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Supplemental Information

Multifunctional Protein Conjugates with Built-in Adjuvant (Adjuvant-Protein-Antigen) as Cancer Vaccines Boost Potent Immune Responses

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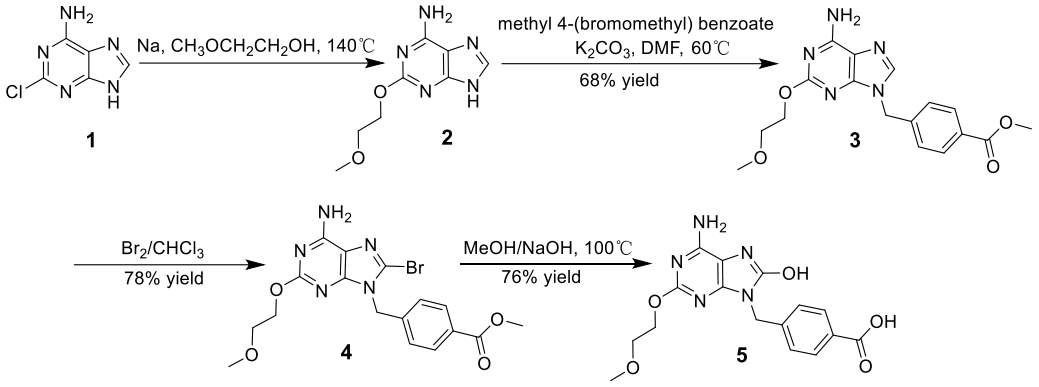
Transparent Methods

1. General information

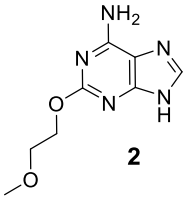
Fmoc L-amino acids and pre-loaded resins were purchased from Nova Biochem. Anhydrous dichloromethane (DCM) was obtained from the drying solvent system (passed through CaH_2) and can be used without further drying. The purchased anhydrous dimethylformamide (DMF) was stored over 4Å molecular sieves. Ethylene glycol monomethyl ether, potassium carbonate, trichloromethane, methanol, sodium hydroxide, piperidine, hydrazine hydrate, potassium bicarbonate, sodium borate, sodium methyl 4-(bromomethyl) benzoate and bromine were purchased from Sinopharm Chemical Reagent (Shanghai, China). Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), trifluoroacetic acid (TFA), *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), 1-hydroxybenzotriazole (HOBt), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) were purchased from Bidepharm (Shanghai, China). *N,N*-Diisopropylethylamine (DIPEA), *N*-methylpyrrolidone (NMP), triisopropylsilane (TIPS) and diethyl squarate were purchased from Energy Chemical (Shanghai, China). Bovine serum albumin (BSA) and aluminum hydroxide (alum) were purchased from Thermo Fisher Scientific Inc. Monophosphoryl Lipid A (MPLA) was purchased from Avanti Polar Lipids, Inc. Pam_3CSK_4 was prepared according to the reported procedure (Chen et al., 2019; Du et al., 2019). Peroxidase-conjugated Affinipure goat anti-mouse kappa antibody IgG (RRID: AB_10015289), IgM (RRID: AB_2338502), and Alexa Fluor® 488-conjugated Affinipure goat anti-mouse IgG (H+L) (RRID: AB_2338840) were purchased from Jackson ImmunoResearch Labs. Peroxidase-conjugated Affinipure goat anti-mouse kappa IgG1, IgG2a, IgG2b, IgG3, IgA and IgE antibodies were purchased from SouthernBiotech. All ^{1}H and ^{13}C-NMR spectra were recorded on Bruker 400 MHz or 600 MHz FT-NMR spectrometers, their signals in deuterated solvents are given δ values from tetramethylsilane (TMS) as an internal standard. Semi-preparative HPLC separations were performed on an Agilent 1260 infinity II prime LC system equipped with a C18 column (Agilent, 250 × 9.4 mm, 5 μm) with a binary mixture of solvent A (100% water with 0.1% trifluoroacetic acid) and solvent B (100% acetonitrile HPLC-grade with 0.1% trifluoroacetic acid) as the mobile phase (flow rates of 4.0 mL/min). UV absorption signals were detected with an UV detector at wavelength of 190-400 nm. The high-resolution mass spectrometry (HRMS) were measured on a Shimadzu LCMS-IT-TOF mass spectrometer or DIONEX UltiMate 3000 & Bruker Compact TOF mass spectrometer by ESI. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS was performed on an AB SCIEX 5800 spectrometer (Shimadzu AXIMA Assurance).

2. Experimental procedures

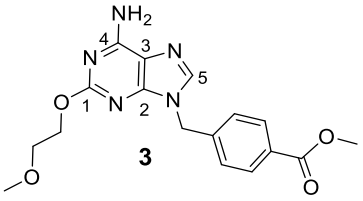
a.Synthesis of TLR7 agonist



Scheme S1. Synthetic route to TLR7 agonist (Gao et al., 2015). Related to Scheme 2.

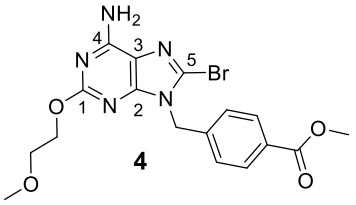


Synthesis of compound 2 (Gao et al., 2015)**.** The sodium salt of ethylene glycol monomethyl ether was prepared by dissolving sodium (4.9 g, 212 mmol) metal in ethylene glycol methyl ether (235 mL, 29.5 mol), and the 2-chloroadenine (**1**) (5.0 g, 29.6 mmol) was added at 140 °C. The reaction mixture was heated for 8 h at this temperature and then cooled to 0 °C, the residue was quenched with 1 M HCl, and concentrated. The crude product was directly applied for next step reaction without purification.



Synthesis of compound 3 (Gao et al., 2015)**.** To a solution of compound **2** (700 mg, 3.34 mmol) in DMF (40 mL) were added methyl 4-(bromomethyl) benzoate (1.5 g, 6.68 mmol) and K_2CO_3 (3.2 g, 23.44 mmol), and stirred for 8 h at 60 °C. The reaction mixture was quenched with 5% citric acid (250 mL), diluted with water (20 mL) and extracted with $CHCl_3$ (3×100 mL), the organic layer was washed with brine, dried ($MgSO_4$), and concentrated. The crude product was purified by silica gel column chromatography (MeOH/DCM = 1:50) to provide compound **3** as a white solid (809 mg, 68%). R_f 0.25 (MeOH/DCM = 1/25). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.10 (s, 1H), 7.95 (d, J =

8.0 Hz, 2H, 2×Ph), 7.42 (d, *J* = 8.1 Hz, 2H, 2×Ph), 7.30 (s, 2H, NH_2), 5.38 (s, 2H, -CH_2-Ph), 4.32 (t, *J* = 4.7 Hz, 2H, CH_2-O), 3.85 (s, 3H, CH_3-O), 3.61 (t, *J* = 4.7 Hz, 2H, CH_2-O), 3.28 (s, 3H, CH_3-O). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ (ppm) 166.4 (C=O), 161.8 (C_1), 157.2 (C_4), 151.6 (C_2), 142.9 (C_5), 140.0, 130.0, 129.3, 128.2 (benzene), 115.5 (C_3), 70.7 (O-$\underline{C}H_2$-), 65.8 (-$\underline{C}H_2$-O), 58.5 (-O$\underline{C}H_3$), 52.6 (-O$\underline{C}H_3$), 46.1 (-$\underline{C}H_2$-Ph). HRMS (ESI) calculated for $C_{17}H_{19}N_5O_4$ $[M+H]^+$: 358.1510, found: 358.1513.



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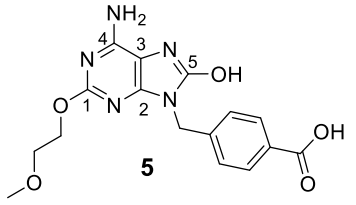
Synthesis of compound 4 (Gao et al., 2015)**.** To a solution of compound **3** (800 mg, 2.2 mmol) in $CHCl_3$ (40 mL) was added bromine (227 μL, 4.4 mmol) at room temperature (rt). The mixture was stirred at rt for 8 h. The residue was quenched with saturated $Na_2S_2O_3$ (25 mL), diluted with water (5 mL) and extracted with $CHCl_3$ (3×25 mL). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The crude product was purified with silica gel column chromatography (MeOH/DCM = 1/60) to give the product as a white solid (747 mg, 78%). R_f 0.6 (MeOH/DCM = 1/25). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ (ppm): 7.96 (d, *J* = 8.1 Hz, 2H, 2×Ph), 7.50 (s, 2H, NH_2), 7.36 (d, *J* = 8.1 Hz, 2H, 2×Ph), 5.36 (s, 2H, -CH_2-Ph), 4.33 (t, *J* = 4.7 Hz, 2H, CH_2-O), 3.85 (s, 3H, CH_3-O), 3.61 (t, *J* = 4.7 Hz, 2H, CH_2-O), 3.29 (s, 3H, CH_3-O). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ (ppm): 166.3 (C=O), 161.8 (C_1), 156.2 (C_4), 152.9 (C_2), 141.7, 130.1, 129.5, 127.8 (Ph), 124.2 (C_5), 115.8 (C_3), 70.6 (O-$\underline{C}H_2$-), 66.0 (-$\underline{C}H_2$-O), 58.5 (-O$\underline{C}H_3$), 52.6 (-O$\underline{C}H_3$), 46.5 ($\underline{C}H_2$-Ph). HRMS (ESI) calculated for $C_{17}H_{18}BrN_5O_4$ $[M+H]^+$: 436.0615, found: 436.0619.



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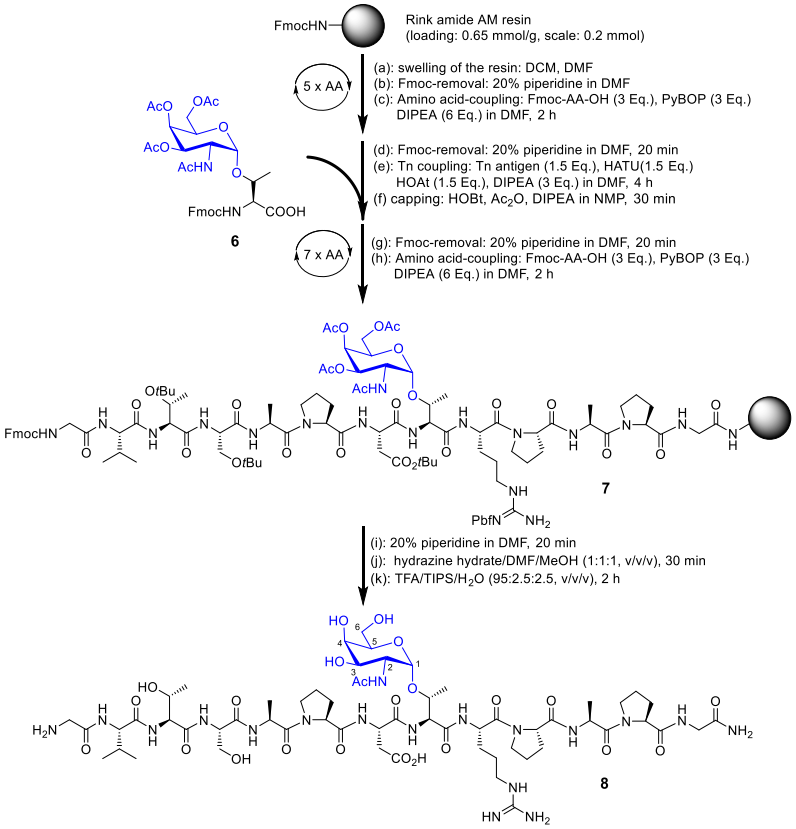
Synthesis of compound 5 (Gao et al., 2015)**.** To a solution of compound **4** (500 mg) was added to MeOH/NaOH (6 M NaOH, v/v = 1/4, 200 mL) at 100 °C. The mixture was stirred at rt for 4 h, neutralized with aqueous 1 N HCl aq., and filtrated. The product was purified with silica gel column chromatography (MeOH/DCM = 1/10 with 0.1% HOAc) to give desired product as a white solid (313 mg, 76%). R_f 0.6 (MeOH/DCM = 1/9 with 0.1% HOAc). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ (ppm): 10.09 (s, 1H, CO_2H), 7.90 (d, *J* = 7.9 Hz, 2H, 2×Ph), 7.37 (d, *J* = 7.9 Hz, 2H, 2×Ph), 6.53 (s, 2H, NH_2), 4.92 (s, 2H, -CH_2-Ph), 4.24 (t, *J* = 4.7 Hz, 2H, CH_2-O), 3.56 (t, *J* = 4.8 Hz, 2H, CH_2-O), 3.25 (s, 3H, CH_3-O). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ (ppm): 167.5 (C=O), 160.3 (C_1), 152.7 (C_4), 149.5 (C_2), 148.2 (C_5), 142.4, 130.4, 130.0, 127.9 (Ph), 98.8 (C_3), 70.6 (O-$\underline{C}H_2$-), 65.7 (-$\underline{C}H_2$-O), 58.5 (-

O<u>C</u>H$_3$), 42.6 (-<u>C</u>H$_2$-Ph). HRMS (ESI) calculated for $C_{16}H_{17}N_5O_5$ $[M+H]^+$: 360.1302, found: 360.1304.

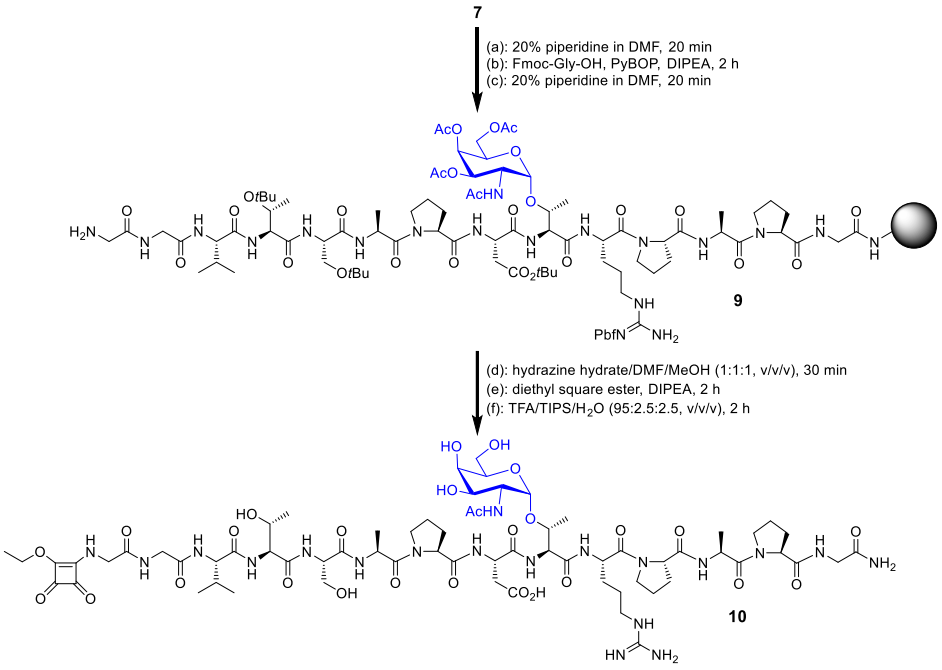
b. General procedure for peptides synthesis

MUC1 glycopeptides were synthesized according to the Fmoc-strategy SPPS starting from Rink amide AM resin (Du et al., 2016; Du et al., 2019). The natural amino acids (3 Eq.) were coupled using PyBOP (78 mg, 0.15 mmol, 3 Eq.) and DIPEA (53 μL, 0.3 mmol, 6 Eq.) in DMF for 1 h. The Fmoc-glycosyl amino acid (Tn antigen) (1.5 Eq.) was coupled using HATU (1.5 Eq.), HOAt (1.5 Eq.) and DIPEA (3 Eq.) in DMF for 4 h, residual free amines were acetylated with capping reagents. The linker of MUC1-glycopeptide with a squaric acid diethyl ester or biotin moiety at the *N*-terminus was achieved by treatment with DIPEA (5 Eq.) or HATU (3 Eq.), HOAt (3 Eq.) and DIPEA (6 Eq.) in DMF for 2 h. After completion of the glycopeptide assembly, the *N*-terminal Fmoc protecting groups or acetyl moieties of glycosyl amino acids were removed using 20% piperidine in DMF or approximately 30% hydrazine in DMF and MeOH. Next, the resin was treated with the cleavage cocktail (trifluoroacetic acid 95%, triisopropylsilane 2.5%, water 2.5%, 10 mL) for 2 h. The resin was filtered, washed three times with trifluoroacetic acid, the residue was concentrated and precipitated with diethyl ether, the crude MUC1 glycopeptides were purified by semi-preparative HPLC and identified with HRMS.



Scheme S2. Synthetic route to MUC1 glycopeptide **8**. Related to Scheme 2.

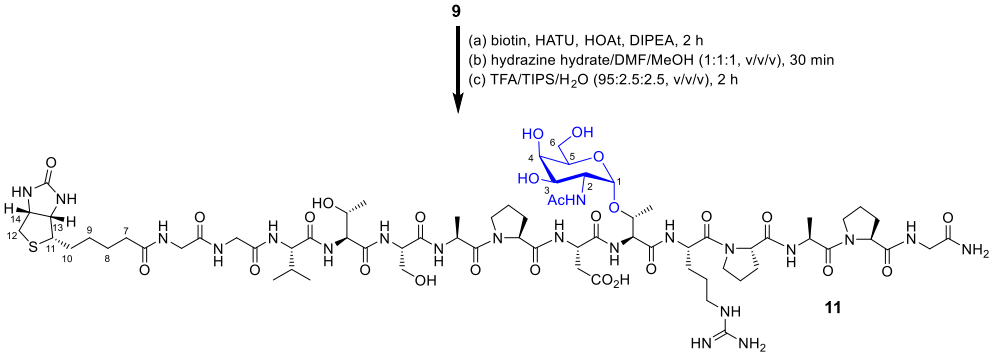
Synthesis of MUC1 glycopeptide 8. MUC1 glycopeptide **8** was synthesized following the Fmoc-SPPS strategy, which was purified by semipreparative HPLC, and the appropriate fractions were lyophilized to afford pure product. Aanalytical HPLC trace of MUC1 glycopeptide **8** (28 mg, 42% yield) (column: Agilent C18), gradient: water/acetonitrile + 0.1% TFA, 0.0 min (95:5) → 15.0 min (10:90) → 20.0 min (0:100) → 20.1 min (95:5) → 30 min (95:5), R_t = 6.699 min. HRMS spectra of MUC1 glycopeptide **8**. HRMS (ESI) calculated for $C_{59}H_{98}N_{18}O_{23}$ $[M+H+Na]^{2+}$: 725.3509, found: 725.3215. ^{1}H NMR (600 MHz, D_2O) δ (ppm): 4.87 (d, 1H, H_1), 4.64–4.63 (m, 2H, D_α, R_α), 4.59–4.57 (m, 2H, 2×A_α), 4.54–4.43 (m, 6H, 3×P_α, S_α, 2×T_α), 4.29–4.27 (m, 1H, V_α), 4.11–4.09 (m, 4H, 2×T_β), 4.05–4.02 (m, 1H, H_3), 3.99–3.88 (m, 5H, 2×G_α, H_5), 3.86–3.63 (m, 12H, S_β, 3×P_δ, H_2, H_4, H_6), 3.50–3.23 (m, 2H, R_δ), 2.96–2.79 (m, 2H, D_β), 2.34–2.29 (m, 3H, 3×$P_{\beta1}$), 2.16–2.08 (m, 1H, V_β), 2.05–2.01 (m, 6H, 3×$P_{\beta2}$, Ac-NH), 2.00–1.87 (m, 6H, 3×P_γ), 1.73–1.71 (m, 4H, R_β, R_γ), 1.42–1.33 (m, 6H, 2×A_β), 1.27–1.23 (m, 6H, 2×T_γ), 0.99–0.97 (m, 6H, V_γ). ^{13}C NMR (150 MHz, D_2O) δ (ppm): 174.7, 174.0, 173.7, 173.7, 173.4, 173.4, 173.1, 173.0, 172.6, 171.4, 171.0, 170.9, 170.7, 167.2, 163.0 (15×C=O), 156.6, 98.5, 75.3, 71.3, 68.4, 67.9, 67.9, 66.9, 61.2, 60.9, 60.6, 60.2, 59.8, 59.5, 58.8, 57.0, 55.1, 51.1, 50.2, 49.5, 47.8, 47.7, 47.6, 47.5, 41.9, 40.4, 40.2, 36.3, 30.0, 29.3, 29.2, 29.1, 27.2, 24.6, 24.5, 24.5, 24.0, 22.2 (Ac-NH), 18.6, 18.3, 17.3, 15.4, 15.0.



Scheme S3. Synthetic route to MUC1 glycopeptide squaric acid monoamide **10**. Related to Scheme 2.

