Bioactive polysaccharide-based pH-sensitive polymers for cytoplasmic delivery of antigen and activation of antigen-specific immunity

Bioactive polysaccharide-based pH-sensitive polymers for cytoplasmic delivery of antigen and activation of antigen-specific immunity

Eiji Yuba*, Ayaka Yamaguchi, Yuta Yoshizaki, Atsushi Harada, and Kenji Kono

Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

***Corresponding author: Eiji Yuba**

Department of Applied Chemistry, Graduate School of Engineering,

Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

Tel: +81-722-54-9913; Fax: +81-722-54-9913; yuba@chem.osakafu-u.ac.jp

Keywords: pH-sensitive liposome / curdlan / β-glucan / mannan / cancer immunotherapy / dendritic cell

Abstract

For establishment of cancer immunotherapy, antigen carriers are needed which have functions not only to deliver antigen into cytosol of dendritic cells (DCs), which induces antigen-specific cellular immune responses, but also to activate DCs. We previously reported cytoplasmic delivery of antigen using liposomes modified with pH-sensitive polymers such as carboxylated poly(glycidol)s or dextran. Modification using these polymers provides stable liposomes with pH-sensitive fusogenic/membrane-disruptive ability. For this study, bioactive polysaccharide-based pH-sensitive polymers were constructed to achieve not only cytoplasmic delivery of antigen but also activation of DCs. Curdlan and mannan were used as bioactive polysaccharides because they are known to activate DCs *via* their respective interactions with Dectin-1 and Dectin-2. Carboxylated curdlan and mannan promoted Th1 cytokine production from DCs, indicating the activation of DCs by these polysaccharide derivatives. These polymer-modified liposomes released their contents at weakly acidic pH and delivered model antigenic proteins into cytosol of DCs. Subcutaneous administration of curdlan derivative-modified or mannan derivative-modified liposomes induced strong antigen-specific immune responses and stronger antitumor effects than those of liposomes modified with dextran derivative. Therefore, bioactive

35 polysaccharide-modified liposomes that achieve both cytoplasmic delivery of antigen

36 and activation of DCs are promising for cancer immunotherapy.

37

1. Introduction

Induction of cancer-specific immunity is crucially important to achieve immunotherapy. Especially, cell-mediated immune response (cellular immunity) is regarded as the most effective immune response to eliminate tumor cells directly [1–3]. For the induction of cellular immunity, the delivery of antigen to cytosol of antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs), and antigen presentation mediated by major histocompatibility complex (MHC) class I molecules are necessary [4–7]. Efficient cytoplasmic delivery carriers of antigen are necessary for the induction of cellular immunity and the establishment of cancer immunotherapy.

To date, various antigen cytoplasmic delivery systems have been developed such as polymeric nanoparticles, polymeric micelles, nanogels, and liposomes [8–14]. Among them, liposomes are useful as antigen carriers because of their biocompatibility and their ease of introducing functionality such as cellular targeting ability and pH-responsive content release properties [13, 14]. In fact, pH-sensitive liposomes have been prepared using a mixture of non-bilayer forming lipids and amphiphiles having carboxylic acid or by the modification of virus-derived fusogenic proteins, pH-sensitive peptide, and pH-sensitive polymers [14–17]. These pH-sensitive liposomes induce destabilization of the endosomal membrane or membrane fusion with endosomal

membranes in intracellular acidic environments (endosomes or lysosomes), which achieve the cytoplasmic delivery of contents [14–17].

We previously reported cytoplasmic delivery of antigen using liposomes modified with carboxylated poly(glycidol)s, which have a backbone resembling biocompatible poly(ethylene glycol) [18–21]. 3-Methyl glutarylated poly(glycidol)-modified liposomes delivered model antigenic protein, ovalbumin (OVA) into cytosol of DCs by membrane fusion with endosomal membrane responding to acidic pH, and induced MHC class I-mediated antigen presentation [22]. Subcutaneous administration of these liposomes induced OVA-specific cellular immunity and therapeutic effects on tumor-bearing mice [23]. As a biodegradable pH-sensitive polymer, 3-methyl glutarylated dextran (MGlu-Dex), which has a polysaccharide backbone and pH-responsive MGlu side chain, was synthesized [24]. MGlu-Dex-modified liposomes also achieved the cytoplasmic delivery of OVA and the induction of cellular immunity [24].

To induce stronger cellular immunity, antigen delivery systems require functions not only for delivery of antigen into DC cytosol but also to activate DCs, which is designated as an adjuvant function. Typically, toll like receptor (TLR) ligands such as CpG-DNA, poly(I:C), and monophosphoryl lipid A (MPLA), which activate

APCs *via* interaction with TLRs expressing in these cells, were introduced to antigen carriers as adjuvant molecules [25–29]. We have also combined immune-activating molecules or systems such as MPLA mentioned above, cationic lipids, and IFN-γ-encoding gene delivery systems with pH-sensitive polymer-modified liposomes to enhance the cellular immune responses [30, 31]. Actually, inclusion of these molecules or systems improved their ability to induce cellular immunity and antitumor effects [26, 29–31]. However, these antigen delivery systems have complicated compositions because antigen delivery and activation of immune response are achieved by each functional molecule. Therefore, antigen carriers that can achieve both antigen delivery and activation by a single functional molecule are sought.

In this study, the inclusion of both antigen delivery function and adjuvant function to antigen carriers was attempted using intrinsically bioactive polysaccharide-based pH-sensitive polymers. For this purpose, curdlan and mannan were chosen as base polymers for the preparation of pH-sensitive polymers with DC activation ability (Fig. 1). Curdlan and mannan are microorganism-derived bioactive polysaccharides that are recognized, respectively, by surface receptors of macrophages or DCs such as Dectin-1 and Dectin-2 [32–34]. Recognition by these lectins leads to the activation of APCs and to the promotion of secretion of various cytokines, thereby

activating immune response (Fig. 1). pH-Sensitive moieties (MGlu units) were introduced to these polysaccharides. Their abilities to deliver antigen to DCs, to activate DCs, and to induce antigen-specific immune responses were investigated and compared with those of conventional pH-sensitive polysaccharide (MGlu-Dex).

2. Materials and Methods

2.1. Materials

Egg yolk phosphatidylcholine (EYPC) was kindly donated by NOF Co. (Tokyo, Japan) for preparation of liposome. Lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA) for fluorescence labeling of liposomal membrane. 3-Methylglutaric anhydride, curdlan from *Alcaligenes faecalis*, mannan from *Saccharomyces cerevisiae* for synthesis of pH-sensitive polymers, OVA, MPLA, fluorescein isothiocyanate (FITC), *p*-Xylene-bis-pyridinium bromide (DPX) for preparation of liposomes, and dextran sulfate sodium salt from *Leuconostoc spp.* for the evaluation of cellular association of liposomes, were purchased from Sigma (St. Louis, MO.). 1-Aminodecane for synthesis of pH-sensitive polymers, pyranine and Triton X-100 for preparation of liposomes were obtained from Tokyo Chemical Industries Ltd.

110 (Tokyo, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride
111 (DMT-MM) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan) for synthesis
112 of pH-sensitive polymers. 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine
113 perchlorate (DiI) was from Life Technologies for fluorescence labeling of liposomal
114 membrane. FITC-OVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in
115 0.5 M $NaHCO_3$ (4 mL, pH 9.0) at 4 °C for three days and subsequent dialysis [23].
116 pH-sensitive dextran derivatives (MGlu-Dex) were prepared as previously reported
117 [24].

118 **2.2. Synthesis of Polysaccharide Derivatives**

119 3-Methyl glutarylated curdlan (MGlu-Curd) was prepared by reaction of
120 curdlan with 3-methylglutaric anhydride. For $MGlu_{65}$-Curd, which refers to curdlan
121 derivatives having 65 mol% of MGlu groups, curdlan (1.0 g, 6.2 mmol of OH groups)
122 and LiCl (1.0 g) were dissolved in distilled *N*,*N*-dimethylformamide (20 mL) and 9.0
123 equiv. of 3-methylglutaric anhydride (7.27 g, 56.7 mmol) was added to the solution.
124 The mixed solution was kept at 100 °C for 24 h with stirring under argon atmosphere.
125 Then, the reaction mixture was evaporated and dialyzed against water for 3 days. The
126 product was recovered by freeze-drying. Other MGlu-Curd polymers and MGlu-Man
127 were also synthesized by reaction of curdlan or mannan with various amounts of

3-methylglutaric anhydride by the same procedure. ^{1}H NMR for MGlu-Curd（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**-), 3.5 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 5.0 (br, glucose 1*H*). ^{1}H NMR for MGlu-Man（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**-), 3.5 – 4.2 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 5.0 – 5.3 (br, glucose 1*H*).

As anchor moieties for fixation of MGlu-Curd and MGlu-Man onto liposome membranes, 1-aminodecane was combined with carboxylic acid groups of MGlu-Curd or MGlu-Man. Each polymer was dissolved in water around pH 7.4, and 1-aminodecane (0.1 equiv. to hydroxy group of polymer) was reacted to carboxylic acid groups of the polymer using DMT-MM (0.1 equiv. to hydroxy group of polymer) at room temperature for 6 h with stirring. The obtained polymers were purified by dialysis in water. The ratios of MGlu units to decyl amide (C_{10}) units for polymers were estimated using ^{1}H NMR. ^{1}H NMR for MGlu-Curd-C_{10}（400 MHz, D_2O+NaOD）: δ 0.8 - 0.9 (br, -CO-NH-CH_2-(CH_2)$_8$-C***H*****3**), 0.9 (s, -CO-CH_2-CH(C***H*****3**)-CH_2-), 1.2 – 1.4 (br, -CO-NH-CH_2-(C***H*****2**)$_8$-CH_3), 1.9 – 2.3 (br, -CO-C***H*****2**-C*H*(CH_3)-C***H*****2**-), 3.2 (br, -CO-NH-C***H*****2**-(CH_2)$_8$-CH_3), 3.5 – 4.0 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 5.0 (br, glucose 1*H*). ^{1}H NMR for MGlu-Man-C_{10}（400 MHz, D_2O+NaOD）: δ 0.8 - 0.9 (br,

-CO-NH-CH_2-$(CH_2)_8$-C***H₃***), 0.9 (s, -CO-CH_2-CH(C***H₃***)-CH_2-), 1.2 – 1.6 (br, -CO-NH-CH_2-(C***H₂***)$_8$-CH_3), 1.9 – 2.3 (br, -CO-C***H₂***-C*H*(CH_3)-C***H₂***-), 3.2 (br, -CO-NH-C***H₂***-$(CH_2)_8$-CH_3), 3.5 – 4.2 (br, glucose 2*H*, 3*H*, 4*H*, 5*H*, 6*H*), 5.0 – 5.3 (br, glucose 1*H*). According to the GPC analysis for polymers on a system equipped with a column (Waters, Ultrahydrogel Linear) with differential refractive index detection (Jasco RI-930) using acetate buffer (pH 6.6) as the eluent, there is no peak derived from low-molecular weight impurities (data not shown).

2.3. Titration

To 10 mL of an aqueous solution of each polymer (carboxylate concentration: 3.0×10^{-4} M) was added an appropriate amount of 0.1 M NaOH solution to make pH 10.0. The titration was carried out by the stepwise addition of 1.0 M HCl and pH of the resultant solution was measured using an automated titration instrument (AUT-701, DKK-TOA Corporation, Tokyo, Japan).

2.4. Precipitation pH

Precipitation pH of polymer was determined by measuring the optical density of aqueous polymer solutions (0.05 mg/mL) at various pH. Polymers were dissolved in 1.0 mL of acetate buffer (30 mM sodium acetate, 120 mM NaCl) adjusted to various pH. After 5 min-incubation at 25 °C, optical densities (OD) of the polymer solutions at 500

nm were measured by using a spectrophotometer (Jasco V-560). Precipitation pH was determined by optical density-pH profile as the pH at which OD drastically rose.

2.5. Cell Culture

DC2.4 cell, which is an immature murine DC line, was provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI-1640 supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine, 100 mM nonessential amino acid, 50 μM 2-mercaptoethanol (2-ME, Gibco) and antibiotics at 37 °C [35]. E.G7-OVA, which is a chicken egg OVA gene-transfected clone of C57BL/6 mice-derived T lymphoma and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) [36].

2.6. Preparation of Liposomes

To a dry, thin membrane of EYPC (10 mg) was added 1.0 mL of OVA/PBS solution (pH 7.4, 4 mg/mL), and the mixture was vortexed at 4 °C. 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 centrifuged with the speed of 55,000 rpm for 2 h at 4 °C twice to remove free OVA from the OVA-loaded liposomes. Polymer-modified liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture with polymers

182 (lipids/polymer = 7/3, w/w). For induction of immune responses, MPLA (4 g/mol
183 lipids) was introduced into liposomal membrane. Loading efficiency of OVA to
184 liposomes was determined and summarized in Table S1.

