Self-assembling peptide nanofiber HIV vaccine elicits robust vaccine-induced antibody functions and modulates Fc glycosylation

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Supplementary Text
Materials and Methods

**Transmission Electron Microscopy (TEM) imaging.** gp120-Q11 vaccine was prepared as described for immunizations, diluted to 0.2 mM of Q11 with DI water, and deposited as a 5 μl droplet onto 400 mesh copper TEM grids (Electron Microscopy Sciences #FCF400-Cu). After 2 minutes, the grids were washed with 10 drops of DI water, stained for 1 minute with 3 μl of 1% uranyl acetate solution while still wet, and dried by wicking away the peptide droplet with Kimwipe. Samples were imaged at 11500x magnification on the FEI Tecnai F30 and analyzed with the FEI TEM Imaging and Analysis (TIA) software.

**Surface plasmon resonance (SPR) for antibody avidity to gp120.** Rabbit total serum IgG was purified with Protein A HP MultiTrap (Cytiva #28903133) as described in total IgG glycan analysis. Avidity screening of purified polyclonal IgGs against CH505T/F gp120 was completed by surface plasmon resonance (SPR) on a Biacore T200 platform (Cytiva Life Sciences) at 25 °C in 1x PBS running buffer (Fisher Scientific #BP39920; 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Immobilization of protein ligands to a Series S CM5 sensor surface (Cytiva #29104988) was performed via amine coupling chemistry at a flow rate of 5 μl/min, following standard procedures recommended by the manufacturer. Briefly, CM5 sensor surfaces were activated with a 7-minute injection of N-Hydroxysuccinimide/1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (NHS/EDC; Cytiva #BR100050), followed by injection of protein ligands, and surface deactivation with a 12-minute injection of ethanolamine-HCl (Cytiva #BR100050). Protein ligands were diluted to 50 μg/ml in 10 mM sodium acetate, pH 4.5 (Cytiva #BR100350) and injected until the immobilization level was >3000 RU. For avidity screening, the purified IgGs from weeks 0, 2, 8, and 14 (100 μg/ml in 1x PBS, pH 7.4) were injected at 30 μL/min over the ligand surfaces for 150 seconds followed by a dissociation phase in running buffer of 300 seconds. Surfaces were regenerated between antibody injections with a 60 second injection of either 10 mM glycine-HCl, pH 2.0 (Cytiva #BR100355) or 25 mM NaOH at 30 μL/min. Data analysis and curve fitting was performed with the BIAevaluation Software (v4.1). Binding profiles were reference corrected by subtraction of the corresponding week 0 sample. Binding response values were reported as the 10 second average beginning 1 second post-injection. Dissociation rate constants (kd) were fit using the 300 second period beginning 4 seconds post-injection. Interactions with response values below 10 RU were excluded from dissociation rate measurement. The avidity score was calculated as the ratio of binding response and dissociation rate constant (kd). Some interactions reported kd values that were either negative or beyond the limit of detection (<1E-05 1/s), therefore avidity scores are reported as the minimum value.

**FcγR cell signaling assay.** In this assay we used mouse BW5147 cell lines stably expressing human FcγRIIla (BW100 cell) or IIb receptor (BW136 cell), both fused to CD3 intracellular domain (CD3ζ). 5 μg/ml of CH505T/F gp120 was coated on Greiner high binding 96 well plates (Millipore-Sigma # M4561-40EA) for BW100 cells, and 1ug/ml for BW136 cells. After overnight incubation at 4°C, the plates were washed with BW media (500 ml RPMI Gibco #11875-093), 50 ml heat-inactivated FBS (Cytiva #SH30910.03), 5 ml 100 mM sodium pyruvate (Gibco #11360-070), 0.5 ml 2-mercaptoethanol (Gibco #21985-023), followed by blocking with 200 μl of BW media. Rabbit sera were 3-fold diluted to yield 5 dilutions starting at 1:10 dilution. Plates were incubated with diluted sera for 1 hour, followed by 3 washes with BW medium. 100,000 BW cells were seeded in each well. After 20 hours of incubation at 37°C, cells were pelleted at 500 x g for 10 minutes. We collected the supernatant of each well. And we performed mouse IL-2 ELISA as previously described in this study. Briefly, 384-well polystyrene high-binding ELISA plates were coated with 3 μg/ml of anti-mouse IL-2 capture antibody (BD
Biosciences #554424) overnight and blocked with superblock. BW cell supernatant was added to
the plate undilutedly, followed by an hour of incubation. Biotinylated rat anti-mouse IL-2
antibody (BD Biosciences #554426, at 1:2000 dilution) was in place for the secondary antibody
and streptavidin-HRP (EMD Millipore #18-152 at 1:8000 dilution) for the tertiary.

