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Peptide-free Synthetic Nicotine Vaccine Candidates with α-Galactosylceramide as Adjuvant

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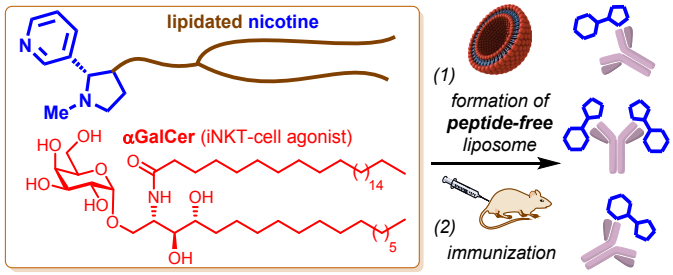
Graphical abstract



ABSTRACT: Peptides are generally needed as T-helper epitopes in nicotine vaccines to induce effective antibody responses, but the highly polymorphic property of MHC molecules may limit opportunities of B cell to receive $CD4^+$ T-cell help. iNKT cells recognize lipid antigens presented by the nonpolymorphic CD1d molecule that is conserved in mammals to a great extent. iNKT cells also display some similar functions to conventional $CD4^+$ T-helper cells, especially they license dendritic cells stimulate antibody isotype switching by B cells. Herein, α-galactosylceramide (αGalCer), a classical iNKT cell agonist, serves as an adjuvant in synthetic nicotine vaccine candidates absent peptide or protein. Our study reveals that αGalCer displays better adjuvant activity than Pam_3CSK_4 (a commonly used lipopeptide TLR agonist). Remarkably, the covalent linker between the nicotine hapten and αGalCer is not critical. Self-assembly of the lipid-tailed nicotine and αGalCer into the liposome represents a structurally simple but immunologically effective way to develop nicotine vaccines. This is the first time to introduce the iNKT cell agonist as an adjuvant to an anti-drug vaccine. This discovery may contribute to improving the efficacy of clinical candidate nicotine vaccines in the future.

KEYWORDS: iNKT cell, α-galactosylceramide, liposomal formulation, covalent conjugation, nicotine vaccine

INTRODUCTION

Tobacco addiction is a main cause of cardiovascular risk and tobacco-related cancer, and nicotine is the main ingredient leading to addiction in tobacco.[1] Approximately 6 million mortalities and immeasurable economic loss are caused by nicotine consumption every year globally. Current therapies to promote smoking cessation mainly focus on nicotine replacement therapies, receptor antagonists, or behavioural intervention, but the overall cessation rate is highly limited, and these therapies might also have potential severe side effects.[2,3] An alternative therapeutic strategy is known as immunopharmacotherapy, which depends on the induction of nicotine specific antibodies that could bind nicotine in the blood circulation, and prevent it from entering the brain, thus reducing nicotine addiction.[4,5]

One of bottlenecks for the development of nicotine vaccine is to trigger the production of high titers of anti-nicotine IgG antibodies with strong affinity.[6] Because of its poor immunogenicity, nicotine need to be covalently attached to macromolecular carriers (such as BSA and KLH proteins) to induce antibody response. Importantly, the proteins are also digested by APCs to produce shorter peptides, which are presented by MHC class II molecules, serving as the crucial Th epitopes to promote antibody class-switching from IgM to IgG. Therefore, peptides (or proteins) are an indispensable component in nicotine vaccines. However, the fact that human MHC class II molecules are highly polymorphic would narrow the population that can respond to a related peptide.[7] Although this problem can be solved by introducing multiple Th epitopes, the syntheses are highly challenging, and can only be managed by experienced organic chemists.

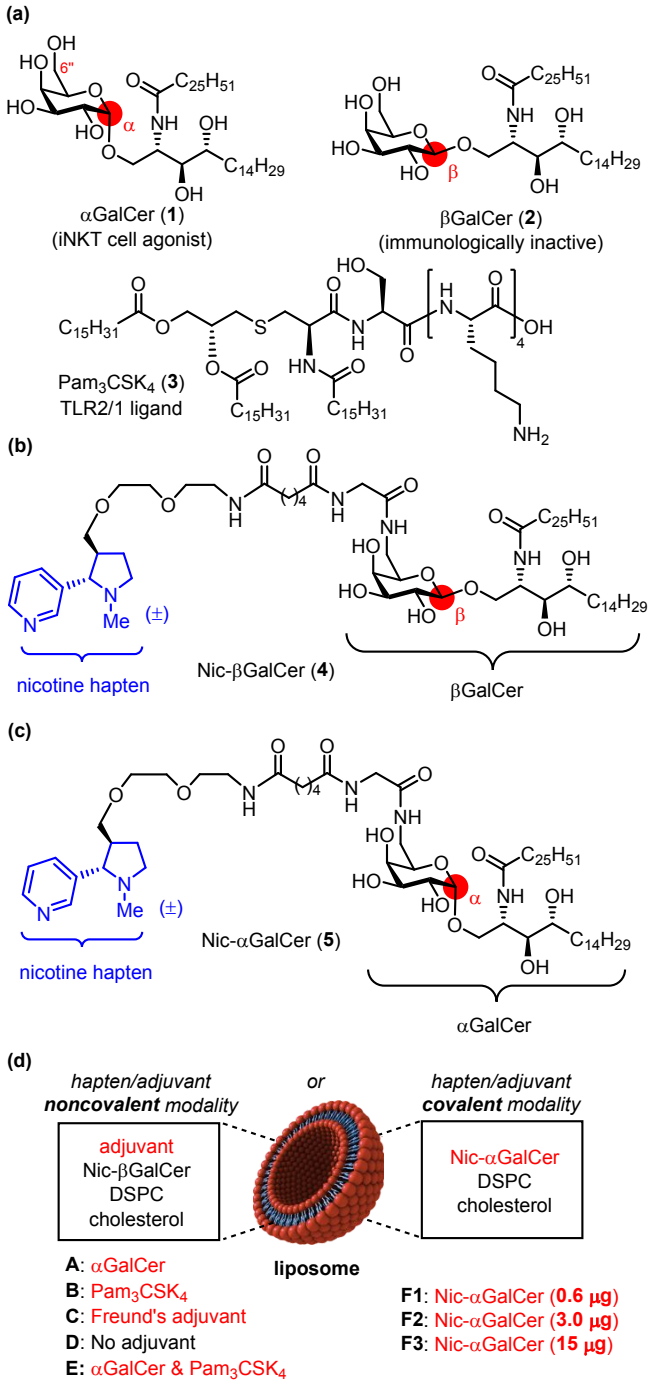
There are several small populations of T lymphocytes that can recognize nonpeptide antigens without the participation of MHC molecules.[8] These populations do not obey the rule that

