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# Glycan profiles of gp120 protein vaccines from four major HIV-1 subtypes produced from different host cell lines under non-GMP or GMP conditions

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## Abstract

Envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1) is an important target for the development of an HIV vaccine. Extensive glycosylation of Env is an important feature that both protects the virus from antibody responses and serves as a target for some highly potent broadly neutralizing antibodies. Therefore, analysis of glycans on recombinant Env proteins is highly significant. Here we present glycosylation profiles of recombinant gp120 proteins from four major clades of HIV-1 (A, B, C, and AE) produced either as research-grade material in 293 and CHO cells or as two independent lots of clinical material under GMP conditions. Almost all potential N-linked glycosylation sites were at least partially occupied in all proteins. The occupancy rates were largely consistent among proteins produced under different conditions, although a few sites showed substantial variability even between two GMP lots. Our data confirmed previous studies in the field showing high abundance of oligomannose on Env protein, with 40-50% of glycans having $Man_5$-$Man_9$ on all four proteins under all production conditions. Overall the differences in occupancy and glycan forms among Env from different subtypes produced under different conditions were less dramatic than anticipated and antigenicity analysis with a panel of six monoclonal antibodies showed that all four gp120s maintained their antibody-binding profiles, including antibodies that recognize glycan forms. Such findings have major implications to the final production of a clinical HIV vaccine including Env glycoprotein components.


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50 **Importance**

51 HIV-1 Env protein is a major target for the development of an HIV-1 vaccine. Env is
52 covered with a large number of sugar-based glycan forms – about 50% of the Env
53 molecular weight is composed of glycans. Glycan analysis of recombinant Env proteins
54 is important to understand its roles in vial pathogenesis and immune responses. The
55 current report presents the first extensive comparison of glycosylation patterns of
56 recombinant gp120 proteins from four major clades of HIV-1 produced in two different
57 cell lines, grown at either laboratory condition or at 50L GMP scale across different lots.
58 Information learned in this study is valuable for the further design and production of HIV-
59 1 Env proteins as the critical components of HIV-1 vaccine formulations.

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61 **Introduction**

62 The envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1) plays
63 the key role in viral entry and serves as a major target for a preventive HIV-1 vaccine.
64 Env is heavily glycosylated with N-glycans comprising about half of its molecular weight.
65 In the course of HIV-1 evolution, either within an individual patient or on the population
66 level, viral gene mutations may lead to the disappearance of certain glycosylation sites
67 and to the appearance of new ones. This shifting glycan shield protects the Env
68 proteins from the engagement of antibodies elicited during the course of viral infection
69 and contributing to the growth of escaping viral mutants in chronic infection (1).
70 Nevertheless, some glycan features are highly conserved even across clades such that
71 glycans contribute to several key antigenic domains recognized by broadly neutralizing
72 antibodies. For example, the highly conserved N332 glycan is important for the binding
73 of monoclonal antibodies (mAbs) PGT128 and 10-1074, while N160 glycan is
74 recognized by mAbs PG9 and PG16 (2, 3). Another conserved feature of HIV glycan
75 shield is high abundance of unusual oligomannose forms, which normally serve as
76 intermediates in mammalian glycan synthesis (4–6).
77
78 Glycosylation pattern can be expected to vary depending on multiple factors impacting
79 glycoprotein synthesis, including the viral strain, the form in which Env is expressed
80 (gp120, gp140, gp160), the cell type, the protein expression levels, and even the
81 metabolic state of the cell (7). In past studies, the exact proportion of oligomannose on
82 Env varied from 17% to 98% with levels of 40-75% being common for both monomeric
83 gp120 and native trimers (5, 8, 9). However, analysis of two batches of membrane-

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anchored Env showed remarkable consistency of forms found at each glycosylation site (9, 10), indicating that glycosylation patterns are generally preserved when the same Env protein is produced under identical conditions and that the differences in oligomannose content reflect either virus- or host cell specific factors.

In previous studies the viral clade-specific differences in the abundance of oligomannose have been attributed to the differences in the total number and regional density of the glycosylation sites, with higher glycan densities correlating with higher oligomannose content (4). On the other hand, comparisons of glycosylation of the same Env proteins expressed in 293 and CHO cells revealed mostly similar oligomannose content, similar occupancy, and similar glycan profiles, but with some notable exceptions (11, 12). It was observed that more complex glycans were present on CHO-derived clade C gp120 as compared to 293-derived protein, particularly at two sites (N386 and N392).

The increasing understanding of the impact of glycans on HIV Env immunogenicity and the increased focus on recombinant Env proteins after the RV144 trial led to the growing appreciation of the importance of these features to the design of Env-based protein vaccines against HIV-1(13). In particular, characterization of glycan profiles of recombinant Env proteins will be important to interpret the resulting antibody responses.

Various approaches of producing and purifying recombinant Envs for laboratory research and for clinical studies can be employed, but there's limited information based

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on well-controlled studies for how different approaches may impact Env glycosylation. While transiently-transfected 293 cells are often used to produce research-grade proteins, the proteins for clinical use are usually produced in stably transfected CHO cells. Clinical material is usually produced in bioreactors that have larger volumes and reach higher cell density than those used in research-grade protein production. Diverse purification processes are used both in the lab and during clinical-grade protein purification, such as antibody-based affinity columns, size-exclusion chromatography, lectin-based columns, or the industry preferred ion-exchange columns. Recently, a few recombinant GMP-grade Env-based vaccines have been characterized by analysis of glycans (14, 15) but the number of Env proteins included in those studies was limited and they did not provide direct comparison between different cell lines or between GMP and non-GMP for the production of the same Env proteins.

