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A Novel Platform for Cancer Vaccines: Antigen-Selective Delivery to Splenic Marginal Zone B Cells via Repeated Injections of PEGylated Liposomes

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Treating cancer with vaccines has been a challenge. In this study, we introduce a novel Ag delivery platform for cancer vaccines that delivers an encapsulated Ag to splenic marginal zone B (MZ-B) cells via the aid of a PEGylated liposome (PL) system. Splenic MZ-B cells have recently attracted interest as alternative APCs. In mice, preimmunization with empty (no Ag encapsulation) PLs triggered the efficient delivery of a subsequent dose of Ag-containing PLs, injected 3 d later, to the spleen compared with a single dose of Ag-containing PLs. In addition, immunization with empty PLs allowed three subsequent sequential injections of OVA-PLs to efficiently induce a CTL response against OVA-expressing murine thymoma (EG7-OVA) cells and resulted in in vivo growth inhibition of subsequently inoculated EG7-OVA cells. However, these sequential treatments require repeated immunizations to achieve their antitumor effect. Therefore, to improve the antitumor effect of our novel vaccine system, an adjuvant, α-galactosylceramide (αGC), was incorporated into the OVA-PLs (αGC/OVA-PLs). As expected, the incorporation of αGC reduced the required number of immunizations with OVA-PLs to the point that a single immunization treatment with empty PLs and an injection of αGC/OVA-PL efficiently triggered a potent CTL induction, resulting in a rejection of the development and a suppression of the growth of tumors that had already developed s.c. Results of this study indicate that a novel Ag delivery platform that grants efficient Ag delivery to splenic MZ-B cells shows promise as a therapeutic modality for conquering tumor growth and/or progression. ***The Journal of Immunology*****, 2018, 201: 2969–2976.**

Cell-mediated immunity plays a crucial role in immune responses against cancer. CTLs are key immune cells in antitumor immunity that recognize tumor-specific Ags on tumor cells, which lead to tumor cell destruction (1). Before CTLs are effective, however, tumor-specific Ags must first be processed and presented by APCs to CTLs within the context of an MHC class I (MHC I) (2). Accordingly, Ag delivery systems to APCs have been designed to trigger efficient CTL-mediated immune responses. In preclinical and clinical situations, many delivery vehicles, such as immunostimulating complex (3), liposomes (4), virus-like particles (5), and polymeric nanoparticles (6), have been adopted to ensure efficient tumor-specific Ag delivery to APCs. These delivery systems retain Ags on the surface of, or inside of, particles that can be easily taken up by APCs by virtue of their size (50–3000 nm). Of note, such exogenous Ags incorporated into delivery vehicles are generally sorted to an MHC class II pathway rather than to MHC I. However, previous reports have demonstrated the ability of dendritic cells to present/process particular Ags (7) and soluble Ag (8, 9) on MHC I molecules via a process known as cross-presentation. Accordingly, based on cross-presentation theory, many researchers have focused on developing cancer vaccines that target dendritic cells (10).

B cell–targeted cancer vaccine has recently been reported as an alternative to dendritic cell–targeted cancer vaccine (11–13). Besides their well-known function as Ab-secreting cells, as with dendritic cells, B cells are known to act as APCs and exhibit the potential to induce CTL immune responses via the cross-presentation of tumor-associated Ags (14, 15). Of particular importance, marginal zone B (MZ-B) cells localized in the spleen highly express CD80/86 costimulatory molecules and efficiently activate T cells (16). Nonetheless, reports emphasizing the limited Ag uptake by B cells have nullified the potential role of B cells as APCs that can activate CTLs (17, 18). These findings raise the assumption that efficient Ag delivery to B cells could restore/augment their APC capacities with the elicitation of potent CTL immune responses against an associated Ag.

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Abbreviations used in this article: 7-AAD, 7-amino-actinomycin; Chol, cholesterol; EG7-OVA, OVA-expressing murine thymoma EL4; FO-B, follicular B; αGC, α-galactosylceramide; HEPC, hydrogenated egg phosphatidylcholine; MDSC, myeloid-derived suppressor cell; MHC I, MHC class I; $mPEG_{2000}$-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000]; MZ-B, marginal zone B; PL, PEGylated liposome; Treg, regulatory T cell.

Upon repeated administration in the same animal within a 2 to 5 d interval, we recently reported a unique phenomenon by which a second dose of PEGylated liposomes (PLs) was transported from the splenic marginal zone to the follicle region where they triggered the elicitation of an Ag-specific immune response (19). Although the exact mechanism underlying such a phenomenon remains under investigation, we assumed that anti-PEG IgM Abs, produced in response to a first dose, bind to a second dose of PLs and subsequently activate the complement system. Such an immune complex of PL/anti-PEG IgM/complement components is then recognized and taken up by MZ-B cells expressing complement receptor followed by transport from the splenic marginal zone to the follicle region in a manner similar to that encountered by blood-borne Ags. This raises the possibility that PLs may act as a novel Ag delivery platform that could efficiently deliver Ag to MZ-B cells with subsequent transport of the Ag to a follicle, which would lead to the elicitation of Ag-specific immune responses.

In this study, therefore, we investigated whether pretreatment with empty PLs could enhance the selective delivery of tumor-specific Ag-containing PLs to MZ-B cells and enhance antitumor immune responses.

Materials and Methods

Materials

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (Chol) was purchased from Wako Pure Chemical Industries (Osaka, Japan), and 7-amino-actinomycin D (7-AAD) was purchased from Becton Dickinson (Franklin Lakes, NJ). CFSE was purchased from Life Technologies (Eugene, OR). OVA (grade V; Sigma-Aldrich, St. Louis, MO), a model Ag, was purchased from Sigma-Aldrich. α-galactosylceramide (αGC; KRN7000), an adjuvant, was purchased from Funakoshi (Tokyo, Japan). All other reagents were of analytical grade.

Animals and cell line

Male C57BL/6N mice were purchased from Japan SLC (Shizuoka, Japan) and were 6–8 wk old at the beginning of each experiment. All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the Tokushima University. EL4 thymoma transfected with OVA (EG7-OVA) cells were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Mediatech, Manassas, VA), 100 U/ml penicillin, 100 μg/ml streptomycin (MP Biomedicals, CA), and 50 μmol/l 2-ME under a humidified atmosphere of 5% CO_2/95% air at 37˚C.

