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Mucosal adjuvant activity of oligomannose-coated liposomes for nasal immunization

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Abstract In the present study, we investigated the effectiveness of liposomes coated with a neoglycolipid consisting of mannotriose and dipalmitoylphosphatidylcholine (Man3-DPPE) as an adjuvant for induction of mucosal immunity. Immunization of BALB/c mice with ovalbumin (OVA)-encapsulated Man3-DPPE-coated liposomes (oligomannose-coated liposomes; OMLs) by a nasal route produced high levels of OVA-specific IgG and IgA antibodies in serum of immunized mice 1 week after the last nasal immunization, whereas no significant serum antibody responses were observed in mice that received OVA in uncoated liposomes or OVA alone. Seven weeks after the last nasal immunization, nasal challenge with an excess amount of OVA in mice that had received OVA/OMLs led to an anamnestic response to the antigen that resulted in 5- to 10-fold increases of antigen-specific serum IgG and IgA antibodies. Only mice immunized nasally with OML/OVA secreted antigen-specific secretory IgA in nasal washes and produced interferon-gamma secreting cells in nasopharyngeal-associated lymphoreticular tissue. Taken together, these results show that nasal administration of OMLs induces mucosal and systemic immunity that are specific for the entrapped antigen in the liposomes. Thus, liposomes coated with synthetic neoglycolipids might be useful as adjuvants for induction of mucosal immunity.

Keywords Neoglycolipids · Oligomannose · Liposome · Mucosal adjuvant · IgA

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Introduction

The majority of pathogens gain access to a human host via mucosal routes. Protection against pathogens is better correlated with the presence of antibodies in local secretions than in serum [1], and antibodies resulting from subcutaneous immunization do not necessarily reach mucosal surfaces [2]. In addition, development of effective mucosal vaccines is required to ameliorate problems associated with current vaccines, such as the need for frequent injections. Therefore, induction of an effective mucosal immune response by vaccination is of considerable importance, and adjuvants and delivery systems are particularly important for this purpose since most antigens are poorly immunogenic when given via mucosal (oral or nasal) routes. Bacterial holotoxins such as cholera toxin and *E. coli* thermolabile enterotoxin can act as adjuvants for induction of mucosal and serum antibodies against an antigen when co-administered with the antigen mucosally, resulting in long-term memory to the antigens [3, 4], but their use is restricted by toxicity [5–7]. CpG oligonucleotides (ODNs), which are recognized by Toll-like receptor (TLR)-9 expressed on antigen-presenting cells (APCs), are also potent mucosal adjuvants that significantly enhance cellular and humoral responses to co-administered antigens when given parenterally or mucosally [8, 9]. However, *in vivo* degradation of ODNs limits their uptake and effectiveness as adjuvants, and repeated injection of CpG-ODNs may have unfavorable immunosuppressive effects [10]. Therefore, there remains a need for development of effective mucosal adjuvants.

We have synthesized a neoglycolipid consisting of mannotriose and dipalmitoylphosphatidylethanolamine (Man3-DPPE) and demonstrated that liposomes coated with Man3-DPPE (oligomannose-coated liposomes, OMLs) induce a Th1 immune response with CTLs specific for

antigens encased in the liposomes following subcutaneous or intraperitoneal administration [11, 12]. No skin damage was caused at the OML injection sites [13], suggesting that OMLs can be used safely as an adjuvant for induction of antigen-specific Th1 immune responses. In the induction of mucosal or systemic immunity, particulate antigens such as liposome-encapsulated antigens may be more effective than soluble antigens because of more efficient endocytosis of the particulate antigens by M cells in mucosal-associated lymphoid tissue [14]. Furthermore, polysaccharides have also been used as intranasal adjuvants for induction of mucosal and systemic immune responses [15, 16]. These results led us to hypothesize that oligosaccharide-coated liposomes such as OMLs could be used as not only an adjuvant for induction of a systemic Th1 immune response via a subcutaneous route but also as a mucosal adjuvant to induce mucosal immune responses. The aim of this study was to clarify whether intranasal administration of OMLs produces mucosal and systemic immunity using ovalbumin (OVA) as a model antigen. Our results indicate that nasal administration of OMLs induces antigen-specific secretory IgA in local tissues and antigen-specific serum IgG and IgA.