T cells can only detect peptides binding with MHC. Invariant natural killer T (iNKT) cell is one of the most studied subset among these unconventional T cells.[9] iNKT cells share many functions with conventional $CD4^+$ T helper cells, especially they license DCs stimulate antibody isotype switching and affinity maturation by B cells.[10-12] Unlike $CD4^+$ T helper cells, the receptors of iNKT cells can recognize lipid antigens presented by CD1d molecules, instead of peptides presented by MHC molecules. Given the non-polymorphic nature of CD1d, iNKT cell can function as helper in a more universal way compared to the majority of $CD4^+$ T cells.[13] The first glycolipid ligand of iNKT cells was α-galactosylceramide (**1**, abbreviated as αGalCer or KRN7000, Scheme 1a), which was originally extracted from a marine sponge and optimized through SAR studies.[14-16] The adjuvanticity of αGalCer have been revealed in many animal models in which the activated iNKT cells act as "universal helpers" to augment both humoral and cellular responses to the co-administered antigens.[17]

Adjuvants have been considered a crucial component in vaccines.[18] Pam_3CSK_4 (**3**, Scheme 1a) is a popular lipopeptide adjuvant that resemble the *N*-terminus of bacterial lipoproteins.[19] Pam_3CSK_4 play an adjuvant role through the cooperation between TLR2 and TLR1, resulting in NF-κB activation. Because lipopeptides tend to be incorporated into liposomes, Pam_3CSK_4 has been widely used in liposomal synthetic vaccines as good inducers of antibody responses, providing appropriate "danger signals".[20] To elicit effective immune responses adjuvanted by Pam_3CSK_4, several approaches of potential benefit have been or can be applied. First, Pam_3CSK_4 is preferably used in a self-adjuvanting manner.[21] The covalent conjugation of Pam_3CSK_4 with antigens is able to induce superior antibody response to a simple mixture of TLR-agonist and free antigen. Indeed, this covalent concept has been extended to the use of other lipid adjuvants, such as monophosphoryl lipid A,[22] muramyl dipeptide,[23] and αGalCer[24] as well. Second, for T-

independent antigens, Th epitope peptides generally has to be incorporated. For example, the liposomal construct lacking the Th epitope was less effective than the corresponding tricomponent construct that consists of Pam_3CSK_4, Th epitope, and B epitope.[25] Third, combination of a TLR agonist and another adjuvant of immunologically distinct mode of action could have synergistic effects.[26]

As part of our ongoing investigations of structurally simple but immunologically effective synthetic vaccines,[27] we report herein the nicotine vaccine candidates with αGalCer as adjuvants. The immune responses induced in mice by the vaccine candidates were characterized by nicotine-specific serum antibody titers, concentrations of cytokines (IFN-γ and IL-4), and antibody affinities. The efficacies of all vaccine candidates were further evaluated by acute pharmacological effects (nicotine-induced antinociception and hypothermia) in mice. We found that the iNKT cell agonist αGalCer more potently induces the anti-nicotine antibody immune responses in mice than the TLR agonist Pam_3CSK_4. Our results also showed that no synergy of the co-adminstrated adjuvants (Pam_3CSK_4 and αGalCer) was observed relative to either Pam_3CSK_4 or αGalCer alone. Next, whether the covalent linker between the nicotine hapten and the glycolipid αGalCer (covalent modality) can further improve vaccine efficacy was investigated. The results suggest that self-assembly of αGalCer with Nic-βGalCer by the noncovalent modality represents a practical means for use of iNKT cell agonists in nicotine vaccines. To the best of our knowledge, αGalCer has been employed for the first time as an adjuvant in the vaccine targeting drugs of abuse.



Scheme 1. (a) The structure of αGalCer (**1**), βGalCer (**2**), and Pam_3CSK_4 (**3**); (b) and (c) The structure of Nic-βGalCer (**4**) and Nic-αGalCer (**5**); (d) liposomal formulations of vaccine candidates **A–F** (The adjuvants in each formulation are shown in bold).

EXPERIMENTAL SECTION

Chemistry

***Se*-phenyl 2-((*tert*-butoxycarbonyl)amino)ethaneselenoate (7).** To Ph_2Se_2 (780 mg, 2.5 mmol) and *N*-Boc-glycine (441 mg, 2.5 mmol) in a solution of DMF (10 mL) was added 2.5 mL of PBu_3 under Ar atmosphere. The reaction mixture was stirred until *N*-Boc-glycine was completely consumed on the TLC. The solvents were removed *in vacuo*. Purification of the resultant residue through flash column chromatography on silica gel (petroleum ether/DCM 9:1 to 1:1) provide **7** as a pale yellow solid (672 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (d, J = 6.7 Hz, 2H), 7.37 (d, J = 6.1 Hz, 3H), 5.45 (s, 1H), 4.04 (d, J = 6.0 Hz, 2H), 1.53 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.96, 155.37, 135.77, 129.12, 128.75, 125.16, 80.57, 53.17, 28.43, 28.29; ESI-MS calcd. for $C_{13}H_{17}NNaO_3Se^+$ $[M + Na]^+$: 338.03, found: 337.95.

