

## Supporting Information

### **Potentiating Immune Response of MUC1-based Antitumor Vaccine Using Peptide-Based Nanovector as a Promising Vaccine Adjuvant**

Yonghui Liu, Youzhi Wang, Fan Yu, Zhenqing Zhang, Zhimou Yang, Wenpeng

Zhang, Peng George Wang, Wei Zhao

### Table of Contents

<b>Materials and instruments.....</b>	<b>1</b>
<b>Analytical data.....</b>	<b>2</b>
<b>Immunological studies.....</b>	<b>7</b>
<b>References.....</b>	<b>10</b>

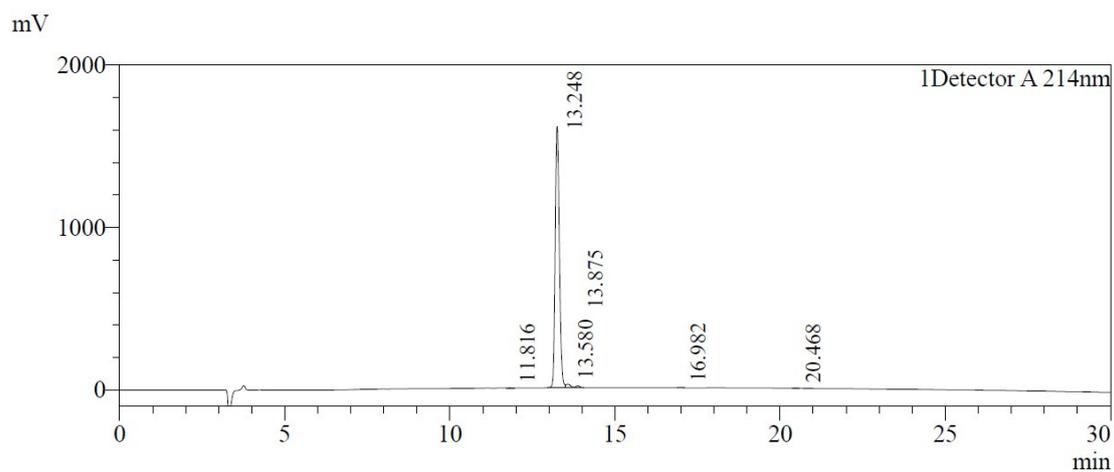
## Materials and instruments

All reagents were purchased from commercial sources and were used without further purification. Fmoc-amino acids were purchased from GL Biochem (shanghai) Ltd. Horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM were purchased from Southern Biotech (AL, USA). High-resolution electrospray-ionization mass spectra (HRESIMS) were obtained on a Varian QFT-ESI mass spectrometer. Matrix-assisted laser desorption/ionization time of flight mass spectra (MALDI-TOF MS) were performed using 2, 5-dihydroxybenzoic acid (DHB) or  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) as matrix on Varian 7.0T FTMS instrument. Reversed-phase HPLC separations were performed on a Waters system 2487 using solution A (0.1% trifluoroacetic acid in 100% acetonitrile) and solution B (0.1% trifluoroacetic acid in 100% water) for elutions. UV absorption signals were detected with an UV detector at a wavelength of 220nm. Semi-preparative HPLC was used for separation and purification of the peptides on a C-18 column (10×250 mm) at a flow rate of 2 mL/min.

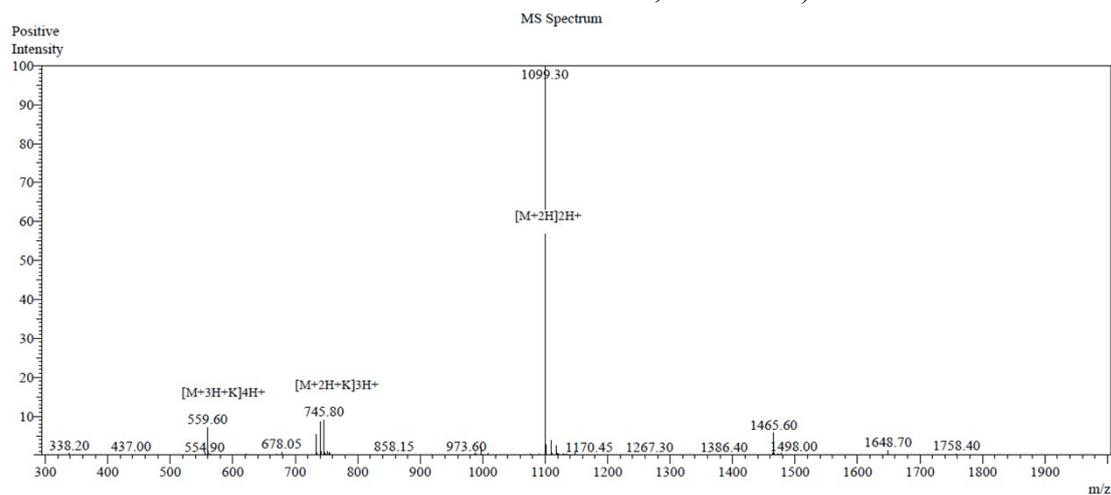
## General protocol of (Glyco)peptides synthesis

