Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Targeted antigen delivery by an anti-Class II MHC VHH elicits focused αMUC1(Tn) immunity

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1. General Information

All animal procedures were performed according to US National Institutes of Health guidelines and approved by the Committee on Animal Care at MIT. 6- to 12- week old female C57BL/6 mice were maintained on normal diet and housed at 5 animals per cage.

All reagents were obtained from commercial sources and used as purchased. Unless noted otherwise, all reactions were carried out at room temperature. LC-MS spectra were recorded on a Waters Xevo system equipped with UPLC-C8 or C18 columns. Flow cytometry analysis was collected on BD FACS Canto II equipped with blue, red and violet lasers. ELISA was recorded on plate reader SpectraMax M3 (Molecular Devices).

2. Glycopeptide synthesis

The peptide GGGCTSAPDT(α-*O*-GalNAc)RPAP with a free N-terminus was synthesized on Rink amide resin (200 mg, 0.1 mmol, loading: 0.5 mmol/g). Commercially available Fmoc-Gly_3-OH and Fmoc-Thr[α-O-GalNAc$(OAc)_4$]-OH (Chem-Impex) were used as purchased. The coupling step was done by adding the mixture of Fmoc protected amino acid building blocks (0.5 mmol), HATU (190 mg, 0.5 mmol), *N*,*N*-diisopropylethylamine (DIPEA) (174 µL, 1.0 mmol) dissolved in DMF (5 ml). After shaking for 2 h, the solution was drained, and the resin was deprotected using 20% piperidine in DMF (10 ml) for 20 min with washing between each step. For glycosylated amino acid Fmoc-Thr[α-O-GalNAc$(OAc)_4$]-OH, reagents were used at 50% of the normal equivalent with overnight reaction time. After completion of the sequence, the peptide was cleaved from resin and deprotected by a mixture of TFA/TIPS/EDT/H_2O (90/5/2.5/2.5, v/v) for 3 h. The cleavage solution was precipitated in cold ether, and pelleted by centrifuge. The pellet was redissolved in H_2O, and directly purified by RP-HPLC on a semi-preparative column (C18 column, Gemini, 5 µm, 10 × 250 mm; Phenomenex) at a flow rate of 3.0 mL/min: solvent A, 0.1% TFA in H_2O; solvent B, 0.1% TFA in CH_3CN. Gradient: 5% → 90% in 30 min. Fractions containing pure product were collected and lyophilized. The temporary acetyl protecting groups on O-GalNAc was removed by redissolving the lyophilized power in 5% hydrazine aqueous solution. The deprotection was monitored by LC-MS, and once the deprotection was complete, the mixture was subject to purification on C18 column by an HPLC system directly. Corresponding fractions were further collected and lyophilized. LC-MS calculated for GGGCTSAPDT(α-O-GalNAc)RPAP: $C_{59}H_{97}N_{19}O_{24}S$ $[M+H]^+$ was 1488.68, found 1488.78.

3. Sortase reaction

The pentamutant version of sortase A, with an improved K_{cat}, was used (Addgene: 51140). Reaction mixtures contained Tris·HCl (50 mM, pH 7.5), $CaCl_2$ (10 mM), NaCl (150 mM), triglycine-containing peptide (500 μM), LPETG-containing VHHs (100 μM), and sortase (2.5 μM). After incubation at 16 °C with gentle agitation for 2~4 h, Ni-NTA beads were added to the reaction mixture with gentle shaking for another 30 min at 4 °C. At the end of incubation, the mixture with beads was directly loaded onto a PD-10 column (GE Healthcare). Fractions corresponding to the desired product fail to stick to Ni-NTA beads and were retrieved in the void volume to yield pure VHH-MUC1(Tn) conjugate. The labeled protein was stored at -80 °C with 10% (v/v) glycerol.

4. Vaccination

Mice were immunized intraperitoneally on day 1, 14, 21 and 28 using 30 μg/mouse/injection of VHH7-MUC1(Tn) conjugate or an equimolar amount of antigen for the other conjugates. The final formulation also contained 25 μg/mouse/injection of αCD40 (1C10, eBioscience) and 50 μg/mouse/injection of polyinosinic–polycytidylic acid sodium salt (P0913 Sigma-Aldrich) as adjuvants. The following adjuvants were used for comparison and administered at 20 μg/mouse of MPLA (Avanti), or 5 ug/mouse of QS21 (Desert King International). For induction of a long-term memory response, animals received booster injections at 60 weeks after the initial immunization. Sera were collected 5 days after the last immunization.

