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Design of pH-Sensitive Polymer-Modified Liposomes for Antigen Delivery and Their Application to Cancer Immunotherapy

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Running Head: pH-Sensitive polymer-based antigen delivery system

Abstract

Accurate antigen delivery into cytosol of antigen-presenting cells, such as dendritic cells, is crucially important to induce cellular immunity for achievement of efficient cancer immunotherapy. Various antigen delivery systems have been studied to date to achieve cytoplasmic delivery of antigens. Among them, pH-sensitive liposome is regarded as a promising carrier because of its pH-responsive membrane disruption or fusion ability, which causes the transfer of encapsulated antigen into cytosol. Recently, highly potent pH-sensitive liposomes have been prepared as antigen delivery systems using liposomes modified with pH-sensitive polymers. The control of pH-responsive fusion ability and intracellular distribution of antigens, the induction of humoral or cellular immunity *in vivo*, the induction of protective immunity against pathogens, and the treatment of tumor-bearing mice have been achieved using these liposomes. The design and function of these pH-sensitive polymer-modified liposomes are outlined in this review.

Keywords: adjuvant / cancer immunotherapy / cytoplasmic delivery / dendritic cell / liposome / membrane fusion / pH-sensitive polymer

Introduction

Cancer immunotherapy, which is a treatment activating the patient's own immune system to fight cancer or removing immunosuppression in tumor microenvironments, has gained much attention as an alternative to standard cancer therapies.[1–3] To date, various cancer immunotherapy methods have been studied, such as adoptive T cell transfer therapy, dendritic cell vaccine, and immune checkpoint inhibitors.[1–7] Among them, activation of the immune system against cancer *via* dendritic cells (DCs) is an attractive treatment because of its selectivity and safety.[4] In 2010, Sipuleucel-T (Provenge; Dendreon Corp.), a DC vaccine against prostate adenocarcinoma, was approved by the US FDA.[5, 6] Since then, DC cancer vaccines have received much attention from numerous researchers.

As an antigen-presenting cell, DC, plays a crucial role in starting and activating immune systems.[8, 9] DC recognizes the tumor antigen and presents to T cells. Activated T cells or B cells subsequently attack the tumor directly or *via* antibody. Two major pathways exist for antigen presentation by DCs (Figure 1). Exogenous antigen is degraded *via* endosome pathway, and is carried onto major histocompatibility complex (MHC) class II molecules, which induces humoral immunity. Endogenous antigen is degraded in proteasomes and is carried onto MHC class I molecules, thereby inducing

cellular immunity. To achieve effective cancer immunotherapy, the induction of cellular immunity is important. To induce cellular immunity, the transfer of exogenous antigen to cytosol and the induction of MHC class I-mediated presentation, which is termed as "cross-presentation", are necessary (Figure 1). Therefore, an efficient cytoplasmic delivery system of exogenous antigen is necessary to induce "cross-presentation" and to achieve cancer immunotherapy.

To date, various delivery systems have been studied for cytoplasmic delivery of antigens.[10–26] Among them, lipid-based delivery systems such as liposomes are attractive because liposomes can achieve cytoplasmic delivery using bio-related functions such as membrane fusion.[10–13, 23–26] Two strategies are used for cytoplasmic delivery. One is direct delivery of exogenous antigen into cytosol, such as direct fusion with cellular membrane by Sendai virus fusion protein-incorporated liposomes or a combination of bubble liposome with ultrasound.[10, 11, 27] Another strategy of cytoplasmic delivery is the promotion of endosomal escape of an antigen, such as influenza virus fusion protein-introduced liposomes (Virosome) or pH-sensitive liposomes.[12, 13, 23–25, 28] However, viral protein-based liposomes might cause unfavorable immune responses derived from viral components. Therefore, the use of pH-sensitive liposomes prepared from synthetic molecules is desired.

Two methods are used for the preparation of pH-sensitive liposomes: the inclusion of pH-sensitive amphiphiles and the modification of pH-sensitive polymers to stable liposomes. In the case of pH-sensitive amphiphiles, non-bilayer forming lipids such as dioleoylphosphatidylethanolamine (DOPE) are used as a lipid component.[24, 25, 29] Therefore, it is difficult to combine the stability of liposome with high sensitivity. However, poly(carboxylic acid)s such as poly(acrylic acid) derivatives have been used as typical pH-sensitive polymers to prepare pH-sensitive polymer-modified liposomes (Figures 2a and 2b).[30–32] These polymers form mixed micelles with lipids at acidic pH, which causes liposomal membrane lysis.[33–35] The hydrophobicity of poly(acrylic acid) derivatives strongly affects their membrane disrupting capability.[36] Especially, poly(propyl acrylic acid) showed higher ability to induce membrane disruption responding weakly acidic pH than poly(ethyl acrylic acid).[36, 37] Poly(propyl acrylic acid) was applied to antigen delivery by direct conjugation to antigenic proteins.[38, 39] These antigen-polymer conjugates or conjugate-based micelles induced "cross-presentation" *in vitro* and cellular immune responses *in vivo*.[38–41] Endosomal membrane lysis activity derived from poly(propyl acrylic acid) might enhance the delivery of antigenic proteins into cytosol of DCs, thereby causing the induction of efficient cross-presentation.

Our group has developed pH-sensitive polymers of another type using

poly(glycidol)s, which have a poly(ethylene glycol) (PEG) like main chain structure.[42, 43] Dicarboxylic acid anhydrides such as succinic anhydride were reacted with hydroxy groups of poly(glycidol)s, resulting in succinylated poly(glycidol) (SucPG, Figure 2c).[42, 43] Long alkyl chains were also introduced to a part of carboxy groups (typically around 10% for hydroxy groups) to fix the polymer onto the liposome membrane. SucPG-modified egg yolk phosphatidylcholine (EYPC) liposomes showed content (calcein) release under acidic pH and delivered calcein to cytosol of CV1 cells.[43] Analyses of intracellular fusion behavior of liposomes based on fluorescence resonance energy transfer (FRET) revealed that SucPG-modified liposomes show membrane fusion ability under acidic pH differently from membrane lysis of poly(acrylic acid) derivatives.[43] The PEG-like main chain structure of SucPG might induce "mild" insertion of polymer chain to liposome membrane under acidic conditions, resulting in induction of membrane defects to cause membrane fusion.[43] In contrast, poly(acrylic acid) derivatives, which have a vinyl main chain structures, might be inserted deeply to the lipid bilayer under the same circumstances, thereby causing the comprehensive destabilization of the lipid membrane.[32]

Based on the intracellular delivery performance of carboxylated poly(glycidol)s, carboxylated poly(glycidol)s-modified liposomes were applied to antigen delivery into

104 cytosol of DC to induce cross-presentation and cellular immunity (Figure 3). This
105 review presents discussion of the effects of carboxylated poly(glycidol) structure on the
106 performance of intracellular delivery of antigen and the induction of cellular immune
107 responses against cancer or an infectious disease model. Carboxylated dextrans were
108 also developed as safer pH-sensitive polymers. Their capability for use as a cancer
109 vaccine was evaluated. To induce stronger immune responses, adjuvant
110 (immune-activating) molecules or functions were introduced to pH-sensitive
111 polymer-modified liposomes. Then their performance was evaluated.
112
113 **Design of pH-sensitive polymers for cytoplasmic delivery**
114 When poly(carboxylic acid)s interact with lipid membrane, hydrophobic
115 interaction of their main chain with hydrophobic domain of lipid bilayers and hydrogen
116 bonds between carboxy groups of polymer and phosphate groups on the surface of lipid
117 membrane are regarded as the main mechanisms for lipid membrane destabilization.[32]
118 Therefore, the structures of carboxylated poly(glycidol)s might strongly affect their
119 fusogenic performance. Hence, poly(glycidol)s having various side chain structures
120 were prepared.[44] With increasing hydrophobicity of side chain structures,
121 membrane-disruption properties of these polymers at acidic pH increased.[44] Especially,