(Glyco)peptides were synthesized according to standard Solid Phase Peptide Synthesis (SPPS) procedure. The peptide synthesis was conducted with natural Fmoc amino acids or D-amino acids (include Fmoc-D-Lys(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-D-Phe-OH) utilizing 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 4.0 equiv), 1-hydroxybenzotriazole (HOBt, 4.0 equiv), N,N-diisopropylethylamine (DIPEA, 8.0 equiv). *O*-glycosylated Fmoc amino acid and 2-Naphthaleneacetic acid were introduced using more reactive 1-[dis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU, 2.0 equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 2.0equiv), DIPEA (4.0 equiv). The acetyl moieties of the Tn antigen were removed by MeONa/MeOH (PH=10~11). All acid-sensitive side chain protecting groups were removed and the (glyco)peptides were detached from the resin by adding 90% TFA, 5% TIPS, 5% H<sub>2</sub>O. The (glyco)peptides were purified by semi-preparative HPLC on a C18 column. The white powder was obtained after freeze-drying. The synthetic process of vaccine candidate C-MUC1(Tn)-Nap glycopeptide was showed in Scheme S1.

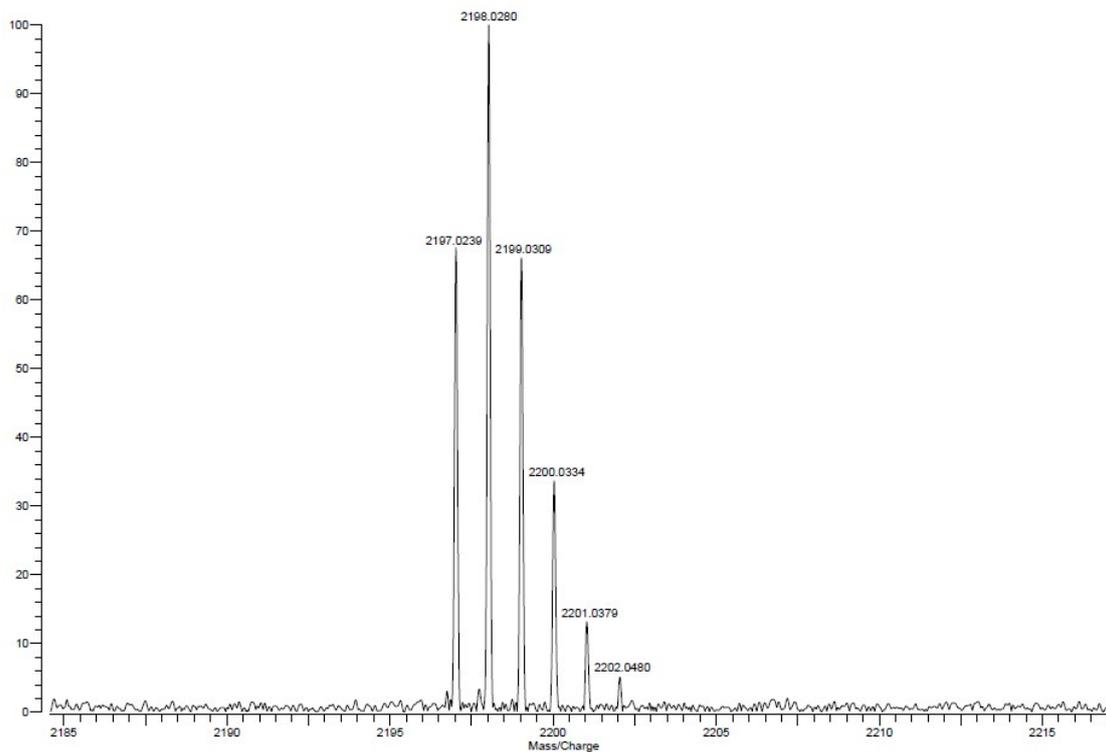




Analytical HPLC:  $R_t$  (retention time) = 13.2 min (20-100% of acetonitrile and 0.1% trifluoroacetic acid over 30 min on a C-18 column,  $\lambda=214$  nm)

