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Galactosylated liposome as a dendritic cell-targeted mucosal vaccine for inducing protective anti-tumor immunity

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ABSTRACT

Mucosal surfaces contain specialized dendritic cells (DCs) that are able to recognize foreign pathogens and mount protective immunity. We previously demonstrated that intranasal administration of targeted galactosylated liposomes can elicit mucosal and systemic antibody responses. In the present study, we assessed whether galactosylated liposomes could act as an effective DC-targeted mucosal vaccine that would be capable of inducing systemic anti-tumor immunity as well as antibody responses. We show that targeted galactosylated liposomes effectively facilitated antigen uptake by DCs beyond that mediated by unmodified liposomes both in vitro and in vivo. Targeted galactosylated liposomes induced higher levels of pro-inflammatory cytokines than unmodified liposomes in vitro. C57BL/6 mice thrice immunized intranasally with ovalbumin (OVA)-encapsulated galactosylated liposomes produced high levels of OVA-specific IgG antibodies in their serum. Spleen cells from mice receiving galactosylated liposomes were restimulated with OVA and showed significantly augmented levels of IFN-γ, IL-4, IL-5 and IL-6. In addition, intranasal administration of OVA-encapsulated beta-galactosylated liposomes resulted in complete protection against EG7 tumor challenge in C57BL/6 mice. Taken together, these results indicate that nasal administration of a galactosylated liposome vaccine mediates the development of an effective immunity against tumors and might be useful for further clinical anti-tumoral applications.
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1. Introduction

The mucosal surfaces, such as the gastrointestinal and respiratory tracts, represent the main entry site for most infectious agents. Thus, mucosal immunity provides the first line of defense against harmful microorganisms. The nasopharynx-associated lymphoid tissue (NALT) contains specialized M-like cells, which are structurally similar to those present in Peyer's patches in the gut [1]. The structural and functional role of M cells is thought to lie primarily in antigen uptake and transportation to underlying lymphocytes and antigen presenting cells (APCs) in the mucosal tissues [2]. Mucosal surfaces also contain specialized dendritic cells (DCs). DCs play a critical role in recognizing environmental pathogens, as well as in initiating and regulating adaptive immune responses [3]. Upon capture of antigen, DCs are rapidly activated by a complex process, become mature and migrate into the secondary lymphoid organs, such as the lymph nodes or the spleen, where they present the processed antigenic peptides in the context of either MHC class I or II molecules to CD4 + or CD8 + T lymphocytes, thereby triggering adaptive immune responses [4,5]. In addition, mature DCs also release a variety of cytokines and chemokines to regulate both the innate and adaptive immune responses [6]. Thus, a number of studies have been aimed at developing effective mucosal vaccines to induce protective immunity against cancer and viral infection [7]. Recently, next-generation mucosal vaccines based on synthetic antigens, such as subunit protein, peptide and DNA, have shown potential for the development of safe and effective vaccination strategies [8]. However, due to their weak immunogenicity and low cellular uptake efficacy by

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APCs in mucosal tissue, these synthetic antigen vaccines require multiple or larger doses to reach a satisfactory level of immune protection [9]. Thus, a more effective delivery system is required to enhance the efficacy of mucosal vaccines.

Liposomes are small particulate vesicles formed from phospholipid bilayers that can be used to encapsulate antigen or immunomodulatory molecules. Past studies have reported that liposomes are capable of improving the delivery of antigens across mucosal membranes [10] and enhancing the immunogenicity of various antigens to boost the cellular or humoral immune response [11–13]. Our previous studies also demonstrated that intranasal immunization of inactivated virus encapsulated in a liposome [14] or co-administrated with immunostimulatory molecules [15] can successfully promote efficient mucosal and systemic antibody responses. Recently, surface modification of the targeting ligands and antibodies, which are specifically recognized by receptors on the surface of APCs, has shown the potential to improve the uptake of liposomes and better induce effective immunity against viruses or tumors [16,17].

C-type lectin receptors (CLRs) expressed by DCs are particularly important for the recognition of glycosylated self-antigens or foreign pathogens. CLRs interact with these antigens mainly through the recognition of carbohydrate structures, such as mannose, fucose and glucan, and promote endocytosis, leading to the processing and presentation of antigens on MHC class I and II molecules [18]. Macrophage galactose-type C-type lectins (MGL) in humans and mice are type II transmembrane glycoproteins with the capacity to bind to galactose, N-acetylgalactosamine and Lewis X as mono- or oligosaccharides [19]. It has been shown that the terminal galactose has a high affinity for mouse MGL receptors on murine macrophages [20]. Furthermore, previous studies have identified MGL expression on immature DCs in humans and mice and have shown that MGL mediates the uptake of antigens containing GalNAc residues [21,22]. Thus, galactose is a potent candidate ligand for targeting APCs to induce effective immunity.

We have recently demonstrated that incorporating galactose-1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (DLPE) into liposomal bilayers as targeted antigen delivery carriers (galactosylated liposomes) resulted in an increase in the uptake and production of cytokines by macrophages [23]. Mice given galactosylated liposomes intranasally showed a significant increase in mucosal secretory immunoglobulin A (s-IgA) and serum IgG antibody responses. DCs are widely accepted as an ideal platform for vaccine design. However, there has been little discussion about the potential of galactosylated liposomes to target DCs. The aim of this study was to evaluate and validate whether galactosylated liposomes could act as an effective DC-targeted mucosal vaccine that could induce systemic antitumor immunity as well as antibody responses. Our results show that galactosylated liposomes effectively facilitated antigen uptake by DCs both in vitro and in vivo. In addition, we established the protective efficacy of ovalbumin (OVA)-encapsulated galactosylated liposomes as a mucosal vaccine against tumor growth.

2. Materials and methods

2.1. Materials

Phosphatidylcholine was purchased from Degussa, Hamburg, Germany. Cholesterol, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), OVA, carbonate–bicarbonate and organic solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). The galactosyl lipids (alpha-galactosyl-DLPE or beta-galactosyl-DLPE) were prepared by conjugation of galactose with DLPE, as described previously [23]. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgA were purchased from Bethyl Laboratories (TX, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco. The mouse cytokine kit interleukin (IL)-4, IL-5, IL-6 and interferon (IFN)-γ, fluorochrome-labeled anti-mouse monoclonal antibodies CD11c, CD80, CD86, major histocompatibility complex class II (MHC-II) and BD Cytofix/Cytoperm Plus (with GolgiPlug) were purchased from BD Biosciences (CA, USA). 3,3′,5,5′-tetramethyl benzidine (TMB) was purchased from Bionovas Biotechnology (Ann Arbor, MI, USA). C57BL/6 female mice (6–8 weeks old) were obtained from the BioLASCO Taiwan Co., Ltd. and acclimated for 1 week prior to the study. All animal experiments were conducted in specific pathogen-free conditions and in compliance with guidelines provided by the Taipei Medical University of Science and Technology for the care and use of animals for research purposes. The E.G7-OVA cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and maintained in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U ml^{-1}) and streptomycin (100 μ ml^{-1}) at 37 °C in 5% CO_2.

2.2. Preparation and characterization of liposomes

Liposomes were prepared as described previously [23]. Briefly, thin films were obtained through the rotary evaporation of chloroform solutions with a 4:4:0.5 lipid molar ratio of phosphatidylcholine:cholesterol:galactosyl lipid (alpha-galactosyl-DLPE or beta-galactosyl-DLPE) using a final lipid concentration of 2 mM. The lipid films were maintained under vacuum for 60 min to remove residual organic solvents. Phosphate-buffered saline (PBS) containing 5 mg ml^{-1} OVA was added to the dried lipid film, and multilamellar vesicles were prepared by intense vortex dispersion. The vesicles were extruded 10 times through a 1 μm pore polycarbonate membrane to form galactosylated liposomes (alpha-gal-liposome or beta-gal-liposome). The amount of entrapped OVA was measured by Bio-Rad protein assay, and non-encapsulated OVA was removed by centrifugation. The OVA-encapsulated unmodified liposomes (bare-liposome) were also prepared according to the above procedure. Equivalent quantities of galactose were added to bare-liposome to form a galactose-mixed-liposome (gal-mixed-liposome).

