). CHex81-Dex-A18-modified liposomes showed almost identical cellular association to DC2.4 cells with CHex77-CS-A8-modified liposomes (Figure 4B), although the same liposomes also showed much higher cellular association to NIH3T3 cells than those of other liposomes (Figure 4C). The effect

of dextran sulfate, an inhibitor for scavenger receptors,[42-44] on cellular association was examined to assess the cellular association mechanism of liposomes. The presence of dextran sulfate markedly suppressed the cellular association of both CHex77-CS-A8-modified liposomes and CHex81-Dex-A18-modified liposomes (Figure 4D), suggesting that these liposomes were taken up *via* recognition of anionic molecules (carboxy groups and sulfo groups) on the liposomes by of DC2.4 cell scavenger receptors.[30] However, CHex81-Dex-A18-modified liposomes showed still higher cellular association to DC2.4 cells even in the presence of dextran sulfate. These results indicate that the strong hydrophobic nature of CHex81-Dex-A18 induced non-specific cellular association, resulting in high cellular association to both DC2.4 cells and NIH3T3 cells. As described in the *Introduction*, CHex-Dex-A-modified liposomes were unable to induce antitumor effects after subcutaneous administration to tumor-bearing mice (Figure S1). CHex-Dex-A-modified liposomes might be taken up by skin-resident cells rather than APCs, resulting in a lack of induction of *in vivo* immune responses. Compared with CHex-Dex-A-modified liposomes, cellular association of CHex77-CS-A8-modified liposomes was cancelled completely in the presence of dextran sulfate (Figure 4D). It showed low cellular association to NIH3T3 cells (Figure 4C) and high cellular association to DC2.4 cells (Figure 4B). Therefore, more CHex77-CS-A8-modified liposomes would be taken

up by APCs in skin tissues after subcutaneous administration than in skin fibroblasts.

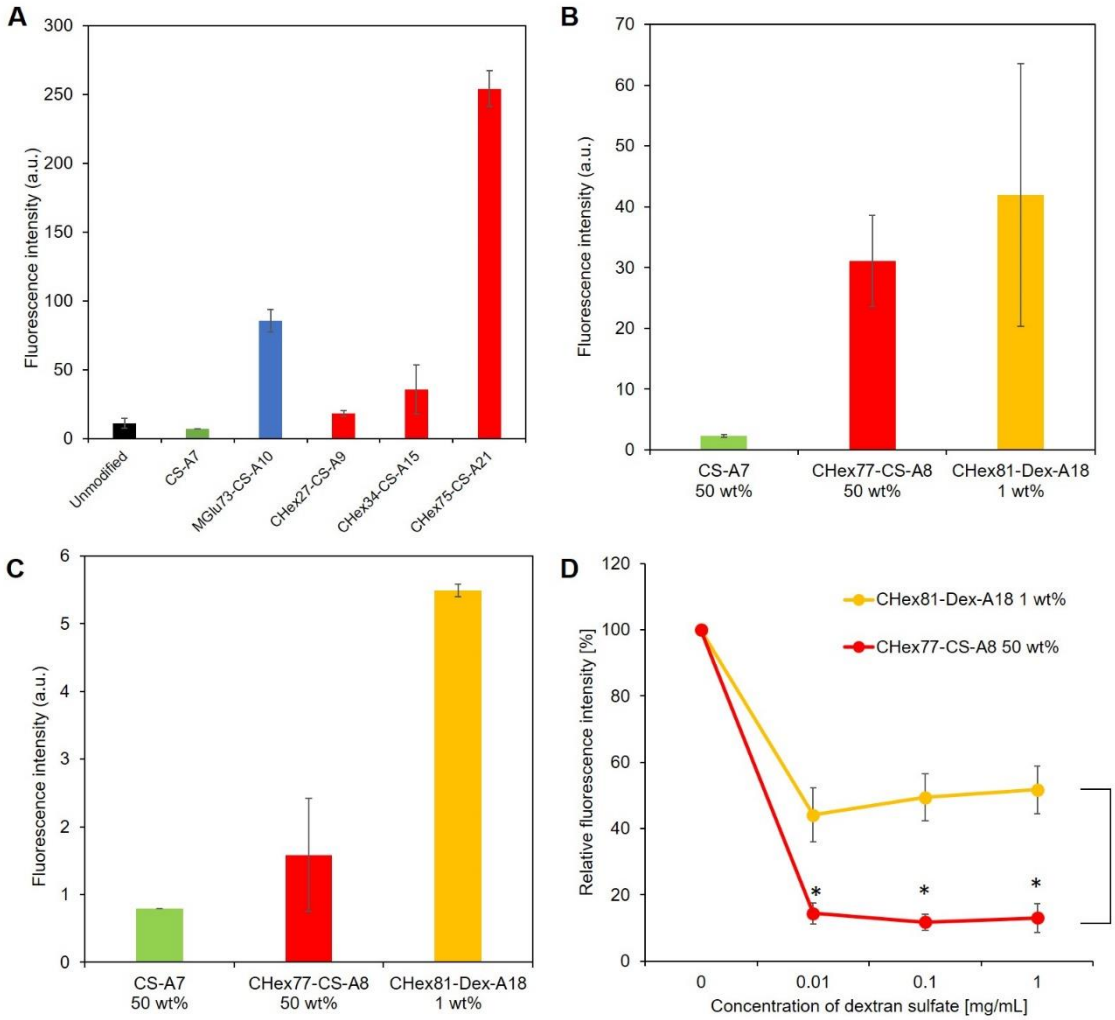


**Figure 4.** Fluorescence intensity for DC2.4 cells (A, B) and NIH3T3 cells (C) treated with DiI-labeled EYPC liposomes modified with or without polysaccharide derivatives. Cells were incubated with liposomes (lipid concentration: 0.1 mM) for 4 h at 37 °C in serum free medium. Data were corrected by DiI fluorescence intensity per lipid in each liposome. (D) Inhibition of cellular association of CHex77-CS-A8-modified liposomes and CHex81-Dex-A18-modified liposomes by dextran sulfate. DC2.4 cells were pre-

incubated with various amounts of dextran sulfate for 1 h before liposome treatment. Relative fluorescence intensity was calculated as the ratios of the amount of association in the presence of inhibitor to that in the absence of inhibitor. *$p < 0.01$.

Furthermore, intracellular distributions of liposomes and the contents were examined. FITC-labeled OVA was encapsulated into DiI-labeled liposomes. These liposomes were applied to DC2.4 cells. Then FITC/DiI fluorescence in the cells was detected using confocal laser scanning microscopy (Figure 5). For cells treated with CS-A7-modified liposomes, both DiI and FITC fluorescence was detected only slightly from the cells under experimental fluorescence sensitivity settings, which reflects their low cellular association to the cells. For the cells treated with MGlu-CS-A-modified liposomes, weak DiI/FITC fluorescence dots were observed within the cells, suggesting that these liposomes were taken up by endocytosis and they were trapped in endo/lysosomes. For CHex-CS-A-modified liposomes, bright yellow fluorescence dots were observed within cells from the merged image after 4 h incubation, indicating that these liposomes were internalized efficiently to the cells and that they were trapped in endo/lysosomes. When these cells were incubated with the liposomes for 24 h, yellow fluorescence dots corresponding to DiI/FITC-OVA molecules entrapped in

endo/lysosomes became red; also leakage of green fluorescence into the cytosol was observed (Figure S3). These results indicate that CHex-CS-A-modified liposomes were taken up by DC2.4 cells and that they released the FITC-OVA inside of endo/lysosomes responding to weakly acidic pH. Then, CHex-CS-A-modified liposomes further destabilized endo/lysosomal membrane over time, leading to the leakage of FITC-OVA molecules into cytosol. For CHex81-Dex-A18-modified liposomes, a part of DiI fluorescence was absorbed onto the cell periphery (Figure S4), which also supports the non-specific cellular association of CHex81-Dex-A18-modified liposomes as discussed in Figure 4. Therefore, CHex-CS-A-modified liposomes are useful not only for selective delivery to APCs but also for cytosolic delivery of antigenic proteins *via* pH-sensitive properties.

