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Synthesis and immunological evaluation of self-adjuvanting glycolipopeptide vaccine candidates

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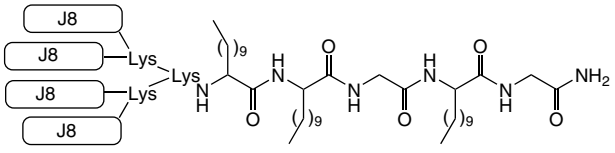
ABSTRACT

Synthesis of four glycolipids with different number of lauroyl groups on glucose or cellobiose as scaffolds is described. Their immunological evaluations either admixed with or covalently linked to J8, a peptide antigen derived from the C-terminus of the antiphagocytic M-protein of group A streptococcus, are also investigated. Administration of mixtures of J8 and glycolipids to B10BR (H-2^k) mice induced low-levels of J8-specific IgG antibodies. While glycolipopeptides, in which J8 was covalently linked to the synthetic glycolipids, demonstrated high-levels of antibody titers comparable with the co-administration of these glycolipopeptides with complete Freund's adjuvant, suggesting clearly the strong potency of the synthesized glycolipids as self-adjuvanting moieties.

1. Introduction

Vaccination is one of the most valuable public health interventions which has dramatically contributed to reduction of deaths from infectious diseases. The global eradication of smallpox announced by WHO in 1980 is generally considered among the great human achievements.[1] However, development of effective vaccines to conquer infectious diseases such as HIV/AIDS,[2] malaria (*Plasmodium falciparum*),[3] group A streptococcus (GAS),[4] and *Mycobacterium tuberculosis*[5] are still demanded. Attempts to develop vaccines against these diseases using traditional methods (live or attenuated microorganisms, and conventional whole proteins) were hindered by the development of cross-reactive antibodies, weak immunogenicity and lack of a human compatible adjuvant. Development and establishment of chemical and biochemical techniques such as solid phase peptide synthesis (SPPS), epitope recognition and recombinant DNA technology resulted in the alternative subunit vaccine approaches using synthetic epitopes (short peptide, plasmid DNA or carbohydrates) which are able to produce epitope-specific antibodies. However, administration of these synthetic epitopes without any adjuvant generally does not elicit early, high and long-lasting immune responses. Adjuvants such as complete Freund's adjuvant (CFA), lipid A, and muramyl dipeptide are limited for human use mainly because of their adverse effects.[6] Recently adjuvants such as mantanide ISA 51[7] and monophosphoryl lipid A (MPL®)[8] are in human clinical trials. Aluminum and calcium salts are the only adjuvant for childhood human vaccine. Therefore research for adjuvant systems is ongoing. In the early 1980s, Hopp et al.[9] demonstrated that conjugation of antigenic peptide and N^{α},N^{ε}-dipalmityl-lysine resulted in the enhancement of peptide immunogenicity, and Rammensee et al.[10] reported that peptides covalently linked to a lipid moiety ($Pam_3CysSerSer$) elicited immune responses without additional adjuvant. To date, a variety of amino acid derivatives such as Pam_2Cys,[11] Pam_3Cys,[11] and lipoamino acids,[12] as lipid moieties have been studied to develop self-adjuvanting lipopeptide-based vaccines[13] with some showing clinical safety in human trials.[14] Several reports have demonstrated that changing the length and number of lipid chains affects adjuvanticity.[15–17] We have developed the self-adjuvanting peptide vaccine system, named lipid-core peptide (LCP),[12,18,19] in which multi copies of peptide antigens are attached on oligolysine branches bearing lipoamino acids as an adjuvant moiety (Fig. 1).



Lipid-core peptide (LCP) vaccine system: LCP-J8
J8: QAEDK VKQSR EAKKQ VEKAL KQLED KVQ

Figure 1. Structure of an example of LCP vaccine system (LCP-J8).

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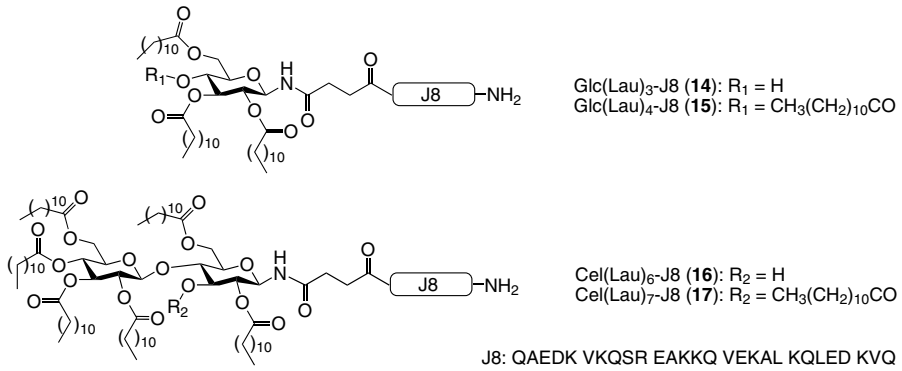


Figure 2. Structure of glycolipopeptide vaccine candidates (**14**–**17**).

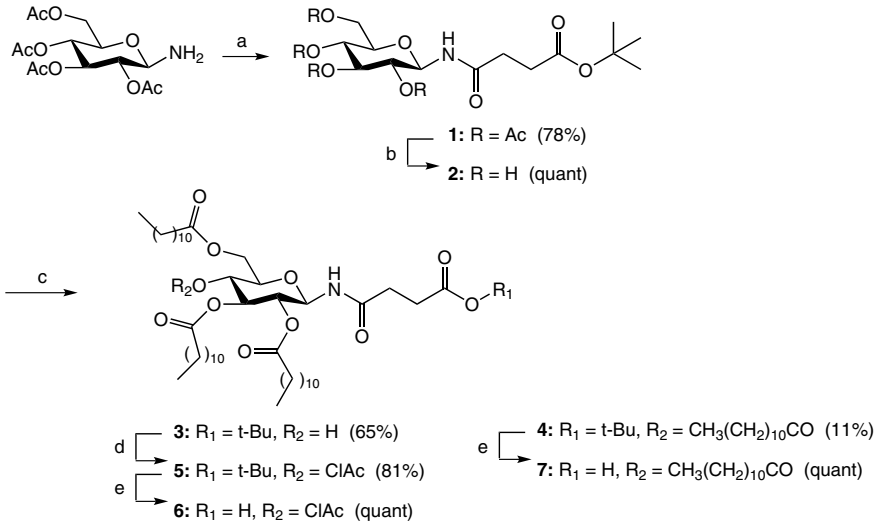
Hence we propose the novel idea to use synthetic glycolipids as built-in adjuvant moieties for glycolipopeptide vaccine development. Carbohydrates are attractive compounds as scaffolds to attach lipids not only because their structural features can provide multiple lipidation points but also they would give information about the effect of lipid orientation on adjuvanticity. Therefore we designed glycolipids bearing aliphatic long-chain acyl groups built on carbohydrate scaffolds, as well as a linker capable to be incorporated into peptide antigens. In this study, J8[20] (QAEDK VKQSR EAKKQ VEKAL KQLED KVQ) was used as a model epitope derived from antiphagocytic GAS M-protein, which is the major causative agent in GAS-associated diseases such as necrotizing fasciitis, rheumatic fever, and rheumatic heart disease.[4] Mono- and disaccharides (glucose and cellobiose, respectively) were chosen to study the effect of changing the number of acyl chains on immune response. In the present paper, we report the synthesis of four glycolipids (**6**, **7**, **12**, and **13**) as well as their immunostimulating activities. To study their potential self-adjuvanting activities, these glycolipids were also covalently bound to J8, as shown in Figure 2.