To evaluate the uptake of liposomes by dendritic cells, DiI-labeled liposomes were also prepared by adding 0.04 mol% DiI (percentage of total lipid moles) to chloroform solutions according to the above procedure. Free DiI and DiI aggregates were removed by centrifugation. The particle size and zeta potential of the resulting preparations (200 μl diluted in 4 ml of distilled deionized water) were confirmed by dynamic light scattering analysis using a particle analyzer (BIC 90 Plus, Brookhaven Instruments, Holtsville, NY, USA).

2.3. Generation of bone-marrow-derived dendritic cells (BMDCs)

Mouse BMDCs were generated according to a published protocol [24]. In brief, bone marrow cells were isolated from C57BL/6 mouse femurs and tibias and passed through a 100 μM cell strainer. The red blood cells were lysed using BD Pharm Lyse lysis buffer. The remaining cells were cultured in RPMI 1640 medium (containing 10% heat-inactivated FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) supplemented with granulocyte macrophage colony-stimulating factor (1000 U ml^{-1}) and IL-4 (500 U ml^{-1}) at 37 °C in 5% CO_2 for 6 days to acquire immature BMDCs. The percentage of CD11c+ cells was verified by flow cytometry, and the cells (final percentage of CD11c+ cells was 85–90%) were used for further in vitro experiments.

2.4. Stimulation of mouse DCs and in vitro cellular uptake assay

Mouse BMDCs were seeded at a density of 10^6 cells per well in a 24-well plate and incubated with bare-liposomes, gal-mixed-liposomes or galactosylated liposomes. After a 24 h incubation, cells were labeled with phycoerythrin (PE) hamster anti-mouse CD80, PE hamster anti-mouse CD86 and PE rat anti-mouse MHC-II monoclonal antibodies. The expression of CD80, CD86 and MHC-II on BMDCs was measured by flow cytometry (BD FACSCalibur System). For the in vitro cellular uptake assay, mouse BMDCs were incubated with various DiI-labeled liposome formulations for 6 and 18 h. Following the incubation, cells were harvested and washed three times with PBS, then analyzed immediately using flow cytometry. The data are presented as the mean fluorescent signal for the 10,000 cells collected.

2.5. Cytokine profile of stimulated cells

Mouse BMDCs were seeded at a density of 10^6 cells per well in a 24-well plate and incubated with various liposome formulations (final OVA amount of 20 μg) at 37 °C under 5% CO_2 at 95% humidity. After 24 h, the supernatants were harvested and the concentrations of IL-1β, IL-6 and IL-12 were measured using mouse IL-1β, IL-6 and IL-12 OptEIA sets according to the manufacturer's instructions.

2.6. Evaluation of uptake of galactosylated liposomes by dendritic cells in NALT

Four mice were given DiI-labeled bare-liposomes, alpha-gal-liposomes or beta-gal-liposomes. After 24 h, single cell suspensions were isolated from the NALT according to the method described previously [25]. The cells were washed twice with PBS and then stained with allophycocyanin-labeled anti-CD11c antibody to detect dendritic cells. Fluorescent signals from DiI in the CD11c+ cells were analyzed, and the uptake of galactosylated liposomes by dendritic cells in the NALT was evaluated.

2.7. Immunization of mice and tumor inoculation

C57BL/6 mice were randomly divided into five groups (10 mice in each group) and immunized intranasally with 20 μg of OVA encapsulated in bare-liposomes, gal-mixed-liposomes, alpha-gal-liposomes, beta-gal-liposomes or PBS (40 μl). This immunization procedure was repeated three times at 1 week intervals. One week after the last immunization, six mice in each group were challenged with a dorsal subcutaneous (s.c.) injection of 2×10^5 E.G7-OVA cells. Tumor size was calculated twice a week using the following formula: tumor volume (mm^3) = length × ($width^2$)/2.

2.8. Determination of OVA-specific antibodies

Blood samples were collected 1 week after the final vaccination. Serum samples were isolated by centrifugation for 30 min at room temperature. MaxiSorpTM 96-well enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Rochester, NY, USA) were coated with OVA suspended in 0.05 M carbonate–bicarbonate buffer at pH 9.6 and held overnight at 4 °C. After washing the plates three times with Tris-NaCl containing 0.05% Tween 20 (pH 8.0), Tris-NaCl with 1% bovine serum albumin (pH 8.0) was added to block nonspecific binding sites. Serum samples were 2-fold serially diluted and then added to the plates. HRP-conjugated goat anti-mouse IgG was subsequently added to the plates to detect the bound anti-mouse IgG. HRP activity was assessed using TMB as the substrate. The reaction was stopped by the addition of 2 M H_2SO_4. The optical density (OD) was measured using a SPECTRA MAX reader (Versamax Microplate Reader, Molecular Devices, Toronto, Canada) at 450 nm. The antibody titer was defined as the reciprocal of the highest dilution that had an OD value that was two times higher than the mean OD of control mice serum.

2.9. In vitro spleen cell responses and intracellular cytokine staining

One week after the third immunization, four mice from each group were sacrificed and single-cell suspensions from the spleens of individual mice were prepared for in vitro spleen cell stimulation and intracellular cytokine staining. The spleen cells were seeded at a density of 5×10^6 cells per well in triplicate in 24-well plates and cultured in RPMI 1640 medium (containing 10% heat-inactivated FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) in the presence of 100 μg ml^{-1} OVA. After a 72 h incubation, the supernatants were collected and kept at −80 °C until they were used for the cytokine assay. Levels of IFN-γ, IL-4, IL-5 and IL-6 were quantified using mouse IFN-γ, IL-4, IL-5 and IL-6 OptEIA sets according to the manufacturer's instructions. Intracellular staining was performed with BD Cytofix/Cytoperm Plus according to the manufacturer's instructions. The spleen cells were seeded at a density of 5×10^6 cells ml^{-1}. Cells were incubated with 100 μg ml^{-1} OVA for 72 h, and 1 μl ml^{-1} GolgiPlug was added during the last 5 h. The cells were stained with PE rat anti-mouse CD8 antibody and allophycocyanin rat anti-mouse IFN-γ antibody for the measurement of IFN-γ producing CD8 + T cells.

2.10. Statistical analysis

Data are reported as mean ± SE. The significance of the data was evaluated using Student's *t* test; a *p*-value < 0.05 was considered significant.

3. Results

3.1. Physicochemical characteristics of galactosylated liposomes

Liposomes were prepared by the thin-film hydration method. To determine the effectiveness of the galactosylated liposomes as a DC-targeted mucosal vaccine, OVA, a well-established model antigen, was encapsulated within each of the liposome formulations and extruded through a membrane with 1 μm pores. As shown in Table 1, the particle sizes are approximately 1000 nm and OVA encapsulation efficiencies are approximately 35–40%. No significant difference was observed in the mean particle diameter or encapsulation efficiency among the four formulations.

3.2. The effect of galactosylated liposomes on the cellular uptake and maturation of dendritic cells in vitro

Delivery of antigens to DCs is thought to be a prerequisite for eliciting an effective immune response. The aim of this study was to evaluate whether galactosylated liposomes could act as an effective DC-targeted mucosal vaccine that could facilitate antigen delivery at mucosal sites and induce effective systemic immune response

Table 1
Physicochemical characteristics of various liposomal formulations.

Formulation	Particle diameter (nm)	Polydispersity index	OVA encapsulation efficiency (%)
Bare-liposome	997.17 ± 15.05	0.233 ± 0.017	36.2 ± 2.7
Gal-mixed-liposome	1078.3 ± 35.6	0.236 ± 0.014	35.7 ± 2.1
Alpha-gal-liposome	988.63 ± 16.35	0.249 ± 0.009	38.6 ± 1.8
Beta-gal-liposome	991.8 ± 19.95	0.221 ± 0.012	40.8 ± 2.3

The liposomes used were multilamellar vesicles with a total lipid concentration of 2 mM. Results are expressed as the means ± SE of three independent experiments.