5. Mouse immunoglobulin isotyping

ELISA plates (high protein binding 96-well plate) were coated overnight at 4 °C with MUC1(Tn)–BSA (5 μg/ml) in 0.1 M bicarbonate/carbonate coating buffer. The plates were washed three times with washing buffer (PBS^-, 2% IFS, 0.5% Tween 20) followed by blocking with blocking buffer (PBS^-, 2.5% BSA, 0.5% Tween 20) for 2 h at room temperature. Next, plates were washed and incubated for 1 h at room temperature with serum dilutions in blocking buffer. Excess serum was removed; plates were washed three times, and incubated with isotype-specific goat anti-mouse Ig antibodies (Sigma) at a 1:1000 dilution in blocking buffer for 1 h at room temperature. Plates were washed three times by washing buffer. Peroxidase-labeled rabbit anti-Goat IgG was diluted 1:1000 in blocking buffer and incubated at room temperature for 1 h. At the end of incubation, the plates were washed three times by washing buffer. 3,3,5,5-tetramethylbenzidine (TMB) ELISA substrate solution (T4319, Sigma-Aldrich) was added to wells. The reaction was quenched by adding an equal volume of 1 M HCl solution. The absorbance was recorded on a microplate reader at 450 nm.

6. Inhibition ELISA

The ELISA plates were coated with MUC1(Tn)–BSA and blocked as the same protocol above. During blocking, sera from immunized animals were diluted (1:100) in the blocking buffer. In another uncoated microtiter plate, the inhibitors (MUC1-Tn and MUC1) were diluted in the blocking buffer to 2X of designated concentration. An equal volume of serum dilution was added to the inhibitor plate to reach the desired final concentration. After 30 min incubation, 100 µl mixed inhibitor solution from each well was transferred to previously coated and blocked plates. After 2 h incubation at room temperature, the plate was washed three times. Secondary antibody (α-mouse IgG-HRP NXA931 GE Healthcare) at 1:10,000 dilution was added and incubated for 1 h. At the end of incubation, the plates were washed and the colorimetric reaction was developed as above.

7. MUC1(Tn) specific T cell and cytokine responses

Splenic DCs were purified with pan-DC microbeads (130-092-465, Miltenyi Biotec) on MACS separation columns. Purified DCs were activated with 1 µg/ml of anti-CD40 and 12.5 µg/ml of poly(I:C) for 1h in cRPMI supplemented with inactivated bovine fetal serum (10% v/v), washed and incubated with 10 ng/ml VHH adducts for 45 min. DCs were then washed and incubated for 18-36 h with purified splenic T-cells (Pan T cell isolation kit, 130-095-130, Miltenyi Biotec) from mice immunized with VHH7-MUC1(Tn). BFA (2 µg/ml) was added to prepare cell mixtures 16 h prior to flow cytometric analysis.

8. Immunoblot

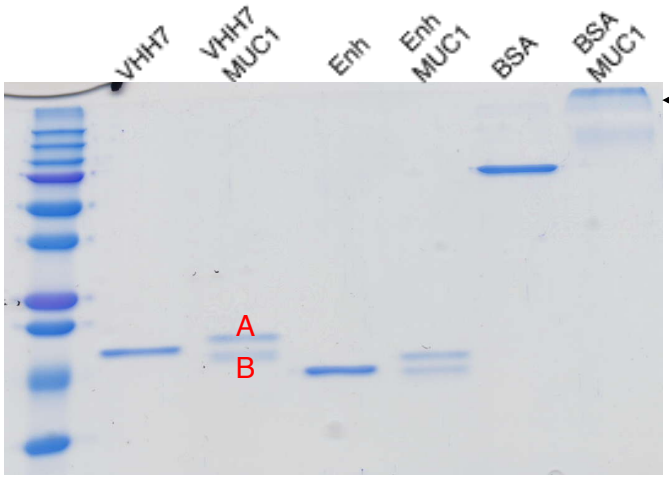
Mouse sera were used for immunoblotting against various concentrations of VHHs and BSA conjugates. Sera were used at 1:2000 dilution; secondary antibodies at 1:10,000 (anti-mouse IgG-HRP NXA931 GE Healthcare). Quantification was done by densitrometry of suitably explosed films (Image Lab, Bio-rad).

9. Long-term memory response

After the initial four immunizations following the standard protocol, mice were given a booster injection after 60 weeks with two injections of the VHH7-MUC1(Tn) construct at one week intervals. Sera were collected 5 days after each injection.