Synthesis of MUC1 glycopeptide squaric acid monoamide 10. MUC1 glycopeptide squaric acid monoamide **10** was synthesized following the Fmoc-SPPS strategy, which was purified by semipreparative HPLC, and the appropriate fractions were lyophilized to

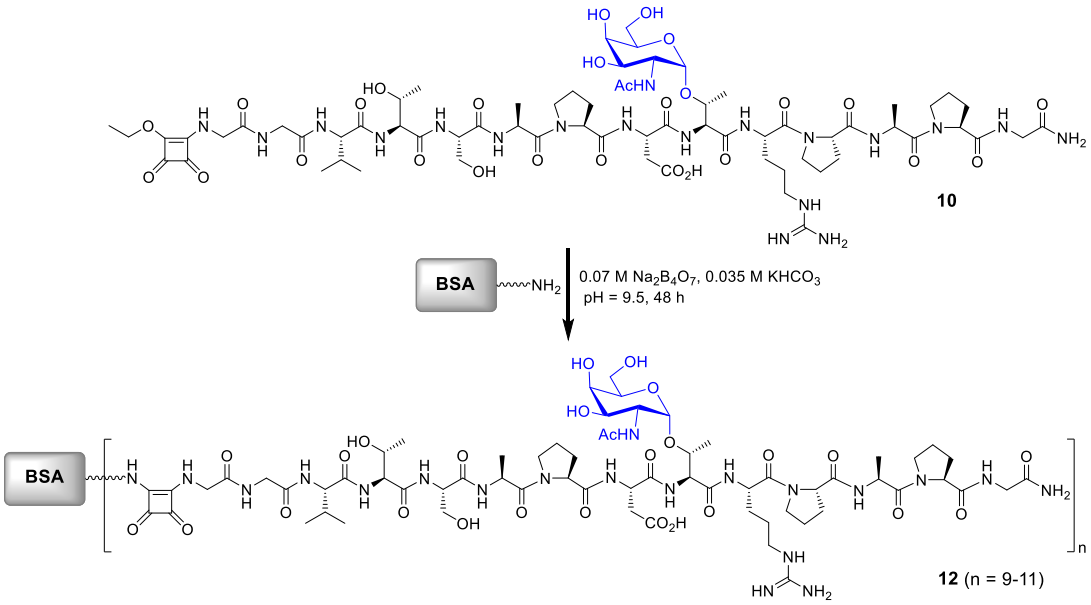
afford pure product. Analytical HPLC trace of MUC1 glycopeptide squaric acid monoamide **10** (26 mg, 51% yield) (column: Agilent C18, gradient: water/acetonitrile + 0.1% TFA, 0.0 min (95:5) → 15.0 min (10:90) → 20.0 min (0:100) → 20.1 min (95:5) → 30 min (95:5), R_t = 7.152 min. HRMS spectra of MUC1 glycopeptide squaric acid monoamide **10** HRMS (ESI) calculated for $C_{67}H_{105}N_{19}O_{27}$ $[M+H+Na]^{2+}$: 816.3713. found: 816.3729. ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 4.81 (d, 1H, H_1), 4.77–4.71 (m, 2H, D_α, R_α), 4.63–4.56 (m, 2H, 2×A_α), 4.48–4.45 (m, 2H, -CH_2-), 4.43–4.34 (m, 6H, P_α, S_α, 2×T_α), 4.23–4.21 (m, 2H, V_α), 4.17 (m, 4H, 2×T_β), 4.12–4.08 (m, 1H, H_3), 3.99–3.83 (m, 7H, 3×G_α, H_5), 3.81–3.60 (m, 12H, S_β, 3×P_δ, H_2, H_4, H_6), 3.26–3.17 (m, 2H, R_δ), 2.95–2.83 (m, 3H, D_β), 2.27–2.20 (m, 3H, 3×$P_{\beta1}$), 2.13–2.11 (m, 1H, V_β), 2.07–1.99 (m, 6H, 3×$P_{\beta2}$, Ac-NH), 1.99–1.86 (m, 6H, 3×P_γ), 1.70–1.67 (m, 4H, R_β, R_γ), 1.47–1.43 (m, 3H, CH_3-), 1.42–1.33 (m, 6H, 2×A_β), 1.26–1.18 (m, 6H, 2×T_γ), 1.00–0.98 (m, 6H, V_γ). ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 188.9, 183.7, 177.4, 174.2, 173.7, 173.5, 173.1, 173.0, 173.0, 172.6, 172.6, 172.2, 172.0, 172.0, 171.9, 170.8, 170.6, 170.6, 170.3, 160.7, 160.4, 157.1, 98.5, 74.2, 71.6, 69.5, 69.5, 68.8, 68.7, 66.6, 61.4, 61.3, 60.7, 59.8, 56.9, 56.3, 56.1, 56.0, 55.9, 55.7, 55.3, 50.5, 50.5, 50.0, 49.8, 48.1, 46.0, 42.0, 41.8, 40.6, 34.7, 30.0, 29.1, 29.0, 28.9, 27.9, 24.7, 24.7, 24.5, 24.4, 22.0 (Ac-NH), 18.3, 18.1, 17.3, 15.7, 15.3.



Scheme S4. Synthetic route to biotin-MUC1 glycopeptide **11**. Related to Figures 3 and 4.

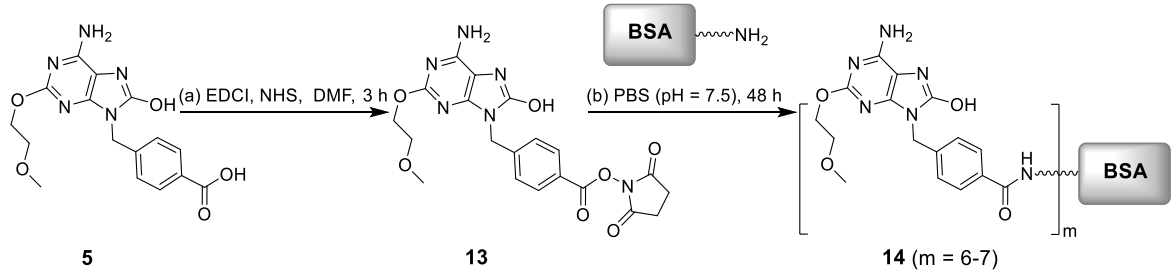
Synthesis of biotin-MUC1 glycopeptide 11. Biotin-MUC1 glycopeptide **11** was synthesized following the Fmoc-SPPS strategy, which was purified by semipreparative HPLC, and the appropriate fractions were lyophilized to afford pure product. Analytical HPLC trace of compound **11** (26 mg, 46% yield) (column: Agilent C18, gradient: water/acetonitrile + 0.1% TFA, 0.0 min (95:5) → 15.0 min (40:60) → 15.1 min (95:5), → 20.0 min (95:5), R_t = 8.498 min. HRMS spectra of biotin-MUC1 glycopeptide **11** HRMS (ESI) calculated for $C_{71}H_{115}N_{21}O_{26}S$ $[M+2Na]^{2+}$: 877.8914, found: 877.8908. ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 4.80 (d, 1H, H_1), 4.77–4.74 (m, 2H, H_{13}, H_{14}), 4.62–4.55 (m, 4H, 2×A_α, D_α, R_α), 4.51–4.41 (m, 6H, 3×P_α, S_α, 2×T_α), 4.37–4.25 (m, 5H, V_α, 2×T_β), 4.21–4.19 (m, 1H, H_3), 4.00–3.87 (m, 7H, 3×G_α, H_5), 3.87–3.65 (m, 12H, S_β, 3×P_δ, H_2, H_4, H_6), 3.65–3.60 (m, 1H, H_{13}), 3.24–3.18 (m, 2H, R_δ), 2.95–2.91 (m, 2H, D_β), 2.87–2.69 (m, 2H, H_{12}), 2.32–2.29 (m, 2H, H_7), 2.26–2.18 (m, 3H, 3×$P_{\beta1}$), 2.12–2.10 (m, V_β), 2.05–1.87 (m, 12H,

3×$P_{\beta 2}$, Ac-NH, 3×P_{γ}), 1.87–1.84 (m, 1H, $H_{10'}$), 1.75–1.73 (m, 2H, H_8), 1.70–1.67 (m, 4H, R_{β}, R_{γ}), 1.48–1.46 (m, 2H, H_9), 1.41–1.34 (m, 6H, 2×A_{β}), 1.33–1.30 (m, 1H, $H_{10'}$), 1.25–1.20 (m, 6H, 3×T_{γ}), 1.01–0.98 (m, 6H, V_{γ}). ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 176.7, 174.7, 174.3, 174.2, 174.2, 173.9, 173.8, 173.8, 173.4, 173.3, 173.1, 172.4, 172.1, 172.1, 171.9, 171.8, 171.5, 165.9, 158.3, 99.8, 75.4, 72.8, 70.0, 69.9, 67.8, 63.0, 62.5, 61.9, 61.4, 61.1, 61.0, 60.2, 58.1, 57.4, 57.2, 57.1, 56.9, 56.7, 56.5, 54.4, 51.8, 51.2, 51.0, 49.7, 49.3, 43.6, 43.4, 43.0, 41.8, 40.8, 36.2, 35.9, 31.1, 30.3, 30.2, 30.1, 29.4, 29.2, 26.3, 25.9, 25.9, 25.7, 25.6, 23.2 (Ac-NH), 19.8, 19.5, 18.7, 16.5, 16.5.



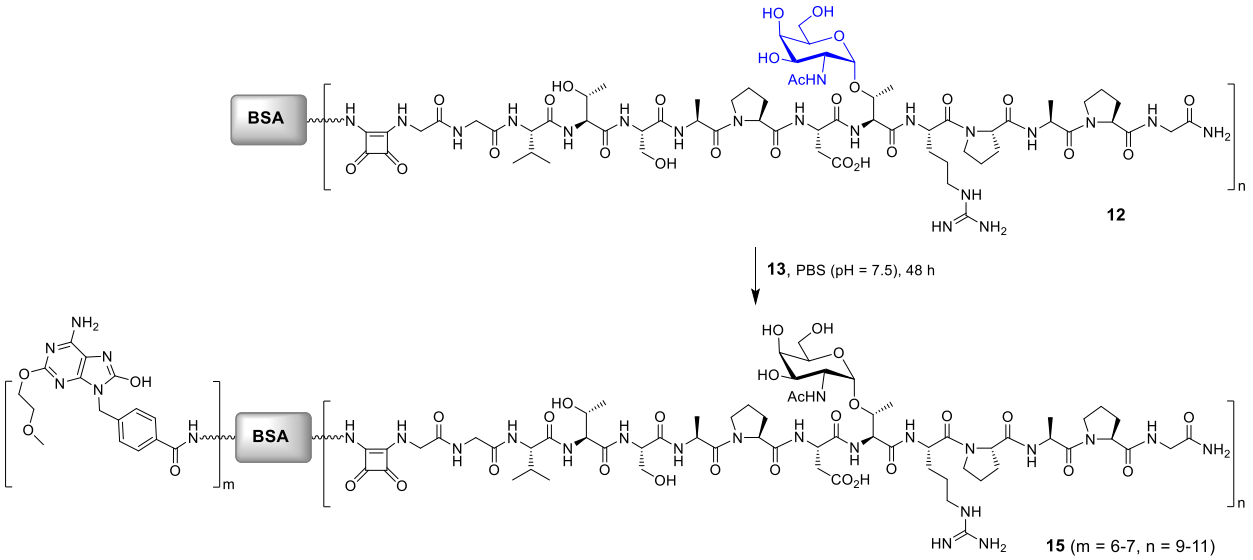
Scheme S5. The synthetic route of BSA-MUC1 conjugate **12**. Related to Scheme 2.

Synthesis of BSA-MUC1 conjugate 12. The glycopeptide squaric acid monoamide **10** (48.4 mg, 0.03 mmol) and BSA (40 mg, 0.6 μmol) were dissolved in 5 mL 0.07 M $Na_2B_4O_7$/0.035 M $KHCO_3$ buffer solution at pH = 9.5. The reaction mixture was stirred at rt for 48 h. The BSA-MUC1 conjugate was purified by size-exclusion gel filtration (Millipore UFC910096 15 M, 10 KD), and lyophilized. The average capacity of MUC1 on BSA was estimated by MALDI-TOF-MS.



Scheme S6. The synthetic route of TLR7a-BSA conjugate **14**. Related to Scheme 2.

Synthesis of TLR7a-BSA conjugate 14. To a solution of compound **5** (11.2 mg, 0.03 mmol) in DMF (1 mL) were added EDCI (17.2 mg, 0.09 mmol) and NHS (10.3 mg, 0.09 mmol), and the reaction mixture was shaken for 3 h. Then a mixture of BSA (20 mg, 0.3 μmol) in PBS (pH = 7.5) was added and shaken for 48 h. The TLR7a-BSA conjugate was purified by size-exclusion gel filtration (10 KD), and lyophilized. The average capacity of TLR7a on BSA was estimated by MALDI-TOF-MS.



Scheme S7. The synthetic route of TLR7a-BSA-MUC1 **15**. Related to Scheme 2.

Synthesis of TLR7a-BSA-MUC1 protein conjugate 15. To a solution of BSA-MUC1 (**12**) (10 mg, 0.12 μmol) in PBS were added compound **13**, and the reaction mixture was shaken for 48 h. The TLR7a-BSA-MUC1 conjugate was purified by size-exclusion gel filtration (10 KD), and lyophilized. The average capacity of TLR7a on BSA-MUC1 (**12**) was estimated by MALDI-TOF-MS.

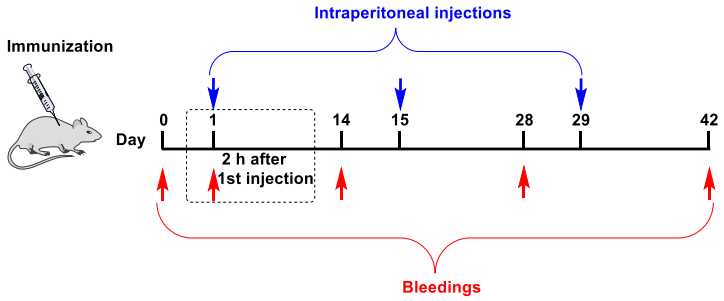
3. Vaccine immunizations

a. Immunization of mice

All animal experiments were performed at Laboratory Animal Centre of Huazhong Agriculture University (Number: SYXK (Wuhan) 2015-0084). Animal experiments were conducted in accordance with the animal ethics guidelines and follow the recommendations concerning laboratory animal welfare. For immunological evaluations, seven groups of female BALB/c mice (age of 6-8 weeks, 5 mice per group, Number: SYXK (Wuhan) 2015-0019) were examined with vaccine candidates, i.e. BSA-MUC1 (Figures S37-S41 and S47) (124 μg), TLR7a-BSA (Figure S48) (119 μg) and MUC1 (Figures S32-

S36) (21 μg), TLR7a (Scheme S1, Figures S30 and S31) (3.6 μg) and BSA-MUC1 (124 μg), TLR7a-BSA-MUC1 (Figure S49) (127 μg), MPLA (17.6 μg) and BSA-MUC1 (124 μg), Pam_3CSK_4 (15 μg) and BSA-MUC1 (124 μg), alum (100 μL) and BSA-MUC1 (124 μg), and PBS as a negative control, respectively.

Mice were immunized by intraperitoneal injection on day 1, day 15 and day 29. The mice were bled on day 0 before initial immunization and on 2 h, day 14, day 28, and day 42 after boost immunizations. Sera collected at 2 h after 1st injection were analysed for the secretion of cytokines IL-6 and IFN-γ. Mouse blood samples were clotted to obtain antisera that were stored at -80 °C before use.



Scheme S8. Immunization strategy. Related to Figures 1-7.

Table S1. The composition of each vaccine candidate, Related to Figures 1-7.[a]

Vaccine candidates	Antigen	Adjuvant
1	BSA-MUC1 (0.0152 μmol, 124 μg)	/
2	MUC1 (0.0152 μmol, 21 μg)	TLR7a-BSA (0.01 μmol, 119 μg)
3	BSA-MUC1 (0.0152 μmol, 124 μg)	TLR7a (0.01 μmol, 3.6μg)
4	TLR7a-BSA-MUC1 (0.0152 μmol, 127 μg)	/
5	BSA-MUC1 (0.0152 μmol, 124 μg)	MPLA (0.01 μmol, 17.6 μg)
6	BSA-MUC1 (0.0152 μmol, 124 μg)	Pam_3CSK_4 (0.01 μmol, 15 μg)
7	BSA-MUC1 (0.0152 μmol, 124 μg)	alum (100 μL)

[a]The amounts of each component in the table are used for one injection per mouse

b. Statistical analyses

Comparison of multiple groups for statistical significance was carried out via one-way ANOVA with Tukey post hoc tests. Statistically significant responses are indicated by asterisks, data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

c. In vivo cytokine assay

Analysis of Cytokine Levels by ELISA. The relative total cytokine levels generated by the vaccine candidates were evaluated using ELISA kits (IFN-γ and IL-6, Biolegend) as per manufacturer's instructions. For this purpose, the high binding 96-well plates (Corning Incorporated, Costar 3590) were coated with capture antibodies (diluted 1:200) dissolved in coating buffer (0.1 M Na_2HCO_3, 0.03 M Na_2CO_3, pH 9.5) at 4 °C overnight. Then, the coated plates were washed four times with PBST (PBS containing 0.05% Tween-20), and blocked by using 1% BSA in PBS (200 μL/well) at 37 ºC for 1 h. After washing four times, the sera samples and standards were diluted with 1% BSA in PBS were added (100 μL/well) and incubated for 2 h at rt. Four additional washing steps were conducted, followed by incubation with the detection antibodies (1:200 dilution) (100 μL/well) for 1 h at rt. After further washing steps, the plates were incubated with Avidin-HRP (1:1000 dilution) (100 μL/well) for 30 min at rt. After final washing steps, the plates were incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (100 μL/well) for 20-30 minutes or until the desired color developed. Then, sulfuric acid was added (100 μL/well). Absorbance was measured at 450 nm with a microplate reader (BioTek, Synergy H1).

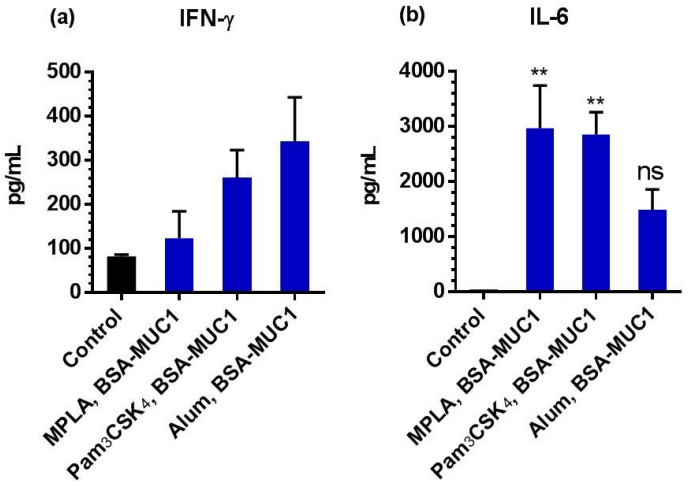


Figure S1. The secretion of cytokines IFN-γ (a) and IL-6 (b) were measured in serum samples from vaccinated mice at 2 h after the first immunization. Data are shown as the mean ± SEM of five mice and are representative of three separate experiments. Related to Figure 1.

d. Analysis of antibody titers and subtypes by ELISA

The antibody titers and antibody isotypes generated by the vaccine candidates were measured by ELISA. The biotinylated MUC1 and avidin (Biosynthesis) were directly dissolved together in the prepared $NaHCO_3/Na_2CO_3$ buffer (50 mM, pH 9.5) with final concentration of 0.125 μg/mL (MUC1) and 1.16 μg/mL (avidin), respectively. Next, 96-well plates were coated with avidin-biotin-MUC1 complex and incubated at 4 °C overnight. Then, the coated plates were washed four times with PBST and blocked with 1% casein in PBS (100 μL/well) at 37 ºC for 1 h. After washing four times, the plates were incubated with the serial diluted sera samples in PBS containing 0.1% casein (100 μL/well) at 37 ºC for 1 h. After another washing steps, the plates were incubated with one of the HRP-linked goat anti-mouse antibody IgG, IgM, IgG1, IgG2a, IgG2b, IgG3, IgA or IgE, 1:5000 dilution in PBST (100 μL/well) at 37 ºC for 1 h. After final washing steps, the plates were washed and TMB (500 μL 0.2 mg/mL) in 9.5 mL 0.05 M phosphate-citrate buffer at pH 5.0 with 32 μL 3% (w/v) urea hydrogen peroxide was added and allowed to react for 5 min. Next, the colorimetric reactions were terminated by 2.0 M sulfuric acid. Absorbance was recorded at 450 nm with a microplate reader. For titer analysis, the OD value was plotted against the sera dilution numbers to obtain a best-fit logarithm line. The dilution number was calculated according to the equation of this line.

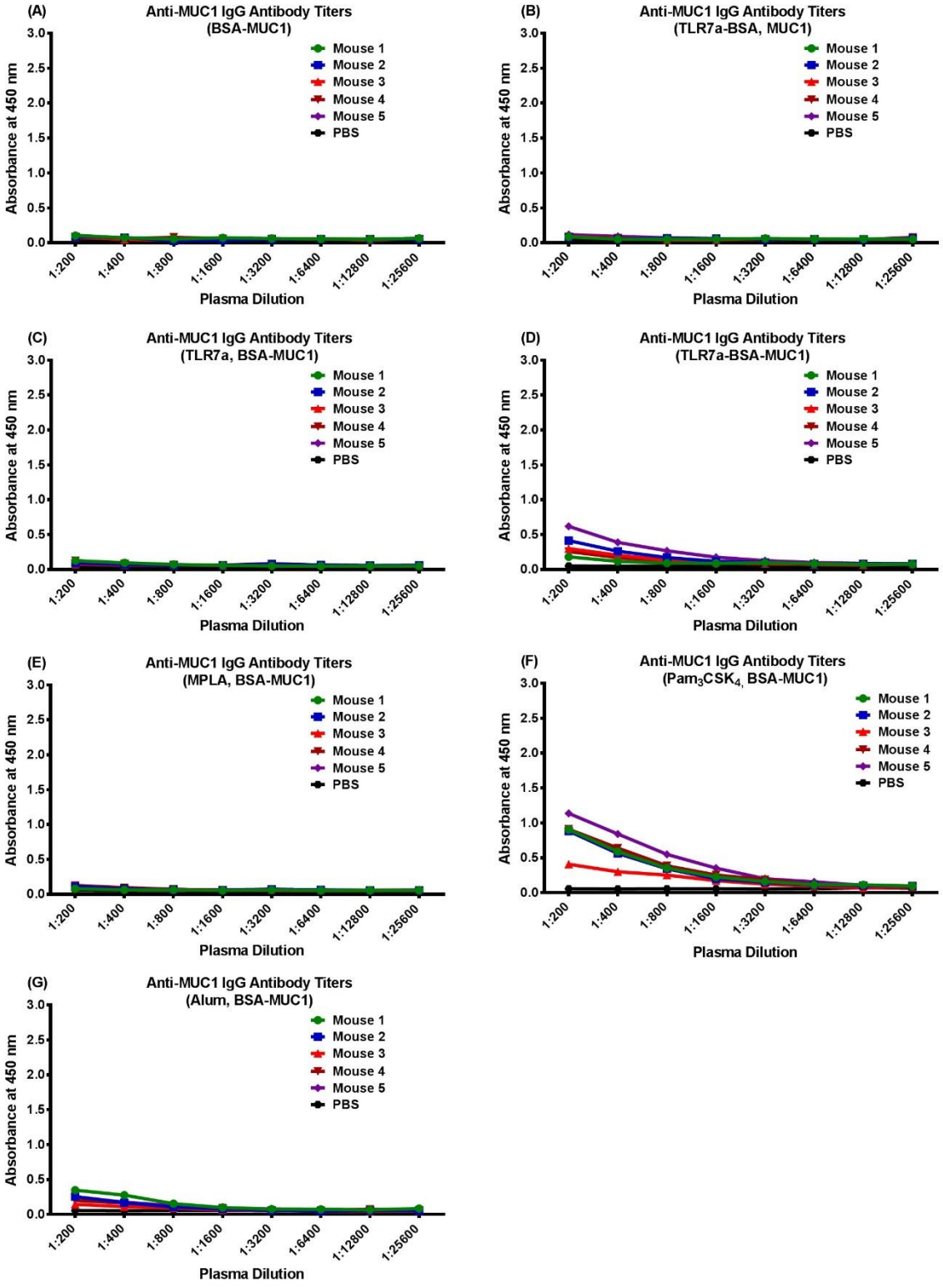


Figure S2. ELISA curves of anti-MUC1 IgG antibody titers in serum samples from vaccinated mice collected on day 14. Related to Figure 3.

