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Characterization of native-like HIV-1 gp140 glycoprotein expressed in insect cells

Tingting Li [a,1], Zhenyong Zhang [a,1], Zhiqing Zhang [b,1], Jiaming Qiao [b], Rui Rong [a], Yuyun Zhang [b], Qiaobin Yao [a], Zekai Li [b], Honglin Shen [a], Fang Huang [a], Wenhui Xue [a], Shuangquan Gao [b], Shaoyong Li [b], Qingbing Zheng [b], Hai Yu [b], Jun Zhang [b], Ying Gu [a,b,*], Shaowei Li [a,b,*], Ningshao Xia [a,b]

[a] State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Life Sciences, Xiamen University, Xiamen 361102, China
[b] National Institute of Diagnostics and Vaccine Development in Infectious Disease, School of Public Health, Xiamen University, Xiamen 361102, China

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ABSTRACT

The trimeric HIV-1 envelope glycoprotein (Env) is critical for vaccine development aimed at achieving broadly-neutralizing antibody responses. The use of various recombinant expression systems and construct designs are associated with the resultant nature of produced proteins, especially in terms of glycosylation, antigenicity, and immunogenicity of the glycoprotein. Here, we explored an otherwise baculovirus cassette than classical one designed to express HIV-1 Env protein, including SOSIP mutation and Foldon moiety involvement. This improved design increased the ratio of the Env trimer fraction from ~40% to ~60% with respect to that of prototypical design, as indicated by high-performance size-exclusion chromatography and sedimentation velocity analysis. In addition, the design prolonged cell viability and enhanced the final yield (approximately 13–15 mg/L) after affinity purification. gp140 produced from insect cells mimicked the native-like trimer and mainly adopted glycosylation pattern of oligomannose glycans. The native-like Env proteins conferred cross-clade neutralizing antibody production in BALB/c mice. In summary, the expression of Env in insect cells by optimizing the baculovirus vector provides an alternative strategy for HIV-1 immunogen production and may benefit future Env-based HIV vaccine design.

1. Introduction

Despite countless efforts, an efficient vaccine to end the human immunodeficiency virus type 1 (HIV-1) epidemic is still not available [1,2]. The greatest challenge in generating an effective HIV-1 vaccine is the immunogen design, which is required to induce the production of protective antibodies, especially broadly-neutralizing antibodies (bNAbs) [3–6]. The envelope glycoprotein (Env) trimer is the only virally encoded surface antigen, and is responsible for mediating receptor binding and membrane fusion during virus entry [7,8]. The precursor of Env, named gp160, is cleaved into gp120 and gp41 that remain heterodimer in non-covalent association and consequently form trimeric spikes residing in viral membrane during HIV maturation. [9,10]. Although a major antigenic target for NAbs, the Env spikes exhibit inherent metastability and evade the host's immune response by presenting Env proteins of different conformational states, thereby confusing the immune system. Meanwhile, the non-neutralizing antibody epitopes presented by the structure-deficient Env proteins impede with the NAb responses to the functional trimer [11–14]. Therefore, the design and evaluation of a functional HIV-1 Env immunogen is critical for vaccine design [1].

In recent years, several groups have focused on a stable, native-like Env design [15–18]. One of most successful strategies is the SOSIP.664 design (using ectodomain of full-length gp160, gp140), which replaced the original cleavage site (REKR) with an optimal cleavage site (RRRRRR), and engineered an intramolecular disulfide bond between gp120 and the gp41 ectodomain ($gp41_{ECTO}$) to facilitate an interaction between the two cleaved subunits [19]. Another residue substitution, isoleucine 559 to proline, stabilized the trimerization in its prefusion conformation [20]. This strategic design could generate desirable soluble, stable and native-like trimers with excellent biochemical properties and structure-mimicking of the native Env spike [21]. Some researchers have reported that the soluble HIV-1 trimer with a native conformation can induce NAbs potently against viruses in rabbits or guinea pigs

* Corresponding authors at: National Institute of Diagnostics and Vaccine Development in Infectious Disease, School of Life Sciences, Xiamen University, Xiamen 361102, China.
E-mail addresses: guying@xmu.edu.cn (Y. Gu), shaowei@xmu.edu.cn (S. Li).
[1] These authors contributed equally to this work.

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[3,22,23]. The SOSIP design has also been critical in the structure determination of HIV Env [24,25].

