The impact of sustained immunization regimens on the antibody response to oligomannose glycans

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Materials

	Vendors/ Sources
Cholesterol	MP Biomedicals
DOPC	Avanti Polar Lipids
CRM_{197} (ecoCRM™)	Fina Biosolutions LLC
QS-21	Desert King

Standard buffers for ELISA assay	
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
Dulbecco's PBS	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4
PBS-T	PBS, 0.05% Tween-20
PBS-T 5% milk (blocking solution)	PBS-T, 5% non-fat dried milk
PBS-T 1% milk (dilution solution)	Prepared from dilution of PBS-T 5% milk
Flat-bottomed ELISA plates (Nunc-Immuno)	Fisher Scientific
2G12 antibody	Polymun Scientific
Goat Anti-human antibody, Horseradish Peroxidase (HRP) conjugate	Invitrogen
3,3′,5,5′-tetramethylbenzidine (TMB)	Abcam, Ab171522
1M sulfuric acid	Fisher Scientific

Procedures

Preparation of CRM-AcBr

CRM_{197} (ecoCRM™, Fina Biosolutions LLC, 1.0 mg, 17 nmol) was dissolved in 0.9 mL of PBS buffer (pH 7.4). Bromoacetamido-PEG_4-NHS ester (BroadPharm, BP-20569, $BrCH_2(CO)NH(CH_2CH_2O)_4$-$(CH_2)_2$-CO-NHS) (1.2 mg, 2.5 µmol, 147 equiv in 0.1 mL PBS buffer pH 7.4) was added for a final protein concentration of 1 mg/mL. The reaction stood at room

temperature for 1.5 h. Excess bromoacetamido-PEG_4-NHS ester was removed by buffer exchange through an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 4 rounds of dilution with PBS pH 8.5). The average molecular weight of the activated CRM-bromoacetamido (CRM-AcBr) was determined by MALDI-TOF analysis. A ~6300-dalton average mass increase indicated an average of ~17 linkers per CRM molecule (**Figure S1**). BCA assay indicated recovery of 1.07 mg of activated protein.

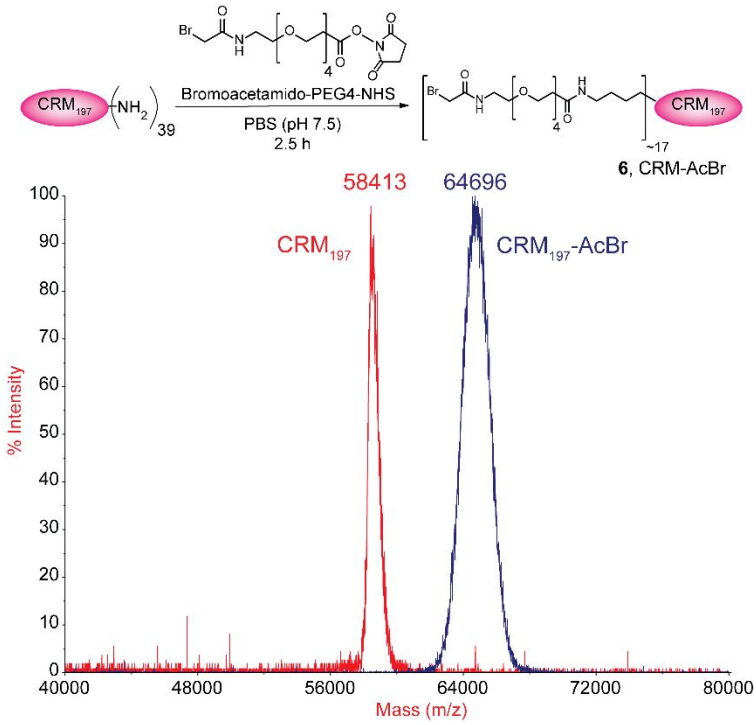


Figure S1. Synthesis of CRM_{197}-bromoacetamido (CRM-AcBr) and MALDI-TOF-MS analysis of CRM-AcBr.