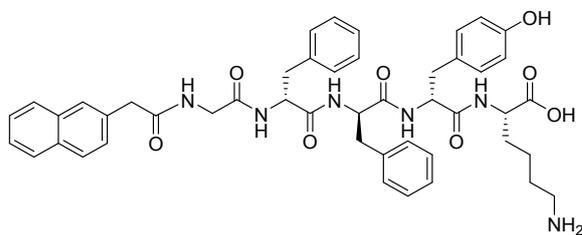


ESI-MS:  $m/z$  for  $C_{103}H_{141}N_{23}O_{31}$   $[M+2H]^{2+}$  calcd 1099.51, found 1099.30;  $[M+2H+K]^{3+}$  calcd 745.99, found 745.80;  $[M+3H+K]^{4+}$  calcd 559.50, found 559.60.

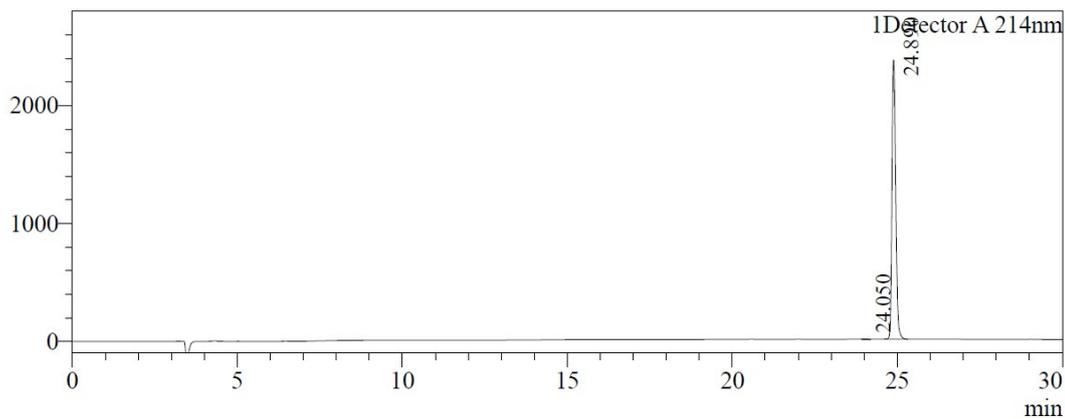


MALDI-TOF-MS:  $C_{103}H_{141}N_{23}O_{31}$   $[M+H]^+$  calcd 2197.0242, found 2197.0239.

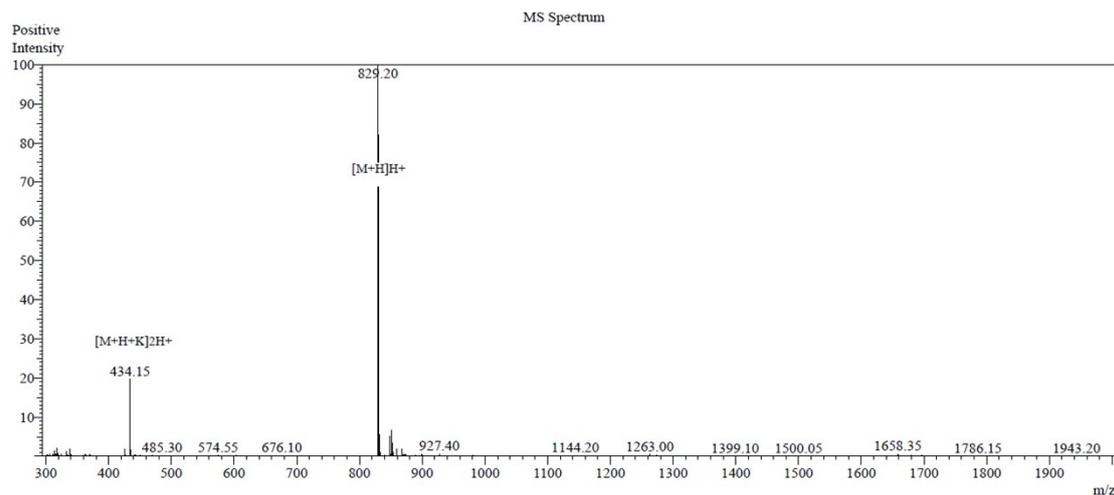
2) Nap-G<sup>D</sup>F<sup>D</sup>F<sup>D</sup>Y<sup>D</sup>K