10. Supplemental figures

a)



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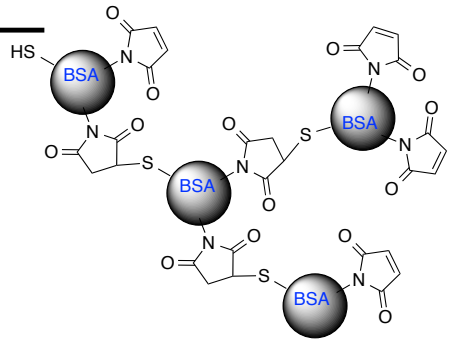

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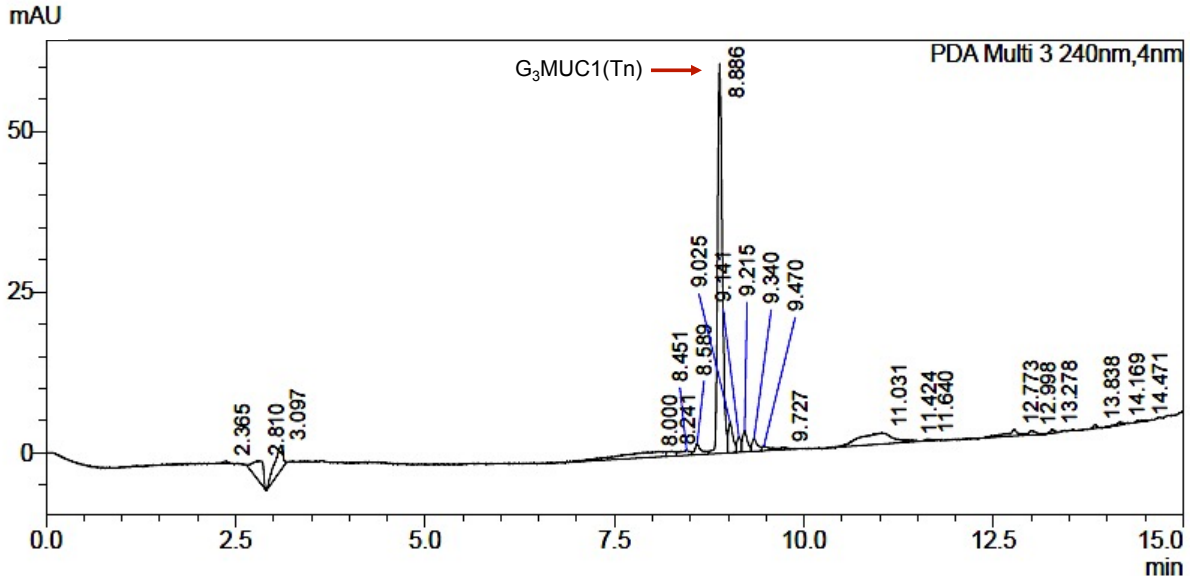

