SUPPORTING INFORMATION

Multivalent Antigen Presentation Enhances the Immunogenicity of a Synthetic Three-Component HIV-1 V3 Glycopeptide Vaccine

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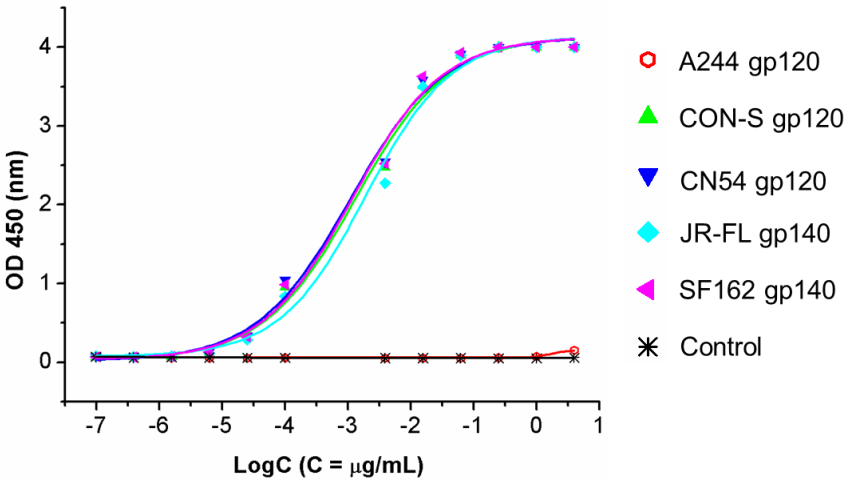

Figure S1. ELISA analysis of the binding of broadly neutralizing antibody 10-1074 antibody to the gp120s and gp140s derived from different HIV-1 strains.

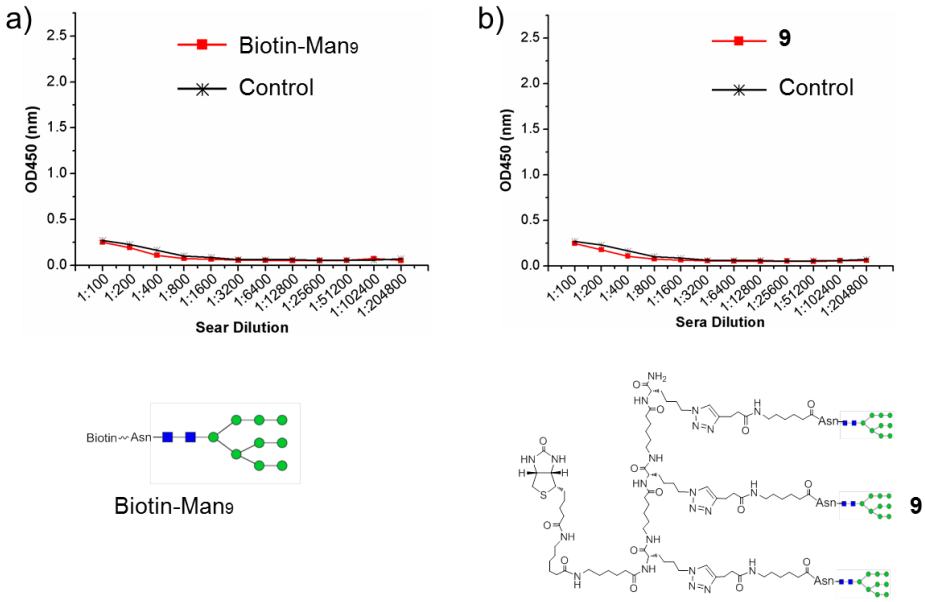

Figure S2. ELISA analysis of the binding of the anti-sera with biotinylated Man9GlcNAc2 glycan and the biotinylated trivalent Man9GlcNAc2 glycans containing the same triazole linker as that of the immunogens. a) binding of the anti-sera induced by the monovalent glycopeptide immunogen (**1**); b) binding of the anti-sera induced by the trivalent glycopeptide immunogen (**4**).