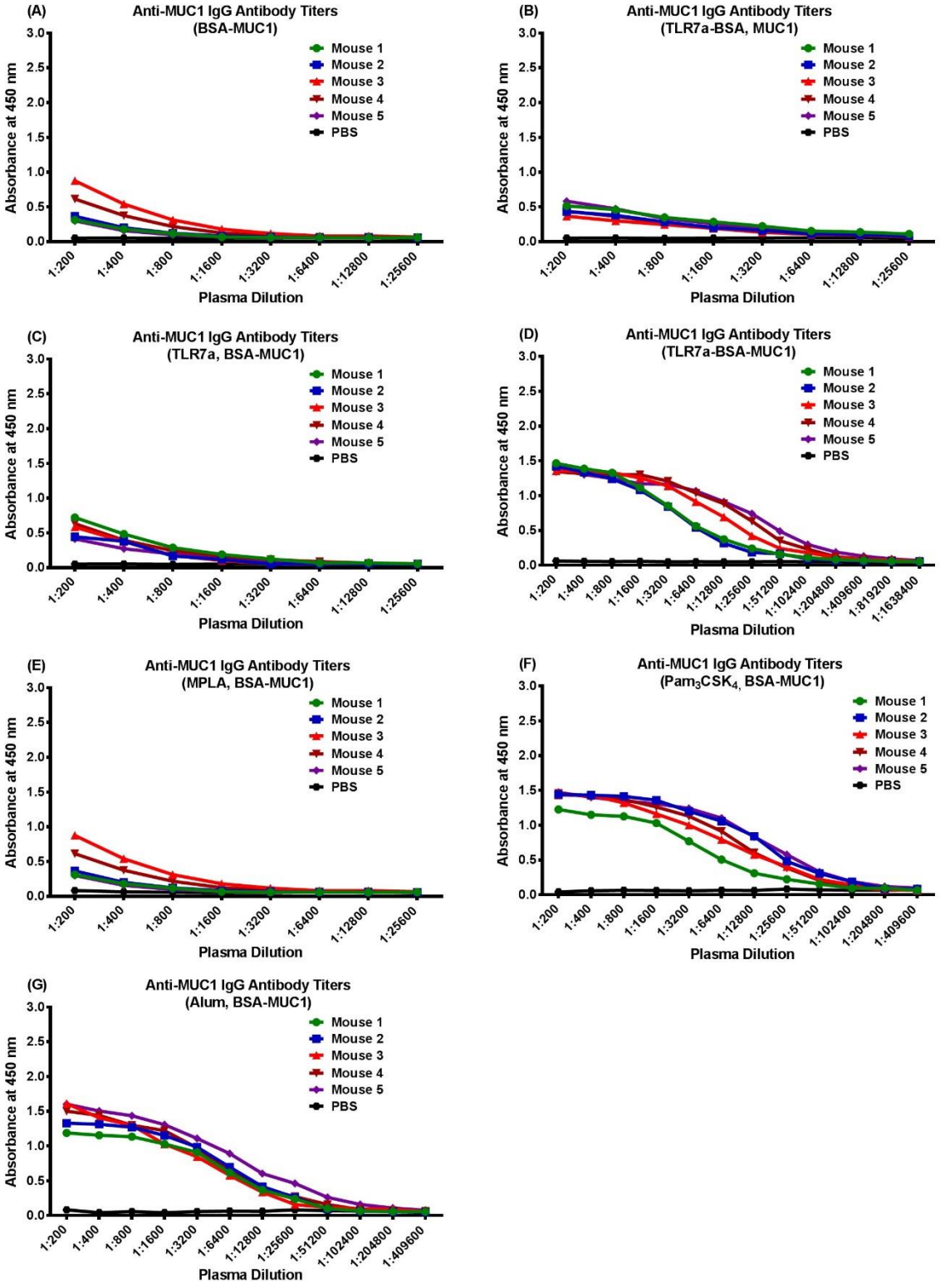


Figure S3. ELISA curves of anti-MUC1 IgG antibody titers in serum samples from vaccinated mice collected on day 28. Related to Figure 3.

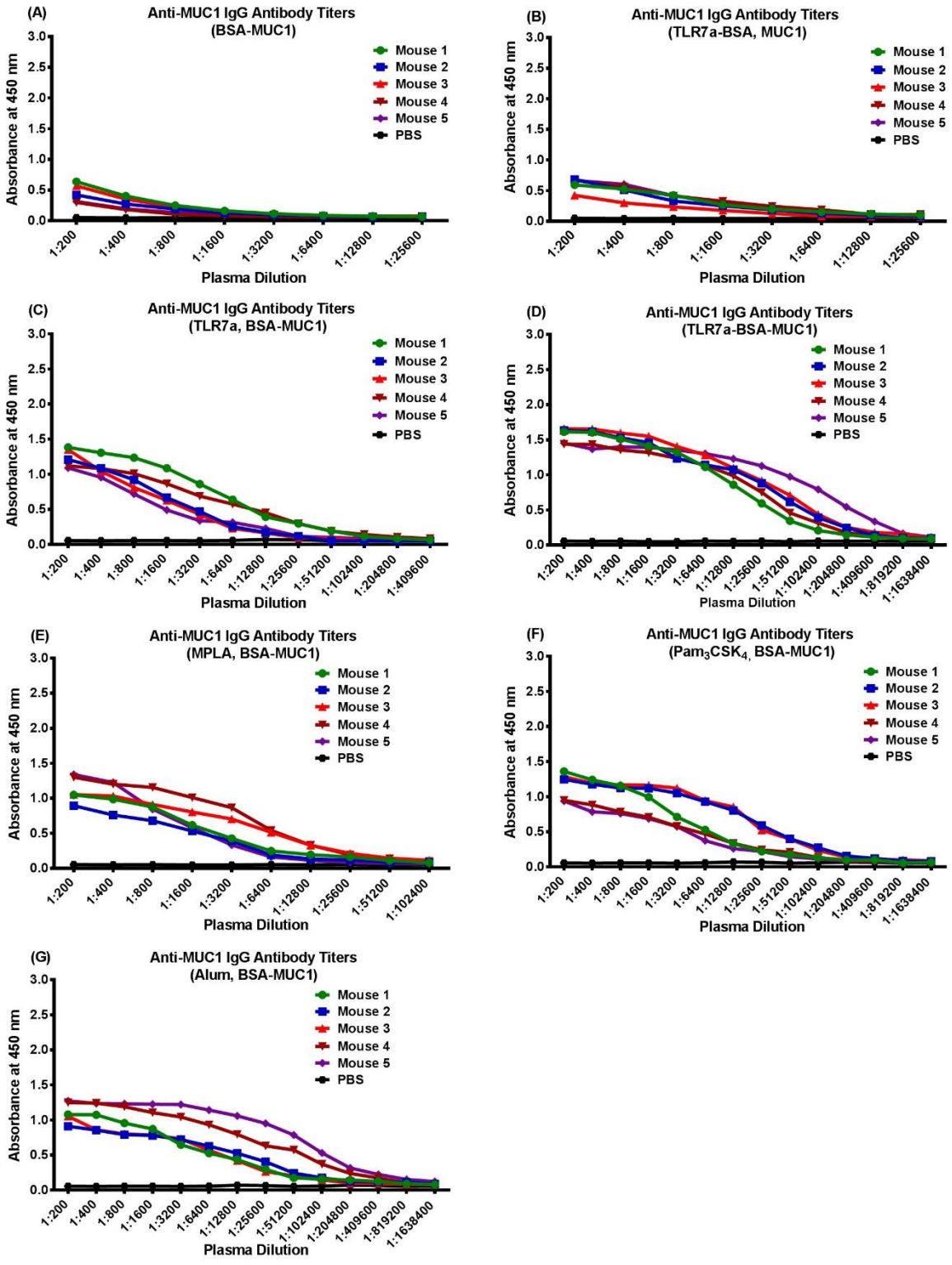


Figure S4. ELISA curves of anti-MUC1 IgG antibody titers in serum samples from vaccinated mice collected on day 42. Related to Figures 2 and 3.

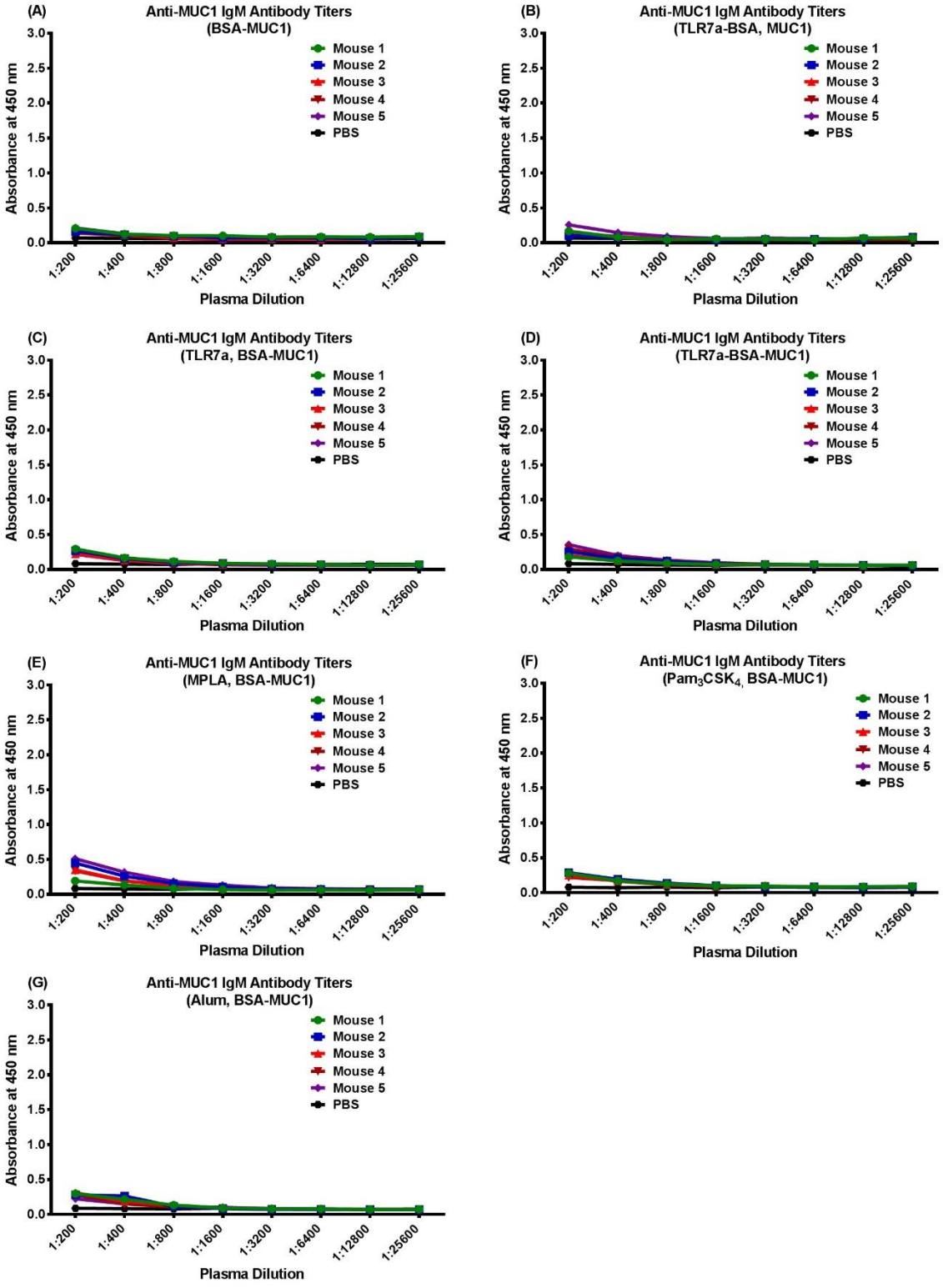


Figure S5. ELISA curves of anti-MUC1 IgM antibody titers in serum samples from vaccinated mice collected on day 14. Related to Figure 3.

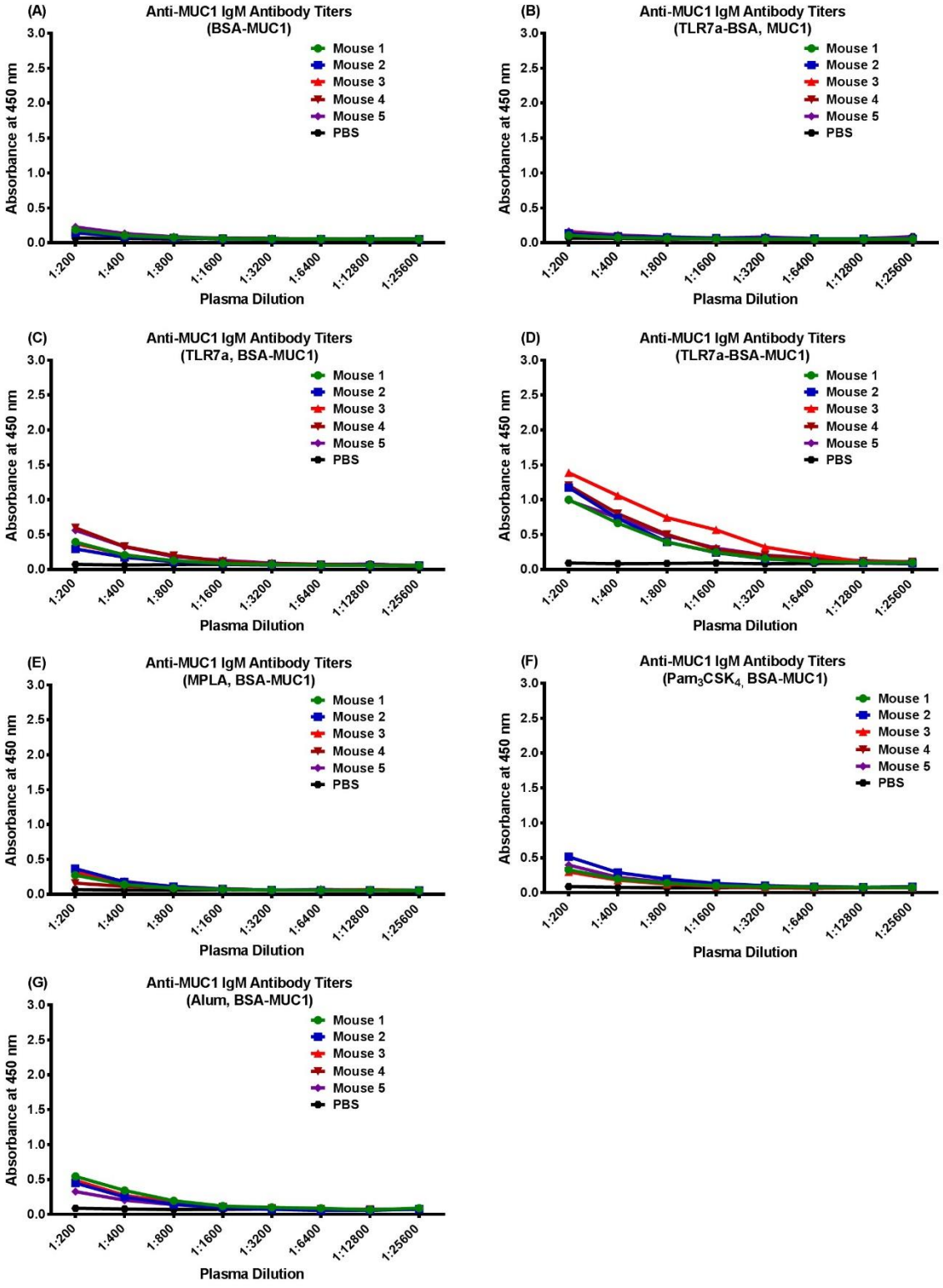


Figure S6. ELISA curves of anti-MUC1 IgM antibody titers in serum samples from vaccinated mice collected on day 28. Related to Figure 3.

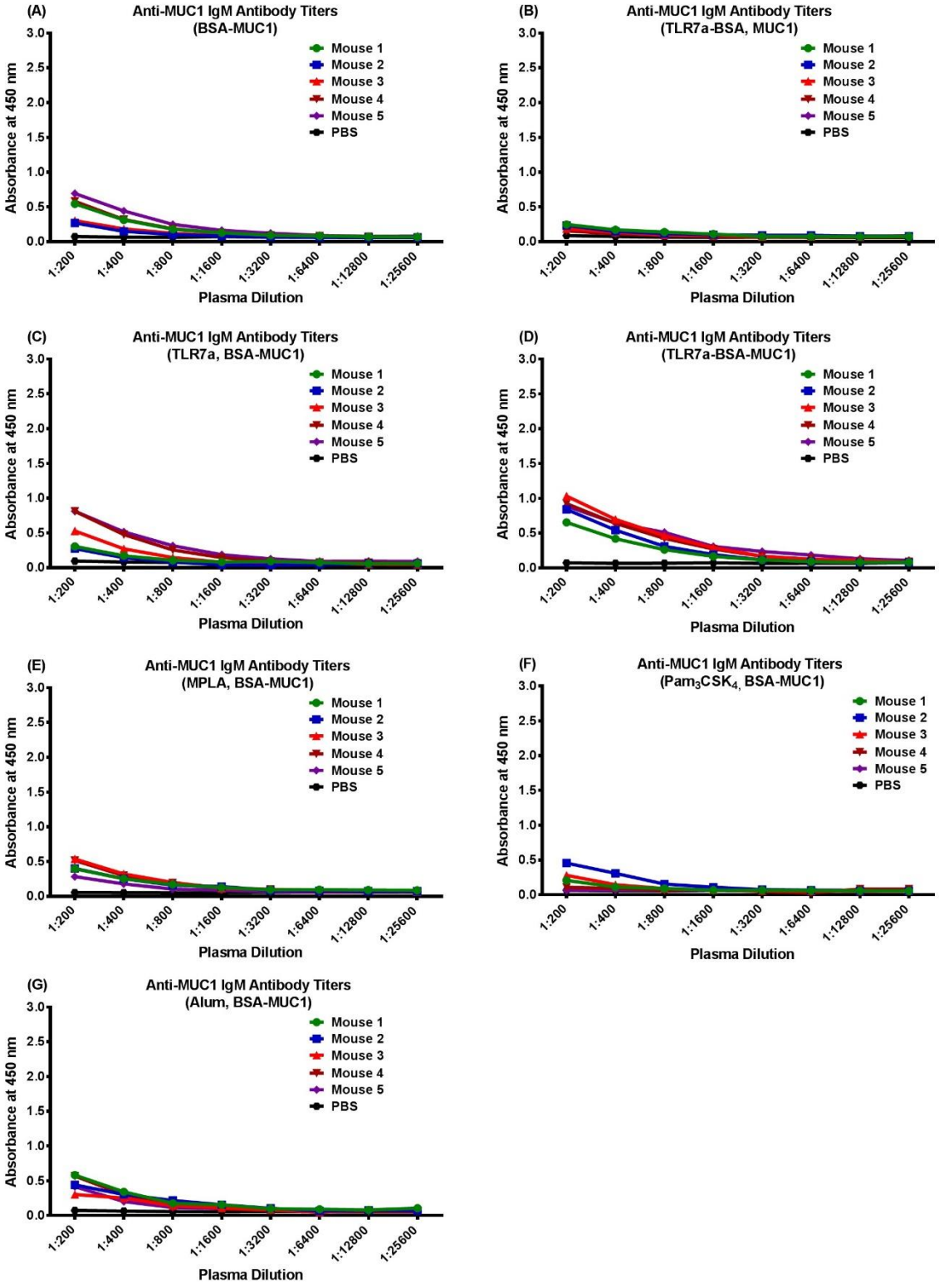


Figure S7. ELISA curves of anti-MUC1 IgM antibody titers in serum samples from vaccinated mice collected on day 42. Related to Figure 3.