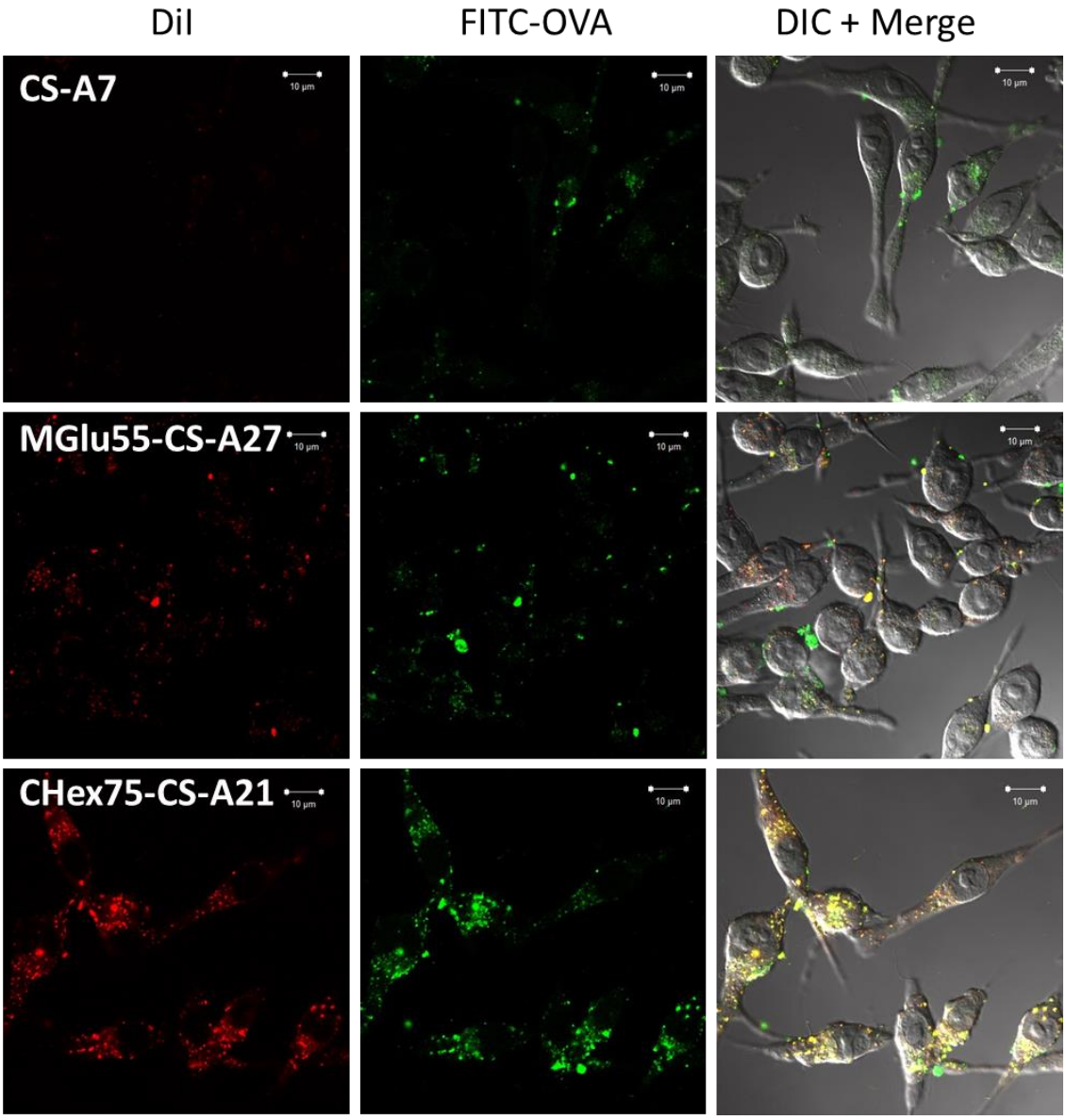


**Figure 5.** Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with FITC-OVA-loaded and DiI-labeled EYPC liposomes modified with CS derivatives for 4 h at 37 °C in serum-free medium. Bar represents 10 μm.

**Adjuvant Properties of Chondroitin Sulfate Derivatives.** Antigen delivery carriers must have an intracellular antigen delivery function and an adjuvant property,

respectively, for the control of antigen presentation processes and maturation of antigen presenting cells.[23] In general, adjuvant molecules such as Toll like receptor ligands have been additionally incorporated to antigen carriers to provide an adjuvant property.[23,28,29] Our earlier studies revealed that pH-sensitive group-introduced polysaccharide derivatives act not only as pH-responsive polymers but also as adjuvant molecules. Moreover, their adjuvant properties are affected strongly by polysaccharide backbone, structures and the amounts of pH-sensitive residues.[30,31] Therefore, adjuvant properties of CS derivatives were investigated by measuring cytokine production from DC2.4 cells treated with CS derivatives (Figure 6) and with CS derivative-modified liposomes (Figure 7). As shown in Figure 6A, MGlu-CS induced almost identical TNF-α production from cells with parent CS, but considerably higher TNF-α production was detected after treatment with CHex-CS. The latter was comparable to that found for CHex-Dex polymers. In addition, CS derivatives having high contents of CHex residues (CHex79-CS and CHex131-CS) showed much higher IL-12 production than CHex-Dex polymers and other polymers (Figure 6B). These results suggest that introduction of CHex residues into polysaccharides can increase their adjuvant properties and suggest that the polysaccharide backbone structure affects their bioactivity. Sulfated polysaccharides including CS are known to induce maturation of APCs.[35-38] Bioactivity derived from CS

might be enhanced by the introduction of CHex residues having a cyclohexyl spacer unit, which resembles the sugar structure.[41] In the cases of CS derivative-modified liposomes, CHex77-CS-A8-modified liposomes showed markedly higher cytokine production than unmodified liposomes, CS-A7-modified liposomes, or CHex-81-Dex-A18-modified liposomes did (Figure 7), indicating that CS derivatives can show adjuvant effects after modification on liposomes. It is noteworthy that modification of CHex-Dex-A to liposomes reduced cytokine production of CHex-Dex polymers strongly compared with the cases of CHex-CS, which might stand as another explanation for the weak antitumor effects observed after subcutaneous administration of CHex-Dex-A-modified liposomes (Figure S1). Actually, CHex77-CS-A8-modified liposomes showed the strongest adjuvant property among the examined liposomes. Therefore, *in vivo* experiments were conducted with tumor-bearing mice.

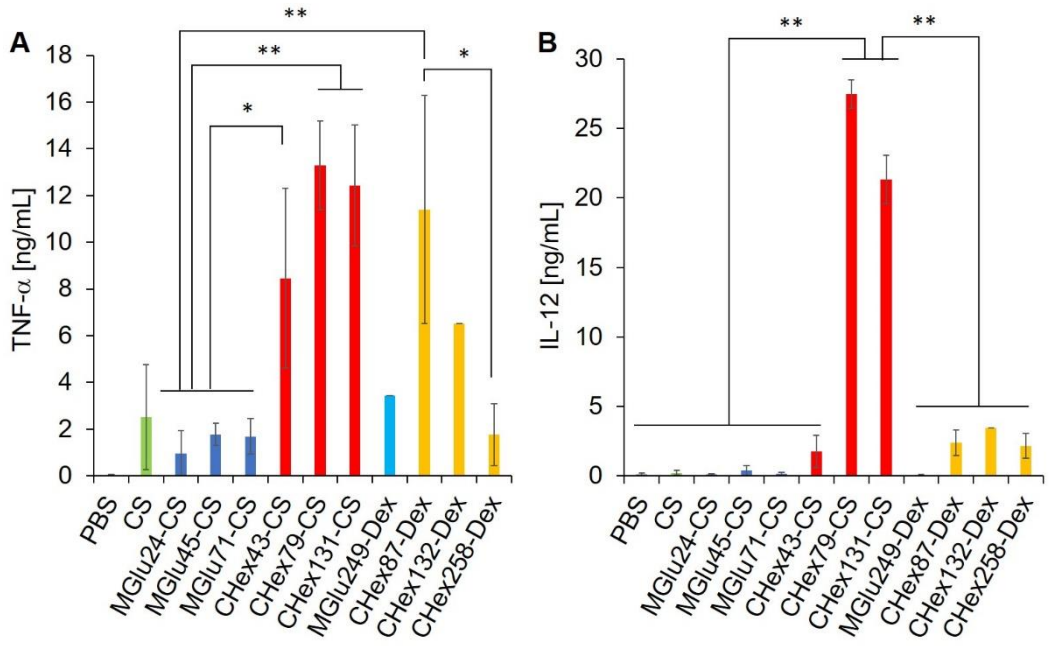


**Figure 6.** TNF-α (A) and IL-12 (B) production from DC2.4 cells treated with polysaccharide derivatives (0.25 mg/mL) for 24 h. *$p < 0.05$. **$p < 0.01$.