Here we expanded the study to characterize glycosylation profiles of four recombinant gp120 proteins from four major clades of HIV-1 (subtypes A, B, C, and AE) produced under GMP condition for a Phase I human clinical trial (HVTN124). We analyze two separate GMP lots of the same four gp120 proteins comparing them to the same four gp120 proteins produced under non-GMP condition in CHO and 293F cells. Our results provide much needed information on the Env glycan patterns among different viral clades and between different preparations of the same protein. Such information is not only valuable for the better understanding on the variation of Env glycan patterns but also critical for the establishment of quality control standards for the production of clinical grade Env-based HIV-1 vaccines.

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## Results

### HIV-1 gp120 Env proteins from four clades and produced under different conditions

The four gp120 glycoproteins included in the current study were selected based on the immunogenicity analysis of a large panel of HIV-1 Env variants (16) and were included in a polyvalent DNA prime-protein boost HIV vaccine formulation currently going through a phase I clinical study at HIV Vaccine Trials Network (HVTN, protocol 124). They represent three primary isolates from clades A, B, and C, as well as a consensus variant from the AE clade. Their amino acid sequences have a low homology to each other in the range of 75-80 % (Fig.1A). They have 23-26 potential N-linked glycosylation sites (PNGSs), which are distributed throughout the sequence in a similar, but distinct manner (Fig.1B).

Research-grade gp120 proteins were produced by transient transfection of 293F cells and from stably-transfected CHO cells in a laboratory setting and the proteins were purified using lectin-based columns. For the GMP manufacturing process, stably transfected CHO cells expressing each of the proteins were grown in 50L bioreactors and the purification process involved ion-exchange columns. Two separate GMP manufacturing runs have been done under identical conditions, which allowed us the opportunity to compare the consistency of glycosylation profiles from one GMP lot to another.

### Glycan analysis of research-grade proteins

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153 Glycan heterogeneity for gp120 proteins produced as research-grade reagents in CHO
154 and 293F cell lines were first analyzed. Digestion with PNGase F was used to release
155 glycans from the gp120 proteins and the released glycans were permethylated and
156 analyzed by NSI-MSn to characterize glycan structural features. Representative
157 profiles for clade B gp120 are shown on Fig. 2A. The types of glycan forms found on
158 proteins produced in CHO and 293F cells were generally very similar and only a few
159 types comprised more than 10% of the total glycans (Fig. 2B). A large proportion of
160 oligomannose forms ($Man_7$-$Man_9$) was detected in both preparations. A diverse group
161 of complex glycans were also present, as well as some hybrid forms. The results for
162 proteins from three other clades were similar (data not shown).
163
164 To study occupancy rate at each PNGS, proteins were digested with several proteases
165 to produce peptides for LC/MS analysis and then consecutively digested with EndoH
166 and PNGase F to allow detection of occupancy by different types of glycans at each
167 PNGS. Digestion with EndoH cleaves N-linked glycans between the two N-
168 acetylglucosamine (GlcNAc) residues in the core region of the glycan chain on high-
169 mannose and hybrid, but not complex, glycans, leaving one GlcNAc still bound to the
170 protein. Treatment with PNGase F removes all glycans that have not been impacted by
171 EndoH treatment and leaves an aspartic acid residue at the site of N-linked
172 glycosylation, which can be distinguished from the original asparagine by mass
173 spectrometry of the peptides. Therefore, consecutive digestion with EndoH and
174 PNGaseF allowed us to distinguish between oligomannose and complex glycans at

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each site. The presence of the original asparagine in the peptide indicates that the PNG site has not been glycosylated.

Analysis of PNGS occupancy of research grade 293F- and CHO-produced proteins showed that most of PNGSs were at least partially occupied in both cases (Fig. 3A). N141, N186 and N339 in clade B protein, N186 and N397 in clade C protein, and N465 in clade AE protein were the only sites that showed less than 20% occupancy in our analysis. A large proportion of oliogomannose glycans was observed for all four gp120 proteins, with higher proportions in glycans described as innate mannose patch.

Glycosylation profiles of proteins produced in 293F and CHO cells were remarkably similar (Fig. 3B). Most of the variation in occupancy was under 30 percentage points. Clade B proteins showed the largest variation with CHO-produced protein being more glycosylated than 293-produced protein. In some cases the changes were not in the total occupancy, but in the relative abundance of oligomannose and complex glycans. For example, N262 in clade B proteins was almost 100% occupied, but 293-produced protein carried an equal mixture of oligomannose and complex glycans, and CHO-produced proteins had almost exclusively oligomannose glycans at this site.

**Glycan profile analysis of four GMP-grade gp120 proteins**

The four GMP-grade gp120 proteins have been produced in stably expressing CHO cell lines at 50L scale and purified using a multi-step chromatography. Two separate lots have been manufactured using the same master cell bank CHO cells and the same

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fermentation and downstream purification process, which allowed us to investigate the lot-to-lot variability of GMP grade gp120 protein preparations. The glycan forms identified from GMP grade gp120 proteins are shown in Fig.4, and the relative amounts of these glycan forms between two GMP lots are show in Fig.5. For most gp120 proteins, oligomannose ($Man_5$-$Man_9$) constituted 40-50% of the total glycans, while complex glycans were about 35-45%, and the rest were hybrid glycans or paucimannose ($Man_3$-$Man_4$). The least-processed $Man_9$ and $Man_8$ forms predominated on clade A and clade B gp120 proteins, while clade C and clade AE proteins showed higher percentages of $Man_5$. Percentage of paucimannose showed the largest variation both between the clades and especially between the two lots of clade C gp120 protein, where in one of the lots paucimannose represented 23% of all glycans. Other proteins showed a more consistent glycan composition for two independent lots. Complex glycans were predominantly (63-87%) sialylated and >85% were core fucosylated (Fig.6).