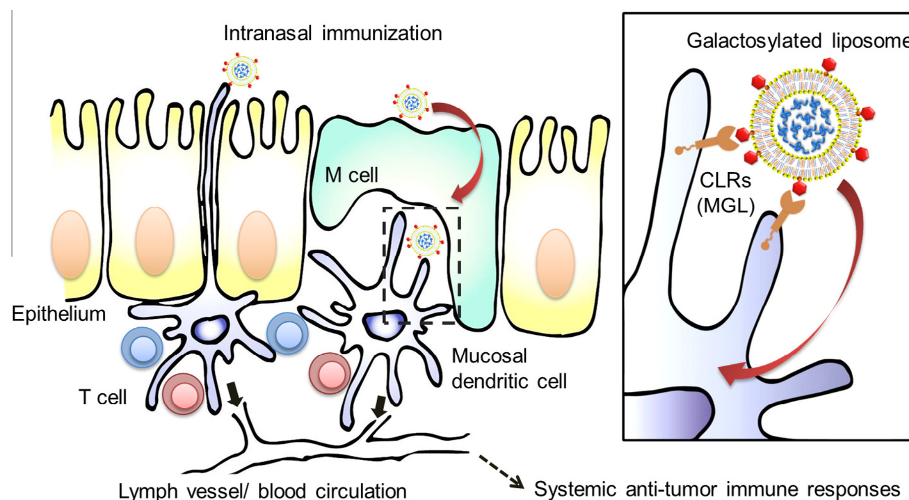


Fig. 1. A scheme of the working hypothesis. Upon intranasal immunization of galactosylated liposomes, specific recognition of galactose molecules by CLRs (MGL) on the surface of dendritic cells at mucosal sites facilitated cellular uptake of liposome and antigen delivery to dendritic cells, and hence induced effective systemic antitumor immune responses.

(Fig. 1). To determine the targeted effect of galactosylated liposomes, we first evaluated the cellular uptake of different liposome formulations by mouse BMDCs in vitro. Fluorescence-activated cell sorting (FACS) analysis allowed for assessment of cell-associated fluorescence and was used to examine the interaction of DiI-labeled liposomes with BMDCs. The results in Fig. 2A–C show that the incorporation of beta-galactosyl-DLPE significantly promoted the uptake of liposome by BMDCs (59.8% and 72.3% of DiI-fluorescent signals were observed after 6 and 18 h incubation with beta-gal-liposomes, respectively). The uptake of liposomes by BMDCs was slightly enhanced by the incorporation of alpha-galactosyl-DLPE (19.7% of DiI-fluorescent signals were observed after 18 h incubation with alpha-gal-liposomes). However, no significant difference was observed between galactose-mixed-liposomes and bare-liposomes.

DC maturation is another key control point for vaccine-induced immune responses. The maturation is characterized by the increased expression of MHC-II molecules, as well as the co-stimulatory molecules CD80 and CD86 on the surface of DCs. Therefore, we next assessed the effect of different liposome formulations on DC maturation. As shown in Fig. 2B–D, BMDCs treated with OVA-encapsulated bare-liposomes and gal-mixed-liposomes showed significant increase in expression of CD86 and MHC II. Furthermore, BMDCs treated with OVA-encapsulated alpha-gal-liposomes and beta-gal-liposomes significantly up-regulated their expression of CD80, CD86 and MHC II on the cell surface compared with bare-liposomes and gal-mixed-liposomes. This result suggests that the efficient uptake of alpha-gal-liposome and beta-gal-liposome could induce the maturation of DCs and induce subsequent immune responses.

3.3. Production of cytokines by DCs stimulated with galactosylated liposomes in vitro

To assess whether galactosylated liposomes induced the secretion of pro-inflammatory cytokines, BMDCs were treated with various liposome formulations for 24 h, and the production of cytokines was determined by ELISA. As shown in Fig. 3A and B, treatment of cells with each liposome formulation resulted in the production of higher levels of IL-1β and IL-6 in comparison with the control group. Furthermore, treating cells with alpha-gal-liposomes and beta-gal-liposomes led to higher levels of IL-1β and IL-6 production compared with bare-liposomes and gal-mixed-liposomes ($p < 0.05$). The production of IL-12 was also significantly elevated in cells treated with alpha-gal-liposomes and beta-gal-liposomes (Fig. 3C). Overall, liposomes incorporated with alpha-galactosyl-DLPE or beta-galactosyl-DLPE enhanced the secretion of pro-inflammatory cytokines and were capable of inducing more efficient immune responses.

3.4. Uptake of galactosylated liposomes by dendritic cells in the NALT

Because resident APCs in the NALT play a critical role in antigen uptake and presentation, we next evaluated the uptake of liposomes by dendritic cells in the NALT. Allophycocyanin-labeled anti-CD11c antibody was used to detect dendritic cells, and DiI fluorescence in CD11c+ cells was analyzed to evaluate the uptake of liposomes by dendritic cells in the NALT. In Fig. 4, the results of flow cytometry showed that 6.16 ± 0.91% and 7.50 ± 0.23% of CD11c+ cells in the NALT obtained from mice receiving alpha-gal-liposomes and beta-gal-liposomes, respectively, were positive for DiI, while only 3.35 ± 0.85% of the cells from mice given bare-liposomes contained DiI fluorescence. These results indicated that antigens encapsulated in alpha-gal-liposomes and beta-gal-liposomes are more effectively delivered into nasal dendritic cells to elicit immune responses.

3.5. Antibody titer analysis

To investigate whether galactosylated liposomes induced a systemic OVA-specific antibody response, OVA, a well-established model antigen, was encapsulated in various liposome formulations, and mice were immunized thrice intranasally. Serum samples were collected after the final administration, and anti-OVA antibodies were measured. Mice that received OVA alone via the intranasal route did not experience any improvement in their systemic antibody response (data no shown). However, improved OVA-specific IgG antibody responses were observed in the serum from mice receiving each of the liposome formulations (Fig. 5). Furthermore, the levels of OVA-specific IgG antibody in the serum of alpha-gal-liposome and beta-gal-liposome receiving mice were significantly higher than those in samples from mice receiving bare-liposomes and gal-mixed-liposomes, suggesting that effective antigen delivery to dendritic cells in the NALT led to effective humoral immune responses.