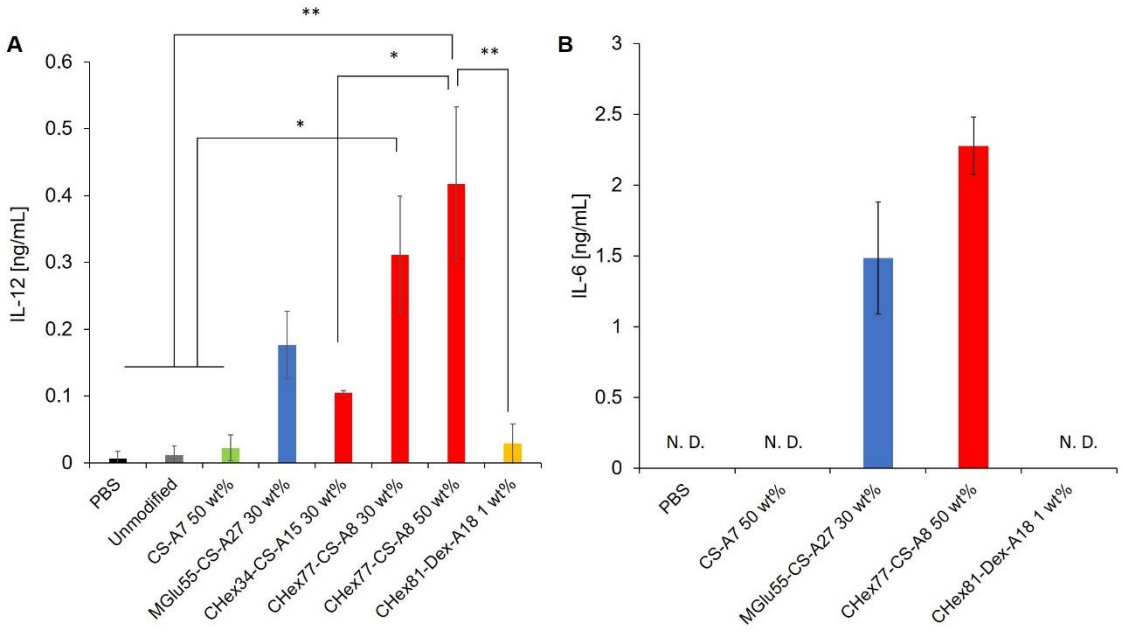


**Figure 7.** IL-12 (A) and IL-6 (B) production from DC2.4 cells treated with liposomes modified with or without polysaccharide derivatives (0.1 mM) for 4 h. *$p < 0.05$. **$p <$

0.01.

**Induction of Antitumor Immunity by Liposomes Modified with Chondroitin Sulfate Derivatives.** OVA-expressing tumor cells (E.G7-OVA cells) were inoculated to the back of mice. Then, OVA-loaded liposomes modified with polysaccharide derivatives were administered subcutaneously into tumor-bearing mice on days 7 and 14 after tumor inoculation. In the case of mice treated with PBS, the tumor volume increased with time (Figure 8A); it reached the endpoint within 20 days (Figure 8B). In the cases of mice administered liposomes modified with CS-A7, MGlu55-CS-A27 or CHex81-Dex-A18, tumor growth was suppressed slightly (Figures 8A and S5). Mouse survival was extended slightly (Figure 8B). In contrast, tumor growth was highly suppressed by the administration of CHex77-CS-A8-modified liposomes. One mouse became completely tumor-free (Figures 8A and S5).

To evaluate the *in vivo* immune response, mice were immunized subcutaneously twice with PBS or liposomes modified with polysaccharide derivatives. Then splenocytes from these mice were cultured in the presence or absence of OVA. After 5 days of culture, IFN-γ secretion in supernatants was measured by ELISA. As shown in Figure S6, in the absence of OVA during *in vitro* culture, IFN-γ secretion could not be detected from any

splenocytes. In contrast, high concentration of IFN-γ were detected in splenocytes from mice treated with CHex77-CS-A8-modified liposomes in the presence of OVA during *in vitro* culture. Furthermore, IFN-γ secretion increased concomitantly with the increase of the OVA dosage of subcutaneous administration. These data suggest that OVA-specific Th1 cells or CTLs were induced dose-dependently in the spleen of mice treated with CHex77-CS-A8-modified liposomes. Induced OVA-specific cellular immune responses would kill OVA-expressing tumor cells in E.G7-OVA tumor-bearing mice, resulting in tumor regression and complete cure (Figures 8 and S5). However, individual difference of CHex77-CS-A8-modified liposome-treated mice in IFN-γ secretion levels was high (Figure S6). This might be reflected to the difference in individual tumor volume change of mice treated with CHex77-CS-A8-modified liposomes (Figure S5). CHex81-Dex-A7-modified liposomes only slightly induced IFN-γ secretion in the spleen at a low dose (10 μg/immunization) but IFN-γ secretion was detected at high dose (100 μg/immunization) (Figure S6). This result suggests that the threshold OVA dose exists in the case of CHex81-Dex-A7-modified liposomes for the induction of cellular immune responses. Most of administered liposomes might be trapped in subcutaneous tissue because of high hydrophobicity of CHex81-Dex-A7. However, at a high dose, some liposomes can be taken up by antigen presenting cells, resulting in the induction of OVA-specific cellular

immunity *in vivo* and suppression of tumor growth in some tumor-bearing mice (Figure S6). Mouse survival was also extended considerably by administration of CHex77-CS-A8-modified liposomes (Figure 8B and Table S2). No marked change in mouse body weight of mice was observed from either treatment (Figure S7).

CHex-Dex-A-modified liposomes were found to have quite high cellular association to DC2.4 cells (Figures 4A and 4B), but they were simultaneously taken up by fibroblasts (Figure 4C), suggesting that these liposomes might be taken up by skin fibroblasts after subcutaneous administration. Furthermore, CHex-Dex-A-modified liposomes only slightly exhibited adjuvant effects (Figure 7) probably because absorption of the liposomes to the cell surface (Figure S4) suppress the cellular bioactivity. By contrast, introduction of same pH-sensitive residue into CS improved these shortcomings of CHex-Dex considerably. CHex-CS-A-modified liposomes showed almost identical pH-sensitive properties to those of CHex-Dex-A-modified liposomes (Figure 3C) and cellular association to DC2.4 cells (Figure 4B). It is noteworthy that CHex-CS-A-modified liposomes exhibited low cellular association to fibroblasts (Figure 4C), probably because charged groups in CS backbone (carboxy groups and sulfo groups) reduce the hydrophobicity and suppress non-specific bindings derived from CHex residues. CHex-CS-A-modified liposomes delivered antigenic proteins into cytosol of DC2.4 cells

(Figure S3) and promoted cytokine production from these cells (Figure 7). These properties of CHex-CS-A-modified liposomes are expected to lead to the induction of antitumor immunity (Figure 8). Therefore, the use of polysaccharides having charged functional groups in their backbone is anticipated as an effective strategy to design multi-functional polysaccharide derivatives having antigen presenting cell-selective delivery performance, cytosolic antigen delivery function and adjuvant property for induction of antigen-specific cellular immunity. Therapeutic effects induced by CHex-CS-A-modified liposomes were still modest. Future studies must examine the increase of therapeutic effects of CS-derivative-modified liposomes by inclusion of additional adjuvant molecules such as MPLA, other TLR agonists or cationic lipids.