Thus all variants of GMP-grade gp120 proteins exhibited high proportion of oligomannose glycans. The comparison of the two GMP lots showed mostly comparable glycan composition for three tested variants of gp120 proteins (clades A, B and AE) and some variation in the amount of paucimannose for clade C variant.

**PNGSs occupancy analysis of GMP-grade gp120 proteins**

Next, PNGS occupancy was mapped for proteins from one of the GMP lots and compared to the CHO-produced research-grade proteins. The overall occupancy profile

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of the GMP-grade gp120 proteins was generally similar to that of research-grade proteins, although the GMP proteins tended to have higher proportion of complex glycans (Fig.7). Analysis of the protein from the second lot did not reveal any major differences with the first lot (data not shown).

The oligomannose glycans were not equally distributed among the PNGSs. N262, N289/N295, N332, and N363 were enriched in oligomannose, corresponding to the intrinsic mannose patch that has been noted for HIV Env previously. While present on all four proteins, the patch was more pronounced for clade A, B, and C proteins, while oligomannose was more evenly distributed on clade AE protein. Clade C had an unusual enrichment in oligomannose at the C-terminus (N406, N442, N448, N463) that was absent in the other three proteins.

**Antigenicity analysis among 4 gp120 proteins**

Finally, we sought to test whether any observed lot-to-lot variation in PNGS occupancy and glycan composition, no matter how minor they may be, had an effect on key antigenic features of these proteins. Using the affinity-measuring Octet Qke system and a panel of probing reagents we tested the preservation of key epitopes on these four gp120 protein: CD4 binding site (IgG-CD4 and mAb VRC01), V2 loop (mAb 2158), V3 region (mAb R16), gp120 bridging sheet and loop F overlapping with CD4bs (mAb R53), and glycan forms (mAb 2G12 and mAb PGT128). The epitope for the 2G12 antibody is believed to include mannose-rich glycans at positions 295, 332, 392, 386, and 448 (17). The PGT128 epitopes includes glycans at positions 332 and 301, as well as the C-terminal end of the V3 loop (18). While we observed differences in affinities of

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these reagents, the differences between two lots of each clade were minimal (Table 1). This includes similar affinity of glycan-dependent 2G12 and PGT128 to the lot 2 of clade C protein, which exhibited the unusually high proportion of paucimannose compared to lot 1. Thus, our results demonstrate that observed differences in glycan profiles did not result in changes in affinity of antibodies targeting key epitopes of four gp120 proteins, including glycan-binding antibodies, indicating that proteins produced under different conditions mostly preserved their antigenic structure.

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253 **Discussion**

254 This report presents the first extensive comparison of glycosylation patterns of
255 recombinant gp120 proteins from four clades of HIV-1 across two different cell lines,
256 grown either at laboratory scale or at 50L GMP conditions, purified using different
257 methods, and across two GMP lots under identical conditions. Our results show that in
258 all four gp120 proteins included in the current study, the majority of PNG sites were
259 occupied by glycans with occupancy rates usually above 50%. We also found that
260 among glycans found on these proteins, oligomannose forms represented 40-50% and
261 were concentrated in the previously described intrinsic mannose patch. At the same
262 time, glycosylation profiles were basically very similar, with a low level of variability
263 under different conditions. These differences did not affect binding by a panel of
264 antibodies targeting key immunologic epitopes of gp120 proteins, indicating that the
265 observed low level glycan differences should not have a major impact on protein
266 immunogenicity.
267
268 The most prominent feature of HIV Env glycans distinguishing them from glycans on
269 host proteins is the presence of oligomannose. In agreement with previous reports, we
270 observed high proportion of oligomannose glycans ($Man_5$-$Man_9$) on all four proteins
271 produced under all conditions confirming that this is a characteristic feature of HIV
272 envelope. Even though previous studies reported a rather wide range of the percentage
273 of oligommannose, we consistently observed that in all cases it constituted 40-50% of
274 glycans. At least one recent study reported that gp120 derived from infectious virions
275 contained 50% oligomannose glycans (19), which suggests that glycosylation patterns

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of native Env are generally preserved on the recombinant gp120 proteins. This preservation is important for HIV vaccine development aiming to elicit antibodies that bind and neutralize HIV virions.

The research-grade gp120 protein was produced in 293F and CHO cells and purified using lectin columns, while the GMP-grade protein was produced in CHO cells grown at 50L scale and purified using ion exchange columns. Nevertheless, we found only minor differences in glycan occupancy and glycan content between proteins produced under different conditions, indicating that these features are primarily determined by viral sequence and not by the producing cells or purification process.

Our comparison of two independent GMP-grade lots of gp120 proteins showed a consistent glycosylation pattern between them, but also did show some differences, including a significant increase in paucimannose content in one of the lots of clade C protein. It should be noted that this particular lot differed from all other GMP lots in having a significantly higher yield of the protein. We do not have enough data to establish the causal relationship between these two observations, but hypothesize that high levels of protein production overwhelmed medial and trans-Golgi glycan-processing machinery in producing cells, resulting in secretion of proteins with glycans that were fully trimmed by cis-Golgi mannosidases but incompletely branched, extended, and capped. Further work is needed to test the factors impacting variability of glycosylation during GMP manufacturing and acceptable variability levels should be

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established. Special attention should be paid to optimizations that boost protein production in cells as that may have an impact on cellular glycosylation machinery.

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## Materials and Methods

### Production of Non-GMP gp120 proteins

The four gp120 glycoproteins used in this report are from HIV-1 clade A isolate 92UG037.8, clade B isolate JRFL, clade C isolate 93MW965.26 and clade AE consensus, respectively (16). The non-GMP research grade HIV-1 gp120 proteins were produced using two protein expression systems: transiently transfected 293F cell expression and stably transfected CHO cell expression system. The codon optimized gp120-coding DNA inserts cloned in the vector pJW4303 were used in both 293F and CHO cells. To produce gp120 proteins from transiently transfect 293F cells, the serum-free 293F cell supernatant was collected at 72 hours after transfection. To express gp120 proteins from CHO cells, the serum-free culture supernatant of stable transfected CHO cells were collected. The harvested gp120 proteins from both 293F and CHO cells in research grade were purified using a Lectin column and verified by SDS-PAGE and Western blot analysis as previously described (20–22).