Another strategy in the HIV vaccine field was the use of a supplementary sequence called "Foldon" from the T4 bacteriophage ferritin to aid trimerization [26]. Since then, however, more advanced and rational structure-guided design has been employed, such as the recent use of an uncleaved, prefusion-optimized (UFO) trimer, which replaces the cleavage site between gp120 and gp41 with various linkers [27]. In summary, the native-like Env trimer provides opportunities for further preclinical candidate vaccine study.

These advances notwithstanding, the current cell lines used for expressing the native-like Env trimer are mostly made in 293 cells and CHO cells [15,28–30] as many highly successful biotech products are made in CHO and similar systems. Although transient cell transfection produces a low yield of the Env trimer, and one group suggested the use of a stable cell line to increase trimer production [30]. Another eukaryotic expression platform, the baculovirus expression vector system (BEVS), is inherently safe, easy to scale-up, and provides a quick production in a serum-free culture system [31,32]. Like mammalian cells, insect cells also permit post-translational modifications, including folding, glycosylation, disulfide-bond formation [33]. To date, BEVS has already supported two licensed viral vaccines: Cervarix for cervical cancer, which is mostly caused by HPV, and FluBlok for the seasonal influenza epidemic [34]. There are still some reports about the production of Env gp140 trimers from insect cells and showed good immunogenicity with NAb responses of gp140 trimers [35,36]. Nevertheless, the glycosylation information on the Env produced in insect cells remains unknown; this is an important consideration, because glycosylation is associated with Env antigenicity and immunogenicity [35,37]. Hence, the biochemical properties, glycans composition related to antigenicity and immunogenicity of the native-like Env protein still need to be addressed in the insect cell expression platform.

As a versatile and powerful expression system, BEVS can be easily manipulated for specific purposes. One optimized strategy minimized the baculovirus genome by removing dispensable genes (*v-cath* and *chiA*) in terms of proteomics analysis [34]. The *v-cath* gene encodes a papain-like cysteine protease called cathepsin, and chitinase, encoded by the *chiA* gene, may help to activate the pro-V-cathepsin when accumulated in the endoplasmic reticulum (ER). This, in turn, leads to proteolysis of recombinant proteins [38]. Thus, the removal of these two non-essential genes resulted in a more efficient baculovirus vector system that showed increased expression of the targeted proteins, presumably by saving energy and reducing proteolysis [38–40]. The *v-cath/chiA* knock-out virus has already been used to express secreted membrane protein but have not report on the env protein.

In this study, we explored the expression of three HIV-1 gp140 constructs using this modified BestBac baculovirus vector system (missing *v-cath* and *chiA*) and characterized the purified Env proteins. The results showed significant enhancement of HIV-1 gp140 protein expression using this modified baculovirus system. The purified gp140 protein was antigenic and glycosylated with oligomannosidic glyans. We assessed the NAb response of three gp140 proteins in BALB/c mice, particularly for cross-clade neutralizing activity against two HIV-1 strains (clade B and clade D). These results may benefit the further investigation of HIV-1 immunogens prepared with insect cells.

2. Materials and methods

2.1. Cloning, protein expression and purification

The HIV-1 NL4-3 Env gene (Accession: AAK08489.2) was amplified by PCR and cloned into a baculovirus transfer vector pAcgp67B (BD Biosciences, CA, USA) using Gibson assembly. The WT.681-His, encoding aa 31–681 (numbered as BXB2 sequence), contains a C-terminal 10-His tag for purification. Based on this construct, the SOSIP.664-10His, encoding aa 31–664 with mutations in A501C, T603C, I559P, and R6 (RRRRRR), was prepared using the Mut Express II Fast Mutagenesis Kit V2 (Vazyme, Nanjing, China). The SOSIP.681-Fd-10His contains the MPER sequence and a Foldon domain to assist in trimerization. In all three constructs, the natural signal peptide was replaced with a gp67 secretion signal peptide.