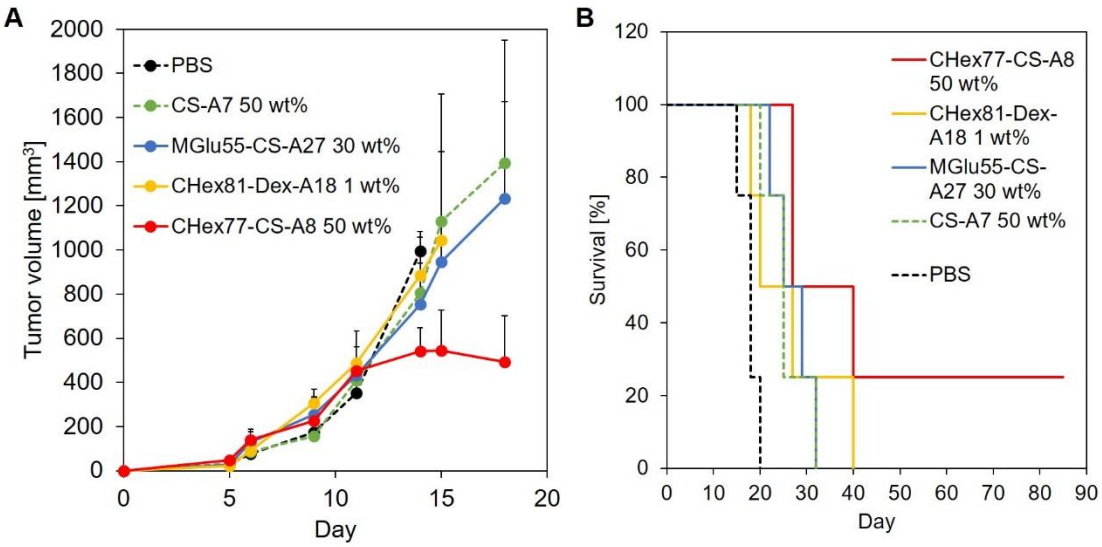


**Figure 8.** Antitumor effects induced by subcutaneous administration of OVA-loaded liposomes. C57BL/6 mice were subcutaneously immunized on days 7 and 14 with PBS,

liposomes modified with polysaccharide derivatives. Changes in tumor volume (A) and survival (B) of mice were monitored after E.G7-OVA cells ($1 \times 10^6$ cells/mouse) inoculation. All treated groups contained four mice.

## Conclusion

This study examined antigen delivery performance and cancer immunity-inducing function of CS derivative-modified liposomes. CHex unit-introduced CS derivative-modified liposomes achieved selective cellular association to dendritic cells, whereas CHex unit-introduced dextran derivative-modified liposomes exhibited high but non-specific cellular association because of their strong hydrophobicity. CHex-CS-modified liposomes delivered model antigenic proteins into cytosol of dendritic cells and promoted cytokine production from the cells, leading to induction of cancer immunity to regress tumor volumes on tumor-bearing mice. These results underscore the importance of backbone structure and charged groups of CHex unit-introduced polymers to design functional polymers that have pH-sensitivity, cell-specificity and adjuvant functions to induce antigen-specific cellular immunity for cancer immunotherapy.

## Materials and Methods

**Materials.** Egg yolk phosphatidylcholine (EYPC) was kindly donated by NOF Co. (Tokyo, Japan). 3-Methylglutaric anhydride, 1,2-cyclohexanedicarboxylic anhydride, *p*-xylene-bis-pyridinium bromide (DPX), fluorescein isothiocyanate (FITC), OVA, fetal bovine serum (FBS) and dextran sulfate sodium salt from *Leuconostoc spp.* were purchased from Sigma (St. Louis, MO.). Chondroitin sulfate sodium salt (Mw: 70,000 determined by SLS Debye plot using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK)), 1-aminodecane, pyranine and Triton X-100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). 4-Dimethylaminopyridine (DMAP) was obtained from nacalai tesque (Kyoto, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was from Life technologies. pH-Sensitive dextran derivatives (CHex87-Dex, CHex132-Dex, CHex258-Dex and CHex81-Dex-A18) were prepared from dextran (Mw: 70,000) as previously reported.[31] FITC-OVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in 0.5 M $NaHCO_3$ (4 mL, pH 9.0) at 4 °C for three days and subsequent dialysis.[22]

**Synthesis of Chondroitin Sulfate Derivatives.** Carboxyl groups of chondroitin sulfate sodium salt was first converted to free acidic form by addition of hydrochloric acid (pH 2.0) and subsequently lyophilized. 3-Methylglutarylated chondroitin sulfate (MGlu-CS) and 2-carboxycyclohexane-1-carboxylated chondroitin sulfate (CHex-CS) were prepared by reaction of chondroitin sulfate with 3-methylglutaric anhydride and 1,2-cyclohexanedicarboxylic anhydride, respectively. A given amount of dimethyl sulfoxide (DMSO) was added to chondroitin sulfate and stirred at 90 °C for 30 min under argon atmosphere. After cooling to room temperature, DMAP and dicarboxylic anhydrides were added to DMSO solution of chondroitin sulfate. The mixed solution was kept at a given temperature for a given time period with stirring under argon atmosphere. Then, saturated sodium hydrogen carbonate aqueous solution was added to the reaction mixture for neutralization and the reaction mixture was dialyzed against water for 3 days. The product was recovered by freeze-drying. $^{1}$H NMR for MGlu-CS（400 MHz, $D_2O$+NaOD）: δ 0.9 (s, -CO-$CH_2$-CH(C*H*$_3$)-$CH_2$-), 1,9–2.3 (br, -CO-C*H*$_2$-C*H*($CH_3$)-C*H*$_2$-, -NH-CO(C*H*$_3$)), 3.3–4.3 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 (br, glucose 1*H*). $^{1}$H NMR for CHex-CS（400 MHz, $D_2O$+NaOD）: δ 1.3–2.2 (m, -cyclo-C*H*$_2$, -NH-CO(C*H*$_3$)), 2.6–3.0 (m, cyclo-CH), 3.3–4.2 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 (br, glucose 1*H*).

As anchor moieties for fixation of MGlu-CS, CHex-CS and CS onto liposome

membranes, 1-aminodecane was combined with carboxy groups of MGlu-CS, CHex-CS and CS. Each polymer was dissolved in water around pH 7.4, and a given amount of 1-aminodecane was reacted to carboxy groups of the polymer using DMT-MM at room temperature for 7-48 h with stirring. The obtained polymers were purified by dialysis in water. The compositions for polymers were estimated using $^{1}$H NMR. $^{1}$H NMR for MGlu-CS-A（400 MHz, $D_2O$+NaOD）: δ 0.9 (s, -CO-$CH_2$-CH(C*H₃*)-$CH_2$-, -CO-NH-$CH_2$-$(CH_2)_8$-C*H₃*), 1.2–1.4 (br, -CO-NH-$CH_2$-(C*H₂*)$_8$-$CH_3$), 1.9–2.3 (br, -CO-C*H₂*-C*H*($CH_3$)-C*H₂*-, -NH-CO(C*H₃*)), 2.5 (br, -CO-NH-C*H₂*-$(CH_2)_8$-$CH_3$), 3.3–4.2 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 (br, glucose 1*H*). $^{1}$H NMR for CHex-CS-A（400 MHz, $D_2O$+NaOD）: δ 0.9 (br, -CO-NH-$CH_2$-$(CH_2)_8$-C*H₃*), 1.3–2.2 (m, -cyclo-C*H₂*, -CO-NH-$CH_2$-(C*H₂*)$_8$-$CH_3$, -NH-CO(C*H₃*)), 2.6–3.0 (m, cyclo-CH), 3.2 (br, -CO-NH-C*H₂*-$(CH_2)_8$-$CH_3$), 3.3–4.2 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.6 (br, glucose 1*H*). $^{1}$H NMR for CS-A（400 MHz, $D_2O$+NaOD）: δ 0.9 (br, -CO-NH-$CH_2$-$(CH_2)_8$-C*H₃*), 1.2–1.4 (m, -CO-NH-$CH_2$-(C*H₂*)$_8$-$CH_3$) 2.0 (s, -NH-CO(C*H₃*)), 2.5 (br, -CO-NH-C*H₂*-$(CH_2)_8$-$CH_3$), 3.3–4.2 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 4.5–4.6 (br, glucose 1*H*).

**Preparation of Liposomes.** A dry, thin membrane composed of a mixture of EYPC and polysaccharides at varying lipid-polymer weight ratios was suspended in phosphate-

buffered saline (PBS) containing OVA (4 mg/mL) by a brief sonication, and the liposome suspension was further hydrated by freezing and thawing, and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was purified with ultracentrifugation for 2 h at 4 °C twice. The concentrations of lipid and OVA in liposome suspension were measured from using phospholipid C test-Wako and Coomassie Protein Assay Reagent, respectively.

**Dynamic Light Scattering and Zeta Potential.** Diameters and zeta potentials of the liposomes (0.1 mM of lipid concentration) were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK). Data was obtained as an average of more than three measurements on different samples.