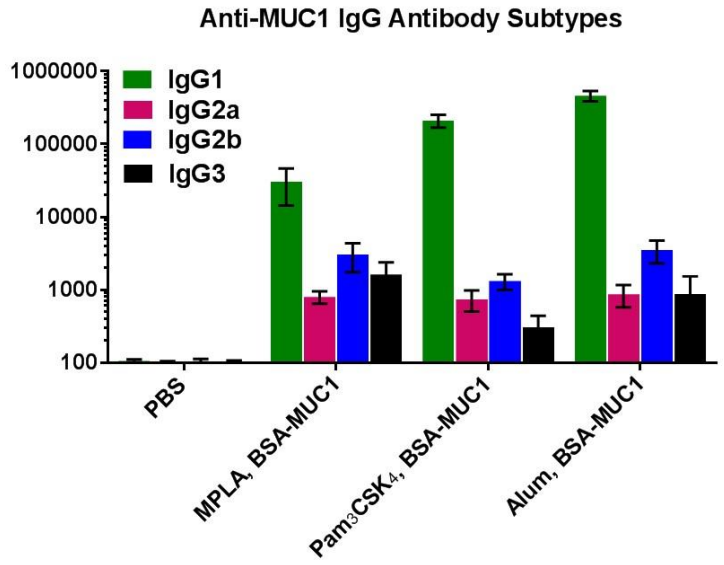


Figure S8. Anti-MUC1 IgG antibody subtype titers were measured in serum samples from vaccinated mice collected on day 42. Data are shown as the mean ± SEM of five mice and are representative of three separate experiments. Related to Figure 4.

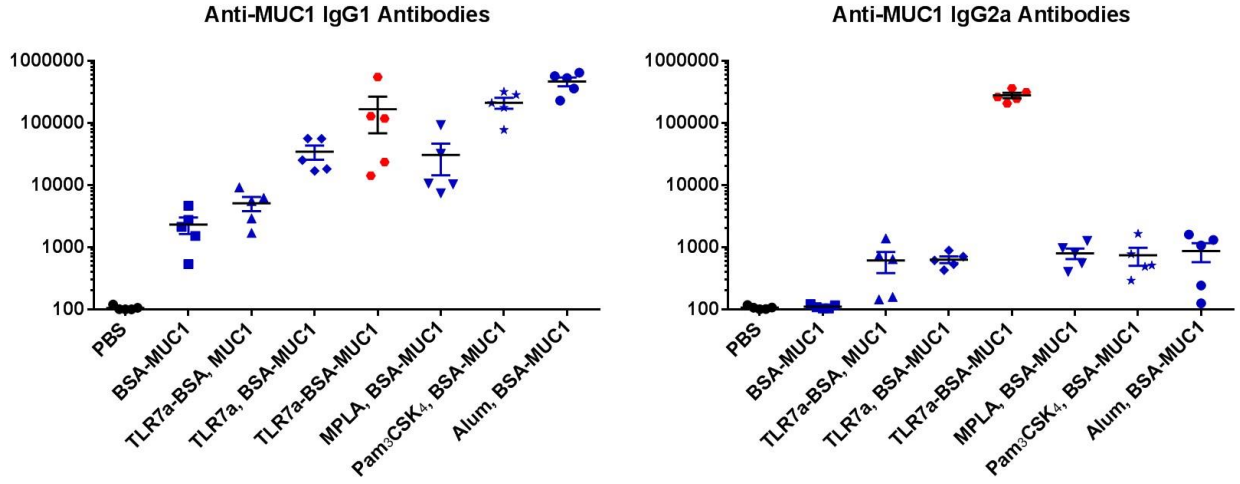


Figure S9. Anti-MUC1 IgG1 and IgG2a antibodies were measured in serum samples from vaccinated mice collected on day 42. Data are shown as the mean ± SEM of five mice and are representative of three separate experiments. Related to Figure 4.

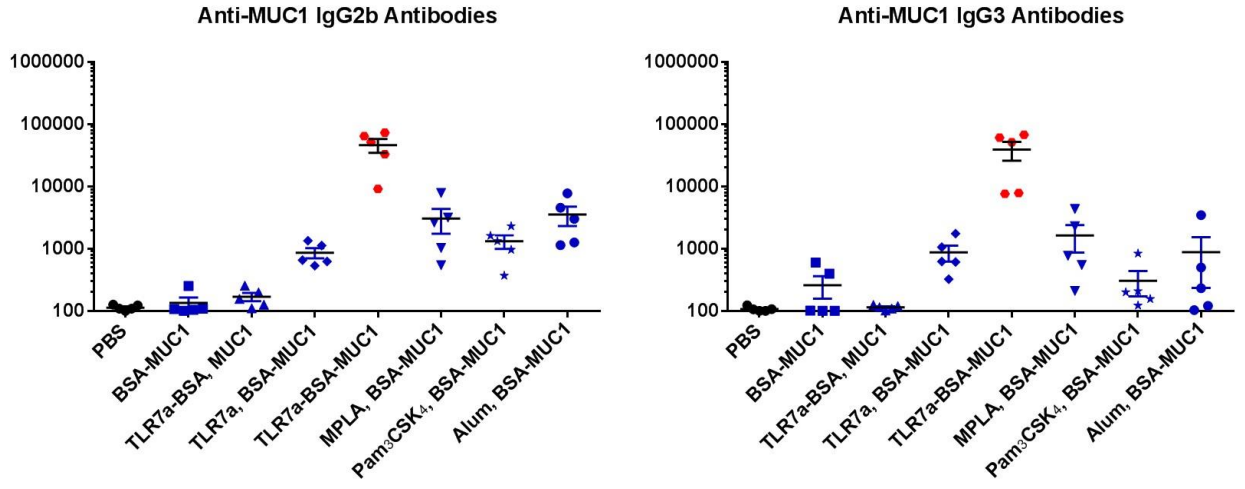


Figure S10. Anti-MUC1 IgG2b and IgG3 antibodies were measured in serum samples from vaccinated mice collected on day 42. Data are shown as the mean ± SEM of five mice and are representative of three separate experiments. Related to Figure 4.

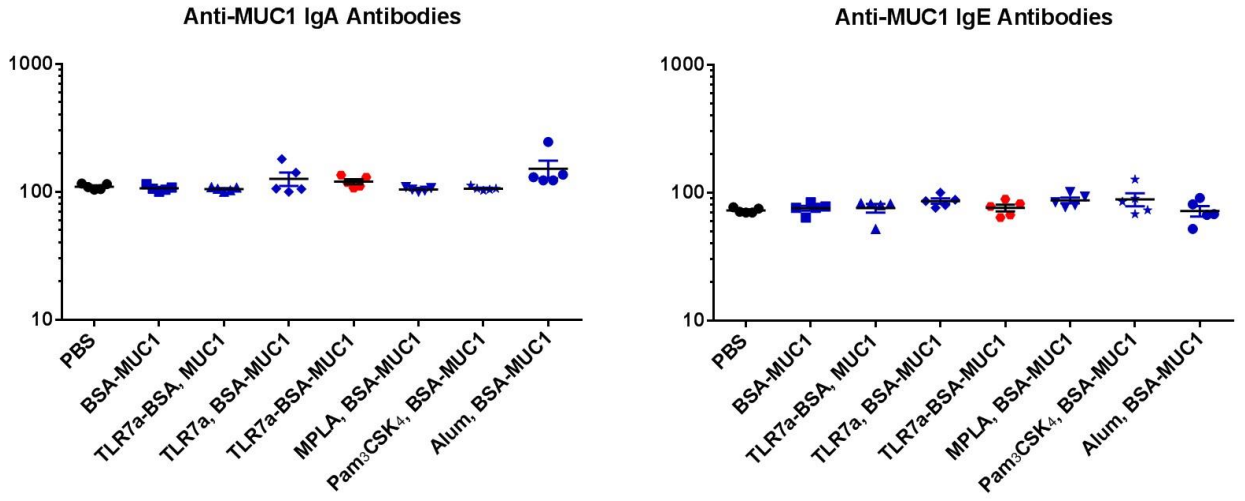


Figure S11. Anti-MUC1 IgA and IgE antibodies were measured in serum samples from vaccinated mice collected on day 42. Data are shown as the mean ± SEM of five mice and are representative of three separate experiments. Related to Figure 4.

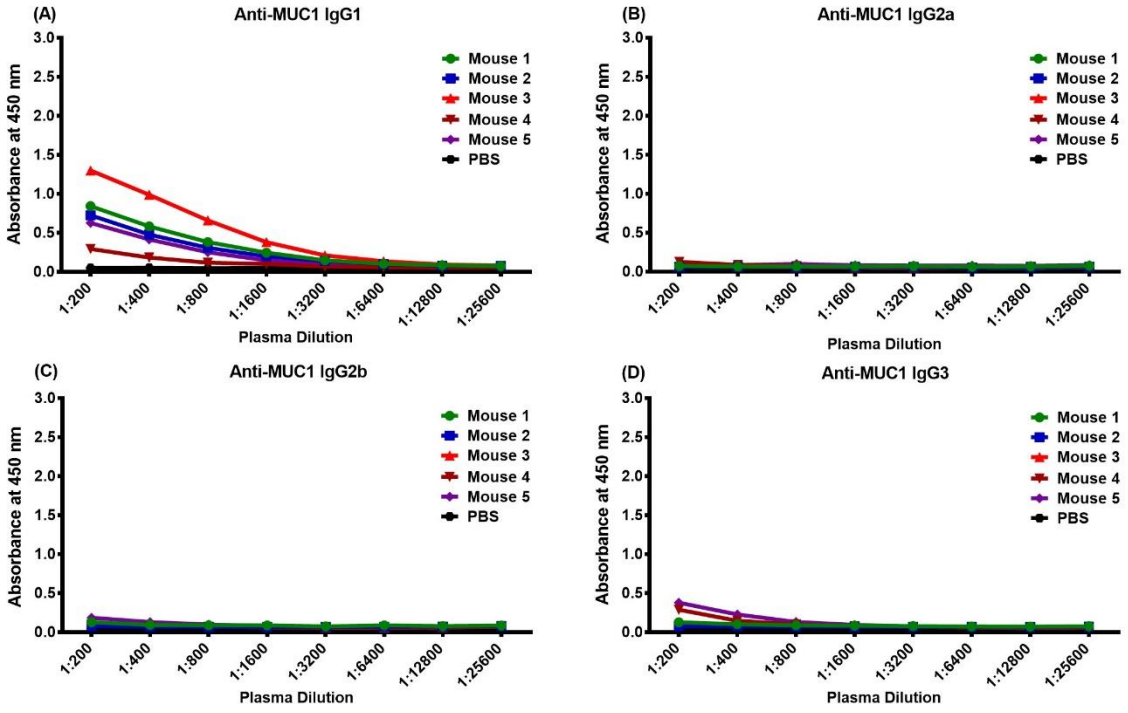


Figure S12. ELISA curves of MUC1-specific (A) IgG1, (B) IgG2a, (C) IgG2b and (D) IgG3 antibodies in plasma 1 (BSA-MUC1) on day 42. Related to Figure 4.

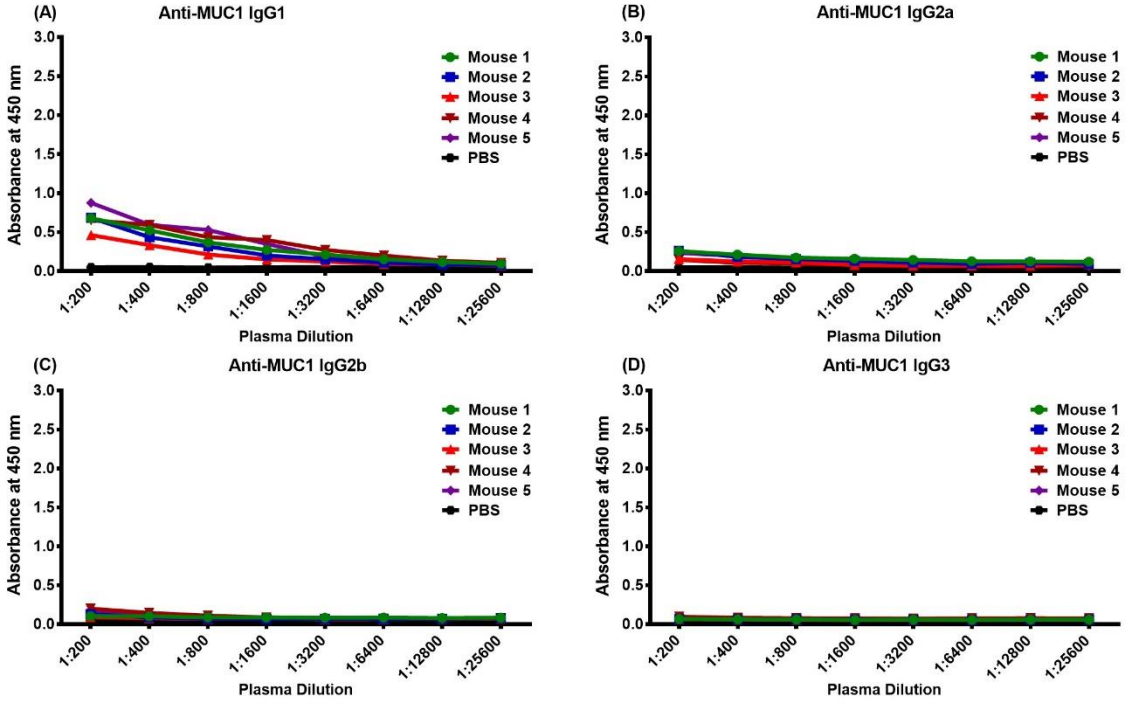


Figure S13. ELISA curves of MUC1-specific (A) IgG1, (B) IgG2a, (C) IgG2b and (D) IgG3 antibodies in plasma 2 (TLR7a-BSA, MUC1) on day 42. Related to Figure 4.

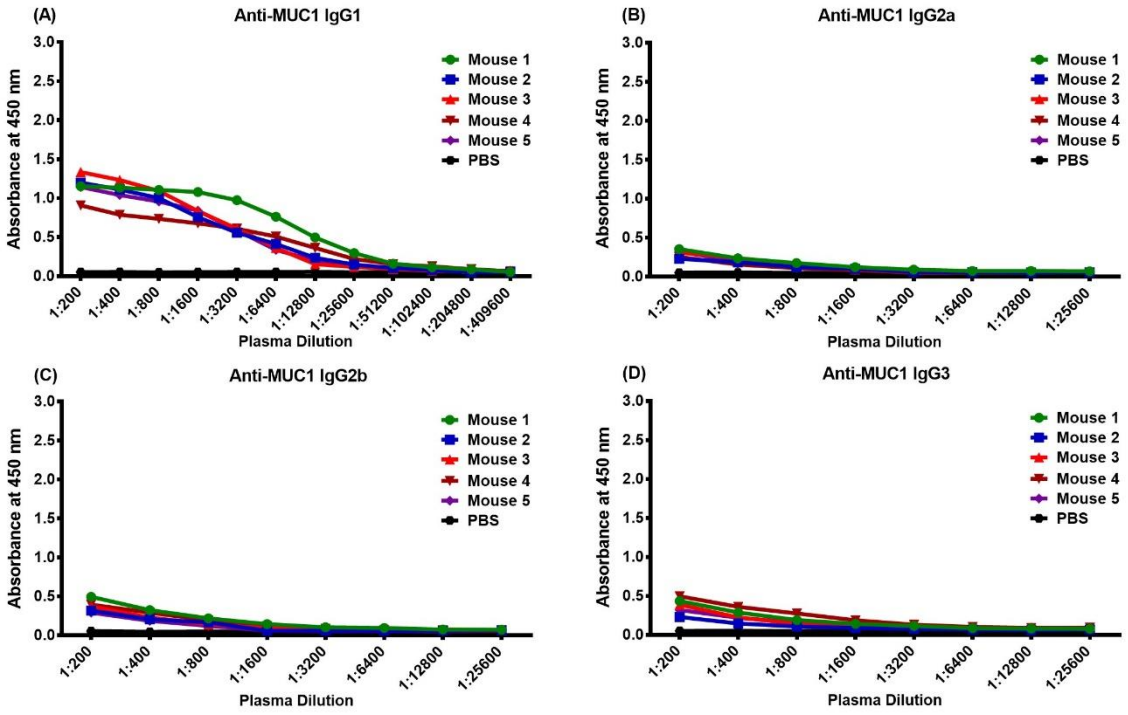


Figure S14. ELISA curves of MUC1-specific (A) IgG1, (B) IgG2a, (C) IgG2b and (D) IgG3 antibodies in plasma 3 (TLR7a, BSA-MUC1) on day 42. Related to Figure 4.

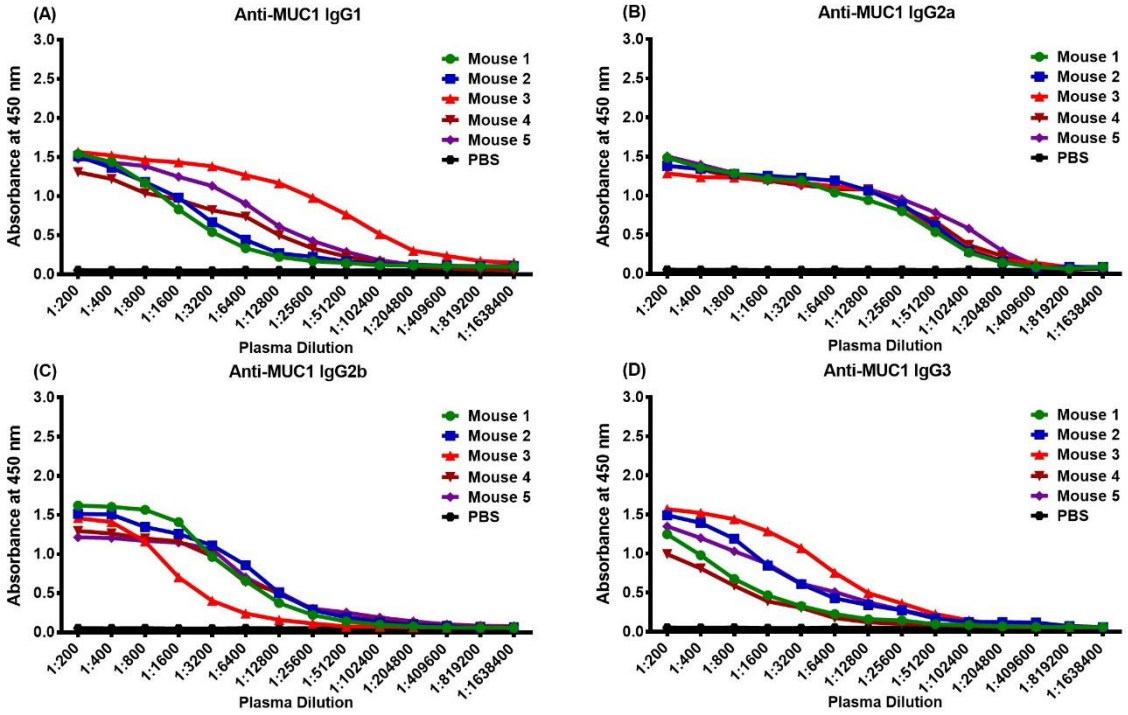


Figure S15. ELISA curves of MUC1-specific (A) IgG1, (B) IgG2a, (C) IgG2b and (D) IgG3 antibodies in plasma 4 (TLR7a-BSA-MUC1) on day 42. Related to Figure 4.

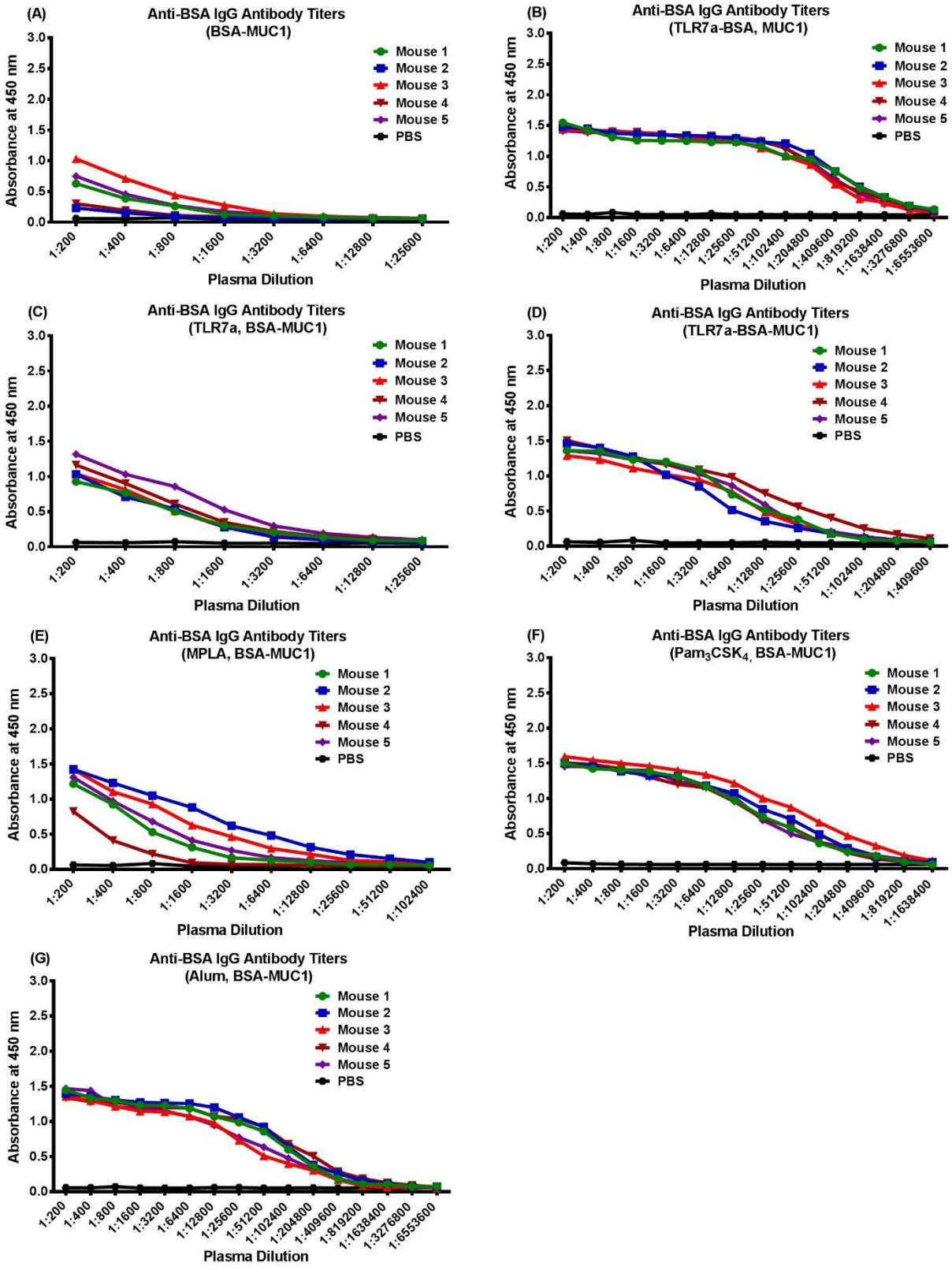


Figure S16. ELISA curves of anti-BSA IgG antibody titers in serum samples from vaccinated mice collected on day 42. Related to Figure 5.

e. Procedures of cellular experiments

Cultures of Cancer Cells. The MCF-7 human breast adenocarcinoma cell lines, the B16-F10 (mouse melanoma tumor cell lines) and B16-MUC1 (human mucin-transfected mouse melanoma tumor cell lines) were obtained from National Infrastructure of Cell Line Resources and used for the in vitro studies. MCF-7 cells maintained in a humidified incubator (Heracell 150i, Thermo Scientific) at 37 ºC with 5% CO_2 and grown using Dulbecco's modified Eagle's medium (DMEM) (Gibco/Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), and 1% (v/v) antibiotics (Gibco/Thermo Fisher Scientific). In addition, the B16-F10 and B16-MUC1 cells were grown in 10% (v/v) FBS and 1% (v/v) antibiotics in RPMI-1640 (Gibco/Thermo Fisher Scientific).