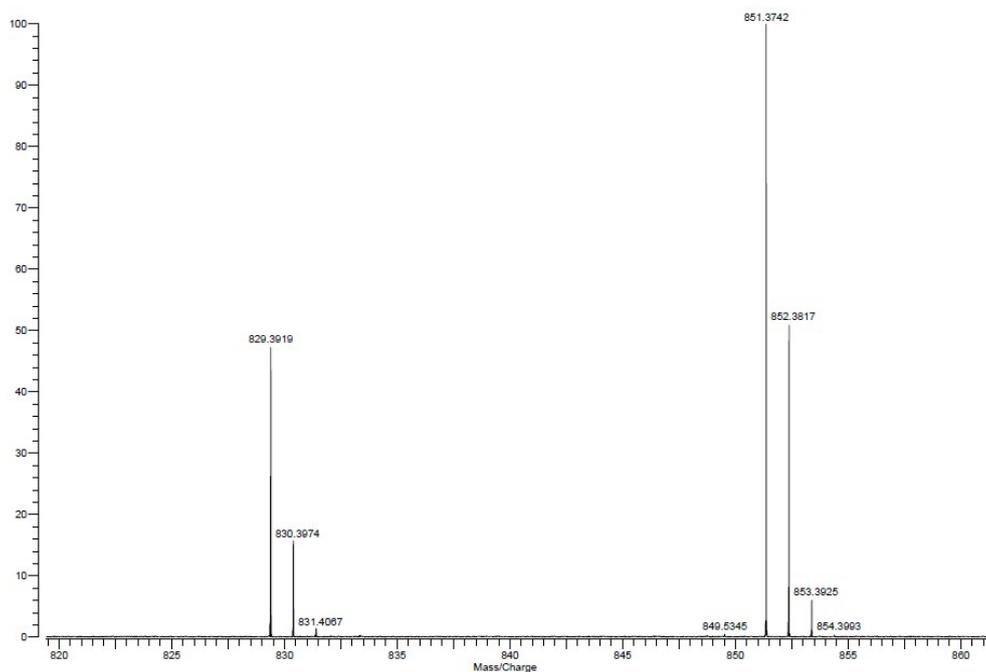
mV



Analytical HPLC:  $R_t$  (retention time) = 24.8 min (20-100% of acetonitrile and 0.1% trifluoroacetic acid over 30 min on a C-18 column,  $\lambda=214$  nm)

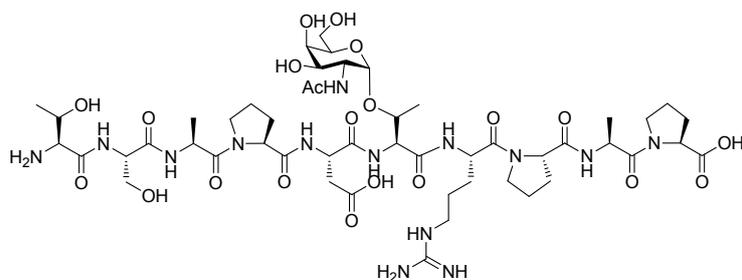


ESI-MS:  $m/z$  for  $C_{47}H_{52}N_6O_8$   $[M+H]^+$  calcd 829.39, found 829.20;  $[M+H+K]^{2+}$  calcd 434.18, found 434.15.

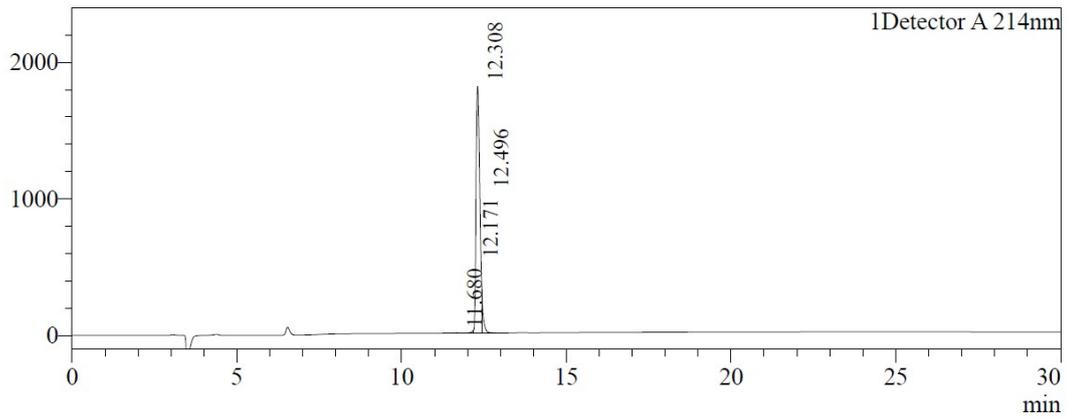


MALDI-TOF-MS:  $C_{47}H_{52}N_6O_8$   $[M+H]^+$  calcd 829.3925, found 829.3919;  $[M+Na]^+$  calcd 851.3744, found 851.3742.