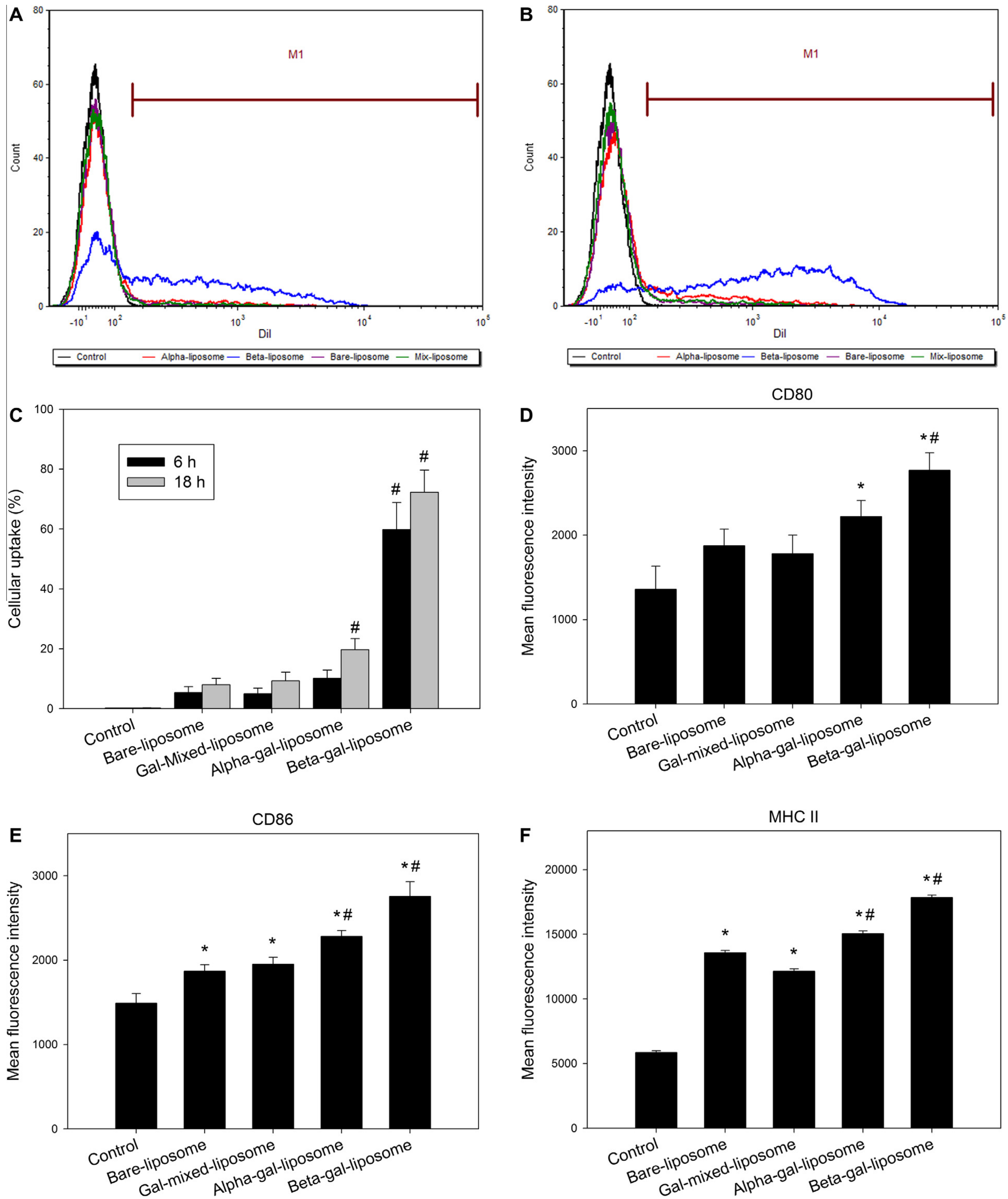


Fig. 2. The effect of galactosylated liposomes on the cellular uptake and maturation of BMDCs. BMDCs were cultured with various DiI-labeled liposome formulations for 6 and 18 h. The uptake of DiI-labeled liposome was measured using flow cytometry. Flow cytometry histograms of DiI-fluorescent signals in BMDCs for 6 and 18 h (A, B) and percentage of DiI-fluorescent signals (C). BMDCs were cultured with medium (control) or different liposome formulations for 24 h. The expression of CD80, CD86 and MHC II was measured using flow cytometry (D–F). *Significant difference ($p < 0.05$) compared with the control group; #significant difference ($p < 0.05$) compared with the bare-liposome group. Similar results were obtained in three separate experiments.

3.6. In vitro cytokine production by splenocytes and IFN-γ producing CD8 + T cell analysis

To assess whether galactosylated liposomes skew the immune response toward a Th1 or Th2 immune response, spleen cells from mice were obtained 1 week after the final immunization and cultured with 100 μg ml^{-1} OVA for 72 h. The quantity of Th1 (IFN-γ) and Th2 (IL-4, IL-5 and IL-6) cytokines in the culture supernatants was determined by ELISA. The results showed that IFN-γ production was significantly increased in spleen cells from

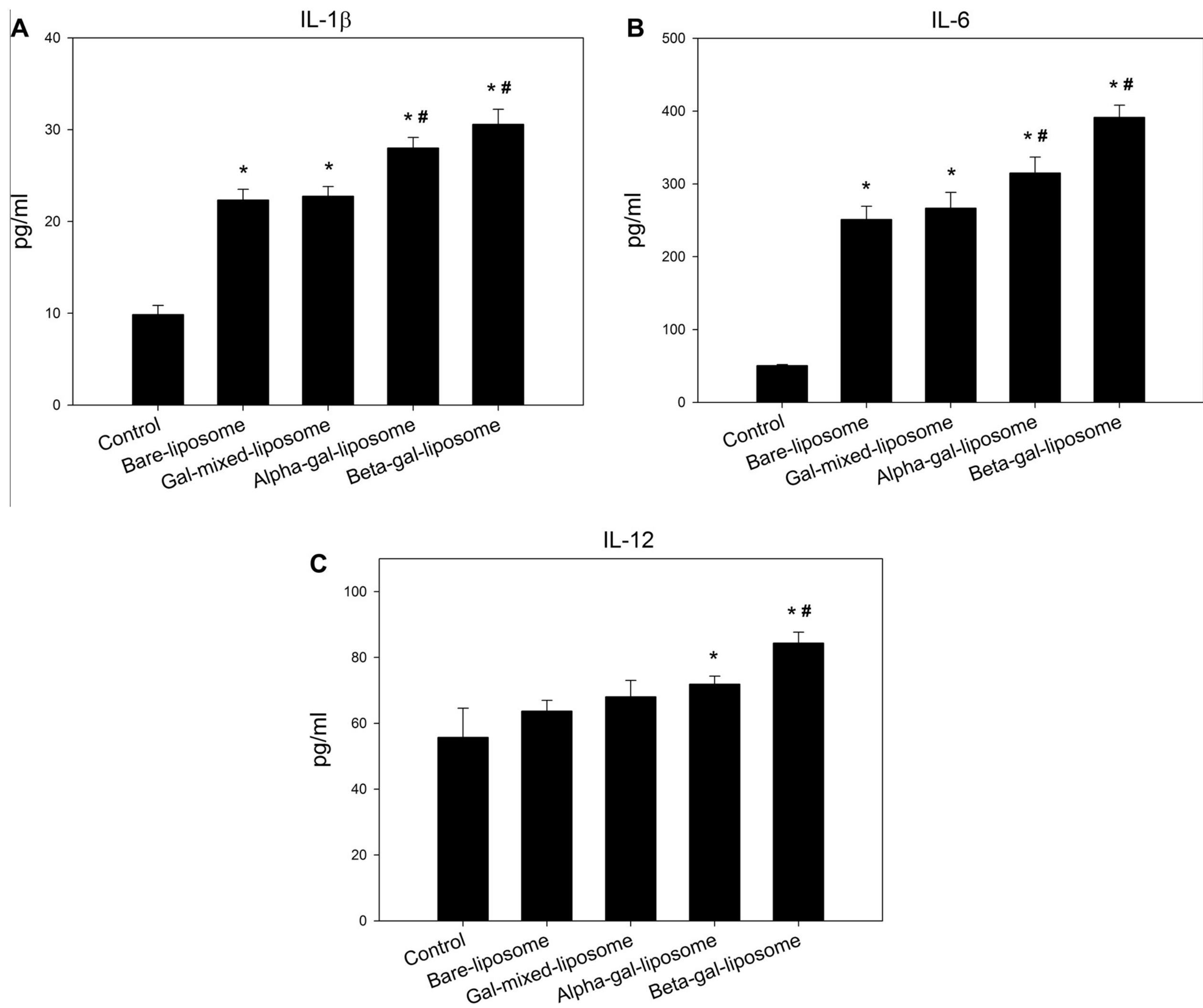


Fig. 3. The effect of galactosylated liposomes on the production of cytokines by BMDCs. BMDCs were treated with various liposome formulations for 24 h. The supernatants were collected and the production of cytokines IL-1β, IL-6 and IL-12 were determined by ELISA. The results are expressed as the means ± SE of triplicate experiments. *Significant difference ($p < 0.05$) compared with the control group; #significant difference ($p < 0.05$) compared with the bare-liposome group.

mice receiving either liposome formulation (Fig. 6A). Furthermore, the levels of IFN-γ induced by alpha-gal-liposomes and beta-gal-liposomes were significant higher than those induced by bare-liposomes and gal-mixed-liposomes. A slight increase in IL-4 secretion was found in the alpha-gal-liposome and beta-gal-liposome groups (Fig. 6B). However, we observed that spleen cell from mice receiving galactosylated liposomes showed significant increase in production of IL-5 and IL-6 (Fig. 6C and D). These results indicated that effective antigen delivery of galactosylated liposomes resulted in higher secretions of Th1 and Th2 cytokine. Next, we investigated the cellular immune response induced by intranasal immunization with galactosylated liposomes. IFN-γ producing CD8 + T cells were analyzed by flow cytometry after in vitro stimulation of spleen cells with whole OVA. As shown in Fig. 7, the number of IFN-γ producing CD8 + T cells was increased in mice immunized with alpha-gal-liposomes ($p = 0.082$) and significantly increased in beta-gal-liposomes ($p < 0.05$) compared with control group. Furthermore, we found the number of IFN-γ producing CD8 + T cells in the beta-gal-liposomes group was significantly increase compared with the bare-liposome group and the gal-mixed-liposome group. These results demonstrate that intranasal administration of galactosylated liposomes induced effective Th1 and Th2 humoral and cellular immune responses.