185 **2.7. Dynamic Light Scattering and Zeta Potential**

186 Diameters of liposome in PBS and zeta potentials of the liposomes in 0.1 mM
187 phosphate buffer (0.1 mM of lipid concentration) were measured using a Zetasizer Nano
188 ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK). Data was obtained as an
189 average of more than three measurements on different samples.

190 **2.8. Release of Pyranine from Liposome**

191 Pyranine-loaded liposomes were prepared as described above except that
192 mixtures of polymers and EYPC were dispersed in aqueous 35 mM pyranine, 50 mM
193 DPX, and 25 mM phosphate solution (pH 7.4) and liposome suspension was purified
194 using a sepharose 4B column. Release of pyranine from liposome was measured as
195 previously reported [20, 24, 37]. Liposomes encapsulating pyranine (lipid
196 concentration: 2.0×10^{-5} M) were added to PBS of varying pH at 37 °C and
197 fluorescence intensity (512 nm) of the mixed suspension was followed with excitation
198 at 416 nm using a spectrofluorometer (Jasco FP-6500, FP-6200). The percent release of
199 pyranine from liposomes was defined as

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

201 where F_i and F_t mean the initial and intermediary fluorescence intensities of the

202 liposome suspension, respectively. F_f is the fluorescent intensity of the liposome

203 suspension after the addition of TritonX-100 (final concentration: 0.1%).

204 **2.9. Cellular Association of Liposomes and Inhibition Assay**

205 Liposomes containing Rh-PE were prepared as described above except that a

206 mixture of polymer and lipid containing Rh-PE (0.6 mol%) was dispersed in PBS.

207 DC2.4 cells (1.5×10^5 cells) cultured for 2 days in 12-well plates were washed with

208 Hank's balanced salt solution (HBSS), and then incubated in serum-free RPMI medium

209 (0.5 mL). The Rh-PE-labeled liposomes (1 mM lipid concentration, 0.5 mL) were added

210 gently to the cells and incubated for 4 h at 37 °C. After the incubation, the cells were

211 washed with HBSS three times. Fluorescence intensity of these cells was determined by

212 a flow cytometric analysis (EPICS XL, Beckman Coulter, Inc). For inhibition assay,

213 free dextran sulfate, curdlan and mannan at various concentrations were pre-incubated

214 to cells for an hour before the incubation of Rh-PE-labeled liposomes for 4 h.

215 Rhodamine fluorescence of each liposome was measured and cellular fluorescence

216 shown in Figure 6 was corrected using liposomal fluorescence intensity.

217 **2.10. Intracellular Behavior of Liposomes**

The FITC-OVA-loaded liposomes containing DiI were prepared as described above except that a mixture of polymer and lipid containing DiI (0.6 mol%) was dispersed in PBS containing FITC-OVA (4 mg/mL). DC2.4 cells (3×10^5 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 (1 mM lipid concentration, 1 mL) were added gently to the cells and incubated for 4 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.). Each liposome showed almost same fluorescence intensity for DiI and FITC. All CLSM images in Figure 8 were taken at same sensitivity setting.

2.11. Cytokine Production from Cells Treated with Polymers or Liposomes

The DC2.4 cells (1.5×10^5 cells) cultured for 2 days in 12-well plates were washed with HBSS, and then incubated in serum-free RPMI medium (0.5 mL). Polysaccharide derivatives (2 mg/mL, 0.5 mL) or liposomes with/without MPLA (4 g/mol lipid) (1 mM lipid concentration, 0.5 mL) were added gently to the cells and incubated for 24 h at 37 °C. After the incubation, supernatants of cultured cells were collected for measurements of TNF-α, IL-10 and/or IL-12 using an enzyme-linked immunosorbent assay kit (ELISA Development Kit, PeproTech EC Ltd.) according to

the manufacture's instruction.

2.12. *In Vitro* Cytokine Release Measured by ELISA and CTL Assay

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. 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. On day 21, mice were sacrificed and splenocytes were suspended in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-ME, and 20 U/mL recombinant murine IL-2 (Peprotech, London, UK). Splenocytes (2×10^6 in 2 mL) were incubated with various concentrations of OVA solution (0, 25, 50 μg/mL) for 5 days. After the incubation, the concentration of IFN-γ was measured using murine IFN-γ ELISA development kit (Peprotech, London, UK) according to the manufacture's instruction. Splenocytes were also stimulated with mitomycin C-treated E.G7-OVA cells at a ratio of 10:1 for 5 days. The stimulated splenocytes were used as effector cells for the cytotoxicity assay. The CTL activity was evaluated at effector cells to target cells (E.G7-OVA) ratio of 25, which was defined as E/T ratio, using a lactate dehydrogenase (LDH) cytotoxicity detection assay (Takara Biomedicals, Tokyo, Japan).

2.13. Treatment of Tumor-Bearing Mice with Liposomes

E.G7-OVA cells (1×10^6 cells) were subcutaneously inoculated into the left backs of C57BL/6 mice under anesthesia with isoflurane. On days 5 and 15, 100 μg of OVA-loaded liposomes with or without MPLA (4 g/mol lipid) were subcutaneously injected into the right backs of the mice under anesthesia. Tumor sizes were monitored from the day of inoculation. Mice immunized with PBS were used as controls to confirm the development of cancer following the first inoculation with E.G7-OVA cells. Mice were sacrificed when tumor volumes become over 2,500 mm^3. All treated groups contained 8 to 12 mice.

2.14. Statistical Analysis

Tukey-Kramer method was employed in the statistical evaluation of the results in Figs. 6, 7, 9-12, S6 and S7. Survival analysis using Log-rank test was performed in Fig. 12.

3. Results and Discussion

3.1. Characterization of Polysaccharide Derivatives

Curdlan derivatives (MGlu-Curd) and mannan derivatives (MGlu-Man) with different contents of MGlu groups as pH-sensitive moiety were synthesized by reacting

curdlan or mannan with various amounts of 3-methylglutaric anhydride (Fig. 2). Decyl (C_{10}) groups were further introduced to MGlu-Curd or MGlu-Man by reaction of decylamine with carboxylic acid groups of MGlu units for fixation of these polymers onto liposome membrane (Fig. 2). The obtained curdlan derivatives and mannan derivatives were characterized using ^{1}H NMR. Figures 3A–3C respectively depict ^{1}H NMR spectra of curdlan, $MGlu_{43}$-Curd, and $MGlu_{41}$-Curd-C_{10}. In comparison of spectra for curdlan (Fig. 3A) and for $MGlu_{43}$-Curd (Fig. 3B), introduction of MGlu groups to curdlan was confirmed from the existence of new peaks corresponding to MGlu groups (0.9 ppm, 2–2.3 ppm) in Fig. 3B. From the integration ratio of peaks of MGlu residues to those of sugar backbone (3.5–4.0 ppm), 43% of hydroxy groups of curdlan were estimated as combined with MGlu residues. Similarly, from the integration ratio between sugar backbone, MGlu residues, and decyl groups (0.9–1.5 ppm), decyl-amidated MGlu residues and MGlu residues were found to be combined to 4% and 41% of hydroxy groups of curdlan, respectively, in products obtained using the reaction of $MGlu_{43}$-Curd and decylamine, which is designated as $MGlu_{41}$-Curd-C_{10}. Mannan derivatives were evaluated using the same procedure (Figs. 3D–3F). Compositions of curdlan derivatives and mannan derivatives prepared for this study are shown in Table 1.

290 In our earlier study, the contents of MGlu residues on dextran derivatives
291 affected their protonation state because of proximity effects between carboxylic acid
292 groups [24]. Therefore, acid-base titration of MGlu-Curd and MGlu-Man was
293 conducted (Fig. 4). MGlu-Curd and MGlu-Man changed their protonation states
294 depending on pH in neutral and weakly acidic regions. However, the protonation
295 profiles of MGlu-Curd having various MGlu contents were almost identical, different
296 from the results found for dextran derivatives [24]. Curdlan is known to form a triple
297 helix structure in the alkaline aqueous solution. The secondary hydroxy groups form
298 mutual hydrogen bonding inside of the helix. Other hydroxy groups exist outside of the
299 helix [38]. The effects of MGlu unit introduction on helix formation of curdlan were
300 evaluated by complexation with Congo red, as previously reported [39]. As shown in
301 Fig. S1A, the absorption spectra of Congo red changed in the presence of curdlan or
302 curdlan derivatives. The maximum absorption wavelength (λ_{max}) shifted from 487 nm
303 (free Congo red) to 515 nm in the presence of curdlan (Fig. S1B), which indicates that
304 Congo red forms the complex with curdlan, which takes a helical structure [38–40]. In
305 the cases of MGlu-Curd with Congo red, the extent of red shift decreased concomitantly
306 with increasing MGlu unit contents (Fig. S1B), suggesting that the helix structure of
307 curdlan was dissociated by the introduction of MGlu units to the OH groups of curdlan.

However, λ_{max} for $MGlu_{77}$-Curd with Congo red remained higher than that of free Congo red, indicating that even MGlu-Curd having high MGlu unit contents partially takes the helical structure that can form a complex with Congo red. Although the detailed structure of MGlu-Curd in the aqueous solution is unclear at present, such a unique conformation of curdlan derivatives might affect the density of carboxylic acid groups, thereby producing the same protonation profiles and almost identical p*K*a values irrespective of MGlu residue contents (Table 2). $MGlu_{43}$-Man and $MGlu_{74}$-Man also exhibited almost identical protonation profiles (Fig. 4). Mannan has α(1, 6)-linked backbone and α(1, 2)-linked and α(1, 3)-linked branches (Fig. 2B). $MGlu_{43}$-Man and $MGlu_{74}$-Man might have almost equal density of carboxylic acid groups in their branches, resulting in almost equal p*K*a values. In the cases of MGlu-Curds, protonation was promoted strongly at around pH 5.0, which might result from the formation of aggregation [41]. To confirm the formation of aggregation, the optical density of MGlu-Curd and MGlu-Man was measured. The solutions of MGlu-Curd and MGlu-Man were transparent at neutral pH, indicating that these polymers were soluble in water. In contrast, these solutions suddenly became turbid under a certain pH, which is defined as the precipitation pH at which these polymers change their characteristics from hydrophilic to hydrophobic and lose their water solubility. As shown in Table 2,

326 the respective values of precipitation pH of $MGlu_{65}$-Curd and $MGlu_{81}$-Curd are 4.8 and
327 5.0, which correspond to the region in which protonation was promoted in titration
328 curves (Fig. 4). In addition, degrees of protonation were 0.94–0.98 at around
329 precipitation pH, indicating that most of the carboxylic acid groups must be protonated
330 for aggregation of these polymers. Compared with MGlu-Curd, $MGlu_{74}$-Man showed
331 lower precipitation pH. The solution of $MGlu_{43}$-Man was transparent under
332 experimental conditions. These results indicate that MGlu-Curd has more hydrophobic
333 properties than MGlu-Man does, probably because of their helical structures (Fig. S1).

334 **3.2. Preparation of Polysaccharide Derivative-Modified Liposomes**

335 Polysaccharide derivative-modified liposomes were prepared by dispersion of
336 mixed thin film composed of EYPC and polysaccharide derivatives having anchor
337 moieties in aqueous solution containing OVA. Then, the liposome suspension was
338 extruded through polycarbonate membranes with pore size of 100 nm and purified by
339 ultracentrifugation. The size and zeta potential of obtained liposomes were evaluated
340 (Table 3). Compared with diameters of unmodified liposomes (157 nm), polysaccharide
341 derivative-modified liposomes showed smaller diameters. After ultracentrifugation,
342 liposome pellet in centrifugation tube was re-dispersed in PBS. Liposome size might be
343 changed during this re-dispersion process depending on their surface properties.