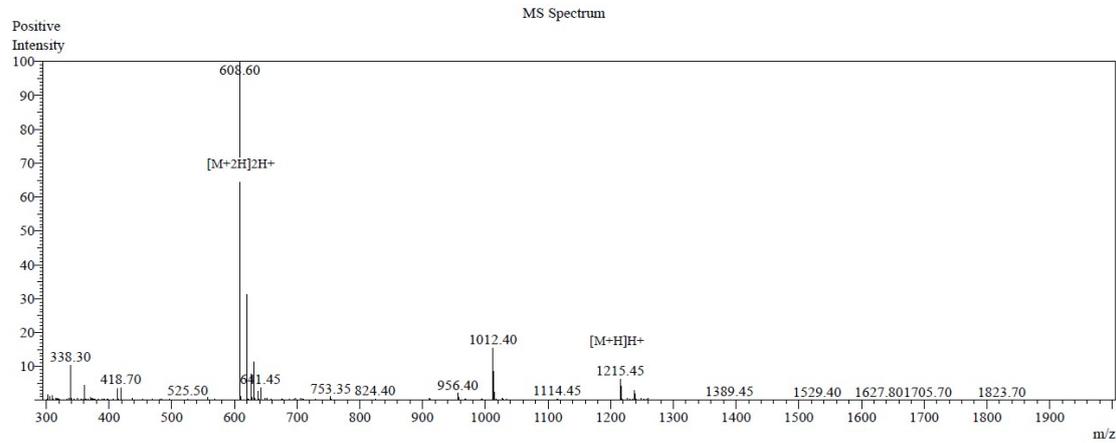
### 3) TSAPDT( $\alpha$ -D-GalNAc)RPAP



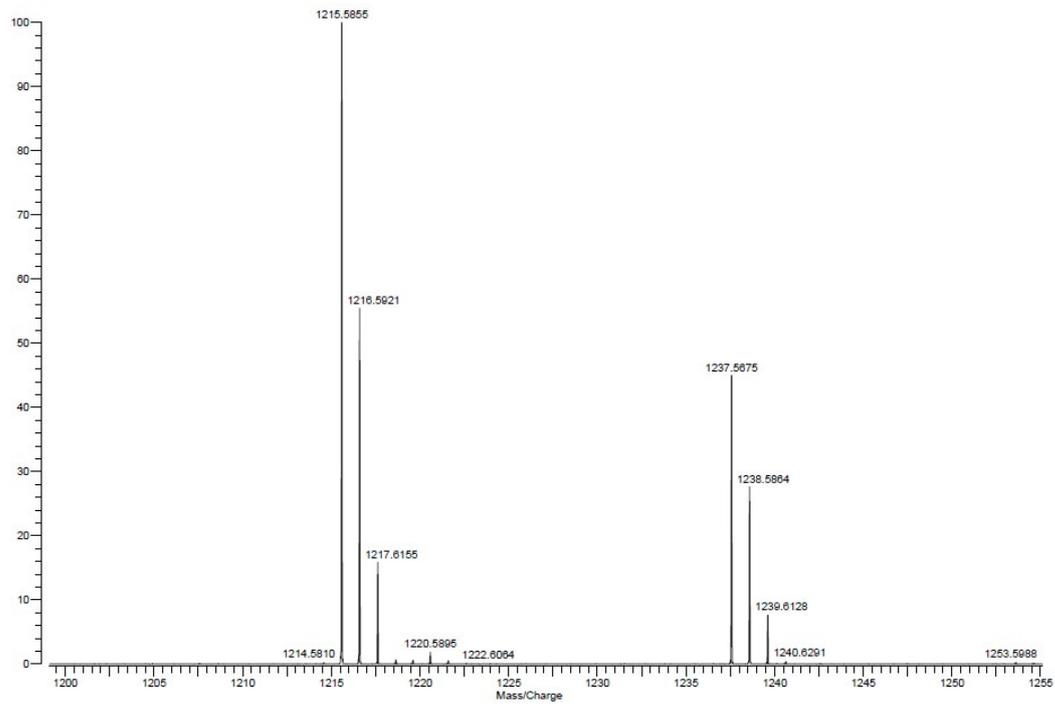
mV



Analytical HPLC:  $R_t$  (retention time) = 12.3 min (10-65% of acetonitrile and 0.1% trifluoroacetic acid over 30 min on a C-18 column,  $\lambda=214$  nm)



ESI-MS:  $m/z$  for  $C_{50}H_{82}N_{14}O_{21}$   $[M+H]^+$  calcd 1215.58, found 1215.45;  $[M+2H]^{2+}$  calcd 608.30, found 608.60.