Materials and methods

Chemicals

Cholesterol, dipalmitoylphosphatidylethanolamine (DPPE), and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma (St Louis, MO). Mannotriose (Man3) with the structure Manα1–6(Manα1–3)Man was purchased from Funakoshi (Tokyo, Japan). Man3-DPPE was prepared in our laboratory by conjugation of these oligosaccharides with DPPE, as described previously [17]. In brief, 20 μmol of oligosaccharide and 70 μmol of DPPE were dissolved in 5 ml of chloroform/methanol/water (10:10:1). Then, 300 μmol of sodium cyanoborohydride dissolved in methanol was added and the reaction mixture was kept at 80°C for 5 h. After desalting using a C18 column (Bond Elute, MEGA BE-C18, Varian, Harbor City, CA), the neoglycolipid was purified by high-performance liquid chromatography (HPLC) on a silica column (Wakosil 5SIL-120, 0.75×30 cm, Wako, Tokyo, Japan) with a linear gradient of chloroform/methanol/water (65:30:5) and chloroform/methanol/water (50:55:18). The purity of Man3-DPPE was determined by high-performance thin-layer chromatography, and was at least 98%.

Preparation of liposomes

Liposomes were prepared as described previously [12, 17]. Briefly, a chloroform:methanol (2:1, v/v) solution containing 1.5 μmol of DPPC, 1.5 μmol of cholesterol, and 0.15 μmol of Man3-DPPE was added to a flask and evaporated to prepare a lipid film. PBS containing 5 mg/ml of OVA (Sigma) 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 (Nucleopore, Pleasanton, CA). The amount of entrapped protein was measured using a modified Lowry protein assay kit (Pierce, Rockford, IL) in the presence of 0.3% (w/v) sodium dodecyl sulfate, using BSA as the standard. Molar ratios of the lipid components of the liposomes were determined using HPLC. Particle sizes of liposomes were determined by dynamic light scattering using a particle size analyzer (LB-550, Horiba, Kyoto, Japan).

Immunization of mice

Female Balb/c mice (6 to 8 week old) were used in the study. Mice (5 per group) were immunized by a nasal or subcutaneous route with 5 μg OVA encased in Man3-DPPE-coated liposomes (OML/OVA), 5 μg OVA encased in uncoated liposomes (Bare/OVA), 5 μg OVA alone, or PBS (100 μl) on days 0, 3, 6, and 9. In some cases, mice were immunized intranasally with 5 μg OVA and 5 μg cholera toxin B (CTB) subunit (List Biological Laboratories, Inc. Campbell, CA). For nasal immunization, mice were given multiple administrations of 10 to 15 μl of immunogen. Seven weeks after the last nasal immunization (on day 58), mice were given 10 μg of OVA in PBS intranasally. Blood was collected weekly from the tails of mice in each group and serum samples were prepared and stored at −30°C until assessment of levels of antibodies. Alternatively, nasal wash, nasopharyngeal-associated lymphoreticular tissue (NALT) and spleen were recovered from mice from each group 1 week after the last immunization (on day 16). Single cell suspensions were prepared from each NALT sample and spleen to evaluate antigen-specific cytokine production. The nasal washes were used for assessment of secreted antibodies specific for OVA.

Determination of antigen-specific antibodies and cytokine production

An enzyme-linked immunosorbent assay (ELISA) was used to determine the relative concentrations of antibodies and cytokines. For the antibody assay, flat-bottom 96-well plates were precoated with 50 μl OVA (2.5 μg/ml) overnight at 4°C. The plates were blocked with 1% BSA in PBS, and 256-fold dilutions of serum samples or 64-fold dilutions of nasal wash samples were dispensed in the wells. After incubation for 1 h, the plates were washed with

0.05% Tween in PBS (Tween-PBS) and the levels of antigen-specific immunoglobulin A (IgA), IgG1, or IgG2a in the samples were determined using rabbit anti-mouse IgA, rabbit anti-mouse IgG1, or rabbit anti-mouse IgG2a antibody (Zymed Laboratories, San Francisco, CA) followed by HRP-conjugated anti-rabbit antibody (MBL, Nagoya, Japan). A HRP-conjugated goat anti-mouse IgG was used for detection of antigen-specific total IgG. For the cytokine assay, single cell suspensions prepared from the spleen of an individual mouse were prepared in Hanks' balanced salt solution containing 2% fetal calf serum (FCS). Erythrocytes were lysed with a Tris-buffered ammonium chloride solution (17 mM Tris-HCl, pH 7.65, containing 150 mM NH_4Cl) and the spleen cells were then washed with RPMI 1640 medium containing 10% FCS, suspended at a density of 5×10^6 cells/ml, dispensed in triplicate onto 24-well plates, and cultured in 1 ml of RPMI 1640 medium containing 10% FCS in the presence or absence of 50 μg/ml OVA. After incubation for 72 h at 37°C in 5% CO_2, supernatants were collected and kept at −80°C until assayed for cytokines. Levels of IFN-γ, IL-4, IL-5, and IL-6 in the supernatants were quantified using commercial ELISA kits (Pierce, Rockford, IL).