344 Polysaccharide derivative-modified liposomes which have many carboxylates on the
345 liposome surface might be re-dispersed more efficiently than unmodified liposomes.
346 Zeta potentials were decreased by modification of polysaccharide derivatives, which
347 indicates that the liposome surfaces are covered with carboxylated polysaccharide
348 derivatives. The liposomes modified with MGlu-Curd-C_{10} or MGlu-Dex-C_{10} exhibited
349 highly negatively charged values of around -50 mV, whereas MGlu-Man-C_{10}-modified
350 liposomes showed low zeta potentials. This might result from insufficient exposure of
351 carboxylic acids on the liposomal surface because of steric hindrance derived from their
352 branched structures (Fig. 2B).
353 The pH-sensitivity of polysaccharide derivatives was evaluated using
354 polymer-modified liposomes. Fluorescence dye pyranine and quencher DPX were
355 encapsulated into liposomes and pH-responsive release behaviors were examined (Fig.
356 5). At neutral pH, all liposomes retained their contents tightly. However, in the weakly
357 acidic pH region (pH 6.5-5.0, which corresponds to early or late endosomes), content
358 release was observed in polysaccharide derivative-modified liposomes, indicating that
359 polysaccharide derivatives became hydrophobic and destabilized the liposomal
360 membrane at these levels of pH. Compared with MGlu-Man-C_{10}-modified liposomes,
361 MGlu-Curd-C_{10}-modified liposomes induced significant content release at narrow pH

362 region, which might reflect the difference of hydrophobicity between MGlu-Curd and
363 MGlu-Man in a weak pH region (Table 2). Moreover, the protonation of MGlu-Curd
364 was promoted at around pH 5.0 (Fig. 4), which might rapidly change MGlu-Curd from
365 hydrophilic to hydrophobic and which destabilizes liposomal membrane efficiently by
366 hydrophobized MGlu-Curds. In our earlier study, MGlu-Dex with higher amounts of
367 MGlu unit induced content release at higher pH regions because of p*K*a increase [24].
368 However, MGlu-Curd-C_{10}-modified liposomes showed similar pH-sensitivity
369 irrespective of the difference of MGlu unit contents, except for $MGlu_{77}$-Curd-C_{10}, which
370 reflects that these $MGlu_{43}$-Curd and $MGlu_{65}$-Curd showed almost equal p*K*a values
371 (Table 2). In addition, the hydrophobicity of MGlu-Curd-C_{10} was evaluated using
372 pyrene fluorescence (Fig. S2) [42]. MGlu-Curd-C_{10} formed hydrophobic domains at a
373 weakly acidic pH region, which causes the liposomal membrane disruption presented in
374 Fig. 5A. $MGlu_{77}$-Curd-C_{10} showed slightly higher hydrophobicity than
375 $MGlu_{41}$-Curd-C_{10} and $MGlu_{59}$-Curd-C_{10} at pH 7.0–6.0, which reflects the
376 pH-responsiveness of $MGlu_{77}$-Curd-C_{10}-modified liposome at a higher pH region than
377 those of $MGlu_{41}$-Curd-C_{10}-modified and $MGlu_{59}$-Curd-C_{10}-modified liposomes.

378 **3.3. Interaction of Polysaccharide Derivative-Modified Liposomes with DCs**

379 Dendritic cells have various lectins that recognize specific polysaccharides or

oligosaccharides derived from bacteria [34]. Therefore, the effects of polysaccharide structures on the cellular association of polysaccharide derivative-modified liposomes were examined. Figure 6 depicts a comparison of fluorescence from the cells treated with Rh-PE-labeled liposomes modified with or without polysaccharide derivatives. For MGlu-Curd-C_{10}-modified liposomes, the cellular association changed depending on MGlu unit contents. $MGlu_{59}$-Curd-C_{10} showed the highest cellular association, which was 13 times higher than that of unmodified liposomes. For MGlu-Man-C_{10}-modified liposomes, the cellular association was low, probably because low zeta potential of these liposomes suppressed interaction with scavenger receptors on dendritic cells, which recognize anionic molecules (Table 3). In comparison with the backbone structure, $MGlu_{59}$-Curd-C_{10}-modified liposomes exhibited higher cellular association than $MGlu_{65}$-Man-C_{10}-modified and $MGlu_{56}$-Dex-C_{10}-modified liposomes. These results show that both MGlu unit contents and backbone structures are important to obtain liposomes with high affinity to DCs.

To reveal the cellular association mechanism of these liposomes, inhibition assay was performed using dextran sulfate, curdlan, and mannan, which are the respective inhibitors for scavenger receptors, Dectin-1, and Dectin-2 [32, 33, 43]. Figures 7A and 7B depict the effects of inhibitors on the cellular association of

398 MGlu-Curd-C_{10}-modified liposomes. Cellular association of MGlu-Curd-C_{10}-modified
399 liposomes was strongly suppressed in the presence of dextran sulfate (Fig. 7A).
400 Reportedly, cellular association of carboxylated polymer-coated liposomes or
401 nanoparticles to macrophages and DCs is inhibited by dextran sulfate because these
402 nanoparticles are recognized by scavenger receptors, which are receptors on
403 macrophages and DCs for the recognition of anionic molecules or surface such as aged
404 erythrocytes or apoptotic cells [44]. Therefore, the result of strong inhibition by dextran
405 sulfate indicates that MGlu-Curd-C_{10}-modified liposomes have high affinity to
406 scavenger receptors on DC2.4 cells. The presence of curdlan also suppressed the
407 cellular association of MGlu-Curd-modified liposomes to some degree but not
408 suppressed the cellular association of MGlu-Dex-modified liposomes, indicating the
409 recognition of MGlu-Curd by Dectin-1 (Fig. 7B). However, the cellular association of
410 liposomes modified with MGlu-Curd having high MGlu contents was not suppressed by
411 curdlan, indicating that the modification of high amounts of MGlu unit to curdlan
412 interrupts the interaction curdlan backbone with Dectin-1. Cellular association of
413 MGlu-Man-C_{10}-modified liposomes were also inhibited by dextran sulfate (Fig. 7C),
414 suggesting that MGlu-Man-C_{10}-modified liposomes also internalized to the cells *via*
415 interaction with scavenger receptors. In contrast, the presence of free mannan showed

416 no effect to the cellular association of MGlu-Man-C_{10}-modified liposomes irrespective
417 of the MGlu unit contents (Fig. 7D). This result indicates that the modification of MGlu
418 units to mannan completely suppresses the interaction of mannan derivatives with
419 Dectin-2. Combined with these results, high performance of MGlu-Curd-modified
420 liposomes in cellular association might result from the efficient recognition of these
421 liposomes by both scavenger receptors and Dectin-1.

422 Next, the intracellular antigen delivery performance of polysaccharide
423 derivative-modified liposomes was examined. FITC-labeled model antigenic protein,
424 OVA-loaded liposomes containing DiI as a fluorescence lipid were applied to DC2.4
425 cells. Then intracellular distributions of OVA and DiI (liposome) were observed using
426 CLSM (Fig. 8, S3). For cells treated with unmodified liposomes, punctate red and green
427 fluorescence was observed within cells even in the case of high concentration of
428 liposomes and FITC-OVA (Fig. S3), indicating that both liposomes and FITC-OVA
429 were trapped in endosomes or lysosomes. In the cases of cells treated with
430 polysaccharide derivative-modified liposomes, punctate red and green fluorescence was
431 also observed. However, some parts of the green fluorescence were diffused throughout
432 cells, except to the nucleus. The colocalization efficiency of FITC fluorescence with DiI
433 fluorescence was calculated from CLSM images (Fig. S4). Liposomes modified with

polysaccharide derivatives, especially $MGlu_{21}$-Curd-C_{10}, $MGlu_{41}$-Curd-C_{10} and $MGlu_{59}$-Curd-C_{10}, showed much lower colocalizaion values than that of unmodified liposomes. These results indicate that polysaccharide derivatives became hydrophobic at weakly acidic pH inside of early or late endosomes and efficiently destabilized their own liposomal membrane and endosomal membrane, consequently delivering of FITC-OVA into cytosol of DCs. MGlu-Curd-C_{10}-modified liposomes with 21, 41, 59% of MGlu unit showed excellent performance of cytoplasmic delivery of FITC-OVA, whereas $MGlu_{77}$-Curd-C_{10}-modified liposomes showed both punctate and diffused fluorescence of FITC-OVA (Fig. 8). $MGlu_{77}$-Curd-C_{10}-modified liposomes showed sharp content release below pH 6.5, which corresponds to the pH region of early endosomes in the cells (Fig. 5A). Therefore, $MGlu_{77}$-Curd-C_{10}-modified liposomes might release FITC-OVA within the early endosomes, but the pH in early endosomes was insufficient for $MGlu_{77}$-Curd-C_{10} to induce destabilization of the endosomal membrane, resulting in observation of punctate green fluorescence inside of cells after 4 h incubation (Fig. 8). In contrast, MGlu-Curd-C_{10}-modified liposomes with 21, 41, 59% of MGlu unit showed content release below at pH 6.0, which corresponds to the pH region of late endosomes in the cells (Fig. 5A). In addition, these polymers formed strong hydrophobic domains below pH 6.0 (Fig. S2). Therefore, these polymers induced

452 both content release from liposomes and endosomal membrane destabilization, resulting
453 in efficient cytoplasmic delivery of FITC-OVA. In the case of MGlu-Man-C_{10}-modified
454 liposomes, cytoplasmic delivery performance was not so high, reflecting their moderate
455 performance of contents released at pH 6.5–5.5 (Fig. 5B). These results indicate that the
456 cytoplasmic delivery performance is affected by pH-sensitive content release
457 performance, which is determined not only by the main chain structure, but also by the
458 MGlu unit contents. The excess destabilization of endosomal membrane might cause
459 the cytotoxicity. Therefore, the cytotoxicity of MGlu-Curd-C_{10}-modified liposomes
460 with high cytoplasmic delivery performance was examined (Fig. S5). As shown in Fig.
461 S5, no cytotoxicity was observed under experimental conditions.

462 **3.4. Activation of DCs by Polysaccharide Derivatives and Liposomes**

463 β-glucans and mannan are recognized by DCs or macrophages, which activates
464 them [32–34]. In addition, curdlan- or mannan-modified poly(D,L-lactide-*co*-glycolide)
465 (PLGA) nanoparticles also activated the macrophages or dendritic cells [45, 46].
466 Therefore, we investigated whether carboxylated polysaccharides retain their capability
467 to activate the immunocompetent cells, or not. DC2.4 cells were incubated with MGlu
468 unit-introduced curdlan, mannan and dextran without anchor moiety overnight. Then
469 the production of Th1 cytokines such as TNF-α and IL-12 was measured. As presented

in Figure 9, cytokine production from the DC2.4 cells increased concomitantly with increasing MGlu unit contents in polysaccharides. These results demonstrate that DCs were activated by these polysaccharide derivatives. The introduction of MGlu units promoted their capability for activation. As described in previous reports of the related literature, macrophage activation by carboxylmethylated curdlan or curdlan sulfate was evaluated [32, 47]. Carboxylmethylated curdlan showed no activation property of APCs [32], although curdlan sulfate slightly increased cytokine production from macrophages [47]. Compared with those earlier studies, the activation properties of MGlu unit-incorporated curdlan were quite high, probably because of the high density of carboxylic acid groups on polysaccharide main chain compared with curdlan sulfate (sulfate contents: 9.23% [47]). In addition, IL-12 production from the cells treated with curdlan and mannan derivatives having medium amounts of MGlu units was apparently higher than that of dextran derivatives (Fig. 9B). This result suggests the importance of backbone structures for the efficient activation of DCs. Actually, IL-12 is an important cytokine for the activation of cellular immune responses. Therefore, MGlu-Curd and MGlu-Man, of which backbones are bioactive polysaccharide, are expected to activate the cellular immune responses.

Activation properties of polysaccharide derivative-modified liposomes were

488 also examined (Fig. 10). Figure 10A shows TNF-α production from DC2.4 cells treated
489 with liposomes modified with or without MGlu-Curd-C_{10}. Reflecting the results of the
490 polymers themselves (Fig. 9), TNF-α production increased concomitantly with
491 increasing MGlu unit contents in curdlan derivative-modified liposomes. Figures 10B-D
492 show the effects of backbone structures on TNF-α, IL-10 and IL-12 production,
493 respectively. Cytokine production from these polysaccharide derivative-modified
494 liposomes was quite higher than that of cells treated with unmodified liposomes. There
495 is no significant difference between the backbone structures of polysaccharides. IL-1β
496 production, which indicates the activation of inflammasome, was also examined (Fig.
497 S6). As shown in Figure S6, IL-1β production was enhanced by carboxylated
498 polysaccharide derivative-modified liposomes, especially MGlu-Curd- and
499 MGlu-Man-modified liposomes, indicating that carboxylated polysaccharide derivatives,
500 especially bioactive curdlan or mannan derivatives might activate inflammasome. In
501 these experiments, MPLA, which is a commonly used lipid type adjuvant [28, 29, 48],
502 is introduced into the liposomal membrane, where its effect on cytokine production was
503 also investigated. MPLA inclusion slightly promoted cytokine production from
504 liposome-treated cells, but its effect was not significant, suggesting that DC2.4 cells
505 might be fully activated by these polysaccharide derivative-modified liposomes,

irrespective of MPLA inclusion.