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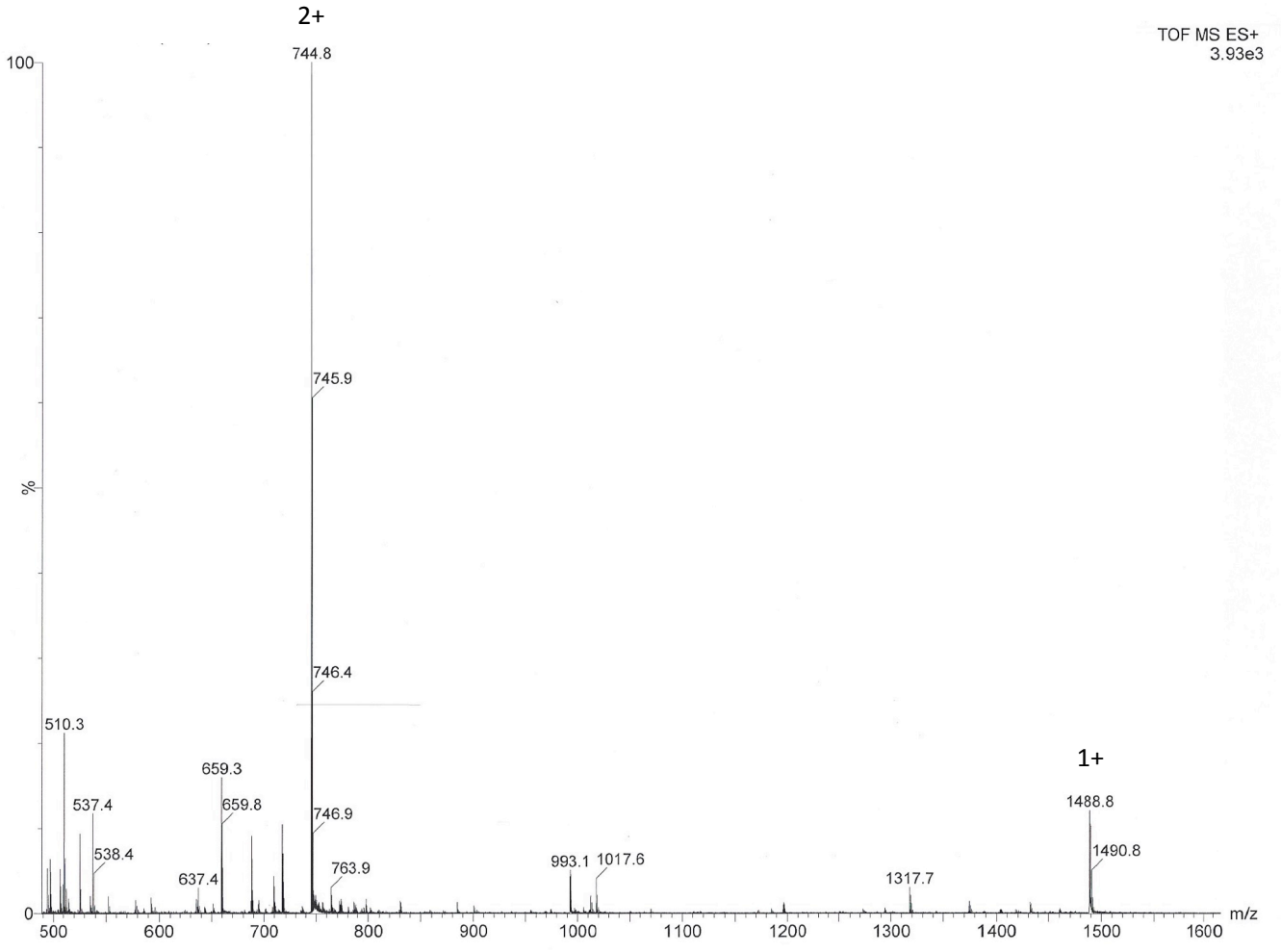
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993.1
1017.6
1317.7
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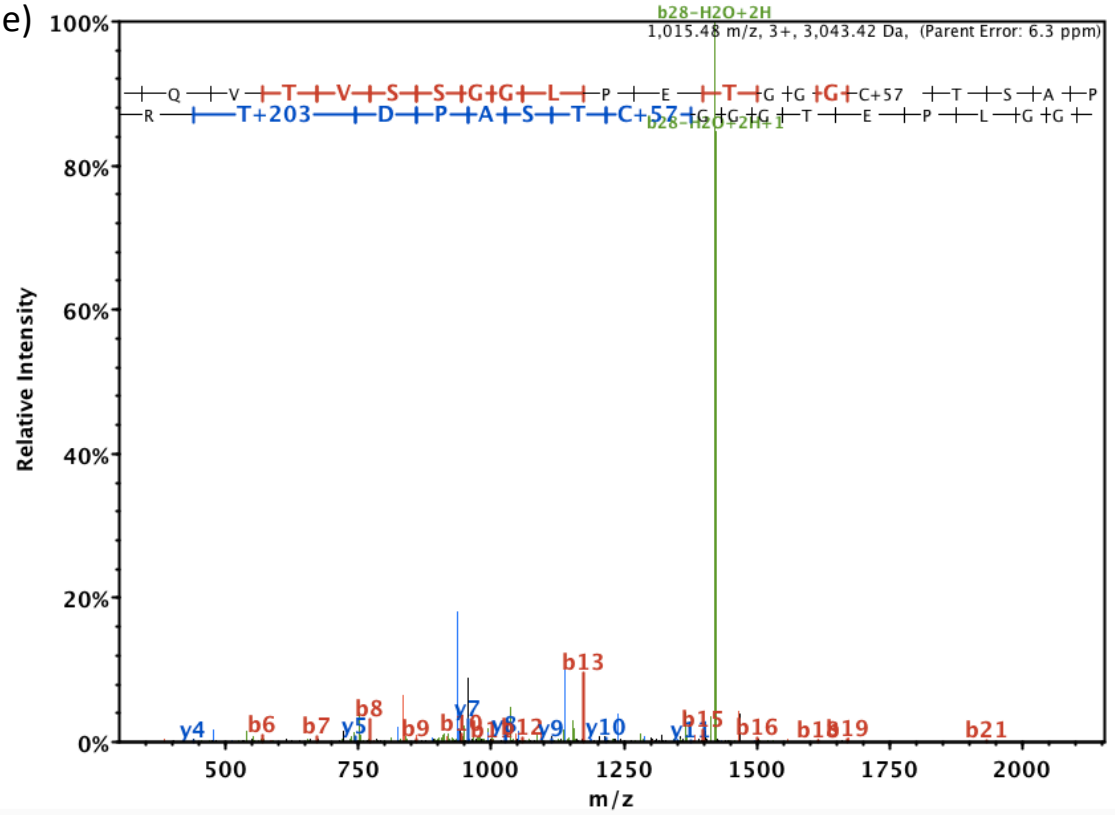
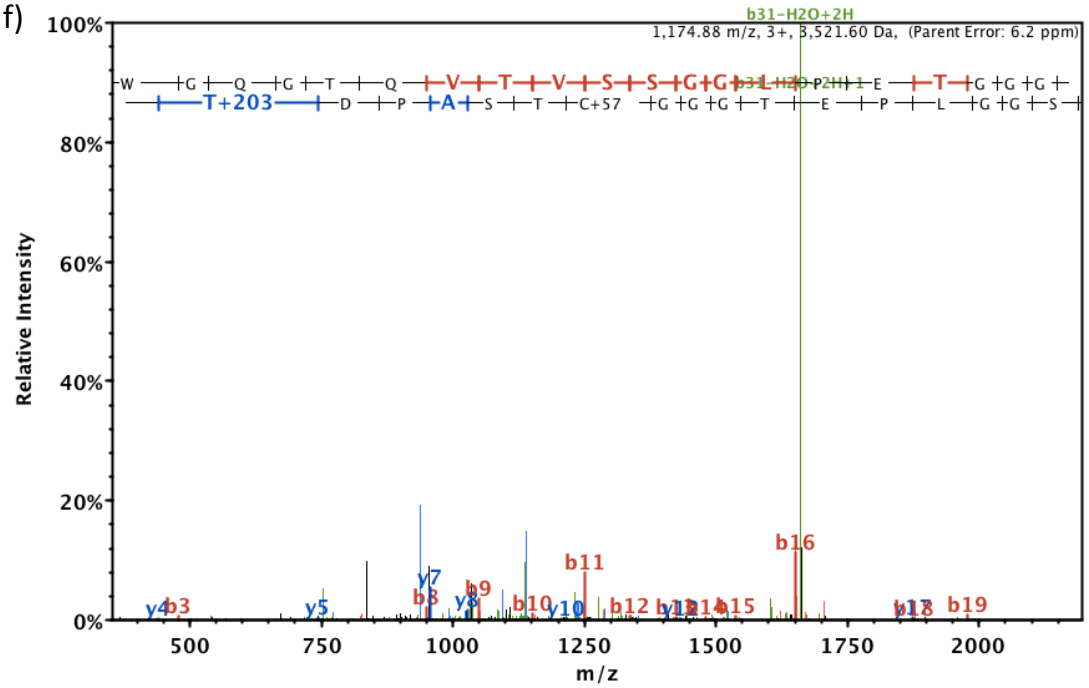
e)