Enzyme-linked immunospot assay (ELISPOT)

The number of IFN-γ secreting cells was detected by an ELISPOT assay using a commercial ELISPOT kit (BD Biosciences, San Diego, CA). Single cell suspensions of NALT or spleen from immunized mice were added in wells in three serial dilutions (1×10^6 to 10^4 cells in 100 μl of RPMI 1640 medium containing 10% FCS) in the presence or absence of 10 μg/ml OVA. Plates were incubated at 37°C in 5% CO_2 for 16 h, washed with Tween-PBS, and then incubated overnight at 4°C with an anti-IFN-γ antibody. After washing the plates with Tween-PBS, spots representing single cells were developed according to the manufacturer's instructions and counted with a dissecting microscope.

Evaluation of uptake of OMLs by dendritic cells in NALT

Balb/c mice were given OMLs or uncoated liposomes (50 μg of cholesterol) with encased Alexa647-conjugated BSA nasally. Twenty-four hours after administration, single cell suspensions were prepared from NALT samples. The cells were washed twice with PBS, treated with Fc block (BD PharMingen, San Diego, CA), and then stained with a phycoerythrin (PE)-labeled anti-CD11c antibody (BD PharMingen) to detect dendritic cells. Fluorescent signals from Alexa647 in CD11c-positive cells were analyzed to evaluate uptake of OMLs by dendritic cells.

Results

Anti-OVA antibodies in serum

Liposomes prepared from DPPC, cholesterol, and Man3-DPPE at the molar ratio of 10:10:1 (oligomannose-coated liposomes, OMLs) and extruded through a membrane with 1-μm pores were used in all experiments. The molar ratios of DPPC, cholesterol, and Man3-DPPE in the Man3-DPPE-coated liposomes (OMLs) and uncoated liposomes (Bare) were 1.00: 1.09±0.10: 0.093±0.009 and 1.00: 0.98±0.08: 0, respectively (mean±SD from 3 independent preparations). The particle sizes (median ± range) of the OMLs and uncoated liposomes were 1122 ± 430 and 984 ± 360 nm, respectively, and the protein concentrations (mean ± SD) of the OMLs and uncoated liposomes were 124.7±13.3 and 122.1±24.2 μg/mg of cholesterol, respectively.

Mice were immunized nasally with OML/OVA, Bare/OVA, OVA, or PBS on days 0, 3, 6, and 9, and the levels of OVA-specific IgG and IgA in serum were determined (Fig. 1). One week after the last immunization (on day 16), both OVA-specific IgG and IgA appeared in serum from mice that received OML/OVA. The levels of these antibodies increased at 3 weeks after the last immunization (on day 30) (Fig. 1a,b). In contrast, OVA-specific IgG and IgA levels in serum of mice that received Bare/OVA and OVA alone were the same as those for mice that received PBS (control). Since Th1 cells induce IgG2a secretion and Th2 cells induce IgG1 secretion in mice [18], we next measured the serum levels of OVA-specific IgG1 and IgG2a in OML/OVA-received mice (Fig. 1c). Antigen-specific IgG1 was secreted in serum on day 16 and the level increased up to day 30, while the level of OVA-specific IgG2a in serum of OML/OVA-received mice was very low and almost the same as that in non-immunized (PBS-received) mice. Increased levels of OVA-specific IgG1 and IgG2a were not observed in sera of mice that received Bare/OVA, OVA, or PBS (data not shown). When the mice were challenged with 10 μg of OVA nasally at 7 weeks after the last immunization (on day 58), the levels of antigen-specific IgG and IgA in serum of OML/OVA-received mice increased by 5–10 fold at 1 week after challenge (on day 65), while the increases in serum antibodies in other groups of mice were almost negligible (Fig. 1d,e).

Antigen-specific production of cytokines from spleen cells

To assess whether intranasal immunization of liposomes induces a Th1 or Th2 cells, production of Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-6) cytokines was determined following *in vitro* stimulation of spleen cells obtained from mice 1 week after the last immunization (on day 16) (Fig. 2). *In vitro* stimulation of spleen cells obtained from

