Supplementary Material

MUC1 Specific Immune Responses Enhanced by Coadministration of Liposomal DDA/MPLA and Lipoglycopeptide

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1. General information and synthetic methods

1.1 General information

All L-amino acids and pre-loaded resins were obtained from GL Biochem (Shanghai, China). Anhydrous dichloromethane (DCM) was obtained from the drying solvent system (passed through CaH$_2$) and can be used without further drying. The purchased anhydrous dimethylformamide (DMF) was stored on the active molecular sieves (4Å). Distearoylglycerophosphocholine (DSPC) was purchased from TCI (Japan) and Energy Chemical (Shanghai, China). Dimethylidioctadecylammonium (DDA) and dioleoylphosphatidylglycerol (DOPG) were purchased from Energy Chemical (Shanghai, China) and Innochem (Beijing, China). Monophosphoryl Lipid A (MPLA) was purchased from Avanti Polar Lipids, Inc. Acetonitrile for HPLC was purchased from Sigma Aldrich (USA). N,N-Diisopropylethylamine (DIPEA) and trisopropylsilane (TIPS) were purchased from Energy Chemical (Shanghai, China). Dimethylformamide (DMF), methanol (MeOH), hydrazine hydrate and piperidine were purchased from Sinopharm Chemical Reagent Co (Shanghai, China). Trifluoroacetic acid (TFA) was purchased from Aladdin (Shanghai, China). Palmitic acid (Pam-OH) was purchased from Macklin Biochemical Co (Shanghai, China). Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 2-(7-azabenzotriazol-1-yl)-N,N',N'-tetramethyluronium hexafluorophosphosphate (HATU) and 3H-[1,2,3]-triazolo[4,5-b]pyridin-3-ol (HOAt) were purchased from Bidepharm (Shanghai, China). Semi-preparative HPLC was performed on an Agilent 1260 Series system equipped with a C18 column (Agilent, 250 × 9.2 mm, 5 μm) at a flow rate of 4.0 mL/min using a mobile phase of solvent A (water with 0.1% trifluoroacetic acid) and solvent B (acetonitrile HPLC-grade with 0.1% trifluoroacetic acid). UV absorption signals were detected with an UV detector at wavelength of 200-280 nm. Lipopeptides were separated on a C8 column (Agilent, 250 × 9.2 mm, 5 μm) also at a flow rate of 4 mL/min. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS was performed on an AB SCIEX 5800 spectrometer. Horseradish peroxidase-conjugated goat anti-mouse IgG, IgM and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) were purchased from Jackson ImmunoResearch (USA). Horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 were purchased from Southern Biotechnology (USA & Canada). IL-6 mouse ELISA kit was purchased from Biolegend (USA & Canada). Standard rabbit complement was purchased from Cedarlane (Canada). Fluorescence microscopy images were captured on an OLYMPUS IX71. Confocal microscopy images were captured on a Leica TCS SP8. The optical density (OD) of enzyme-linked immunosorbert assay (ELISA) was measured on a BioTek SYNERGY H1. Fluorescence activated cell sorting (FACS) was analyzed on a BD Accuri flow cytometer.
1.2 General synthetic scheme for MUC1 (1)

Scheme S1. Preparation of MUC1 (1).

MUC1 (1) was synthesized using the Fmoc-SPPS strategy, starting from Rink amide AM resin. The crude product was dissolved in 30% acetonitrile/water and further purified by semi-preparative HPLC. The compound 1 was determined by analytical HPLC (see Figure S6) (column: Agilent C18, gradient: water/acetonitrile + 0.1% TFA, 0 min (95:5) → 10 min (15:85) → 20 min (95:5), 20 min. Rt = 6.631 min; yield 20 mg (35%). ESI-MS: (see Figure S7): Calcd for C91H146N28O33 [M+2H]^{2+} m/z = 1080.03, [M+2K]^{2+} m/z = 1118.03; observed: 1080.81 [M+2H]^{2+}; 1118.88 [M+2K]^{2+}. 
1.3 General synthetic scheme for P1-MUC1 (2)

Scheme S2. Preparation of P1-MUC1 (2).

P1-MUC1 (2) was synthesized using the Fmoc-SPPS strategy, starting from the resin-bound glycopeptide S2. The crude product was dissolved in 50% acetonitrile/water and purified by semi-preparative HPLC. The compound 2 was determined by analytical HPLC (see Figure S8) (column: Agilent C8, gradient: water/acetonitrile + 0.1% TFA, 0 min (65:35) → 15 min (0:100) → 20 min (65:35), 20 min. Rt = 9.749 min; yield 32 mg (26%); ESI-MS: (see Figure S9): Calcd for C109H179N29O35 [M+2H]^{2+} m/z = 1228.66, observed: 1228.64 [M+2H]^{2+}. 
1.4 General synthetic scheme for P2-MUC1 (3)

Scheme S3. Preparation of P2-MUC1 (3).