3,4-Di-*O*-benzyl-1-*O*-(2,3-di-*O*-benzyl-4-hydroxyl-6-(2-((*tert*-butoxycarbonyl)amino) ethylamino)-β-D-galactopyranosyl)-2-hexacosylaminooctadecane-1,3,4-triol (8). To the solution of **6**[26] (50 mg, 0.04 mmol) in wet solvents of MeOH/THF (9:1, 10 mL) was added PMe_3 (0.4 mL, 0.4 mmol). The reaction mixture was stirred at rt for 4 h. After the solvents was removed *in vacuo*. the residue was placed under high vacuum for 24 h to further remove trimethylphosphine oxide. The resultant amine was applied directly without column purification. To the solution of amine and DIPEA (15 μL) in DCM (3 mL) was treated with the solution of **7** (19 mg, 0.06 mmol) in DCM (3 mL) at rt. After stirred at rt for 2 h, the reaction mixture was concentrated under reduce pressure to offer the residue which was purified by flash column chromatography on silica gel (DCM/MeOH 20:1) to give **8** as a white solid (90.3 mg, 96% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.23 (m, 20H), 7.04 – 6.98 (m, 1H), 6.06 (d, J = 8.7 Hz, 1H), 5.59 (s, 1H), 4.78 (d, J = 11.2 Hz, 1H), 4.72 – 4.64 (m, 4H), 4.60 (d, J = 11.8 Hz, 1H), 4.55 (d, J = 3.4 Hz, 2H), 4.29 (d, J = 7.7 Hz, 1H), 3.88 (d, J = 3.5 Hz, 1H), 3.85 – 3.74 (m, 5H), 3.70 (dd, J = 17.0, 5.5 Hz, 2H), 3.64 – 3.56 (m, 2H), 3.43 (ddd, J = 16.0, 9.1, 3.6 Hz, 2H), 3.25 (ddd, J = 13.1, 8.6, 3.9 Hz,

1H), 2.59 (s, 1H), 1.81 (q, J = 7.0 Hz, 2H), 1.71 – 1.61 (m, 2H), 1.42 (s, 9H), 1.23 (d, J = 16.1 Hz, 70H), 0.88 (t, J = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.90, 170.16, 155.71, 138.22, 138.16, 137.42, 128.33, 128.29, 128.23, 128.18, 127.92, 127.83, 127.70, 127.60, 127.52, 104.36, 81.10, 80.33, 79.61, 78.78, 75.13, 72.90, 72.40, 72.00, 69.03, 67.15, 50.59, 44.12, 40.22, 36.69, 32.04, 30.60, 29.83, 29.49, 29.15, 28.47, 27.78, 25.96, 25.71, 22.84, 14.32. ESI-MS calcd. for $C_{57}H_{111}N_3NaO_{11}^+$ $[M + Na]^+$: 1397.00, found: 1397.30.

Nic-βGalcer (4). To a degassed solution of **8** (53 mg, 0.039 mmol) in DCM/MeOH (4 mL, 2:1) was treated with a catalytic amount of $Pd(OH)_2/C$ (25 mg, 20% Pd on carbon, normally contain 50% water). The suspension was stirred under H_2 atmosphere until **8** was consumed. After restoring Ar atmosphere, the mixture was filtered by a Celite pad which was prewashed by MeOH followed by DCM. Upon the removal of solvent *in vacuo*, the residue was subjected to the purification of flash column chromatography on silica gel (DCM/MeOH 10:1) to give **S2** (see the structure in the Supporting Information) as a white solid (32.3 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ 1:1) δ 4.24 (s, 1H), 4.19 (d, J = 7.2 Hz, 1H), 4.07 (d, J = 5.6 Hz, 1H), 3.77 – 3.66 (m, 4H), 3.63 – 3.42 (m, 7H), 2.21 (t, J = 7.6 Hz, 2H), 1.62 (m, 2H), 1.46 (s, 9H), 1.27 (s, 70H), 0.89 (t, J = 6.6 Hz, 6H). ESI-MS calcd. for $[M + H]^+$: 1014.82, found: 1015.06. A solution of **S2** in DCM (4 mL) was treated with anhydrous trifluoroacetic acid (1 mL, 0.013 mmol) at 0 °C. The resultant mixture was stirred at 0 °C for 15 min followed by at rt for 30 min. The solvents were removed *in vacuo* to afford amine **S3** as a crude product. To the solution of amine **S3** in DCM/MeOH (4 mL, 1:1) was added the solutions of **9** in DCM (1 mL) and Et_3N (50 μL) at rt. After stirred at rt for 5 h, the reaction mixture was concentrated under reduced pressure and then purified on a LH-20 column using DCM/MeOH (1:1) as the eluent to offer Nic-βGalCer (**4**) after lyophiliztion as a white solid (6.9 mg, 53% yield over two steps). ^{1}H NMR (400 MHz,

$CDCl_3/CD_3OD$ 1:1) δ 8.55 (dd, *J* = 14.4, 3.5 Hz, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.47 (dd, *J* = 7.9, 4.7 Hz, 1H), 4.24 (s, 1H), 4.21 (d, *J* = 6.9 Hz, 1H), 4.11 (dd, *J* = 10.3, 4.6 Hz, 1H), 3.86 (d, *J* = 6.3 Hz, 1H), 3.77 (d, *J* = 2.9 Hz, 1H), 3.64 (q, *J* = 7.1 Hz, 4H), 3.60 – 3.45 (m, 11H), 3.38 (s, 6H), 2.44 – 2.11 (m, 10H), 1.65 (s, 9H), 1.27 (s, 70H), 0.89 (t, *J* = 6.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.31, 174.03, 173.95, 170.33, 148.94, 148.35, 135.94, 123.80, 103.29, 73.70, 72.65, 72.41, 71.70, 71.47, 70.63, 69.75, 69.39, 68.97, 68.48, 56.75, 55.16, 49.78, 48.60, 48.38, 48.17, 47.96, 47.74, 47.53, 47.32, 47.11, 46.08, 42.06, 39.23, 38.76, 38.58, 35.75, 35.01, 34.73, 31.39, 31.28, 29.14, 29.05, 29.00, 28.92, 28.81, 28.70, 28.68, 25.48, 25.29, 24.55, 24.38, 21.98, 16.90, 13.07. ESI-MS calcd. for $[M + H]^+$: 1304.01, found: 1304.30.