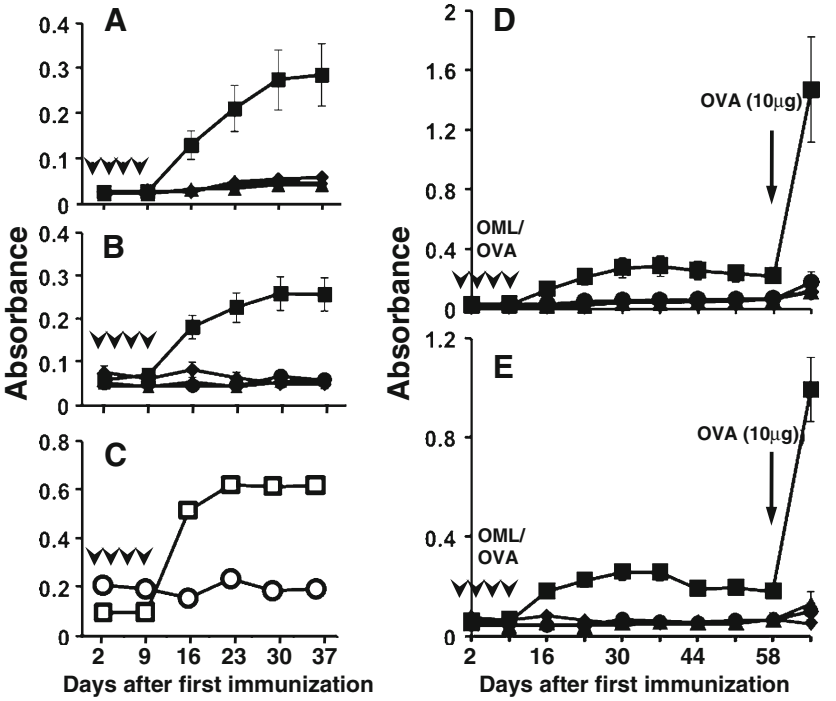

Fig. 1 Serum antibody response in mice immunized with OVA in OMLs by an intranasal route. Mice were immunized intranasally with OVA in OMLs (OML/OVA) (■), OVA in bare liposomes (Bare/OVA) (●), OVA alone (▲), or PBS (♦) on days 0, 3, 6, and 9 (indicated as arrowheads), with 5 μg of OVA given on each day. On day 58, mice were given 10 μg of OVA nasally (indicated as arrows). Serum was collected from each mouse weekly and the levels of OVA-specific IgG (panels A and D) and OVA-specific IgA (panels B and E) were determined in 256-fold diluted samples. Panel C showed the levels of the antigen-specific serum IgG1 (□) and IgG2a (○) of mice that received OML/OVA. Each bar represents the mean ± SD of three independent experiments

mice that received OML/OVA and Bare/OVA resulted in production of higher levels of IL-5 and IL-6 in comparison with cells from mice that received OVA alone or PBS ($P< 0.001$). Although production of IL-4 from spleen cells was not observed in any mice, these results indicate that intranasal administration of particulate antigens such as liposome-entrapped antigens induces Th2 cytokines, regardless of the presence of a carbohydrate coating. In contrast, intranasal administration of OML/OVA led to production of IFN-γ from spleen cells following *in vitro* stimulation with OVA. The IFN-γ level produced from spleen cells of OML/OVA-received mice was low, but significantly higher than those for mice that received Bare/OVA and OVA alone.

Comparison of adjuvant activity of OMLs to that of cholera toxin B

It is well known that bacterial holotoxins such as cholera toxin act as strong adjuvants for induction of mucosal and serum antibodies against an antigen [3]. In addition, CTB, which has no enzymatic activity but has GM1-binding properties, can also serve as an adjuvant for antigens administered by an intranasal route [19]. Therefore, we compared the adjuvant activity of OMLs to that of CTB to evaluate the adjuvant efficiency of OMLs. One week after the last immunization (on day 16), the levels of antigen-specific serum IgG and serum IgA in OML-received mice were almost the same as those in CTB-treated mice (Fig. 3). In addition, the levels of IFN-γ and IL-5 produced from spleen cells of OML-received mice were similar to those from spleen cells of CTB-treated mice following *in vitro* stimulation with OVA. These results indicate that OMLs have almost equivalent efficiency as a mucosal adjuvant to that of 5 μg of CTB.

Anti-OVA IgA in nasal washes

The above results indicate that intranasal administration of OMLs induces systemic immunity with secretion of OML-entrapped antigen-specific serum IgA, but it was unclear whether OML administration could also induce mucosal immunity. The antigen-specific IgA isotype, especially secretory IgA (S-IgA), is the primary immunoglobulin involved in protection of mucosal surfaces. To determine if nasal immunization with OMLs can elicit antigen-specific mucosal immunity, we measured the levels of secreted anti-OVA antibody in nasal washes from immunized mice 1 week after the last immunization (on day 16) (Fig. 4). Antigen-specific secretory IgGs were not detected in nasal washes from any mice. However, the levels of antigen-specific IgA in nasal washes from mice given OVA/OML intranasally were significantly higher than those in samples from mice that received OVA alone or PBS intranasally, suggesting that mucosal immunization with OMLs induced OML-entrapped antigen specific S-IgA in local mucosal tissues. In contrast, subcutaneous administration of OML/OVA did not induce antigen-specific S-IgA in the nasal washes of the immunized mice.