122 3-methylglutarylated poly(glycidol) (MGluPG, Figure 2d) exhibited strong membrane
123 fusion activity at weakly acidic pH corresponding to endosomal pH regions, which is
124 suitable performance for pH-responsive intracellular delivery system. Actually,
125 MGluPG-modified liposomes achieved cytoplasmic delivery of calcein to HeLa cells at
126 quicker timing than those of SucPG-modified liposomes.[44]

127 SucPG-modified and MGluPG-modified liposomes were applied to antigen
128 delivery to DCs.[45] Ovalbumin (OVA) was encapsulated as a model antigenic protein in
129 liposomes composed of EYPC and DOPE. OVA-loaded liposomes were added to DC2.4
130 cells, a murine dendritic cell line. Compared with polymer-unmodified liposomes, both
131 SucPG-modified and MGluPG-modified liposomes showed five-times-higher cellular
132 association (Figure 4a).[45] DCs and macrophages are known to engulf microorganisms or
133 apoptotic cells having anionic components *via* scavenger receptors.[46, 47] Considering
134 that SucPG-modified and MGluPG-modified liposomes have negatively charged
135 surfaces attributable to their carboxy groups in polymer side chains, these liposomes are
136 likely to be taken up by DC2.4 cells *via* interaction with scavenger receptors. Actually,
137 the addition of dextran sulfate, an inhibitor of interaction between negatively charged
138 compounds and scavenger receptors, strongly suppressed association of the
139 SucPG-modified liposome-based nanoparticles to DC2.4 cells.[48] In general, negatively

charged surfaces suppress interaction between cells and nanoparticles.[49, 50] Therefore, these pH-sensitive polymer-modified liposomes were taken up by macrophages or DCs in preference to other cells in the body that have no scavenger receptors. Such properties of carboxylated polymer-modified liposomes are particularly beneficial because they achieve selective association of the liposomes to antigen-presenting cells in the body. According to analyses of intracellular distribution of liposomes and FITC-labeled OVA, OVA molecules were delivered into cytosol of DC2.4 cells by MGluPG-modified liposomes, which showed stronger fusion activity than SucPG-modified liposomes did.[45]

Considering the membrane fusion mechanism by viral fusogenic proteins, three-dimensional structures of these proteins might play an important role in generating a defect in lipid membrane and subsequently induce membrane fusion.[51, 52] Therefore, fusogenic polymers having three-dimensional structures might be expected to show stronger fusion activity. For that reason, hyperbranched poly(glycidol)s (HPGs) having various degrees of polymerization (DPs) were used as a main chain of pH-sensitive polymer (Figure 2e).[53] MGlu-HPG, an analogous polymer of linear MGluPG, formed more hydrophobic domains at weakly acidic pH than MGluPG having the same DP.[53] Reflecting this result, MGlu-HPG showed higher membrane disruption ability at weakly

158 acidic pH than MGluPG did.[53] These results indicate that the hyperbranched polymer
159 backbone might be more beneficial for the induction of membrane fusion than the linear
160 polymer backbone. In addition, cellular association increased concomitantly with
161 increasing DP of MGlu-HPG (Figure 4b), indicating that bulkier polymer-modified
162 liposomes might interact with scavenger receptors on DCs by multivalent interactions
163 because of their three-dimensional structures (Figure 4c).[53] After internalization into
164 cells, MGlu-HPG-modified liposomes showed intracellular fusion activity and delivered
165 OVA into cytosol of DC2.4 cells more efficiently than MGluPG-modified liposomes
166 (Figure 4d).[53]

167 As another type of pH-sensitive polymer, pH-sensitive polymer-lipids were
168 prepared for pH-sensitization of liposomes.[54] Compared with random anchor polymers,
169 polymers having phospholipid moiety can introduce the pH-sensitive polymer to the
170 liposome without disruption of the lipid membrane structure. Polymer-lipids of two
171 types were synthesized, MGluPG-PE and CHexPG-PE, which are distearyl
172 phosphatidylethanolamines having 3-methylglutarylated or
173 2-carboxycyclohexane-1-carboxylated poly(glycidol) groups, respectively (Figure 2f).
174 Polymer-lipid-incorporated liposomes were prepared.[54] The polymer-lipid liposomes
175 showed content release at specific pH regions depending on their side chain structures

and polymer-lipid contents.[54] In addition, polymer-lipid liposomes showed high cellular association with DC2.4 cells and delivered contents into the cytosol of cells.[54] Especially, CHexPG-PE exhibited excellent delivery performance, even at lower polymer-lipid contents.[54] Hydrophobicity of the side chain in pH-sensitive polymers strongly affects their pH-sensitivity and intracellular delivery performance.

Application of pH-sensitive polymer-modified liposomes to antigen carrier

pH-Sensitive polymer-modified liposomes achieved cytoplasmic delivery of contents into DCs. Cytoplasmic delivery of antigen into DCs induces MHC class I-mediated presentation (cross-presentation), resulting in induction of cellular immunity (Figure 1). Therefore, the application of these liposomes to induction of antigen-specific immune responses was investigated.

First, the antigen presentation pathway was evaluated using T cells (CD8-OVA1.3 cells or OT4H.1D5 cells), which respectively recognize MHC class I or class II-dependent antigen presentation (Figure 5a).[55, 56] OVA was used as a model antigen and monophosphoryl lipid A (MPLA) was incorporated to liposomes as an adjuvant.[57] Bone marrow-derived dendritic cells (BMDCs) treated with MGluPG-modified liposomes induced higher levels of antigen presentation than that of

194 unmodified liposomes-treated BMDCs (Figure 5).[45, 58] Especially, MGluPG-modified
195 liposomes induced MHC class I-mediated antigen presentation more efficiently than
196 unmodified liposomes (Figure 5b). These results indicate that higher cellular association
197 of liposomes and efficient cytoplasmic delivery of antigen by MGluPG-modified
198 liposomes promoted MHC class I-mediated antigen presentation.

199 Next, OVA-loaded liposomes were administered nasally to mice. Then,
200 OVA-specific antibody production and the induction of OVA-specific cytotoxic T
201 lymphocytes (CTLs) in spleen were evaluated.[45, 59–61] Mucosal surfaces are the main
202 route for pathogens to enter the body. The induction of both mucosal and systemic
203 immunity against pathogens is important to guard against the pathogen's infection.[62–66]
204 Nasal administration of pH-sensitive polymer-modified liposomes to mice induced
205 OVA-specific antibody in serum and intestine.[60, 61] In addition, splenocytes produced
206 Th1 cytokines (IFN-γ) and induced cytotoxic activity against OVA-expressing cells
207 (E.G7-OVA cells) but not against cells without OVA expression (EL4 cells).[45, 61] These
208 results indicate that pH-sensitive polymer-modified liposomes can induce protective
209 immunity against pathogens invading mucosal tissues. To evaluate the usefulness of
210 polymer-modified liposomes as mucosal vaccines, *Salmonella enteritidis*
211 antigen-containing liposomes modified with MGluPG were administered to the eyes of

chickens for control of *Salmonella* infection.[61] Intraocular immunization with *S. enteritidis* antigen-containing MGluPG-modified liposomes induced antigen-specific IgG and IgA production in the serum and intestine. Importantly, MGluPG-modified liposomes induced higher IgA production than commercial *S. enteritidis* vaccine did.[61] Bacteria in fecal waste and cecum were apparently fewer in liposome-immunized chickens than in unimmunized controls.[61] These results indicate that pH-sensitive polymer-modified liposomes induced antigen-specific antibody in mucosal tissues and Th1-polarized immune responses *in vivo* through efficient cross-presentation of antigen, which achieved efficient protection against pathogens.