Preparation of liposome

PLs composed of HEPC/$mPEG_{2000}$-DSPE/Chol (1.85:0.15:1, molar ratio) were prepared as described previously (20). To detect the liposome distribution in spleen and their association with spleen cells, the liposomes were labeled with the hydrophobic fluorescent dye DiI (1 mol% of liposomal phospholipid). To enhance antitumor immune responses, αGC was incorporated into the liposomes (HEPC/$mPEG_{2000}$-DSPE/Chol/αGC equal to 1.85:0.15:1:0.015, molar ratio). Encapsulation of OVA or Alexa Fluor 647–labeled OVA into these liposomes was performed using a freeze/thaw method. Freezing (−81˚C, 2 h) and thawing (37˚C, 15 min) was repeated three times. Unencapsulated OVA was removed by size-exclusion chromatography (Sepharose CL-4B; GE Healthcare, Uppsala, Sweden). The mean diameter of the resultant liposomes was determined using a Nicomp 370 HPL submicron particle analyzer (Particle Sizing Systems, Santa Barbara, CA) and was ∼110 ± 13 nm. Encapsulated OVA in the liposomes was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Berkeley, CA), and phospholipid concentrations in the liposome preparations were determined via colorimetric assay (21). The loading amount of OVA was ∼20–40 μg OVA/μmol phospholipids.

Prophylactic immunization

For prophylactic immunization studies, empty PL (0.01 μmol phospholipids/kg) was i.v. injected into mice as a prior treatment. Three days later, the treatment with OVA-containing PLs (OVA-PL) (10 μg OVA per mouse) was begun. The combined treatment with empty PL and OVA-PLs was carried out three times every 2 wk. For another prophylactic immunization study, empty PLs (0.01 μmol phospholipids/kg) were i.v. injected into mice as a prior treatment. Three days later, αGC/OVA–containing PLs (αGC/OVA-PLs) (10 μg OVA per mouse) were i.v. injected once. Emulsion of OVA and CFA, a positive control, was s.c. injected instead of OVA-PLs without a prior treatment of PLs. Antitumor activity of the prophylactic immunization was evaluated in EG7-OVA tumor–bearing mice. EG7-OVA cells (10^6 cells per mouse) were s.c. inoculated at either day 7 or 14 after the last injection of either OVA-PLs or αGC/OVA-PLs. The tumor volume was measured every 3 d using a caliper. Tumor volume was calculated using the following formula:

$$\text{Tumor volume } (\text{mm}^3) = 0.5 \times (\text{length}) \times (\text{width})^2.$$

Therapeutic immunization

EG7-OVA cells were s.c. inoculated into mice. When the tumor volume reached 50–100 mm^3, empty PLs were i.v. injected as a primary treatment. Three days later, αGC/OVA-PLs (10 μg OVA per mouse) were i.v. injected once. Tumor-bearing mice given αGC/OVA-PLs (10 μg OVA per mouse) without the prior treatment served as the positive controls. During treatments, tumor growth was monitored as described above.

In vitro CTL assay

Spleen samples were collected from mice and suspended into RPMI 1640 medium by being pressed through a cell strainer (Greiner Bio-One, Frickenhausen, Germany). RBCs in the suspension were lysed via treatment with ammonium chloride lysis buffer (0.83% NH_4Cl) for 3 min. The obtained spleen cells (4×10^6 cells/ml) were cultured in the presence of OVA (100 μg/ml) for 5 d to restimulate OVA-specific CTLs, and the resultant cells were used as effector cells. EG7-OVA cells, which are target cells expressing OVA, were inactivated with mitomycin C (100 μg/ml, 37˚C, 1 h) and labeled with CFSE (100 μM, 37˚C, 10 min). Both effector and target cells were cocultured for 12 h at various cell ratios (E:T ratio equal to 12.5:1, 25:1, and 50:1). Following staining with 7-AAD, apoptosis of the target cells ($CFSE^+$, $7\text{-}AAD^+$) was analyzed via a Guava EasyCyte Mini flow cytometer, (Millipore, MA). The data were analyzed using WinMDI software version 2.9 (The Scripps Research Institute, San Diego, CA).

Analysis of splenic immune cells

A spleen cell suspension was prepared as described above. Frequencies of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) were measured as indicators of the immune suppressive environment. Spleen cells were incubated with combinations of Abs (Treg, PE-labeled anti-mouse CD4 and Alexa Fluor 488–labeled anti-mouse CD25; MDSC, PE-labeled anti-mouse Ly-6G, and FITC-labeled anti-mouse CD11b [eBioscience, Santa Clara, CA]). To detect NKTs, which are activated by αGC, the cells were stained with FITC-labeled anti-mouse CD3 Ab (eBioscience) and T-Select Mouse CD1d Tetramer (Medical & Biological Laboratories, Nagoya, Japan).

Ag uptake and presentation by MZ-B cells and follicular B cells

For uptake study, empty PL (0.01 μmol phospholipids/kg) was i.v. injected into mice as a prior treatment. Three days later, either DiI-labeled PL (5 μmol phospholipids/kg) or fluorescence-labeled OVA-containing PL (10 μg OVA per mouse) was i.v. injected. At 24 h, spleen was collected from mice and a spleen cell suspension was prepared. The spleen cells were stained with FITC-labeled anti-mouse CD21/35 (eBioscience) and Alexa Fluor 647–labeled anti-mouse CD23 (BioLegend). The uptake of liposomes and OVA was analyzed by a Gallios flow cytometer (Beckman Coulter). The data were analyzed using Kaluza software (Beckman Coulter). MZ-B cells were gated on $CD21^{high}$ and $CD23^-$ population, and follicular B (FO-B) cells were gated on $CD21^{low}$ and $CD23^+$ population.

For Ag presentation study, empty PL (0.01 μmol phospholipids/kg) was i.v. injected into mice. Three days later, αGC/OVA-PL (10 μg OVA per mouse) was i.v. injected. At 24 h, spleen was collected, and the spleen cell suspension was stained with PE-labeled anti-mouse H-2Kb bound to SIINFEKL Ab (BioLegend) in combination with anti-CD21/35 and anti-CD23 Ab. Ag presentation by MZ-B cells and FO-B cells was analyzed by flow cytometry.