2. Results and discussion

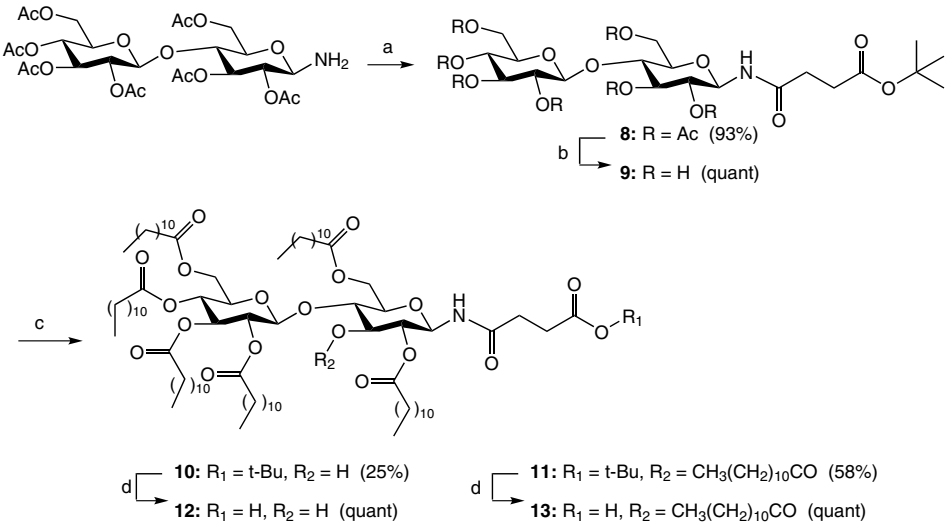
2.1. Chemistry

Glycolipids (**6**, **7**, **12**, and **13**) in the current study were designed to bear a carboxyl group to be incorporated into the N-terminus of peptide antigens on resin (Schemes 1 and 2). Peracetylated glycosyl amines were prepared from glucose and cellobiose according to the Esteves' method[21] and Choudhury's method, [22] respectively; which were then coupled to mono-*tert*-butyl succinate[23] using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylhexafluorophosphate (HBTU) and Et_3N in dry tetrahydrofuran (THF). After deacetylation, the given free hydroxyl groups were acylated with lauroyl chloride. In order to obtain both full and partial acyl-glycosamide derivatives, 3.5 and 7.5 equivalents of lauroyl chloride were used against the mono- and disaccharide, respectively, followed by column chromatography to separate full and partial acyl-glycosamide derivatives (**3**: 65% yield, **4**: 11% yield, **10**: 25% yield, and **11**: 58% yield). To avoid the expected side reaction following conjugation onto peptide, both partially acetylated compounds **3** and **10** were treated with chloroacetyl chloride; however **10** did not react indicating the low reactivity of its OH at position 6 which could be due to steric hindrance. The free OH group at position 6 of compound **10** was left unprotected because it was thought that it would not be affected during the following coupling reaction with J8. *tert*-Butyl group was removed by 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) to generate carboxyl group prior to coupling with peptide.

Since glycolipid compounds were found to be unstable under HF cleavage conditions for the Boc-chemistry (data are not shown), the peptide epitope J8 was assembled on Rink amide resin by Fmoc-SPPS using HBTU as a coupling reagent and 20% piperidine in *N*,*N*-dimethylformamide (DMF) as an Fmoc-deprotection re-



Scheme 1. Synthesis of monosaccharide derivatives **6** and **7**. Reagents: (a) mono-*tert*-butyl succinate,[23] HBTU, THF, Et_3N (pH 8), moleculersieve; (b) 0.1 M $NaOCH_3$/MeOH; (c) lauroyl chloride, pyridine, DMAP, column chromatography; (d) chloroacetyl chloride, pyridine, DMAP; (e) 50% TFA in DCM.



Scheme 2. Synthesis of disaccharide derivatives **12** and **13**. Reagents: (a) mono-*tert*-butyl succinate,[23] HBTU, THF, Et_3N (pH 8), moleculersieve; (b) 0.1 M $NaOCH_3$/MeOH; (c) lauroyl chloride, pyridine, DMAP, column chromatography; (d) 50% TFA in DCM.

agent. After assembly of J8 sequence, glycolipids (**6**, **7**, **12**, and **13**) were coupled to its N-terminus using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop) in DCM/dimethylsulfoxide (DMSO). Dechloroactylation[24] was performed by treatment with thiourea in DMF. Glycolipopeptides were cleaved from resin by treatment with TFA/DCM/triisopropylsilane (TIS) (8:8:1) for 3 h at room temperature, precipitated with ice-cold Et_2O, filtrated and then dissolved in MeCN/H_2O (1:1) containing 0.1% TFA followed by lyophilization. All compounds were identified using ESI-MS and purified by RP-HPLC linear gradients of 10–100% solvent B (0.1% TFA in MeCN) in solvent A (0.1% TFA in water) and C18 column for J8 and C4 column for glycolipopeptides (**14**–**17**), respectively.

2.2. Immunological assessment

The immunological experiment was designed to explore both the ability of the glycolipid moieties to impart a self-adjuvanting activity to the glycolipopeptide vaccine candidates (glycolipopeptides **14–17** were administered with and without CFA) and its adjuvanticity when physically mixed with a short peptide (glycolipids **6** and **12**, were tested with and without J8 peptide antigen). Two positive control groups (J8 mixed with CFA and self-adjuvanting lipopeptide LCP-J8,[12,18] in which four copies of J8 epitopes are attached on Lys-based dendrimer bearing three lipoamino acids at C-terminus, Fig. 1), and two negative control groups (PBS and CFA) were used. Mice (B10BR (H-2^k)) received three boosts of each compound at days 21, 31, and 41 post-primary immunization. During experiment no apparent sign of toxicity was observed. Sera were collected prior to each boost, and 9 days after the final boost, to assess the levels of J8-specific serum IgG elicited using an enzyme-linked immunosorbent assay (ELISA).[18]

Low-level of J8-specific antibody titers were observed following priming with glycolipopeptides (**14–17**), on the other hand the admixture of J8 with glycolipid **6** or **12** elicited low-level J8-specific antibody titers after the second boost. The antibody