Conjugation of glycopeptide g10F6 to CRM-AcBr

Glycopeptide g10F6[1] (1.2 mg, 95 nmol) in 285 µL of water was treated with 1.9 µL of 500 mM TCEP·HCl/ 1M pH 7.8 Tris-HCl buffer (950 nmol, 10 equiv). Complete removal of the cysteine

protecting group (-StBu disulfide) was confirmed by UPLC-ESI-MS after the reaction stood overnight at room temperature under N_2. Excess TCEP was removed by buffer-exchange through an Amicon centrifugal filter (3-kDa cutoff, Ultra-0.5, 20 min in the first round of filtration, and 30 min for the second round) with PBS buffer (pH 8.5). The deprotected glycopeptide was added to freshly-prepared CRM_{197}-AcBr (0.7 mg, 10.8 nmol, average ~17 linkers/CRM_{197}, 184 nmol of bromoacetamide groups) in a total volume of 393 μL PBS buffer (pH 8.5). The solution stood overnight under N_2 at room temperature in the dark. The CRM_{197}-g10F6 glycopeptide conjugate was purified by removing excess glycopeptide and salts through an Amicon centrifugal filter (30 kDa cutoff, Ultra-0.5, 5 min for at least 4 rounds of filtration, dilution with water). MALDI-TOF analysis indicated distribution of conjugates with median loading of ~5.5 glycopeptides per CRM molecule (median MW ~132 KDa) (**Figure S2)**. The unreacted bromoacetyl groups were then capped with β-mercaptoethanol (11.9 μL from 10 mM stock, 119 nmol) in the dark for 1h in 0.5 mL PBS (pH 8.5) and the capped protein was then buffer-exchanged to water again by Amicon centrifugal filters (30 kDa cutoff, Ultra-0.5, 5 min for at least 4 rounds of filtration). BCA quantification assay detected 1.25 mg protein without the carbohydrate content, corresponding to

1.88 mg (1.25/0.67) total mass of CRM_{197}-Ac-g10F6 glycopeptide conjugate.

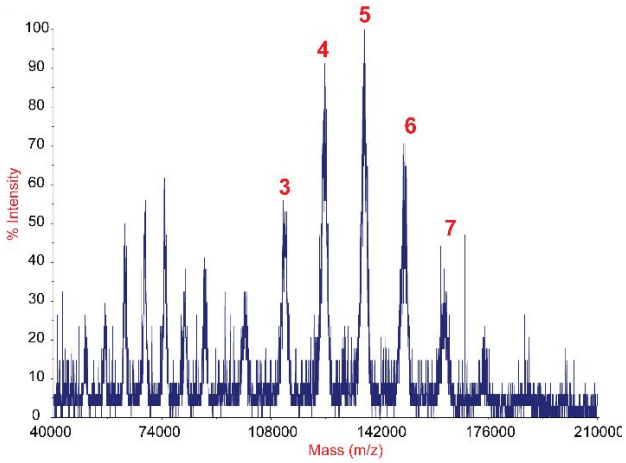


Figure S2. MALDI-TOF MS analysis of CRM-Ac-g10F6 glycopeptide conjugate. Smaller peaks to the left are doubly charged ions.

Preparation of QS-21 liposome adjuvant

A liposome formulation of QS-21 saponin adjuvant was formulated with cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as described by Collin and coworkers.[2] Briefly, DOPC (50 mg, 1.2 mL from 40mg/mL of DOPC stock solution) and cholesterol (12.5 mg, 1.2 mL from 10 mg/mL cholesterol stock solution) were dissolved in 2.3 mL chloroform in a 25 mL round bottom flask. The solvent was carefully evaporated *in vacuo* to yield a transparent lipid film, which was then left under vacuum for 1 h. The film was then hydrated in Dulbecco's PBS buffer (5 mL) at 37 ºC for at least 1 h. The hydrated liposomes were extruded for 10 cycles through 0.4 µm polycarbonate membrane filters, then 10 cycles through 0.2 µm filters, and finally 15 cycles through 0.1 µm filters into 12 mL polypropylene tubes. Commercially available lyophilized QS-21(3 mg) was added to 6 mL of DPBS -/- and allowed to sit for 1 hour at room temperature for complete dissolution of QS-21. The QS-21 solution (2 mg, 4 mL) was added to the 12 mL polypropylene tube containing the liposome suspension (4 mL). The tube was gently inverted five

times and stored at 4 ºC. For transmission electron microscopy (TEM), samples were applied to copper grids coated with continuous carbon, negatively stained with 2% uranyl acetate (JT Baker Chemical Co., Phillipsburg, NJ), and imaged using an FEI Morgagni transmission electron microscope (FEI, Hillsboro, OR) operating at 80 kV and equipped with a 1k × 1k charge coupled device (CCD) camera (Gatan, Pleasanton, CA). TEM **(Figure S3)** showed the liposomes to be primarily 100-250 nm in size, with no substantial change observed after two weeks storage at 37 °C.