Adoptive transfer of MZ-B cells and FO-B cells

Empty PL (0.01 μmol phospholipids/kg) was i.v. injected into mice. Three days later, αGC/OVA-PL (10 μg OVA per mouse) was i.v. injected. At 1 h, spleen was collected, and spleen cell suspension was prepared. MZ-B cells and FO-B cells were isolated using an MZ and FO B Cell Isolation Kit (Miltenyi Biotec). Isolated MZ-B cells or FO-B cells (5×10^5 cells per mouse) were adoptively transferred to naive mice via i.v. injection. Seven days later, spleen was collected, and OVA-specific CTL was stained with FITC-labeled anti-mouse CD8 Ab and T-Select H-2Kb OVA Tetramer-SIINFEKL-PE (Medical & Biological Laboratories). OVA-specific CTL in spleen was analyzed by flow cytometry.

Statistical analysis

All values are expressed as the mean ± S.D. Statistical analysis was performed using a two-tailed unpaired *t* test and one-way ANOVA followed by a Tukey post hoc test using GraphPad InStat software (GraphPad Software, La Jolla, CA). The level of significance was set at $p < 0.05$.

Results

Effect of prophylactic immunization on the induction of CTLs and tumor growth

In this study, OVA and EG7-OVA cells were used as a model Ag and a model target tumor cell, respectively, to prove our research concept. First, we investigated whether immunization with OVA-PLs following a prior treatment with empty PLs could induce a CTL response against OVA. A single injection of OVA-PLs followed a prior treatment with empty PLs, and these treatments neither triggered a CTL response against EG7-OVA cells in vitro (Supplemental Fig. 1A) nor suppressed the growth of an s.c. inoculated EG7-OVA tumor in vivo (Supplemental Fig. 1B).

In order to attain a strong immune response, mice were then subjected to three biweekly immunizations with OVA-PL following a prior treatment with empty PLs. As shown in Fig. 1A, three biweekly immunizations triggered a potent apoptotic response against EG7-OVA cells in vitro. Obviously, such an apoptotic response was not observed without a prior treatment with empty PL. This indicated that sequential immunizations that combine an i.v. injection of empty PLs followed by subsequent injections of OVA-PL induces OVA-specific CTLs. The potential for prophylactic applications was investigated in EG7-OVA tumor–bearing mice. EG7-OVA cells were s.c. inoculated into mice at day 7 after the last injection of OVA-PLs. In this in vivo experiment, OVA emulsified with CFA (OVA–CFA) was used as a positive control. Without prior treatment with empty PL, three repeated immunizations with OVA-PLs showed no significant suppression of tumor growth by comparison with the control (nontreated) group (Fig. 1B). As expected, however, a sequential treatment with empty PLs (×3) followed by OVA-PLs (×3) showed potent tumor growth inhibition comparable to that achieved following three treatments with OVA–CFA.

To verify the contribution of EG7-OVA–specific CTLs to the potent in vivo tumor growth-suppressive effect achieved with our immunization system, an in vitro CTL assay was performed on spleen cells collected 29 d following the last OVA-PL treatment. As shown in Fig. 1C, spleen cells were collected either from mice that had received three immunizations with our system or with an OVA–CFA-induced potent apoptotic response against EG7-OVA cells in vitro. The results indicated that both forms of immunization elicited a strong CTL immune response against EG7-OVA cells that predominantly resulted in tumor growth suppression.

Contribution of immunosuppressive cells to the prevention of tumor development in prophylactically immunized mice

Immune cells such as Treg and MDSCs can counteract the antitumor immune response of CTLs (22). Accordingly, to gain insight into the negative contribution of protumor immunity manifested by either Treg or MDSCs to the CTL-mediated antitumor immune response induced by our prophylactic immunization, the populations of Treg and MDSCs in the spleen following immunizations were evaluated. The percentage of either Treg (Fig. 2A) or MDSCs (Fig. 2B) in the spleen was unchanged under any immunization treatment. These results indicate that the antitumor effect and prevention of tumor development that was obtained by our prophylactic immunization was independent of protumor immunity.

Effect of αGC incorporation in OVA-PL on the antitumor activity of our immunization system

αGC is known to activate NKTs and, thus, modulates the antitumor immune response of CTLs (23). In order to decrease the

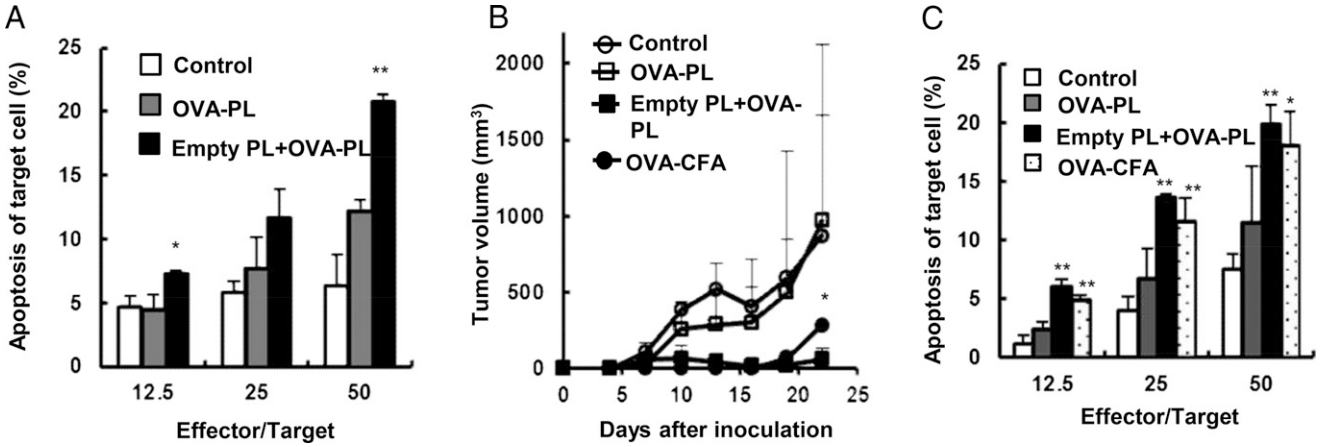

FIGURE 1. Combining an empty PL dose and an OVA-PL dose for prophylactic immunization and its effect on the induction of CTL and on tumor growth. Three days post-preimmunization with or without an i.v. injection of empty PL, mice were immunized with an i.v. injection of OVA-PL. This immunization schedule was repeated three times every 2 wk. As a positive control, OVA emulsified with CFA (OVA–CFA) was s.c. injected three times every 2 wk without prior treatment with empty PL. (**A**) On day 7 following the last immunization, spleen cells were collected and stimulated with OVA. The spleen cells (Effector cell) were cocultured with EG7-OVA cells (Target cell), and then apoptosis of EG7-OVA cells was assessed by flow cytometry. (**B**) On day 7 following the final immunization, EG7-OVA cells were s.c. inoculated into the mice and the tumor growth was recorded. (**C**) On day 22 after tumor inoculation (day 29 after final immunization), the spleen cells were collected. In vitro CTL assay was carried out as described above. The data are presented as the mean ± SD ($n = 4$). *$p < 0.05$, **$p < 0.01$ versus control.