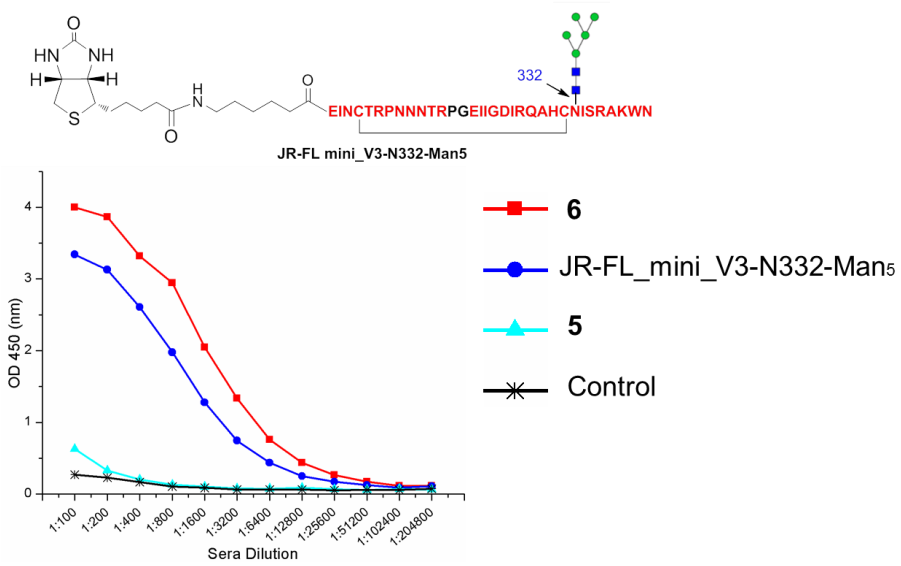

Figure S3. ELISA analysis of the binding of the V3 $Man_5GlcNAc_2$ glycopeptide and $Man_9GlcNAc_2$-glycopeptide **6** with the pretreated anti-sera induced by immunogen **1**.

General Procedures and Methods

Safety statement: No unexpected or unusually high safety hazards were encountered.

General procedures

Analytical reverse-phase HPLC was carried out on a Waters 626 HPLC system equipped with a dual absorbance UV detector. All the V3 peptides and glycopeptides were run on a C18 column (YMC-Triart C18, 4.6 X 250 mm, 5 µm) at a flow rate of 1 mL/min using a linear gradient of 15-45% MeCN containing 0.1% TFA over 30 min. The lipopeptides were run on a CN column (YMC-Pack CN, 4.6 X 250 mm, 5 µm) using a linear gradient of 20-70% MeCN containing 0.1% TFA over 50 min. ESI-MS spectra were measured on a Micromass ZQ-4000 single-quadrupole mass spectrometer. For the V3 peptides and glycopeptides, preparative reverse-phase HPLC was carried out on a Waters 600 HPLC system equipped with a dual absorbance UV detector using a C18 column (Waters XBridge, Prep Shield RP 10 X 250 mm, 5µm) at a flow rate of 4 mL/min. The preparative HPLC purification of the lipopeptides was performed on a CN column (YMC-Pack CN, 10 X 250 mm, 5 µm).

Synthesis of lipopeptide 2

Lipopeptide **2** synthesis was performed under microwave synthesis conditions using a CEM Liberty Blue microwave peptide synthesizer. Synthesis was based on Fmoc chemistry using PAL-PEG-PS resin (0.18 mmol/g) on a 0.1 mmol scale. Couplings were performed using 6 equiv. of Fmoc-protected amino acids, 6 equiv. of TBTU and 12 equiv. of DIPEA in DMF at 45 °C for 20 min. Fmoc deprotection was carried out with 20% piperidine in DMF containing 0.1 M HOBt. The Lys-N_3 was first coupled to the resin, and then Fmoc-ε-Acp-OH was attached. The P30 sequence was coupled stepwise followed by Fmoc-ε-Acp-OH coupling. Then the SKKKK

sequence was coupled and finally the Pam_3Cys-OH was coupled. The resin was washed with DMF (3X) and DCM (3X) then cleavage was carried out using cocktail R (TFA/Thioanisole/Ethanedithiol/Anisole = 90/5/3/2). The resin was then filtered and the solution was added to cold diethyl ether for peptide precipitation. The crude peptide was dissolved in glacial acetic acid and then lyophilized. The crude peptide was purified by RP-HPLC on a CN column to give the purified **2** (172 mg, yield 35%). ESI-MS: Calcd., M = 4884.9; found (*m/z*): 698.71.94 $[M + 7 H]^{7+}$, 815.16 $[M + 6 H]^{6+}$, 977.99 $[M + 5 H]^{5+}$, 1222.23 $[M + 4 H]^{4+}$. RP-HPLC retention time, t_R = 39.8 min.

The synthesis of V3 glycopeptide alkyne **3** and biotin labelled V3 peptide **5** was carried out following the previously described procedures [1]. The synthesis of the biotin labelled mono-, bi- and trivalent V3 glycopeptides (**6**-**8**) and the biotinylated labelled trivalent Man9GlcNAc2 glycans (**9**) was carried out following the previously reported procedure [2].