MALDI-TOF-MS:  $C_{50}H_{82}N_{14}O_{21}$   $[M+H]^+$  calcd 1215.5857, found 1215.5855;  
 $[M+Na]^+$  calcd 1237.5677, found 1237.5675.

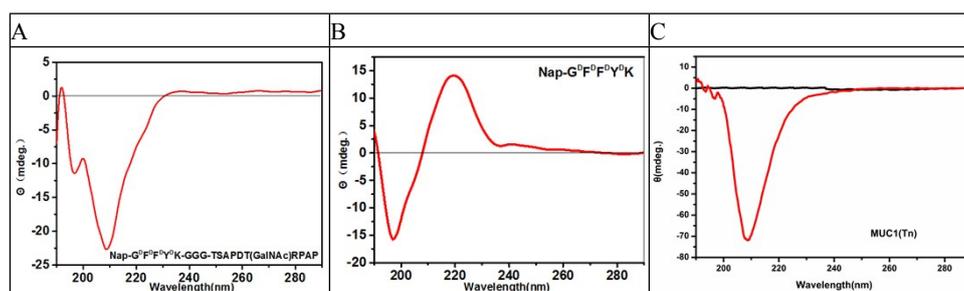
This is consistent with previous report.<sup>1</sup>

### TEM sample preparation

(Glyco)peptide was dissolved in PBS solution in injection concentration. 5  $\mu$ L of each sample was dropped to copper grids with carbon support films. Removed the excess solution with filter paper and washed the copper grid three times with 5  $\mu$ L double distilled water. Uranyl acetate was used to dye the sample. The grids were allowed to dry in air overnight, and then observed with JEM100CXII system operating at 100 kV.

### Circular Dichroism Spectrum

Circular dichroism (CD) spectra was performed on BioLogic MOS-450 under room temperature (22–25 °C).



**Fig. S1** (A) CD spectrum of C-MUC1(Tn)-Nap conjugate. (B) CD spectrum of Nap-G<sup>D</sup>F<sup>D</sup>F<sup>D</sup>Y<sup>D</sup>K nanomaterials. (C) CD spectrum of MUC1(Tn)

### Immunological studies

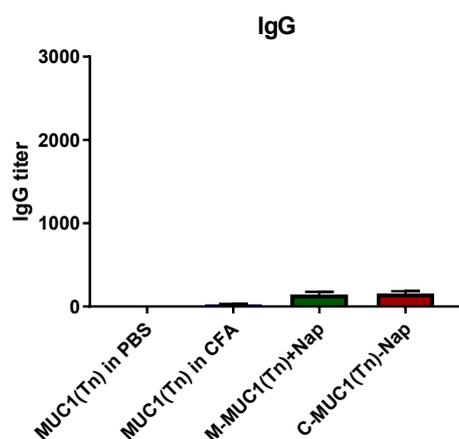
#### Vaccination

All the mice experiments were performed at Beijing Institute of Pharmacology and Toxicology, following the protocol of the Institutional Animal Care and Use Committee, which was in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. Female Bab/c mice (6-8 weeks old, 5 mice/group) were vaccinated via subcutaneous injections of vaccine candidates containing 10  $\mu$ g glycopeptide antigen TSAPDT( $\alpha$ -GalNAc)RPAP in 100  $\mu$ L PBS on days 0, 14, 28, 42. Blood sample were collected via tail vein 14 days after the last vaccination and the anti-sera were prepared for further analysis.

#### Enzyme-Linked Immunoabsorbent Assay (ELISA)

High-binding 96-Well ELISA plates were coated with glycopeptide\_TSAPDT( $\alpha$ -GalNAc)RPAP (20  $\mu$ g/mL) in a coating buffer and incubated overnight at 4 °C. The

plates were washed three times with PBST (0.1% Tween-20 in PBS buffer). The plates were then blocked with 1% BSA in PBST for 1 h at room temperature. After blocking, the mouse sera diluted with 1% BSA in PBST were added to the plates at the setting dilutions and incubated 2 h at room temperature. The plates were washed three times and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, IgM at corresponding working concentration for 1 h at room temperature. The plates were then washed four times and TMB substrate was added. After 30 min, 0.5 M H<sub>2</sub>SO<sub>4</sub> solution was added to quench the reaction. The absorbance was measured at 450 nm.