Plasmids were co-transfected with linearized Bestbac 1.0 (with *v-cath/chiA* genes) or 2.0 DNA (deficient in *v-cath/chiA* genes) (Expression Systems, CA, USA) into *Spodoptera frugiperda* 9 (Invitrogen) insect cells, according to the protocol provided by the manufacturer (Expression Systems). The transfection supernatant was harvested and amplified 2 times to obtain a high titer of the recombinant viruses. Hive Five cells (BTI-TN-5B1-4) (Thermo Fisher Scientific, MA, USA) were cultured in ESF921 medium (Expression Systems) and infected with recombinant virus at an multiplicity of infection (MOI) of 5 in the exponential growth phase (2×10^6 - cells/ml, 95% viability) at 28 °C for 72 h or 120 h for recombinant virus 1.0 or 2.0, respectively. The viability and diameter of the infected cells were assessed using an automated Countstar cell counter (ALIT Life Science, Shanghai, China). The supernatant was then dialyzed against phosphate-buffered saline (PBS), pH 7.4, and purified with TALON resin (GE Healthcare, Boston, USA) by the elution with 150 mM imidazole. The protein concentrations of the final purified samples were measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), and the purities were calculated from the image of SDS-PAGE gel using Quantity One software (BioRad Laboratories, CA, USA). The Env protein was concentrated and stored in PBS at −80 °C.

2.2. SDS-PAGE and western blot

Protein samples were mixed with loading buffer (50 mM Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue, 8% glycerol), boiled for 10 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of protein for each sample were loaded onto two SDS-PAGE gels, one for western blotting and one for Coomassie staining. The proteins were electrophoresed for 70 min at 120 V in a BioRad MINI-PROTEAN Tetra system (BioRad Laboratories), and the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad) for 30 min at room temperature. For western blotting, separated proteins were transferred onto a nitrocellulose membrane (Whatman, Dassel, Germany) using a Trans-Blot Turbo transfer system (Bio-Rad). The membrane was blocked with 5% skim milk for 40 min and then incubated for 1 h with an HIV-1-specific mouse mAb 3A7 antibody (1:5000 dilution). Unbound antibody was removed by five 5-min washes and the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (DAKO). Membranes were washed again and then developed using a mixture of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Pierce Biotechnology; Rockford, IL).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Purified proteins were coated onto 96-well microtiter plates at 200 ng/well in PBS at 37 °C for 4 h. The background was blocked with 1 × Enzyme dilution buffer (PBS + 0.25% casein + 1% gelatin + 0.05% proclin-300) at 37 °C for 2 h. Two-fold serially diluted human antibody or serum was added to the wells (100 μl) and incubated at 37 °C for 1 h. Horseradish peroxidase (HRP)-labeled mouse anti-human antibody or anti-mouse antibody (Abcam, Cambridge, UK) was used as secondary antibody at 1:5000 for

30 min. Wells were washed again and the reaction catalyzed using o-phenylenediamine (OPD) substrate at 37 °C for 10 min. The OD_{450nm} (reference, OD_{620nm}) was measured on a microplate reader (TECAN, Männedorf, Switzerland), with a cut-off value 0.1. The EC_{50} was calculated by sigmoid trend fitting using GraphPad Prism software. For endpoint ELISA, titers were defined as the reciprocal serum dilution that reached an absorbance of two-fold over the background values. For double-antibodies Sandwich ELISA, calibration of the quantification of gp140 protein using 293 cell-derived gp140 [41] served as the standard calibrator. The concentrations of gp140 proteins in supernatant were measured every 24 h post infection according to quantification assay established.

2.4. Size-Exclusive chromatography (SEC)

Purified proteins were subjected to HPLC (Waters; Milford, MA) using a TSK Gel G5000PW_{XL} 7.8 × 300 mm column (TOSOH, Tokyo, Japan) equilibrated in PBS, pH 7.4. The system flow rate was maintained at 0.5 mL/min and eluted proteins were detected at 280 nm. The standards (GE healthcare, Boston, USA) used to calculate the molecular weight consisted of thyroglobulin (670 kDa), ferritin (440 kDa), and catalase (232 kDa).

2.5. Analytical ultracentrifuge (AUC) assay

The AUC assay was performed using a Beckman XL-Analytical ultracentrifuge (Beckman Coulter, Fullerton, CA), as described elsewhere [42]. The sedimentation velocity (SV) was carried out at 20 °C with diluted proteins (1 mg/ml) in PBS. The AN-60 Ti rotor speed was set to 30,000 rpm according to the molecular weight of the control proteins. Data was collected using SEDFIT computer software, kindly provided by Dr. P. C. Shuck (NIH, Bethesda, MA, USA). Multiple curves were fit to calculate the sedimentation coefficient (S) using continuous sedimentation coefficient distribution model [c(s)], and then the c(s) used to estimate protein molar mass (MW).