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	58.0	29.5			G	3,045.4	1,523.2	3,028.4	3,027.4	30
2	186.1	93.5	169.1		Q	2,988.4	1,494.7	2,971.4	2,970.4	29
3	243.1	122.1	226.1		G	2,860.3	1,430.7	2,843.3	2,842.3	28
4	344.2	172.6	327.1	326.1	T	2,803.3	1,402.2	2,786.3	2,785.3	27
5	472.2	236.6	455.2	454.2	Q	2,702.3	1,351.6	2,685.2	2,684.2	26
6	571.3	286.1	554.3	553.3	V	2,574.2	1,287.6	2,557.2	2,556.2	25
7	672.3	336.7	655.3	654.3	T	2,475.1	1,238.1	2,458.1	2,457.1	24
8	771.4	386.2	754.4	753.4	V	2,374.1	1,187.5	2,357.1	2,356.1	23
9	858.4	429.7	841.4	840.4	S	2,275.0	1,138.0	2,258.0	2,257.0	22
10	945.5	473.2	928.4	927.5	S	2,188.0	1,094.5	2,171.0	2,170.0	21
11	1,002.5	501.7	985.5	984.5	G	2,101.0	1,051.0	2,083.9	2,082.9	20
12	1,059.5	530.3	1,042.5	1,041.5	G	2,043.9	1,022.5	2,026.9	2,025.9	19
13	1,172.6	586.8	1,155.6	1,154.6	L	1,986.9	994.0	1,969.9	1,968.9	18
14	1,269.6	635.3	1,252.6	1,251.6	P	1,873.8	937.4	1,856.8	1,855.8	17
15	1,398.7	699.8	1,381.7	1,380.7	E	1,776.8	888.9	1,759.7	1,758.8	16
16	1,499.7	750.4	1,482.7	1,481.7	T	1,647.7	824.4	1,630.7	1,629.7	15
17	1,556.8	778.9	1,539.7	1,538.7	G	1,546.7	773.8	1,529.7	1,528.7	14
18	1,613.8	807.4	1,596.8	1,595.8	G	1,489.7	745.3	1,472.6	1,471.6	13
19	1,670.8	835.9	1,653.8	1,652.8	G	1,432.6	716.8	1,415.6	1,414.6	12
20	1,830.8	915.9	1,813.8	1,812.8	C+57	1,375.6	688.3	1,358.6	1,357.6	11
21	1,931.9	966.4	1,914.9	1,913.9	T	1,215.6	608.3	1,198.6	1,197.6	10
22	2,018.9	1,010.0	2,001.9	2,000.9	S	1,114.5	557.8	1,097.5	1,096.5	9
23	2,089.9	1,045.5	2,072.9	2,071.9	A	1,027.5	514.3	1,010.5	1,009.5	8
24	2,187.0	1,094.0	2,170.0	2,169.0	P	956.5	478.7	939.4	938.5	7
25	2,302.0	1,151.5	2,285.0	2,284.0	D	859.4	430.2	842.4	841.4	6
26	2,606.2	1,303.6	2,589.1	2,588.1	T+2...	744.4	372.7	727.4	726.4	5
27	2,762.3	1,381.6	2,745.2	2,744.2	R	440.3	220.6	423.2		4
28	2,859.3	1,430.2	2,842.3	2,841.3	P	284.2	142.6			3
29	2,930.3	1,465.7	2,913.3	2,912.3	A	187.1	94.1			2
30	3,045.4	1,523.2	3,028.4	3,027.4	P	116.1	58.5			1