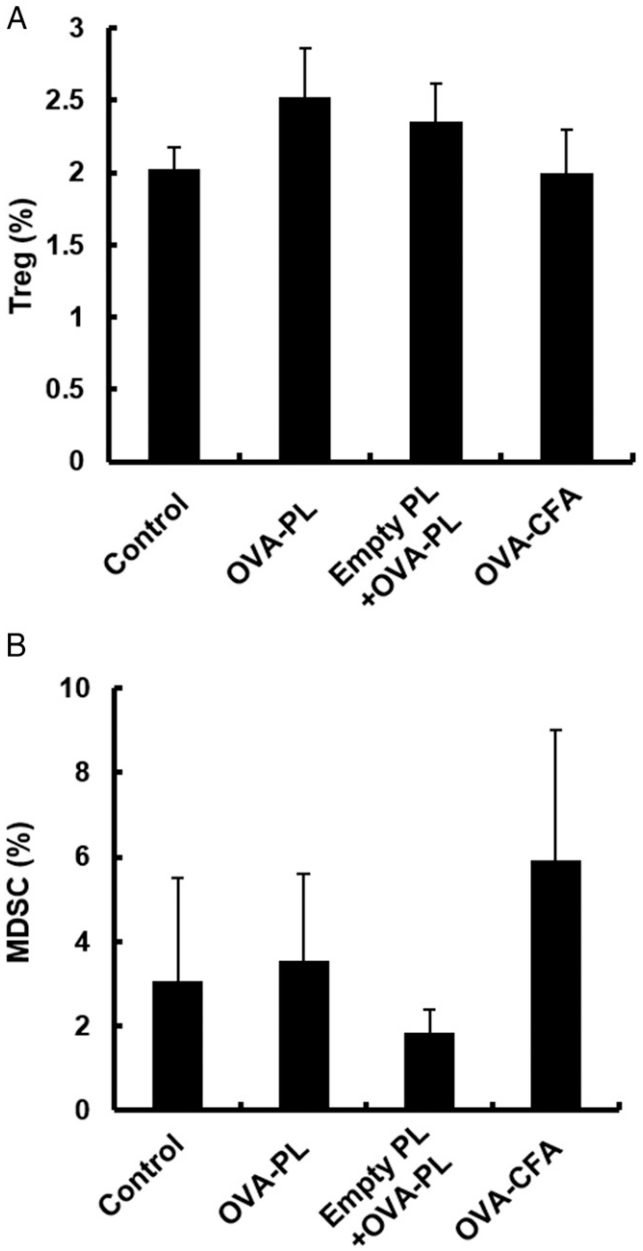

FIGURE 2. Contribution of immunosuppressive cells to the prevention of tumor development by empty PL (×1) plus OVA-PL (×3). Three days following preimmunization with or without an i.v. injection of empty PL, mice were immunized with an i.v. injection of OVA-PL. This immunization schedule was repeated three times every 2 wk. As a positive control, OVA emulsified with CFA (OVA–CFA) was s.c. injected three times every 2 wk without prior treatment with empty PL. On day 7 following the last immunization, EG7-OVA cells were s.c. inoculated into the mice. Twenty-two days later, the spleen cells were collected, and the populations of Treg and MDSC cells were analyzed by flow cytometry. (**A**) Frequency of Treg. (**B**) Frequency of MDSCs. The data are presented as the mean ± SD (n = 4).

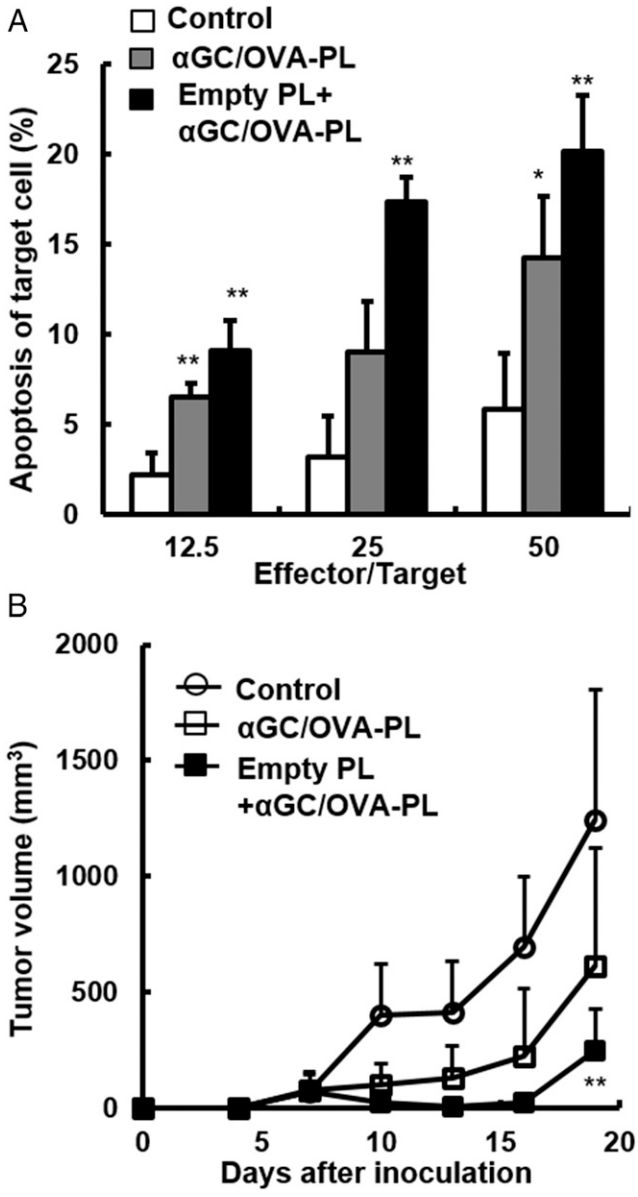

FIGURE 3. Effect of αGC incorporation in OVA-PL on antitumor activity. Three days following preimmunization with or without an i.v. injection of empty PL, mice were immunized with a single i.v. injection of αGC/OVA-PL. (**A**) On day 14 after αGC/OVA-PL injection, the spleen cells were collected and then stimulated with OVA. The spleen cells were cocultured with EG7-OVA cells, and apoptosis of EG7-OVA cells was then assessed. (**B**) On day 14 after αGC/OVA-PL injection, EG7-OVA cells were s.c. inoculated into the mice, and the tumor growth was recorded. The data are presented as the mean ± SD (n = 4). *$p < 0.05$, **$p < 0.01$ versus control.