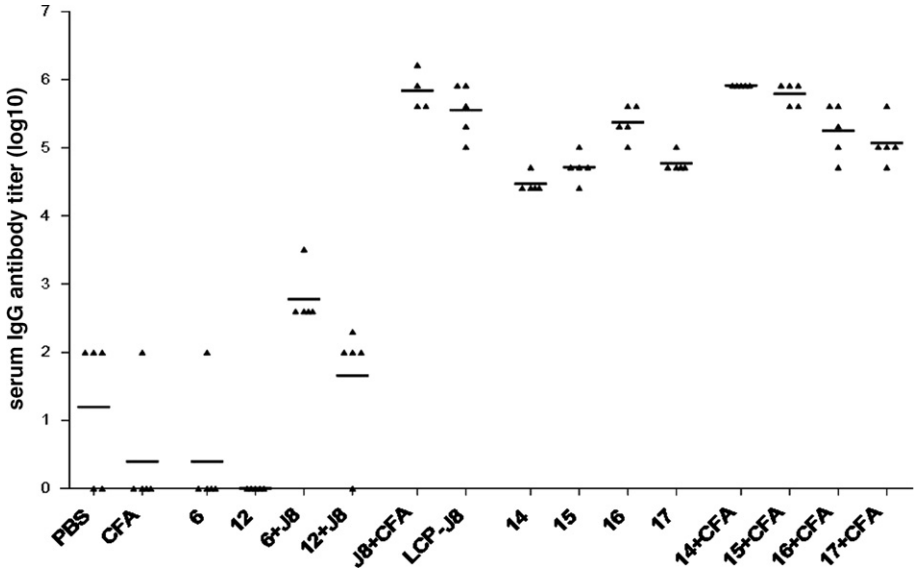


Figure 3. J8-specific serum IgG antibody titers (log10) at the final bleed (day 50) elicited in response to immunization of B10.BR (H-2^k) mice at the tail base with glycolipopeptides **14**–**17**, as determined by ELISA.

titers were increased after each boost (data are not shown). Antibody titers against J8 antigen (days 50) are shown in Figure 3. Mice administered mixtures of J8 and glycolipids **6** or **12**, J8/glycolipid (1:1), showed low-level of J8-specific antibody titers (J8 with **6** or **12** vs CFA or PBS, $p > 0.05$) compared with when admixed with CFA (J8 with **6** or **12** vs J8 with CFA, $p < 0.001$). Meanwhile the specific antibody titers against J8 were dramatically enhanced in mice administered the glycolipopeptides (**14–17**) without CFA and these titers were as immunogenic as a positive controls (**14**, **15**, **16** or **17** vs J8 with CFA, $p > 0.05$). In general big vaccine molecules bearing multiple copies of epitopes such as multiple antigen peptide (MAP)[25] and LCP[12,18] show higher immunogenicity than monovalent peptide antigens. However, synthesis of such big molecules commonly encounters difficulty in purification to homogeneity and characterization. In the current study, our novel glycolipopetides containing one copy of J8 (**14–17**) demonstrated the same level of J8-specific IgG antibody titers elicited by the LCP-J8 bearing four copies of J8 (**14**, **15**, **16** or **17** vs LCP-J8, $p > 0.05$), which would improve the ease of synthesis and purification as well as the cost of vaccine production. Moreover, synergetic effect of mixing glycolipopeptides (**14–17**) with CFA on J8-specific antibody responses was studied. The level of antibody titers induced by glycolipopeptides alone bear comparison with the antibody titer levels when these glycolipopeptides were emulsified as 1:1 in CFA (**14** vs **14** + CFA, **15** vs **15** + CFA, **16** vs **16** + CFA, **17** vs **17** + CFA, $p > 0.05$). Among the glycolipopeptides, disaccharide derivatives (**16** and **17**) were slightly more immunogenic than the monosaccharide derivatives (**14** and **15**). These differences among the four glycolipopeptides suggest the intervening effects of both the number and orientation of lipids on immunological activity. This needs further investigation to determine the contribution of each factor.

The structure of lipid A, a phosphoglycolipid of Gram-negative bacterial outer cell membranes, is highly conserved among species in terms of β(1–6)-linkage glucosamine disaccharide unit and phosphate groups.[26] Despite its strong adjuvanticity, lipid A is not suitable for human use due to its toxicity. The dephosphorylation of lipid A decreases its toxicity as well as its immunostimulating activity probably due to insolubility.[27] Glycolipids (**6**, **7**, **12**, and **13**), which are structurally different from lipid A, did not show strong adjuvanticity when physically mixed with J8, this could be also due to the solubility issue. However the covalent linkage between synthesized glycolipids and peptide antigen could improve not only the solubility of glycolipids but also the immunogenicity of the incorporated peptide antigen resulting in potent self-adjuvanting glycolipopeptide vaccine candidates.

3. Conclusion

Since the adjuvanticity of alums was demonstrated in 1926,[28] plenty of compounds were developed to be used as adjuvants but failed, mostly due to toxicity. Self-adjuvanting subunit lipopeptides are currently being investigated for human use[14] as an alternative vaccine strategy and show several advantages as ease of purification to homogeneity, elicitation of antigen-specific antibodies, and no need of additional adjuvant. The current study is the first to demonstrate the utility of alternative synthetic glycolipid compounds, which can be simply synthesized from glucose or cellobiose and induce strong immunogenicity when coupled to short peptide antigens. Further studies using different carbohydrate scaffolds and/or different alkyl chains, hemolysis and toll-like receptor affinity assays are currently underway to meliorate and evaluate the mechanism.

4. Experimental

4.1. Materials and methods

^{1}H NMR spectra were recorded at 297 K on a Bruker 500 MHz instrument using $CDCl_3$, CD_3OD or aceton-d_6 as solvents and TMS as an internal standard, unless stated otherwise. Coupling constants are given in Hz. Column chromatography was performed on silica gel columns (size A, 28×2; B, 30×2.5; and C, 43×4 cm; silica gel 0.040–0.063 mm. Mass spectra were obtained on a quatropol-electrospray-MS (Pekin Elmer API 3000 instrument) in the positive ion mode using MeCN/H_2O mixtures. Concentration of solutions was performed at reduced pressure at temperatures <40 °C. Analytical RP-HPLC was performed on a Shimadzu instrument (LC-10AT liquid chromatograph, SC_L-10A system controller, SPD-6A UV detector, a SIL-6B auto injector with a SCL-6B system controller, and a Vydac analytical C18-column (218TP54: 5 μm, 4.6×250 mm) or Vydac analytical C4 column (214TP54: 5 μm, 4.6×250 mm) using a 0–100% linear gradient of solvent B over 30 min with 1 mL/min flow rate. Following running methods were used: method 1; C18 column and 0.1% TFA, 90% MeCN in H_2O (solvent B), method 2; C18 column and 0.1% TFA, 90% MeOH in H_2O (solvent B), method 3; C4 column and 0.1% TFA, 90% MeCN in H_2O (solvent B), method 4; C4 column and 0.1% TFA, 90% isopropanol in H_2O (solvent B). HPLC purification was done on a Waters HPLC system (Model 600 controller, 490E UV detector, F pump, and 0.46×15 cm Vydac preparative C18 column (218TP1022: 10 μm, 22×250 mm) or C4 column (214TP1022: 10 μm, 22×250 mm)) using a MeCN/H_2O gradient. He-gas was applied for degassing of HPLC-solvents.