**Circular dichroism analysis.** An AVIV Model 435 circular dichroism spectrometer was
used with a 0.1 cm path length quartz cell. Q11 and gp120-Q11 nanofiber solutions were
prepared in the same way as immunization. The unadjuvanted solutions were further diluted to
0.2 mM with 0.137 M potassium fluoride solution (equivalent to 1×PBS). The STR8S-C-
adjuvanted solutions were prepared as previously indicated in vaccination preparation, and
subsequently diluted for 20-fold in potassium fluoride solution before measurement. The
wavelength range was 195-320 nm, the scanning speed was 1 nm/s, and the bandwidth was 1
nm. Each spectrum shown is the average of three scans with solvent background subtracted.

**Thioflavin T assay.** Thioflavin T (ThT) was used to confirm the presence of β-sheet
structures before and after gp120 conjugation and the addition of STR8S-C adjuvant. ThT (Alta
Aesar #J61043) was dissolved in 1× PBS to 0.05 mM immediately prior to the experiment.
gp120-Q11 was prepared as already described and diluted to a nanofiber concentration of
0.67 mM and a protein concentration of 0.06 mg/mL. Q11 was prepared at 2 mM and diluted to
0.67 mM immediately prior to the experiment. gp120 was diluted to 0.08 mg/mL. After dilution,
STR8S-C adjuvant was added to appropriate samples at 15% v/v. A 20 μL sample was combined
with 180 μL ThT solution in a 96 well plate (Corning Costar #3915) for 1 minute prior to reading.
Fluorescence intensity was read with excitation 440 nm and emission 488 nm using a Molecular
Devices Spectramax M2 spectrophotometer.
Fig. S1. Glycan nomenclature, and Fc glycan analysis procedure

Asn297 residue in the CH2 domain of IgG constant region is post-translationally modified by glycan. The glycan precursor is first trimmed down to the core glycan Man3GlcNAc4 (glycans connected by solid lines) in the Golgi body and then decorated (glycans connected by dash lines) via (1) fucose (red triangle); (2) single or double galactose (yellow circle); (3) bisecting N-acetylglucosamine (GlcNAc) (blue square); (4) sialylation (purple diamond) of galactose via α2-6 or α2-3 linkage. The symbol nomenclature follows standard symbol nomenclature for glycans (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). (Image was created with Biorender.com)
Fig. S2. Quantification of gp120 content in gp120-Q11 vaccine by SDS-PAGE

(A) gp120-Q11 and recombinant gp120 standard were heat-denatured and analyzed by polyacrylamide gel electrophoresis in a non-reducing condition. gp120 standard with different concentrations and gp120-Q11 with unknown gp120 concentration were loaded as indicated. (B) J image analyzer (NIH) was used to quantify the intensity of each band on the gel. The band intensities of gp120 standard in different concentrations were used to build a standard curve for quantifying the concentration of gp120 in the gp120-Q11 vaccine.
Fig. S3. Q11 β-sheet structure analysis