Synthesis of three-component trivalent immunogen 4

Lipopeptide **2** (1.0 mg, 0.2 µmol), V3 alkyne glycopeptide **3** (4.0 mg, 0.7 µmol) and CuOAc (4 µg, 0.03µmol) were dissolved in 100 µL DMF. Then the mixture was incubated at 40 °C for 18 h. The reaction mixture was diluted with 1 mL water and then lyophilized. The crude product was purified by RP-HPLC on a CN column to give the desired immunogen **4** (2.5 mg, 56%). ESI-MS: Calcd., M = 22429; found (*m/z*): 1247.0 $[M + 18 H]^{18+}$, 1320.4 $[M + 17 H]^{12+}$, 1402.7 $[M + 16 H]^{16+}$, 1496.2 $[M + 15 H]^{15+}$, 1603.0 $[M + 14 H]^{14+}$, 1726.3 $[M + 13 H]^{13+}$, 1870.1 $[M + 12 H]^{12+}$, 2039.8 $[M + 11 H]^{11+}$, 2243.8 $[M + 10 H]^{10+}$. RP-HPLC retention time, t_R = 31.5 min.

Rabbits immunization

The synthetic three-component trivalent immunogen **4** was incorporated into liposome following the previously described procedure [3]. New Zealand White rabbits in a group of 3 were immunized via intramuscular and subcutaneous injections containing 50 μg of **4**. After priming, three booster injections were given at interval of 21 days, and bleeds were taken 7 days postimmunization. The combined sera from each group were used for ELISA analysis.

ELISA binding analysis with HIV-1 gp120s and trimeric gp140s

The 96-well ELISA microtiter plates were coated with gp120 (2 μg/mL in PBS) and incubated at 4 ºC overnight. The plates were washed with PBS/0.05% Tween-20 and blocked with 2% sodium caseinate (w/v) in PBS at room temperature for 1 h. Then plates were washed three times and titrated against rabbit antisera in 1% sodium caseinate. The plates were incubated at 37 ºC for 1 h. After washing three times, a solution (100 μl/well) of 1:3000 diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H + L) antibody or horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) antibody in 1% PBS was added to the plates. The plates were incubated for 1 h at 37 ºC. After washing three times, a solution of 3, 3', 5, 5'-tetramethylbenidine (TMB) was added. Color was allowed to develop for 5 min, and then quenched by adding a solution of 1 M H_3PO_4. The readout was measured at a wavelength of 450 nm.

ELISA binding analysis with synthetic V3 peptide, glycopeptides, and the trivalent glycan construct

To subtract the peptide specific antibodies in the antisera, streptavidin magnetic beads (1 mg, New England BioLabs) were incubated with the synthetic 33er V3 peptide **5** (0.5 mg/ml, 500 μL) at 37 ºC for 30 min. The beads were washed three times with PBS buffer. Then 1 mL antisera induced by immunogen **4** were added and incubated with the beads for 30 min at 37 ºC. The supernatant was separated by applying magnet and used for ELISA analysis.

The 96-well ELISA microtiter plates were coated with 5 μg/mL Neutravidin in PBS (100 μL/well) and incubated at 4 ºC overnight. The plates were washed with PBS/0.05% Tween-20 and blocked with 2% sodium caseinate (w/v) in PBS at room temperature for 1 h. After washing three times, 2 μg/mL of the biotinylated peptide (**5**), glycopeptides (**6**, **7**, and **8**), or the trivalent glycan construct (**9**) were dissolved in 1% casein PBS (100 μL/well) was added and incubated at 37 ºC for 1 h. Controls were set up by adding PBS without V3 glycopeptide to the wells. Then plates were washed three times and titrated against rabbit antisera in 1% sodium caseinate. The plates were incubated at 37 ºC for 1 h. After washing three times, a solution (100 μl/well) of 1:3000 diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H + L) antibody or horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) antibody in 1% PBS was added to the plates. The plates were incubated for 1 h at 37 ºC. After washing three times, a solution of 3, 3', 5, 5'-tetramethylbenidine (TMB) was added. Color was allowed to develop for 5 min, and then quenched by adding a solution of 1 M H_3PO_4. The readout was measured at a wavelength of 450 nm.

References

(1) Cai, H.; Orwenyo, J.; Giddens, J. P.; Yang, Q.; Zhang, R.; LaBranche, C. C.; Montefiori, D. C.; Wang, L. X. Synthetic Three-Component HIV-1 V3 Glycopeptide Immunogens Induce Glycan-Dependent Antibody Responses. *Cell Chem Biol* **2017,** *24*, 1513-1522 e1514.

(2) Cai, H.; Orwenyo, J.; Guenaga, J.; Giddens, J.; Toonstra, C.; Wyatt, R. T.; Wang, L. X. Synthetic multivalent V3 glycopeptides display enhanced recognition by glycan-dependent HIV-1 broadly neutralizing antibodies. *Chem Commun (Camb)* **2017,** *53*, 5453-5456.

(3) Ingale, S.; Wolfert, M. A.; Gaekwad, J.; Buskas, T.; Boons, G. J. Robust immune responses elicited by a fully synthetic three-component vaccine. *Nat Chem Biol* **2007,** *3*, 663-667.