3.5. Induction of *In Vivo* Immune Responses

Next, the induction of immune response *in vivo* by polysaccharide derivative-modified liposomes was examined. OVA-loaded liposomes were administered subcutaneously to mice twice. Seven days after the second immunization, splenocytes were collected and stimulated *in vitro* for 5 days in the presence of OVA. Furthermore, IFN-γ secretion from splenocytes was measured using ELISA (Fig. 11A). In the cases of splenocytes from mice treated with polysaccharide derivative-modified liposomes, high amounts of IFN-γ were produced compared with the case of PBS. Production levels increased concomitantly with increasing OVA concentrations used for *in vitro* culture, indicating that OVA-specific Th1-dominant immune responses were induced by polysaccharide derivative-modified liposomes. Especially, MGlu-Curd-C_{10}-modified liposomes induced the highest production of IFN-γ, which might result from efficient cellular association and cytoplasmic delivery of OVA into antigen-presenting cells by MGlu-Curd-C_{10}-modified liposomes (Figs. 6 and 8).

The CTL activity in the spleen was also evaluated. Splenocytes stimulated *in vitro* for 5 days were co-cultured with E.G7-OVA cells, which are OVA-expressing tumor cells, as a target cell. Then, cytotoxic effects against E.G7-OVA cells were

524 measured (Fig. 11B). Compared with splenocytes from PBS-treated mice, splenocytes
525 from mice treated with polysaccharide derivative-modified liposomes showed higher
526 cytotoxicity against E.G7-OVA cells, indicating that OVA-specific CTLs were induced
527 efficiently by immunization of polysaccharide derivative-modified liposomes.
528 Reflecting the results of IFN-γ secretion, MGlu-Curd-C_{10}-modified liposomes exhibited
529 higher CTL response, suggesting that IFN-γ secretion from Th1 cells or CTLs activated
530 cellular immune response efficiently.
531 As another strategy for induction of cellular immunity, mice were treated with
532 the mixture of OVA solution and liposomes modified with polysaccharide derivatives
533 and their immunity-inducing performance was evaluated (Fig. S7). Unexpectedly, the
534 mixture of OVA and polysaccharide derivative-modified liposomes also induced IFN-γ
535 secretion from splenocytes as the case of OVA-loaded liposomes (Fig. 11A). Especially,
536 $MGlu_{59}$-Curd-C_{10}- and $MGlu_{65}$-Man-C_{10}-modified liposomes induced much higher
537 cellular immune responses than that of $MGlu_{56}$-Dex-C_{10}-modified liposomes.
538 Polysaccharide derivative-modified liposomes could promote the production of Th1
539 cytokines from dendritic cells (Figs. 9 and 10). In addition, the transfer of OVA
540 molecules to cytosol might be promoted if both OVA molecules and liposomes are taken
541 up in the same endosomes. Cytoplasmic transfer of OVA and the production of Th1

542 cytokines by mixture of OVA and polysaccharide derivative-modified liposomes might
543 induce the OVA-specific cellular immune responses. These unexpected results are
544 promising because various antigenic proteins or peptides can be combined with
545 polysaccharide derivative-modified liposomes by just mixing for induction of
546 antigen-specific cellular immunity.

547 **3.6. Therapeutic Effect of Polysaccharide Derivative-Modified Liposomes**

548 Finally, therapeutic effects on tumor-bearing mice by immunization with
549 polysaccharide derivative-modified liposomes were investigated. E.G7-OVA cells were
550 inoculated to mice at 5 days and 15 days after tumor cell inoculation, OVA-loaded
551 liposomes were administered subcutaneously to mice. Then tumor growth and survival
552 were monitored (Fig. 12). This experiment also examined the effects of MPLA
553 inclusion to liposomes on their therapeutic effect. For mice treated with PBS, the tumor
554 volume increased with time. However, in the cases of mice treated with polysaccharide
555 derivative-modified liposomes, tumor volumes decreased at around 7 days after from
556 immunization, indicating the induction of strong cellular immune responses against
557 E.G7-OVA cells by these liposomes, as presented in Fig. 11B. In the absence of MPLA,
558 MGlu-Curd-C_{10}-modified liposomes showed higher tumor regression and more
559 prolonged survival than either MGlu-Dex-C_{10}-modified ($p = 0.0533$) or

MGlu-Man-C_{10}-modified liposomes (p = 0.0495) (Figs. 12A, 12C and Table S2), which might derive from the synergy of efficient intracellular delivery of OVA to DCs (Figs. 6 and 8), the activation of DCs (Fig. 9). In the presence of MPLA, all polysaccharide derivative-modified liposomes exhibited much stronger antitumor effects than those of liposomes without MPLA. Most tumors nearly disappeared (Fig. 12B). Mice survival was also prolonged efficiently. 40–50% of mice became completely tumor-free during 60 days (Fig. 12D). In this case, MGlu-Man-C_{10}-modified liposomes showed the strongest antitumor effects in the polysaccharide derivatives. This result suggests that MPLA is a suitable adjuvant to enhance the immunity-inducing effects of MGlu-Man-C_{10}-modified liposomes. The combination of proper adjuvant with polysaccharide-modified liposomes might produce more efficient antigen delivery systems. It is noteworthy that, even in the absence of MPLA, MGlu-Curd-C_{10}-modified liposomes showed strong tumor regression comparable to MPLA-containing liposomes (Fig. 12A). In addition, there is no significant difference in survival between MGlu-Curd-C_{10}-modified liposome with or without MPLA (p = 0.364, Table S2). Therefore, MGlu-Curd-C_{10}-modified liposomes are beneficial as adjuvant-free antigen delivery systems.

4. Conclusion

This study developed bioactive polysaccharide-based pH-sensitive polymers. MGlu unit-introduced curdlan and mannan efficiently destabilized liposomal membrane at weakly acidic pH and promoted Th1 cytokine production from dendritic cells. MGlu-Curd-C_{10}-modified or MGlu-Man-C_{10}-modified liposomes were taken up efficiently by DCs mainly *via* scavenger receptors. They delivered model antigenic protein into cytosol of DCs. Subcutaneous administration of these liposomes to mice induced OVA-specific cellular immune responses and CTLs in spleen, resulting in the strong therapeutic effects on tumor-bearing mice. Therefore, bioactive polysaccharide-based pH-sensitive polymers and these polymer-modified liposomes are promising as potent antigen delivery systems to achieve both antigen delivery and activation of immune systems for cancer immunotherapy.

Acknowledgments

This work was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture in Japan (15H03024, 25750177).

595 **References**

596 [1] S.A. Rosenberg, J.C. Yang, N.P. Restifo, Cancer immunotherapy: moving beyond

597 current vaccines, Nat. Med. 10 (2004) 909–915.

598 [2] F. Wang, E. Bade, C. Kuniyoshi, L. Spears, G. Jeffery, V. Marty, et al., Phase I trial

599 of a MART–1 peptide vaccine with incomplete Freund's adjuvant for resected high–risk

600 melanoma, Clin. Cancer Res. 5 (1999) 2756–2765.

601 [3] S.A. Rosenberg, M.E. Dudley, Adoptive cell therapy for the treatment of patients

602 with metastatic melanoma, Curr. Opin. Immunol. 21 (2009) 233-240.

603 [4] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature

604 392 (1998) 245-252.

605 [5] I. Mellman, R.M. Steinman, Dendritic cells: specialized and regulated antigen

606 processing machines, Cell 106 (2001) 255-258.

607 [6] N.C. Fernadez, A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter,

608 et al., Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate

609 anti-tumor immune responses in vivo, Nat. Med. 5 (1999) 405-411.

610 [7] O.P. Joffre, E. Segura, A. Savina, S. Amigorena, Cross-presentation by dendritic

611 cells, Nat. Rev. Immunol. 12 (2012) 557-569.

612 [8] J. Panyam, W.Z. Zhou, S. Prabha, S.K. Sahoo, V. Labhasetwar, Rapid

613 endo-lysosomal escape of poly(DL-lactide-*co*-glycolide) nanoparticles: implications for
614 drug and gene delivery, FASEB J. 16 (2002) 1217–1226.
615 [9] T. Akagi, M. Baba, M. Akashi, Biodegradable nanoparticles as vaccine adjuvants
616 and delivery systems: regulation of immune responses by nanoparticle-based vaccine,
617 Adv. Polym. Sci. 247 (2012) 31–64.
618 [10] E.M. Bachelder, T.T. Beaudette, K.E. Broaders, J.M.J. Fréchet, M.T. Albrecht, A.J.
619 Mateczun, et al., In vitro analysis of acetalated dextran microparticles as a potent
620 delivery platform for vaccine adjuvants, Mol. Pharm. 7 (2010) 826–835.
621 [11] A. Boudier, A. Aubert-Pouëssel, N. Mebarek, A. Chavanieu, J. Quentin, D. Martire,
622 et al., Development of tripartite polyion micelles for efficient peptide delivery into
623 dendritic cells without altering their plasticity, J. Control. Release 154 (2011) 156-163.
624 [12] T. Nochi, Y. Yuki, H. Takahashi, S. Sawada, M. Mejima, T. Kohda, et al., Nanogel
625 antigenic protein-delivery system for adjuvant-free intranasal vaccines, Nat. Mater. 9
626 (2010) 572-578.
627 [13] J. Kunisawa, T. Nakanishi, I. Takahashi, A. Okudaira, Y. Tsutsumi, K. Katayama,
628 et al., Sendai virus fusion protein mediates simultaneous induction of MHC class
629 I/II-dependent mucosal and systemic immune responses via the
630 nasopharyngeal-associated lymphoreticular tissue immune system, J. Immunol. 167

631 (2001) 1406-1412.

632 [14] L. Bungener, K. Serre, L. Bijl, L. Leserman, J. Wilschut, T. Daemen, et al.,

633 Virosome-mediated delivery of protein antigens to dendritic cells, Vaccine 20 (2002)

634 2287-2295.

635 [15] R. Reddy, F. Zhou, L. Huang, F. Carbone, M. Bevan, T.B. Rouse, pH sensitive

636 liposomes provide an efficient means of sensitizing target cells to class I restricted CTL

637 recognition of a soluble protein, J. lmmun. Methods 141 (1991) 157-63.

638 [16] T. Nakamura, R. Moriguchi, K. Kogure, N. Shastri, H. Harashima, Efficient MHC

639 class I presentation by controlled intracellular trafficking of antigens in

640 octaarginine-modified liposomes, Mol. Ther. 16 (2008) 1507-1514.

641 [17] N. Murthy, J.R. Robichaud, D.A. Tirrell, P.S. Stayton, A.S. Hoffman, The design

642 and synthesis of polymers for eukaryotic membrane disruption, J. Control. Release 61

643 (1999) 137-143.

644 [18] K. Kono, K. Zenitani, T. Takagishi, Novel pH-sensitive liposomes: liposomes

645 bearing a poly(ethylene glycol) derivative with carboxyl groups, **Biochim. Biophys.**

646 **Acta** 1193 (1994) 1-9.

647 [19] K. Kono, T. Igawa, T. Takagishi, Cytoplasmic delivery of calcein mediated by

648 liposomes modified with a pH-sensitive poly(ethylene glycol) derivative, Biochim.

649 Biophys. Acta 1325 (1997) 143-154.

650 [20] N. Sakaguchi, C. Kojima, A. Harada, K. Kono, Preparation of pH-sensitive

651 poly(glycidol) derivatives with varying hydrophobicities: their ability to sensitize stable

652 liposomes to pH, Bioconj. Chem. 19 (2008) 1040-1048.

653 [21] E. Yuba, A. Harada, Y. Sakanishi, K. Kono, Carboxylated hyperbranched

654 poly(glycidol)s for preparation of pH-sensitive liposomes, J. Control. Release 149

655 (2011) 72-80.

656 [22] E. Yuba, C. Kojima, A. Harada, Tana, S. Watarai, K. Kono, pH-Sensitive fusogenic

657 polymer-modified liposomes as a carrier of antigenic proteins for activation of cellular

658 immunity, **Biomaterials 31** (2010) 943-951.

659 [23] E. Yuba, A. Harada, Y. Sakanishi, S. Watarai, K. Kono, A liposome-based antigen

660 delivery system using pH-sensitive fusogenic polymers for cancer immunotherapy,

661 **Biomaterials 34 (2013)** 3042-3052.

662 [24] E. Yuba, N. Tajima, Y. Yoshizaki, A. Harada, H. Hayashi, K. Kono, Dextran

663 derivative-based pH-sensitive liposomes for cancer immunotherapy, **Biomaterials 35**

664 **(2014)** 3091-3101.

665 [25] R.D. Weeratna, M.J. McCluskie, Y. Xu, H.L. Davis, CpG DNA induces stronger

666 immune responses with less toxicity than other adjuvants, Vaccine 18 (2000)

667 1755-1762.