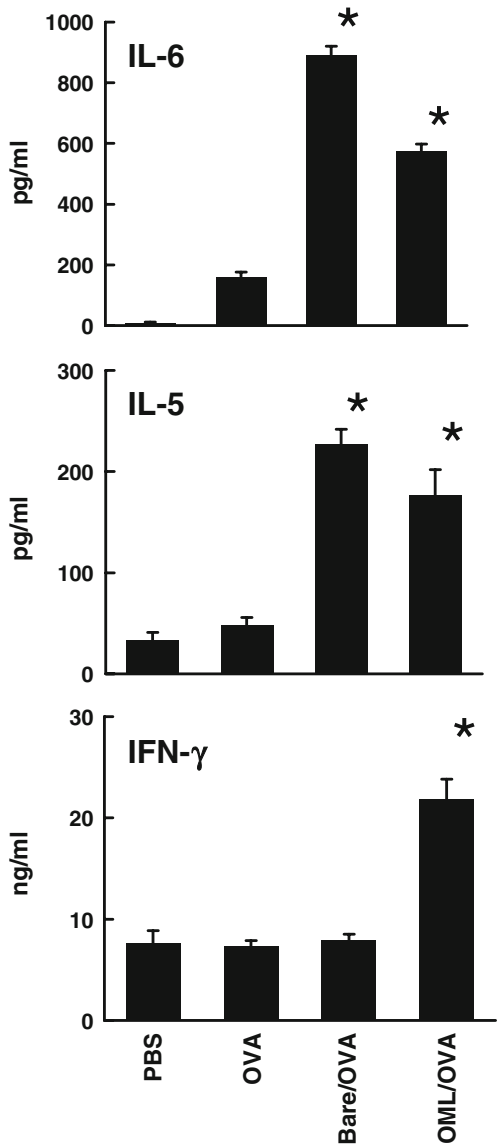

Fig. 2 Cytokine production from spleen cells after *in vitro* stimulation. The spleen was obtained from each mouse 1 week after the last nasal immunization (on day 16). Single cell suspensions from spleens of mice in the designated groups were prepared and cultured in triplicate for 48 h in the presence of OVA (50 μg/ml). Culture supernatants were assayed for IL-5, IL-6, and IFN-γ by ELISA. Each bar represents the mean ± SD of three independent experiments. * $P<$ 0.01 compared to mice that received PBS

Induction of IFN-gamma secreting cells in NALT

Secretory component (SC) acts as a transmembrane polymeric immunoglobulin receptor in epithelial cells and binds to polymeric IgA to facilitate secretion of S-IgA [20]. IFN-γ produced by Th1 cells upregulates production of SC at mucosal sites [20, 21]. Although we showed that nasal administration of OML/OVA induced IFN-γ production from spleen cells following *in vitro* stimulation (Fig. 2), it was not clear if IFN-γ was produced in local mucosal tissues. Therefore, the numbers of IFN-γ secreting cells in inductive lymphoid tissues such as NALT and spleen were measured by an ELISPOT assay 1 week after the last immunization (on day 16) (Fig. 5). A significant number of antigen-specific IFN-γ secreting cells was found in the single cell suspension of NALT only from mice that received OML/OVA nasally after *in vitro* stimulation of the cells. Some antigen-specific IFN-γ secreting cells were also detected in spleen cells from mice that received OML/OVA nasally. In contrast, nasal administration of Bare/OVA or OVA alone did not induce IFN-γ secreting cells in NALT or spleen. Subcutaneous administration of OML/OVA did not induce IFN-γ secreting cells in NALT, but did induce a significant number of these cells in spleen.

Effective uptake of OMLs by dendritic cells in NALT

Enhanced immunization by OMLs may be due to effective uptake of OMLs by APCs in nasal tissues. To evaluate this possibility, OMLs or uncoated liposomes with encased Alexa647-conjugated BSA were administered nasally to mice, and single cell suspensions were prepared from NALT samples 24 h after administration. Fluorescent signals from Alexa647 in CD11c-positive cells were analyzed to evaluate uptake of liposomes by dendritic cells in NALT. As shown in Fig. 6, 7.6% of CD11c-positive cells in NALT obtained from OML-received mice showed strong signals for Alexa647, while less than 1% of cells from mice given uncoated bare liposomes showed Alexa647 fluorescence. These results suggest that antigens encased in OMLs are more effectively delivered into nasal dendritic cells, compared to antigens administered in uncoated liposomes.