**Release of Pyranine from Liposome.** Pyranine-loaded liposomes were prepared as described above except that mixtures of polymers and EYPC were dispersed in aqueous 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4). Liposomes encapsulating pyranine (lipid concentration: $2.0 \times 10^{-5}$ M) were added to PBS of varying pH at 37 °C and fluorescence intensity (512 nm) of the mixed suspension was followed with excitation at 416 nm using a spectrofluorometer (Jasco FP-6500, FP-6200). The

percent release of pyranine from liposomes was defined as

Release (%) = $(F_t - F_i) / (F_f - F_i) \times 100$

where $F_i$ and $F_t$ mean the initial and intermediary fluorescence intensities of the liposome suspension, respectively. $F_f$ is the fluorescent intensity of the liposome suspension after the addition of TritonX-100 (final concentration: 0.1%).

**Cell Culture.** Murine embryo fibroblast-derived NIH3T3 cell were obtained from RIKEN BRC (Ibaraki, Japan) and were grown in DMEM supplemented with 10% FBS and antibiotics at 37 °C. Immature murine dendritic cell line DC2.4 cell was provided from Kenneth L. Rock (University of Massachusetts Medical School, Worcester, MA, USA) and was grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mM nonessential amino acid, 50 μM 2-mercaptoethanol and antibiotics at 37 °C.[45] E.G7–OVA, which is a chicken egg OVA gene–transfected murine T lymphoma and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) and was grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 0.5 mg/mL G418 and antibiotics at 37 °C.[46]

**Cellular Association of Liposomes and Inhibition Assay.** Liposomes containing DiI were prepared as described above except that a mixture of polymer and lipid containing DiI (0.1 mol%) was dispersed in PBS containing OVA. DC2.4 cells ($7.5 \times 10^4$ cells) cultured for 2 days in a 24–well plate or NIH3T3 cells ($5.0 \times 10^4$ cells) cultured overnight in a 24–well plate were washed with PBS, and then incubated in serum-free medium (0.25 mL). The DiI-labeled liposomes (0.2 mM lipid concentration, 0.25 mL) were added gently to the cells and incubated for 4 h at 37 °C. After the incubation, the cells were washed with HBSS or PBS three times. Fluorescence intensity of these cells was determined by a flow cytometric analysis (CytoFlex, Beckman Coulter, Inc.). DiI fluorescence of each liposome was measured and cellular fluorescence shown in Figure 4 was corrected using liposomal fluorescence intensity. For inhibition assay, various concentrations of dextran sulfate were pre-incubated to cells for an hour before the incubation of DiI-labeled liposomes for 4 h.

**Intracellular Behavior of Liposomes.** FITC-OVA-loaded liposomes containing DiI were prepared as described above except that a mixture of polymer and lipid containing DiI (0.1 mol%) was dispersed in PBS containing FITC-OVA (4 mg/mL). DC2.4 cells ($1.5 \times 10^5$ cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with HBSS,

and then incubated in serum-free RPMI medium (1 mL). The FITC-OVA-loaded liposomes (0.2 mM lipid concentration, 1 mL) were added gently to the cells and incubated for 4 h or 24 h at 37 °C. After the incubation, the cells were washed with HBSS three times. Confocal laser scanning microscopic (CLSM) analysis of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.).

**Cytokine Production from Dendritic Cells.** DC2.4 cells ($3 \times 10^5$ cells) cultured for 2 days in a 6–well plate were washed with HBSS, and then incubated in serum–free RPMI–1640 medium. Polysaccharide derivatives in PBS (final concentration: 0.25 mg/mL) or OVA-loaded liposomes (final concentration: 0.1 mM) were added gently to the cells, followed by incubation for 24 h or 4 h at 37 ℃. After incubation, supernatants were collected and cytokine (TNF–α, IL–6 and IL–12) production was measured using an enzyme–linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to the manufacture's instruction.

**Animal Experiments.** Female C57BL/6 mice (H–$2^b$, 7 weeks old) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

**Treatment of Tumor-Bearing Mice with Liposomes.** E.G7-OVA cells ($1 \times 10^6$ cells/mouse) were subcutaneously inoculated into the left back of C57BL/6 mice under anesthesia with isoflurane. On days 7 and 14, 100 μg of OVA-loaded liposomes were subcutaneously injected into the right backs of the mice under anesthesia with isoflurane. Tumor sizes were monitored from the day of tumor inoculation. Mice immunized with PBS were used as a control to confirm the development of tumor following the first inoculation of E.G7-OVA cells. Mice were sacrificed when tumor volumes become over 2,500 $mm^3$. All treated groups contained four mice.

**Statistical Analysis.** Student t-test and Tukey-Kramer test were performed for the statistical analysis of the results in Figures 4D and Figures 6 and 7, respectively. Survival data in Figures 8B was evaluated using Log-rank test (Table S2).

**Associated content**

**Supporting information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

Antitumor effects on tumor-bearing mice induced by subcutaneous administration of pH-sensitive dextran derivative-modified liposomes (Figure S1); $^{1}$H NMR spectra of chondroitin sulfate derivatives (Figure S2); Intracellular distribution of DiI-labeled CHex-CS-A-liposomes containing FITC-OVA in DC2.4 cells for long incubation time (Figure S3); Intracellular distribution of DiI-labeled CHex-Dex-A-liposomes in DC2.4 cells (Figure S4); Individual tumor volume change of mice in Figure 8 (Figure S5); IFN-γ production from splenocytes from mice immunized with liposomes (Figure S6); Change in body weight of mice in Figure 8 (Figure S7); Polymer contents per lipid after purification (Table S1); Statistical analysis of Figure 8B (Table S2) (PDF)

**Author information**

**Author Contributions**

E.Y. and A.H. conceived and designed the research. M.O. and M.M. performed

experiments. M.O., M.M. and E.Y. wrote the paper. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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**Abbreviations**

CS, chondroitin sulfate; Dex, dextran; MGlu, 3-methyl glutarylated; CHex, 2-carboxycyclohexane-1-carboxylated; APC, antigen presenting cell; MPLA, monophosphoryl lipid A; CTL, cytotoxic T lymphocyte, EYPC, egg yolk phosphatidylcholine; CLSM, confocal laser scanning microscopy

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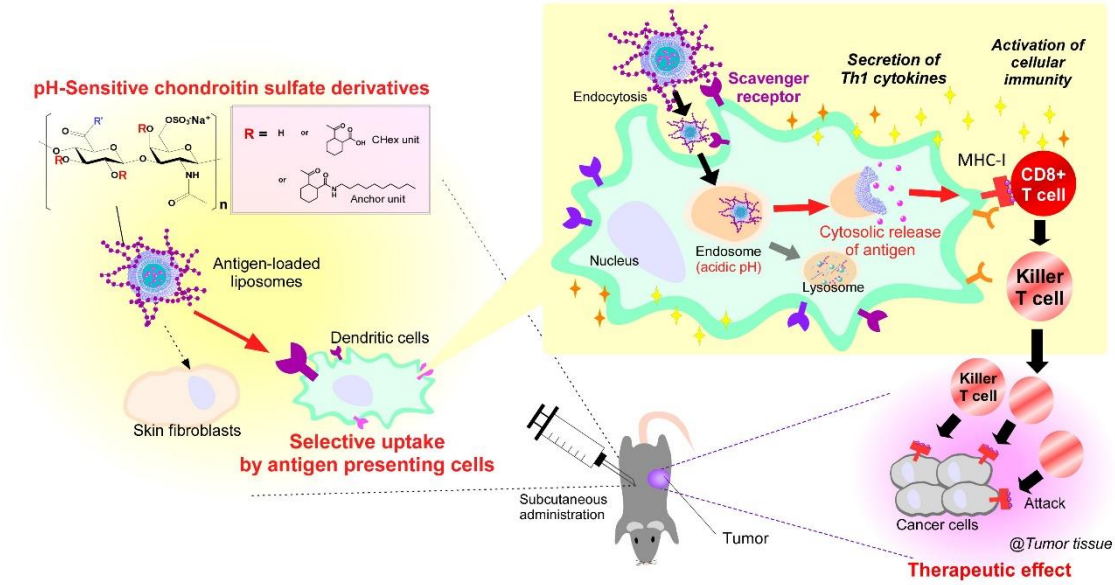
## Table of Contents



*Supporting Information*

**Chondroitin Sulfate-Based pH-Sensitive Polymer-Modified Liposomes for Intracellular Antigen Delivery and Induction of Cancer Immunity**