Application of pH-sensitive polymer-modified liposomes to cancer immunotherapy

The feasibility of pH-sensitive polymer-modified liposomes as antigen delivery carriers were investigated for their use in cancer immunotherapy. Subcutaneous administration of these liposomes to mice induced cellular immune responses in the spleen, as observed for the case of the nasal administration of the same liposomes (Figure 6).[58] Administration of MGlu-HPG-modified and MGluPG-modified liposomes generated antigen-specific CTL at the same efficiency irrespective of the difference of their antigen delivery efficiency *in vitro* (Figures 6b and 4b). Probably, both liposomes

are taken up by antigen-presenting cells in the body enough to induce strong cellular immunity. Consequently, protective immunity against OVA-expressing E.G7-OVA cells was induced, considerably improving mice survival.[58] An example of the therapeutic effects of these liposomes for tumor-bearing mice is depicted in Figure 7. In the experiment, E.G7-OVA cells were injected to mice. Then the tumor volumes were monitored 7 days after OVA-loaded liposomes were administered to these mice. Results show that administration of MGluPG-modified or MGlu-HPG-modified liposomes suppressed tumor growth and regressed the tumor volumes to a significant degree (Figure 7a). In fact, 50–75% of mice were cured completely (Figure 7b).[58] These results indicate that strong cellular immunity induced by pH-sensitive polymer-modified liposomes efficiently killed OVA-expressing tumor cells, leading to tumor rejection and regression. According to the results of cytotoxic activity of splenocytes against EL4 cells (OVA-non-expressing cells), around 10% of non-specific cytotoxicity was observed (Fig. 6c). However, OVA-loaded MGluPG-modified liposomes showed no antitumor effects against EL4-tumor-bearing mice as previously reported.[67] These results clearly indicate that non-specific cytotoxic activity was not sufficient to obtain antitumor effects and E.G7-OVA tumor cells were killed by OVA-specific CTLs induced by pH-sensitive polymer-modified liposomes.

As described above, the membrane-disrupting capabilities of pH-sensitive polymers are influenced by the hydrophobicity of the introduced pH-sensitive moieties. For example, CHexPG-PE liposomes showed sharp destabilization at very weakly acidic pH of around 6.5–7.0, whereas MGluPG-PE liposomes exhibited destabilization at around pH 5.[54] When these polymer-lipid-incorporated liposomes containing OVA were administered subcutaneously to mice, the antigen-specific cellular immunity was induced in mice for both cases.[54] However, immunization with CHexPG-PE liposomes induced stronger therapeutic effects than those with MGluPG-PE liposomes.[54] In fact, we observed complete rejection of OVA-expressing E.G7-OVA cells and marked regression of E.G7-OVA tumors for mice treated with CHexPG-PE liposomes.[54] Strong and highly sensitive properties to very weakly acidic pH for the liposomes might engender the efficient induction of antigen-specific cellular immunity.

From the viewpoint of clinical applications, safer materials are desired for liposome preparation. Therefore, instead of synthetic polymer (poly(glycidol)), biopolymer-based pH-sensitive polymer, 3-methylglutarylted dextran (MGlu-Dex; Figure 2g) was developed.[68] MGlu-Dex-modified liposomes efficiently delivered antigen into the cytosol of DC2.4 cells and induced antitumor effects against tumor-bearing mice.[68] The effects of MGlu group contents and molecular weights of

dextran on their immune-inducement effects were also evaluated. Results show that medium amounts of MGlu group-introduced dextran (MGlu56-Dex) showed the strongest antitumor effects and that the molecular weights of dextran did not affect their immune-inducement effects.[68]

Towards more effective antigen delivery system

Delivery systems require not only cytoplasmic delivery functions but also activation functions of DCs to produce more effective antigen delivery systems. Hence, the combination of activation molecules or systems (adjuvant molecules, or systems) with pH-sensitive polymer-modified liposomes was evaluated to induce more effective cancer immunity (Figure 3).

To date, various adjuvant molecules have been introduced to antigen delivery systems. Typically, toll like receptor (TLR) ligands, which are molecules derived from bacteria or virus components and which are recognized by TLRs expressing in immune-competent cells, are used as adjuvant molecules.[69–71] For example, the incorporation of CpG-DNA, poly(I:C), and MPLA, which are known respectively as TLR9, TLR7, and TLR4 ligands, enhanced the immune-inducement effects of antigen delivery systems. In our studies, MPLA inclusion to MGlu-HPG-modified liposomes as

284 a part of the lipid membrane component strongly promoted their antitumor immunity
285 compared with liposomes without MPLA.[72] However, considering that TLR ligands are
286 obtained from bacteria or viruses, these molecules might present some variations from
287 the perspectives of biological activities, thereby presenting the possibility of unexpected
288 side effects originating from their structural variety and molecular purity. Therefore, to
289 support their practical use, other adjuvants must be considered for the modification of
290 liposome-based vaccines. One candidate adjuvant might be cationic lipids, which are
291 known to have adjuvant function by activation of intracellular signaling *via* interaction
292 with various membrane proteins.[73] 1,2-Dioleoyl-3-dimethylammoniumpropane
293 (DOTAP) is the most-studied cationic lipid that might be used as an adjuvant
294 molecule.[73–76] Actually, DOTAP is recognized by G protein-coupled receptors (GPCRs)
295 on cellular membranes of DCs and activates MAP kinases, which induce up-regulation
296 of co-stimulatory molecules CD80 or CD86 on DCs.[73] Reactive oxygen species (ROS)
297 generated by cationic lipids or cationic liposomes also play a role in inducing multiple
298 signaling pathways for the production of cytokines and up-regulation of co-stimulatory
299 molecules.[77] Furthermore, cationic lipids having an amidine group are known to have
300 adjuvant functions *via* interaction not only with GPCRs but also TLR4.[73, 78] Inspired by
301 these studies, 3, 5-didodecyloxybenzamidine (TRX, Figure 3) was selected as a cationic

302 lipid and was incorporated into MGlu-HPG-modified liposomes to increase their
303 immune-inducement effects.[72] In fact, TRX inclusion into MGlu-HPG-modified
304 liposomes increased the cellular association of liposome and promoted the production of
305 various cytokines (IFN-γ, IL-10, TNF-α, IL-6) from DCs depending on their TRX
306 contents.[72] Moreover, TRX inclusion was found to change the intracellular distribution
307 of liposomes and antigen: TRX-containing MGlu-HPG-modified liposomes delivered
308 OVA not only to cytosol but also endosome/lysosome.[72] Positively charged TRX might
309 enhance the association of negatively charged MGlu-HPG chains on the surface of
310 liposomal membranes through electrostatic interaction. Therefore, the polymer chains
311 might interact with the liposomal membranes rather than the endosomal membrane,
312 resulting in OVA delivery in endosomes. This fact suggests that intracellular
313 distributions of OVA in cytosol, endosomes, and lysosomes can be controlled by
314 adjusting cationic lipid contents in the liposomes. According to the results of antigen
315 presentation pathway and analysis of antibody production, not only CTL activation but
316 also Th1-polarized immune responses were induced by TRX-containing
317 MGlu-HPG-modified liposomes, which might correspond to efficient
318 endosome/lysosome delivery of OVA.[72] Th1 cells support CTL activation through
319 secretion of Th1 cytokines such as IFN-γ. Reflecting these results, antitumor effects on