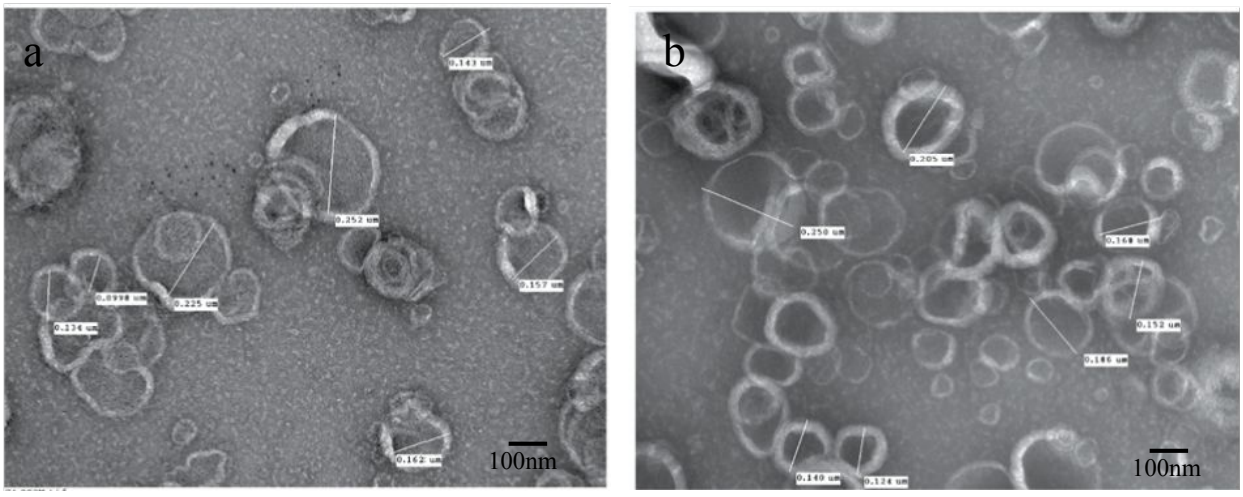


Figure S3. Negative stain Transmission Electron Microscopy (TEM) images of liposome/QS-21 adjuvant combined with g10F6-CRM immunogen. a) Day 0. b) After incubation at 37º C for 14 days. Liposomes were stained with 2% uranyl acetate.

Rabbit immunization regimen

Three groups of six female New Zealand White rabbits were housed at Toxikon Inc. Animals were immunized as described below with CRM_{197}-Ac-g10F6 glycopeptide conjugate (prepared as described above) and QS-21 liposomes (formulated as described above).

Bolus group: Animals received bilateral subcutaneous bolus injections (200 μL split between two sites) comprising conjugate containing total 50 μg of glycopeptide and liposomes containing total 50 μg of QS-21 adjuvant per animal. Diluent was sterile phosphate-buffered saline (Dulbecco's PBS). This immunization was repeated at 4 weeks and 8 weeks. BG505T332N SOSIP boost injections were performed at weeks 16, 20 and 24 with 50 μg SOSIP protein and liposome containing 50 μg QS-21, in 400 μL distributed between two sites.

Exponential group: Animals received bilateral subcutaneous, exponentially increasing doses of conjugate + QS-21 liposomes on days 0, 2, 4, 6, 8, 10 and 12. Regardless of dose, the injection volume was diluted to 200 μL total for two sites; the exponentially increasing amounts of conjugate and QS-21 liposomes in these injections contained the quantities of glycopeptide and QS-21 indicated below (Table S1). Diluent was sterile phosphate-buffered saline (Dulbecco's PBS). This immunization cycle was repeated at 4 weeks and 8 weeks. BG505T332N SOSIP boost injections were performed as bolus injections at weeks 16, 20 and 24 with 50 μg SOSIP protein and liposome containing 50 μg QS-21, in 400 μL distributed between two sites.