4.2. General procedure for synthesis of *N*-(*O*-peracetyl-glycosyl)-succinamide *tert*-butyl ester (1 and 8)

Peracetylated glycosylamine (tetra-*O*-acetyl-β-glucopyranosyl-1-amine[21] 347.3 mg, or hepta-*O*-acetyl-β-cellobiosyl-1-amine[22] 635.5 mg: 1 mmol) was dissolved in dry THF (8 mL), then a solution of mono-*tert*-butyl-succinate[23] (192 mg, 1.1 mmol), molecular sieve 4 Å (200 mg) and HBTU (4.7 mg, 1.1 mmol) in dry DMF (3 mL) was added at 0 °C. Immediately the pH was adjusted to pH 8 by adding Et_3N and the mixture was stirred overnight at room temperature under N_2. The mixture was filtered over Celite® and co-evaporated with toluene under reduced pressure. The organic residue was dissolved in ethyl acetate (200 mL) and washed with 10% citric acid (3× 50 mL), satd. $NaHCO_3$-solution (3× 25 mL), and brine (3× 20 mL), dried ($MgSO_4$) and concentrated under reduced pressure. In the case of glucose derivative, the residue was crystallized from ethyl acetate/hexane to afford white crystals **1** (393 mg, 78% yield). The residue derived from cellobiose was purified on a column of silica gel, (1:2 → 1:1 toluene/ethyl acetate) to give a white precipitate **8** (735 mg, 93% yield).

4.2.1. *N*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-succinamide *tert*-butyl ester (1)

ESI-MS: (calcd, $C_{22}H_{33}O_{12}N$) 503.51 *m/z* (%): $[M+Na]^+$: 526.6 (100). ^{1}H NMR ($CDCl_3$): δ 6.44 (d, 1H, NH), 5.26 (t, 1H, $J = 9.4$ Hz, H-3), 5.22 (t, 1H, $J = 9.2$ Hz, H-1), 5.01 (t, 1H, H-4), 4.88 (t, 1H, H-2), 4.24 (dd, 1H, $J = -12.3$, 4.4 Hz, H-6a), 4.02 (dd, 1H, $J = 1.9$ Hz, H-6b), 3.76 (ddd, 1H, H-5), 2.32–2.63 (m, 4H, $2CH_2$), 2.03, 2.02, 1.98, 1.97 (4 s, 12H, 4 Ac), 1.38 (s, 9H, ^{t}Bu).

4.2.2. *N*-(2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-β-D-cellobiosyl)-succinamide *tert*-butyl ester (8)

ESI-MS: (calcd, $C_{34}H_{49}O_{20}N$) 791.76 *m/z* (%): $[M+H]^+$: 792.8 (100), $[M+Na]^+$: 814.7 (20). ^{1}H NMR ($CDCl_3$): δ 6.36 (d, 1H, NH),

5.22 (t, 1H, J = 9.1 Hz, H-3), 5.15 (t, 1H, J = 9.0 Hz, H-1), 5.09 (t, 1H, J = 9.3 Hz, H-3′), 5.02 (t, 1H, J = 9.4 Hz, H-4′), 4.87 (t, 1H, H-2′), 4.80 (t, 1H, H-2), 4.46 (d, 1H, J = 7.9 Hz, H-1′), 4.40 (dd, 1H, J = −12.2, 1.3 Hz, H-6b), 4.32 (dd, 1H, J = −12.5, 4.5 Hz, H-6a′), 4.07 (dd, 1H, J = −12.2, 4.6 Hz, H-6a), 3.99 (dd, 1H, J = 2.1 Hz, H-6b′), 3.71 (t, 1H, H-4), 3.67 (ddd, 1H, H-5), 3.61 (ddd, 1H, H-5′), 2.30–2.58 (m, 4H, $2CH_2$), 2.07, 2.04, 2.02, 2.00, 1.99, 1.96, 1.93 (7 s, 21H, 7 Ac), 1.37 (s, 9H, ^{t}Bu).

4.3. General de-O-acetylation procedure (2 and 9)

A solution of *N*-(*O*-peracetyl-glycosyl)-succinamide *tert*-butyl ester (**1** 500 mg, or **8** 790 mg: 1 mmol) in dry MeOH (7 mL) was stirred with 1 M methanolic NaOMe (0.7 mL) for 4 h at room temperature. The solution was deionized by addition of Dowex 50-AG WX8 (H^+) cation-exchange resin to pH 7, filtered, and evaporated and a white powder of **2** (330 mg) and **9** (480 mg), respectively, were obtained (97% yield).

4.3.1. *N*-(β-D-Glucopyranosyl)-succinamide *tert*-butyl ester (2)

ESI-MS: (calcd, $C_{14}H_{25}O_8N$) 335.36 *m/z* (%): $[M+H]^+$: 336.4 (60), $[M+Na]^+$: 358.5 (100). ^{1}H NMR (DMSO-d_6): δ 8.39 (d, 1H, NH), 4.87–5.07 (m, 3H, OH-2, OH-3, OH-4), 4.66 (t, 1H, J = 8.8 Hz, H-1), 4.49 (t, 1H, OH-6), 3.61 (ddt, 1H, H-6a), 3.40 (dd, 1H, H-6b), 3.15 (t, 1H, J = 8.0 Hz, H-3), 2.90–3.10 (m, 3H, H-2, H-4, H-5), 2.26–2.50 (m, 4H, $2CH_2$), 1.37 (s, 9H, ^{t}Bu).

4.3.2. *N*-(β-D-Cellobiosyl)-succinamide *tert*-butyl ester (9)

ESI-MS: (calcd, $C_{20}H_{35}O_{13}N$) 497.50 *m/z* (%): $[M+H]^+$: 498.7 (100), $[M+NH_4]^+$: 515.7 (50), $[M+Na]^+$: 520.5 (20). ^{1}H NMR (DMSO-d_6): δ 8.42 (m, 1H, NH), 5.25 (s, 1H, OH-2′), 5.01 (m, 3H, 3 OH), 4.71 (m, 2H, H-1, OH), 4.57 (m, 2H, 2 OH), 4.24 (m, 1H, H-1′), 3.08–4.30 (m, 7H, H-6b′, H-2, H-3, H-3′, H-4, H-5, H-5′), 3.03 (d, 1H, H-4′), 2.97 (d, 1H, H-2′), 2.26–2.50 (m, 4H, $2CH_2$), 1.37 (s, 9H, ^{t}Bu).

4.4. General procedure for lauroylation (3, 4, 10, and 11)

To a solution of *N*-glycosyl-succinamide *tert*-butyl ester derivatives (**2** 101 mg, 0.3 mmol or **9** 500 mg 1.0 mmol, 500 mg) in DCM/pyridine (2:1, 3 mL) and *N*,*N*-dimethylamino pyridine (DMAP) (cat.), lauroylchloride (3.5 equiv 1.05 mmol, 26 μL for glucose derivatives; 7.5 equiv 7.58 mmol, 1.8 mL for cellobiose derivatives) was added dropwise at 0 °C and stirred at room temperature for 48 h. The solution was co-evaporated with toluene (3× 20 mL), the residue dissolved in ethyl acetate (100 mL) and washed with 20% citric acid (3× 20 mL), satd. $NaHCO_3$-solution (3× 30 mL), brine (3× 20 mL), dried ($MgSO_4$) and evaporated. In the case of glucose derivatives, two major products **3** and **4** with the R_f value of 0.33 and 0.60 using hexane/ethyl acetate (3:1), respectively, were purified on a column of silica gel, (3:1 → 1:1 hexane/ethyl acetate) to afford compounds **3** (65% yield) and **4** (11% yield) as colorless syrup. In the case of cellobiose derivatives, two compounds **10** and **11** with the R_f value of 0.40 and 0.70 using hexane/ethyl acetate (3:1), respectively, were purified on a column of silica gel, (5:1 → 3:1 hexane/ethyl acetate) to afford compounds **10** (409 mg, 25% yield) and **11** (1.04 g, 58% yield) as colorless syrups.