Determination of Antibody Binding to Cancer Cells by FACS Analysis. The reactivity of the antibody binding induced by vaccine candidate towards MCF-7, B16-MUC1 and B16-F10 cells was detected staining the cells with mice antisera followed by FACS analysis (Cai et al., 2014). Hence, cancer cells were subjected to a digestion step with 0.25% Trypsin-EDTA (Gibco/Thermo Fisher Scientific) for 1 min, then transferred to different conical centrifuge tubes (1×10^6 cells per tube) and centrifuged at 1,500 rpm for 2 min. After removing the culture medium, cancer cells were incubated with 500 μL of mice antisera (1:50 dilution) (2 μL per mouse, 10 μL per group) in FACS buffer (1% FBS, 0.1% NaN_3 and 1% BSA in PBS) at 0 °C for 1 h. After washing three times with 1% FBS in PBS (centrifugation at 1500 rpm, then the supernatant was removed), cancer cells were incubated with Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) secondary antibody in flow cytometry buffer (1:50 dilution) (100 μL per tube) at 0 °C for 1 h. After further washing steps (centrifugation at 1500 rpm, then the supernatant was removed), 300 μL of flow cytometry buffer was added, the cells were detected using a flow cytometry (BD Accuri™).

Determination of Antibody Binding to Cancer Cells by Confocal Microscopy Analysis. Cancer cells were stained with mice antisera to determine their potential in recognizing the MUC1 targets. Initially, cancer cells were subjected to a digestion step with trypsin, transferred to confocal dishes (1×10^6 cells per dish), followed by maintaining in a humidified incubator at 37 ºC for 12 h. After washing five times with 1% BSA in PBS buffer (1 min each wash), cancer cells were incubated with mice antisera (1:50 dilution) at 0 °C for 1 h. Subsequently, the culture medium was removed and cancer cells were washed five times with 1% BSA in PBS buffer, cancer cells were incubated with Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) secondary antibody in 1% BSA in PBS buffer (1:50 dilution) at 0 °C for 30 min. After washing, the cancer cells were visualized using confocal microscope (Leica TCS SP8, Wetzlar, Germany) with a 63X oil objective.

Determination of Cell Viability of MCF-7 Cells by MTT Protocol. The purpose of this experiment is to investigate whether the antibodies are able to mediate complement lysis via activation of CDC. MCF-7 cells were incubated in DMEM containing 2% FBS and planted on a 96-well cell culture plate (8000 cells per well). After washing three times with PBS solution, MCF-7 cells were incubated with the mice antisera in PBS containing 1% BSA (1:50 dilution) (50 μL/well) at 37 ºC for 2 h. After another washing steps, the prepared rabbit sera (1:5 dilution) (Cedarlane Labs) in 1% BSA/PBS exited as complement supplier (RC: rabbit complement; RC-inactive: inactivated rabbit complement after treatment at high temperature) were added (50 μL/well). After incubation for 4 h, without washing, the prepared 0.5% MTT (Aladdin) in PBS solution was added (20 μL/well) and incubated at 37 ºC. After incubation for 2 h, DMSO (Leagene) was added (150 μL/well) and the absorption was analyzed at the wavelength of 490 nm. The cell viabilities of MCF-7 cells were measured with the following formula:

Cell viability (%) = (Experimental/Control) × 100

CTL Assay. Groups of BALB/c mice (n = 5) were immunized three times (days 1, 15, and 29) by subcutaneous injection of vaccine candidates into the back of the neck. Fourteen days after the third immunization, spleens were obtained from mice and processed into a single cell suspension, then employed as effector cells for CTL assay. The freshly isolated splenocytes (1×10^6 cells/well) were added and co-incubated with the MCF-7 cells (1×10^6 cells/well) in RPMI-1640 (Gibco/Thermo Fisher Scientific) for 12 h. Finally, the effector cell-mediated cytotoxicity to target cells was examined by lactate dehydrogenase (LDH) assay according to the manufacture's protocol (Beyotime Biotechnology). Each plate was centrifuged at 250 g for 4 min, then 120 μL of the cell-free supernatant was transferred to the wells of another 96-well enzymatic assay plate containing LDH assay reagents (60 μL/well). The 96-well plates were incubated at rt protected from light for 30 min. The absorptions of these plates were read at 490 nm wavelength using a microplate reader. In the meantime, the spontaneous LDH release values were determined by incubating tumor cells alone or splenocytes alone, respectively. The maximum LDH release values were determined by incubating tumor cells in RPMI-1640 containing lysis solution without FBS. The percentage of cell lysis was calculated according to the following formula:

Cytotoxicity (%) = (Experimental – Effector Spontaneous – Target Spontaneous/Target Maximum – Target Spontaneous) × 100

f. Determination of antibody binding to tumor cells by FACS analysis

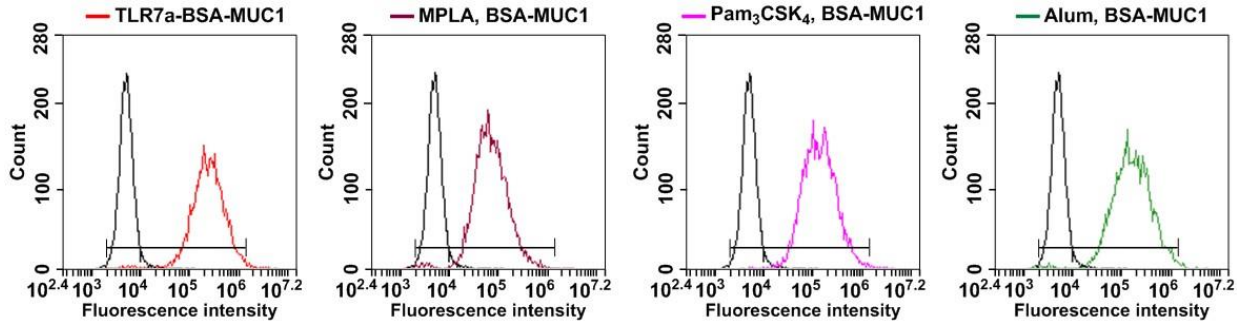


Figure S17. FACS analysis of the binding of vaccinated mouse serum samples to MCF-7 cells. Incubation with PBS group sample (black) served as a control. The images are representative of five independent experiments. Related to Figure 6.

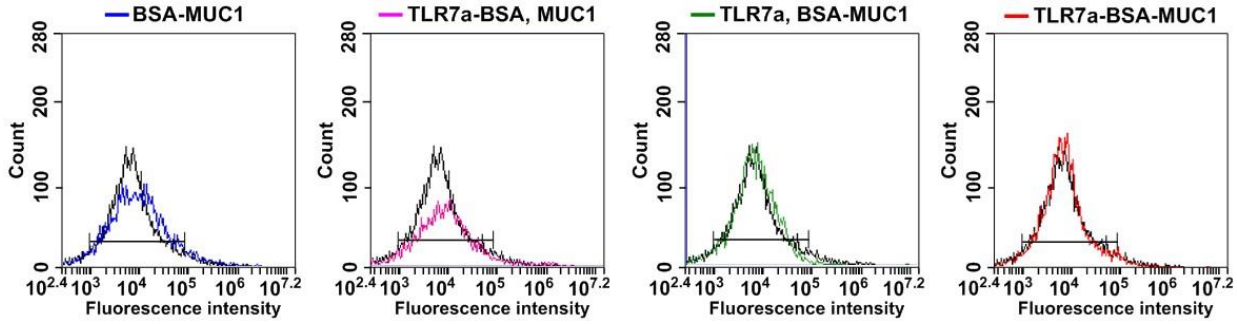


Figure S18. FACS analysis of the binding of vaccinated mouse serum samples to B16-F10 cells (Li et al., 2019). **Incubation with PBS group sample (black) served as a control.** The images are representative of five independent experiments. Related to Figure 6.

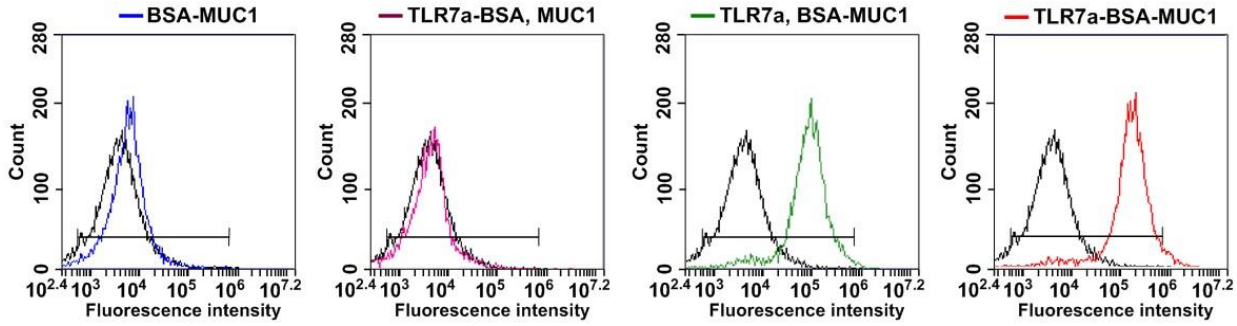


Figure S19. FACS analysis of the binding of vaccinated mouse serum samples to B16-MUC1 cells. Incubation with PBS group sample (black) served as a control. The images are representative of five independent experiments. Related to Figure 6.

g. Determination of antibody binding to tumor cells by confocal microscopy

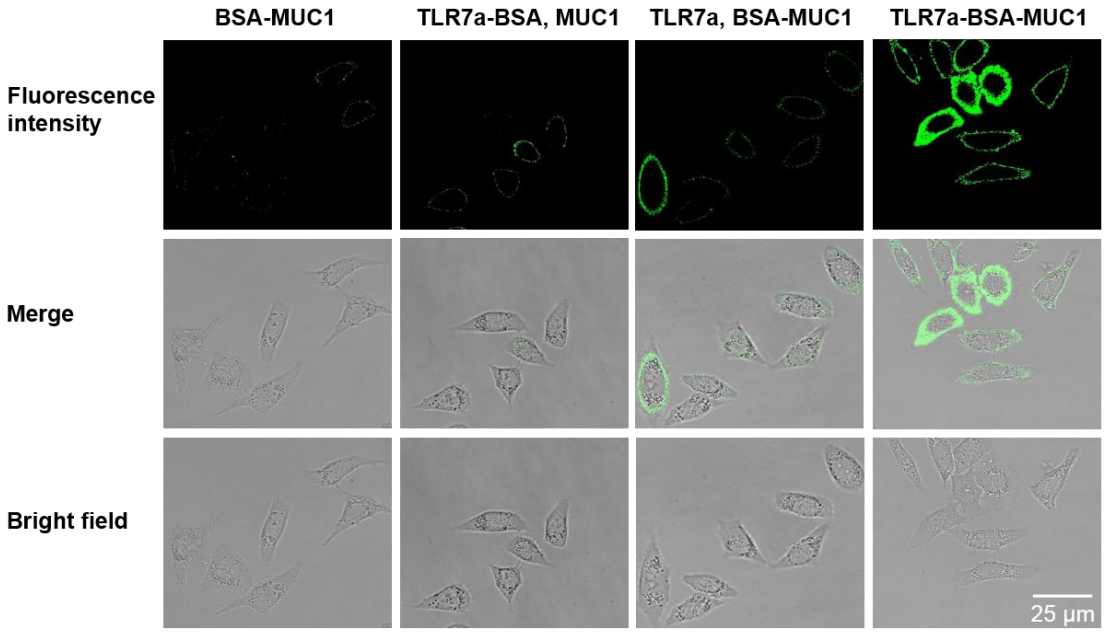


Figure S20. Confocal fluorescence microscopy images of MCF-7 cells incubated with serum samples from vaccinated mice (magnification: 63×). The images are representative of five independent experiments. Scale bar = 25 μm. Related to Figure 6.

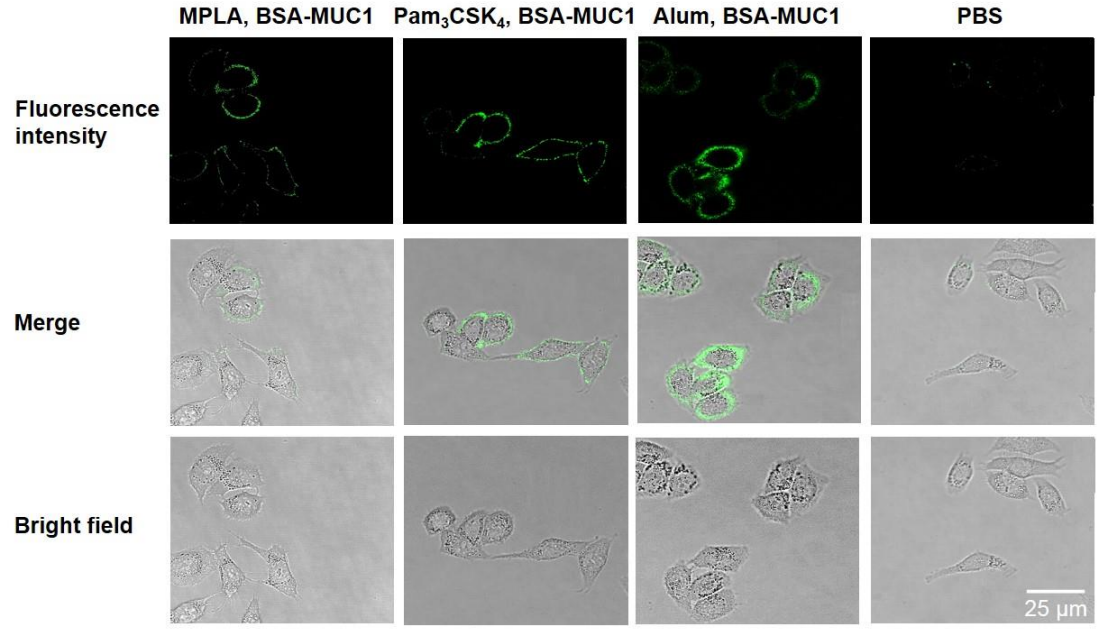


Figure S21. Confocal fluorescence microscopy images of MCF-7 cells incubated with serum samples from vaccinated mice (magnification: 63×). Incubation with PBS group sample served as a control. The images are representative of five independent experiments. Scale bar = 25 μm. Related to Figure 6.

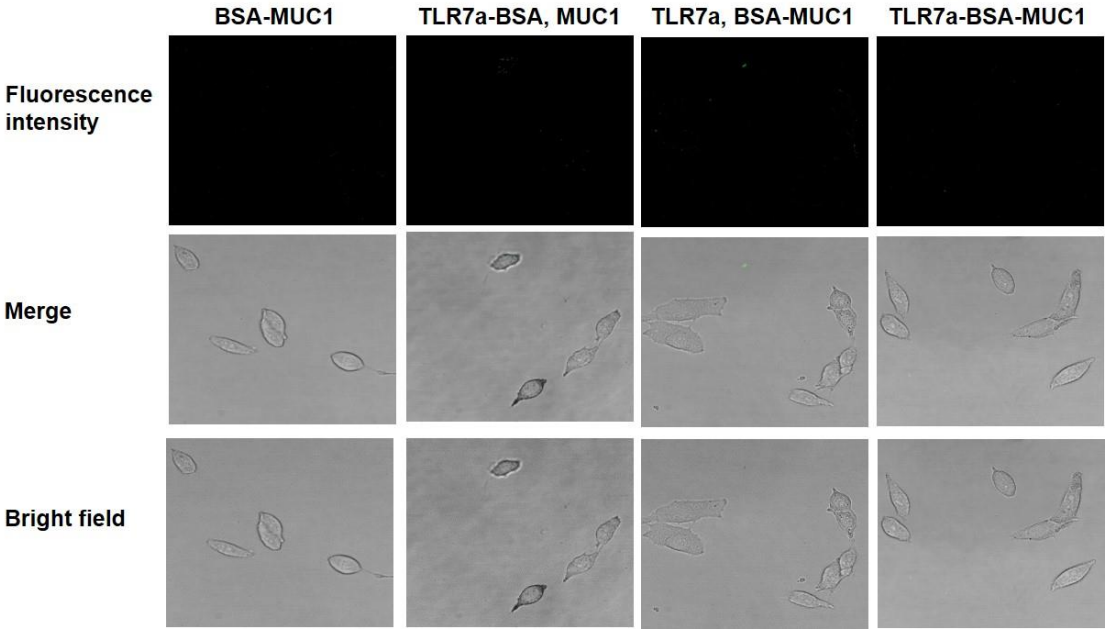


Figure S22. Confocal fluorescence microscopy images of B16-F10 cells incubated with serum samples from vaccinated mice (magnification: 63×). The images are representative of five independent experiments. Related to Figure 6.

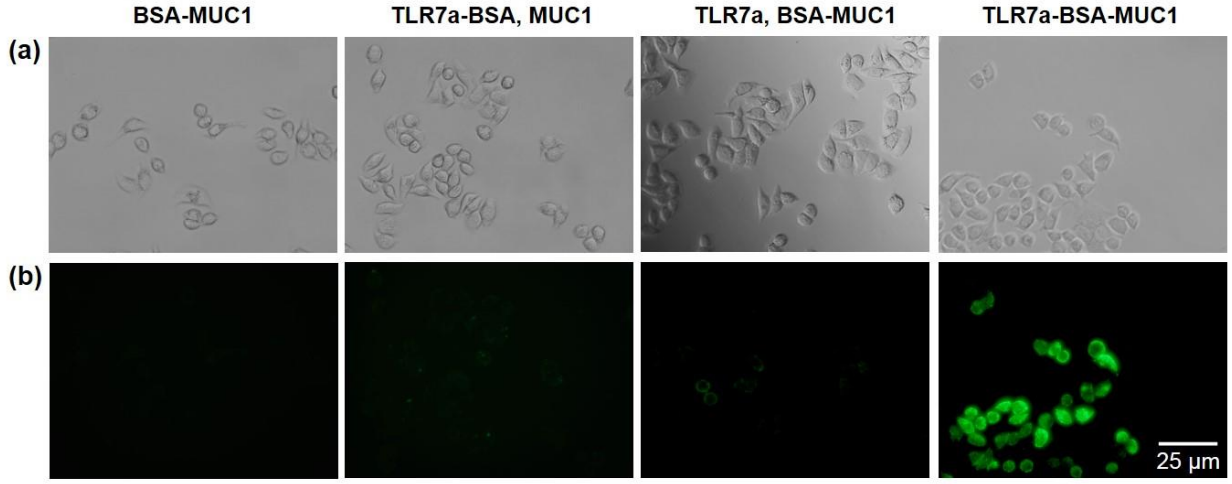


Figure S23. Fluorescence-microscopy staining of MCF-7 cells. a): bright field images. b): fluorescent images. The images are representative of five independent experiments. Scale bar = 25 μm. Related to Figure 6.