Nic-αGalcer (5). The preparation of Nic-αGalCer (**5**) from **10** was similar to that of **Nic-βGalCer** from **6.** The crude product was purified through LH-20 chromatography column using DCM/MeOH (1:1) as the eluent to give Nic-αGalCer (**5**) after lyophiliztion as a white solid (11.7 mg, 61% yield over two steps). ^1H NMR (400 MHz, $CDCl_3/CD_3OD$ 1:1) δ 8.58 – 8.40 (m, 2H), 7.92 (d, *J* = 7.9 Hz, 1H), 7.41 (dd, *J* = 7.9, 4.9 Hz, 1H), 4.76 (d, *J* = 3.5 Hz, 1H), 4.09 (d, *J* = 5.2 Hz, 1H), 3.77 – 3.60 (m, 7H), 3.56 – 3.35 (m, 13H), 3.25 (dd, *J* = 12.6, 3.8 Hz, 4H), 2.39 (s, 2H), 2.21 (d, *J* = 6.7 Hz, 3H), 2.12 (dd, *J* = 9.3, 5.8 Hz, 5H), 1.55 (d, *J* = 7.0 Hz, 9H), 1.17 (s, 70H), 0.79 (t, *J* = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$ 1:1) δ 174.25, 174.04, 173.90, 170.10, 149.08, 148.88, 136.04, 123.91, 98.95, 73.76, 71.44, 71.25, 70.53, 69.75, 69.41, 69.34, 69.15, 68.91, 68.30, 68.21, 66.21, 56.71, 55.02, 49.91, 48.34, 48.12, 47.91, 47.70, 47.49, 47.27, 47.06, 41.96, 39.33, 38.53, 38.28, 35.72, 34.99, 34.71, 31.63, 31.24, 29.14, 29.05, 29.02, 28.96, 28.79, 28.71, 28.67, 28.64, 25.31, 25.22, 25.06, 24.55, 24.34, 21.95, 16.86, 13.04. ESI-MS calcd. for $C_{73}H_{134}N_6NaO_{13}^+$ $[M + Na]^+$: 1325.99, found: 1326.19.

Immunological test

Immunization of mice. Female BALB/c mice (6-8 weeks age) in this study were purchased from the Center for Animal Experiments of Wuhan University. All mice were used according to the animal ethics guidelines. The mice were divided into 8 groups (5 mice per group) and bred in the Center for Animal Experiments of Wuhan University. The Mice were immunized by intraperitoneal injection on the 1st, 15th, and 29th days. The mice were bled (day 0) before initial immunization and on 14th, 28th, and 42nd days after boost immunizations. Th sera collected at 2 and 24 h after 1st injection was analysed for the cytokine profiles (IFN-γ and IL-4). Mouse blood samples were clotted to obtain antisera that were stored at -80 °C before use. The vaccine efficacy upon nicotine challenge was evaluated on day 56 by nicotine-induced antinociception (hot plate procedure) and hypothermia.

ELISA procedure. A 96-well microtiter plate (Costar type 3590, Corning Inc.) was first coated with Nic-BSA (18 nicotine hapten per BSA protein), which had been dissolved in 0.1 M $NaHCO_3$ buffer (pH = 9.6). Each microwell contains 0.1 μg of Nic-BSA in 100 μL of $NaHCO_3$ buffer solution. Next, the plate was kept at 4 °C overnight and washed 3 times with PBST (0.05% Tween-20 in PBS). The solution of 1.5% (w/v) BSA in PBS was added to each well and incubated at 37 °C for 1 h. After washed again with PBST, the plate was treated with serially diluted sera (100 μL/well), incubated for 1 h at 37 °C and washed (3 times) with PBST. A diluted solution (1:5000) of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific), IgG1, IgG2a, IgG2b, IgG3, IgA or IgE in 0.1% BSA/PBS (100 μL per well) was added to each microwell respectively. After incubation (1 h at 37 °C) and a final wash (3 times), substrate solutions (freshly prepared with 9.5 mL critic buffer at pH 5.0, 0.5 mL 2 mg/mL tetramathyl benzidine (TMB), and 32 μL 3% (w/v) urea hydrogen peroxide) were added to the wells (100 μL per well). The plate was placed in the dark for 15 min for the color development

and terminated by a solution of 2 M H_2SO_4. The optical density (OD) value was then recorded at 450 nm. The antibody titer was described as the highest dilution indicating an 0.1 absorbance, after subtracting the background. Pre-immunization sera (day 0) have been used as negative control.

Antibody affinity by competition ELISA. The relative affinity of anti-nicotine antibody elicited by the vaccine candidates was measured by a competition ELISA method.[28] The sera were diluted in 0.1% BSA in PBS until the absorbance values reach approximately 1.0 at 450 nm following the IgG-HRP antibody development assay. The nicotine tartrate was serially diluted from 100 mM to 2 nM using 0.1% BSA in PBS. Equal volumes (65 μL) of diluted sera and nicotine were added to a non-absorbent 96-well plate and incubated for 1 h at 37 °C, allowing equilibrium be achieved. The mixture of serum/nicotine were then added (100 μL/well) to the ELISA plates coated with Nic-BSA (The antigen-coated ELISA plates was prepared identically as "ELISA procedure"). The plates were incubated for 30 min at 37 °C and then washed 3 times with PBST. Next, detection of IgG antibody binding was carried out as described in "ELISA procedure". The OD readings at 450 nm of diluted serum without nicotine was B_0, and B/B_0 were plotted against the molar concentration of nicotine and the 50% inhibition (IC_{50}) was the concentration of competitors where the binding rate was 50%.

***In vivo* cytokine assay.** The cytokine levels in sera were evaluated using ELISA kits (IFN-γ and IL-4, Biolegend) according to the manufacturer's protocol.

Hot plate assessment. Two weeks following the last bleeding, the antinociception effect against nicotine (0.5 mg/kg, injected subcutaneously) was evaluated by the hot plate test following the reported procedure with slightly modification.[29] A mouse was placed in a glass beaker (11 cm diameter × 16 cm) whose bottom was heated to 55 °C. The timing latency to exhibit one of the following nociceptive responses was recorded: licking of paw, shaking/withdrawal of paw, or

jumping. The typical baseline latency was between 8-15 s, and a 35 s cutoff was imposed to protect the mice from damage. After response, mice were taken out from the beaker. The antinociceptive response for the hot-plate test is calculated as percentage of maximum possible effect (%MPE), where %MPE = (test-control) / [(35 s for the hot plate) – control)] × 100.

Hypothermia evaluation. Rectal temperature was determined by inserting a 1-cm thermistor probe into the rectum and temperature read from the digital thermometer. The measurements were carried out at 5, 15, and 30 min after injection of nicotine. Baseline values were recorded just before injection.