3.7. Protective effect of intranasal administration of galactosylated liposomes against EG7 tumor challenge

To evaluate whether galactosylated liposomes could be used as a DC-targeted mucosal vaccine for the induction of protective immune response against EG7 tumor challenge, mice were immunized intranasally with various liposome formulations three times at 1 week intervals. One week after the final immunization, mice were subcutaneously injected with 2×10^5 E.G7-OVA cells in the dorsal region, and tumor growth was evaluated in each mouse for 5 weeks. As shown in Fig. 8, the tumor growth in mice immunized with galactosylated liposomes was markedly reduced, whereas mice immunized with bare-liposomes or mixed-liposomes showed only partial inhibition of tumor growth. All mice immunized with beta-gal-liposomes completely rejected the E.G7-OVA tumor cells until 35 days after tumor inoculation. These results indicate that the DC-targeting and immunostimulatory effects of galactosylated liposomes could induce a systemic immune response that protects mice against tumor challenge.

4. Discussion

Nasal-associated lymphoid tissue is found in the murine upper respiratory tract and is considered equivalent to the Waldeyer's

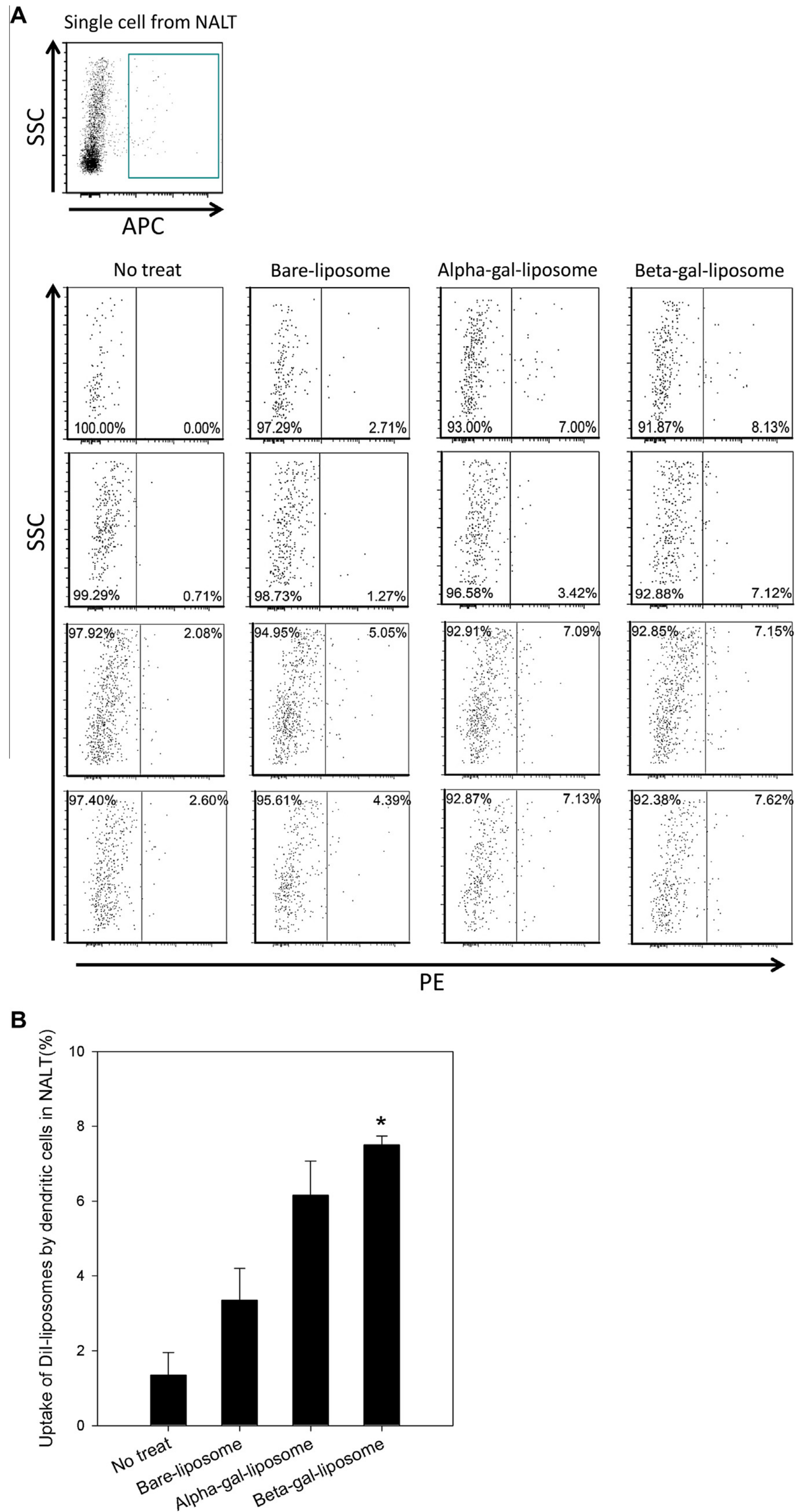


Fig. 4. Uptake of galactosylated liposomes by dendritic cells in the NALT. C57BL/6 mice were given DiI-labeled bare-liposomes, alpha-gal-liposomes or beta-gal-liposomes intranasally. After 24 h, single cell suspensions were isolated from the NALT and analyzed by FACS to evaluate DiI-labeled liposomes in CD11c+ cells (DCs). The CD11c+ cells were first gated based on allophycocyanin fluorescence (upper panel), and then uptake of liposomes in gated cells was analyzed based on DiI (PE) fluorescence. (A) Uptake of DiI-liposomes by dendritic cells in NALT. Similar results were obtained in four separate experiments. (B) The percentage of uptake of DiI-liposomes by dendritic cells was scored and represented in a histogram with a bar as the mean ± SE of four independent experiments. *Significant difference ($p < 0.05$) compared with the bare-liposome group.

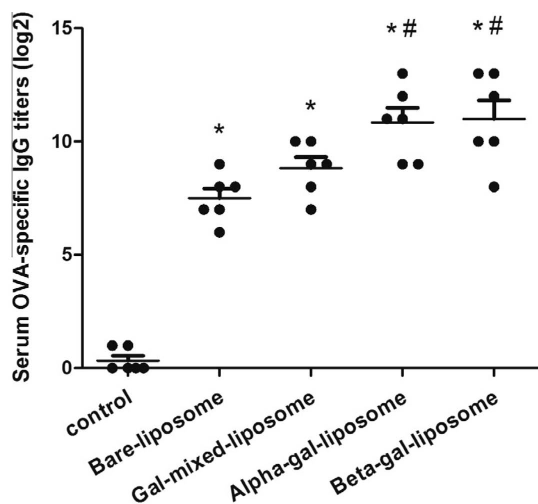


Fig. 5. Effect of galactosylated liposomes on the serum OVA-specific IgG antibody response. C57BL/6 mice were given various liposome formulations. One week after the final administration, serum samples were collected and anti-OVA antibodies were measured by ELISA. The antibody titer was defined as the reciprocal of the highest dilution that had an OD value that was two times higher than the mean OD of control mice serum. The results are expressed as the means ± SE. *Significant difference ($p < 0.05$) compared with the control group; #significant difference ($p < 0.05$) compared with the bare-liposome group.

ring in humans [26]. The NALT consists of a specialized follicle-associated epithelium, which contains antigen-sampling M cells [27] as well as large numbers of B cells, T cells and APCs, such as dendritic cells and macrophages [28]. Thus, the NALT contains all of the lymphoid cells that are required for the initiation and regulation of antigen-specific immune responses and is believed to be the ideal route for vaccination. However, because of its close proximity to a large number of commensal and dietary antigens in the environment, the mucosal immune system multifacetedly regulates the balance between pathogen surveillance and tolerance. Thus, mucosal vaccines require potent adjuvants, vectors or delivery platforms for inducing effective immune responses [29].