f)



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	130.0	65.5		112.0	E	3,523.6	1,762.3	3,506.6	3,505.6	33
2	293.1	147.1		275.1	Y	3,394.5	1,697.8	3,377.5	3,376.5	32
3	479.2	240.1		461.2	W	3,231.5	1,616.2	3,214.5	3,213.5	31
4	536.2	268.6		518.2	G	3,045.4	1,523.2	3,028.4	3,027.4	30
5	664.3	332.6	647.2	646.3	Q	2,988.4	1,494.7	2,971.4	2,970.4	29
6	721.3	361.2	704.3	703.3	G	2,860.3	1,430.7	2,843.3	2,842.3	28
7	822.3	411.7	805.3	804.3	T	2,803.3	1,402.2	2,786.3	2,785.3	27
8	950.4	475.7	933.4	932.4	Q	2,702.3	1,351.6	2,685.2	2,684.2	26
9	1,049.5	525.2	1,032.4	1,031.5	V	2,574.2	1,287.6	2,557.2	2,556.2	25
10	1,150.5	575.8	1,133.5	1,132.5	T	2,475.1	1,238.1	2,458.1	2,457.1	24
11	1,249.6	625.3	1,232.6	1,231.6	V	2,374.1	1,187.5	2,357.1	2,356.1	23
12	1,336.6	668.8	1,319.6	1,318.6	S	2,275.0	1,138.0	2,258.0	2,257.0	22
13	1,423.6	712.3	1,406.6	1,405.6	S	2,188.0	1,094.5	2,171.0	2,170.0	21
14	1,480.7	740.8	1,463.6	1,462.7	G	2,101.0	1,051.0	2,083.9	2,082.9	20
15	1,537.7	769.3	1,520.7	1,519.7	G	2,043.9	1,022.5	2,026.9	2,025.9	19
16	1,650.8	825.9	1,633.7	1,632.8	L	1,986.9	994.0	1,969.9	1,968.9	18
17	1,747.8	874.4	1,730.8	1,729.8	P	1,873.8	937.4	1,856.8	1,855.8	17
18	1,876.9	938.9	1,859.8	1,858.9	E	1,776.8	888.9	1,759.7	1,758.8	16
19	1,977.9	989.5	1,960.9	1,959.9	T	1,647.7	824.4	1,630.7	1,629.7	15
20	2,034.9	1,018.0	2,017.9	2,016.9	G	1,546.7	773.8	1,529.7	1,528.7	14
21	2,092.0	1,046.5	2,074.9	2,074.0	G	1,489.7	745.3	1,472.6	1,471.6	13
22	2,149.0	1,075.0	2,132.0	2,131.0	G	1,432.6	716.8	1,415.6	1,414.6	12
23	2,309.0	1,155.0	2,292.0	2,291.0	C+57	1,375.6	688.3	1,358.6	1,357.6	11
24	2,410.1	1,205.5	2,393.0	2,392.1	T	1,215.6	608.3	1,198.6	1,197.6	10
25	2,497.1	1,249.1	2,480.1	2,479.1	S	1,114.5	557.8	1,097.5	1,096.5	9
26	2,568.1	1,284.6	2,551.1	2,550.1	A	1,027.5	514.3	1,010.5	1,009.5	8
27	2,665.2	1,333.1	2,648.2	2,647.2	P	956.5	478.7	939.4	938.5	7
28	2,780.2	1,390.6	2,763.2	2,762.2	D	859.4	430.2	842.4	841.4	6
29	3,084.3	1,542.7	3,067.3	3,066.3	T+2...	744.4	372.7	727.4	726.4	5
30	3,240.4	1,620.7	3,223.4	3,222.4	R	440.3	220.6	423.2	422.3	4
31	3,337.5	1,669.2	3,320.5	3,319.5	P	284.2	142.6		266.1	3
32	3,408.5	1,704.8	3,391.5	3,390.5	A	187.1	94.1		169.1	2
33	3,523.6	1,762.3	3,506.6	3,505.6	P	116.1	58.5			1