number of OVA-PL doses, which could provide a clinical advantage and further increase the antitumor potential of our immunization system, αGC was incorporated into OVA-PLs. On day 3 following prior treatment with empty PLs, αGC/OVA-PLs were i.v. injected once. An in vitro CTL assay was performed on spleen cells obtained on day 14 after αGC/OVA-PL injection. A single immunization with αGC/OVA-PL without prior empty PL treatment significantly increased the spleen cell–mediated cytotoxicity against EG7-OVA cells (Fig. 3A), presumably via inducing a remarkable CTL immune response. As expected, prior treatment with empty PLs followed by a single injection of αGC/OVA-PLs significantly triggered a potent apoptotic response against EG7-OVA cells, presumably via further induction of CTLs against EG7-OVA. To confirm the induction of OVA-specific CTL, the cells were detected using OVA-tetramer. The OVA-specific CTL was increased following immunization with αGC/OVA-PL with prior empty PL treatment (Supplemental Fig. 2A).

The antitumor effect of prophylactic immunization was also investigated. The growth of EG7-OVA cells in the immunized mice was delayed by prophylactic immunization in the following rank

order: empty PL (×1) + αGC/OVA-PL (×1) > αGC/OVA-PL (×1) > no immunization (control) (Fig. 3B). Collectively, incorporation of αGC into OVA-PLs potentially increased the antitumor immune response and allowed a reduction in the number of OVA-PL immunization doses with no effect on the overall therapeutic efficacy.

Contribution of immunosuppressive cells to the prevention of tumor development in mice treated with our prophylactic immunization (empty PLs plus αGC/OVA-PLs)

To address the contribution of Treg and MDSCs to a CTL-mediated antitumor immune response elicited by prophylactic immunization, the number of Treg and MDSCs in the spleen after treatment with αGC/OVA-PLs with or without prior empty PL treatment was investigated. Immunization by either αGC/OVA-PLs or empty PLs plus αGC/OVA-PLs did not affect the Treg population in the spleen (Fig. 4A). To identify Treg accurately, the cells were stained with anti-FOXP3 Ab in addition to anti-CD4 and anti-CD25 Ab. The population of Treg ($CD4^+$, $CD25^+$, $FOXP3^+$) was not changed (Supplemental Fig. 2B). In contrast, either αGC/OVA-PLs or empty PLs plus αGC/OVA-PLs significantly decreased the population of MDSCs in the spleen (Fig. 4B). These results might indicate that incorporation of αGC into OVA-PLs not only induces CTL activity but also suppresses protumor immunity, particularly that of MDSCs, resulting in enhanced antitumor immunity against EG7-OVA cells.

Therapeutic effect of immunization from empty PLs plus αGC/OVA-PLs on EG7-OVA in tumor-bearing mice

As shown in Fig. 3B, immunization with αGC/OVA-PLs following treatment with empty PLs prevented the development of tumors that were inoculated after the immunization, which showed significant prophylactic potential. In contrast, cancer vaccines generally are expected to show not only prophylactic potential that can prevent tumor development but also therapeutic efficacy that will suppress tumor growth or even the complete eradication of tumors. Therefore, the therapeutic efficacy of our immunization system was evaluated against well-established tumors in mice (Fig. 5). Our immunization system showed potent growth-suppressive activity against tumors that had developed prior to immunization. This effect was maintained for a prolonged period following immunization. A single injection of αGC/OVA-PLs also efficiently suppressed tumor growth. The effect was transient, however, and the tumors began to regrow after a short period of immunization (day 9 postimmunization). Nonetheless, these results suggest that our immunization system demonstrated both prophylactic and therapeutic potential against tumors.

Contribution of NKT cells to the therapeutic effect of our combined immunization, empty PLs plus αGC/OVA-PLs

Activation of NKT has triggered both direct antitumor and CTL-mediated antitumor responses (23). αGC is frequently adopted as an NKT cell-mediated immune adjuvant in anticancer therapies (24). Accordingly, the induction of NKT was evaluated in tumor-bearing mice on day 3 following immunization because previous reports have revealed that NKT reached its maximum level on day 3 after αGC stimulation (25). Interestingly, immunization with either αGC/OVA-PLs or αGC/OVA-PLs following prior treatment with PLs induced a comparable increase in the number of NKT (Fig. 6). These results indicate the contribution of immunization-induced NKT to overall antitumor therapeutic efficacy.