### Production of GMP grade gp120 proteins

The codon optimized gp120 gene inserts from the same four clades (A, B, C and AE) as described above for research grade proteins were transfected into CHO DG44 cells (Invitrogen, CA), respectively, and used to establish the master cell banks. Each of these four gp120 expressing stable CHO GD44 cells were grown in 50L bioreactors and the cell culture supernatants were collected after 8-10 days of fermentation and purified through a down stream purification process including the anion-exchange, cation-exchange and size-exclusion steps under the GMP conditions. The purity of each

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gp120 proteins is at the range of 96-98% based on their release certificates. The same purified gp120 proteins are currently being tested in a Phase I clinical trial under HVTN124 protocol at six major US medical centers.

**Detection of occupancy of N-linked glycosylation sites on gp120 proteins**

An aliquot of each gp120 protein was buffered to alkaline pH, reduced, alkylated, and digested with a combination of proteases including Lys-C (Promega), Arg-C (Promega), Glu-C (Promega), and trypsin (Promega). Following digestion, the proteins were deglycosylated by Endo-H (Promega) followed by PNGaseF (Glyko®, Prozyme) treatment in the presence of $^{18}$O-water. The resulting peptides were separated on an Acclaim PepMap RSLC C18 column (75 µm x 15 cm) and eluted into the nano-electrospray ion source of an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (Thermo Fisher Scientific) with a 240-min linear gradient consisting of 0.8-80% acetonitrile over 180 min at a flow rate of 200 nL/min. Full MS analysis was conducted in the Orbitrap and automated MS/MS analysis using collision-induced dissociation was conducted in the ion trap. Resulting data was analyzed using a combination of Proteome Discover/Sequest and ProteoIQ/Provalt to generate a 1% false discovery rate for protein assignments. Site occupancy was calculated using spectral counts assigned to the $^{18}$O-Asp-containing (PNGaseF-cleaved) and/or HexNAc-modified (EndoH-cleaved) peptides and their unmodified counterparts. The positivity cut-off for spectral counts was set at 10% of the spectral count for the most abundant peptide in each LC/MS run. Peptides with spectral counts below the positivity cut-off were not included in the analysis.

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348 **N-linked glycan profiling analysis for gp120 proteins**

349 A 20 μg aliquot of each gp120 sample was denatured by boiling in SDS. Upon cooling,
350 the SDS was removed by precipitation as its potassium salt. Denatured proteins were
351 buffered, recombinant peptide-N-glycanase F (PNGaseF) was added, and the mixture
352 was incubated overnight to release N-linked glycans. The released N-linked glycans
353 were freed from residual enzyme, deglycosylated protein, and other contaminants by
354 passage over C18 Sep-pak. The released, purified glycans were permethylated using
355 methyliodide ($CH_3I$) under basic conditions in an aprotic solvent (DMSO), followed by
356 recovery through organic extraction. For mass spectrometric analysis, one-half of the
357 total permethylated glycans released from 20 μg of protein (10 μg equivalent of protein)
358 was supplemented by the addition of 10 pmol of an exogenous glycan standard
359 (maltotetraose) that had previously been permethylated with isotopically heavy
360 methyliodide ($^{13}CH_3I$). The sample glycans spiked with standard were directly infused
361 into an LTQ/Orbitrap mass spectrometer fitted with a nanospray ionization interface
362 (NSI-MSn; Orbitrap Discovery, Thermo-Fisher). Glycans were detected in full MS mode
363 and by total ion mapping (TIM), in which automated CID is performed on small,
364 overlapping m/z windows. TIM allows the unbiased detection of ions that give
365 fragmentation patterns consistent with glycan structural topologies (23). Glycan signal
366 intensities were recovered from extracted full MS spectra (Xtract, Thermo-Fisher) and
367 glycan identities were assigned based on exact mass and CID fragmentation.
368 Graphical representations of monosaccharide residues are presented in accordance
369 with the broadly accepted Symbolic Nomenclature For Glycans, SNFG, and glycan

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analysis was performed in keeping with the MIRAGE guidelines for glycomic studies (24, 25).

**IgG-CD4 protein and gp120-specific monoclonal antibodies (mAbs)**

The IgG-CD4 protein used in the Octet Qke assays was a fusion protein of human CD4 domains 1 and 2 with human IgG1 Fc at the C-terminus produced by transient transfection of 293F cells and His-tagged purification. The gp120 CD4 binding site (CD4bs) monoclonal antibody VRC01 (26) was produced from transiently transfected 293F cells using the molecular clones coding for VRC01 heavy and light chains obtained from NIH AIDS Reagent Program and purified with a Protein A column. The gp120 glycan-specific human mAb 2G12 (27) was purchased from Polymun. The gp120 glycan specific mAb PGT128 (18) was provided by Wayne Koff from International AIDS Vaccine Initiative (IAVI). The gp120-V2 specific human mAb 2158 (28) was purchased from Dr. Susan Zolla-Pazner's lab at Mount Sinai School of Medicine. The gp120 V3 and C4 specific rabbit mAbs R16 and R53 as reported (29, 30) were produced from transiently transfected 293F cells using paired heavy and light chain molecular clones and purified using a Protein A column. The IgG-CD4 protein and monoclonal antibodies produced by this study were verified before use.