P2-MUC1 (3) was synthesized using the Fmoc-SPPS strategy, starting from the resin-bound glycopeptide S3. The crude product was dissolved in 50% acetonitrile/water and purified by semi-preparative HPLC. The compound 3 was determined by analytical HPLC (see Figure S10) (column: Agilent C8, gradient: water/acetonitrile + 0.1% TFA, 0 min (65:35) → 15 min (0:100) → 20 min (65:35), 20 min. Rₜ = 10.677 min; yield 19 mg (15%); ESI-MS: (see Figure S11): Calcd for C₁₃₁H₂₂₁N₃₁O₃₇ [M+2H]²⁺ m/z = 1411.82; observed: 1412.92 [M+2H]²⁺.
1.5 General synthetic scheme for P3-MUC1 (4)

**Scheme S4.** Preparation of P3-MUC1 (4).

P3-MUC1 (4) was synthesized using the Fmoc-SPPS strategy, starting from Rink amide AM resin. The crude product was dissolved in 50% acetonitrile/water and further purified by semi-preparative HPLC. The compound 4 was determined by analytical HPLC (see Figure S12) (column: Agilent C8, gradient: water/acetonitrile + 0.1% TFA, 0 min (20:80) → 15 min (0:100) → 20 min (0:100), 15 min. Rt = 13.531 min; yield 16 mg (10%); ESI-MS: (see Figure S13): Calcd for C_{153}H_{263}N_{33}O_{39} [M+2H]^2^+ \ m/z = 1594.98, observed: 1595.57 [M+2H]^2^+. 
1.6 General synthetic schemes for MUC1 glycopeptide squaric acid monoamide (5) and BSA-MUC1 (6)

**Glycopeptide squaric acid monoamide (5)** was synthesized using the Fmoc-SPPS strategy, starting from the resin-bound glycopeptide S5. The compound 5 was dissolved in 30% acetonitrile/water and purified by semi-preparative HPLC. The compound 5 was determined by analytical HPLC (see Figure S14) (column: Agilent C18, gradient: water/acetonitrile + 0.1% TFA, 0 min (95:5) – 12 min (30:70) – 15 min (0:100), 15 min. Rf = 7.782 min; yield 27 mg (40%); ESI-MS: (see Figure S15): Calcd for C101H158N30O38 [M+2H]²⁺ m/z = 1200.06; observed: 1199.07 [M+2H]²⁺.

**BSA-MUC1 (6).** Linking the resulting squarate monoamide (5) (3.0 μmol, 50 Eq.) to bovine serum albumin (BSA) (4.0 mg, 0.06 μmol) were then achieved in 0.07 M Na₂B₄O₇/0.035 M KHCO₃ buffer solution at pH 9.5,³ providing the desired conjugate 6 after dialysis. The antigen loading level of 6 was estimated by MALDI-TOF mass spectrometry and indicated on average fifteen molecules of glycopeptide per molecule of BSA (see Figure S16). The BSA-MUC1 (6) was used as coating reagent for ELISA.

Scheme S5. Preparation of BSA-MUC1 (6).
2. Vaccine formulation and physical characteristics of liposomes

2.1 Vaccine formulation and immunization

The composition of each vaccine candidate is shown in Table S1. Vaccines A-D: the liposomes of mixture of adjuvant (each mouse MPLA 17 µg), antigen (MUC1 for A, P1-MUC1 for B, P2-MUC1 for C, and P3-MUC1 for D), and auxiliary lipid DDA in a molar ratio of 10:10:80 were dissolved in a mixture of DCM/MeOH (1:1, v/v, 2 mL); Vaccines E-G: the liposomes of mixture of adjuvant (MPLA 17 µg), antigen (P2-MUC1 28 µg), and auxiliary lipid (DSPC for E, DOPG for F, no auxiliary lipid for G) in a molar ratio of 10:10:80 were dissolved in a mixture of DCM/MeOH (1:1, v/v, 2 mL); Vaccines H-K: the liposomes of mixture of antigen (P2-MUC1 28 µg) and auxiliary lipid (DDA for H, DSPC for I, DOPG for J, no auxiliary lipid for K) in a molar ratio of 10:80 were dissolved in a mixture of DCM/MeOH (1:1, v/v, 2 mL), and the solvents were removed under reduced pressure through rotary evaporation, which generated a thin lipid film on the flask wall. This film was hydrated and subjected to freeze/thaw cycles to produce multilamellar vesicles, then the Tris buffer (20 mM, pH 7.4) was added and shaking the mixture under an argon atmosphere at 25 °C for 1 h. The milky suspension was finally sonicated for 20 min to obtain the optimal liposomes.