Evaluation of the vaccine safety through weight change. The safety of all vaccine candidates was preliminarily evaluated by checking the weight changes of the mice,[30] and the mice were weighed at 0, 28th, 42nd and 56th days before bleedings.

Statistical analyzes. Comparisons were carried out via one-way ANOVA followed by a Turkey's posthoc test. Differences were judged to be insignificant when *P*-values are greater than 0.05. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

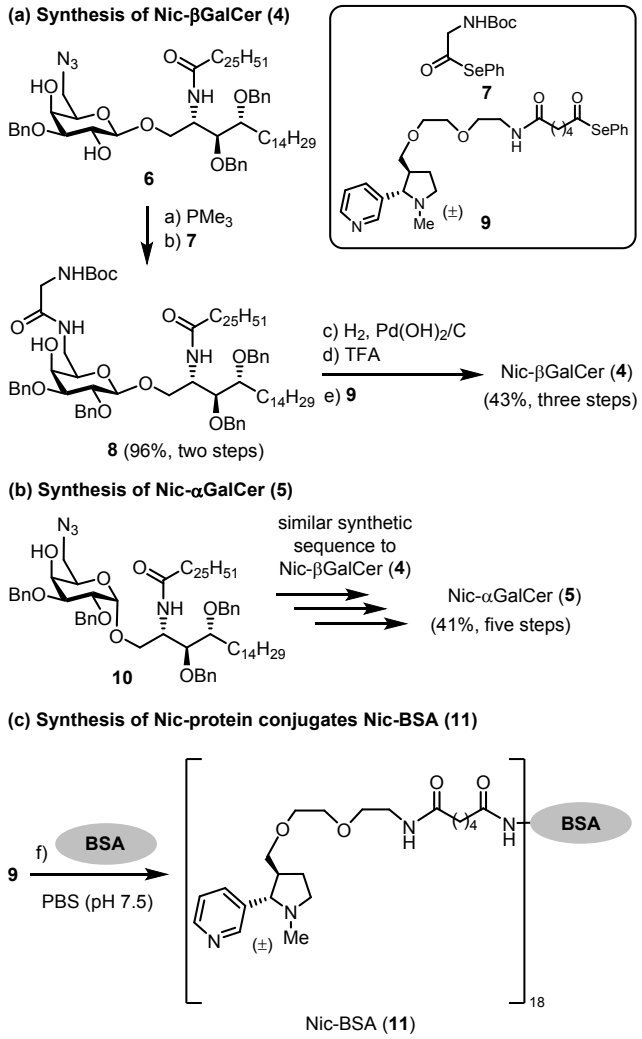
RESULTS

Design rationale for noncovalent vaccine modality

We began by comparing the adjuvant activity of αGalCer with the commonly used adjuvants. The simple liposomal formulation developed by Savage and co-workers was employed.[31,32] Self-assembly of the lipid-tailed hydrophilic antigen is able to provide the crucial multivalency for cross-linking of B cell receptors, eliminating the need for covalent attachment to carrier proteins. Due to the amphipathic property, the glycolipid adjuvant can be inserted to the liposomal formulation. In this model, there is no covalent linker between adjuvant and antigen.

There are two key issues need to be addressed with regard to the design of such vaccine formulation: choosing a suitable lipid anchor that is immunologically inactive and a conjugation site on nicotine hapten. As illustrated in Scheme 1b, Nic-βGalCer (**4**) was designed to present a nicotine hapten. The choice of βGalCer (**2**) as lipid anchor is according to our previous research on the anti-STn cancer vaccine.[27] The covalent conjugation of βGalCer with STn antigen only induces weak anti-STn IgG antibody response, but allows the formation of liposomes for multivalent antigen presentation. A 3'-substituted nicotine analogue was chosen as the hapten, because this regiochemical modification around nicotine molecule has been adopted in the nicotine vaccines (NicVAX[33] and NicQβ[34]) that entered to clinical trials.

Scheme 1d exhibits the vaccine formulations investigated in our present work. The formulations **A**, **B**, and **C** contained αGalCer, Pam_3CSK_4, and the Freund's adjuvant, respectively. The complete Freund's adjuvant contains several TLR agonists originated from inactivated *Mycobacteria*, which stimulate extremely strong immune responses in a murine model, but its use is prohibited in humans due to severe adverse reactions. The construct **D** lacking adjuvants was used as a negative control. In order to investigate whether there is co-operation between Pam_3CSK_4 and αGalCer, the formulation **E** was prepared. In each formulation, DSPC and cholesterol are added. Both of them are non-immunomodulatory, but the addition of them in liposomal vaccines has been shown to facilitate the induction of strong antibody responses in small animals.[35]



Scheme 2. (a) and (b) Synthesis of **4** and **5**; (c) Preparation of protein conjugate Nic-BSA (**11**).

Synthesis of Nic-βGalCer and protein conjugates Nic-BSA

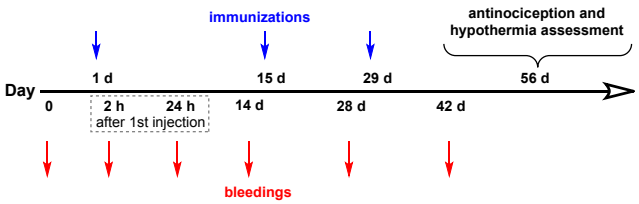
As shown in Scheme 2a, the preparation of **4** began with the amide formation between the glycine selenoester **7** and the 6'-NH_2 derivative of βGalCer prepared by the reduction of azide **6**. The attempt to treat **6** via simultaneous reduction of N_3 and deprotection of Bn group led to an unidentified mixture. The hydrogenlysis of **8** and subsequent deprotection of Boc group provided the amine that reacts with the nicotine selenoester **9** to afford **4**. The purity of **4** was confirmed by HPLC. The protein conjugate Nic-BSA (**11**) were prepared by coupling **9** with BSA.[36] According

to our previous study on protein conjugation, the selenoester displays better combination of stability and reactivity than the commonly used active esters (*N*-hydroxysuccinimide, *p*-nitrophenyl, and polyfluorophenyl esters).[36, 37] The incorporation number per BSA molecule is 18, which was determined by the MALDI-TOF mass spectrometry. **11** was applied as the coating antigen for ELISA of anti-nicotine antibodies (Scheme 2c).