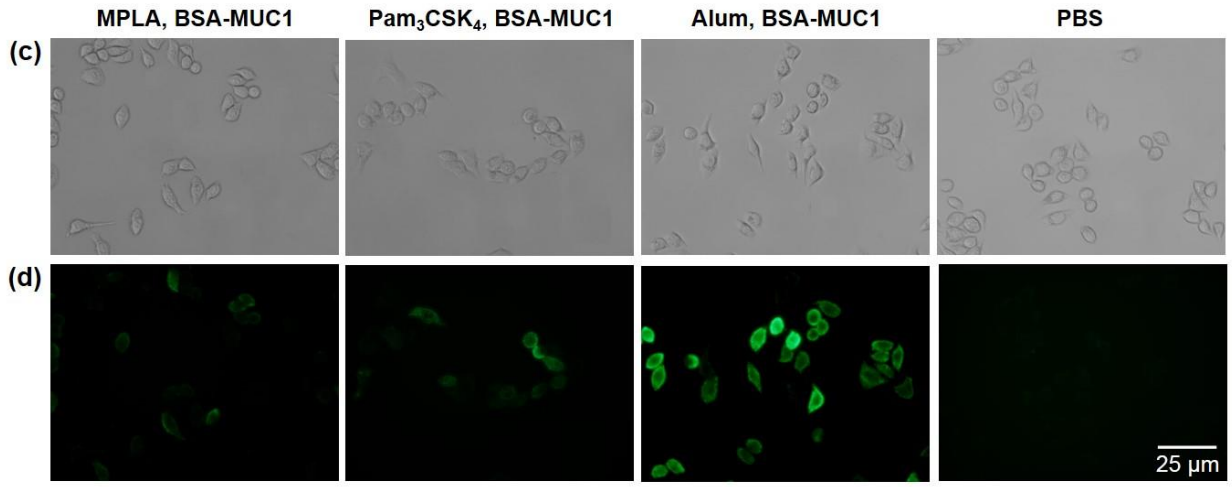


Figure S24. Fluorescence-microscopy staining of MCF-7 cells. c): bright field images. d): fluorescent images. Incubation with PBS group sample served as a control. The images are representative of five independent experiments. Scale bar = 25 μm. Related to Figure 6.

h. Preliminary evaluation of the safety of weight change

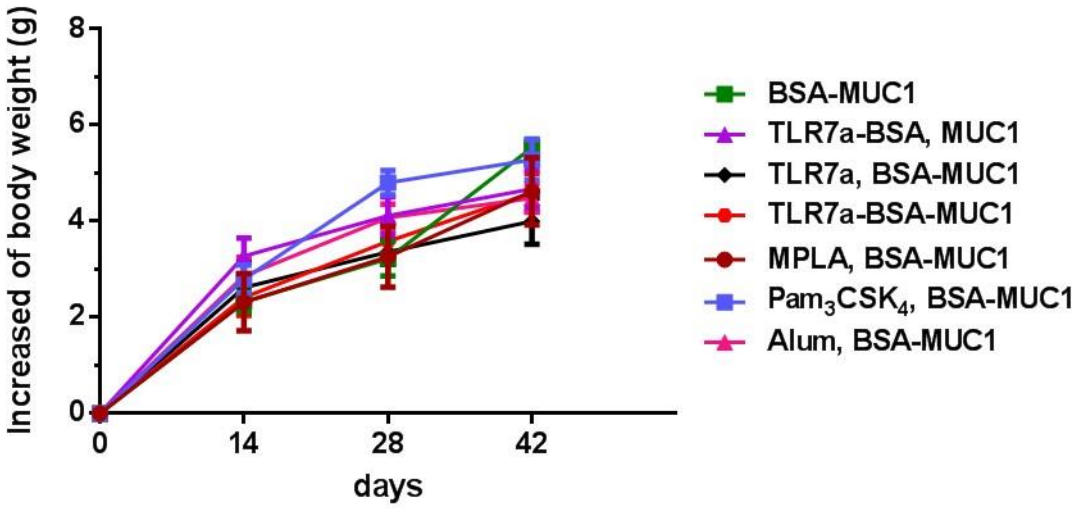


Figure S25. Assessment of the safety of MUC1-bsaed vaccines by monitoring the weight changes of immunized mice. Related to Figure 7.

4. References

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5. Analytical data of compounds

a. Compound 3

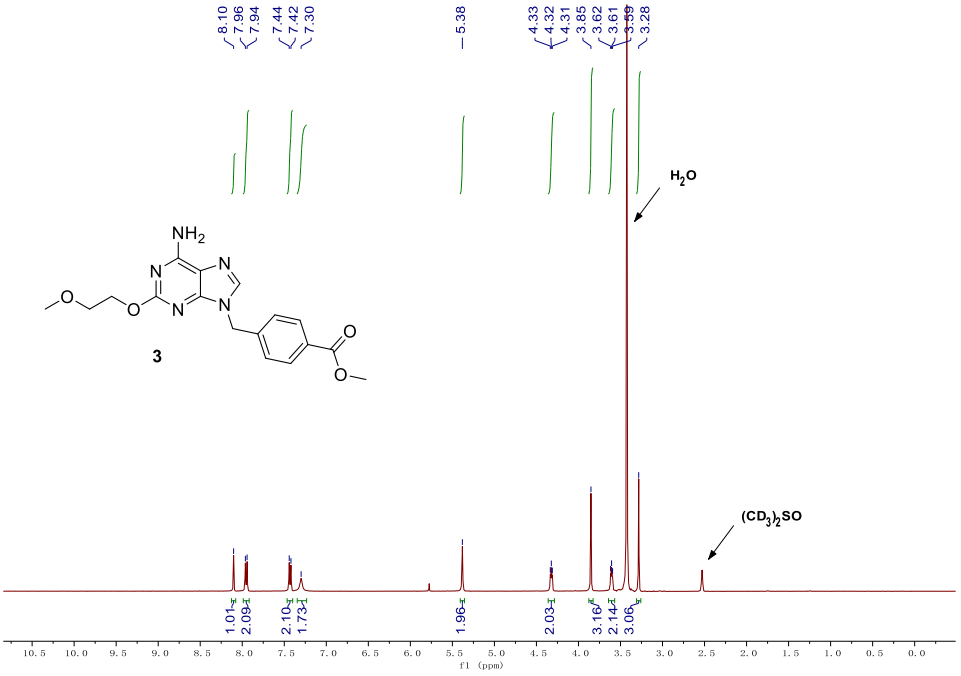


Figure S26. ^{1}H NMR spectrum for compound **3**. Related to Scheme 2.

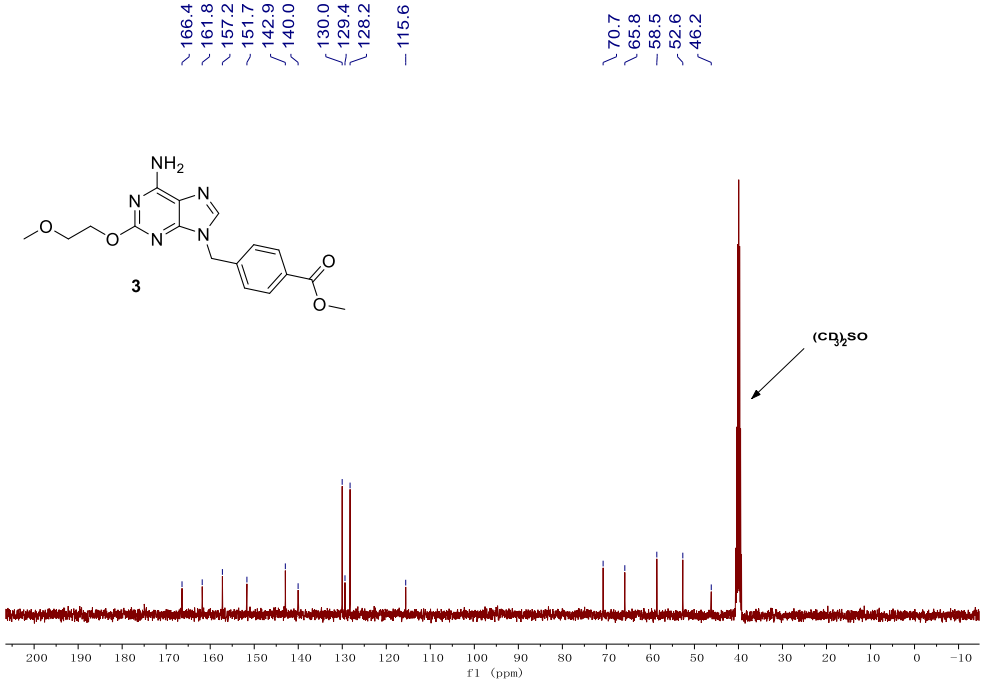


Figure S27. ^{13}C NMR spectrum for compound **3**. Related to Scheme 2.

b. Compound 4

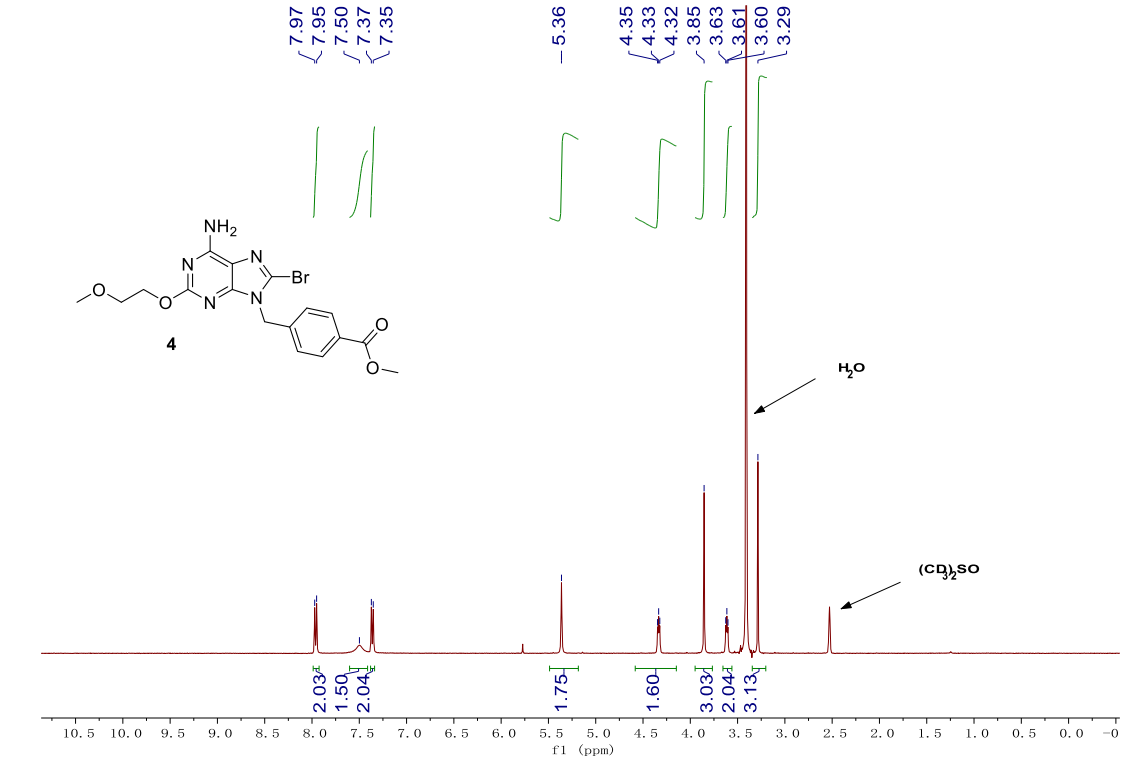


Figure S28. ^{1}H NMR spectrum for compound **4**. Related to Scheme 2.

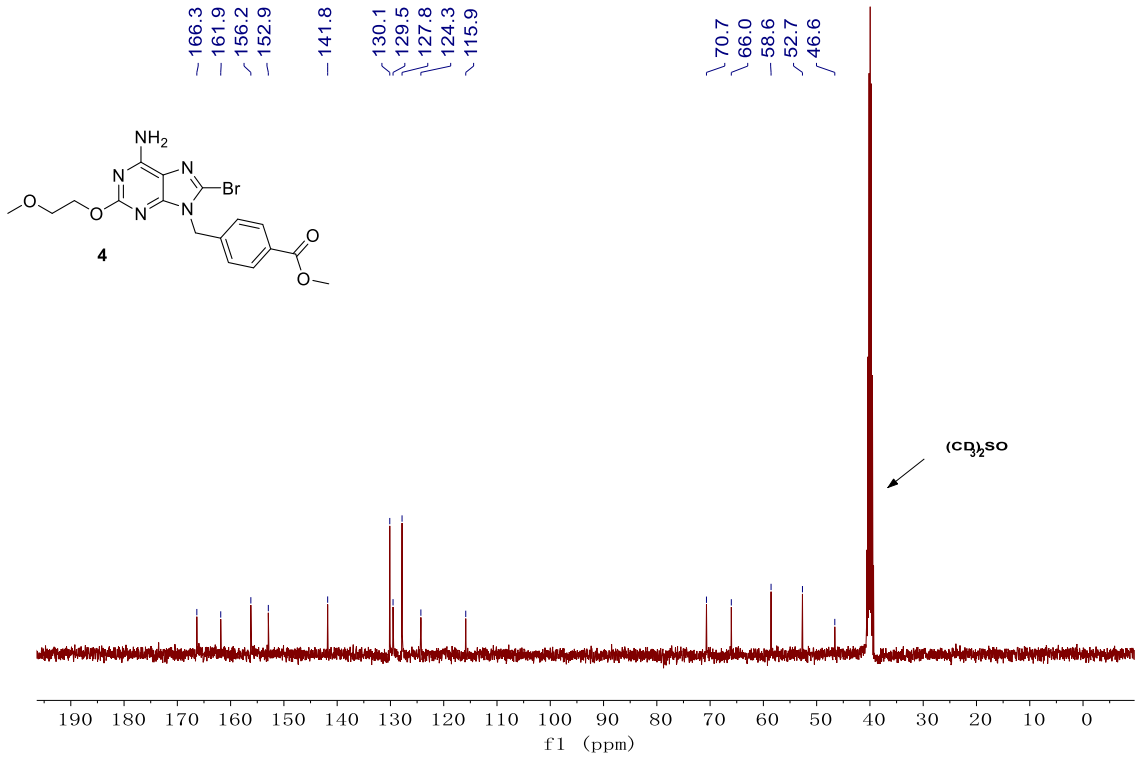


Figure S29. ^{13}C NMR spectrum for compound **4**. Related to Scheme 2.

c. Compound 5

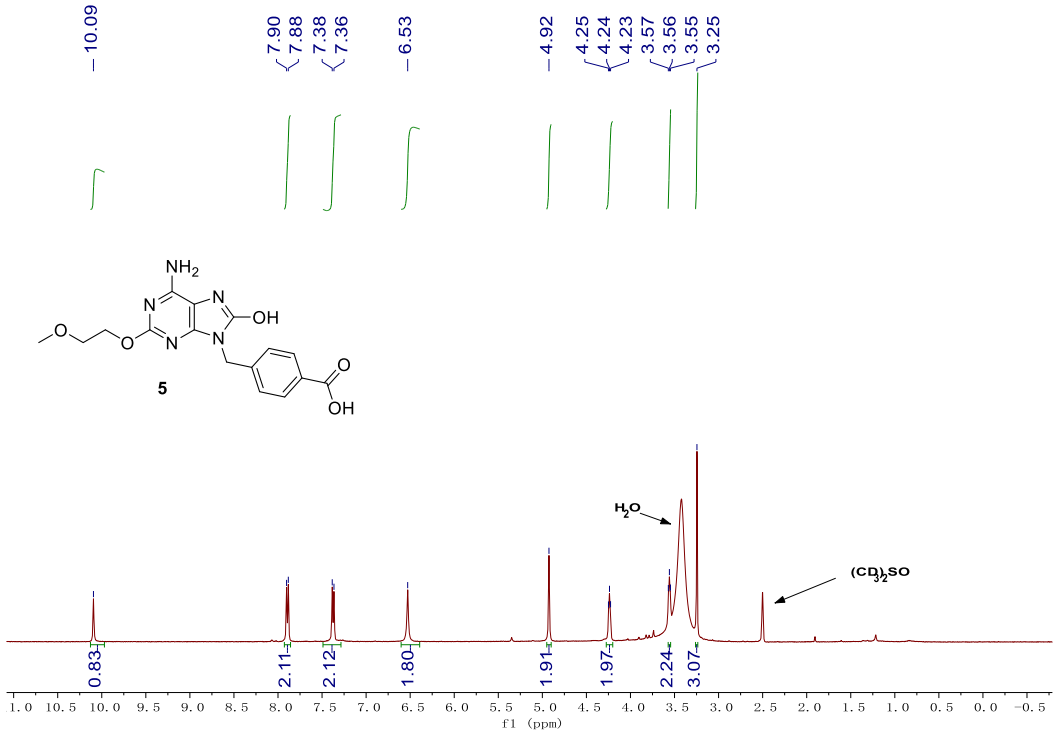


Figure S30. ^{1}H NMR spectrum for compound **5**. Related to Scheme 2.

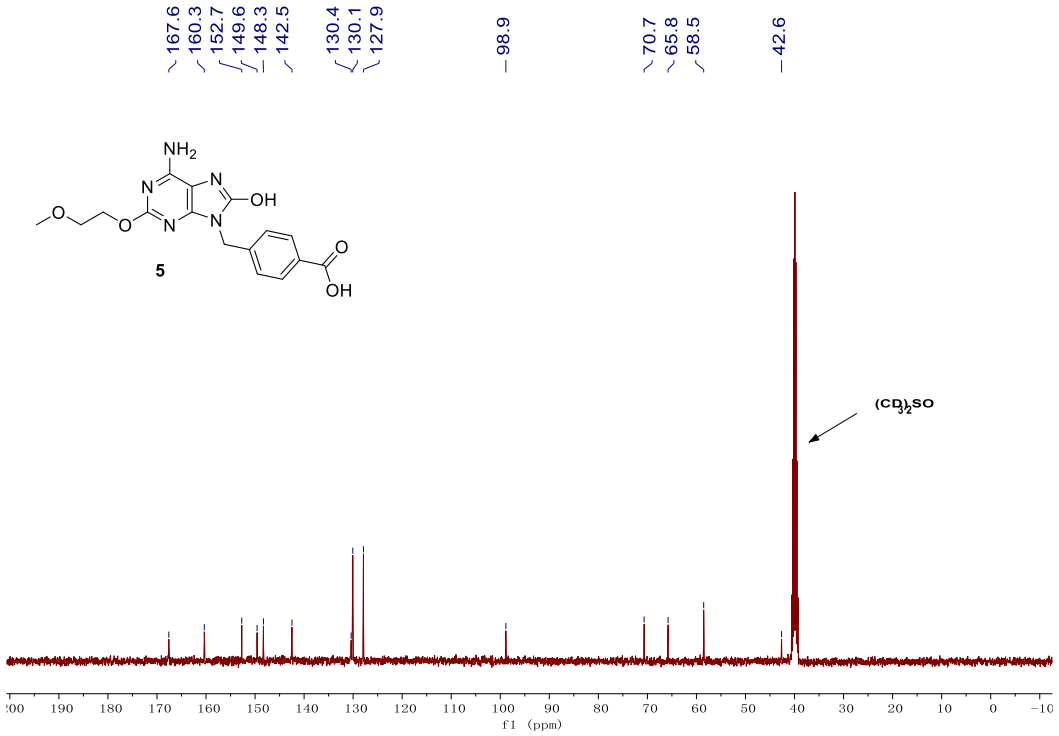


Figure S31. ^{13}C NMR spectrum for compound **5**. Related to Scheme 2.

d. MUC1 glycopeptide 8

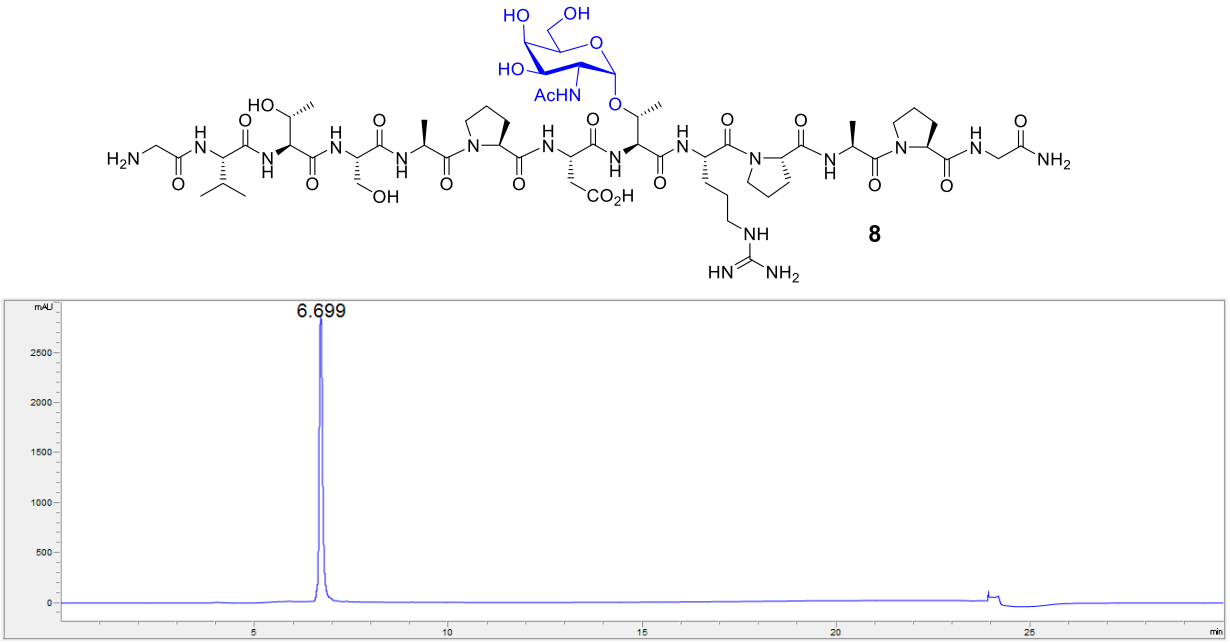


Figure S32. Analytical HPLC trace of MUC1 glycopeptide **8**. (28 mg, 42% yield). Analytic gradient is 5% to 90% of solution B (acetonitrile with 0.1% trifluoroacetic acid) in solution A (water with 0.1% trifluoroacetic acid) in 30 min on the analytic C18 column. Related to Scheme 2.