**Antigenicity analysis of gp120 proteins with Octet Qke**

The antigenicity of gp120 proteins was tested using IgG-CD4 and gp120-specific monoclonal antibodies by Octet Qke (ForteBio) based on biolayer interferometry. The IgG-CD4 or each gp120-specific human mAb was individually loaded on to Protein G sensors at 20 µg/mL and the individual gp120-specific rabbit mAb was loaded to Protein

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A sensors at 10 µg/mL diluted in ForteBio kinetics buffer. After capture, tips were washed in kinetics buffer and a baseline measurement recorded. The tips were then incubated in wells containing serial dilutions of individual gp120 protein (600nM-0.4nM) to measure the association rate (Kon) and dissociation rate (Koff). The antibody binding kinetics and KD values (Koff/Kon) were collected by the ForteBio Data Analysis software package v7.1 using a 1:1 fitting model for IgG-CD4 and mAbs VRC01, PGT128, 2G12, 2158 and R16, and using a 2:1 fitting model for mAb R53.

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545

546 **Figure legends**

547

548 Figure 1. A. Sequences of the gp120 proteins belonging to clade A (92UG037.8), B
549 (JRFL), C (93MW965.26), and AE (consensus) aligned to the reference strain HXB2.
550 Identical amino acids are shown as dots, gaps are indicated with dashes, numbers
551 correspond to the HXB2 sequence. Variable regions V1-V5 of gp120 are indicated
552 above the sequences. PNGSs predicted based on the consensus glycosylation
553 sequence are shown in red font and marked with stars above the sequences. B.
554 Summary of glycosylation site distribution among the four gp120 proteins. N indicates
555 the presence of the PNGS in the sequence.

556

557 Figure 2. A. Representative MS spectra of glycans identified on research grade gp120
558 proteins produced in CHO cells (top) and 293F cells (bottom); The gp120 protein from
559 clade B (strain JR-FL) protein is shown here as a representative example. Glycan forms
560 corresponding to the most abundant peaks are shown using the standard glycan icons.
561 Mass peaks corresponding to oligomannose and complex glycan forms are indicated.
562 B. Quantitative comparison of relative proportions of various glycan forms (in %) on
563 CHO- and 293F-derived gp120-B proteins. Glycan structures found at proportions
564 greater than 10% of total glycans are highlighted in grey.

565

566 Figure 3. A. PNGS occupancy analysis of gp120 proteins expressed in 293F cells (top
567 row) and CHO cells (bottom row). The percent of glycan occupancy at each PNGS are

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shown for each of the gp120 proteins (clades A, B, C and AE) as indicated on the top of each panel. Green and purple bars indicate the percent of oligomannose glycans and complex glycans, respectively, and grey bars means the site was not occupied by a glycan. ND indicates peptides not detected. B. Differences (%) in glycan occupancy between the 293F- and CHO-produced proteins at each PNGS for each of the gp120 proteins. Percentages for oligomannose and complex glycans were compared at each site and the difference was plotted based on which protein had larger amount of that glycan. For example, at position N187 in clade A protein, 293F-derived protein had 25 percentage points more complex glycans than CHO-derived protein, while CHO-derived protein had 18 percentage points more oligomannose glycans than 293F-derived protein.

Figure 4. MS spectra of glycans released from four gp120 proteins (A, B, C and AE) produced under GMP conditions. Glycan forms corresponding to the most abundant peaks are shown using the standard glycan icons. Spectra corresponding to oligomannose and complex glycan forms are highlighted below.

Figure 5. Quantitation of the types of glycan forms released from two independent lots (Lot 1 & Lot 2) of gp120 proteins (A, B, C and AE) produced under GMP conditions. Numbers indicate the percentages of the corresponding glycan forms or structural classes as color coded for Man5 (M5), Man6 (M6), Man7 (M7), Man8 (M8), Man9 (M9), Pauci (Man 3 and Man4), Hybrid, and Complex.

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Figure 6. The analysis of sialylation (left panel) and core fucosylation (right panel) modifications of complex glycans of each gp120 protein (A, B, C or AE) produced from 2 different GMP lots. Only complex glycans were included in the analysis. "1" and "2" under each bar of the graph indicate lot 1 and lot 2 GMP-grade gp120 proteins, respectively. "A", "B", "C" and "AE" at the bottom of the graph indicate gp120 protein from clades A, B, C and AE, respectively.

Figure 7. A. PNGS occupancy analysis of GMP-grade gp120 proteins. The percent of glycan occupancy at each PNGS is shown for each of the gp120 proteins (clades A, B, C and AE) as indicated on the top of each panel. Green and purple bars indicate the oligomannose glycans and complex glycans, respectively, and grey bars means the site was not occupied by a glycan. ND indicates peptides not detected. B. Differences (%) in glycan occupancy in gp120 proteins produced in CHO cells under research-grade conditions and under GMP conditions at each PNGS. The plots were generated as described in Figure 3B.

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609 **Table 1.** Binding affinity of two GMP lots of gp120 proteins, $K_d$ (M)