Day	QS-21	g10F6
0	0.1 μg	0.1 μg
2	0.26 μg	0.26 μg
4	0.68 μg	0.68 μg
6	1.8 μg	1.8 μg
8	4.6 μg	4.6 μg
10	12 μg	12 μg
12	31 μg	31 μg
cumulative	50 μg	50 μg

Table S1. Exponential dose quantities used in rabbit immunization

Osmotic pump group: Animals received bilateral continuous infusions of glycopeptide conjugate via subcutaneously implanted Alzet® mini-pumps. Each 2-week 100 μL pump was loaded with

conjugate containing 12.5 μg glycopeptide and 12.5 μg of QS-21 (in liposome formulation); pumps were implanted bilaterally into subcutaneous pockets created in the dorsum of each animal on weeks 0, 4, and 8. At the end of each two-week pump infusion, each rabbit received bilateral subcutaneous bolus injections of conjugate containing 12.5 μg of glycopeptide and 12.5 μg of QS-21, so that each immunization cycle totaled 50 μg of immunogen and 50 μg of QS-21. Serum samples were collected one week after the completion each 2-week infusion. BG505T332N SOSIP boost injections were performed as bolus injections at weeks 16, 20 and 24 with 50 μg SOSIP protein and liposome containing 50 μg QS-21, in 400 μL distributed between two sites.

ELISA protocol

All steps were performed as follows unless stated otherwise. High-protein-binding flat-bottomed Maxisorp ELISA plates were coated with 120-2000 ng/mL antigen in coating buffer (100 μL/well) and incubated at 4 °C overnight. Glycopeptide conjugates and carrier proteins were coated at 200 ng/mL concentration, and SOSIP trimer was coated at 120 ng/mL (for sera post-SOSIP boost) or 2 μg/mL (for sera prior to SOSIP boost). The wells were washed twice with PBS-T (200 μL) and then blocked for 1 h at room temperature with 5% fat-free milk PBS-T (200 μL/well). After washing again twice with PBS-T, the wells were then incubated with either 3-fold, 4-fold or 5-fold serial dilutions of rabbit serum (starting at different concentrations: either 1:10 or 1:100) in 1% fat-free milk in PBS-T for 2 h at room temperature. The wells were washed 3 times before incubating with 100 μL of an HRP-conjugated sheep anti-rabbit antibody at 1: 10,000 dilution for 1h at room temperature. After 3 washes, the wells were developed by adding 100 μL of TMB solution for 3-35 min. The reaction was stopped by adding 100 μL of 1 M sulfuric acid and absorbance was measured at 450 nm wavelength. All measurements were performed in triplicate, except that the prebleed sera were tested in duplicate.