668 [26] S.Y. Kim, M.B. Heo, G.S. Hwang, Y. Jung, Y. Choi do, Y.M. Park, et al.,

669 Multivalent polymer nanocomplex targeting endosomal receptor of immune cells for

670 enhanced antitumor and systemic memory response, Angew. Chem. Int. Ed. Engl. 54

671 (2015) 8139-8143.

672 [27] C. Wischke, J. Zimmermann, B. Wessinger, A. Schendler, H.H. Borchert, J.H.

673 Peters, et al., Poly(I:C) coated PLGA microparticles induce dendritic cell maturation, Int.

674 J. Pharm. 365 (2009) 61-68.

675 [28] C.R. Alving, M. Rao, N.J. Steers, G.R. Matyas, A.V. Mayorov, Liposomes

676 containing lipid A: an effective, safe, generic adjuvant system for synthetic vaccines,

677 Expert Rev. Vaccines 11 (2012) 733-744.

678 [29] Y. Guo, D. Wang, Q. Song, T. Wu, X. Zhuang, Y. Bao et al., Erythrocyte

679 membrane-enveloped polymeric nanoparticles as nanovaccine for induction of

680 antitumor immunity against melanoma, ACS Nano 9 (2015) 6918-6933.

681 [30] Y. Yoshizaki, E. Yuba, N. Sakaguchi, K. Koiwai, A. Harada, K. Kono, Potentiation

682 of pH-sensitive polymer-modified liposomes with cationic lipid inclusion as antigen

683 delivery carriers for cancer immunotherapy, **Biomaterials 35** (2014) 8186-8196.

684 [31] E. Yuba, Y. Kanda, Y. Yoshizaki, R. Teranishi, A. Harada, K. Sugiura, T. Izawa, J.
685 Yamate, N. Sakaguchi, K. Koiwai, K. Kono, pH-Sensitive polymer-liposome-based
686 antigen delivery systems potentiated with interferon-γ gene lipoplex for efficient cancer
687 immunotherapy, **Biomaterials 67** (2015) 214-224.
688 [32] K. Kataoka, T. Muta, S. Yamazaki, K. Takeshige, Activation of macrophages by
689 linear (1→3)-β-D-glucans: Implications for the recognition of fungi by innate immunity,
690 J. Biol. Chem. 277 (2002) 36825-36831.
691 [33] E.P. McGreal, M. Rosas, G.D. Brown, S. Zamze, S.Y. Wong, S. Gordon, et al.,
692 The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for
693 high mannose, Glycobiology 16 (2006) 422-430.
694 [34] V. Sukhithasri, N. Nisha, L. Biswas, V. Anil Kumar, R. Biswas, Innate immune
695 recognition of microbial cell wall components and microbial strategies to evade such
696 recognitions, Microbiol. Res. 168 (2013) 396-406.
697 [35] Z. Shen, G. Reznikoff, G. Dranoff, K.L. Rock, Cloned dendritic cells can present
698 exogenous antigens on both MHC class I and class II molecules, J. Immunol. 158
699 (1997) 2723–2730.
700 [36] M.W. Moore, F.R. Carbone, M.J. Bevan, Introduction of soluble protein into the
701 class I pathway of antigen processing and presentation, Cell 54 (1988) 777-785.

[37] D.L. Daleke, K. Hong, D. Papahadjopoulos, Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay, Biochim. Biophys. Acta 1024 (1990) 352-366.

[38] K. Miyoshi, K. Uezu, K. Sakurai, S. Shinkai, Proposal of a new hydrogen-bonding form to maintain curdlan triple helix, Chem. Biodivers. 1 (2004) 916-924.

[39] K. Ogawa, T. Dohmaru, T. Yui, Dependence of complex formation of (1-->3)-beta-D-glucan with congo red on temperature in alkaline solutions, Biosci. Biotechnol. Biochem. 58 (1994) 1870-1872.

[40] K. Ogawa, M. Hatano, Circular dichroism of the complex of a (1-3)-/β-D-glucan with Congo Red, Carbohydr. Res. 67 (1978) 527-535.

[41] M. Furukawa, R.S. Farinato, E. Kokufuta, Potentiometric titration behavior of poly(acrylic acid) within a cross-linked polymer network having amide groups. Colloid Polym. Sci. 286 (2008) 1425-1434.

[42] K. Kalyanasundaram, J.K. Thomas, Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar systems, J. Am. Chem. Soc. 99 (1977) 2039-2044.

[43] T. Thelen, Y. Hao, A.I. Medeiros, J.L. Curtis, C.H. Serezani, L. Kobzik, et al., The class A scavenger receptor, macrophage receptor with collagenous structure, is the

major phagocytic receptor for Clostridium sordellii expressed by human decidual macrophages, J. Immunol. 185 (2010) 4328-4335.

[44] M.L. Albert, S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, et al. Immature dendritic cells phagocytose apoptotic cells *via* $\alpha_v\beta_5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes, J. Exp. Med. 188 (1998) 1359-1368.

[45] M. Tukulula, R. Hayeshi, P. Fonteh, D. Meyer, A. Ndamase, M.T. Madziva, et al., Curdlan-conjugated PLGA nanoparticles possess macrophage stimulant activity and drug delivery capabilities. Pharm. Res. 32 (2015) 2713-2726.

[46] A. Haddadi, S. Hamdy, Z. Ghotbi, J. Samuel, A. Lavasanifar, Immunoadjuvant activity of the nanoparticles' surface modified with mannan. Nanotechnology 25 (2014) 355101.

[47] P. Li, X. Zhang, Y. Cheng, J. Li, Y. Xiao, Q. Zhang, et al., Preparation and in vitro immunomodulatory effect of curdlan sulfate, Carbohydr. Polym. 102 (2014) 852–861.

[48] V. Mata-Haro, C. Cekic, M. Martin, P.M. Chilton, C.R. Casella, T.C. Mitchel, The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4, Science 316 (2007) 1628-1632.

737 **Table 1. Compositions of Polysaccharide Derivatives**

Polymer †	-OH (mol%)*	-MGlu (mol%)*	-Anchor (mol%)*
$MGlu_{32}$-Curd	68	32	-
$MGlu_{43}$-Curd	57	43	-
$MGlu_{65}$-Curd	35	65	
$MGlu_{81}$-Curd	19	81	-
$MGlu_{43}$-Man	57	43	-
$MGlu_{74}$-Man	26	74	-
$MGlu_{9}$-Dex**	91	9	-
$MGlu_{35}$-Dex**	65	35	-
$MGlu_{65}$-Dex**	35	65	-
$MGlu_{85}$-Dex**	15	85	-
$MGlu_{21}$-Curd-C_{10}	76	21	3
$MGlu_{41}$-Curd-C_{10}	55	41	4
$MGlu_{59}$-Curd-C_{10}	35	59	6
$MGlu_{77}$-Curd-C_{10}	17	77	6
$MGlu_{36}$-Man-C_{10}	60	36	4
$MGlu_{65}$-Man-C_{10}	29	65	6
$MGlu_{56}$-Dex-C_{10}**	27	56	7

738 *Determined by ^{1}H NMR. **Synthesized as previously reported [24].

739 † $MGlu_X$-Curd refers to the polymer with X mol% of MGlu groups. $MGlu_Y$-Curd-C_{10} refers to
740 the polymer with Y mol% of MGlu groups and decyl (C_{10}) groups as indicated mol% in a
741 column in –Anchor (mol%).

742

743 **Table 2. p*K*a and Precipitation pH for Polysaccharide Derivatives**

Polymer	p*K*a	Precipitation pH	Degree of protonation at precipitation pH
$MGlu_{32}$-Curd	-	N.D.	-
$MGlu_{43}$-Curd	5.85	4.4	0.98
$MGlu_{65}$-Curd	5.78	4.8	0.94
$MGlu_{81}$-Curd	5.96	5.0	0.95
$MGlu_{43}$-Man	6.40	N.D.	-
$MGlu_{74}$-Man	6.53	3.8	0.99
$MGlu_{65}$-Dex	6.26*	4.5*	0.95*

744 *Previously reported [24].

745 **Table 3. Hydrodynamic Sizes and Zeta Potentials of Liposomes**

Polymer	Hydrodynamic size (nm)	Zeta potential (mV)
Unmodified	157 ± 4	-11.6 ± 5
$MGlu_{21}$-Curd-C_{10}	109 ± 3	-46.9 ± 5
$MGlu_{41}$-Curd-C_{10}	121 ± 6	-47.3 ± 4
$MGlu_{59}$-Curd-C_{10}	131 ± 2	-50.5 ± 1
$MGlu_{77}$-Curd-C_{10}	121 ± 4	-46.5 ± 2
$MGlu_{36}$-Man-C_{10}	123 ± 3	-20.0 ± 2
$MGlu_{65}$-Man-C_{10}	100 ± 1	-31.7 ± 2
$MGlu_{56}$-Dex-C_{10}*	109 ± 4	-46.5 ± 2

746 *As previously reported [24]

747

748 **Figure captions**

749 **Figure 1.** Design of liposomes modified with bioactive polysaccharide derivatives for
750 induction and activation of antigen-specific immunity. These liposomes are taken up by
751 dendritic cells *via* endocytosis and trapped in endosome. Its weakly acidic environment
752 triggers destabilization of the liposome, which induces release of antigen molecules in
753 endosome and their transfer to cytosol *via* fusion with endosome. Antigen molecules in
754 cytosol cause antigen-specific cytotoxic T lymphocytes (CTL) *via* presentation by MHC
755 class I, resulting in the induction of cellular immunity. Liposomes modified with
756 bacteria-derived polysaccharide derivatives, MGlu-Curd or MGlu-Man are also
757 recognized *via* Dectin-1 and Dectin-2, respectively, which causes the promotion of Th1
758 cytokine production and the activation of cellular immunity.

759 **Figure 2.** (A) Synthetic route for polysaccharide derivatives having carboxy groups and
760 alky chains as anchor units to liposomal membrane. (B) Structures of polysaccharides
761 used in this study.

762 **Figure 3.** ^{1}H NMR spectra of (A) curdlan, (B) $MGlu_{43}$-Curd, (C) $MGlu_{41}$-Curd-C_{10}, (D)
763 mannan, (E) $MGlu_{74}$-Man, and (F) $MGlu_{65}$-Man-C_{10} in D_2O/NaOD.

764 **Figure 4.** Acid-base titration curves for (A) $MGlu_{50}$-Curd (open diamonds),
765 $MGlu_{65}$-Curd (gray triangles), $MGlu_{81}$-Curd (closed squares), and (B) $MGlu_{43}$-Man

766 (closed diamonds), and $MGlu_{74}$-Man (open squares).

767 **Figure 5.** pH-sensitive contents release behaviors of curdlan (A) or mannan (B)

768 derivative-modified liposomes. Pyranine release after 30 min-incubation from

769 liposomes modified with $MGlu_{21}$-Curd-C_{10} (closed diamonds), $MGlu_{41}$-Curd-C_{10}

770 (closed circles), $MGlu_{59}$-Curd-C_{10} (closed squares), $MGlu_{77}$-Curd-C_{10} (closed triangles),

771 $MGlu_{36}$-Man-C_{10} (open squares), $MGlu_{65}$-Man-C_{10} (open circles), and without polymer

772 (open diamonds) were shown. Lipid concentrations were 2.0×10^{-5} M. Each point is the

773 mean ± SD (n = 3).

774 **Figure 6.** Fluorescence intensity for DC2.4 cells treated with Rh-PE-labeled EYPC

775 liposomes modified with or without polysaccharide derivatives having various amounts

776 of MGlu groups. DC2.4 cells were incubated with liposomes (lipid concentration: 0.5

777 mM) for 4 h at 37 °C in serum free medium. Cellular auto fluorescence was corrected.

778 *p <0.05. **p <0.01.