| GMP gp120 proteins | gp120 specific mAb | | | | | | IgG-CD4 |
|---|---|---|---|---|---|---|---|
| | CD4bs | Glycan | | V2 | V3 | C4 | |
| | VRC01 | 2G12 | PGT128 | 2158 | R16 | R53 | |
| Clade A Lot 1 | 6.04E-09 | 3.11E-10 | 7.68E-10 | 6.85E-09 | 5.59E-10 | 6.76E-08 | 5.48E-09 |
| Clade A Lot 2 | 5.96E-09 | 1.80E-10 | 7.62E-10 | 6.46E-09 | 2.81E-10 | 2.76E-08 | 3.62E-09 |
| Clade B Lot 1 | 3.11E-08 | <1.0E-12 | 2.46E-09 | 4.55E-09 | 3.49E-10 | 1.16E-08 | 7.44E-09 |
| Clade B Lot 2 | 2.83E-08 | <1.0E-12 | 2.10E-09 | 3.26E-09 | 4.56E-10 | 1.32E-08 | 4.87E-09 |
| Clade C Lot 1 | 4.53E-08 | 5.54E-09 | 3.25E-08 | 2.61E-08 | 3.80E-10 | 5.63E-09 | 1.57E-07 |
| Clade C Lot 2 | 5.21E-08 | 2.24E-09 | 2.03E-08 | 3.31E-08 | 4.27E-10 | 4.98E-08 | 1.15E-07 |
| Clade AE Lot 1 | 5.25E-08 | 8.02E-09 | 3.71E-08 | 3.05E-08 | 5.87E-10 | 8.89E-09 | 2.46E-08 |
| Clade AE Lot 2 | 3.53E-08 | 8.62E-09 | 2.62E-08 | 3.32E-08 | 3.22E-10 | 5.63E-09 | 1.84E-08 |

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Fig 1. gp120 sequence alignment

A.




B.

| | N88 | N130 | N136 | N139 | N141 | N143 | N148 | N156 | N160 | N186 | N187 | N197 | N230 | N234 | N241 | N262 | N276 | N289 | N295 | N301 | N332 | N334 | N339 | N356 | N363 | N386 | N392 | N394 | N397 | N403 | N406 | N442 | N448 | N461 | N463 | N465 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | N | | N | | N | | N | N | N | N | N | N | | N | N | N | N | N | N | N | N | | | N | N | N | | N | N | | N | | N | | N | |
| B | N | | N | N | N | | | N | N | N | N | | | | N | N | N | | N | N | N | | N | N | N | N | N | | N | | N | | N | | N | |
| C | N | N | N | | N | | N | N | N | N | N | N | N | | N | N | N | N | | N | N | | N | N | | N | | | N | N | N | N | N | | N | |
| AE | N | N | N | | | N | N | N | N | N | | N | | N | N | N | N | N | N | N | | N | | N | | N | N | | N | | N | | N | N | | N |

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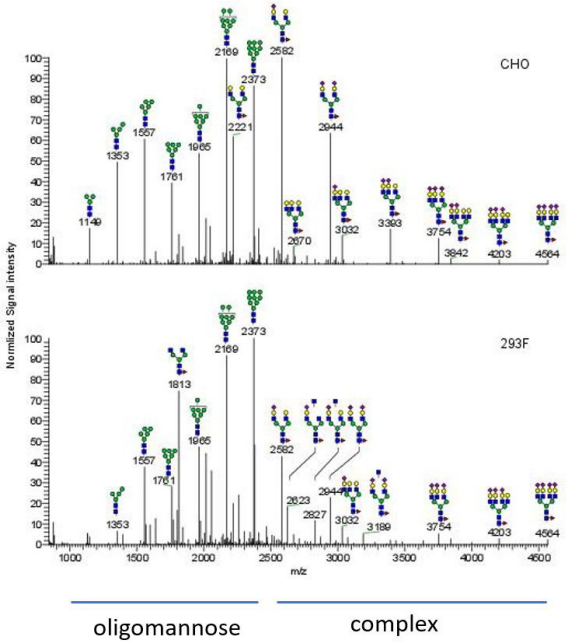
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Fig 2. Glycoform analysis of clade B gp120 proteins expressed in CHO and 293F cells.

A.




B.

| Glycan | CHO | 293 |
|---|---|---|
| M3 | 2.4 | 0.7 |
| M4 | 7.0 | 1.2 |
| M5 | 8.5 | 7.0 |
| M6 | 5.5 | 5.2 |
| M7 | 7.6 | 8.8 |
| M8 | 14.0 | 17.0 |
| M9 | 12.2 | 18.6 |
| N2M3N2F | 2.0 | 13.8 |
| LacNAc2M3N2F | 8.7 | 3.7 |
| LacNAc3M3N2F | 1.2 | 0.9 |
| NA1LacNAc1GN2M3N2F | 14.1 | 7.9 |
| NA1G1N3M3N2F | 0.6 | 3.4 |
| NA1LacNAc1GN2M3N2F | 0.3 | 2.1 |
| NA2G2N2M3N2F | 8.9 | 4.2 |
| NA1LacNAc2GNM3N2F | 1.9 | 1.6 |
| NA2G2N3M3N2F | 0.1 | 1.0 |
| NA2LacNAc1G2N2M3N2F | 2.4 | 0.4 |
| NA3G3N3M3N2F | 1.7 | 1.0 |
| NA2LacNAc2G2N2M3N2F | 0.3 | 0.5 |
| NA3LacNAc1G3N3M3N2F | 0.3 | 0.5 |
| NA4G4N4M3N2F | 0.3 | 0.4 |
| Total | 100 | 100 |