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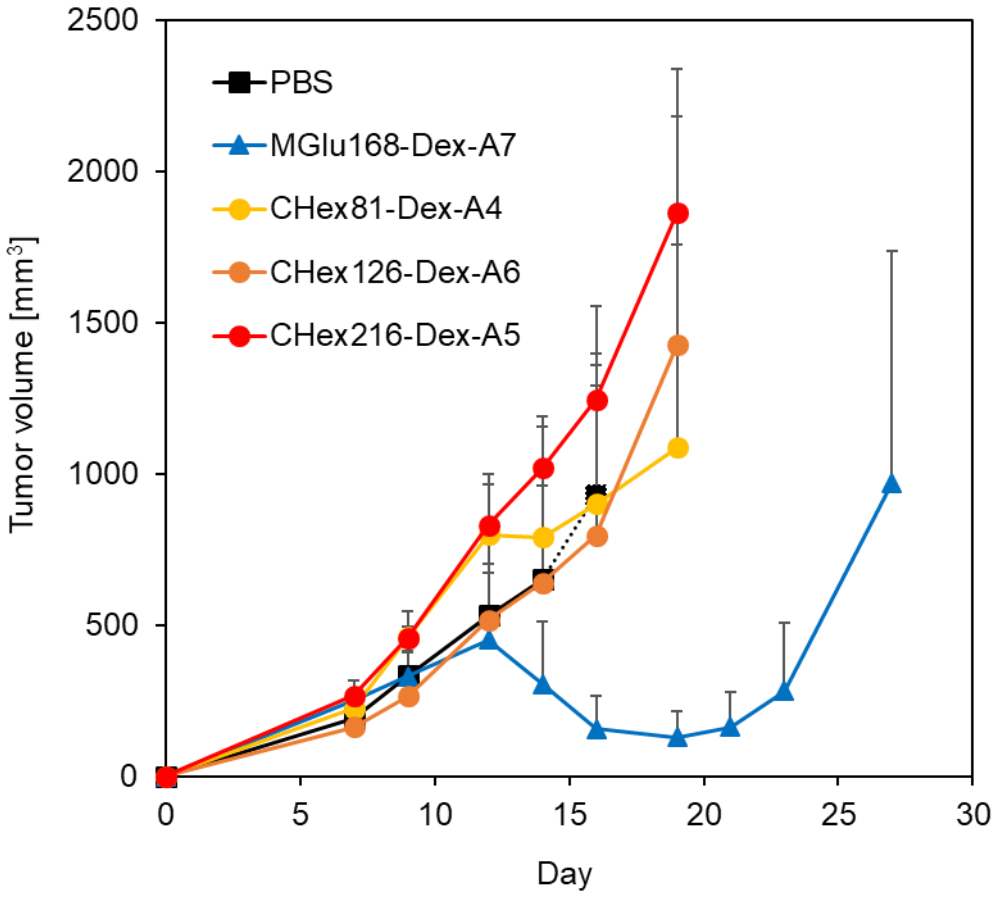


**Figure S1.** Antitumor effects induced by subcutaneous administration of OVA-loaded liposomes modified with dextran derivatives. C57BL/6 mice were immunized on days 7 and 14 with liposomes containing monophosphoryl lipid A as an adjuvant (OVA 100 μg). Changes in tumor volume of mice were monitored after E.G7-OVA cells ($1 \times 10^6$ cells/mouse) inoculation. All treated groups contained four mice.

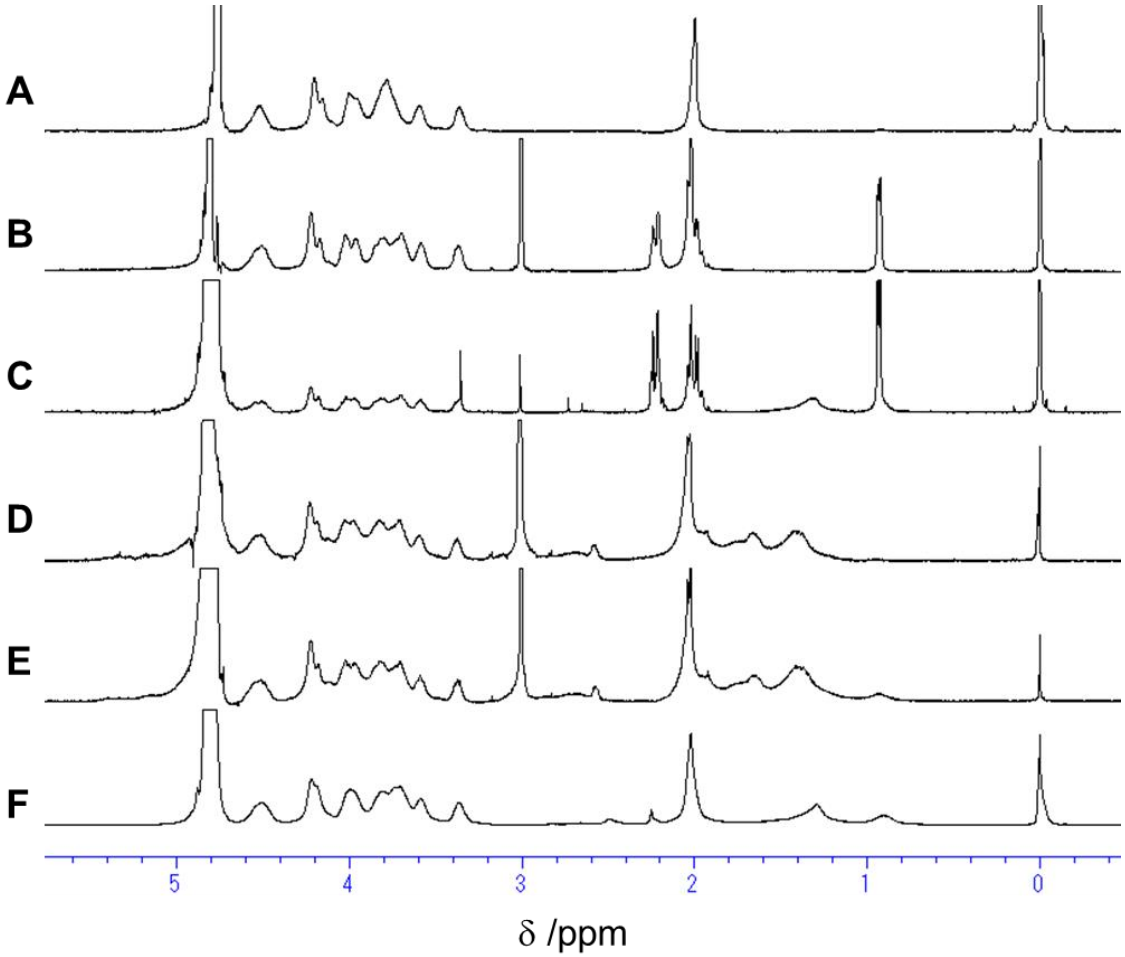


**Figure S2.** $^{1}H$ NMR spectra of (A) chondroitin sulfate (CS), (B) MGlu24-CS, (C) MGlu73-CS-A10, (D) CHex42-CS, (E) CHex27-CS-A9, and (F) CS-A7 in $D_2O$/NaOD.