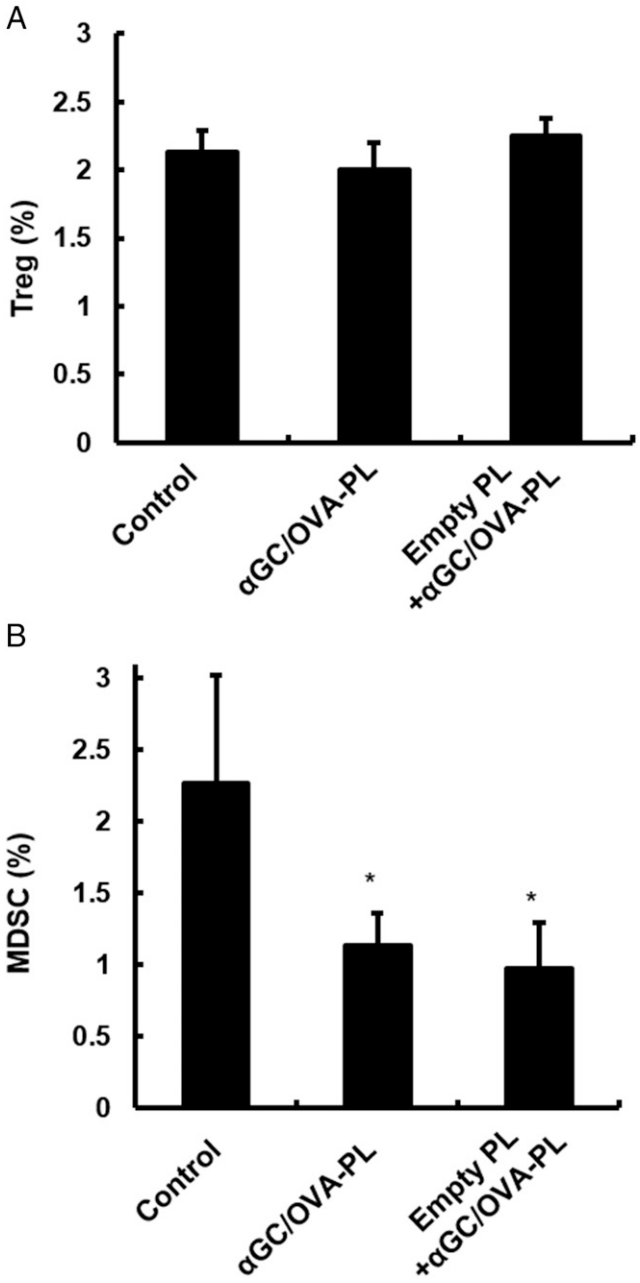

FIGURE 4. Contribution of immunosuppressive cells to the prevention of tumor development via empty PL and αGC/OVA-PL. Three days following preimmunization with or without an i.v. injection of empty PL, mice were immunized with a single i.v. injection of αGC/OVA-PL. On day 14 following immunization, EG7-OVA cells were s.c. inoculated into the mice. Nineteen days later, the spleen was collected and stained with various Abs prior to flow cytometry analysis. (**A**) Frequency of Treg in the spleen. (**B**) Frequency of MDSCs in the spleen. The data are presented as the mean ± SD ($n = 4$). *$p < 0.05$ versus control.

MZ-B cells selectively took up αGC/OVA-PL, presented Ag onto MHC I, and induced OVA-specific CTL

Finally, to confirm the contribution of MZ-B cells to the induction of antitumor immune responses, we investigated the delivery of Ag to MZ-B cells and Ag presentation by MZ-B cells. The liposomes labeled with DiI, a lipid trace marker, was i.v. injected into mice, and the uptake of the liposomes was analyzed at 24 h with flow cytometry. The uptake of second dose liposomes by MZ-B cells was significantly increased compared with the first dose (Fig. 7A). Second dose liposomes were also taken up by FO-B cells, but

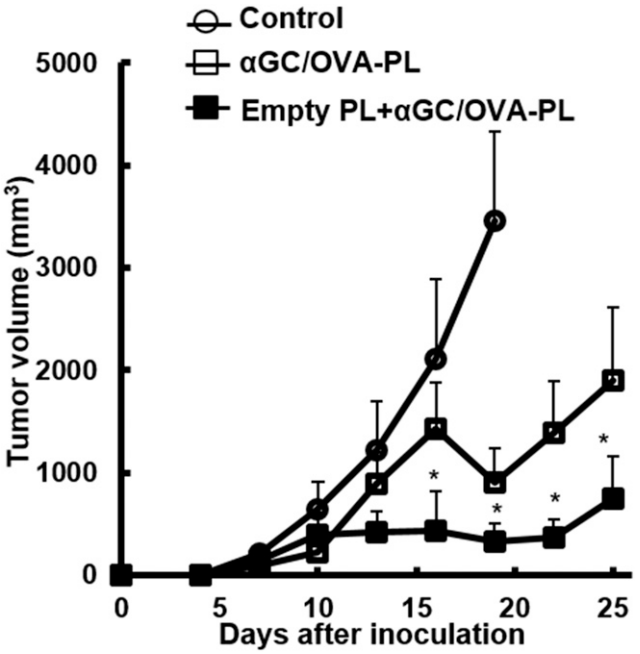

FIGURE 5. Therapeutic effect of combined immunization of empty PL and αGC/OVA-PL in a tumor-bearing mice model. EG7-OVA tumor–bearing mice were treated with or without an i.v. injection of empty PL. Three days following preimmunization, the mice were i.v. injected with a single i.v. dose of αGC/OVA-PL. Tumor growth was continuously monitored. The data are presented as the mean ± SD (n = 5). *$p < 0.05$ versus αGC/OVA-PL.

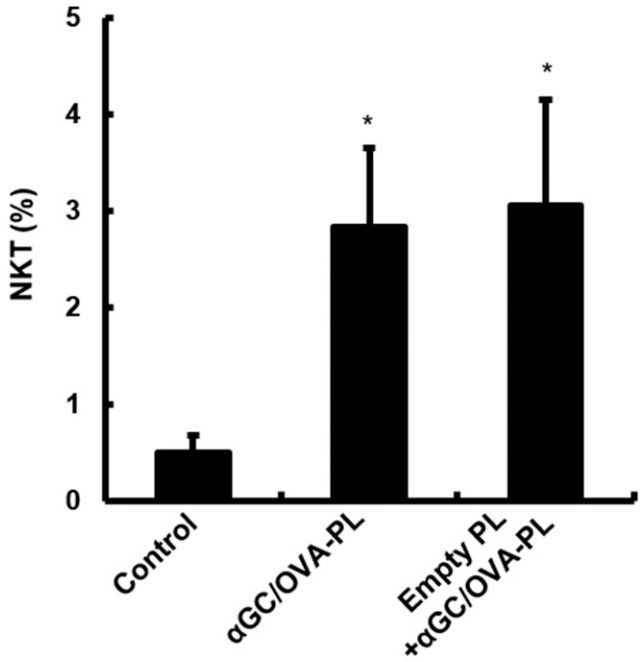

FIGURE 6. Contribution of NKT to the tumor growth-suppressive effect of immunization combining empty PL and αGC/OVA-PL. EG7-OVA tumor–bearing mice were treated with or without an i.v. injection of empty PL. Three days following preimmunization, mice were i.v. injected with a single i.v. dose of αGC/OVA-PL. On day 3 following immunization, the frequency of the occurrence of NKT in the spleen was measured by flow cytometry. The data are presented as the mean ± SD (n = 3). *$p < 0.05$ versus control.