| |
|---|
| >10 |
| ≤10 |

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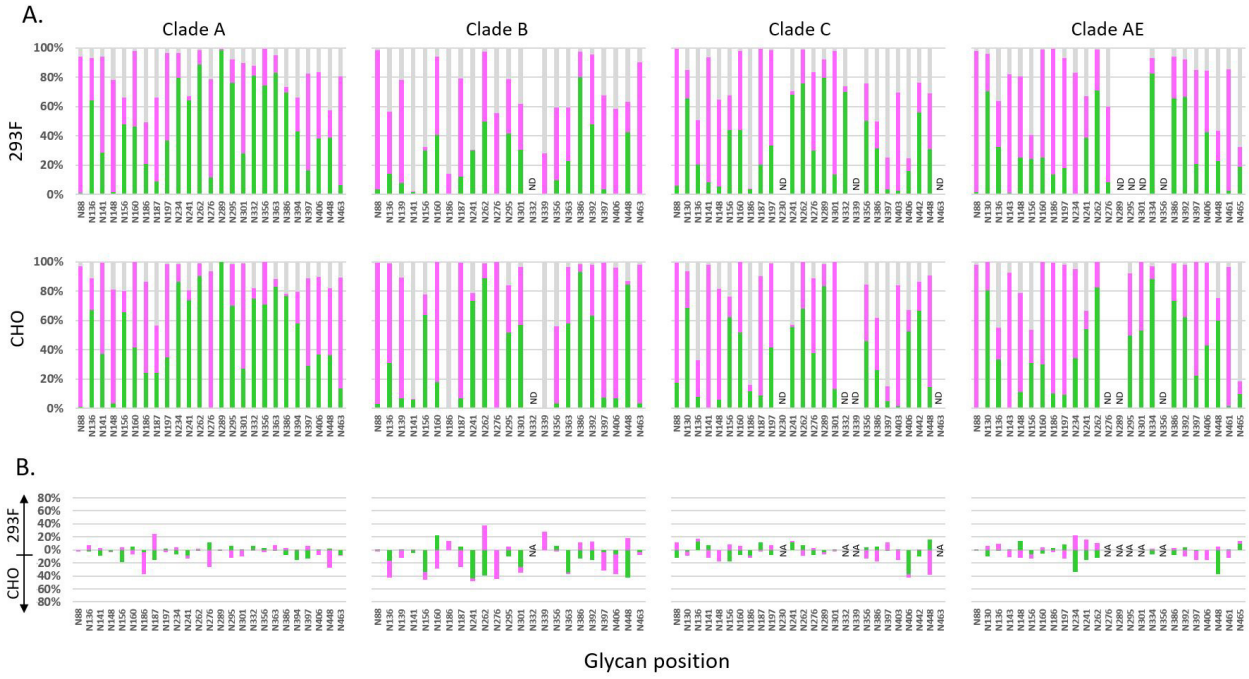
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Fig 3. A. PNGS occupancy analysis of gp120 proteins expressed in CHO and 293F cells. B. Differences in glycosylation between 293- and CHO-produced proteins.



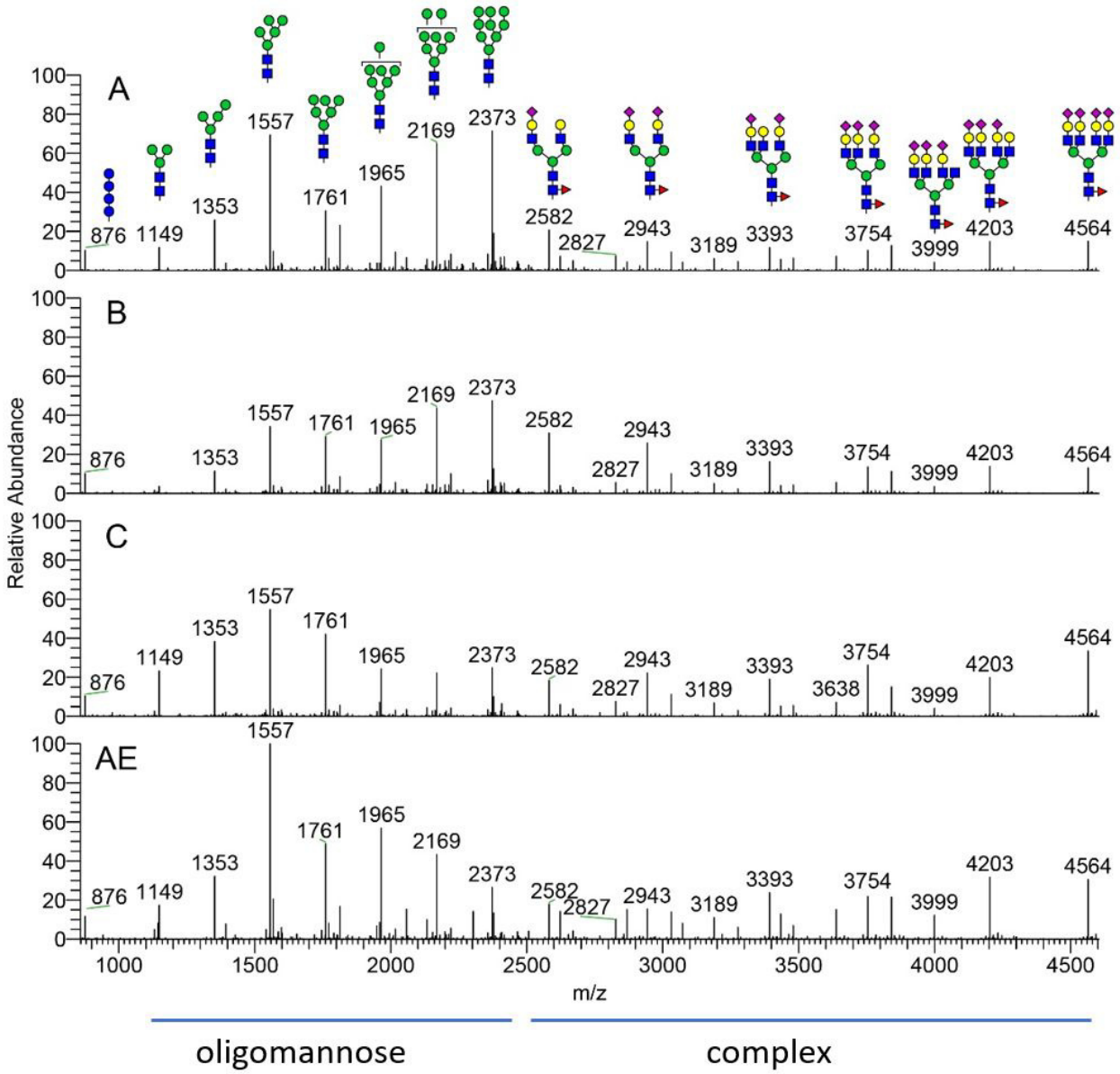
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Fig 4. Glycan profiles of gp120 proteins produced by GMP manufacturing.