Vaccine formulation and immunization

αGalCer (1 μg) and Nic-βGalCer (3 μg, contains 0.38 μg of the nicotine hapten) was administered in the liposomal formulation (**A**) prepared with cholesterol and DSPC in a molar ratio of 0.5:1:5:4 by the reported method.[22,38] An equimolar amount of Pam_3CSK_4[39,40] (0.0011 μmol, 1.7 μg) instead of αGalCer was employed in **B**. No adjuvant was used in **D**. In the construct **E**, both αGalCer (1 μg) and Pam_3CSK_4 (1.7 μg) were introduced. After hydration of a lipid film that contained all comonents in a HEPES buffer, the liposomes **A**, **B**, **D**, and **E** were prepared by 20-minute sonication.[21,41] The dynamic light scattering (DLS) confirmed that the liposomes are uniformly sized with a diameter of approximately 600 nm (Figure S1 in Supporting Information). In the formulation **C**, the complete/incomplete Freund's adjuvant was employed based on the manufacturer's instructions (Table S1 in Supporting Information).

Female BALB/c mice (6−8 weeks old) were divided to 8 groups (5 mice per group), and immunized with the vaccine candidates **A**-**E** via intraperitoneal injections. Scheme 3 demonstrates the vaccine schedule. During the immunization study, no mouse showed signs of significant weight loss (Figure S15 in Supporting Information).



Scheme 3. Study design for nicotine vaccine candidates in mice. Mice were immunized on the 1st, 15th, and 29th days, and the blood was taken before the initial immunization (day 0) as well as on 14th, 28th, and 42nd days after the boost immunizations. The sera collected at 2 and 24 h after 1st injection were analyzed for cytokine profile (IFN-γ and IL-4). Nicotine-induced antinociception (the hot plate procedure) and hypothermia were evaluated on the 56th day.

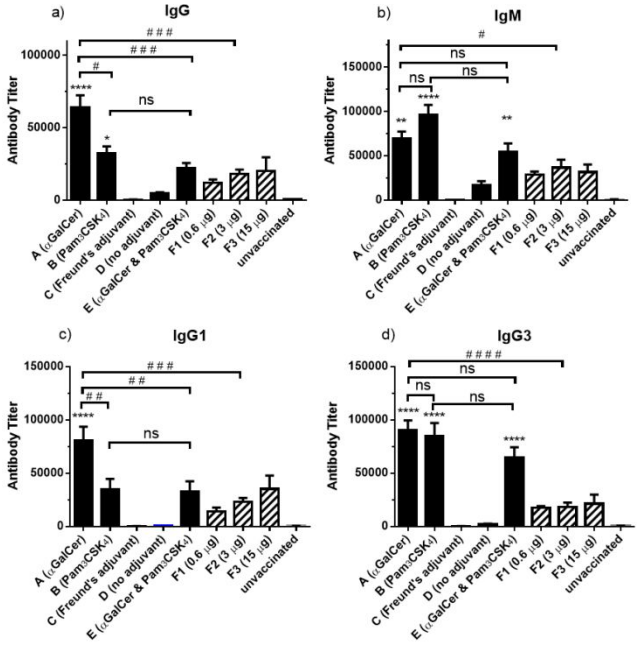


Figure 1. The titers of anti-nicotine antibodies (IgG, IgM, IgG1, and IgG3) elicited by the vaccine candidates (solid bars for noncovalent modality **A**-**E**, hatched bars for covalent modality **F1**, **F2**, **F3**) after three immunizations (day 42). The data are indicated as the average of two independent experiments ± SEM. Significantly different compared to **D** group: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Significantly different: $^{\#\#}$, $P < 0.01$; $^{\#}$, $P < 0.05$; ns, no significant difference.

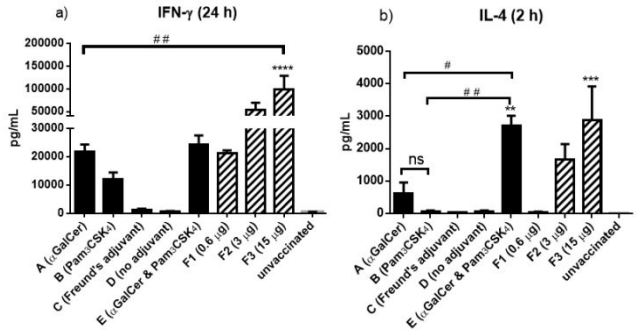


Figure 2. IFN-γ (a) and IL-4 (b) production induced by the vaccine candidates (solid bars for noncovalent modality **A**-**E**, hatched bars for covalent modality **F1**, **F2**, **F3**). The serum concentrations of cytokines were evaluated by ELISA at the indicated time points after 1st

injection. The data are indicated as the average of two independent experiments ± SEM. Significantly different compared to **D** group: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Significantly different: $^{\#\#}$, $P < 0.01$; $^{\#}$, $P < 0.05$; ns, no significant difference.

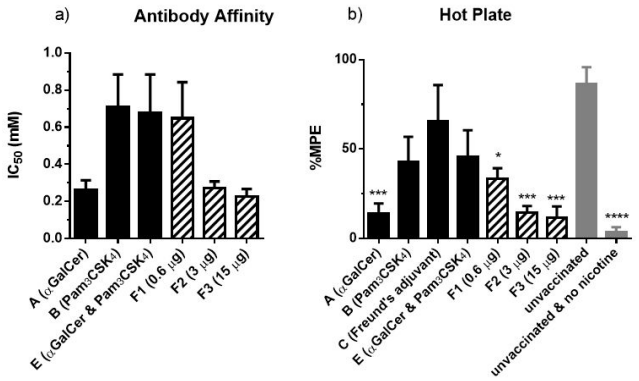


Figure 3. (a) Relative affinities of nicotine specific antibody measured by competition ELISA. The serum collected on the 42nd day was subjected to a competitive ELISA using serially diluted (-)-nicotine as the competitor. The data are shown as the average of two independent experiments ± SEM. Difference between individual bleeds is insignificant ($P > 0.05$). (b) Antinociception effects to nicotine measured by responses to hot plates. Mice were challenged at day 56 with nicotine (0.5 mg/kg) by the subcutaneous route. The mice were placed on a surface with a steady temperature of 55 °C and the time between placement and hind paw licking or jumping (whichever occurred first) was recorded as nociceptive latencies. A 35-s cut-off was used to prevent tissue damages. "% MPE": percent maximal possible effect. "Unvaccinated & no nicotine": the mice that were unvaccinated and were not injected with nicotine as well. The data are shown as the average ± SEM. Significantly different compared to "unvaccinated" group: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