Liposomes have excellent immunostimulatory properties, including the capacity for sustained release of antigen over a prolonged time and preferential internalization by APCs; thus, the higher antigen delivery efficacy might promote the maturation and antigen presentation of APCs [30]. Antigen-encapsulated liposome formulations have been extensively used to enhance the effectiveness of mucosal vaccines in the oral and nasal delivery of antigens [31]. Recently, directly targeting APCs has been considered as a novel strategy to improve the efficiency of vaccines [32]. APCs have been shown to display various pattern-recognition receptors (PRRs), such as C-type lectin receptors (CLRs) and Toll-like receptors, which are critical for recognizing pathogen-associated molecular patterns and inducing immune responses. Among the PRRs, CLRs are particularly important for the recognition and uptake of glycosylated antigens [33]. In addition, several studies have shown that CLRs directly or indirectly trigger distinct signaling pathways by inducing specific cytokine secretion profiles, which then determine T cell polarization [34]. Their ability to mediate cellular uptake by APCs and regulate the immune response makes CLRs potentially useful for targeting APCs. MGLs are type II transmembrane glycoproteins with the ability to bind galactose, N-acetylgalactosamine and Lewis X as mono- or oligosaccharides [19]. Recent studies have reported that MGL is expressed on immature DCs in humans and mice [21,22]. For this reason, MGL was suggested to mediate the uptake of antigens and initiate the immune response in immature DCs. In our previous study, we prepared galactosyl lipids by conjugating a galactose molecule with DLPE. The galactosyl lipid was incorporated into the lipid bilayer of liposomes to form galactosylated liposomes [23]. Our results demonstrated that galactosylated liposomes can effectively target murine macrophages to enhance antigen uptake and cytokine production by murine macrophages. A significant increase in mucosal s-IgA and serum IgG antibody responses was also observed in mice receiving galactosylated liposomes intranasally. These results demonstrated the effectiveness of galactosylated liposomes as targeting carriers in intranasal immunization. To further expand the utility of galactosylated liposomes for targeting antigen delivery and for immune stimulation, the aim of the present study was to investigate the targeting effect of galactosylated liposomes on DCs and to evaluate whether galactosylated liposomes can act as a dendritic cell-targeted mucosal vaccine for inducing protective anti-tumor immunity.

The particle size of each liposome formulation is approximately 1000 nm. Therefore, the influence of particle size can be excluded as a confounding factor. To investigate the targeting effects of galactosylated liposomes, BMDCs were treated with various liposome formulations. After 6 and 18 h co-culture, galactosylated liposomes, especially beta-gal-liposomes, significantly facilitated the efficiency of uptake by BMDCs (Fig. 2). However, no significant difference was observed between the gal-mixed-liposomes and bare-liposomes. We next evaluated the uptake of galactosylated liposomes in vivo. Mice were intranasally treated with various DiI-labeled liposomes. Twenty-four hours after single-dose administration, similar results (Fig. 4) were observed as in the in vitro assays. DCs in the NALT took up galactosylated liposomes more effectively than liposomes without galactose modifications, suggesting that the recognition of galactose residues facilitated the galactosylated liposome uptake. Upon encapsulation of galactosylated liposomes, antigen could easily be delivered to DCs in a short time or with a low dose. It has been indicated that repeated low-dose antigen concentration exposure on the mucosa results in the induction of tolerance [35]. Thus, galactosylated liposomes have the potential capability to induce more efficient immune responses than conventional liposomes.

There are two signals involved in the activation of T cells: (i) the recognition of the major MHC–peptide complex by the T-cell receptor; and (ii) the interaction of B7-1 (CD80) or B7-2 (CD86) with the co-stimulatory receptor CD28 [36]. After capture of antigen, DCs lost their ability to acquire antigen but demonstrated an increased ability to present antigen and enhanced expression of B7 and MHC molecules [37]. Thus, the expression of B7 and MHC molecules tends to be a marker for DC maturation and the ability to activate T cells. Many researches have demonstrated that unmodified liposome facilitated antigen delivery to DC. In this study, we observed that BMDCs treated with galactosylated liposomes had higher expression of CD80, CD86 and MHC II molecules compared with bare-liposome (Fig. 2B–D). These results indicate both the effective antigen delivery by targeted galactosylated liposomes and the liposome-enhanced maturation of DCs. The cytokines secreted by DCs also affect the maturation and development of DCs, thus influencing the subsequent immune regulations [38]. The distinct cytokines secreted by mature DCs ultimately determine their Th1/Th2 polarizing capacities. IL-1 and IL-6 are considered the major pro-inflammatory cytokines that play important roles in the protection against pathogens during an infection [39]. It has been reported that IL-12 induces IFN-γ secretion from natural killer (NK) cells and T cells activates NK and T cells, enhances the cytotoxic activity of NK cells and favors cytotoxic T lymphocyte generation [40]. Thus, we evaluated cytokine secretion by DCs. The results showed that stimulating BMDCs with galactosylated liposomes significantly increased IL-1β, IL-6 and IL-12 secretion into the supernatant (Fig. 3). These results suggest that galactosylated liposomes are capable of inducing more efficient immune responses than conventional liposomes. According to these in vitro results, we believe that