The β-sheet secondary structure of Q11 was examined after gp120 conjugation by (A) Circular Dichroism (CD) and (B) Thioflavin T (ThT) assay. CD demonstrated that β-sheet structure of Q11 nanofiber was preserved after gp120 conjugation. gp120-Q11 appears to have a structure that is intermediate between the Q11 and gp120-βtail structure. ThT also confirmed the presence of β-sheet structure of Q11 nanofiber after conjugating to gp120. Moreover, strong signal was also detected after gp120-Q11 was mixed with STR8S-C adjuvant, indicating that gp120 conjugation or STR8S-C did not compromise Q11’s nanofiber structure.
After 3 doses (wk14) of gp120-Q11 vaccine with STR8S-C adjuvant, gp120-Q11 slightly raised the IgG response to the Q11 peptide as compared to the pre-vaccination time point (wk0). Yet, such level of anti-Q11 IgG was significantly lower than the anti-gp120 response induced by gp120-Q11 (Wilcoxon signed rank exact test).

Fig. S4. IgG response to Q11 scaffold and gp120
Fig. S5. Raw fluorescence histogram of infected-cell binding assay
To show the raw FITC signal in the infected-cell binding assay, one rabbit each from gp120-Q11 and gp120 vaccine groups were plotted together at each time point shown in Figure 2D. To rule out non-specific antibody binding to the cells, we included a control using mock-infected cells. And the assay background was measured by only incubating with secondary FITC-conjugated antibody (2ary only).
Fig. S6. Contribution of variables in principle component analysis of vaccine-induced antibody functions

Contribution of the top 20 variables to dimension 1 (Dim1) and 2 (Dim2) in the PCA plot shown in Figure 3E. The red dash represents the expected average contribution of each variable.
Fig. S7. Step-by-step procedure of antigen-specific IgG and total serum IgG Fc glycosylation analysis

To pull down antigen-specific antibodies, serum was first cleaned up with uncoupled beads to remove non-specific binding proteins, followed by incubation with antigen-coupled beads. Bead-bound IgG was digested with an IgG-specific protease such as IdeZ. Purified Fc region was deglycosylated, and the released glycans were labeled before analyzed with Gly-Q Glycan Analysis System. For total serum IgG glycan analysis, serum IgG was first purified with protein A, followed by Fc digestion with IgG-specific protease. Fc region was then purified by protein A. (Image was created with Biorender.com)
Fig. S8. Contribution of variables in principle component analysis of rabbit gp120-specific and total IgG glycosylation in Figure 4B

The contribution of the top 10 variables to Dim1 and 2 in the PCA of (A) gp120-specific IgG glycosylation and (B) total IgG glycosylation in rabbits was shown. The red dash represents the expected average contribution of each variable.
The glycosylation profiles of rabbit total serum IgG prior to vaccination (wk0 total IgG) and after 3 immunizations (wk14 total IgG) were characterized. A similar Fc glycosylation profile was observed between gp120-Q11- and gp120-immunized rabbits.

Fig. S9. Glycosylation profile of rabbit serum total IgG

The glycosylation profiles of rabbit total serum IgG prior to vaccination (wk0 total IgG) and after 3 immunizations (wk14 total IgG) were characterized. A similar Fc glycosylation profile was observed between gp120-Q11- and gp120-immunized rabbits.
Fig. S10. Contribution of variables in principle component analysis of mouse gp120-specific and total IgG glycosylation in Figure 5C

The top 10 contributors to Dim 1 and 2 in the PCA of (A) mouse gp120-specific IgG glycosylation and (B) mouse total serum IgG glycosylation.
Fig. S11. Glycosylation profile of mouse serum total IgG

Immunization of gp120-Q11 and gp120 induced a similar glycosylation profile of total serum IgG in mice.
Median [Min, Max]