| Vaccines | Auxiliary lipid (80 nmol) | Adjuvant (10 nmol) | Antigen (10 nmol) |
|----------|---------------------------|--------------------|------------------|
| A        | DDA (50 µg)               | MPLA (17 µg)       | MUC1 (22 µg)     |
| B        | DDA (50 µg)               | MPLA (17 µg)       | P1-MUC1 (25 µg)  |
| C        | DDA (50 µg)               | MPLA (17 µg)       | P2-MUC1 (28 µg)  |
| D        | DDA (50 µg)               | MPLA (17 µg)       | P3-MUC1 (32 µg)  |
| E        | DSPC (63 µg)              | MPLA (17 µg)       | P2-MUC1 (28 µg)  |
| F        | DOPG (62 µg)              | MPLA (17 µg)       | P2-MUC1 (28 µg)  |
| G        | \                         | MPLA (17 µg)       | P2-MUC1 (28 µg)  |
| H        | DDA (50 µg)               | \                  | P2-MUC1 (28 µg)  |
| I        | DSPC (63 µg)              | \                  | P2-MUC1 (28 µg)  |
| J        | DOPG (62 µg)              | \                  | P2-MUC1 (28 µg)  |
| K        | \                         | \                  | P2-MUC1 (28 µg)  |

Table S1. The composition of each vaccine candidate. The amounts of each component in the table are used for one injection per mouse.
Scheme S6. Study design for MUC1 vaccine candidates in mice. Mice were immunized on the 1st, 15th, and 29th days, and the blood was taken on day 0 before initial immunization, and 14th, 28th, and 42nd days before the boost immunizations.

2.2 Physical characteristics of the liposome formulations

Figure S1. (A) DLS results of vaccine candidates. (B) Zeta potential results of vaccine candidates, values are presented as the mean ± SD (n=3).
3. Immunological evaluation

3.1 Analysis of the binding of antisera to MCF-7 cells

Figure S2. Flow cytometry was used to analyze the binding of antisera induced by all vaccines to MCF-7 tumor cells. Cells incubated with pre-immune sera (black) served as control.
3.2 Fluorescent microscopy

Figure S3. Fluorescence-microscopy staining of breast cancer cells (MCF-7). Top row A-L: bright field images. Bottom row A1-L1: fluorescent images. A-L and A1-L1: the cells were incubated with sera of A-F for 1 h at 4 °C.
3.3 The titers of anti-MUC1 antibodies IgG (14, 28, 42 day)

![Graph showing IgG Antibody Titer over time for different treatments.]

**Figure S4.** The titers of anti-MUC1 antibodies IgG (14, 28, 42 day) elicited by the vaccine candidates. The data are indicated as the average of two independent experiments ± SEM. *, *P* < 0.05; ***, *P* < 0.001; ****, *P* < 0.0001. ns, no significant difference.

3.4 Preliminary evaluation of the safety of weight change

![Graph showing increased body weight over time for different treatments.]

**Figure S5.** Assessment of the safety of MUC1 vaccines by monitoring the weight change.
4. Biological testing methods

Mouse immunization schedule
BALB/c mice (five per group) were vaccinated via intraperitoneal injection of vaccines (A-I) on days 1, 15 and 29 of a 2-week cycle by intraperitoneal injection, sera were collected on 2 h, 24 h and days 14, 28, 42 after the first immunization. All the animal experiments were conducted strictly in accordance with the principles of welfare and ethics of laboratory animals.