4.4.1. *N*-(2,3,6-Tri-*O*-lauroyl-β-D-glucopyranosyl)-succinamide *tert*-butyl ester (3)

ESI-MS: (calcd, $C_{50}H_{91}O_{11}N$) 882.28 *m/z* (%):$[M+H-{}^tBu]^+$: 826.9 (10), $[M+H]^+$: 882.6 (100), $[M+NH_4]^+$: 899.9 (20), $[M+Na]^+$: 905.2 (5). ^{1}H NMR ($CDCl_3$): δ 6.32 (d, 1H, NH), 5.19 (t, 1H, J = 9.1 Hz, H-1), 5.14 (t, 1H, J = 9.2 Hz, H-3), 4.82 (t, 1H, H-2,) 4.50 (dd, 1H, J = −12.3, 3.8 Hz, H-6a), 4.21 (dd, 1H, J = 1.9 Hz, H-6b), 3.59 (ddd, 1H, H-5), 3.45(t, 1H, J = 9.5 Hz, H-4), 2.22–2.60 (m, 10H, $2CH_2$, 3 α-CH_2), 1.50–1.64 (m, 6H, 3β-CH_2), 1.18–1.32 (m, 48H, $24CH_2$), 0.85 (t, 9H, $3CH_3$).

4.4.2. N-(2,3,4,6-Tetra-*O*-lauroyl-β-D-glucopyranosyl)-succinamide *tert*-butyl ester (4)

ESI-MS: (calcd, $C_{62}H_{113}O_{12}N$) 1064.59 *m/z* (%): $[M+H-{}^tBu]^+$: 1010.3 (100), $[M+H]^+$: 1065.2 (80), $[M+Na]^+$: 1087.3 (30). ^{1}H NMR ($CDCl_3$): δ 6.40 (d, 1H, NH), 5.29 (t, 1H, J = 9.5 Hz, H-3), 5.21 (t, 1H, J = 9.2 Hz, H-1), 5.05 (t, 1H, H-4,) 4.90 (t, 1H, H-2), 4.21 (dd, 1H, J = −12.3, 4.3 Hz, H-6a), 4.04 (dd, 1H, J = 1.3 Hz, H-6b), 3.77 (ddd, 1H, H-5), 2.16–2.59 (m, 12H, $2CH_2$, 4 α-CH_2), 1.46–1.63 (m, 8H, 4β-CH_2), 1.40 (s, 9H, ^{t}Bu), 1.17–1.31 (m, 64H, $32CH_2$), 0.85 (t, 12H, $4CH_3$).

4.4.3. *N*-(2,2′,3′,4′,6,6′-Hexa-*O*-lauroyl-β-D-cellobiosyl)-succinamide *tert*-butyl ester (10)

ESI-MS: (calcd, $C_{92}H_{167}O_{19}N$) 1591.35 *m/z* (%): $[M+H]^+$: 1593.2 (100). ^{1}H NMR ($CDCl_3$): δ 6.30 (d, 1H, NH), 5.18 (t, 1H, J = 9.5 Hz, H-3′), 5.10 (t, 1H, J = 9.1 Hz, H-1), 4.98–5.02 (m, 2H, H-2′, H-4′), 4.74 (t, 1H, H-2), 4.51 (d, 1H, J = 8.0 Hz, H-1′), 4.23 (dd, 1H, J = −11.8, 1.7 Hz, H-6b), 4.20 (dd, 1H, J = −12.2, 2.3 Hz, H-6b′), 4.05 (dd, 1H, J = −12.2, 6.8 Hz, H-6$^{a\prime}$), 3.96 (dd, 1H, J = 4.6 Hz, H-6a), 3.73–3.81 (m, 2H, H-3, H-5′), 3.61 (ddd, 1H, H-5), 3.47 (dd, 1H, H-4), 2.14–2.57 (m, 16H, $2CH_2$CONH, 6 α-CH_2), 1.44–1.64 (m, 12H, 6β-CH_2), 1.40 (s, 9H, ^{t}Bu), 1.18–1.32 (m, 96H, $48CH_2$), 0.85 (t, 18H, CH_3).

4.4.4. *N*-(2,2′,3′,4,4′,6,6′-Hepta-*O*-lauroyl-β-D-cellobiosyl)-succinamdie *tert*-butyl ester (11)

ESI-MS: (calcd, $C_{104}H_{189}O_{20}N$) 1773.66 *m/z* (%): $[M+H]^+$: 1774.6 (80), $[M+Na]^+$: 1796.5 (100). ^{1}H NMR ($CDCl_3$): δ 6.23 (d, 1H, NH), 5.26 (t, 1H, J = 9.4 Hz, H-3), 5.15 (t, 1H, J = 9.3 Hz, H-1), 5.10 (t, 1H, J = 9.4 Hz, H-3′), 5.02 (t, 1H, H-4′), 4.89 (t, 1H, H-2′), 4.80 (t, 1H, H-2), 4.42 (d, 1H, J = 7.9 Hz, H-1′), 4.42 (dd, 1H, J = −12.2, 1.3 Hz, H-6b), 4.27 (dd, 1H, J = −12.5, 4.9 Hz, H-6a′), 4.10 (dd, 1H, J = −12.2, 4.5 Hz, H-6a), 4.00 (dd, 1H, J = 2.1 Hz, H-6b′), 3.71 (t, 1H, H-4), 3.64 (ddd, 1H, H-5), 3.58 (ddd, 1H, H-5′), 2.13–2.57 (m, 18H, $2CH_2$CONH, 7 α-CH_2), 1.42–1.66 (m, 14H, 7β-CH_2), 1.40 (s, 9H, ^{t}Bu), 1.18–1.30 (m, 112H, $56CH_2$), 0.86 (t, 21H,CH_3).

4.5. *N*-(4-*O*-Chloroacetyl-2,3,6-tri-*O*-lauroyl-β-D-glucopyranosyl)-succinamide *tert*-butyl ester (5)

Chloroacetyl chloride (19 μL, 0.24 mmol) in DCM (0.6 mL) was added dropwise to a solution of **3** (102 mg, 0.12 mmol) and DMAP (cat.) in DCM/pyridine (1.3 mL, 10:1) at −78 °C and stirred for 16 h at −20 °C. The solution was diluted with DCM (100 mL) and washed with satd. $NaHCO_3$-solution (3× 30 mL), brine (3× 20 mL) and co-evaporated with toluene (3× 30 mL). A yellow residue was purified on a column of silica gel, (5:1 → 1:1 hexane/ethyl acetate) to afford compound **5** (93 mg, 81% yield) as colorless syrup. ESI-MS: (calcd, $C_{52}H_{92}O_{12}NCl$) 958.78 *m/z* (%): $[M+H]^+$: 959.0 (60), $[M+Na]^+$: 981.8 (100). ^{1}H NMR ($CDCl_3$): δ 6.44 (d, 1H, NH), 5.31(t, 1H, J = 9.5 Hz, H-3), 5.22 (t, 1H, J = 9.2 Hz, H-1), 5.07 (t, 1H, H-4,) 4.91 (t, 1H, H-2), 4.28 (dd, 1H, J = −12.6, 4.0 Hz, H-6a), 4.06 (dd, 1H, J = 1.6 Hz, H-6b), 3.98 (d, 1H, J = −14.7 Hz, CH_2Cl (A)), 3.93 (d, 1H, CH_2Cl (B)), 3.81 (m, 1H, H-5), 2.16–2.58 (m, 10H, $2CH_2$, 3 α-CH_2), 1.46–1.62 (m, 6H, 3β-CH_2), 1.39 (s, 9H, ^{t}Bu), 1.16–1.31 (m, 48H, $24CH_2$), 0.84 (t, 9H, $3CH_3$).