αGalCer is more effective than Pam_3CSK_4 in inducing anti-nicotine responses

Figure 1 gave the ELISA results of the antisera obtained on the 42nd day from the mice immunized with **A**-**E**. Compared to the nonadjuvant-loaded **D**, the immunization of **A** or **B** resulted in significant enhancements of anti-nicotine IgG antibodies; the IgG titer initiated by **A** is about twice as high as that of **B**. To our surprise, given the extremely powerful adjuvant activity of the Freund's adjuvant, no significant IgG production was observed in **C**. This result parallels the previous reports on anticancer synthetic liposomal vaccines which the emulsification process required by the use of Freund's adjuvant may destroy the structure of liposomes.[42,43]

The pattern of IgG subclasses was also checked. The sera from the mice immunized with **A** stimulated the almost equal levels of IgG1 (Th2) and IgG3 (Th1) subclasses, and no significant titers of IgG2a or IgG2b were observed, indicating a Th1/Th2 mixed type antibody response, consistent with the polarization of IFN-γ (Th1)/IL-4 (Th2) cytokine profile (Figure 2).[44] In contrast, **B** triggered the similar level of IgG3 but the lower level of IgG1 relative to **A**, displaying a skewed Th1 antibody response, which is also reflective in cytokine production: the high level of IFN-γ but almost no production of IL-4.[45,46] Because the murine IgG3, instead of IgG1, can be generated without T cell helps,[32,47] the lack of Th epitopes in **B** may explain the relatively low titer of the IgG1 antibody.

In addition to antibody titers, the affinities of these antibodies were also measured by the competition ELISA (Figure 3a) according to the reported methods.[48] Compared to **B**, the antibodies stimulated by **A** exhibits higher affinity (lower IC_{50}), although the difference is statistically insignificant. To evaluate antibody function, antinociception and hypothermia caused by nicotine were determined.[29] As shown in Figure 1h, the αGalCer-adjuvanting cohorts (**A**) exhibited less antinociception effect than **B**. Consistent to the results of the hot plate assay, **A** also more effectively attenuate nicotine-induced hypothermia than **B**. The hypothermia effect was measured by the rectal body temperature (Figure S14 in Supporting Information). Therefore, αGalCer-adjuvanting cohorts (**A**) exhibited more significantly blunted responses to nicotine than **B**.

No synergy between αGalCer and Pam_3CSK_4 in stimulating antibody responses

Because Pam_3CSK_4 and αGalCer initiate immune response through two immunologically distinct modes of action, these two adjuvants could have synergistic effects to enhance the humoral

response.[26,49] Therefore, we compared the antibody responses elicited by the immunizations using liposomal formulations containing αGalCer alone (**A**), Pam_3CSK_4 alone (**B**), or the mixture of these two (**E**) to find whether the cooperation between two adjuvants could be observed. Although the production of IL-4 induced by the immunization of **E** did enhance relative to the use of **A** or **B** (Figure 2), the augmentation of the IgG titer with individual adjuvants was not observed (Figure 1), nor did the improvement of the antibody affinity and the antinociception effect be detected (Figure 3), indicating that there is no cooperation between αGalCer and Pam_3CSK_4 in stimulating antibody responses. The results are consistent with the previous reports that αGalCer and the TLR agonists do not have synergistic effect on the amplification of antibody responses.[50,51]

Noncovalent modality stimulates antibody response as effectively as covalent modality

It has been shown that the direct conjugation of iNKT cell agonist glycolipids to antigens is crucial for excellent immune responses.[38,52-57] Therefore, Nic-αGalCer (**5**) was prepared via the similar synthetic sequence to **4** (Scheme 2b). The procedures of vaccine formulation and immunization are similar to those of the noncovalent modality. As indicated in Figure 1a and 1b, the immunization with the covalent modality candidates (**F1**, **F2**, and **F3**) induced both IgG and IgM anti-nicotine antibodies in a dose-dependent manner. However, the titer of IgG antibody triggered by **F3** that containing the most Nic-αGalCer is still less than half of that induced by **A**. The patterns of IgG subclasses of **F1**, **F2**, and **F3** are similar to that of **A** (Figure 1c and 1d), except that their titers are lower than those of **A**, and demonstrate a Th1/Th2 mixed-type response. In term of the cytokine profile (Figure 2), **A** exhibited comparable potency to **F1**. Given the content of αGalCer in **F1** is approximately equal to that of **A**, the modification at the 6''-position of αGalCer caused by conjugation probably does not have a detrimental effect on the stimulation of cytokines. The cytokine production stimulated by **F1**, **F2**, and **F3** also exhibited a dose-dependent manner.

Impressively, the administration of **F1** induced mouse lymphocytes to produce the Th1 cytokines selectively, which is in agreement to the study by Mori group that the low concentration of glycolipids results in more selective cytokine generation.[58]

The affinities of the antibodies stimulated by the covalent modality candidates do not appear to be dose-dependent, and the antibodies initiated by **F2** and **F3** display the comparable affinities (Figure 3a). It is worth noting that the enhancement of the antibody affinities by means of the covalent modality was not achieved relative to the noncovalent modality. The other remarkable observation is that the antibody affinity is not closely correlated with the secretion of IFN-γ and IL-4 cytokine, which however is an important indicator for the immunological evaluation of iNKT cell agonists. For the effects of antinociception and hypothermia, slightly but insignificant difference was observed between **A** and **F** candidates (Figure 3b). Taken together, the covalent linking of the nicotine hapten to αGalCer does not give rise to a greater vaccine efficacy compared to the noncovalent modality.