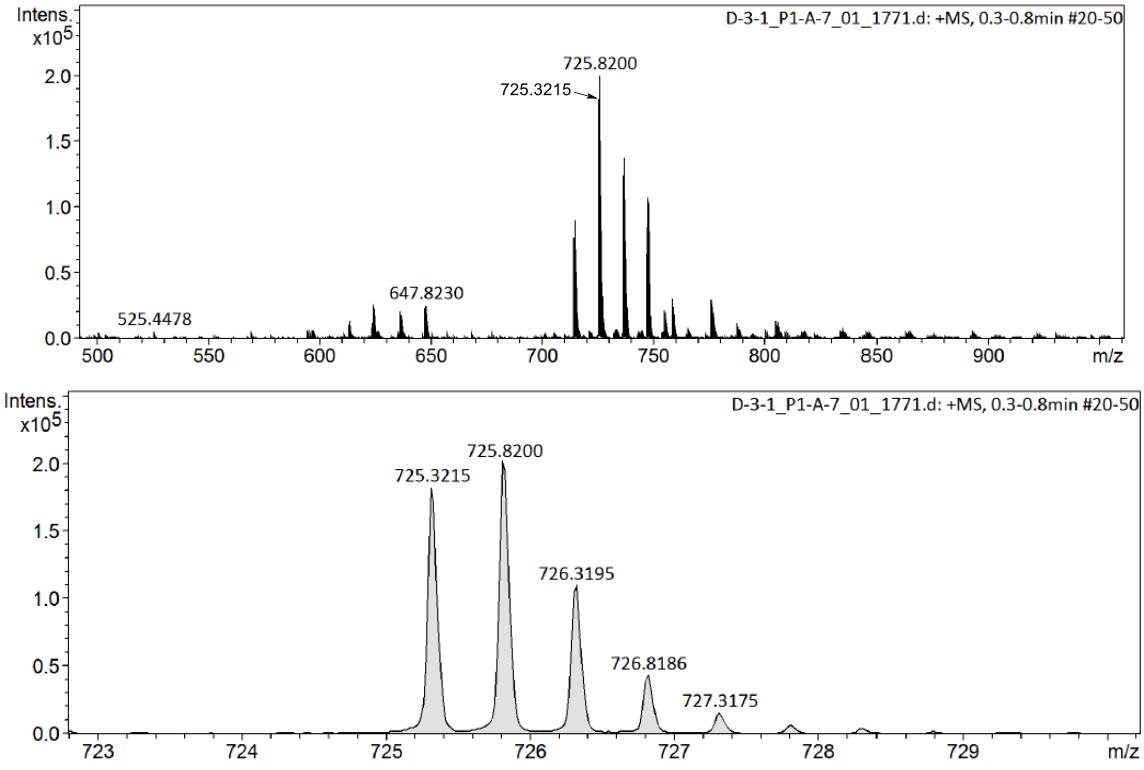


Figure S33. ESI-Q-TOF MS data of MUC1 glycopeptide **8**. Related to Scheme 2.

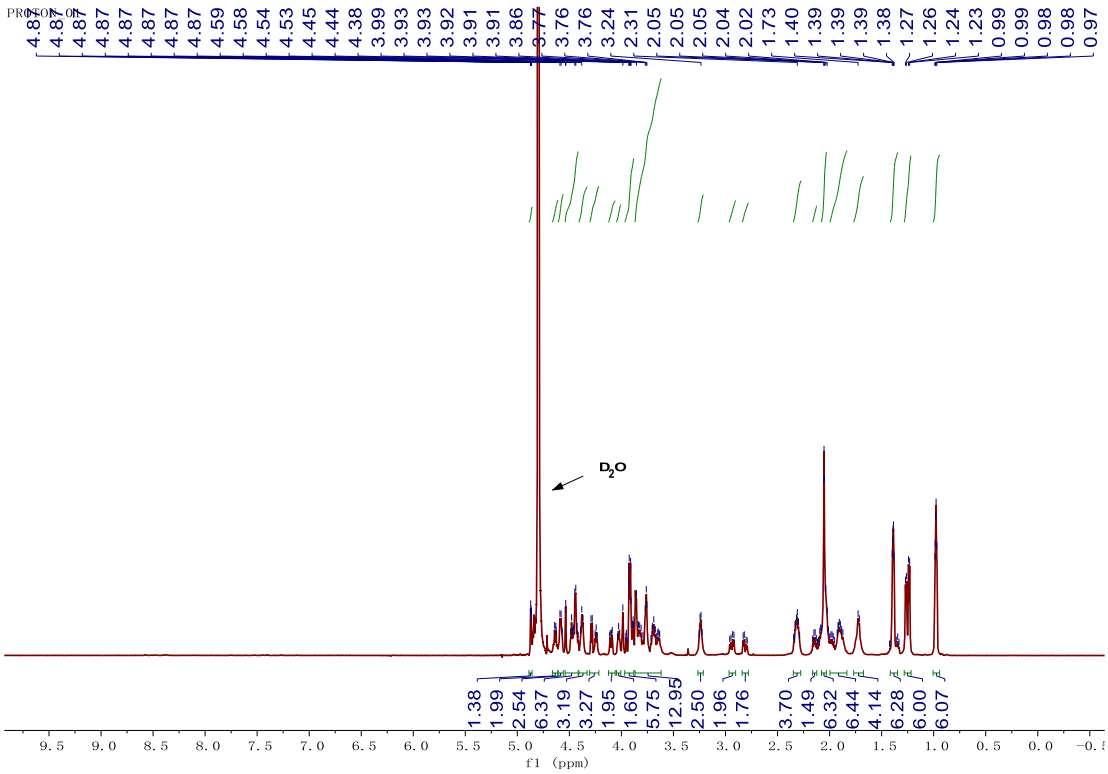


Figure S34. ^{1}H NMR spectrum for MUC1 glycopeptide **8**. Related to Scheme 2.

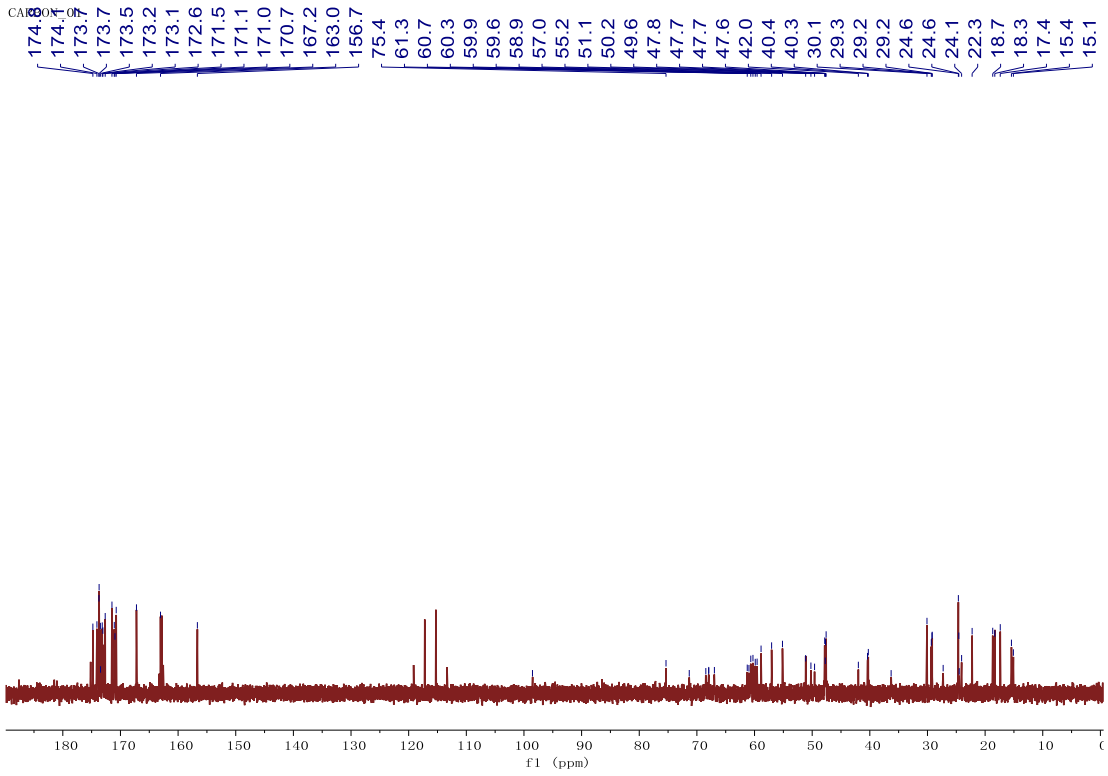


Figure S35. ^{13}C NMR spectrum for MUC1 glycopeptide **8**. Related to Scheme 2.

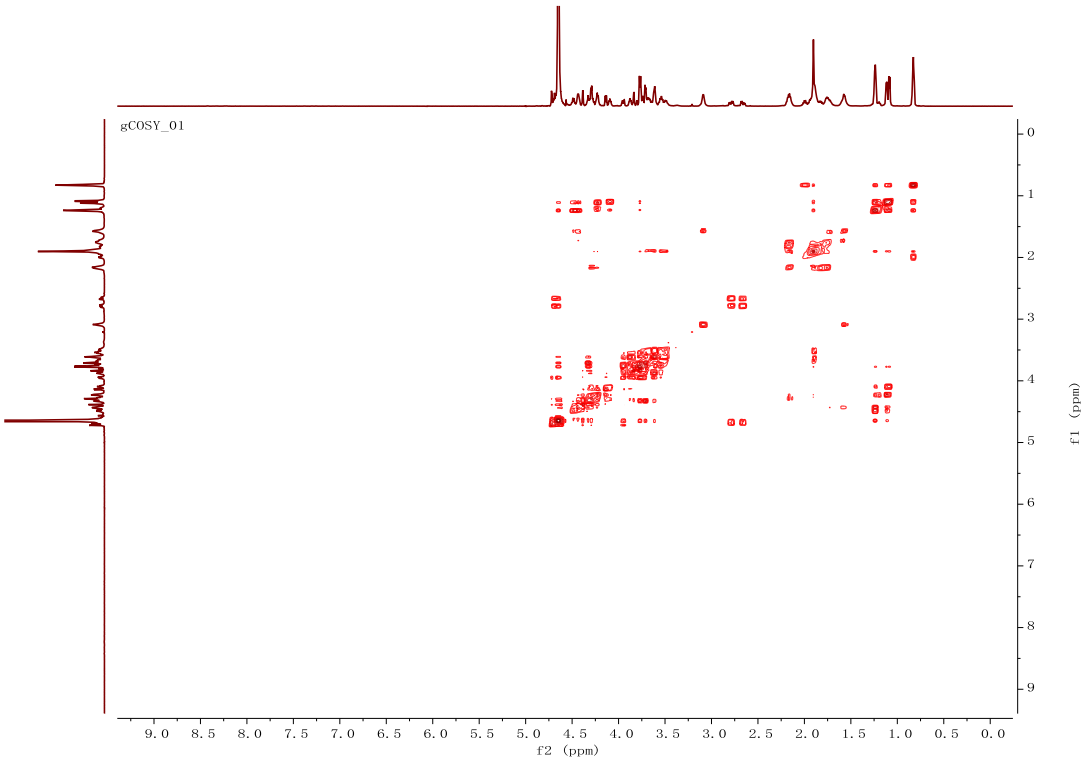
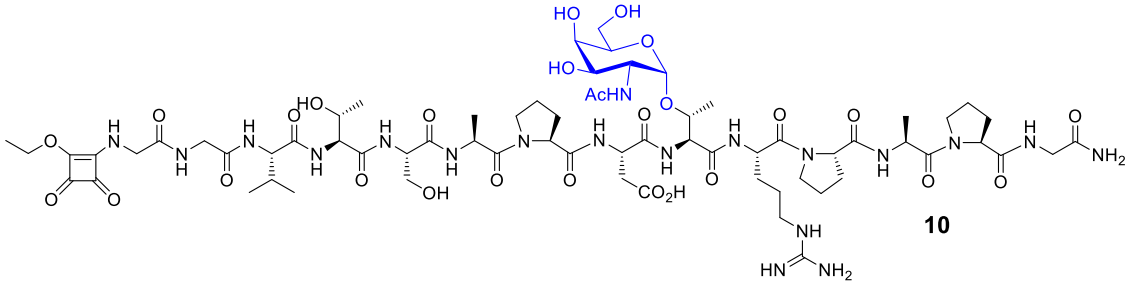


Figure S36. COSY of MUC1 glycopeptide **8**. Related to Scheme 2.

e. MUC1 glycopeptide squaric acid monoamide 10



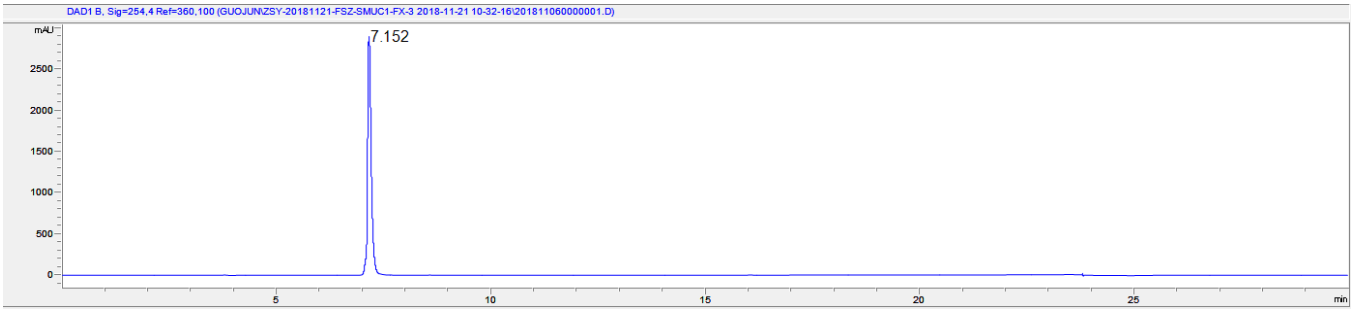


Figure S37. Analytical HPLC trace of MUC1 glycopeptide squaric acid monoamide **10**. (26 mg, 51% yield). Analytic gradient is 5% to 90% of solution B (acetonitrile with 0.1% trifluoroacetic acid) in solution A (water with 0.1% trifluoroacetic acid) in 30 min on the analytic C18 column. Related to Scheme 2.

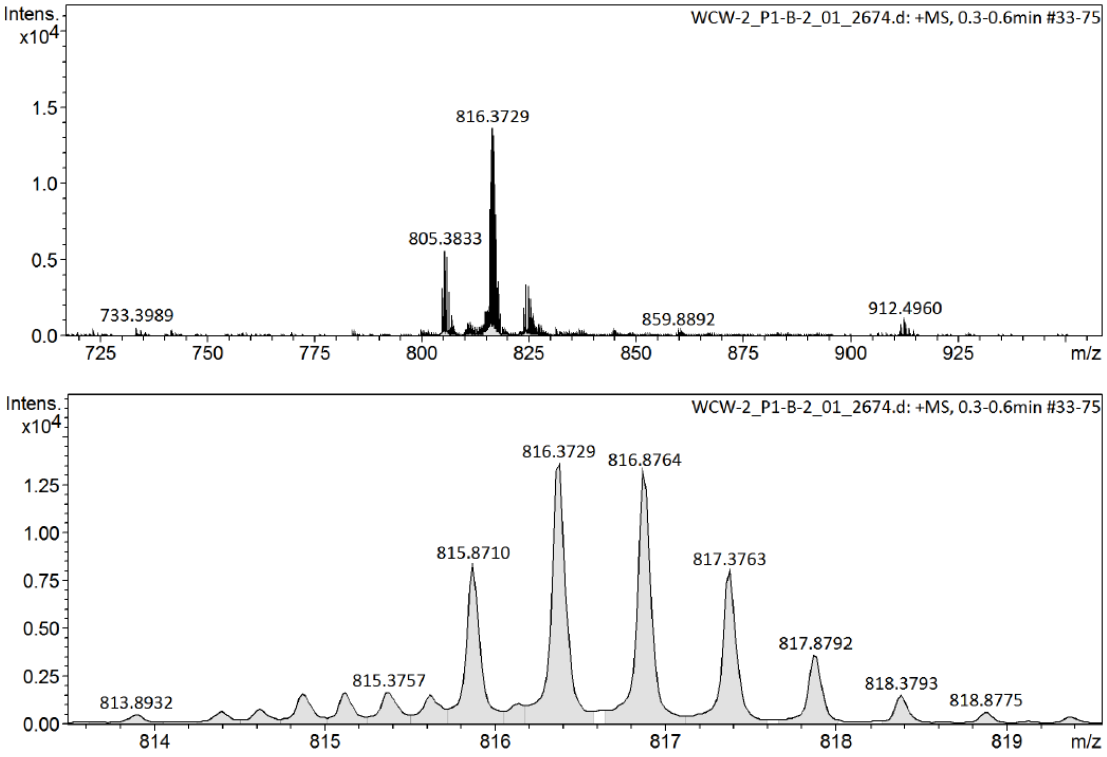


Figure S38. ESI-Q-TOF MS data of MUC1 glycopeptide squaric acid monoamide **10**. Related to Scheme 2.

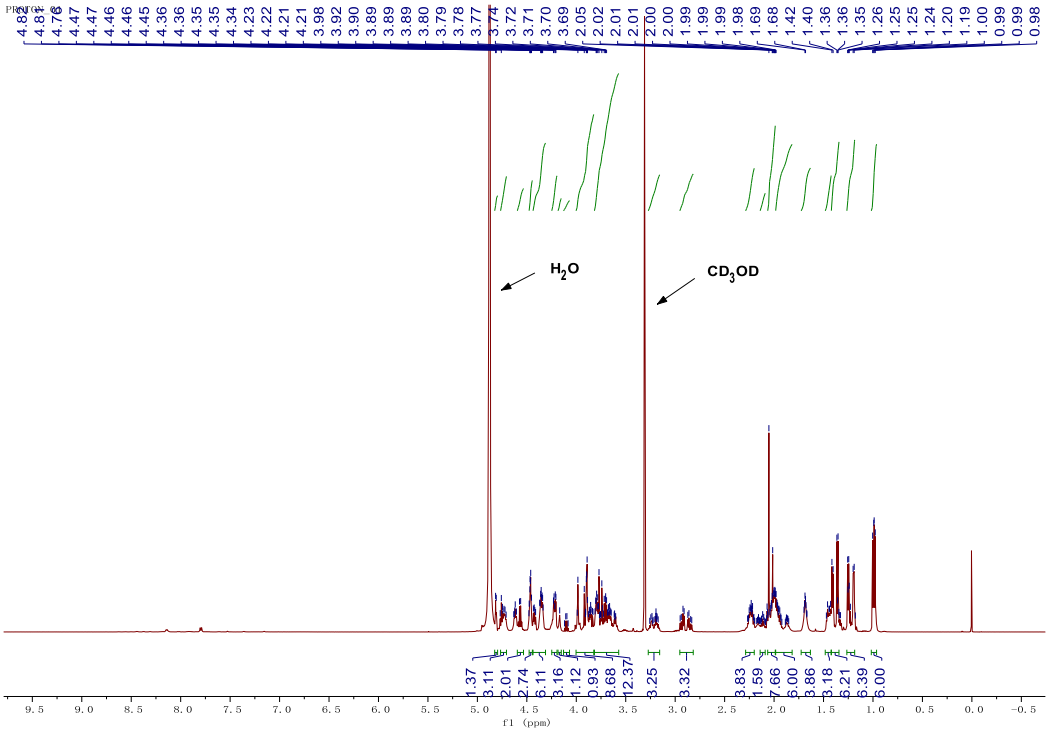


Figure S39. ^{1}H NMR spectrum for MUC1 glycopeptide squaric acid monoamide **10**. Related to Scheme 2.

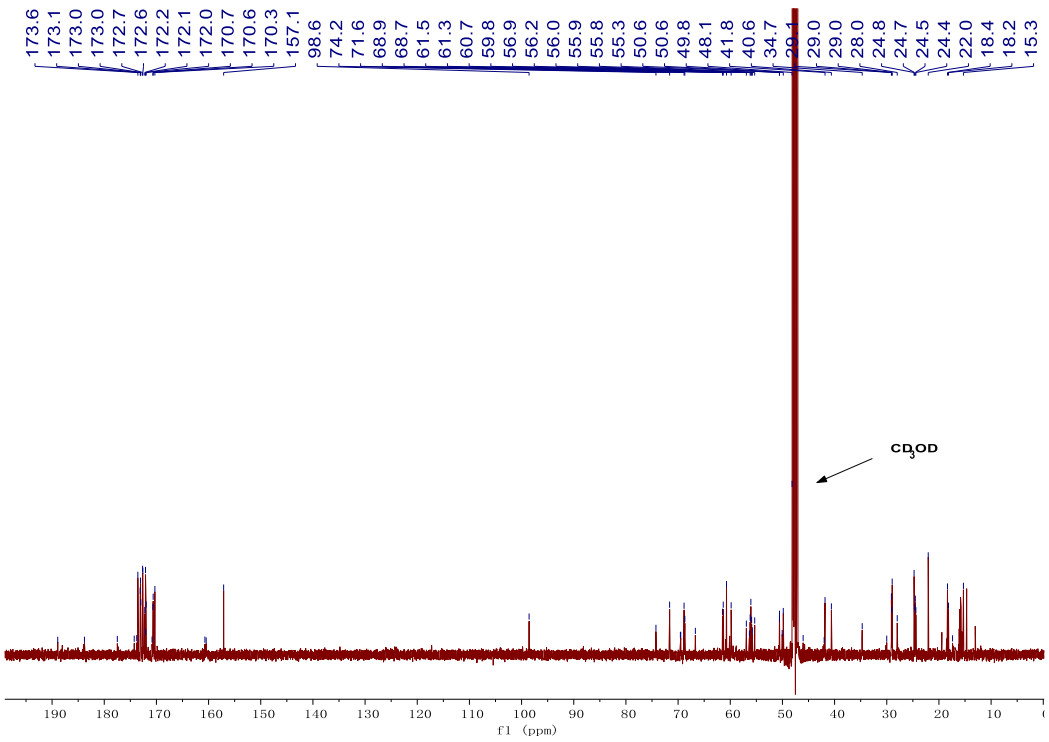


Figure S40. ^{13}C NMR spectrum for MUC1 glycopeptide squaric acid monoamide **10**. Related to Scheme 2.