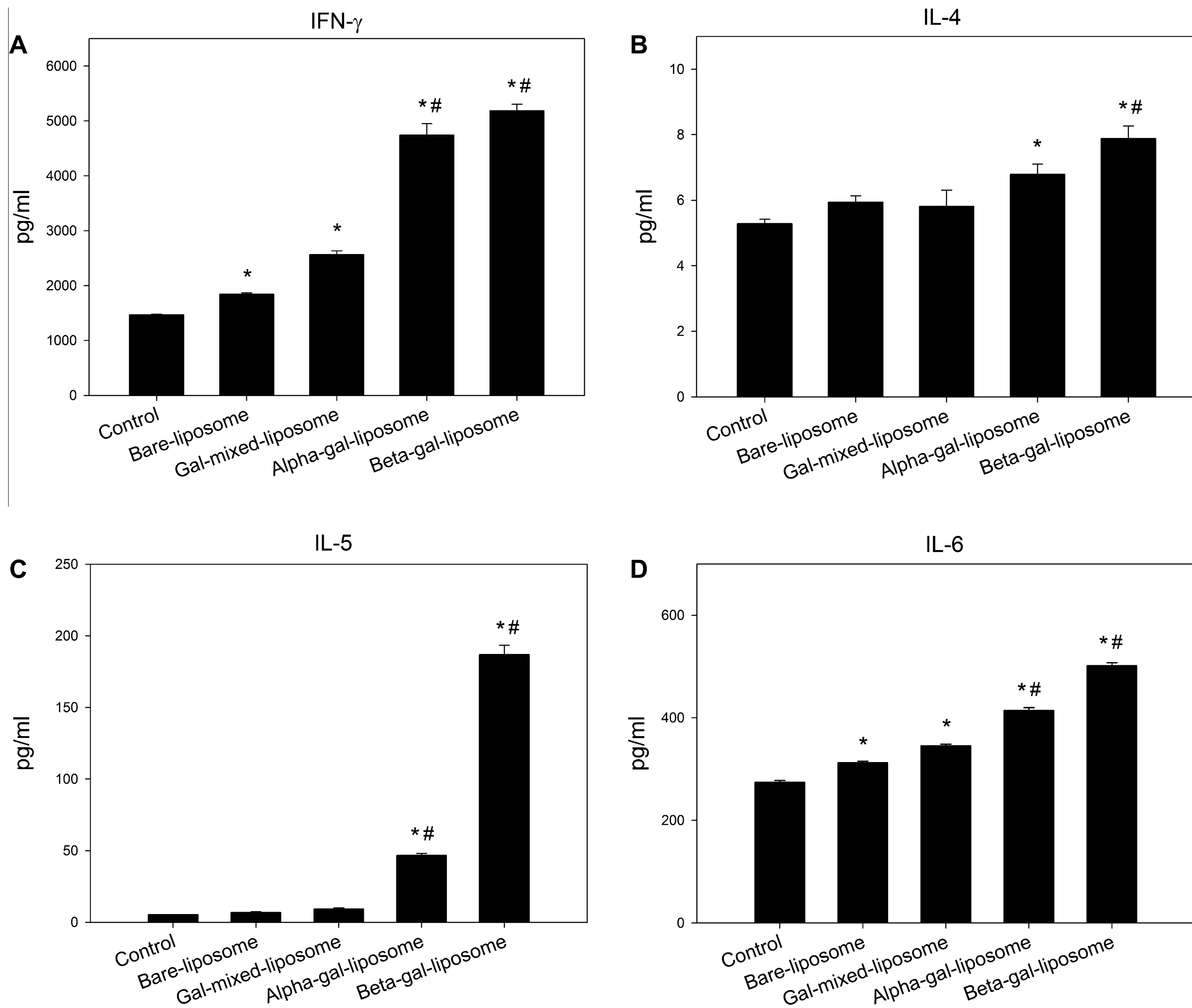
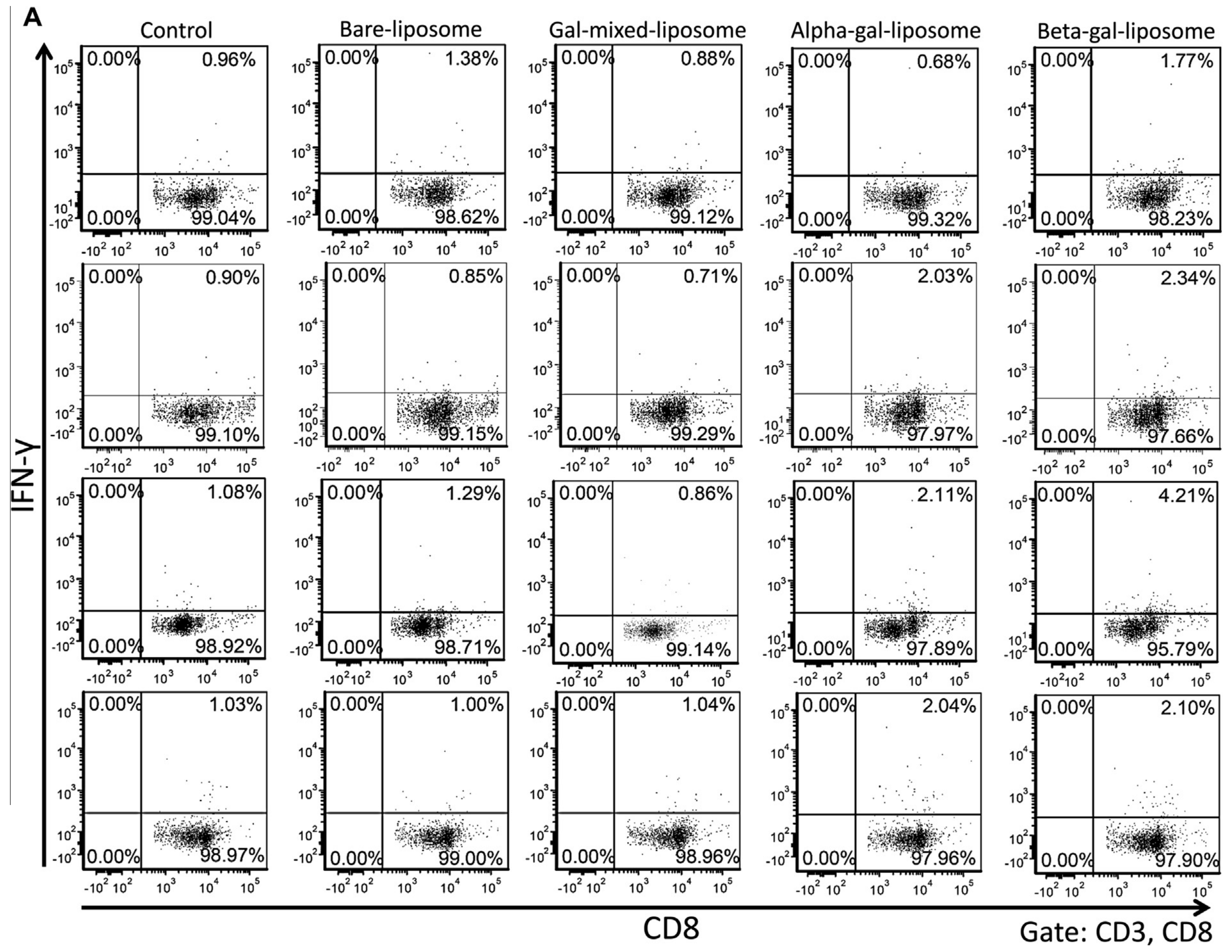


Fig. 6. Cytokine production from spleen cells after in vitro stimulation. Spleen cells from mice receiving different liposome formulations were obtained 1 week after the final immunization and cultured with 100 μg ml^{-1} OVA for 72 h. The supernatants were collected and the production of cytokines IFN-γ, IL-4, IL-5 and IL-6 was measured. The results are expressed as the means ± SE. *Significant difference ($p < 0.05$) compared with the control group; #significant difference ($p < 0.05$) compared with the bare-liposome group.

galactosylated liposomes indeed have the potential to act as a targeted mucosal vaccine.

Cancer immunotherapy is the use of the immune system to reject cancer. There is a large volume of published studies describing the adjuvant effect of liposomes in the induction of s-IgA, which provides more cross-protection against viral infection than that afforded by systemic immunity. However, in an effectively therapeutic or protective antitumor immune response, both a humoral immune response and a cell-mediated immune response must be launched. Thus, many approaches have attempted to modify the liposomes or co-administer various immunostimulators with the liposomes for effective cancer immunotherapy [8]. Recently, Ishii and Kojima [41] demonstrated that intranasal administration of mannose-modified liposomes without other adjuvants induced antigen-specific serum IgG and IgA as well as IFN-γ and IL-5 secretion by spleen cells. In the current study, our results have shown that nasal administration with OVA-encapsulated alpha- or beta-galactosylated liposomes induced higher serum IgG titers than bare-liposomes or gal-mixed-liposomes (Fig. 5). Furthermore, following an immunization boost, we observed a significant increase in serum OVA-specific antibodies over that of the primary immunization (data no shown), demonstrating that liposomes are able to induce an anamnestic immune response. The levels of IFN-γ and IL-4 were quantified to evaluate the Th1 and Th2 immune responses. Spleen cells from mice immunized with galactosylated liposomes produced significantly increased amounts of IFN-γ after in vitro stimulation with OVA. These results indicate that intranasal immunization of galactosylated liposomes can trigger a better Th1 immune response. We further analyzed the IFN-γ producing CD8 + T cells after stimulating spleen cells with OVA. We observed that the number of IFN-γ secreting CD8 + T cells was significantly increased in galactosylated liposome-immunized mice compared with those in the bare-liposome and mixed-liposome groups. Based on these results, we suggested that efficient antigen delivery of galactosylated liposomes up-regulates pro-inflammatory cytokine (IL-1β, IL-6 and IL-12) secretion and increases the expression of co-stimulatory molecules and MHC II molecules on DCs, which are essential for the induction of a Th1/Th2 immune response in mice. As a result, galactosylated liposomes effectively enhance the immune response without an additional immunostimulator.