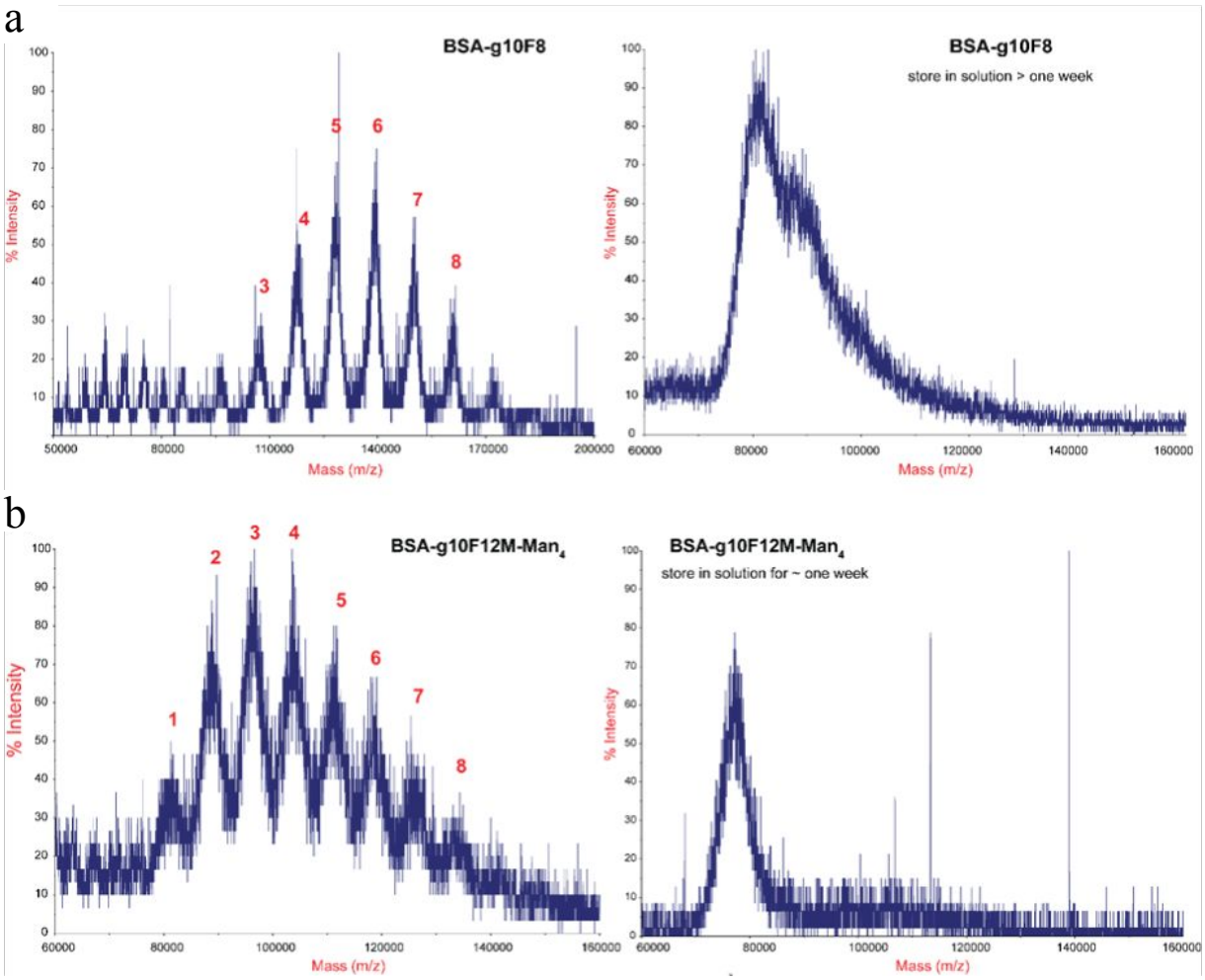
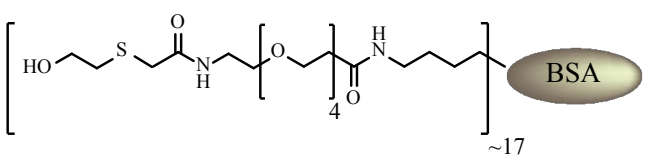


Figure S4. Instability of the thiol-maleimide linkage. a) MALDI-TOF mass spectra of maleimide-linked g10F8-BSA conjugate[1] before and after one week of storage in water at 4 °C. b) MALDI-TOF mass spectra of maleimide-linked Man_4-g10F12M-BSA conjugate[1] before and after one week of storage in water at 4 °C.

a



b

Bolus	Exponential	Pump
--*	--*	--*
1:256	--*	--*
--*	--*	1:85
--*	--*	--*
--*	--*	--*
--*	--*	--*

Figure S5. Rabbit serum response to linker used in the CRM_{197}-g10F6 glycopeptide conjugates. a) capped BSA+linker used in ELISA to detect anti-linker response. b) Post-dose 2 serum ELISA EC_{50} values (serum dilutions). * (OD_{50} below 0.1 at minimum serum dilution of 1:50).