Figure S1. Characterization of MUC1-Tn conjugates.

a) SDS-PAGE (15%) analysis of MUC1(Tn) conjugates;

b) Proposed crosslinking model when using commercial available maleimide-activated BSA as carrier protein;

c) Analytic run of the purified G_3MUC1(Tn) peptide. [Phenomenex Kinetex, 5 μ, C18, 100Å, 250*4.6mm. Gradient: 5% B 2min; 5% - 60% B 2-12 min; 5% B 12-15min]

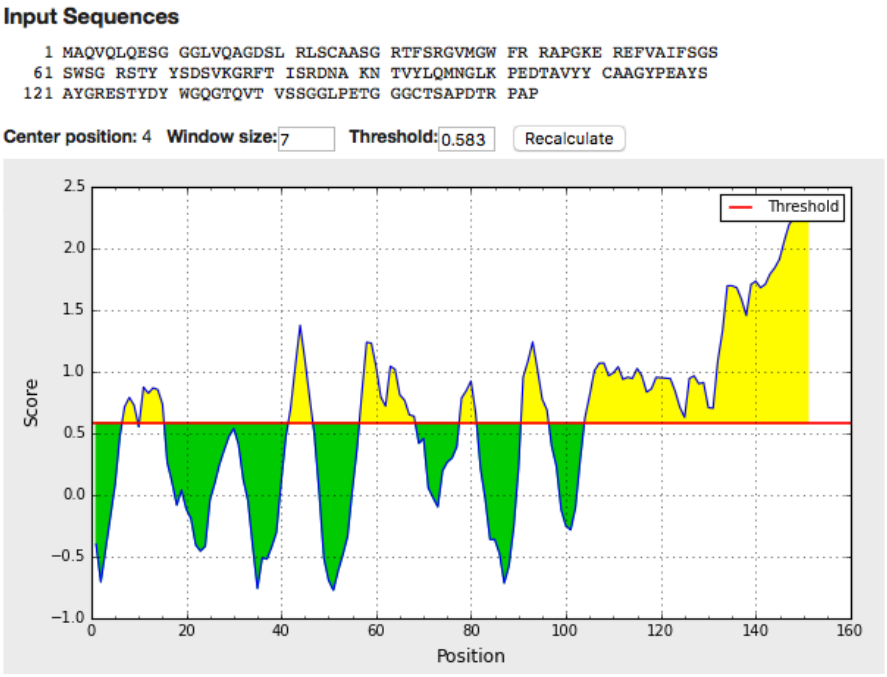
d) Mass of G_3MUC1(Tn) peptide;

e) Key fragmentation spectrum and the corresponding fragmentation table of the identified C-terminal sequence (W)GQGTQVTVSSGGLPETGGGCTSAPDT(HexNAc)RPAP from band A (peptide threshold >97%, $_{\Delta}$Mass< 1Da);

f) Key fragmentation spectrum and the corresponding fragmentation table of the identified C-terminal sequence (F)EYWGQGTQVTVSSGGLPETGGGCTSAPDT(HexNAc)RPAP from band B (peptide threshold >99%, $_{\Delta}$Mass< 1Da)

[Tandem MS experiment and data analysis: Band A and B were recovered from SDS-PAGE, digested separately by chymotrypsin. The digested samples were subjected to MS/MS analysis on a Thermo Fisher Orbitrap instrument. The resulting tandem mass spectra from the samples were matched to peptide sequences generated from a database of *E. coli.* proteins together with the target sequence to reduce the possibility of false positive. The tandem mass analysis was provided by the Proteomics Core Facility at Whitehead Institute. Data visualization was presented by Scaffold Viewer 4.4.1];

a)



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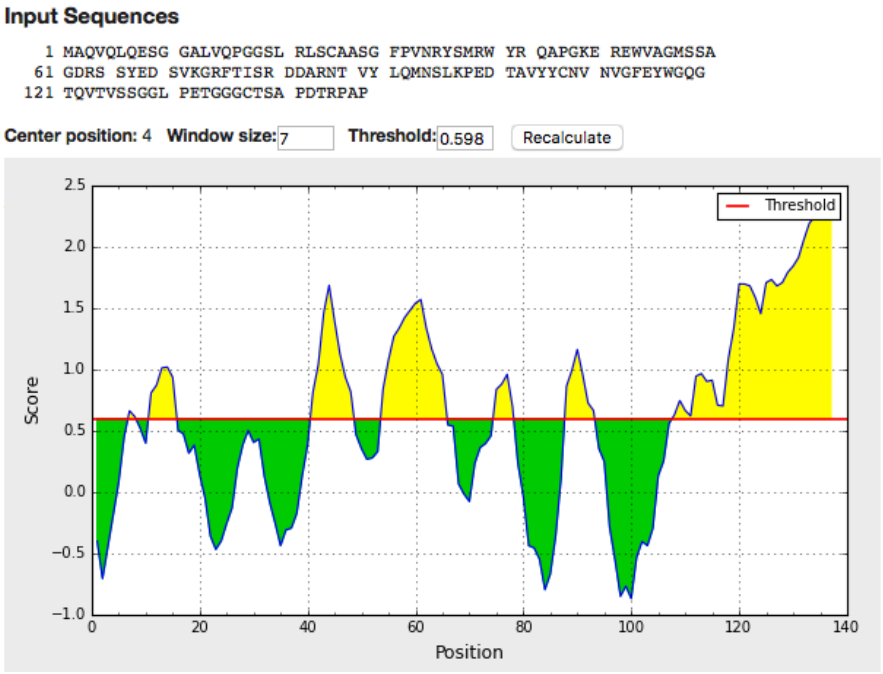