320 tumor-bearing mice were found to be improved by TRX inclusion to
321 MGlu-HPG-modified liposomes.[72]

322 It has been demonstrated that Th1 cytokines such as IFN-γ activate cellular
323 immune response efficiently *via* the promotion of antigen presentation.[79] Therefore, the
324 combination of cytoplasmic antigen delivery and IFN-γ delivery is an attractive strategy
325 for the induction of strong cellular immune responses. However, the half-life of IFN-γ
326 protein administered to the body is too short and systemic delivery of IFN-γ induces
327 remarkable side effects.[80] Therefore, transfection of IFN-γ-encoding gene to DCs or
328 tumor cells has been studied instead of systemic delivery of IFN-γ protein.[81–83] In our
329 study, the IFN-γ gene was simultaneously delivered as hybrid complexes between
330 liposomes and lipoplexes, which were previously reported as efficient non-viral gene
331 carriers for DCs, or as separate administration of liposomes and lipoplexes without
332 pre-mixing.[48, 84] MGluPG-modified liposomes containing OVA were complexed with
333 TRX-based lipoplexes containing IFN-γ gene *via* electrostatic interaction. Confocal
334 laser scanning microscopic analysis using fluorescence-labeled complexes has revealed
335 that hybrid complexes delivered both antigen and gene into the cytosol of DC2.4 cells
336 by fusion activity with endosomal membranes derived from MGluPG-modified
337 liposomes.[84] Treatment with hybrid complexes induced IFN-γ production from DC2.4

338 cells, which indicates that IFN-γ gene was transfected to DC2.4 cells by hybrid
339 complexes.[84] The OVA-loaded MGluPG-modified liposomes or hybrid complexes were
340 administered to E.G7-OVA tumor-bearing mice and then, tumor growth was monitored.
341 Unexpectedly, antitumor effects induced by hybrid complexes were almost identical to
342 those of MGluPG-modified liposome.[84] Next, OVA-loaded MGluPG-modified
343 liposomes and IFN-γ gene-containing lipoplexes were administered without pre-mixing
344 at the same site (Combination delivery). In the case of Combination delivery, antitumor
345 effects were enhanced strongly compared with MGluPG-modified liposome and all
346 treated mice became completely tumor-free during 60 days.[84] For comparison,
347 liposomes and lipoplexes were administered at distant sites in tumor-bearing mice
348 (Separate delivery). Separate delivery induced almost identical antitumor effects to
349 those of liposomes and effects were less than those obtained with Combination delivery,
350 which suggests that injection of antigen and IFN-γ gene at the same site is important to
351 obtain their synergetic effects.[84] Actually, the induction of CTLs in spleen was enhanced
352 by Combination delivery.[84] In addition, immunofluorescence staining of the tumor
353 section revealed that infiltration of CTLs into tumor tissues at an early stage of tumor
354 (Day 10) was promoted to a greater degree by Combination delivery than by
355 MGluPG-modified liposomes (Figure 8a).[84] According to the results of H&E staining of

356 tumor sections, more tumor cells were killed at early timing by Combination delivery
357 than liposomes (Figure 8b). These results indicate that Combination delivery of antigen
358 and IFN-γ gene is an effective strategy for the enhancement of antitumor immunity.
359
360 **Summary and outlook**
361 In this review, pH-sensitive polymer-based antigen delivery systems for the
362 induction of antigen-specific immune response were overviewed. The pH-sensitive
363 polymer structures strongly affected their pH-sensitivity, cellular association,
364 intracellular delivery performance, properties of DC activation, and *in vivo*
365 immune-inducing functions. Highly hydrophobic side chain structures are suitable for
366 the efficient intracellular delivery of antigens and for the induction of antitumor
367 immunity. Dextran-based pH-sensitive polymers were also developed as safer functional
368 polymers. For dextran-based polymers, the contents of pH-sensitive units strongly
369 affected their properties of antigen delivery and induction of antitumor immunity.
370 Therefore, not only the side chain structure, but also their contents on polymer chains
371 should be optimized further to obtain more effective pH-sensitive polymer-based
372 antigen carriers. The inclusion of cationic lipids to pH-sensitive polymer-modified
373 liposomes has promoted their immune-inducing effects considerably. Not only cationic

374 lipids, but varying adjuvant molecules such as TLR ligands can be incorporated into the
375 liposomes. Specific combinations of TLR ligands are known to induce synergetic
376 immune-activating effects.[85, 86] Therefore, the inclusion of multiple TLR ligands to
377 cationic lipid-containing liposomes might generate highly potent antigen delivery
378 systems. Notably, the combination of cytokine gene delivery system (lipoplexes) and
379 liposomes achieved the complete cure of tumor-bearing mice. The combination of other
380 cytokine or chemokine genes such as IL-12, TGF-β, or CCR7 might create various
381 immune-inducing systems or immunosuppression cancelling systems in tumor
382 microenvironments.

383 Our recent and current studies have used a model antigen (chicken egg albumin,
384 OVA), which has strong immunogenicity against mouse, for the evaluation of immune
385 responses of pH-sensitive polymer-modified liposomes. For more practical evaluation,
386 we have started the use of human cancer antigenic peptides such as glypican-3
387 (GPC3)-derived peptide, which is overexpressed in hepatocellular carcinoma, or
388 insulin-like growth factor II mRNA-binding protein 3 (IMP-3)-derived peptide, which is
389 overexpressed in cases of head-and-neck malignant tumor, lung cancer, and esophageal
390 cancer, for the evaluation of pH-sensitive polymer-modified liposomes.[87, 88] Actually,
391 CHexPG-PE liposomes containing any of the respective peptides induced much more

efficient cross-presentation than free peptide solution on human autologous DCs.[87, 88] These latest studies indicate clearly that our pH-sensitive polymer-modified liposomes present the potential for immune-inducing functions, not only for model antigenic proteins but also for clinically used cancer antigenic peptides. We believe that further optimizations of pH-sensitive polymer structures, adjuvant molecules inclusion, and the combination of cytokine gene delivery systems on pH-sensitive polymer-modified liposomes will provide practical levels of antigen delivery systems for use in cancer immunotherapy.

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681 **Titles and legends to figures**

682 **Figure 1.** Antigen presenting pathway of dendritic cell. Cellular immunity is generally
683 induced *via* MHC class I presentation of endogenous antigen. It is known that a part of
684 exogenous antigen escape from endosome and presented by MHC class I molecules
685 ("Cross-presentation"). To promote the cross-presentation process, pH-sensitive
686 molecules have been studied for destabilization of endosomal membrane or induction of
687 fusion with endosomal membrane.

688 **Figure 2**. Structures of pH-sensitive polymers: (a) poly(2-ethylacrylic acid), (b)
689 poly(2-propylacrylic acid), (c) SucPG, (d) MGluPG, (e) MGlu-HPG with DP of 40, (f)
690 MGluPG-PE and CHexPG-PE, and (g) MGlu-Dex.

691 **Figure 3.** Design of liposomal vaccine having pH-sensitive activity and immune
692 activation function. Antigen-loaded liposomes were modified with pH-sensitive
693 polymers and adjuvant molecules/systems (such as TLR ligands, cationic lipids, or
694 cytokine (IFN-γ) gene delivery systems). After internalization *via* endocytosis,
695 liposomes induce fusion with endosomal membrane responding to acidic pH inside of
696 endosomes, which causes the transfer of most of antigen into cytosol as shown in the
697 fluorescence microscopic image. As a result, cross-presentation is promoted, leading to
698 the induction of cellular immunity. In addition, dendritic cells are activated by

699 stimulation *via* interaction between adjuvant molecules and receptors, which causes
700 activation of immune responses. Otherwise, IFN-γ produced by Th1 cells or IFN-γ
701 gene-transfected dendritic cells also activate cellular immunity. Microscopic image in
702 the figure shows DC2.4 cells treated with FITC-OVA-loaded MGlu-HPG liposomes
703 labeled with Rhodamine-PE. Green fluorescence shows the location of FITC-OVA and
704 red fluorescence shows the location of liposomes.