**Fig. S2** The titers of IgG of antisera immunized with vaccine candidates. Microtiter plates coated with NapG<sup>DP</sup>F<sup>DP</sup>F<sup>DP</sup>Y<sup>DP</sup>K. Antibody titers were defined as the biggest dilution numbers of the sera yielding an optical density of 0.1 or higher than the negative control sera, and calculated from linear regression analysis by using absorbance versus dilution numbers. Bars represent mean ± SD.

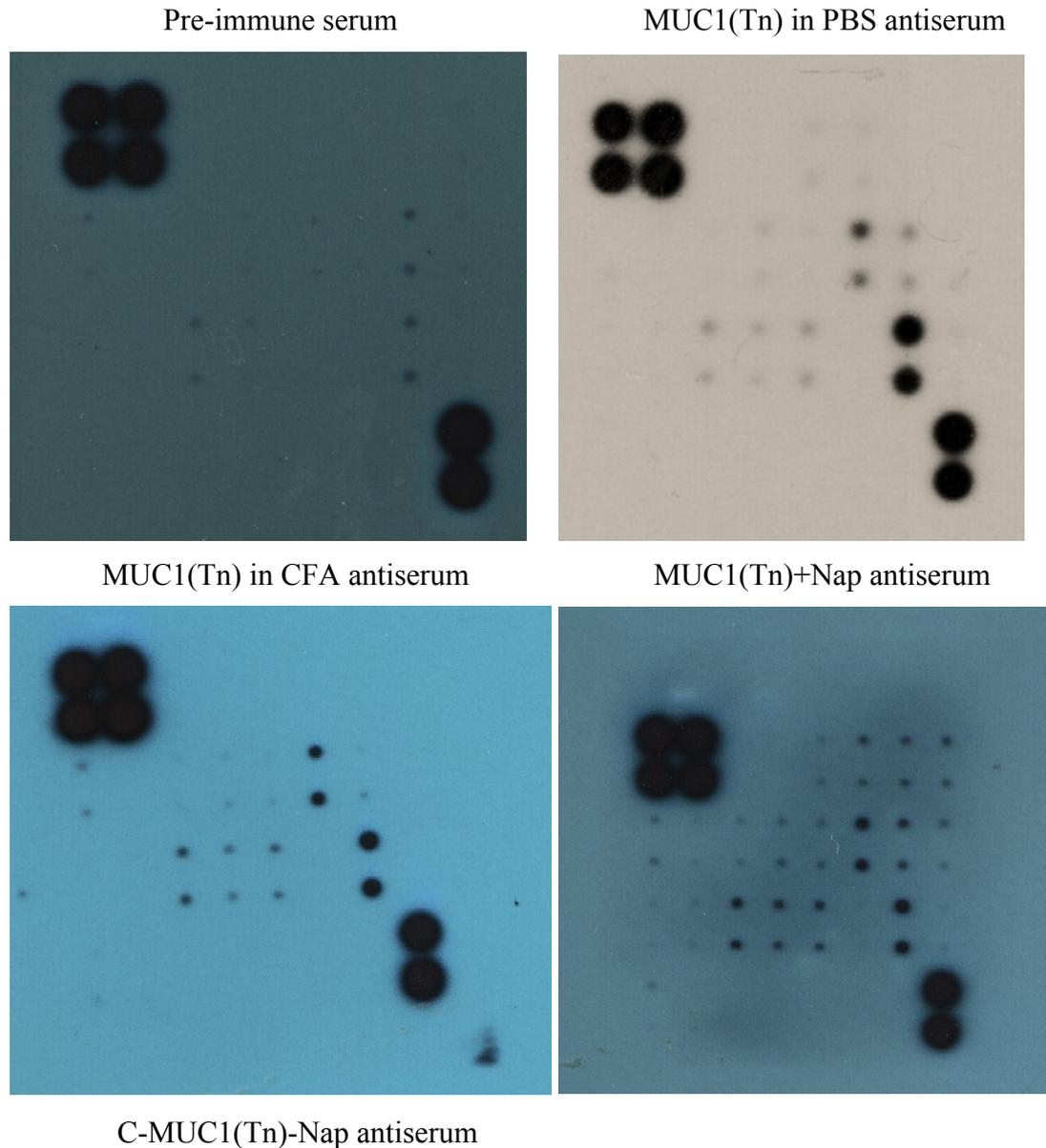
### Mice sera cytokine profile

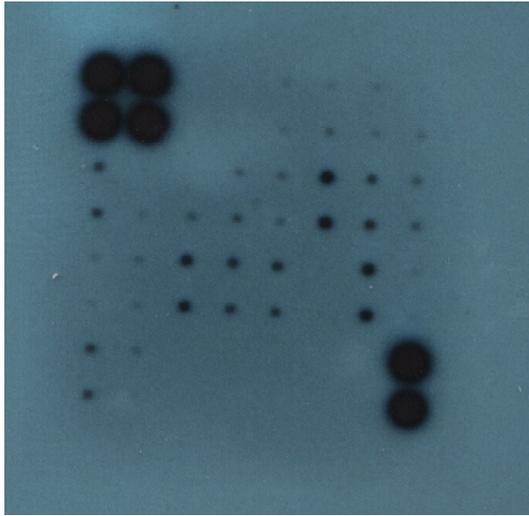
The cytokine profile in the sera after immunization was screened using Mouse Cytokine Antibody Array (Abcam, USA). The experiment was carried out as described in the manual. The cytokine antibody array membranes were incubated with mouse antisera (1:10 diluted in blocking buffer) after being blocked by blocking buffer for 30 min. The biotin-conjugated anti-cytokine antibodies mixture was then applied to incubate with each membrane at room temperature for 2 h. The member was washed, following by incubation with HRP-conjugated streptavidin. The membranes were sent to exposure on X-ray film after the addition of chemiluminescence detection buffer. After exposure, the X-ray film were scanned and the images were processed by Image J software (NIH, USA) to obtain the densitometry data of each image. The densitometry data from different membranes was subtracted for the background and normalized to the positive

control spot on each membrane before the further comparison between membranes. For each treatment group, antisera from three to five mice were assayed separately and the results were presented as mean  $\pm$  SD in the figures.

### Raw data for the cytokine release assays

Compared with pre-immune mouse sera, the relative expression levels of IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IFN- $\gamma$ , MCP-1, MCP-5, RANTES and TNF- $\alpha$  in the antiserum were evaluated, the following figure is a set of representative results.





POS	POS	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
POS	POS	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12p70	IL-13