4.6. General procedure for *tert*-butyl ester-hydrolysis (6, 7, 12, and 13)

Each *tert*-Butyl ester derivative (0.1 mmol: **4**: 110 mg, **5** 96 mg, **10**: 160 mg, or **11**: 180 mg) was stirred in TFA/DCM (1:1, 4 mL) at room temperature for 16 h. Solvents and TFA was removed by

blowing out with a N_2-stream then the residue was co-evaporated with toluene (3 × 20 mL) and purified on a column of silica gel, (3:1 → 1:1 toluene/hexane, 1% AcOH) to afford carboxylic acid derivatives (**6** 86 mg, **7** 95 mg, **12** 145 mg, or **13** 160 mg) as slightly yellow colorless syrups (90–95% yield).

4.6.1. 4-*O*-Chloroacetyl-2,3,6-tri-*O*-lauroyl-β-D-glucopyranosylamino-4-oxbutanoic acid (6)

ESI-MS: (calcd, $C_{48}H_{84}O_{12}NCl$) 902.66 *m/z* (%): $[M+H]^+$: 903.2 (100), $[M+NH_4]^+$: 920.0 (30). EA: Calcd for $C_{48}H_{84}NO_{12}Cl$: C, 63.87; H, 9.38; N, 1.55. Found: C, 63.80; H, 9.31; N, 1.46. ^{1}H NMR ($CDCl_3$): δ 6.52 (d, 1H, NH), 5.32 (t, 1H, J = 9.6 Hz, H-3), 5.23 (t, 1H, J = 9.0 Hz, H-1), 5.08 (t, 1H, H-4,) 4.92 (t, 1H, H-2), 4.30 (dd, 1H, J = −12.6, 3.9 Hz, H-6a), 4.07 (dd, 1H, J = 1.6 Hz, H-6b), 3.99 (d, 1H, J = −14.7 Hz, CH_2Cl (A)), 3.94 (d, 1H, CH_2Cl (B)), 3.83 (m, 1H, H-5), 2.39–2.71 (m, 4H, 2CH_2), 2.18–2.32 (m, 6H, 3 α-CH_2), 1.46–1.60 (m, 6H, 3β-CH_2), 1.18–1.30 (m, 48H, 24CH_2), 0.85 (t, 9H, 3CH_3).

4.6.2. 2,3,4,6-Tetra-*O*-lauroyl-β-D-glucopyranosylamino-4-oxbutanoic acid (7)

ESI-MS: (calcd, $C_{58}H_{105}O_{12}N$) 1008.48 *m/z* (%): $[M+H]^+$: 1009.1 (100), $[M+Na]^+$: 1031.2 (30) $[M+NH_4]^+$: 1026.5 (70). EA: Calcd for $C_{58}H_{105}NO_{12}$: C, 69.08; H, 10.49; N, 1.39. Found: C, 69.15; H, 10.18; N, 1.30. ^{1}H NMR ($CDCl_3$): δ 6.45 (d, 1H, NH), 5.30 (t, 1H, J = 9.5 Hz, H-3), 5.21 (t, 1H, J = 9.2 Hz, H-1), 5.06 (t, 1H, H-4,) 4.90 (t, 1H, H-2), 4.24 (dd, 1H, J = −12.5, 4.2 Hz, H-6a), 4.07 (dd, 1H, J = 1.5 Hz, H-6b), 3.78 (ddd, 1H, H-5), 2.39–2.73 (m, 4H, 2CH_2), 2.16–2.32 (m, 8H, 4α-CH_2), 1.46–1.63 (m, 8H, 4β-CH_2), 1.17–1.31 (m, 64H, 32CH_2), 0.85 (t, 9H, 3CH_3).

4.6.3. 2,2′,3′,4′,6,6′-Hexa-*O*-lauroyl-β-D-cellobiosylamino-4-oxbutanoic acid (12)

ESI-MS: (calcd, $C_{88}H_{159}O_{19}N$) 1535.20 *m/z* (%): $[M+H]^+$: 1536.1 (100), $[M+Na]^+$: 1558.2 (40). EA: Calcd for $C_{88}H_{159}NO_{19}$: C, 68.85; H, 10.44; N, 0.91. Found: C, 68.90; H, 10.30; N, 0.85. ^{1}H NMR ($CDCl_3$): δ 6.38 (d, 1H, NH), 5.18 (t, 1H, J = 9.5 Hz, H-3′), 5.10 (t, 1H, J = 9.0 Hz, H-1), 4.98–5.02 (m, 2H, H-2′, H-4′), 4.74 (t, 1H, H-2), 4.51 (d, 1H, J = 8.0 Hz, H-1′), 4.25 (dd, 1H, J = −12.1, 1.5 Hz, H-6b), 4.20 (dd, 1H, J = −12.2, 2.3 Hz, H-6b′), 4.05 (dd, 1H, J = −12.2, 6.8 Hz, H-6a′), 3.97 (dd, 1H, J = 4.7 Hz, H-6a), 3.79 (t, 1H, J = 8.8 Hz, H-3), 3.76 (ddd, 1H, H-5′), 3.61 (ddd, 1H, H-5), 3.47 (dd, 1H, H-4), 2.38–2.73 (m, 4H, 2CH_2CO), 2.14–2.34 (m, 12H, 6 α-CH_2), 1.44–1.63 (m, 12H, 6β-CH_2), 1.18–1.32 (m, 96H, 48CH_2), 0.85 (t, 18H, CH_3).

4.6.4. 2,2′,3,3′,4′,6,6′-Hepta-*O*-lauroyl-β-D-cellobiosylamino-4-oxbutanoic acid (13)

ESI-MS: (calcd, $C_{100}H_{181}O_{20}N$) 1717.54 *m/z* (%): $[M+H]^+$: 1718.6 (100), $[M+Na]^+$: 1740.7 (30). EA: Calcd for $C_{100}H_{181}NO_{20}$: C, 69.93; H, 10.62; N, 0.82. Found: C, 70.01; H, 10.60; N, 0.88. ^{1}H NMR ($CDCl_3$): δ 6.31 (d, 1H, NH), 5.26 (t, 1H, J = 9.5 Hz, H-3), 5.14 (t, 1H, J = 9.2 Hz, H-1), 5.10 (t, 1H, J = 9.3 Hz, H-3′), 5.02 (t, 1H, H-4), 4.89 (t, 1H, H-2′), 4.79 (t, 1H, H-2), 4.42 (m, 2H, H-1′, H-6a), 4.28 (dd, 1H, J = −12.2, 4.7 Hz, H-6a′), 4.10 (dd, 1H, J = −12.2, 4.2 Hz, H-6b), 3.99 (dd, 1H, J = 1.5 Hz, H-6b′), 3.47 (dd, 1H, H-4), 3.64 (ddd, 1H, H-5), 3.58 (ddd, 1 H, H-5′), 2.35–2.71 (m, 4H, 2CH_2CO), 2.12–2.35 (m, 14H, 7 α-CH_2), 1.42–1.65 (m, 14H, 7β-CH_2), 1.16–1.33 (m, 112H, 56CH_2), 0.85 (t, 21H, CH_3).