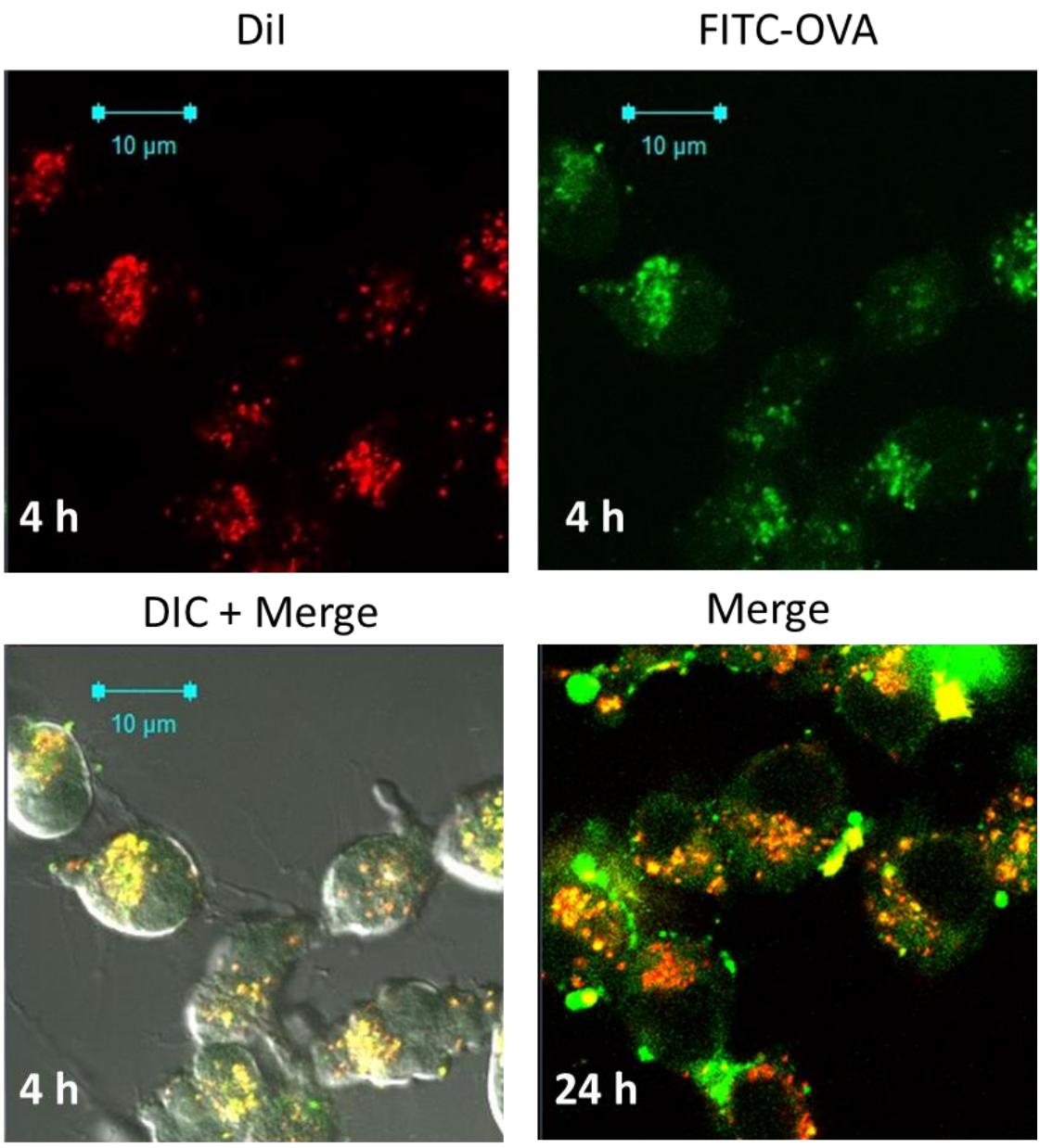


**Figure S3.** CLSM images of DC2.4 cells treated with FITC-OVA-loaded and DiI-labeled liposomes modified with CHex75-CS-A21 for 4 h or 24 h at 37 °C in serum-free medium. Bar represents 10 μm.

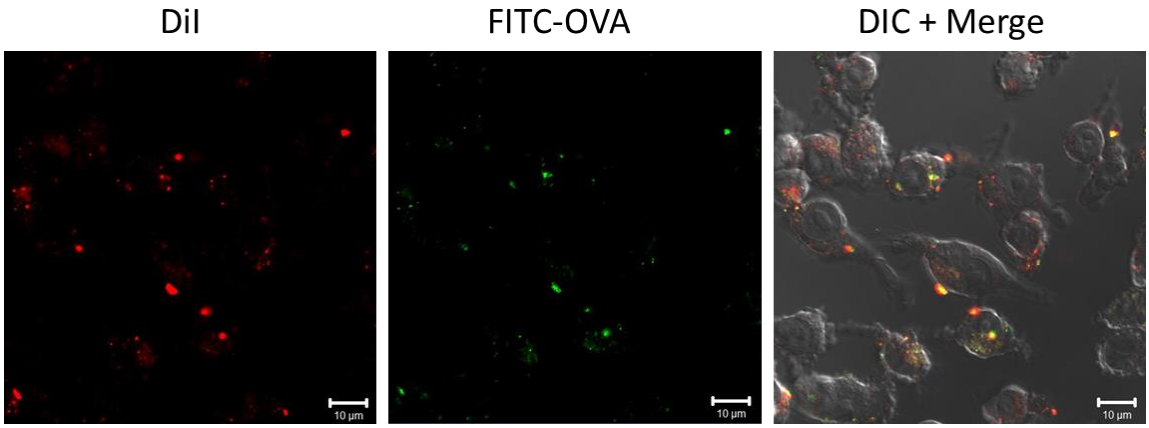


**Figure S4.** CLSM images of DC2.4 cells treated with calcein-loaded and DiI-labeled liposomes modified with CHex81-Dex-A18 for 4 h at 37 °C in serum-free medium. Bar represents 10 μm.

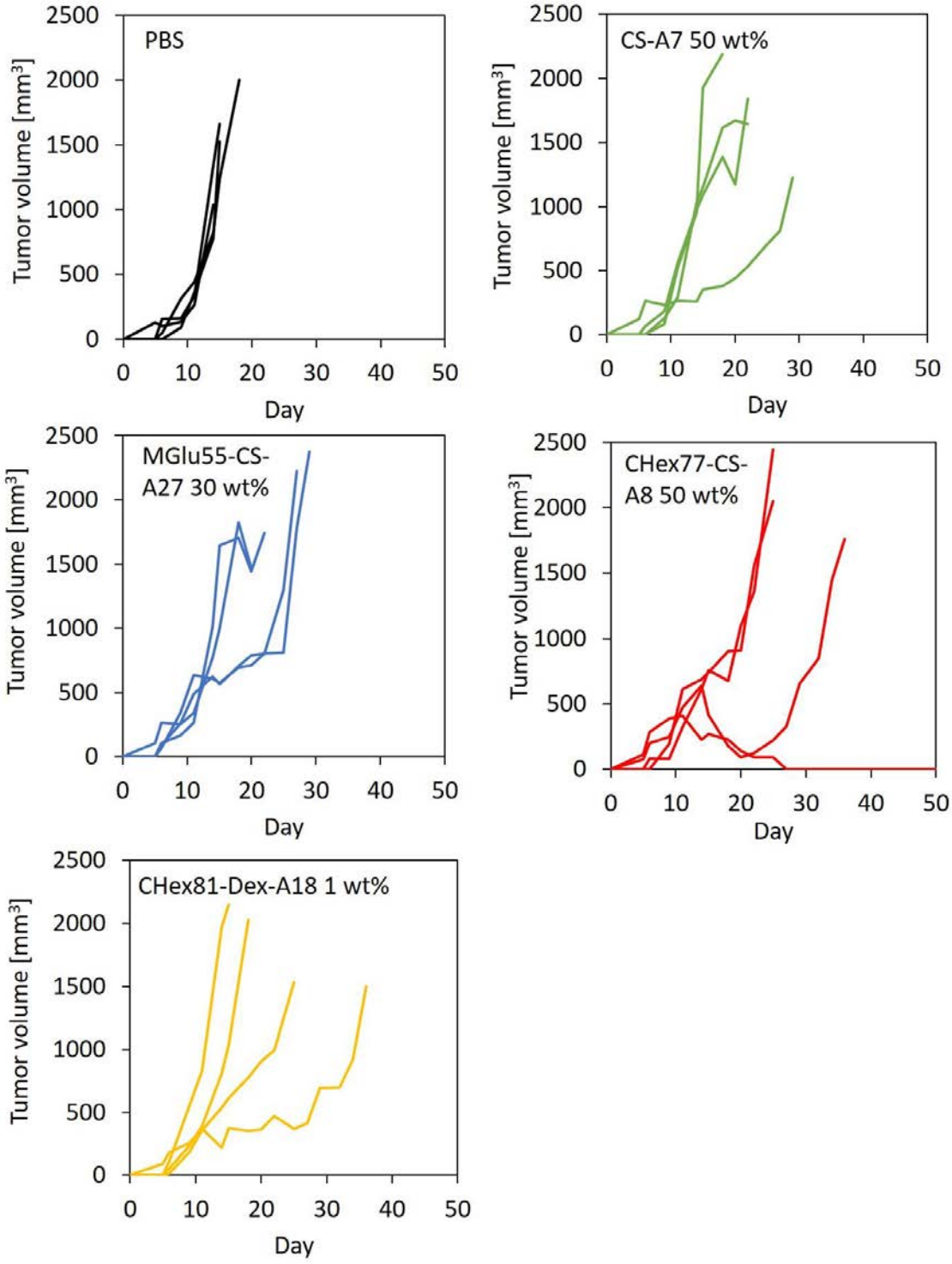


**Figure S5.** Individual tumor volumes of C57BL/6 mice treated with PBS or liposomes modified with various polysaccharide derivatives in Figure 8.