705 **Figure 4.** (a, b) Mean fluorescence intensity of DC2.4 cells treated with liposomes
706 modified with SucPG, MGluPGs and MGlu-HPGs having various DPs. Cellular
707 association of liposomes to DC2.4 cells was promoted by carboxylated polymer
708 modification (a) and increased with increasing DP of MGlu-HPG (b). (c) Schematic
709 illustration for the interaction of MGlu-HPG-modified liposomes with DCs. (d)
710 Confocal laser scanning microscopic (CLSM) images of DC2.4 cells treated with
711 Rh-PE-labeled and FITC–OVA-loaded liposomes modified with MGlu-HPG60,
712 MGluPG76 or without polymers. Intracellular localization of Rh-PE (red) and FITC–
713 OVA (green) was observed using a CLSM. Scale bar represents 10 μm. Partially
714 reproduced from Yuba *et al.*[45, 53] with permission. Copyright (2010 and 2011) Elsevier.

715 **Figure 5.** Presentation of OVA-derived epitope peptides *via* MHC molecules in
716 BMDCs. (a) Schematic illustration of experimental procedure. BMDCs were incubated

717 with free OVA (diamonds), OVA-loaded MGluPG-modified liposomes (triangles) and
718 unmodified liposomes (squares) at varying OVA concentrations for 3 h. Concentrations
719 of IL-2 in the medium after co-culture of OVA-treated BMDCs with (b) CD8-OVA1.3
720 (specific for $OVA_{257–264}$/H-2 K^b complex, MHC class I pathway) and (c) OT4H.1D5
721 (specific for $OVA_{265–277}$/I-A^b complex, MHC class II pathway) cells for 24 h as a
722 function of OVA concentration during the BMDCs treatment were shown. Partially
723 reproduced from Yuba *et al.*[45] with permission. Copyright (2010) Elsevier.

724 **Figure 6.** OVA-specific CTL induction in spleen after 7 days from subcutaneous
725 immunization with PBS (closed diamonds), OVA solution (open diamonds),
726 unmodified liposomes (closed triangles), MGluPG-modified liposomes (open triangles),
727 MGlu-HPG-modified liposomes (closed squares) and Complete Freund's adjuvant
728 (CFA, open squares). (a) Schematic illustration of experimental procedure. Cytotoxic
729 activity was measured by a lactate dehydrogenase (LDH) assay at various effecter
730 cell/target cell (E/T) ratios. Amount of OVA administered was 100 μg per mouse.
731 E.G7-OVA cells (b) and EL4 cells (c) were used as target cells. Partially reproduced
732 from Yuba *et al.*[58] with permission. Copyright (2013) Elsevier.

733 **Figure 7.** Antigen-specific antitumor effect induced by subcutaneous administration of
734 pH-sensitive polymer-modified liposomes. The E.G7-OVA cells were subcutaneously

735 inoculated into the left backs of C57BL/6 mice and then liposomes with or without
736 pH-sensitive polymers containing 100 μg of OVA were subcutaneously administered
737 into the right backs of the mice on days 7 and 14. Mice immunized with PBS were
738 shown as controls. All treated groups contained four mice. (a) Change in tumor volume
739 and (b) survival curves of mice were shown. Partially reproduced from Yuba *et al.*[58]
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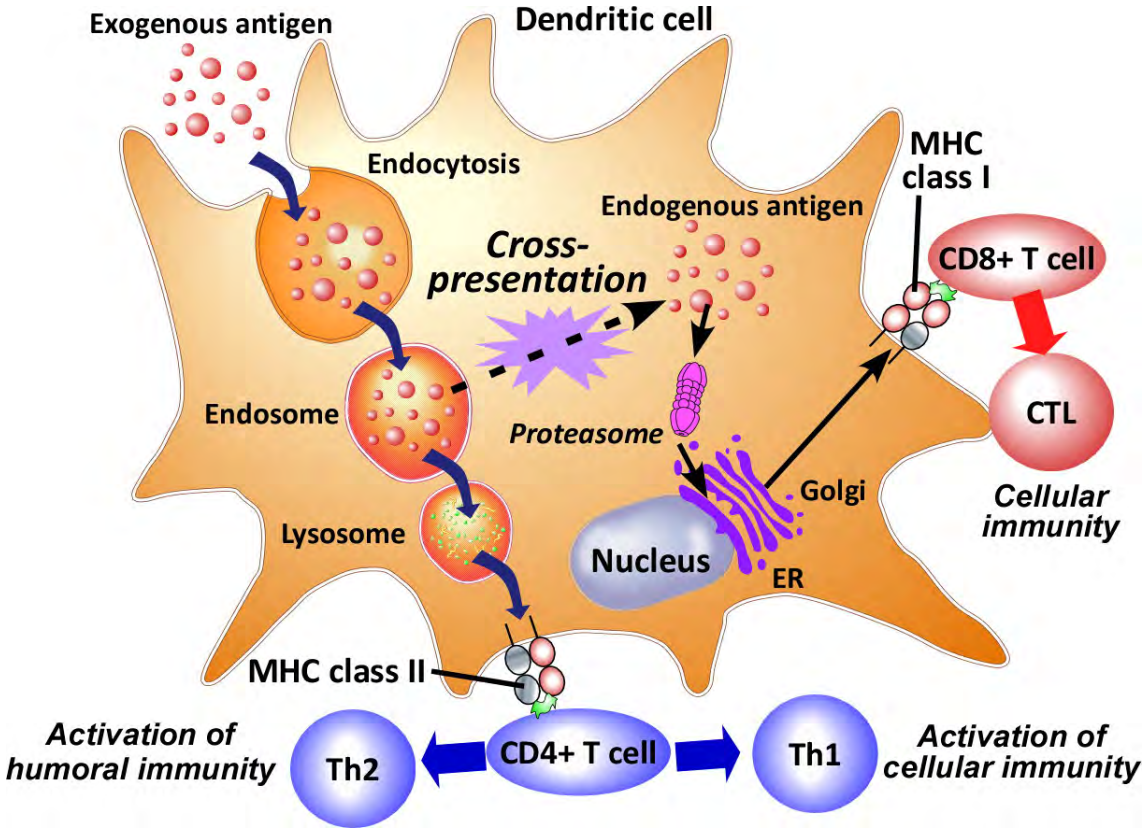
741 **Figure 8.** (a) Immunofluorescence analysis of tumor sections from mice subcutaneously
742 administered with PBS, OVA-loaded MGluPG-modified liposomes, or combination of
743 OVA-loaded MGluPG-modified liposomes and the IFN-γ gene-containing lipoplexes on
744 days 5 and/or 12. CD8 positive cells in tumor sections were stained using anti-mouse
745 CD8 antibody and Cy3-anti-rat IgG as a secondary antibody (Red). Cellular nucleus
746 were stained by DAPI (Blue). (b) H&E staining for tumor sections from mice
747 subcutaneously administered with PBS, OVA-loaded MGluPG-modified liposomes, or
748 combination of OVA-loaded MGluPG-modified liposomes and the
749 IFN-γ gene-containing lipoplexes on days 5 and/or 12. Magnified images for regions (1:
750 normal tumor cells, 2/4: damaged cells with deformed nuclei, 3: denucleated necrotic
751 cells, 5: fibrotic tissues and fibroblast-like cells) in the middle images are shown in the
752 bottom. Partially reproduced from Yuba *et al.*[83] with permission. Copyright (2015)