the uptake by FO-B cells was lower than that of MZ-B cells. In addition, fluorescence-labeled OVA encapsulated in liposomes were injected as second dose liposomes. The uptake of OVA showed a similar pattern to liposomes (Fig. 7B). Next, we examined Ag presentation by MZ-B cells. Following i.v. injection of αGC/OVA-PL, presentation of OVA-derived peptide onto MHC I was determined with flow cytometry. Presentation of OVA-peptide on MZ-B cells was significantly increased following immunization with αGC/OVA-PL with prior empty PL treatment (Fig. 7C). In contrast, presentation of OVA-peptide on FO-B cells was not observed. Next, we investigated whether adoptive transfer of MZ-B cells could induce OVA-specific CTL. Following immunization with αGC/OVA-PL with prior empty PL treatment, MZ-B cells and FO-B cells were isolated and then transferred to naive mice. Seven days after adoptive transfer, OVA-specific CTL was determined with flow cytometry. Adoptive transfer with MZ-B cells significantly induced OVA-specific CTL, but FO-B cells did not (Fig. 7D). Collectively, MZ-B cells selectively took up αGC/OVA-PL following prior treatment with empty PL, presented OVA-peptide onto MHC I, and, finally, induced OVA-specific CTL.

Discussion

In the current study, we demonstrated the feasibility of combined immunization that consists of a prior i.v. injection of empty PLs (×3) plus Ag (OVA)-containing PLs (OVA-PLs) (×3) to ensure efficient Ag delivery to splenic MZ-B cells. The combined immunization triggered the elicitation of antitumor immunity, which enhanced Ag-specific CTL induction and suppressed tumor development (Fig. 1). In addition, incorporation of the NKT ligand αGC into OVA-PL significantly augmented the antitumor potential of the combined immunization (Fig. 3) and allowed a reduction in the number of immunization doses, which is of great clinical relevance to cancer patients. Of interest, an improved immunization system, empty PLs (×1) plus αGC/OVA-PLs (×1), not only prevented tumor development but also suppressed tumor growth in vivo (Fig. 5). Collectively, our combined immunization system might represent a unique prophylactic and therapeutic antitumor vaccine.

The ability to efficiently deliver Ags to APCs with subsequent activation of CTLs is considered a key determinant for the development of cancer vaccines. Various strategies, such as the conjugation of ligands to Ags or the formation of an immune complex, have been adopted to enhance Ag targeting to APCs (11, 26). In the current study, we exploited our previous observation that anti-PEG IgM produced in response to a first dose of empty PLs triggers the efficient delivery of a second dose of PLs to splenic MZ-B cells following complement activation (27). In this study, we further confirmed both liposomes and OVA were selectively delivered to MZ-B cells rather than FO-B cells (Fig. 7A, 7B). This ensures the efficient delivery of Ags to splenic MZ-B cells via the use of a liposomal platform. Splenic MZ-B cells are superior to other B cell subsets in terms of Ag presentation (16). In fact, we observed that MZ-B cells are superior to FO-B cells in terms of Ag presentation following immunization with αGC/OVA-PL (Fig. 7C). Furthermore, those cell fractions are more abundant than dendritic cells (28). Consequently, efficient delivery of OVA-PLs or αGC/OVA-PLs to splenic MZ-B cells triggered by prior treatment with empty PLs is expected to increase Ag delivery to MZ-B cells with subsequent CTL induction (Figs. 1A, 3A, 7D). Nevertheless, the possibility that other APCs contribute to the induction of CTLs cannot be nullified. Splenic dendritic cells aggressively take up trinitrophenyl-OVA in the presence of anti-trinitrophenyl Ab and, consequently, induce CTLs against OVA (29). In a similar manner, Ag delivery to splenic marginal zone metallophilic macrophages is known to enhance