DISCUSSION

The major challenge for nicotine vaccines is to induce high titers of specific IgG antibodies that bind the free nicotine in the blood and hinder them from crossing the blood brain barrier.[5] The rational use of adjuvants plays a critical role in addressing this challenge.[59] αGalCer has not been investigated in vaccines against drugs of abuse and addiction. To the best of our knowledge, this is the first vaccine candidate that targets addiction drugs employing the iNKT cell agonist as an adjuvant. The key findings from our study are as follows.

First, αGalCer displays better adjuvant activity than Pam_3CSK_4, Pam_3CSK_4 has also been widely used in synthetic vaccines, preferably in a manner of self-adjuvanting.[21] However, without

Th epitope peptides, the vaccines only stimulate inadequate antibody class-switching from IgM to IgG.[60] Because iNKT cells can provide help to B cells without the participation of $CD4^+$ T cells, Th epitope is not indispensable. As a result, αGalCer is able to play multiple roles including liposomal carrier, stimulation of APCs, and Th epitope-like function. Generally, combination of several adjuvant properties to a single molecule provide the potential to enhance the vaccine efficacy.[18,29] Notably, it is known that a single administration of αGalCer tends to cause iNKT cell anergy which resistant to further aGalCer activation within at least one month.[61] However, iNKT cell anergy might not be a serious problem in our liposomal vaccines. The antibody titers after the 2nd and 3rd injections do not decrease but increase relative to those after 1st injection (Figure S12 in Supporting Information). Our data of antibody response agree with the report on the synthetic vaccine against Tn, a typical tumor-associated carbohydrate antigen.[38] Anergy has frequently been observed when αGalCer is administrated in solution as a soluble form, probably the liposomal formulation can more selectively deliver αGalCer to professional APCs, thus preventing or reducing this phenomenon.[62]

Second, there is no synergy of αGalCer and Pam_3CSK_4 in stimulating antibody responses. Cerundolo and coworkers reported the cooperative actions between iNKT cell and TLR agonists contribute to DC maturation and cytotoxic T cell stimulation.[49] More relevantly, it was reported that in *M. tuberculosis* infection model, co-administration of Pam_3CSK_4 with IFN-γ was able to up-regulate CD1d on macrophages.[63] However, the combination of αGalCer and Pam_3CSK_4 did not elevate anti-nicotine IgG titers compared to the treatment of each adjuvant alone, in spite of the distinct mechanisms through which two adjuvants function. These results are in agreement with the reports about the lack of cooperation between αGalCer and TLR ligands for humoral responses.[50,51] Given some examples of synergistic TLR and iNKT cell collaborations in cellular

immunity,[64-68] perhaps humoral immune responses are separable from DC-dependent cytotoxic T cell responses.[50]

Third, the covalent attachment of nicotine hapten to αGalCer may not be key to induce an effective immune response; in contrast, the noncovalent modality involving an admixture of the lipid-tailed antigen and the glycolipid adjuvant represents a more practical means for vaccine construction. Generally, conjugation of an adjuvant to an antigen has exhibited higher efficacy than unconjugated mixtures.[69,70] Because adjuvants and antigens usually have different pharmacokinetic properties, simply mixing them without using covalent linker cannot ensure that they are recognized concurrently by APC. However, the covalent modality is not the only approach to guarantee antigen and adjuvant arrive at APC simultaneously. The noncovalent modality is an alternative approach in which the antigen is appended a "hydrophobic foot" and then combines with an adjuvant. One vaccine against pneumococcal bacteria (PBS150/PBS57) prepared by this noncovalent design is able to trigger better responses than Prevnar, a conjugate vaccine approved by FDA in 2010.[31] Particularly, the direct comparison between covalent and noncovalent modalities in which a model antigen, 4-hydroxy-3-nitrophenyl (NP) moiety was used, demonstrates that the immunization of the noncovalent modality stimulates higher anti-NP IgG antibody titers than that of the covalent one.[71] From the perspective of synthesis, the noncovalent modality is more practical. The lipid anchor need not to be βGalCer, and a structurally simple lipid would also meet the requirement.[72] Another advantage of this noncovalent modality is that no modification of adjuvants is required, thus avoiding decrease or even disappearance of adjuvant activity caused by covalent conjugation.[38] Additionally, the ratio of hapten to adjuvant in covalent modality is fixed at 1: 1, which in contrast can be altered in noncovalent modality. Given the fact that iNKT cell anergy is closely correlated to high dose of αGalCer, [61] it is likely that the optimal

ratio of hapten to adjuvant is much greater than one, which can be regarded as the third advantage of noncovalent modality. Taken together, our present study suggests that the covalent linker between the nicotine hapten and αGalCer might not be indispensable for an effective nicotine vaccine.

Finally, the cytokine profiles (IFN-γ and IL-4) of the αGalCer-adjuvanting vaccine candidates, which are commonly used criteria for the iNKT cell agonists, do not correlate closely with the vaccine efficacies in our system. Further study on the use of iNKT cell agonists (particularly Th1 and Th2-skewing glycolipids) in nicotine synthetic vaccines is required for the optimization of a vaccine construct.

CONCLUSIONS

Our study highlights the use of iNKT cell agonist αGalCer by means of noncovalent and peptide-free vaccine modality in the liposomal synthetic nicotine vaccine, which is synthetically simple but capable of triggering effective anti-nicotine antibody responses. In view of the nonpolymorph of CD1d, these αGalCer-adjuvanting vaccine candidates has the potential to be universally effective in all people. Although these findings are based on nicotine vaccines, they should be also applicable for the development of vaccines against other addictive drugs.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, compound characterization spectra (^{1}H NMR, ^{13}C NMR, MS spectra), vaccine formulation, characterization of liposomes by dynamic light scattering, biological

assays (antibody titers, antibody affinity, studies with antinociception and hypothermia). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

αGalCer, α-galactosylceramide; APC, antigen-presenting cells; BSA, bovine serum albumin; DCs, dendritic cells; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; IL-4, interleukin-4; iNKT, invariant natural killer T; MHC, major histocompatibility complex; MPE, maximum possible effect; MPLA, monophosphoryl lipid A; Nic, nicotine; NP, 4-hydroxy-3-nitrophenyl; OVA, ovalbumin; Th, T helper; TLR, toll-like receptor.

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