753 Elsevier.



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756 *Yuba, Figure 1.*

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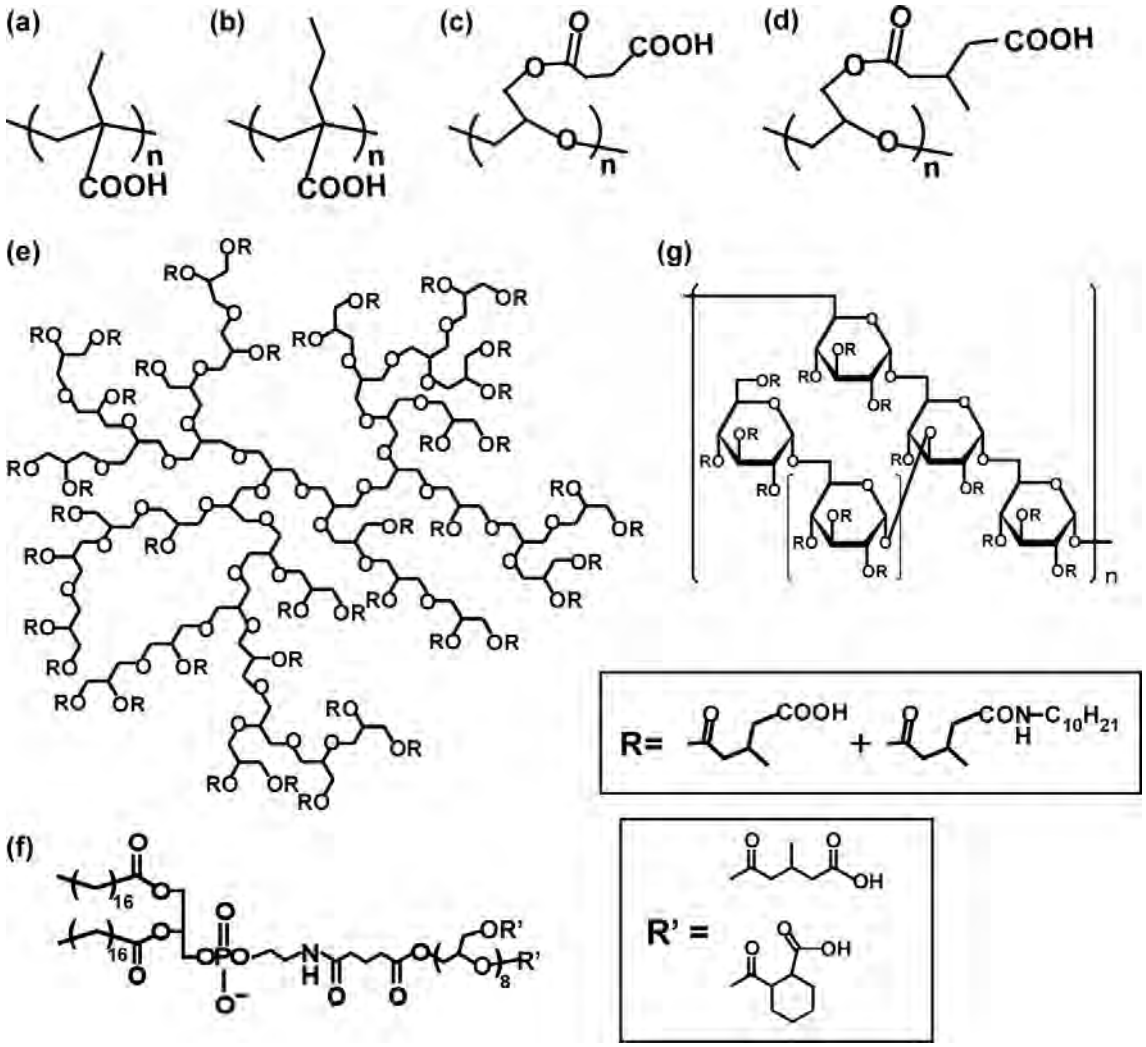
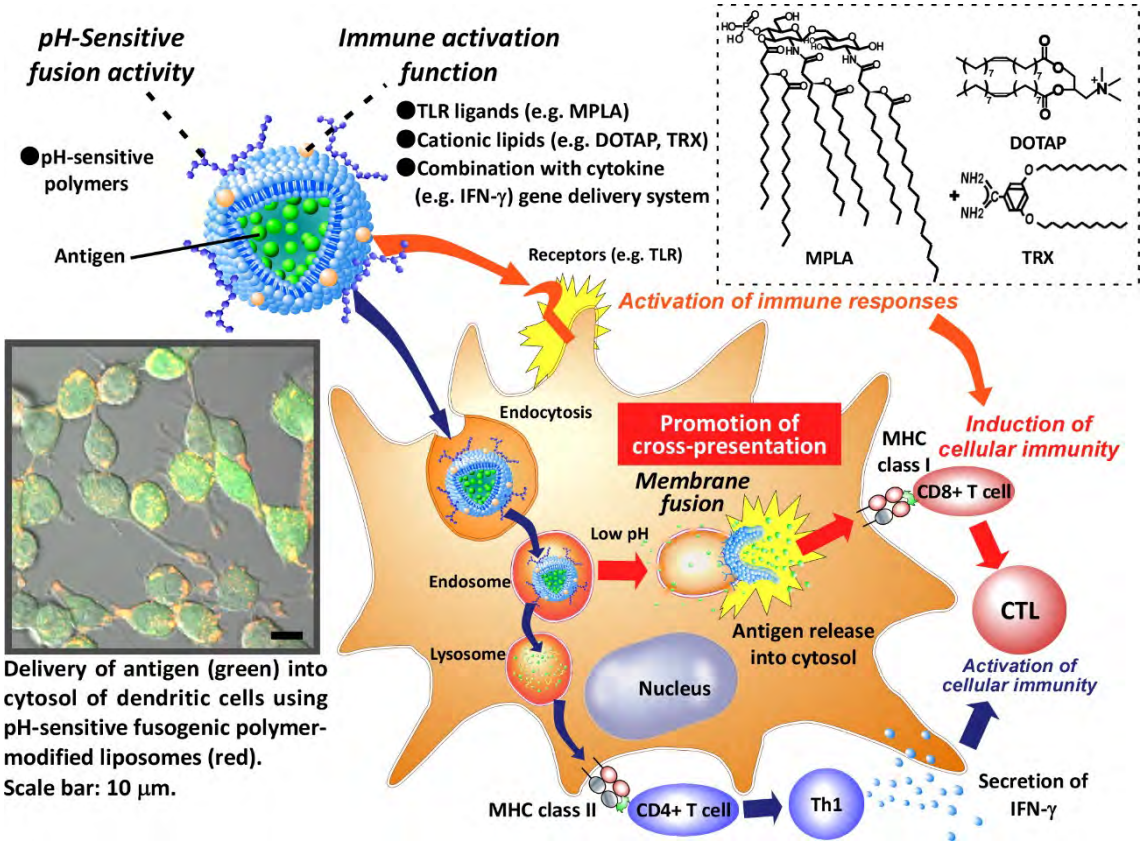
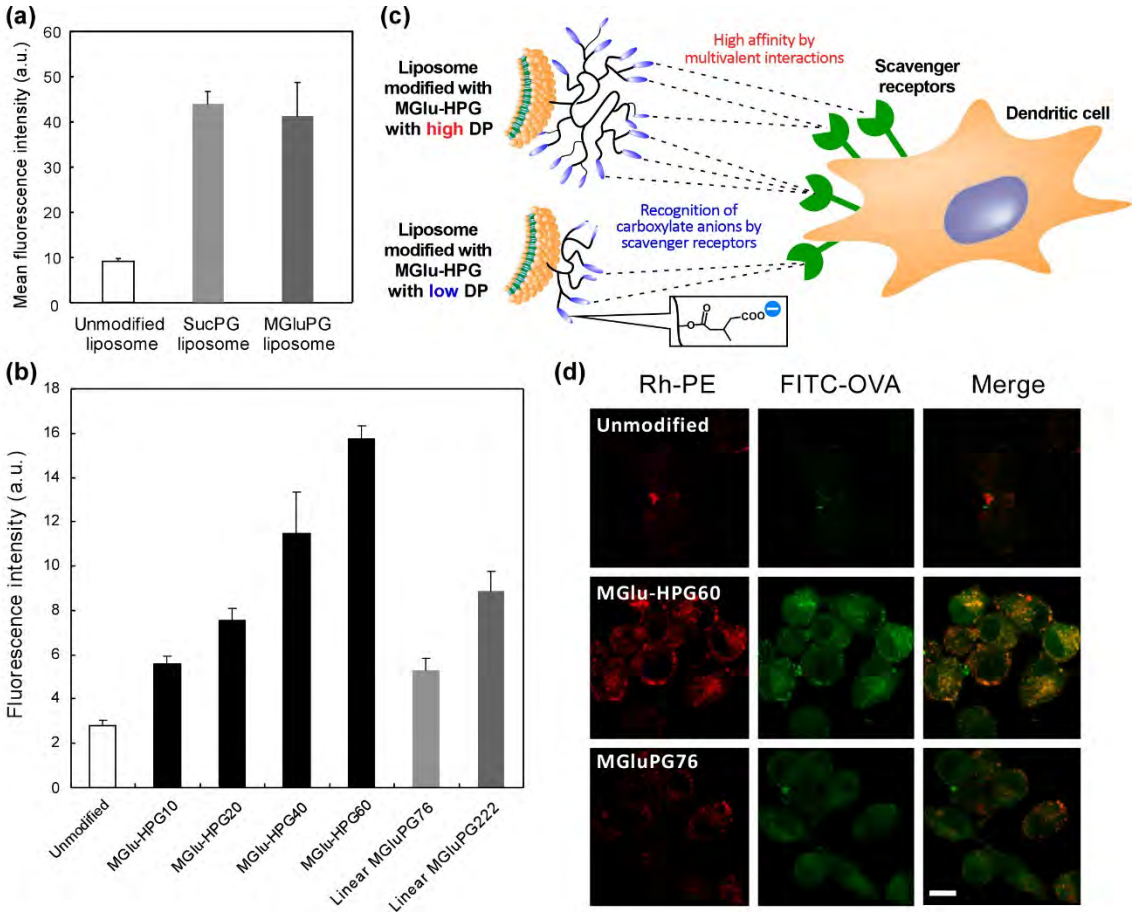