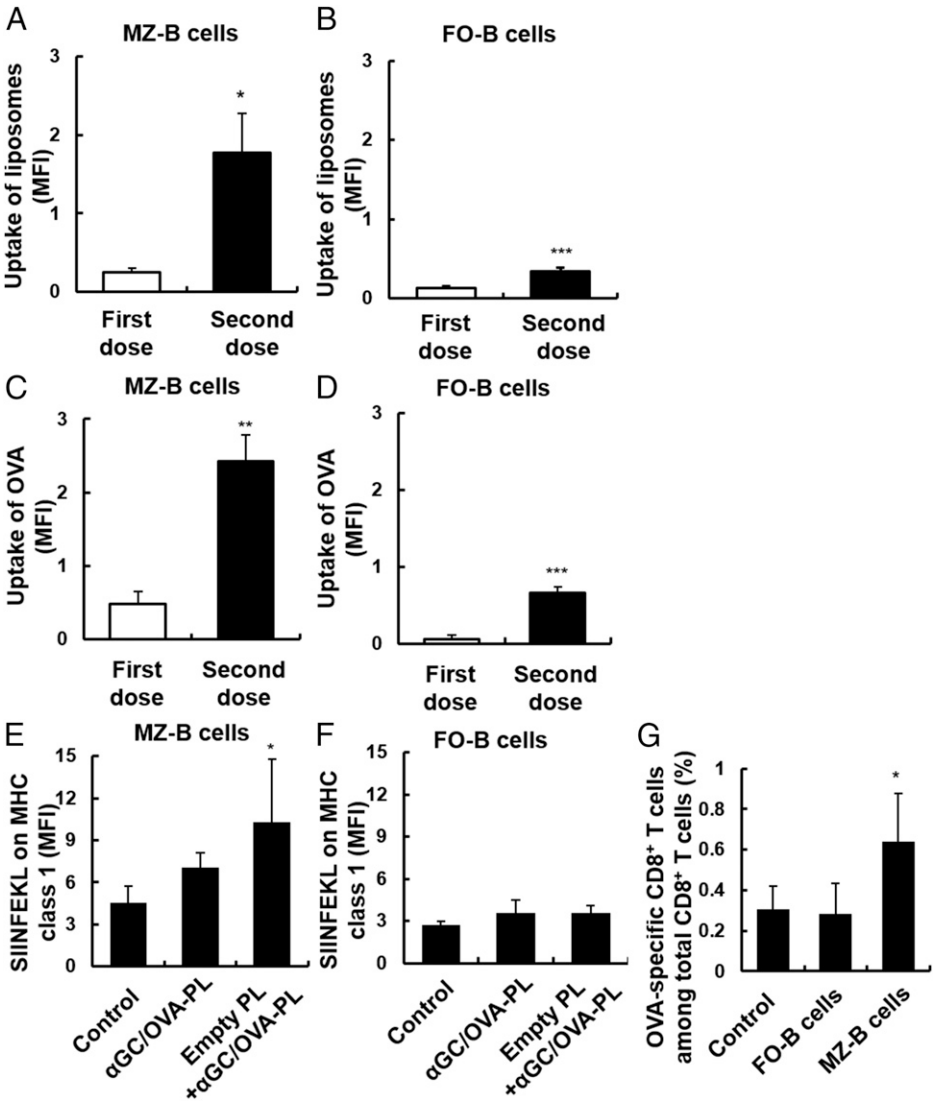

FIGURE 7. MZ-B cells selectively took up Ag, presented Ag, and induced Ag-specific CTL. Three days following an i.v. injection of empty PL, mice were injected with either (**A** and **B**) fluorescence-labeled liposomes or (**C** and **D**) fluorescence-labeled OVA encapsulated in PL. At 24 h, spleen was collected, and spleen cells were stained with various Abs. The uptake of liposomes by (A and C) MZ-B cells and (B and D) FO-B cells was analyzed by flow cytometry. (**E**–**G**) Three days following preimmunization with or without an i.v. injection of empty PL, mice were immunized with a single i.v. injection of αGC/OVA-PL. (E and F) At 24 h, spleen was collected, and spleen cells were stained with various Abs. OVA peptide (SIINFEKL) on MHC I was analyzed by flow cytometry. (G) At 1 h, spleen was collected, and MZ-B cells and FO-B cells were isolated. These cells were adoptively transferred into naive mice. Seven days later, OVA-specific T cells in spleen were analyzed by flow cytometry. The data are presented as the mean ± SD ($n = 4$–5). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

Ag-specific CTLs (30). In our preliminary study, however, we confirmed that only MZ-B cells and neither dendritic cells nor marginal zone metallophilic macrophages contributed to the uptake of a second dose of PLs following prior treatment with a first dose of empty PLs (Supplemental Fig. 3). Consequently, in our combined immunization, splenic MZ-B cells were assumed to predominantly contribute to Ag presentation and CTL induction.

Effective antitumor immunity requires activation of both the innate and adaptive immune systems to circumvent the immune evasion strategies adopted by tumors. Incorporation of an adjuvant, αGC, into OVA-PLs induced efficient antitumor immunity following only a single injection (Fig. 3), compared with a 3-fold immunization with OVA-PLs (Fig. 1). αGC, an NKT ligand, is known to activate innate and adaptive immunity via the production of large amounts of INF-γ (31). Indeed, we observed an induction of NKT (Fig. 6) and an abundant secretion level of IFN-γ (data not shown) following immunization with αGC/OVA-PLs. Therefore, it is plausible that the enhanced antitumor activity triggered by αGC/OVA-PLs could be mediated via the dual activations of the innate immunity manifested by the activation of NKT and by the adaptive immunity represented by enhanced CTL induction. Of note, NKT is induced more rapidly than CTLs, and both can kill tumor cells directly. However, each of these only exhibit their effector functions for a short period of time (32). After treatments, the proportion of NKT in the spleen was the same in either the presence or absence of prior treatments with empty PLs (Fig. 6). To confirm the major contribution of CTL to antitumor effect, $CD8^+$ T cells were depleted before immunization (Supplemental Fig. 4). The antitumor effect obtained by immunization completely disappeared with depletion of $CD8^+$ T cells. Accordingly, the antitumor effect obtained by treatment that combines with empty PLs plus αGC/OVA-PLs could mainly depend on CTL-mediated tumor killing induced by both MZ-B cells and $CD8^+$ T cells rather than NKT-mediated killing activity alone.

Protumor immunity inhibits CTL activation and promotes tumor growth. Treg and MDSCs are the key effector cells of protumor immunity, and these are blamed for compromising the efficacy of cancer vaccines via their inhibitory effect on CTL functions (33). In cancer immunotherapy, an adjuvant is a critical component in vaccine formulations that both stimulates the desired immune responses and dampens the effect of tumor-induced suppressive immune populations (Treg and MDSCs) circulating in tumor-bearing individuals. Nonetheless, some cancer vaccines themselves could induce MDSCs, which are meant to attenuate the overall efficacy of the adopted vaccine (34). In this study, the combined treatment of empty PLs and αGC/OVA-PLs did not affect the numbers of Treg (Fig. 4A), but it did decrease the level of MDSCs (Fig. 4B). In fact, accumulating evidence demonstrates

the ability of some vaccine adjuvants to convert tumor MDSCs into APCs. Ko et al. (35) have demonstrated the potential of αGC-mediated NKT activation to trigger the conversion of immature myeloid cells into mature APCs. Accordingly, in our experiments, αGC/OVA-PLs seemed to trigger the conversion of MDSCs into dendritic cells, which thereby temporarily decreased the number of MDSCs. Although decrease of MDSCs might be useful for maintaining CTL function, contribution of MDSCs to the antitumor effect is still unclear.

To achieve success, clinical cancer immunotherapy outcomes must promote strong immune responses against tumor-specific Ags. Cell-targeted vaccines represent a promising strategy to induce appropriately strong immune responses. Dendritic cell–targeted cancer vaccines have achieved hopeful results in preclinical animal studies. Nonetheless, the clinical response rate of these vaccines has been dismal over the past decade. Difficulty in selecting the ideal targets of dendritic cell subsets and the inefficient targeting of cells have posed challenges to the effectiveness of such cell-targeted vaccines in both preclinical and clinical settings. Recently, B cell–targeted cancer vaccines have attracted interest as alternatives to dendritic cell–targeted cancer vaccines. Unfortunately, similar to dendritic cell–targeted cancer vaccines, identifying the optimal B cell subsets in tandem with ensuring efficient targeting to B cells has been an obstacle in the development of efficient B cell–targeted cancer vaccines. In this study, we focused on MZ-B cells that are reported as the most promising B cells in terms of Ag presentation capacity. By using the immune response to empty PLs, cancer Ags within a second dose of PLs were efficiently delivered to MZ-B cells, which led to both prophylactic and therapeutic antitumor effects. Accordingly, the selective tumor-specific Ag targeting of splenic MZ-B cells might represent a promising strategy for the development of well-defined cell-targeted vaccines for the prevention and treatment of cancer.

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Disclosures

The authors have no financial conflicts of interest.

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