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p71	IL-12p71	IL-14
IL-17	IFN- gamma	MCP-1	MCP-5	RANTES	SCF	sTNF RI	TNF- $\alpha$
IL-18	IFN- gamma	MCP-1	MCP-5	RANTES	SCF	sTNF RI	TNF- $\alpha$
Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	POS
Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	POS

**Fig. S3** The membrane map for the experiments of expression levels of cytokines

### FACS assay

MUC1 expressing human breast cancer cells MCF-7 were cultured with DMEM culture fluid containing fetal bovine serum (FBS, 10%) at 37°C. The tumor cells were digested with 0.25% (w/v) trypsin solution, following by washing with PBS solution containing 1% FBS. The cells suspensions were then incubated with antiserum (1:50 in PBS containing 1% FBS) at 4°C for 1h. After washing with PBS (1% FBS), the cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody (diluted 1:1000 in PBS containing 1% FBS, Sigma) at 4°C for 1h. After washing with PBS (1% FBS), the cells were suspended in washing buffer (1 mL) and filtered through 200 mesh sieve, FACS analysis was performed on BD FACS Aria III flow cytometry.

### **Complement-dependent Cytotoxicity**

MCF-7 cells suspension (100  $\mu$ L per well) were planted into 96-well cell plate, and were then cultured for 12 h (37°C, 5% CO<sub>2</sub>). Antisera (diluted 10 times with culture medium, 50  $\mu$ L per well) were then added and incubated for 1 h (37°C, 5% CO<sub>2</sub>). Following by adding rabbit complement (diluted 2 times with culture medium, 50  $\mu$ L per well), the cells were then cultured for 12 h (37°C, 5% CO<sub>2</sub>). Adding CCK-8 solution (10  $\mu$ L per well), the optical absorption was then measured at the wavelength of 450 nm after the cells were cultured for 2 h. The experiment was repeated for three times. Cells which were cultured in culture medium adding CCK-8 only were used as control. The cytotoxicity was calculated according to the following formula.

$$\text{Cytotoxicity (\%)} = [\text{control OD} - \text{experimental OD}] / \text{control OD} \times 100$$

S1. S. Ingale, M. A. Wolfert, J. Gaekwad, T. Buskas and G. J. Boons, *Nat. Chem. Biol.*, 2007, **3**, 663-667.