Discussion

Liposomes formulated with antigen enhance the effectiveness of mucosal vaccines and have been used extensively in oral and nasal delivery of antigens in mice [22]. Many liposomes used to date for induction of mucosal immunity are conjugated with viral fusion proteins [23] or adjuvants such as cholera toxin, lipid A, and CpG [24–26] to improve antigen uptake and presentation. Liposomes conjugated with polysaccharides such as mannan and chitosan have also been used for induction of mucosal immunity [27], but little is known about the mucosal adjuvant activity of liposomes coated with synthetic oligosaccharide derivatives such as neoglycolipids.

We have recently developed a new technology for accumulation of anti-cancer drugs at extranodal lymphoid tissue in the omentum to control intraabdominal metastatic foci using Man3-DPPE coated liposomes (OMLs) as a

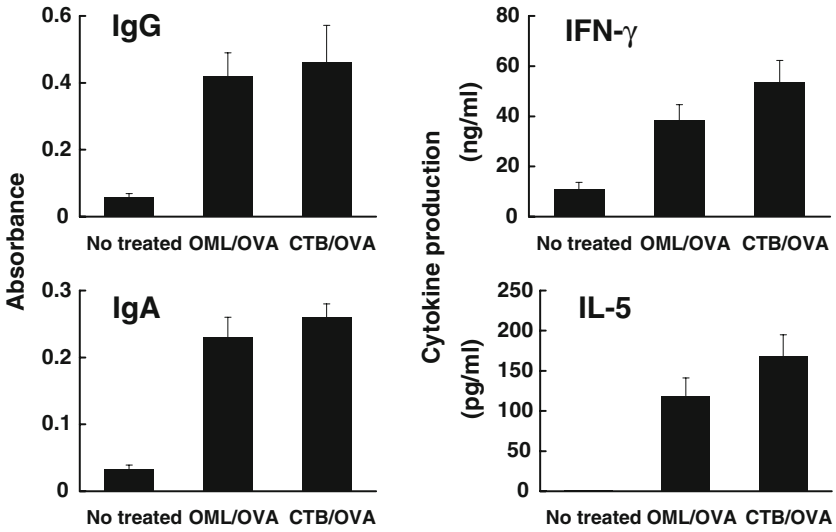

Fig. 3 Comparison of immune responses between OML-received and CTB-treated mice. Mice were immunized intranasally with OML/OVA or CTB/OVA on days 0, 3, 6 and 9, with 5 μg of OVA given on each day. On day 16, sera were collected and the levels of OVA-specific IgG and IgA were determined in 256-fold diluted samples. On the same day, single cell suspensions were prepared from spleens of mice and cultured for 48 h in the presence of OVA. The culture supernatants were assayed for IL-5 and IFN-γ by ELISA. Each bar represents the mean ± SD of three independent experiments

carrier for the anti-cancer drugs [28, 29]. This novel OML-based drug delivery system can also be used for induction of systemic immune responses, as shown by the production of IFN-γ and the cytotoxic activity of *in vitro* stimulated spleen cells obtained from immunized mice. Subcutaneous administration of OMLs also induced an antigen-specific cellular immune response. These results demonstrate the effectiveness of OMLs as a subcutaneous and intraperitoneal adjuvant for induction of a cellular immune response specific for a liposome-entrapped antigen. To expand the utility of OMLs for antigen delivery and activation of immune responses, the main aim of the present study was to define whether OMLs could be used as an adjuvant to induce mucosal immunity following administration by mucosal routes.

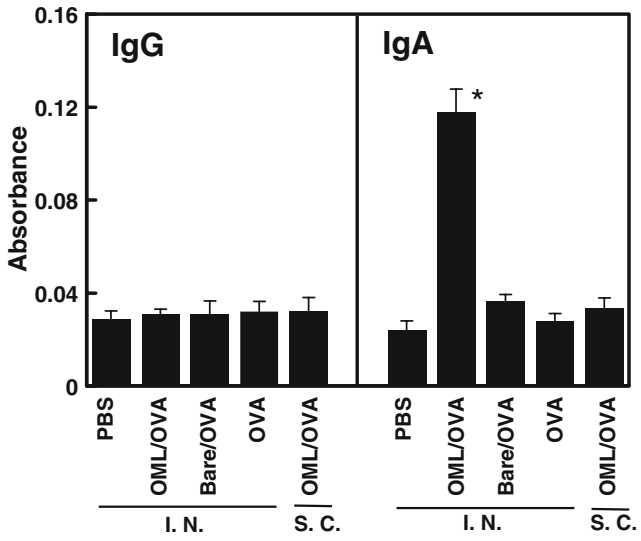

Fig. 4 Secretion of IgA in nasal washes. Nasal washes were collected from mice immunized intranasally (I.N.) with OML/OVA, Bare/OVA, OVA alone, or PBS or from mice administered OML/OVA subcutaneously (S.C.) at 1 week after the last immunization (on day 16). Antigen-specific IgG and IgA in each nasal wash (64-fold dilution) were determined by ELISA. Each bar represents the mean ± SD of three independent experiments. * $P<0.01$ compared to mice that received PBS