779 **Figure 7.** Inhibition of cellular association of liposomes modified with

780 $MGlu_{21}$-Curd-C_{10} (closed diamonds), $MGlu_{41}$-Curd-C_{10} (closed circles),

781 $MGlu_{59}$-Curd-C_{10} (closed squares), $MGlu_{77}$-Curd-C_{10} (closed triangles),

782 $MGlu_{36}$-Man-C_{10} (open squares), $MGlu_{65}$-Man-C_{10} (open circles), and $MGlu_{56}$-Dex-C_{10}

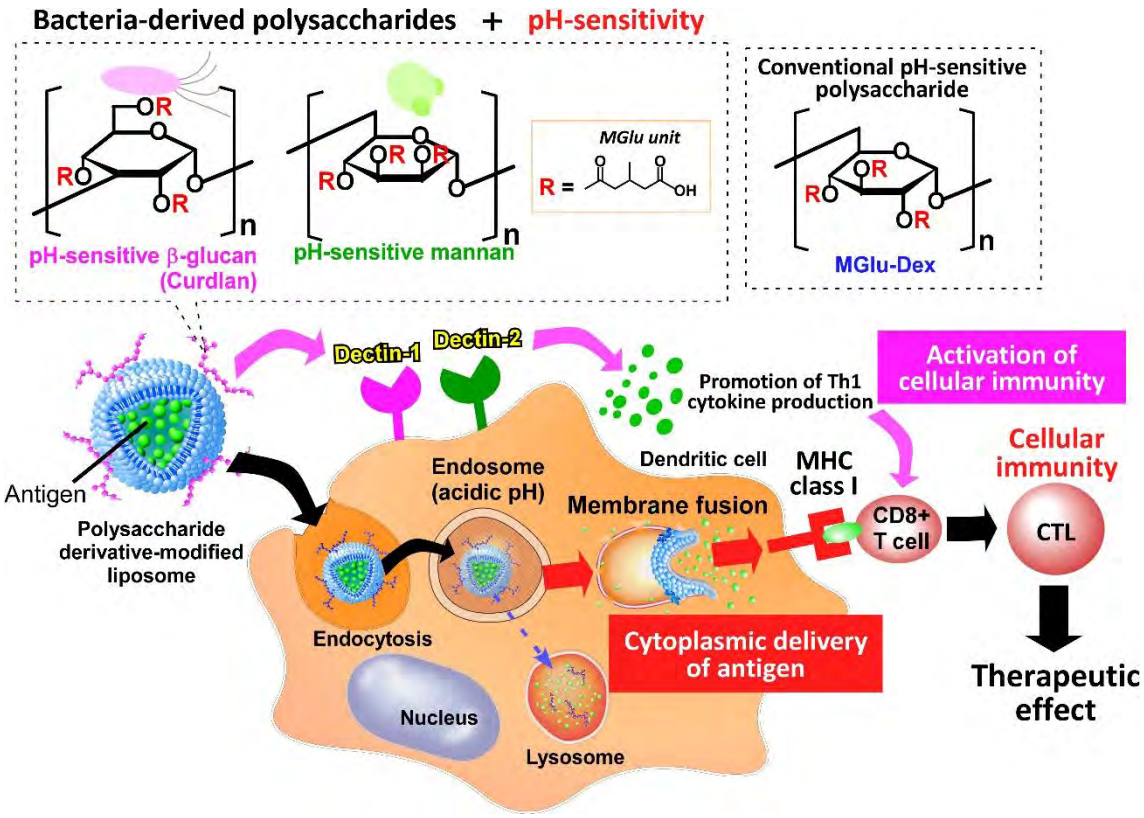
783 (closed inverted triangles). Various concentrations of free dextran sulfate (A, C), free

784 curdlan (B) and free mannan (D) were incubated with DC2.4 cells for 1 h before sample
785 apply. Relative fluorescence intensity was calculated as the ratios of the amount of
786 association in the presence of ligands to that in the absence of ligands. *p <0.05 and **p
787 <0.01 comapred to the case in the absence of ligands.
788 **Figure 8.** Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated
789 with DiI-labeled and FITC-OVA-loaded liposomes modified with indicating
790 polysaccharide derivatives or without polymer for 4 h at 37 °C in serum-free medium.
791 Bar represents 10 μm.
792 **Figure 9.** (A) TNF-α and (B) IL-12 productions from DC2.4 cells treated with curdlan
793 (diamonds), mannan (triangles) and dextran (squares) derivatives having various
794 contents of MGlu groups (1 mg/mL) for 24 h. *p <0.05 and **p <0.01 comapred to the
795 parent polysaccharide.
796 **Figure 10.** Cytokine production from DC2.4 cells treated with polysaccharide
797 derivatives-modified liposomes (0.5 mM lipid concentration) for 24 h. (A) Effect of
798 MGlu group contents for MGlu-Curd-C_{10}-modified liposomes. (B, C) Effects of main
799 chain structures and inclusion of MPLA. *p <0.05. **p <0.01.
800 **Figure 11.** (A) IFN-γ production from splenocytes of OVA-immunized mice.
801 Splenocytes were isolated from C57BL/6 mice immunized with PBS (white bars), OVA

802 encapsulated liposomes modified with $MGlu_{59}$-Curd-C_{10} (gray bars), $MGlu_{65}$-Man-C_{10}
803 (striped bars) and $MGlu_{56}$-Dex-C_{10} (closed bars) twice. Splenocytes (2×10^6/mL in 2
804 mL) were incubated with OVA (0, 25 or 50 μg/mL) for 5 days. Concentration of IFN-γ
805 in the medium was measured by ELISA. (B) OVA-specific CTL induction in spleen of
806 mice after 7 days from subcutaneous immunization with polysaccharide
807 derivative-modified liposomes twice. Cytotoxic activity was measured at effecter
808 cell/target cell (E/T) ratio of 25 using the LDH assay. Aliquots of OVA (100 μg) was
809 administered per mouse. E.G7-OVA cells were used as target cells. Each plots shows
810 the data from each mouse and bars represent the mean value. *p <0.05. **p <0.01. † p
811 =0.14.

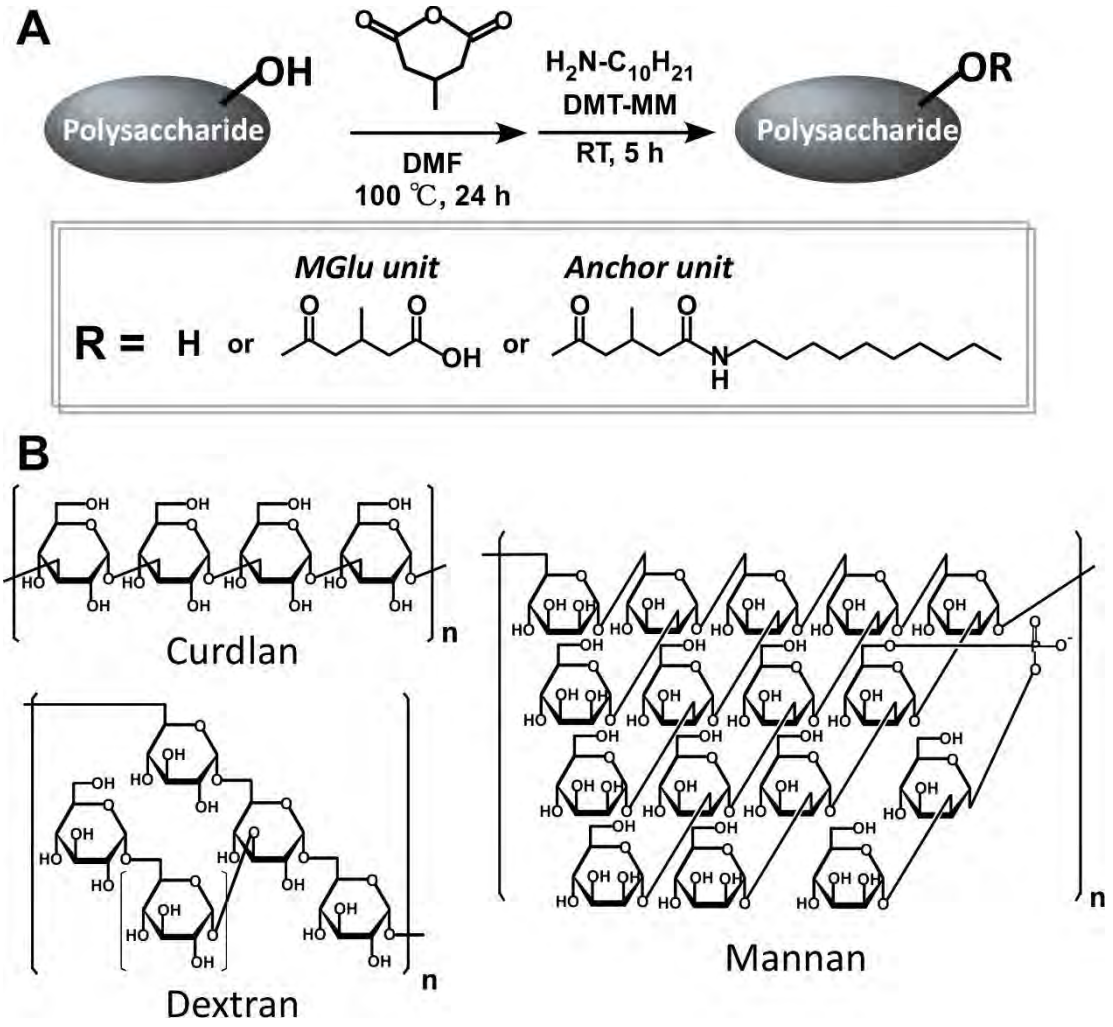
812 **Figure 12.** Antitumor effects induced by subcutaneous administration of OVA-loaded
813 liposomes without (A, C, open symbols) or with (B, D, closed symbols) MPLA.
814 E.G7-OVA cells were subcutaneously inoculated into the left backs of C57BL/6 mice
815 and tumor volume was monitored. Tumor volumes (A, B) and survival (%) (C, D) of
816 tumor-bearing mice were followed from tumor cell inoculation.
817 $MGlu_{56}$-Dex-C_{10}-modified liposomes (squares), $MGlu_{59}$-Curd-C_{10}-modified liposomes
818 (diamonds), and $MGlu_{65}$-Man-C_{10}-modified liposomes (triangles) containing 100 μg of
819 OVA were subcutaneously administered into the right backs of the mice twice on day 5

820 and day 15. Mice administered with PBS (circles) were used as controls to confirm the
821 development of tumor. Typical results of tumor volume change using four mice were
822 shown in Figs. 12A and B. Eight to twelve mice were used for each treated group. *p
823 <0.05 and **p <0.01 comapred to PBS-treated group. Results for Log-rank test is
824 shown in Table S2.
825