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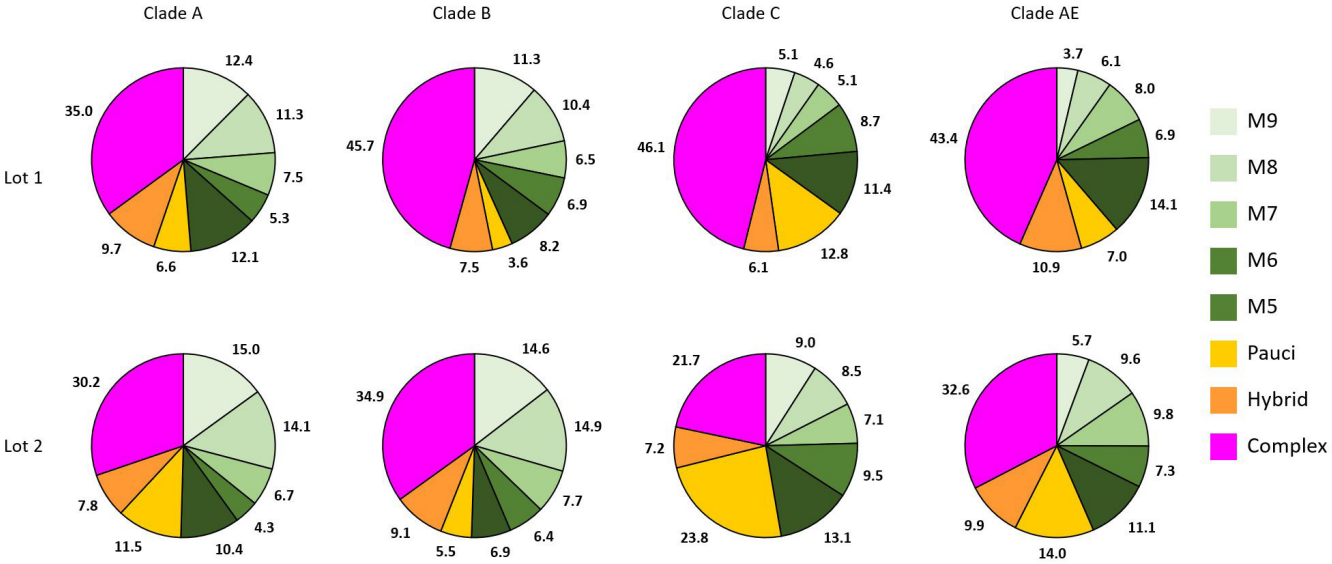
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Fig 5. Glycan profiles of gp120 proteins produced by GMP manufacturing.



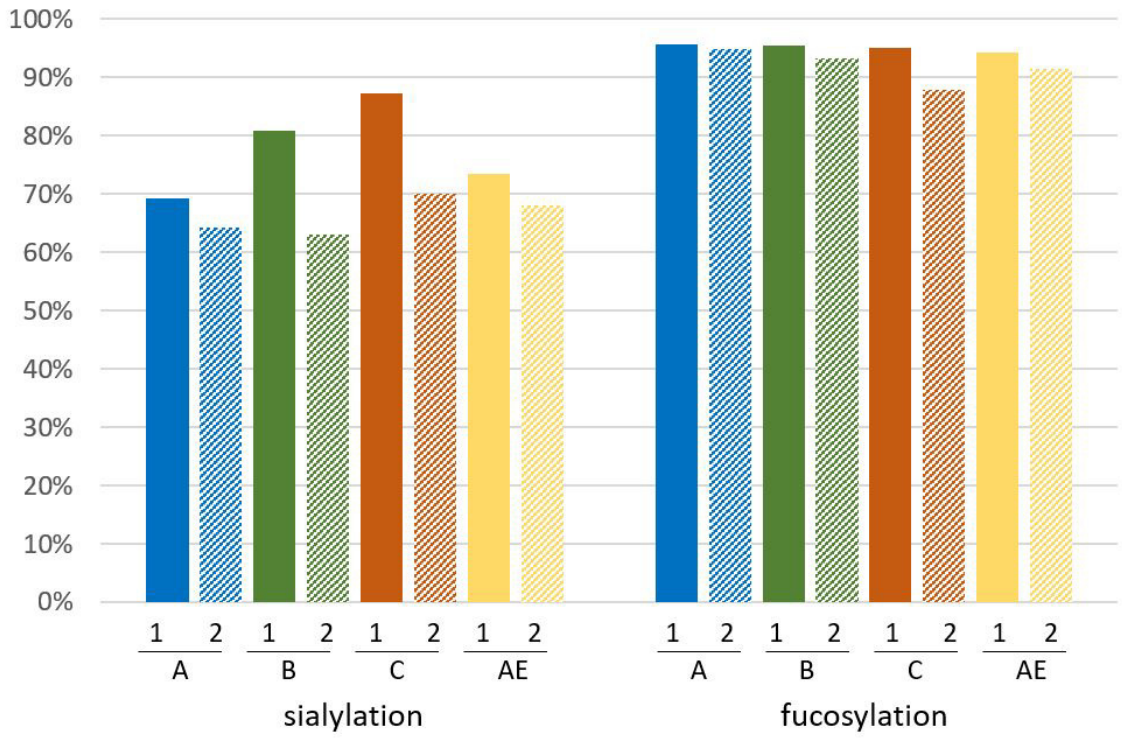
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Fig 6. Sialylation and core fucosylation of GMP grade gp120s.




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Fig 7. Glycan occupancy analysis of GMP-grade proteins.




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