4.7. Solid phase synthesis

Following side protecting groups of Fmoc amino acid derivatives were used: Gln(Trt), Lys(Boc), Asp(O^{t}Bu), Glu(O^{t}Bu), Arg(Pbf), and Ser(^{t}Bu). Peptide synthesis was started with 710 mg of Rink amide resin (loading value: 0.70 mmol/g) on 0.5 mmol scale. Fmoc-deprotection was performed using 20% piperidine in DMF (1 × 5 min and 2 × 30 min). Each amino acid coupling reaction was carried out using 4.1 equimolars of amino acid derivative, 4.0 equimolars of HBTU, and 5.0 equimolars of *N*,*N*-diisopropylethylamine (DIPEA). After assemble of J8 (QAEDK VKQSR EAKKQ VEKAL KQLEDL VQ),[18] the peptide-resin with full-protection was dried (2.075 g: 1.365-g mass increase). For each glycolipopeptide derivative, 300 mg of the fully protected J8-resin (theoretical substitution value: 0.213 mmol/g) were re-swollen in DMF, treated with 20% piperideine (1 × 5 min and 2 × 30 min), and washed with DMF. Glycolipid derivative (**6**: 75 mg, **7**: 84 mg, **12**: 130 mg or **13**: 145 mg; 82.5 μmol) and PyBop (494 mg, 95 μmol) were dissolved in mixed DCM/DMSO (1:1) in the presence of DIPEA (166 μL, 95 μmol), and after 5-min activation, the reaction was left at room temperature for 6 h. The chloroacetyl group was removed by 3-h treatment with 20% thiourea, 20% 2,6-lutidine in DMF.[22] After draining and washing, the resin was treated with TFA/DCM/TIS (8:8:1) at RT for 3 h. After blowing out of solvents, crude compounds were precipitate with ice-cold ether, filtrated, and redissolved in 50% MeCN in H_2O. After lyophilization, J8 (148.7 mg), **14** (134.78 mg), **15** (115.4 mg), **16** (97.42 mg), and **17** (97.86 mg) were obtained. 50 mg of each were purified by HPLC on C4 or C18 columns.

4.7.1. J8

Purification yield 45.4%. RP-HPLC: t_R = 16.47 min (method 1), t_R = 16.65 min (method 2), t_R = 14.61 min (method 3). ESI-MS: MW 3281.72 g/mol, $[M+2H]^{2+}$ *m/z* 1642.2 (calcd, 1641.9), $[M+3H]^{3+}$ *m/z* 1095.3 (calcd, 1094.9), $[M+4H]^{4+}$ *m/z* 821.8 (calcd, 821.4).

4.7.2. Glc(Lau)$_3$-J8 (14)

Purification yield 27.0%. RP-HPLC: t_R = 23.5 min (method 3), t_R = 18.8 min (method 4). ESI-MS: MW 4098.87 g/mol, $[M+2H]^{2+}$ *m/z* 2047.5 (calcd, 2045.9), $[M+3H]^{3+}$ *m/z* 1365.4 (calcd, 1364.3), $[M+4H]^{4+}$ *m/z* 1024.1 (calcd, 1023.46).

4.7.3. Glc(Lau)$_4$-J8 (15)

Purification yield 34.4%. RP-HPLC: t_R = 25.0 min (method 3), t_R = 20.4 min (method 4). ESI-MS: MW 4272.16 g/mol, $[M+2H]^{2+}$ *m/z* 2138.4 (calcd, 2137.1), $[M+3H]^{3+}$ *m/z* 1426.0 (calcd, 1425.1), $[M+4H]^{4+}$ *m/z* 1069.9 (calcd, 1069.0).

4.7.4. Cel(Lau)$_6$-J8 (16)

Purification yield 30.4%. RP-HPLC: t_R = 20.0 min (method 4). ESI-MS: MW 4798.9 g/mol, $[M+2H]^{2+}$ *m/z* 2400.6 (calcd, 2400.5), $[M+3H]^{3+}$ *m/z* 1601.5 (calcd, 1600.6), $[M+4H]^{4+}$ *m/z* 1200.3 (calcd, 1200.7), $[M+5H]^{5+}$ *m/z* 961.5 (calcd, 960.8).

4.7.5. Cel(Lau)$_7$-J8 (17)

Purification yield 30.0%. RP-HPLC: t_R = 20.3 min (method 4). ESI-MS: MW 4981.22 g/mol, $[M+2H]^{2+}$ *m/z* 2491.5 (calcd, 2491.6), $[M+3H]^{3+}$ *m/z* 1662.9 (calcd, 1661.07), $[M+4H]^{4+}$ *m/z* 1247.5 (calcd, 1246.3).

4.8. Mice and subcutaneous immunization

All protocols were approved by the Queensland Institute of Medical Research Animal Ethics Committee and were carried out according to Australian National Health and Medical Research Council guidelines. Immunization was performed as previously reported elsewhere.[18] Female B10BR (H-2^k) mice (4- to 6-week-old, Animal Resource Centre, Perth, Western Australia, Australia) were used for immunization. Mice (n = 5/group) were injected subcutaneously at the tail base on days 0, 21, 31, and 41 with 30 μg of immunogens in a total volume of 50 μL of sterile-filtered PBS. A po-

sitive control received 30 μg of J8 emulsified in a total volume of 50 μL of CFA/PBS (1:1). Two negative controls were administered a 50 μL of either CFA/PBS (1:1) or PBS alone. All controls were boosted with 50 μL of PBS.

4.9. Collection of sera

Blood was collected from the tail artery of each mouse 1 day prior to each injection and 9 days after the last immunization. The blood was left to clot at 37 °C for 1 h, and then centrifuged for 10 min at 3000 rpm to remove clots. Sera were then stored at −20 °C.

4.10. Elisa

ELISA was performed to determine serum IgG antibodies against J8 epitope as previously described.[29] Briefly, serial dilutions of sera were produced in 0.5% skim milk/PBS–Tween 20 buffer, starting at 1:100 concentration with 2-fold dilutions. Optical density was read at 450 nm in a microplate reader following the addition of peroxidase-conjugated goat anti-mouse IgG, and *O*-phenylenediamine. The antibody titer was defined as the lowest dilution with an optical density more than three standard deviations greater than the mean absorbance of control wells containing normal mouse serum.

4.11. Statistics

Statistical analysis of antibody titers between groups was performed using a one-way ANOVA followed by the *t*-Student post-hoc test. GraphPad Prism 4 software was used for statistical analysis, with $p < 0.05$ taken as statistically significant.