Yuba, Figure 2.

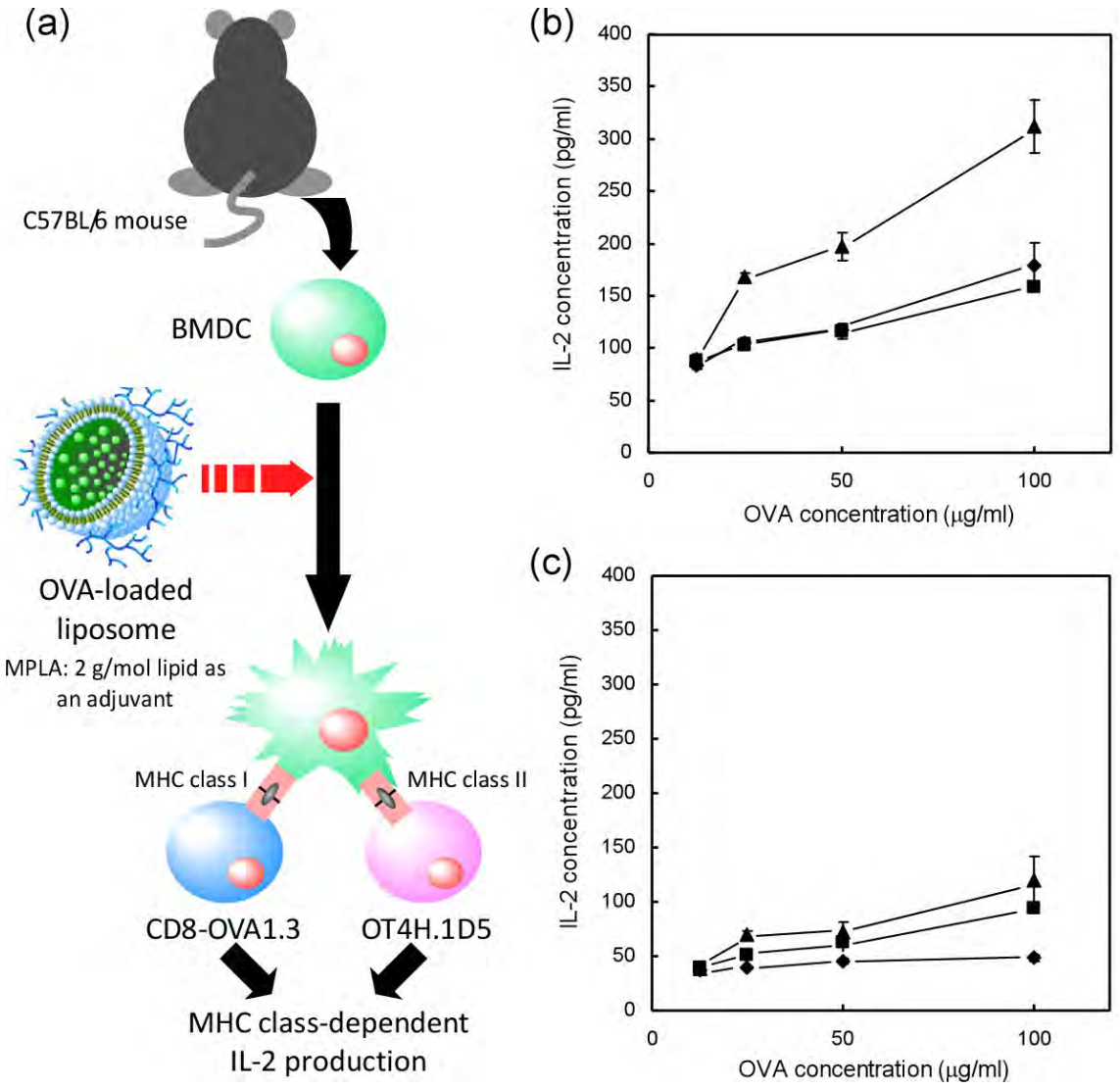


Delivery of antigen (green) into cytosol of dendritic cells using pH-sensitive fusogenic polymer-modified liposomes (red). Scale bar: 10 μm.