Fig. S2. Bepipred linear B-epitope prediction (http://tools.immuneepitope.org/bcell/). a) B cell epitope prediction based on VHH7-MUC1 sequence; b) B cell epitope prediction based on Enh-MUC1 sequence.

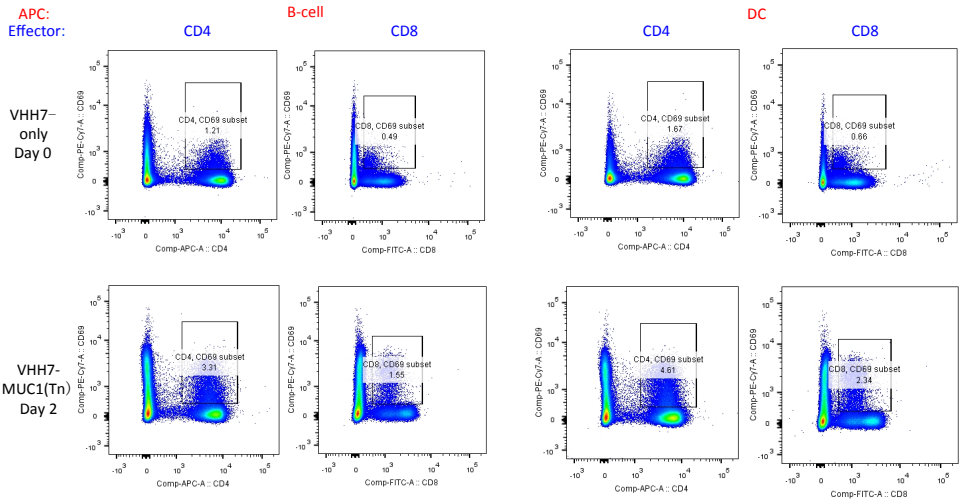

Figure S3. VHH7 vaccine construct induces 4 times increase in the expression of the T-cell early activation marker CD69.

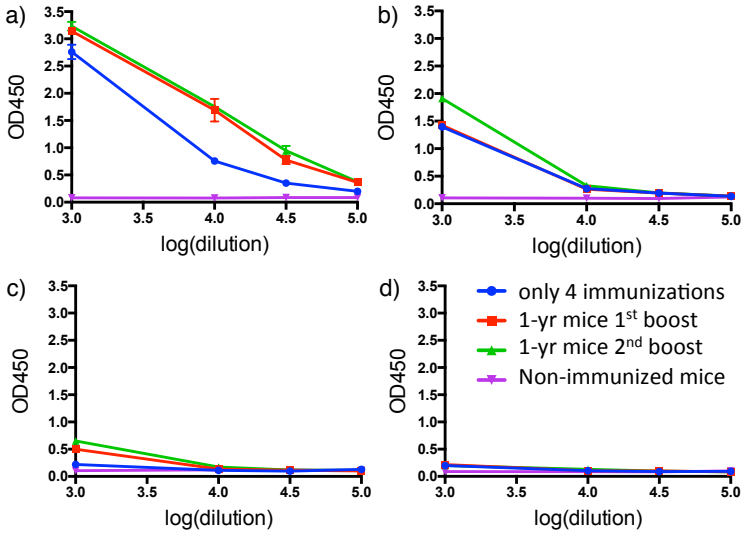

Figure S4. VHH7-MUC1(Tn) conjugate effectively induces long-term memory responses. Titers of IgG1 (a); IgG2a (b); IgG2b (c); IgG3 (d) were determined by ELISA on plates coated with BSA-MUC1(Tn) conjugate and incubated with mouse serum, isotype-specific goat anti-mouse Ig isotypes, and rabbit anti-goat HRP as 1^{o}, 2^{o} and 3^{o} antibodies respectively. 3,3,5,5-tetramethylbenzidine (TMB) was added to all plates. (error bars: mean ± SD, n = 3)