Protocol for ELISA
The antibody titers and antibody isotypes generated by the vaccine candidates were measured by ELISA. The biotinylated MUC1 (0.125 μg/mL) and avidin (1.16 μg/mL) were directly dissolved in the prepared NaHCO₃/Na₂CO₃ buffer (50 mM, pH 9.5) with a final concentration of 0.125 μg/mL and 1.16 μg/mL, then 96-well ELISA plates (costar 3590) were coated with avidin-biotin-MUC1 complex solution and incubated at 4°C overnight. Then the plates were washed three times with 0.05% Tween PBS and blocked with PBS containing 3% BSA for 1 hour at 37 °C. After the plates were washed three times with 0.05% PBST, the diluted antisera were added to each well (100 μL/well) and incubated for 1 h at 37 °C. After washing, the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ-chain specific), IgM (μ-chain specific), IgG1, IgG2a, IgG2b and IgG3 (1:2000 dilution) in 0.1% BSA/PBS were added to each well (100 μL/well) respectively, and incubated for 1 h at 37 °C. After washing, the freshly prepared substrate solutions [9.5 mL critic buffer at pH 5.0, 0.5 mL 1.6 mg/mL tetramethyl benzidine (TMB), and 32 μL 3% (w/v) urea hydrogen peroxide] were added to each well (100 μL/well) in the dark for 5 minutes, then 2 M H₂SO₄ (50 μL/well) was added to quench the reaction. Optical density was then measured at a wavelength of 450 nm with a microplate reader (Synergy H1). The titer of antibody was defined as the highest dilution, indicating that the absorbance was 0.1, minus the background. Data were analyzed with the software GraphPad (San Diego, CA).

Cell culture
Human breast tumor cells (MCF-7) were obtained from ATCC (HTB 22) (Rockville, Maryland, USA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) + 1% penicillin/streptomycin + 10% fetal bovine serum (FBS, Invitrogen). Cell dissociation during passaging was carried out in 0.05% trypsin/EDTA solution (Gibco, Life Technologies, Carlsbad, USA). All cells were maintained at 37°C in humidified atmosphere with 5% CO₂.

Analysis of the binding of antisera to human breast cancer cells
MCF-7 cells were incubated with sera of vaccines A-I (diluted 1:50) in fluorescence-activated cell sorting (FACS) buffer (contained PBS (1x), 10% FBS and 0.1% sodium azide) for 1 h at 4 °C. Then cells were washed three times with 1% BSA/PBS and incubated with secondary antibodies (Alexa Fluor 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L), Jackson ImmunoResearch, diluted 1:50) for 30 min at 4°C. After washing three times with 1% BSA/PBS, the cells were added the FACS buffer and analyzed by flow cytometry

Fluorescent microscopy
MCF-7 cells were grown to 80% confluence and seeded overnight with Dulbecco’s modified Eagle’s medium (DMEM). After washing with 1% BSA/PBS buffer, the cells were incubated with sera (diluted 1:50) of all vaccines for 1 h at 4 °C. Then culture medium was removed and cells were incubated with secondary antibodies (Alexa Fluor 488-conjugated AffiniPure Goat Anti-Mouse IgG
(H+L), Jackson ImmunoResearch, diluted 1:50) for 30 min at 4 °C. The cell images were obtained with laser scanning by confocal microscope (Leica TCS SP8) and fluorescence microscope (OLYMPUS IX71), respectively.

**Cell cytotoxicity**

To assess whether the induced antibodies of the antisera from vaccinated mice can mediate complement lysis via activation of the complement dependent cytotoxicity (CDC). For this purpose, MCF-7 cells were incubated with these antisera (at a antisera dilution of 1:50) (50 μL per well) from vaccinated mice, after washing with PBS solution, the prepared rabbit sera (at a rabbit sera dilution of 1:50) in 1% BSA/PBS were added as complement supplier (RC: rabbit complement; RC-inactive: inactivated rabbit complement). MCF-7 cells were incubated with antisera diluted to 1:50 in 1% BSA/PBS (50 μL per well). After washing, the rabbit sera diluted to 1:5 in 1% BSA/PBS, and 50 μL/well of the solution were added. After incubation for 4 hours, 0.5% MTT solution in PBS (MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was added (20 μL/well) and incubated for 2 h. After removing the medium, DMSO was added (150 μL/well) and the absorption was analyzed at the wavelength of 490 nm. The survival rate of cells was measured with the following formula. All assays were conducted in five independent experiments.

5. **Supplementary references**

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6. HPLC, ESI-MS and MALDI-TOF-MS spectrometry

Figure S6. HPLC chromatogram of compound 1.

Figure S7. ESI-MS data of compound 1.
Figure S8. HPLC chromatogram of compound 2.

RT = 9.749 min

Figure S9. ESI-MS data of compound 2.

$[M+2H]^{2+} \ m/z = 1228.64$
Figure S10. HPLC chromatogram of compound 3.

Figure S11. ESI-MS spectra of compound 3.
Figure S12. HPLC chromatogram of compound 4.

Figure S13. ESI-MS data of compound 4.
Figure S14. HPLC chromatogram of compound 5.

Figure S15. ESI-MS data of compound 5.
Figure S16. MALDI-TOF-MS data of BSA and BSA-MUC1.