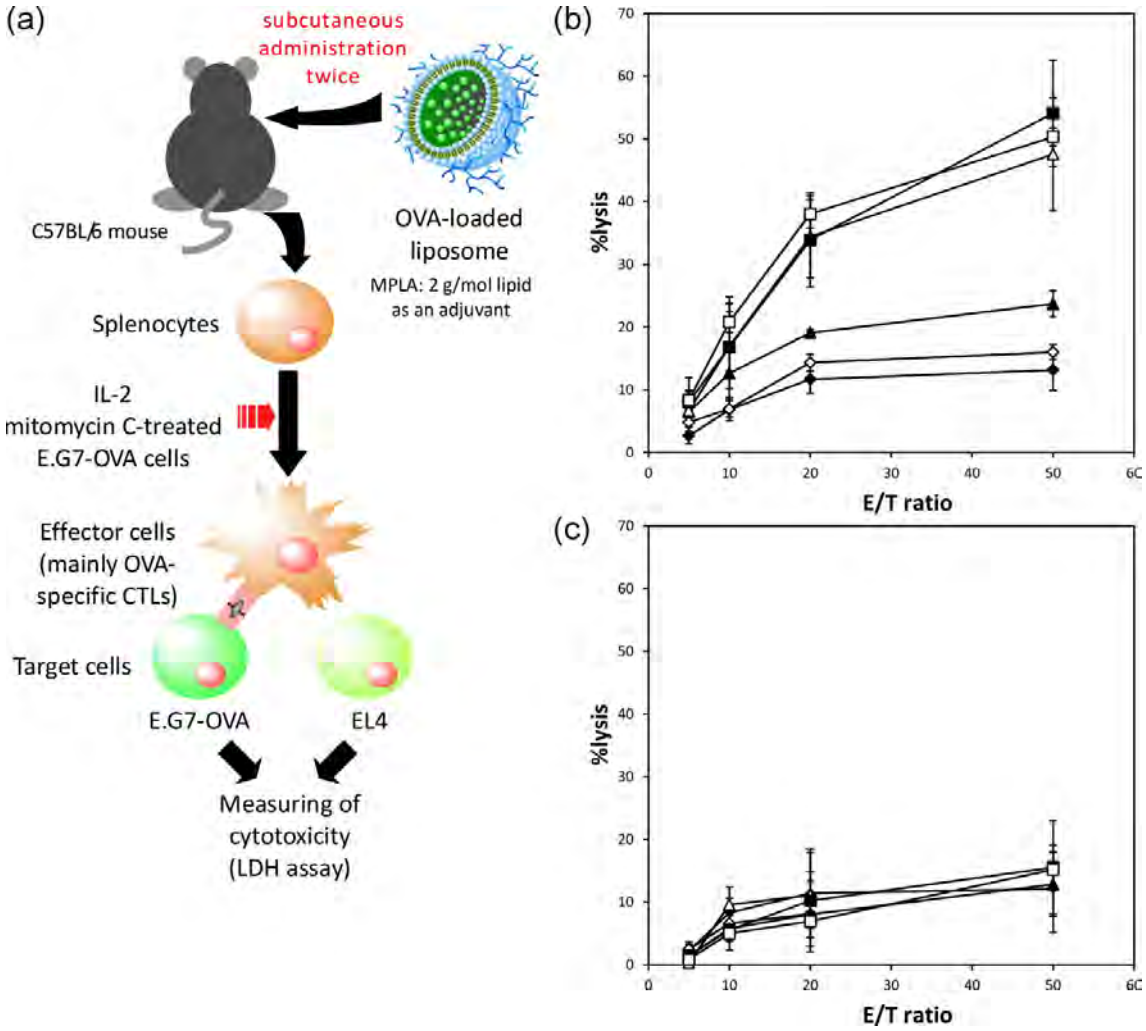
Yuba, Figure 3.



Yuba, Figure 4.



Yuba, Figure 5.



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772 *Yuba, Figure 6.*

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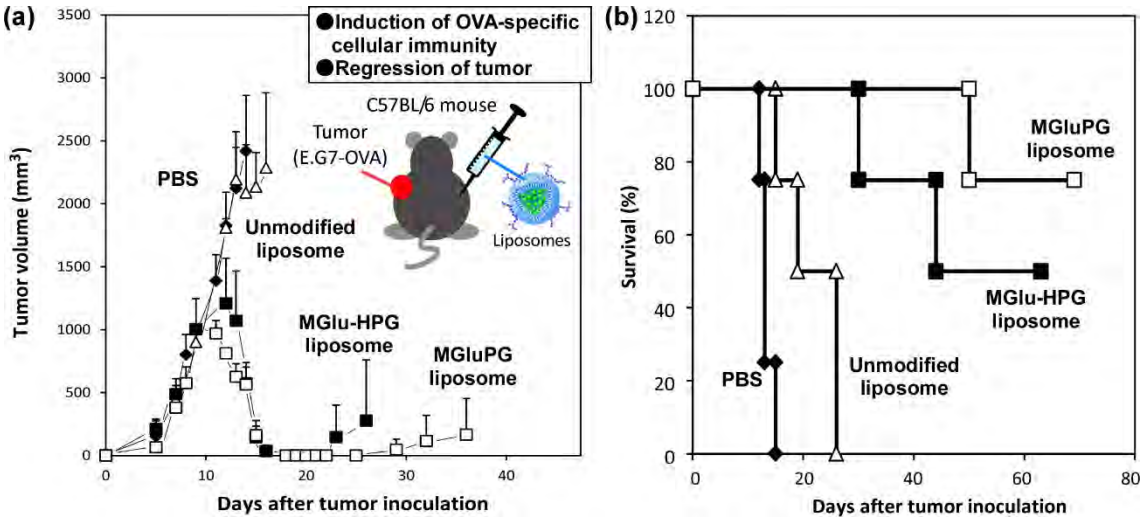

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776 *Yuba, Figure 7.*

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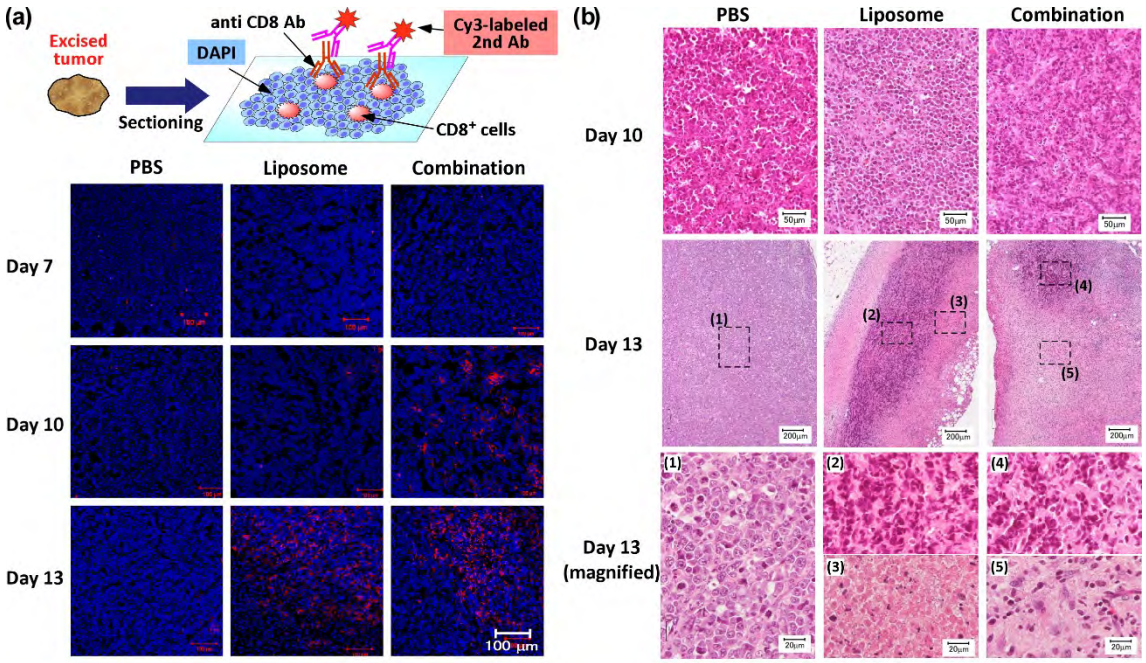

Yuba, Figure 8.