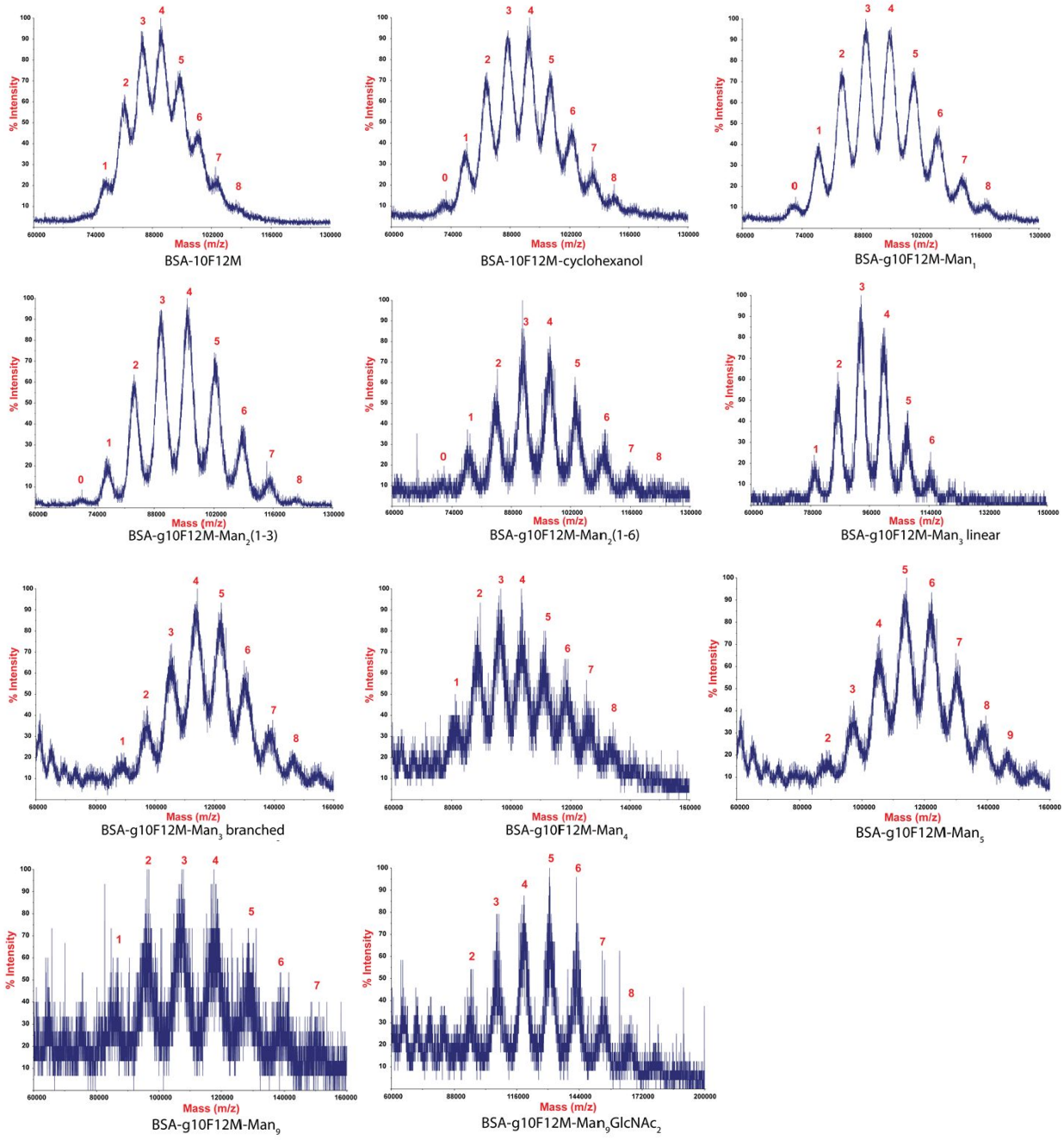


Figure S6. MALDI-TOF MS of BSA-Ac-glycopeptide conjugates used in microspecificity study. The red numbers indicate the number of glycopeptides per BSA protein molecule. More closely clustered peaks at lower m/z are doubly charged ions. Conjugates were prepared analogously to CRM197-Ac-g10F6 using glycopeptides that were reported previously.[1]

Production of BG505 SOSIP Env for boosting

BG505 SOSIP.664 (T332N) used for boosting was co-expressed with furin in 293 Freestyle cells (ThermoFisher) following standard protocols.[3] SOSIP was purified from the media by Galanthus Nivalis Lectin affinity chromatography (Vector Labs) followed by size exclusion chromatography (Superdex 200, 16/60, GE).