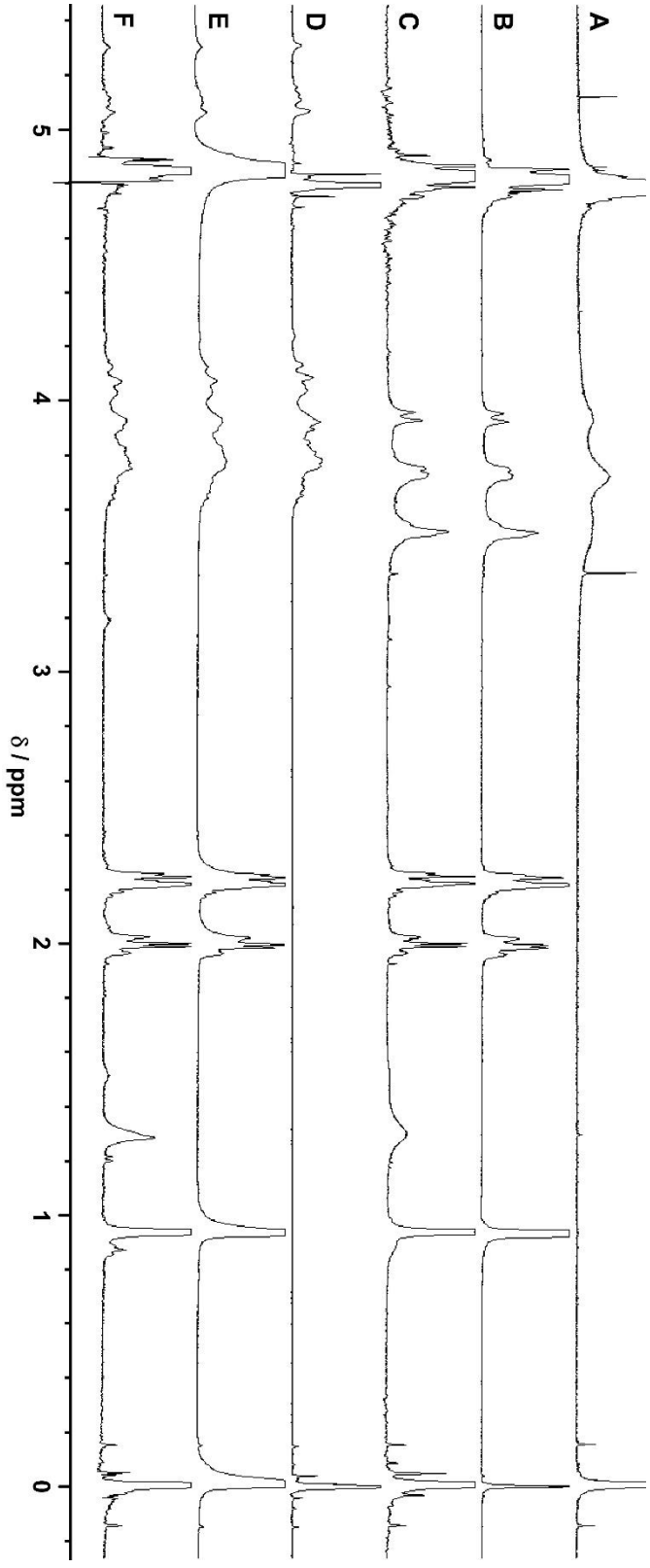
826

827 *Yuba, et al., Figure 1.*

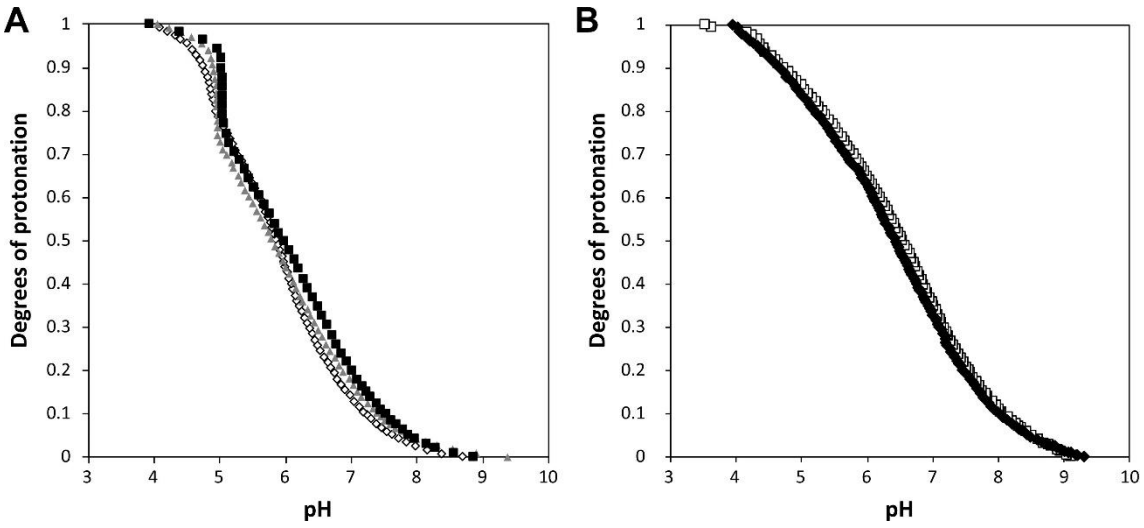


828

829 *Yuba, et al., Figure 2.*

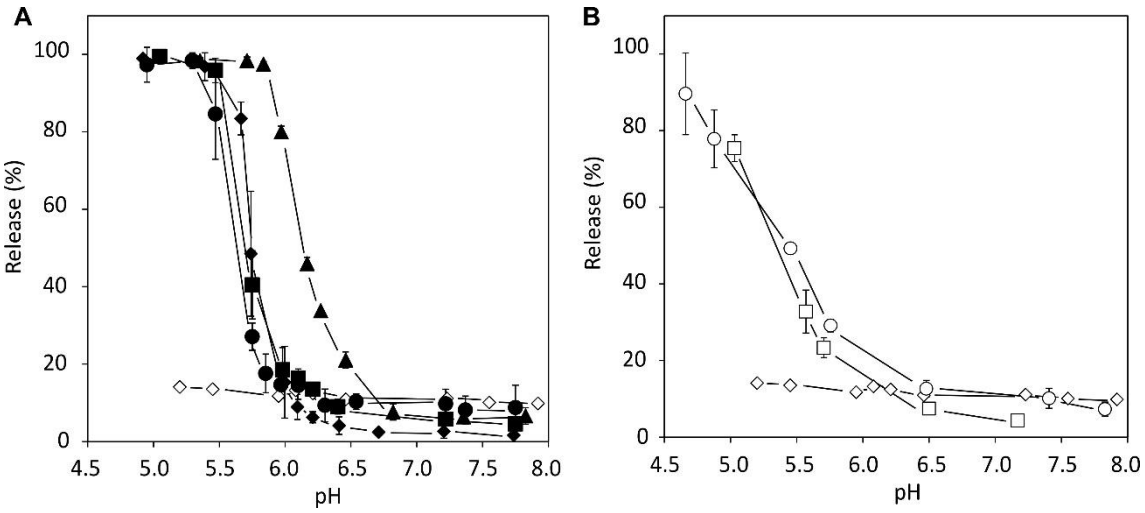


Yuba, et al., Figure 3.