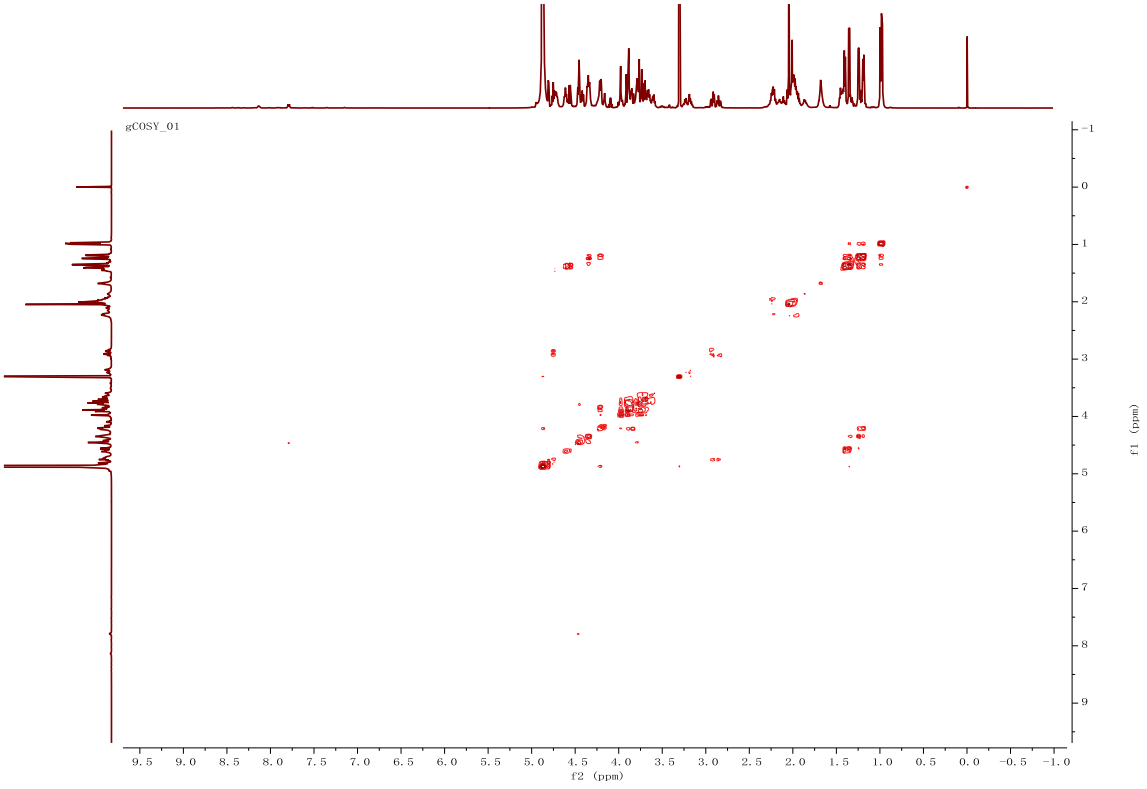


Figure S41. COSY of MUC1 glycopeptide squaric acid monoamide **10**. Related to Scheme 2.

f. Biotin-MUC1 glycopeptide 11

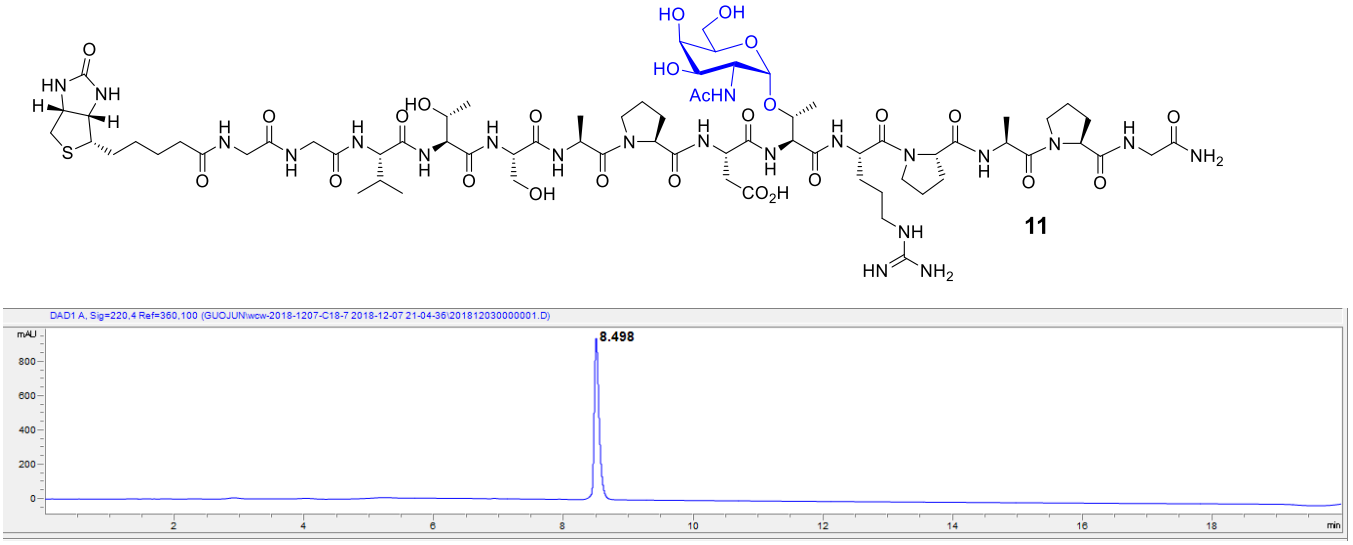


Figure S42. Analytical HPLC trace of biotin-MUC1 glycopeptide **11**. (26 mg, 46% yield). Analytic gradient is 5% to 60% of solution B (acetonitrile with 0.1% trifluoroacetic acid) in solution A (water with 0.1% trifluoroacetic acid) in 20 min on the analytic C18 column. Related to Figures 3 and 4.

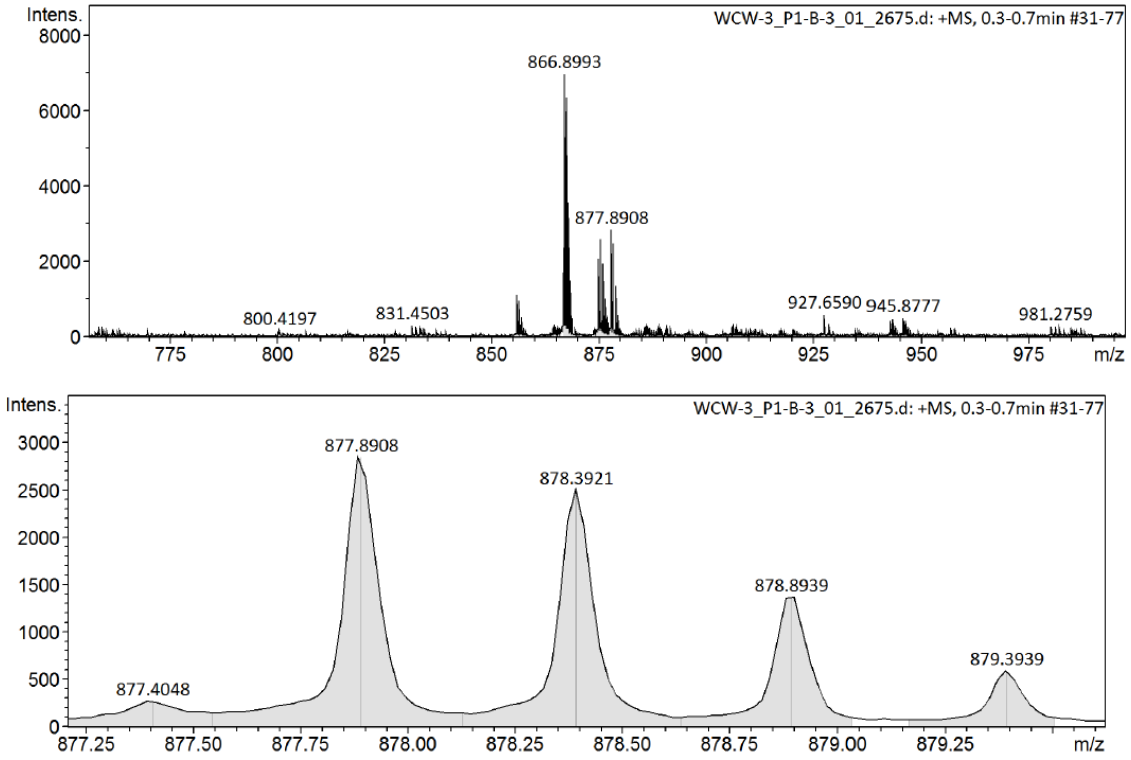


Figure S43. ESI-Q-TOF MS data of biotin-MUC1 glycopeptide **11**. Related to Figures 3 and 4.

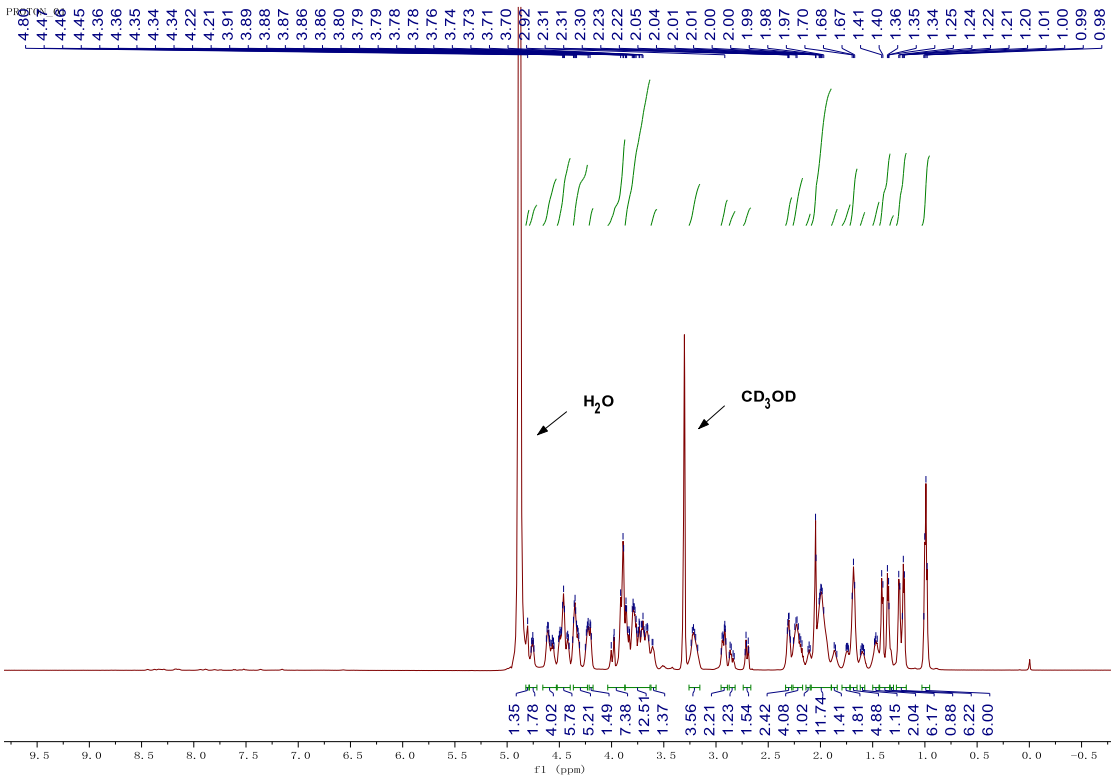


Figure S44. ^{1}H NMR spectrum for biotin-MUC1 glycopeptide **11**. Related to Figures 3 and 4.

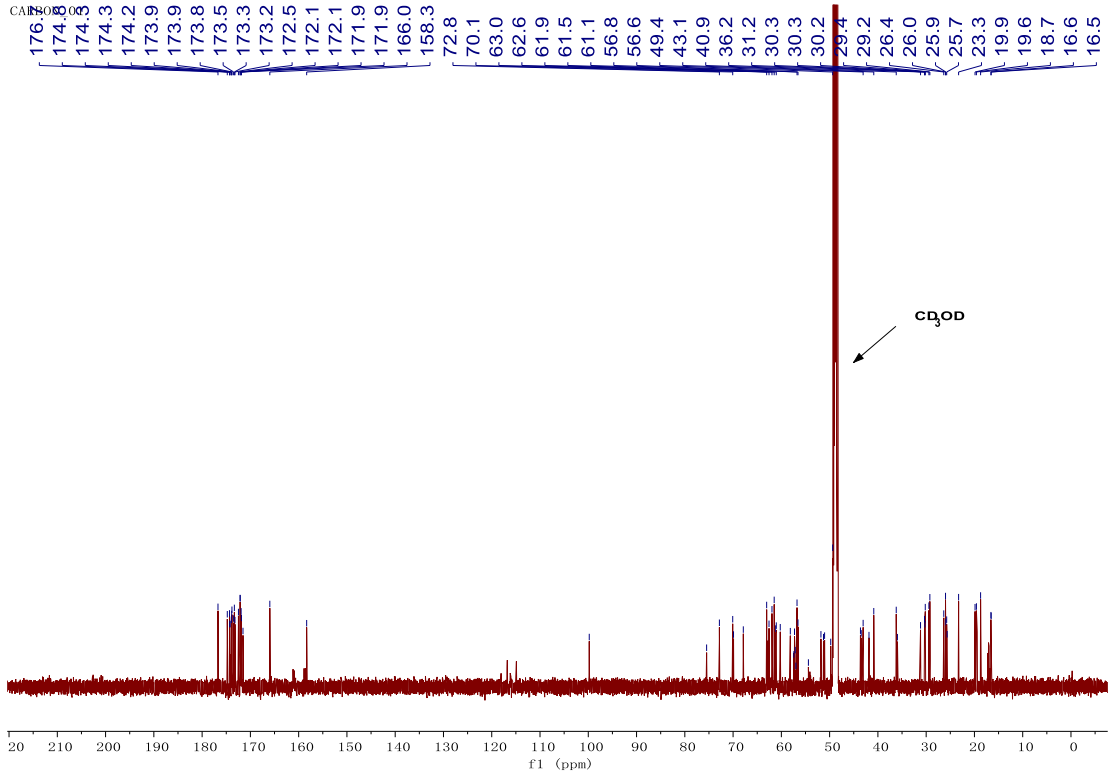


Figure S45. ^{13}C NMR spectrum for biotin-MUC1 glycopeptide **11**. Related to Figures 3 and 4.

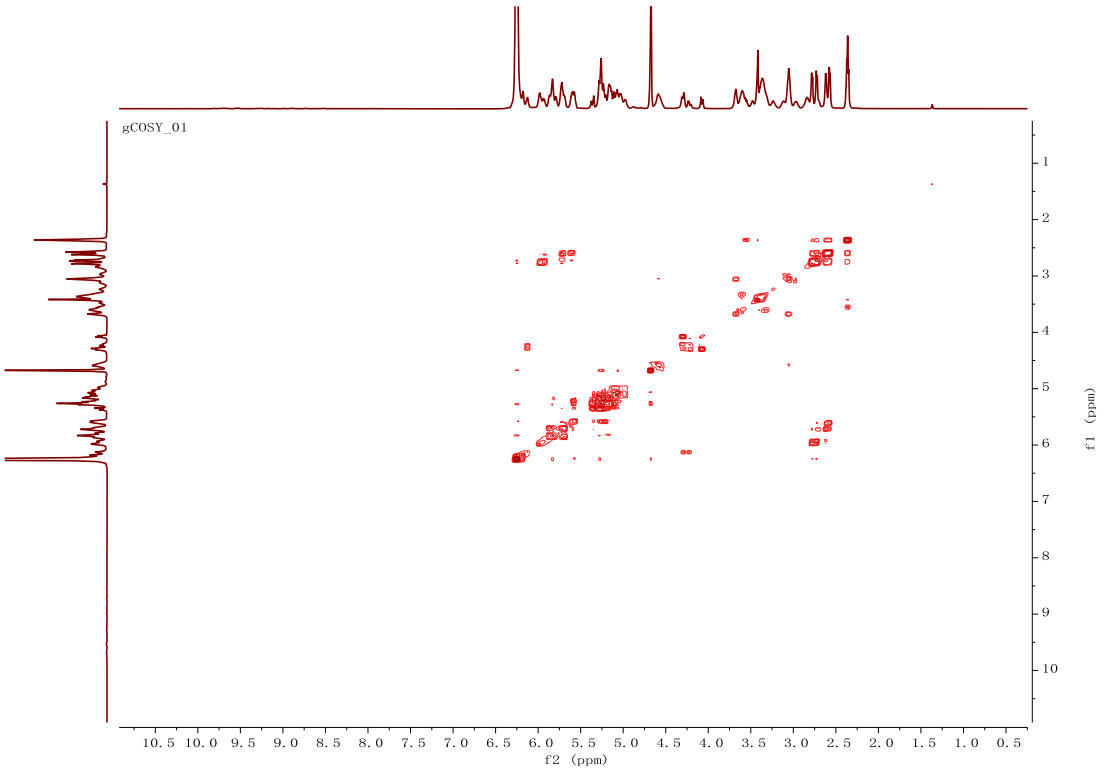


Figure S46. COSY of biotin-MUC1 glycopeptide **11**. Related to Figures 3 and 4.

g. BSA-MUC1 (12)

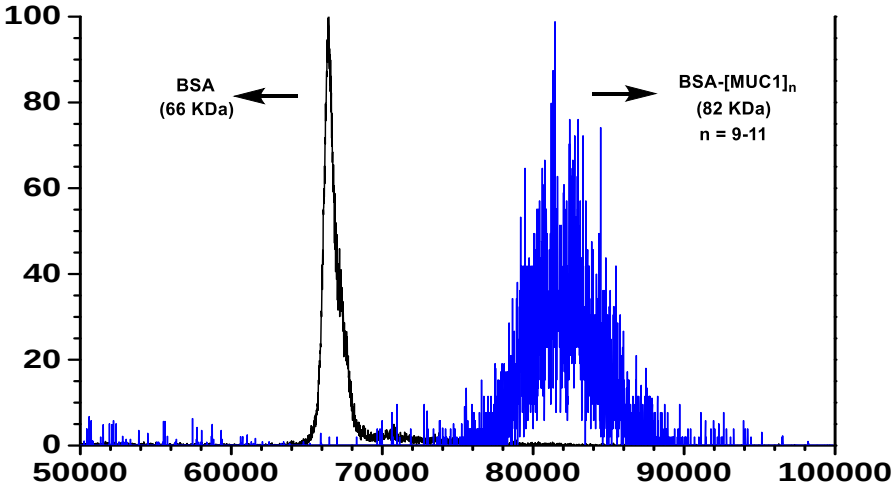


Figure S47. MALDI-TOF-MS analysis of BSA-MUC1 (**12**). Sinapic acid containing 0.1% TFA was used as the matrix. Related to Scheme 2.

h. TLR7a-BSA (14)

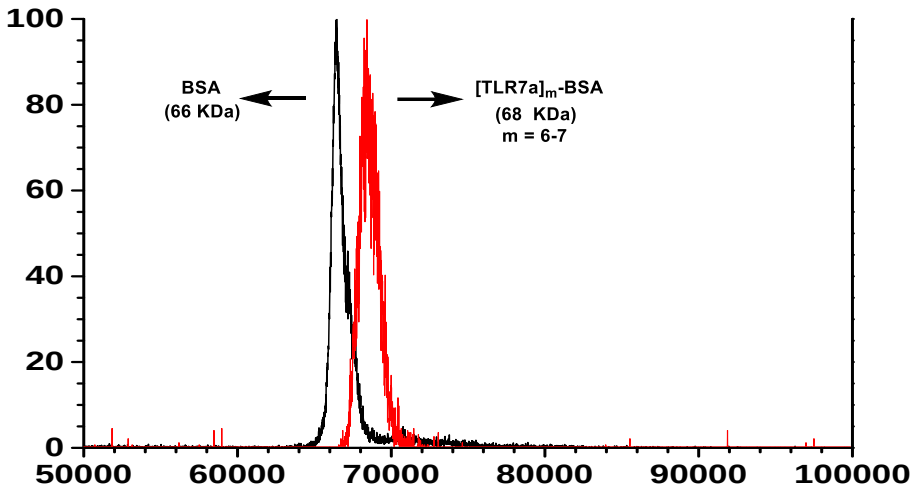


Figure S48. MALDI-TOF spectrum of BSA calibration standard and conjugated product TLR7a-BSA (**14**). Sinapic acid containing 0.1% TFA was used as the matrix. Related to Scheme 2.

i. TLR7a-BSA-MUC1 (15)

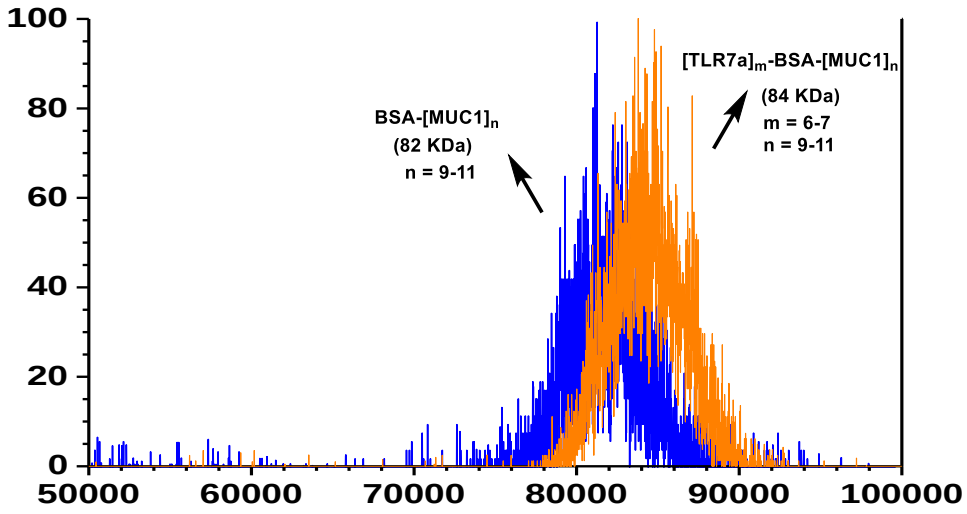


Figure S49. MALDI-TOF spectrum of BSA-MUC1 calibration standard and conjugated product TLR7a-BSA-MUC1 (**15**). Sinapic acid containing 0.1% TFA was used as the matrix. Related to Scheme 2.