TZM-bl neutralization assay procedure

Neutralizing antibody activity in serum samples was measured in duplicate in 96-well culture plates by using Tat-regulated luciferase (Luc) reporter gene expression to quantify reductions in virus infection in TZM-bl cells. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. Assays were performed with HIV-1 Env-pseudotyped viruses essentially as previously described.[4] Serum samples were heat-inactivated at 56^{o}C for 1 hr, then diluted over a range of 1:20 to 1:43740 in cell culture medium and pre-incubated with virus (~150,000 relative light unit equivalents) for 1 hr at 37 ^{o}C before addition of cells. Following a 48 hr incubation, cells were lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer). Neutralization titers are the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells.

		ID_{50} in TZM-bl Cells[1]					
		SVA-MLV	MW965.26	BG505/T332N	JR-FL	JR-FL.V1_3Q	CH0848.3.d949.10.17.N133D.N138T
		Neg Ctrl	Clade C	Clade A	Clade B	Clade B	Clade C
			Tier 1A	Tier 2	Tier 2	Tier 1A	Tier 2
Study ID	Animal ID	ID#8075	ID#7847	ID#9212	ID#730	ID#9113	ID#9210
Bolus	1101	<20	<20	<20	<20	<20	<20
Bolus	1102	<20	<20	<20	<20	<20	<20
Bolus	1103	<20	<20	<20	<20	<20	<20
Bolus	1104	<20	<20	<20	<20	<20	<20
Bolus	1105	<20	<20	<20	<20	<20	29*
Bolus	1106	<20	<20	<20	<20	<20	<20
Exponential	2101	<20	<20	<20	<20	<20	<20
Exponential	2102	<20	<20	<20	<20	<20	<20
Exponential	2103	<20	<20	<20	<20	<20	<20
Exponential	2104	<20	<20	<20	<20	<20	<20
Exponential	2105	<20	<20	<20	<20	<20	<20
Exponential	2106	<20	<20	<20	<20	<20	<20
Pump	3101	<20	<20	<20	<20	<20	<20
Pump	3102	<20	<20	<20	<20	<20	<20
Pump	3103	<20	<20	<20	<20	<20	<20
Pump	3104	<20	<20	<20	<20	<20	<20
Pump	3105	<20	<20	<20	<20	<20	<20
Pump	3106	<20	<20	<20	<20	<20	<20
CH01-31 (positive control)		>25	1.63	0.02	0.02	<0.01	0.43

[1]*Values are the serum dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).*

*****Both replicates exhibited concentration-dependent signal with RLU decreasing at higher serum concentrations.*

Table S2. TZM-bl Neutralization assay results of post-dose 3 sera (after three immunizations with CRM-Ac-g10F6 glycopeptide conjugate, prior to SOSIP boost).

		ID_{50} in TZM-bl Cells[1]		
		SVA-MLV	CH0848.3.d949.10.17.N133D.N138T	BG505/T332N
		Neg Ctrl	Clade C	Clade A
			Tier 2	Tier 2
Group	Animal ID	ID#8075	ID#9479	ID#9212
Bolus	1101	<20	<20	<20
Bolus	1102	<20	<20	**89**
Bolus	1103	<20	<20	<20
Bolus	1104	<20	<20	**218**
Bolus	1105	<20	<20	**259**
Bolus	1106	<20	<20	**241**
Exponential	2101	<20	<20	<20
Exponential	2102	<20	<20	**87**
Exponential	2103	<20	<20	**42**
Exponential	2104	<20	<20	**179**
Exponential	2105	<20	<20	**413**
Exponential	2106	<20	<20	**40**
Pump	3101	<20	<20	**371**
Pump	3102	<20	<20	**104**
Pump	3103	<20	<20	**820**
Pump	3104	<20	<20	**128**
Pump	3105	<20	<20	**36**
Pump	3106	<20	<20	**270**
CH01-31 (positive control)		>25	0.383	0.049

[1]*Values are the serum dilution or antibody concentration (in ug/ml) at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).*

Table S3. TZM-bl Neutralization assay results of post-dose 6 sera (after three SOSIP boost immunizations).

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