Many studies have indicated that tumor cell lysates could be used to induce a sufficient anti-tumor immune response. Therefore, particle-based antigen delivery systems were developed in tumor-associated antigen (TAA)-based cancer immunotherapy for



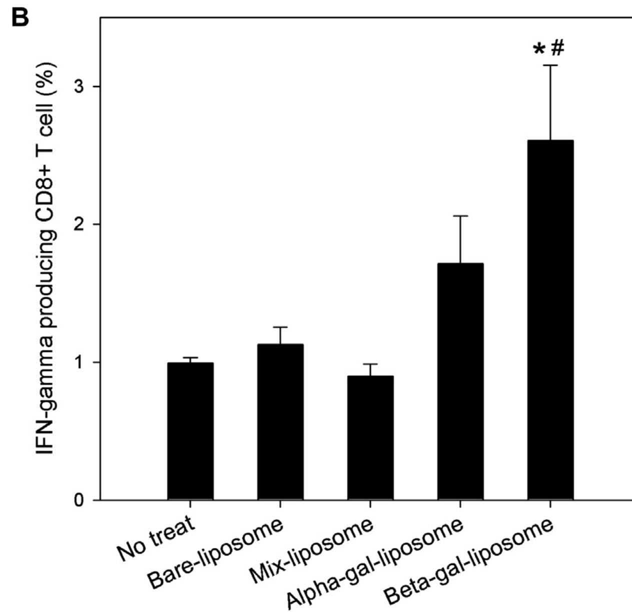


Fig. 7. IFN-γ producing CD8 + T cell analysis. Spleen cells (5×10^6 cells ml^{-1}) from mice receiving different liposome formulations were incubated with 100 μg ml^{-1} OVA for 72 h and 1 μl ml^{-1} GolgiPlug was added during the last 5 h. Intracellular staining was performed with BD Cytofix/Cytoperm. (A) IFN-γ producing CD8 + T cells were analyzed by FACS. Similar results were obtained in four separate experiments. (B) The percentage of IFN-γ producing CD8 + T cells was scored and represented in a histogram with the bar as the mean ± SE of four independent experiments. *Significant difference ($p < 0.05$) compared with the control group; #significant difference ($p < 0.05$) compared with the bare-liposome group.

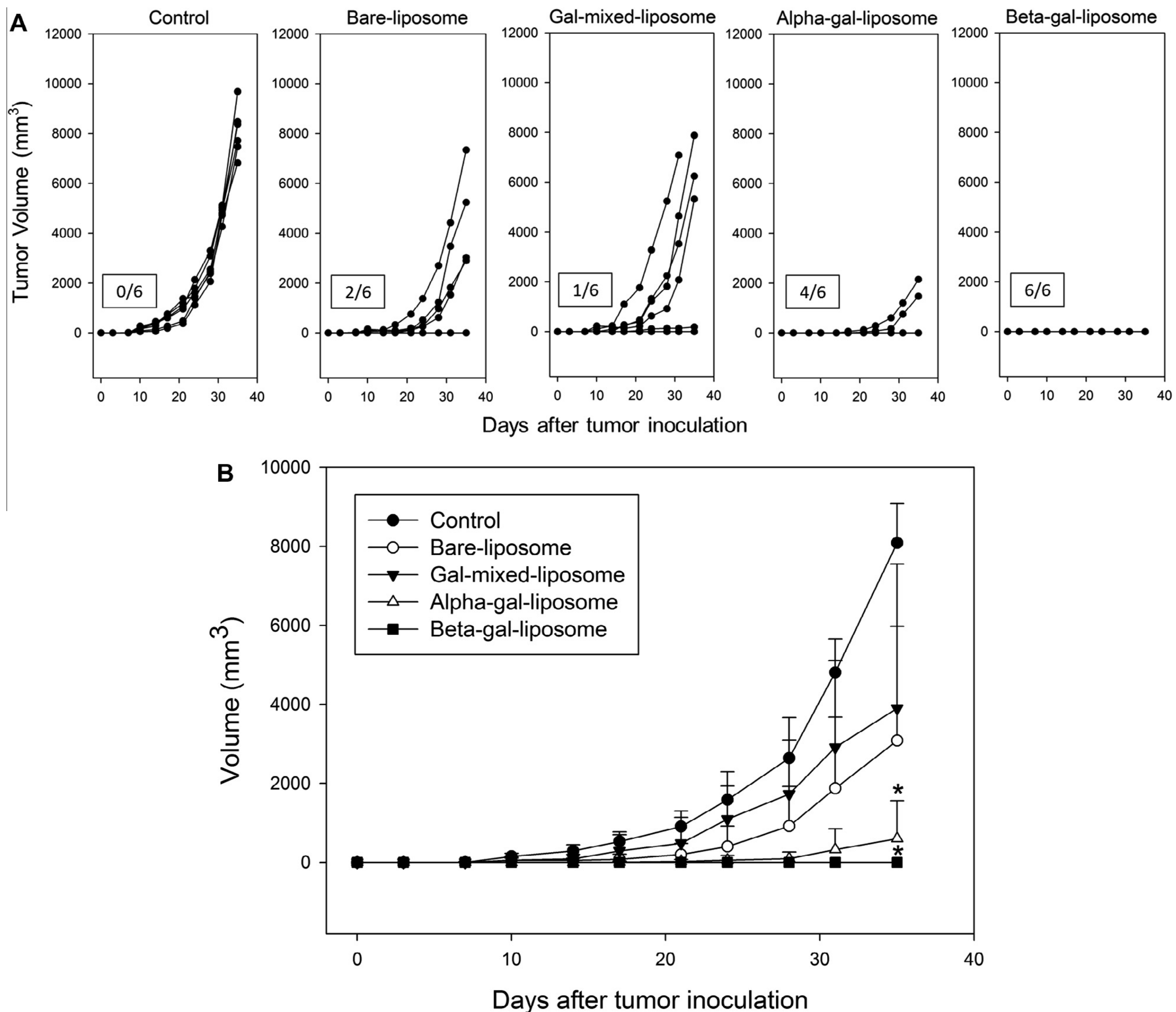


Fig. 8. Growth of E.G7-OVA tumors in mice immunized intranasally with galactosylated liposomes. C57BL/6 mice were immunized with bare-liposomes, gal-mixed-liposomes, alpha-gal-liposomes, beta-gal-liposomes or PBS (control). (A) Individual tumor growth curves from mice injected s/c/with 2×10^5 E.G7-OVA cells 1 week after the final immunization. Boxed fractions indicate complete rejections after tumor inoculation. (B) The average tumor volume in each group. The results are expressed as means ± SE. *Significant difference ($p < 0.05$) compared with the control group.

protection from or elimination of tumors that express TAA. The E.G7-OVA cell line, an EL4 cell line that stably transfected with cDNA of OVA to allow constitutive production of OVA with an H-2Kb-restricted CTL epitope [42], is a suitable model system for studying MHC class I restricted responses of cytotoxic T lymphocytes in mice. In this study, we used an s.c. E.G7-OVA tumor protection model to evaluate galactosylated liposomes inducing an in vivo antitumor effect. In Fig. 8, 5/6 and 6/6 mice receiving alpha-gal-liposomes and beta-gal-liposomes, respectively, completely rejected the tumor challenge. By contrast, only 2 and 1 mice receiving bare-liposomes and gal-mixed-liposomes, respectively, completely rejected the tumor challenge after tumor inoculation. These results indicate that intranasally immunized galactosylated liposomes might be more effectively taken up by DCs in the NALT, subsequently triggering DCs to mature and migrate to the systemic circulation, thus resulting in the induction of local and systemic immune responses. Thus, vaccination with galactosylated liposomes, especially beta-gal-liposomes, was able to achieve more effective protective anti-tumor effects.

5. Conclusion

In this study, we report that galactosylated liposomes effectively facilitated antigen uptake by DCs in vitro and in vivo, leading to an increase of cytokine production. Moreover, mice receiving intranasally administrated galactosylated liposomes showed significant increases in serum IgG antibody levels and activity of spleen cells, which led to an efficient anti-OVA immune response against EG7 tumor challenge. Our results suggest that galactosylated liposomes could elicit not only antibody immune responses but also systemic anti-tumor immunity. Recently, many studies have focused on designing cancer vaccines for the treatment of mucosal tumors. Sandoval et al. have reported that the growth of orthotopic head and neck or lung cancers was inhibited when a cancer vaccine was delivered via the intranasal mucosal route but not by the intramuscular route [43]. Thus, we suggest that intranasal vaccination with galactosylated liposomes, especially beta-gal-liposomes, was able to achieve more effective protective effects that will be useful for the treatment of mucosal tumors.

Acknowledgments

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1, 2 and 4 is difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.09.019.

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