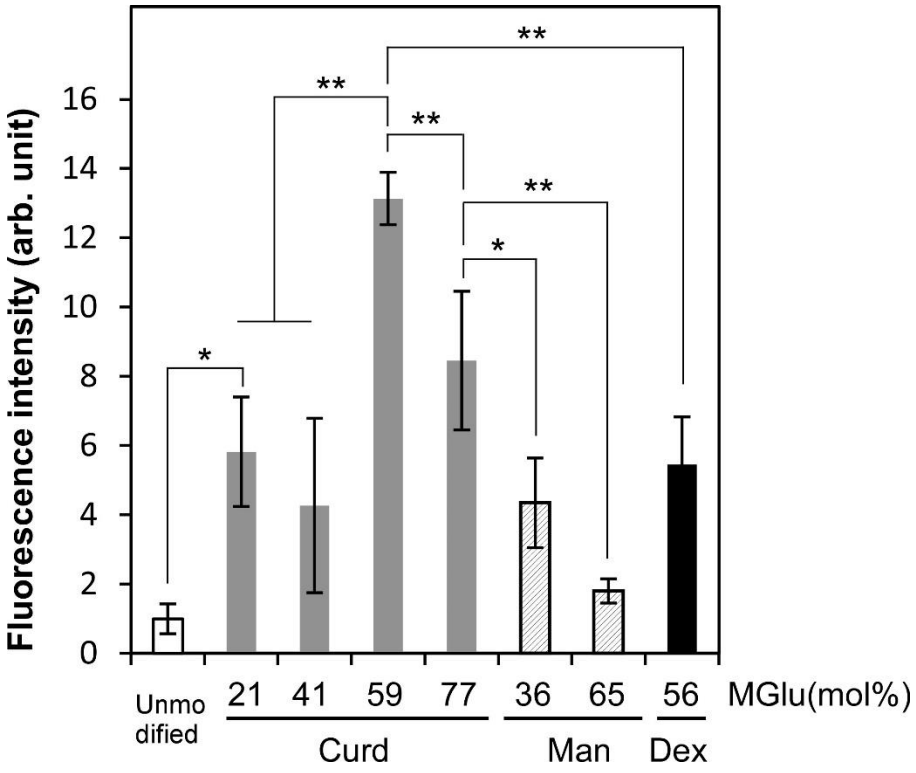
832

833 *Yuba, et al., Figure 4.*



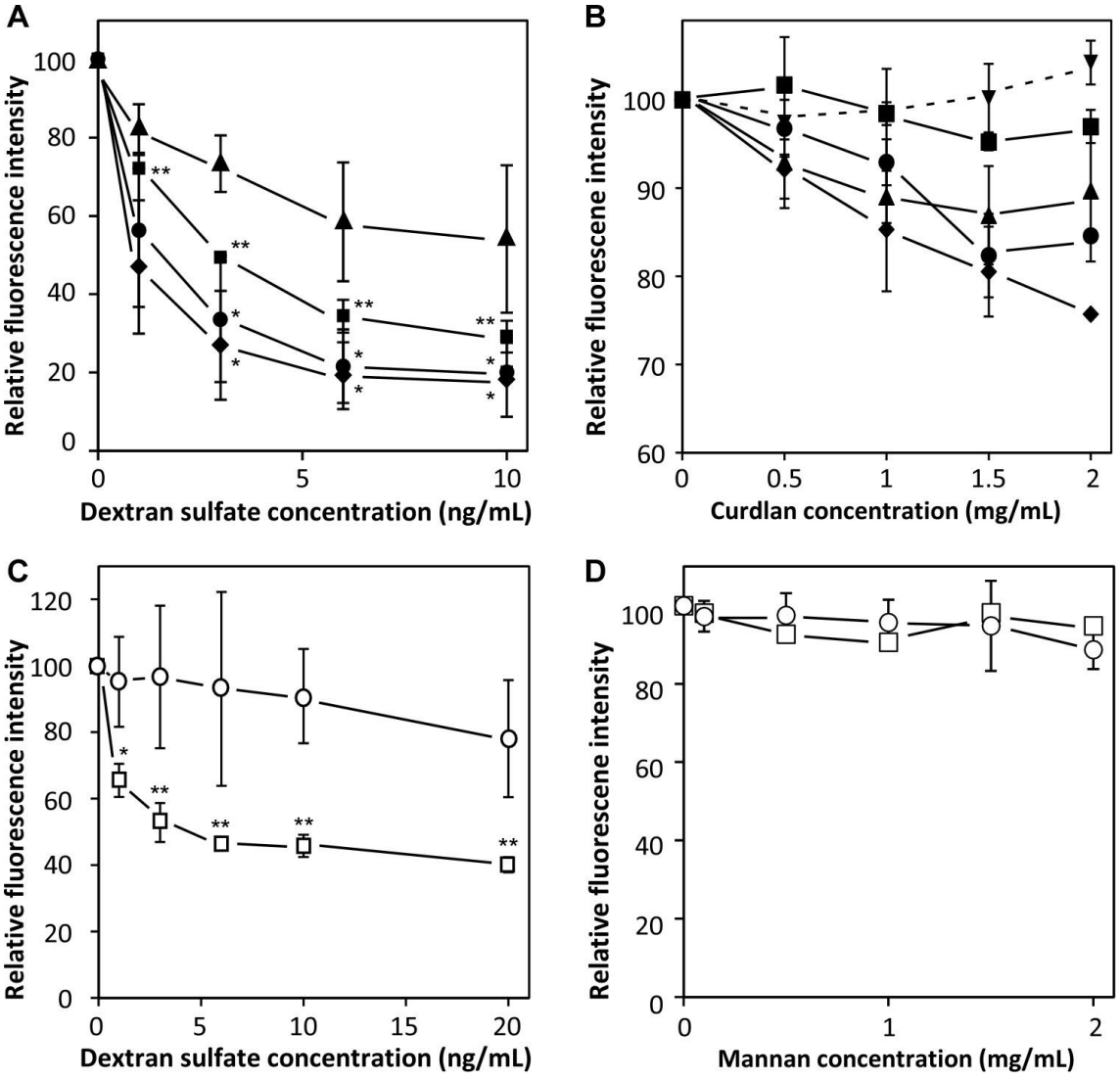
834

835 *Yuba, et al., Figure 5.*



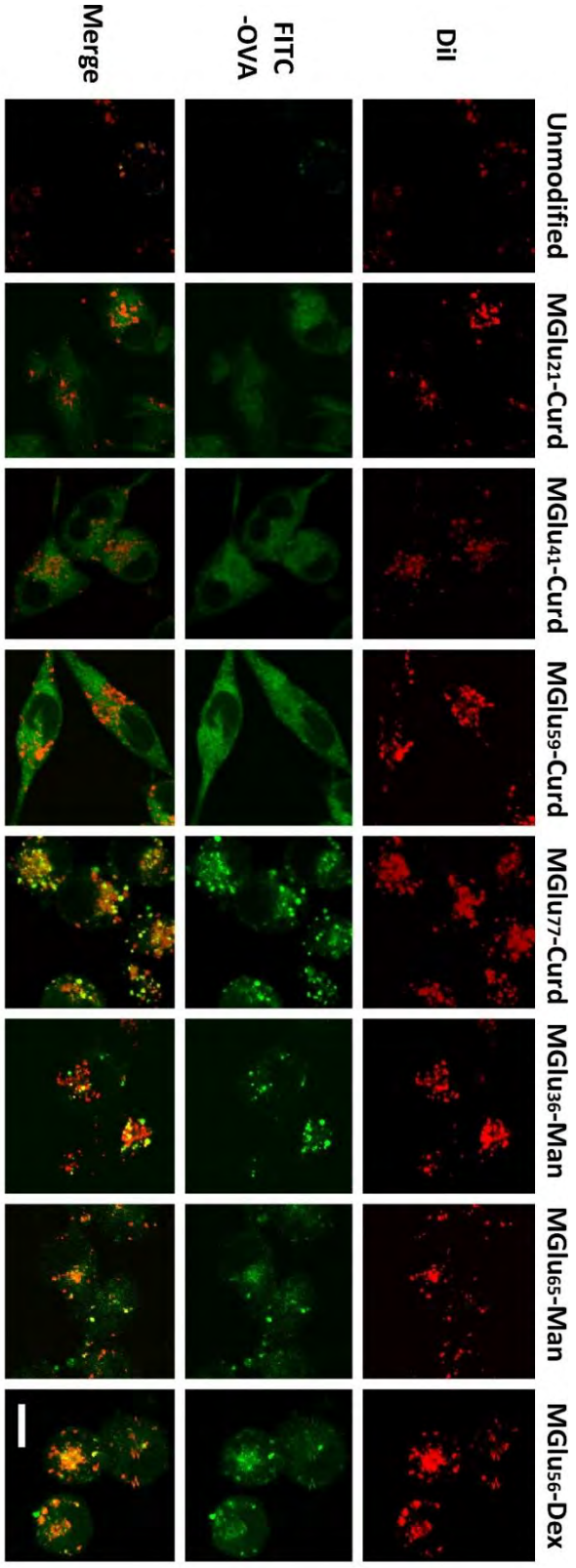
836

837 *Yuba, et al., Figure 6.*



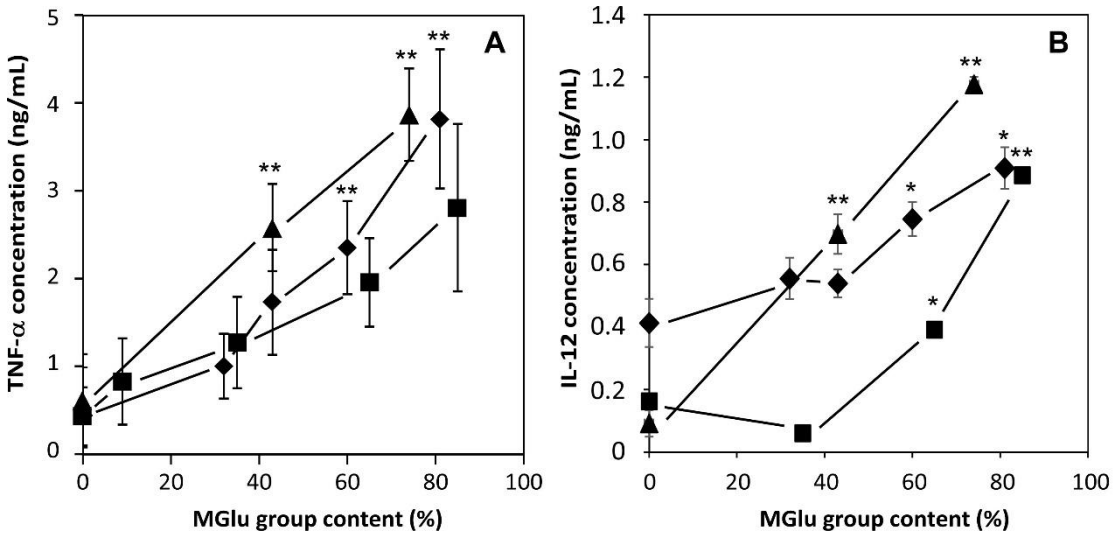
838

839 *Yuba, et al., Figure 7.*



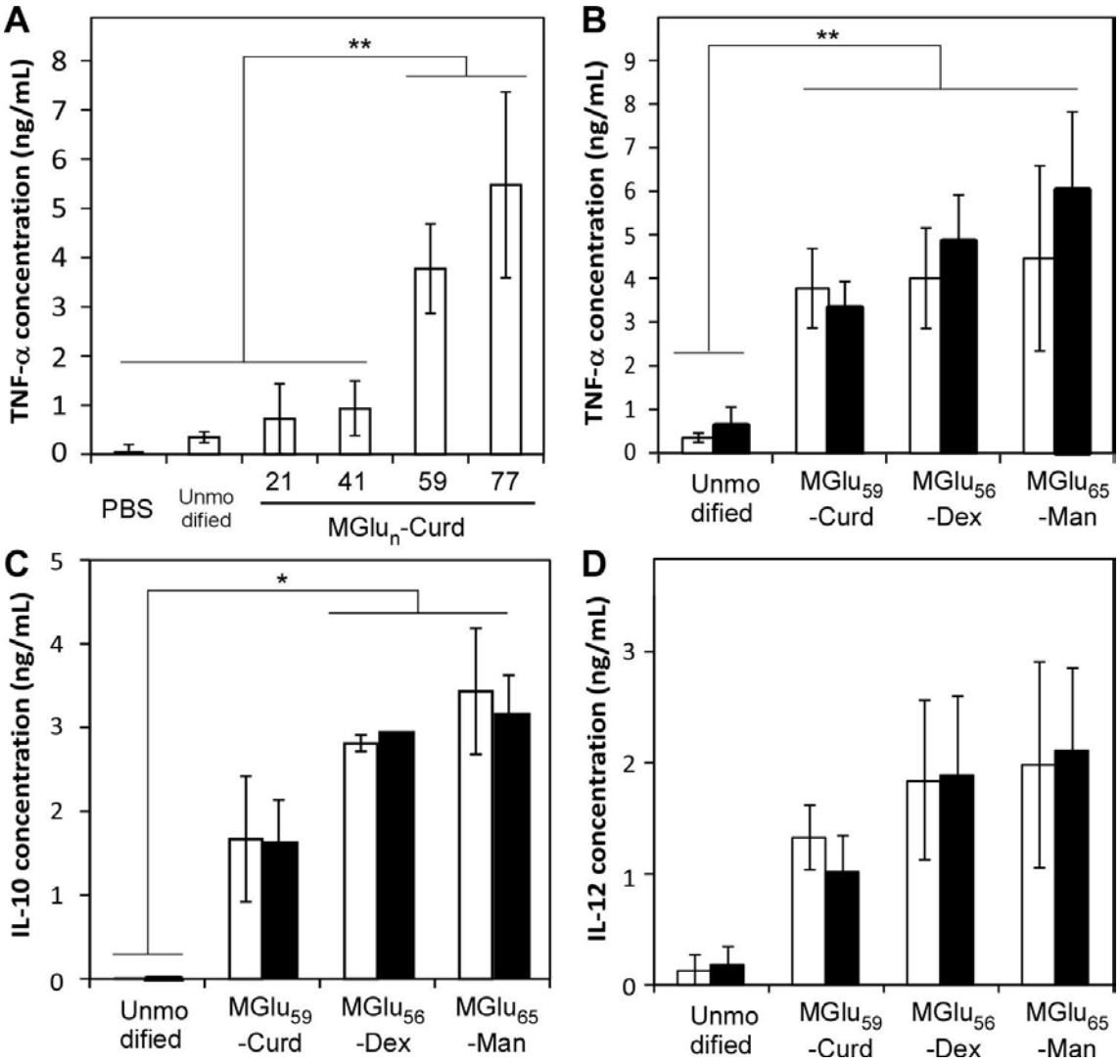
840

841 *Yuba, et al., Figure 8*



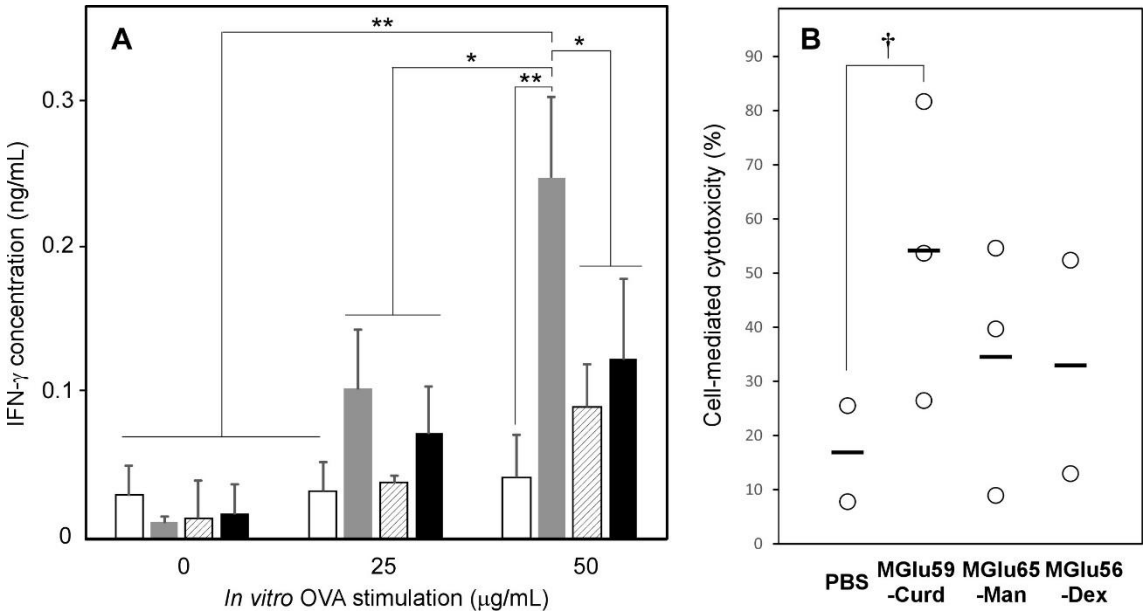
842

843 *Yuba, et al., Figure 9.*



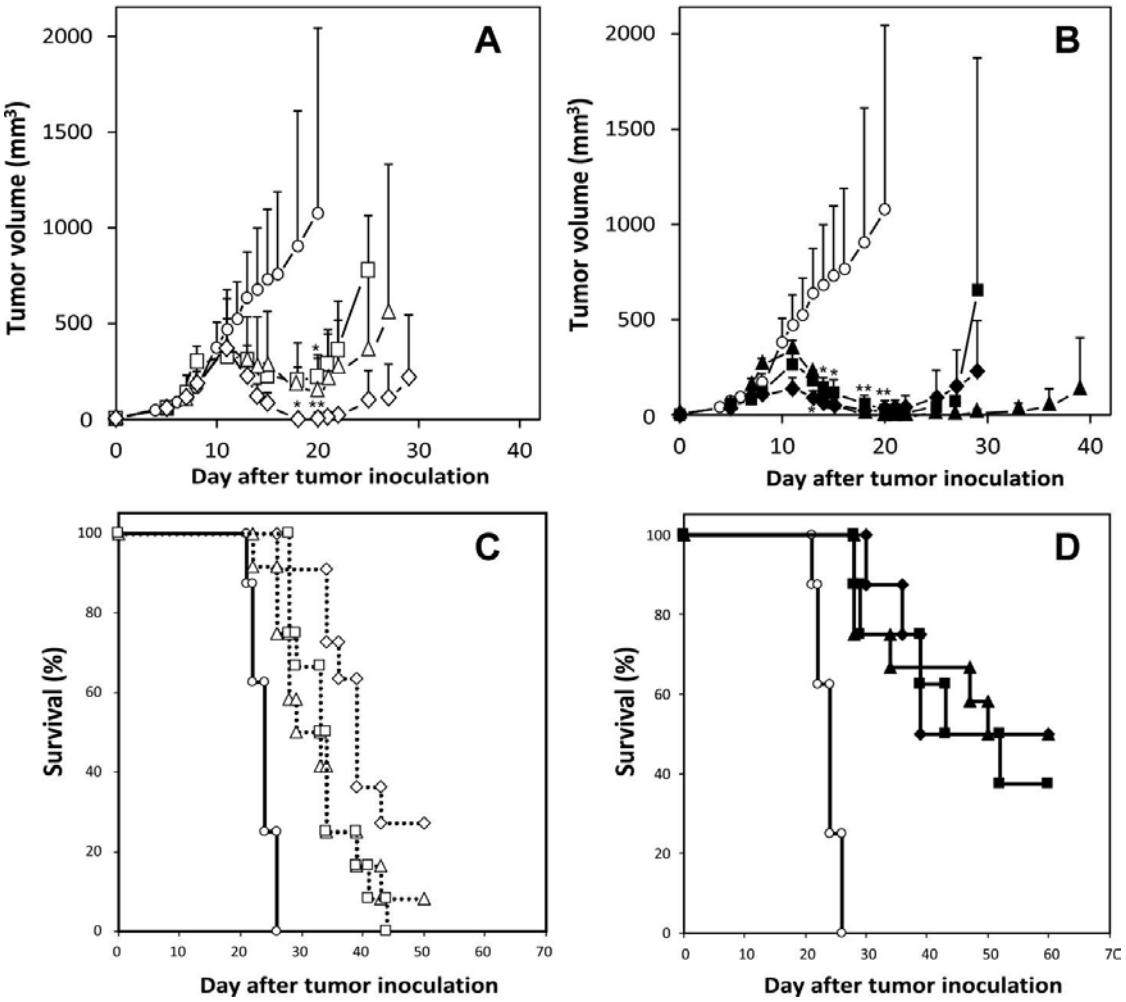
844

845 *Yuba, et al., Figure 10.*



846

847 *Yuba, et al., Figure 11.*



848

849 *Yuba, et al., Figure 12.*

850