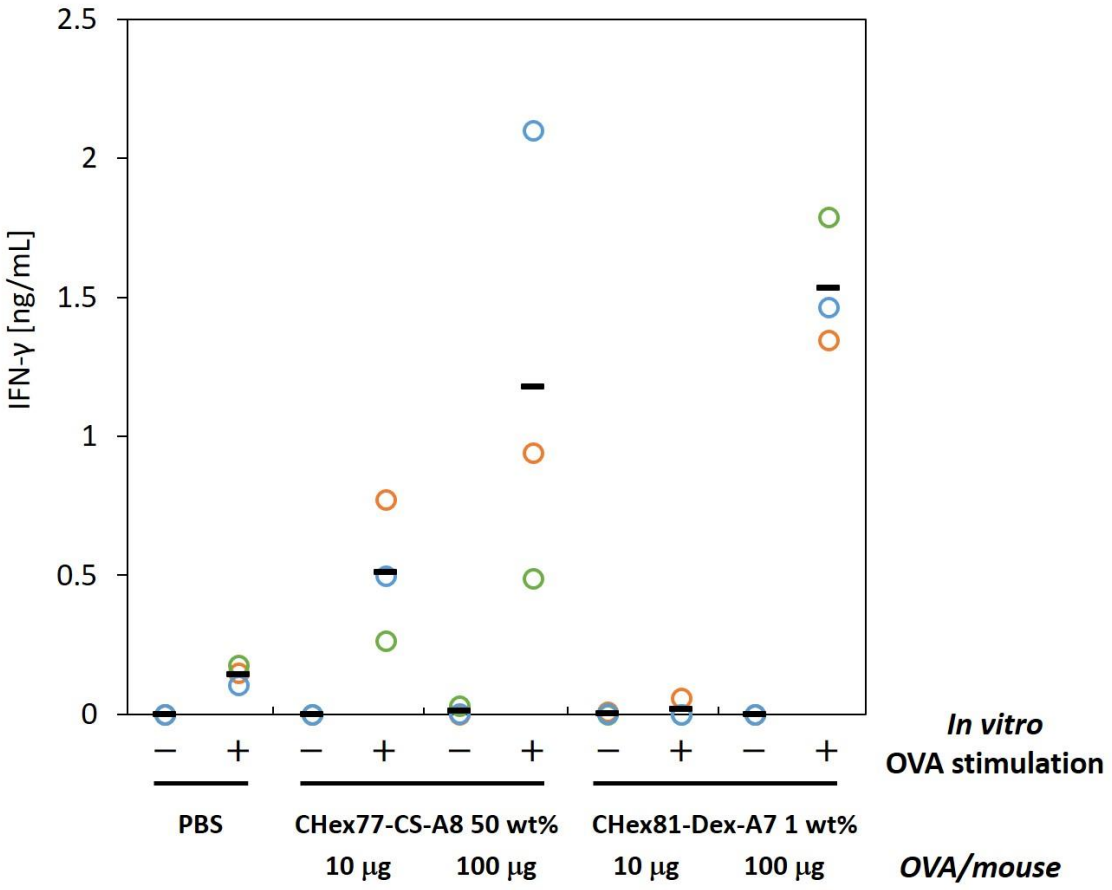


**Figure S6.** *In vitro* stimulation of splenocytes from mice immunized with 10 μg or 100 μg OVA-loaded liposomes at days 7 and 14. 7 days after second immunization, splenocytes ($2 \times 10^6$/2 mL) isolated from immunized C57BL/6 mice were cultured with (+) or without (−) 50 μg/mL of OVA for 5 days. IFN-γ production in the supernatant was measured by ELISA.

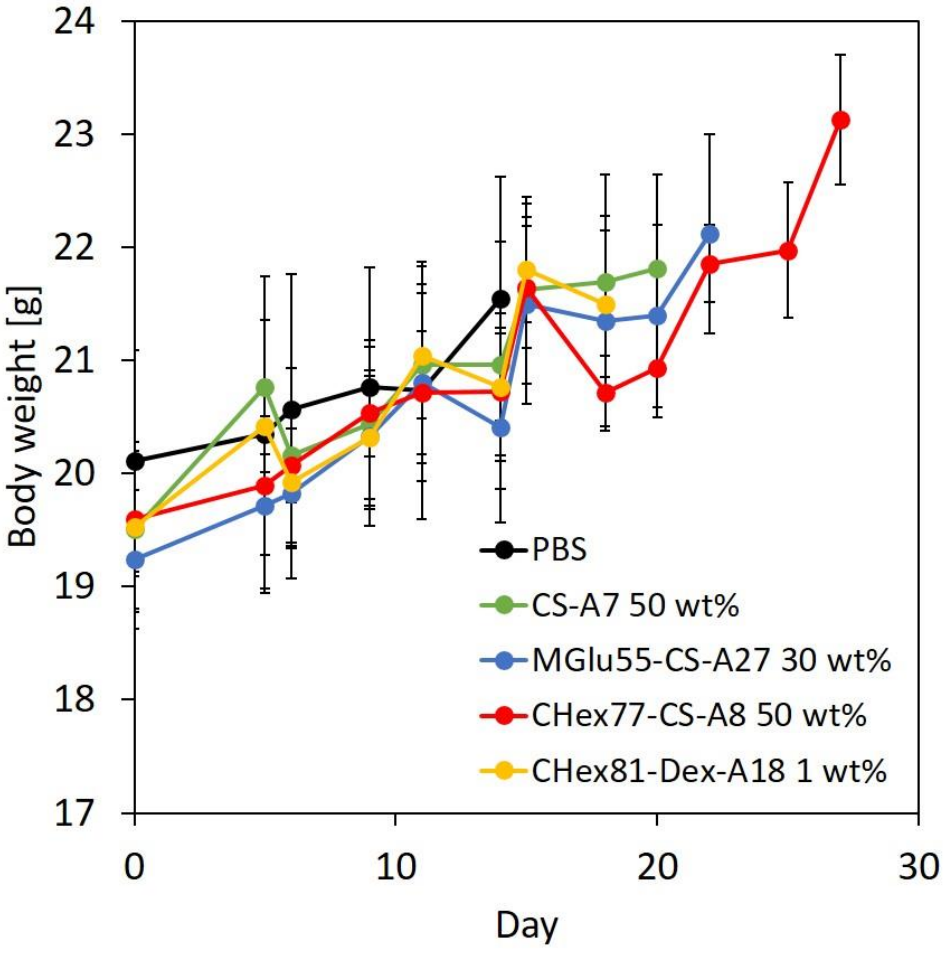


**Figure S7.** Changes in body weight of mice in Figure 8 were monitored after E.G7-OVA cells ($1 \times 10^6$ cells/mouse) inoculation. All treated groups contained four mice.

**Table S1. Polymer Contents per Lipid after Purification**

| Liposome | Polymer content per lipid [wt%] | | Recovery rate [%] |
|---|---|---|---|
| | In feed | After purification* | |
| CS-A7 | 50 | 18.3 ± 0.3 | 36.6 ± 0.6 |
| MGlu55-CS-A27 | 30 | 22.6 ± 1.6 | 75.3 ± 5.4 |
| CHex77-CS-A8 | 50 | 14.9 ± 5.9 | 29.8 ± 12 |

*Determined by phenol-sulfuric acid assay.

**Table S2. Survival Analysis by Log-Rank Test for Figure 8**

| Comparison | *p* value |
|---|---|
| PBS vs **CS-A7** | 0.018* |
| PBS vs **MGlu55-CS-A27** | 0.007** |
| PBS vs CHex81-Dex-A18 | 0.084 |
| PBS vs **CHex77-CS-A8** | 0.007** |
| CS-A7 vs MGlu55-CS-A27 | 0.083 |
| CS-A7 vs CHex81-Dex-A18 | 0.791 |
| CS-A7 vs CHex77-CS-A8 | 0.071 |
| MGlu55-CS-A27 vs CHex81-Dex-A18 | 0.172 |
| MGlu55-CS-A27 vs CHex77-CS-A8 | 0.184 |
| CHex81-Dex-A18 vs CHex77-CS-A8 | 0.184 |

*$p$ <0.05, **$p$ <0.01.