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References and notes

1. Strassburg, M. A. *Am. J. Infect. Control* **1982**, *10*, 53.
2. Tonks, A. *BMJ* **2007**, *334*, 1346.
3. Komisar, J. L. *Front Biosci.* **2007**, *12*, 3928.
4. (a) Carapetis, J. R.; Steer, J. R.; Mulholland, E. K.; Weber, M. *Lancet Infect. Dis.* **2005**, *5*, 685; (b) Batzloff, M. R.; Pandey, M.; Olive, C.; Good, M. F. *Immunol. Res.* **2006**, *35*, 233.
5. Fattorini, L. *Minerva Med.* **2007**, *98*, 109.
6. Gupta, R. K.; Relyveld, E. H.; Lindblad, E. B.; Bizzini, B.; Ben-Efrain, S.; Gupta, C. K. *Vaccine* **1993**, *11*, 293.
7. Gonzalez, G.; Crombet, T.; Torres, F.; Catala, M.; Alfonso, L.; Osorio, M.; Neninger, E.; Garcia, B.; Mulet, A.; Perez, R.; Lage, R. Epidermal growth factor-based cancer vaccine for non-small-cell lung cancer therapy. *Ann. Oncol.* **2003**, *14*, 461.
8. Baldrick, P.; Richardson, D.; Elliott, G.; Wheeler, A. W. Safety evaluation of monophosphoryl lipid A (MPL): an immunostimulatory adjuvant. *Regul. Toxicol. Pharmacol* **2002**, *35*, 398.
9. Hopp, T. P. *Mol. Immunol.* **1984**, *21*, 13.
10. Deres, K.; Schild, H.; Wiesmüller, K. H.; Jung, G.; Rammensee, H. G. *Nature* **1989**, *342*, 561.
11. Zeng, W.; Ghosh, S.; Lau, Y. F.; Brown, L. E.; Jackson, D. C. *J. Immunol.* **2002**, *169*, 4905.
12. Toth, I.; Flinn, N.; Gibbons, W. A.; Good, M.; Hayman W, Brown, F. In *Peptides: Chemistry, Structure and Biology*; Kaumaya, P. T. P., Hodges R. S., Eds.; Mayflower Scientific Ltd, 1995; pp 810.
13. (a) Brown, L. E.; Jackson, D. C. *Curr. Drug Deliv.* **2005**, *2*, 383; (b) Olive, C.; Toth, I.; Jackson, D. *Mini Rev. Med. Chem.* **2001**, *1*, 429; (c) Moyle, P. M.; Toth, I. *Curr. Med. Chem.* **2008**, *15*, 506.
14. (a) Vitiello, A.; Ishioka, G.; Grey, H. M.; Rose, R.; Famess, P.; LaFound, R.; Yuan, L.; Chisari, F. V.; Fueze, J.; Bartholomeuz, R.; Chesnut, R. W. *J. Clin. Invest.* **1995**, *95*, 341; (b) Durier, C.; Launay, O.; Meiffrédy, V.; Sa, Y.; Salmon, D.; Lévy, Y.; Guillet, J.-G.; Pialoux, G.; Aboulker, J.-P. *Aids* **2006**, *20*, 1039.
15. (a) Johnson, D. A.; Keegan, D. S.; Sowell, C. G.; Livesay, M. T.; Johnson, C. L.; Taubner, L. M.; Harris, A.; Myers, K. R.; Thompson, J. D.; Gustafson, G. L.; Rhodes, M. J.; Ulrich, J. T.; Ward, J. R.; Yorgensen, Y. M.; Cantrell, J. L.; Brookshire, V. G. *J. Med. Chem.* **1999**, *42*, 4640; (b) Stöver, A. G.; Da Silva Correia, J.; Evans, J. T.; Cluff, C. W.; Elliott, M. W.; Jeffery, E. W.; Johnson, D. A.; Lacy, M. J.; Baldridge, J. R.; Probst, P.; Ulevitch, R. J.; Persing, D. H.; Hershberg, R. M. *J. Biol. Chem.* **2004**, *27*, 4440.
16. Adbel-Aal, A.-B. M.; Batzloff, M. R.; Fujita, Y.; Barozzi, N.; Faria, A.; Simerska, P.; Moyle, P. M.; Good, M. F.; Toth, I. *J. Med. Chem.* **2008**, *51*, 167.
17. (a) Ghielmetti, M.; Reshner, A.; Zwicker, M.; Padovan, E. *Immunobiology* **2005**, *210*, 211; (b) Chua, B. Y.; Zeng, W.; Lau, Y. F.; Jackson, D. C. *Vaccine* **2007**, *25*, 92.
18. (a) Olive, C.; Batzloff, M. R.; Horváth, A.; Wong, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. *Infect. Immun.* **2002**, *70*, 2734; (b) Olive, C.; Batzloff, M.; Horváth, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. *Infect. Immun.* **2003**, *71*, 2373; (c) Olive, C.; Hsien, K.; Horváth, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. *Vaccine* **2005**, *23*, 2298.
19. Simerska, P.; Abdel-Aal, A.-B. M.; Fujita, Y.; Moyle, P. M.; McGeary, R. P.; Batzloff, M. R.; Olive, C.; Good, M. F.; Toth, I. *J. Med. Chem.* **2008**, *51*, 1447.
20. (a) Hayman, W. A.; Brandt, E. R.; Relf, W. A.; Cooper, J.; Saul, A.; Good, M. F. *Int. Immunol.* **1997**, *9*, 1723; (b) Relf, W. A.; Cooper, J.; Brandt, E. R.; Hayman, W. A.; Anders, R. F.; Pruksakorn, S.; Currie, B.; Saul, A.; Good, M. F. *Pept. Res.* **1996**, *9*, 12.
21. Esteves, A. P.; Rodrigues, L. M.; Silva, M. E.; Gupta, S.; Oliveira-Campos, A. M. F.; Machalicky, O.; Mendonça, A. J. *Tetrahedron* **2005**, *61*, 8625.
22. Choudhury, A. K.; Kitaoka, M.; Hayashi, K. *Eur. J. Org. Chem.* **2003**, *2003*, 2462.
23. Lui, F.; Zha, H. Y.; Yao, Z. J. *J. Org. Chem.* **2003**, *68*, 6679.
24. Naruto, M.; Ohno, K.; Naruse, N.; Takeuchi, H. *Tetrahedron Lett.* **1979**, *20*, 251.
25. (a) Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5409; (b) Tam, J. P.; Lu, V. A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9084.
26. (a) Rietschel, E. T.; Kirikae, T.; Schade, F. U.; Uimer, A. J.; Holst, O.; Brade, H.; Schmidt, G.; Mamat, U.; Grimmeche, H.-D.; Kusumoto, S.; Zähringer, U. *Immunobiology* **1993**, *187*, 169; (b) Jiang, Z.-H.; Budzynski, W. A.; Qui, D.; Yalamati, D.; Koganty, R. R. *Carbohydr. Res.* **2007**, *342*, 784.
27. (a) Holst, O.; Borowiak, D.; Weckesser, J.; Mayer, H. *Eur. J. Biochem.* **1983**, *137*, 325; (b) Takada, H.; Kotani, S.; Tsujimoto, M.; Ogawa, T.; Takahashi, I.; Harada, K.; Katsukawa, C.; Tanaka, S.; Shiba, T.; Kusumoto, S.; Imoto, M.; Yoshimura, H.; Yamamoto, M.; Shimamoto, T. *Infect. Immun.* **1985**, *48*, 219.
28. Glenny, A. T.; Pope, C. G.; Waddington, H.; Wallace, U. *J. Pathol. Bacteriol.* **1926**, *29*, 38.
29. Pruksakorn, S.; Galbraith, A.; Houghten, R. A.; Good, M. F. *J. Immunol.* **1992**, *149*, 2729.