The results showed that nasal administration of OVA entrapped in Man3-DPPE-coated liposomes (OMLs) induces systemic humoral immune responses, as shown by production of OVA-specific serum IgG and IgA, while that of OVA entrapped in carbohydrate-uncoated, bare liposomes or OVA alone did not induce these antibody responses. Therefore, induction of systemic humoral immune responses by nasal administration of antigen-containing liposomes strongly depends on the carbohydrate residues on the liposomes. Subcutaneous immunization of OMLs induces antigen-specific Th1 immune responses in mice [10, 16], whereas nasal administration of OMLs seemed predominantly to induce Th2 immune responses based on the higher amount of OVA-specific IgG1 compared to OVA-specific IgG2a in serum. However, antigen-specific Th1 cells might also be induced in OML-received mice, even if the mice had a skewed Th2 type humoral immune response, because IFN-γ was produced from spleen cells of OML-received mice following *in vitro* stimulation. Mucosal administration of OMLs might induce an anamnestic immune response to the antigen, since the levels of both antigen-specific serum IgG and IgA increased significantly when OML-received mice were given OVA alone nasally 7 weeks after the last OML/OVA immunization. In addition to induction of systemic humoral immune responses, mucosal immunity with secretion of antigen-specific S-IgA in local mucosal tissues was also induced in OML-received mice. S-IgA is primarily involved in protection of mucosal surfaces, through inhibition of adherence and invasion of mucosal pathogens

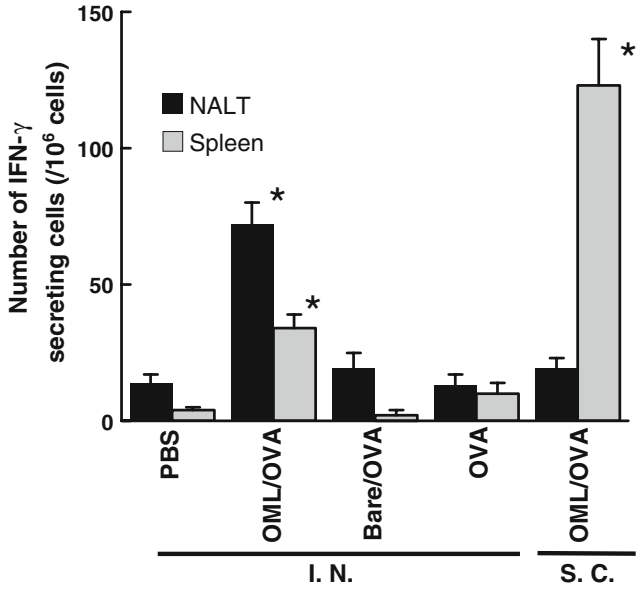

Fig. 5 Induction of cytokines in NALT. A. Production of cytokines from a single cell suspension of NALT prepared 1 week after the last intranasal immunization (on day 16). Cells (1×10^6) were cultured in triplicate for 48 h in the presence or absence of OVA (50 μg/ml). Culture supernatants were assayed for IL-5, IL-6, and IFN-γ by ELISA. Each bar represents the mean ± SD of three independent experiments. * $P<0.01$ compared to mice that received PBS. B. Induction of IFN-γ secreting cells in NALT. NALT and spleen were obtained from each mouse 1 week after the last intranasal (I.N.) or subcutaneous (S.C.) immunization (on day 16). For detection of IFN-γ secreting cells, single cell suspensions of NALT or spleen from immunized mice were added to wells in three serial dilutions (10^6 to 10^4 cells in the medium) in the presence or absence of 50 μg/ml OVA and incubated at 37°C in 5% CO_2 for 16 h. Then the plates were washed and incubated overnight at 4°C with an anti-IFN-γ antibody. After washing the plates, spots representing single cells were developed and counted with a dissecting microscope. Each bar represents the mean ± SD of three independent experiments. * $P <$ 0.05 compared to mice that received PBS

and neutralization of the virulence of toxins [30]. Therefore, nasally administered OMLs may also be useful as an adjuvant to induce protective mucosal immunity against mucosal pathogens such as influenza virus.