### Glycan feature

| Glycan feature | gp120 (n=4)       | gp120-Q11 (n=3)       | Overall (n=7)       |
|----------------|--------------------|-----------------------|---------------------|
| Fucosylated    | 43.9 [34.3, 47.9]  | 54.0 [50.1, 55.1]     | 47.9 [34.3, 55.1]   |
| Sialylated     | 7.62 [7.16, 10.7]  | 9.86 [7.34, 16.0]     | 7.76 [7.16, 16.0]   |
| Bissected      | 7.55 [6.13, 9.45]  | 10.5 [8.22, 11.9]     | 8.69 [6.13, 11.9]   |
| Agalactosylated| 13.7 [8.49, 17.3]  | 19.3 [16.3, 20.1]     | 16.3 [8.49, 20.1]   |
| Mono-galactosylated | 17.8 [15.8, 19.3] | 20.5 [18.7, 24.0]   | 19.2 [15.8, 24.0]   |
| Di-galactosylated | 26.3 [24.1, 32.6] | 25.4 [22.3, 26.3]    | 26.2 [22.3, 32.6]   |
| Galactosylated  | 43.8 [40.4, 51.9]  | 45.9 [41.0, 50.2]     | 45.4 [40.4, 51.9]   |

**Fig. S12. Glycosylation profile of gp120-specific IgG in mice immunized with unadjuvanted gp120 or gp120-Q11**

(A) The glycosylation of gp120-specific serum IgG from mice immunized with unadjuvanted gp120-Q11 (n=4) or gp120 (n=3) was profile. Likely due to the small group sizes, none of the glycan features showed significant difference between the two vaccine groups. Notably, unadjuvanted gp120-Q11 induced an IgG glycosylation profile in mice similar to gp120-Q11 immunization with STR8S-C adjuvant, with higher levels of fucosylation, agalactosylation, mono-galactosylation but lower di-galactosylation. (B) As one animal in each of the two vaccine groups showed low S/N ratio in the glycosylation analysis, we re-plotted without these two animals. Notably, after excluding low quality data from these two animals, the trend of higher fucosylation, agalactosylation, mono-galactosylation and lower di-galactosylation in gp120-Q11 is more prominent. (C) Due to the small number of animals in each group, statistical analysis will
be inconsequential. Therefore, we analyzed the median and the range of each glycan feature in each vaccine group and the two groups combined.

**Fig. S13. Impact of IgG fucosylation on Fc receptor binding and cell signaling at week 14 post-vaccination**

(A) Spearman correlation showed negative correlation of several fucosylated glycan species with antibody binding to FcγRIIIa at week 14. Two FcγRIIIa variants (phenylalanine158 (F158, low affinity) and valine158 (V158, high affinity)) were included. (B) The binding of gp120-specific IgG to FcγRIIIa and FcγRIIb was measured at 1:100 serum dilution using a multiplex bead-based antibody binding assay (BAMA). Wilcoxon rank sum tests were used for comparison between the vaccine groups. (C) BW5147 cell lines stably expressing FcγR-CD3ζ chimeric receptors were used to measure cellular signaling induced by rabbit gp120-specific IgG in gp120-Q11 and gp120 groups. Median is presented as boxes with range shown as error bars (Wilcoxon rank sum test).
Fig. S14. Correlation between Fc glycosylation and ADCP

(A) Fc glycosylation was poorly correlated with ADCP assayed against CH505T/F gp120 and 1086.C gp120. gp120-specific IgG glycan analysis was performed using autologous gp120 (CH505T/F).
Fig. S15. gp120-Q11 and gp120 vaccines induced similar levels of G1F[6], A2(2,6), A2FB(2,6), G0FB, G2F, G0, G1[6], A1(2,6), and A2F(2,6)

Wilcoxon test was used to test the difference between gp120-Q11 and gp120 vaccine groups in the relative abundance of each glycoform in gp120-specific IgG. Results were normalized based on week 0 total IgG data. Difference was considered statistically significant only when unadjusted P values < 0.05 both before and after normalization and FDR P value < 0.1 (P value before normalization with week0 > 0.05). (Glycan schematics were created with Biorender.com)
Fig. S16. Antibody avidity to CH505T/F gp120 and epitope specificity induced by gp120-Q11 and gp120

(A) Surface plasmon resonance (SPR) was used to measure serum IgG’s avidity to CH505T/F gp120. $k_d$ of serum antibody binding below detection limit was set to the highest $k_d$ detected plus 1, and avidity was set to 0. (Non-parametric repeated measures) (B) Antibody binding to subtype C (1086.C and consensus sequence) linear epitopes at week 14 was measured with BAMA (Generalized linear models).