The efficiency of adjuvant activity of OMLs was similar to that of CTB, which serves as an adjuvant for antigens administered by the intranasal route [19], based on the similar levels of serum IgG and IgA, and IFN-γ and IL-5 produced by spleen cells from OML-received and CTB-treated mice. Efficient nasal immunization requires supplemental stimulation via cytokines or co-stimulatory molecules [31, 32]. However, we have shown that OML treatment upregulates co-stimulatory molecules and MHC class II molecules on cells; that OML-stimulated macrophages preferentially produce IL-12, which is essential for induction of a Th1 immune response; and that APCs containing OMLs effectively present encased antigen-derived peptides via MHC class I and II molecules and can activate both CD4-positive and CD8-positive T cells [11, 33]. Therefore, OMLs seem to enhance the immunization efficiency without additional stimulation, although we did not test the effect of additional stimulation on the adjuvant activity of OMLs in this study.

NALT contains specialized M-like cells similar to those present in Peyer's patches in the gut, and the M cells are thought to play an important role in antigen uptake and transportation to underlying APCs in mucosal tissues [34]. Microparticles such as liposomes are taken up by M cells of NALT [14]. Since the levels of IL-5 and IL-6 produced from spleen cells of mice that received OMLs were similar to those in mice that received bare liposome (Fig. 2), M cells of NALT might take up both bare liposomes and OMLs and deliver liposomes to the underlying APCs. IL-5 and IL-6 produced by Th2 cells support IgA responses in mouse [21], but only mice that received OMLs had antigen-specific serum IgA and S-IgA in nasal washes. IFN-γ produced by Th1 cells also supports secretion of S-IgA in mucosa through enhancement of production of SC. In the present study, we found IFN-γ secreting cells in both NALT and spleen only in mice that received OMLs. It is unclear whether IFN-γ produced in NALT can really act on SC-producing cells located at submucosal sites, but a

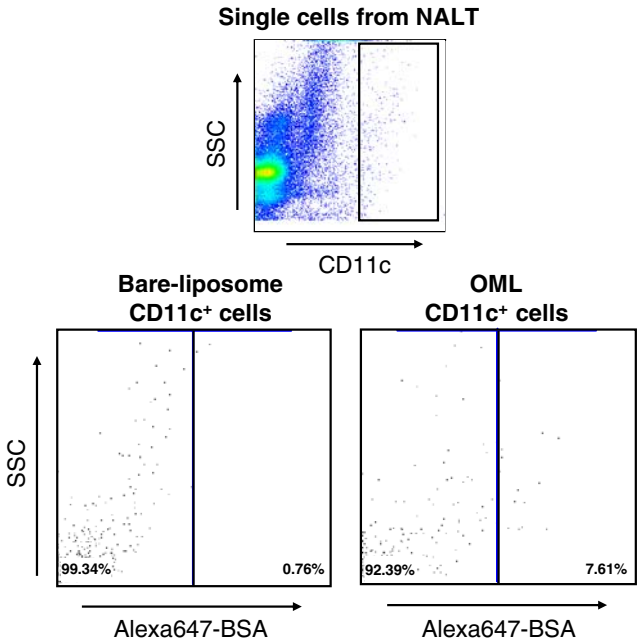

Fig. 6 Uptake of OMLs by dendritic cells in NALT. Mice were given OMLs or uncoated liposomes (Bare-liposome) with encased Alexa647-conjugated BSA nasally. Twenty-four hours after administration, single cell suspensions were prepared from NALT samples, stained with PE-labeled anti-CD11c, and analyzed by FACS to evaluate incorporation of liposomes in CD11c-positive cells. The CD11c-positive cells were first gated based on PE fluorescence (indicated by the rectangle, upper panel), and then uptake of liposomes in gated cells was analyzed based on Alexa647 fluorescence

carbohydrate-dependent mechanism might be involved in induction of IFN-γ-secreting cells and in induction of antigen-specific serum IgA and S-IgA. In this study, we showed that APCs in NALT took up OMLs more effectively than liposomes without a carbohydrate coating (Fig. 6). APCs express an array of C-type lectins, which have roles in antigen uptake and in induction of immune responses [35], and recently we have shown that OMLs can be recognized and ingested by a mannose-binding lectin, specific ICAM-3 grabbing nonintegrin related 1 (SIGNR1) [36]. Therefore, OMLs might be taken up and transported by M cells in a carbohydrate-independent manner and subsequently phagocytosed by underlying macrophages or dendritic cells in a carbohydrate-dependent manner, resulting in initiation of systemic and mucosal immune responses with secretion of S-IgA.

In conclusion, our results showed that liposomes containing synthetic neoglycolipids consisting of natural oligosaccharides and a natural phospholipid (phosphatidylethanolamine), are effective and attractive vaccine delivery vehicles for development of a mucosal vaccine. Optimization of the carbohydrate density on the liposome surface, particle size, and the sugar component of the neoglycolipids is required for successful development of new carbohydrate-based vaccines for induction of an effective mucosal immune response.

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