2.6. Animal immunization

The experimental protocols were approved by the Xiamen University Laboratory Animal Management Ethics Committee. All manipulation was strictly conducted in compliance with animal ethics guidelines and approved protocols. The mouse group size was 5 per group. Eight-week-old BALB/c mice were immunized by an initial intramuscular injection (300 μl) of 100 μg HIV Env proteins at different sites: left and right posterior limb—WT.681-His, SOSIP.664-His, SOSIP.681-Fd-His—with 50% Freund's complete adjuvant (Sigma-Aldrich) in week 0. For the next two immunizations in weeks 2 and 4, the 300-μl injection comprised 100 μg HIV Env proteins with 50% Freund's incomplete adjuvant (Sigma-Aldrich). Blood samples were collected before each injection and were centrifuged at 13,000 × g for 10 min. Serum samples were preserved at −20 °C.

2.7. Virus production

The infection clones, pNL4-3 and p94UG114, were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Program. The 293FT cells were seeded in a 10-cm dish at a density of 5×10^6 cells for 6 h. Cells were transfected with 45 μg of the infection clone plasmid using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The culture medium was removed and replaced with fresh medium 12 h post-transfection. After 72 h, the supernatant containing HIV-1 was harvested and filtered through a 0.4-μm filter.

2.8. Neutralization assay

One day before infection, TZM-bl cells were plated into the wells of a 96-well plate at 1.5×10^5/well in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal calf serum (FCS), 10% Gibco® Minimum Essential Medium (MEM) nonessential amino acids, penicillin and streptomycin (both at 100 U/ml), and incubated at 37 °C in an atmosphere containing 5% CO_2 for 48 h. 100 $TCID_{50}$ (50% tissue culture infective doses) of virus was incubated for 1 h at room temperature, and 3-fold serially diluted. This mixture was then added to the cells and incubated at 37 °C for 48 h. The medium was removed and the TZM-bl cells were fixed with glutaraldehyde (0.2%) and stained with an X-gal substrate. The HIV-infected cell spots were counted using an Immunospot Series Analyzer (Cellular Technology, Cleveland, OH). Percentage neutralization was determined with the following formula: %Neutralization = (Control − Treatment) × 100/control.

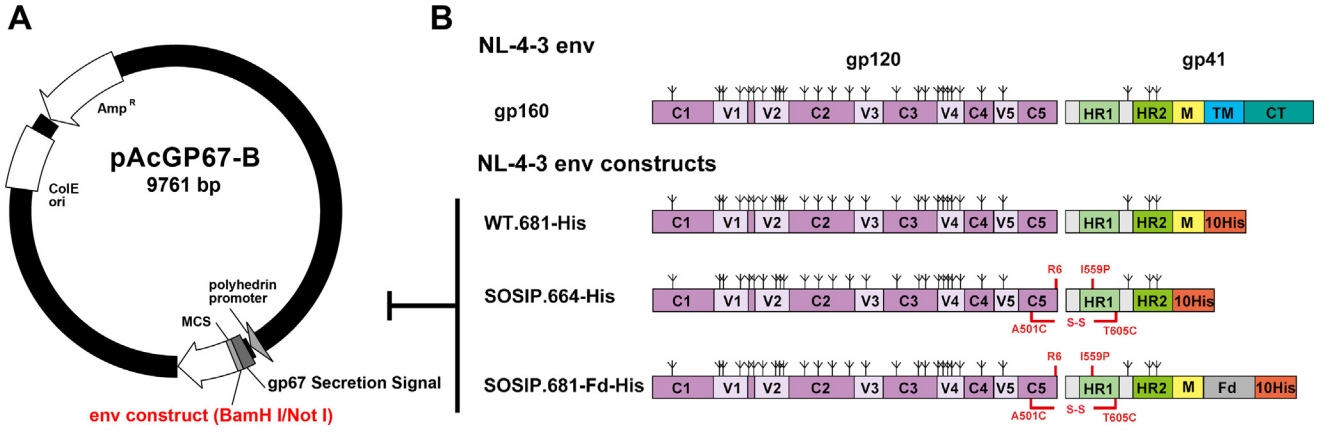


Fig. 1. Schematic map of the HIV-1 NL4-3 constructs compared with full-length gp160. (A) The transfer vector was based on pAcgp67B, which contains the gp67 signal sequence. Target genes were inserted into multiple cloning sites between BamH I and Not I. (B) Linear representations of three NL4-3 gp140 constructs used in this study. The constant (C1-C5) and variable (V1-V5) regions in gp120; the heptad repeat (HR1, HR2); and the membrane-proximal external (M), transmembrane (TM), cytoplasmic (CT) domains in gp41 are indicated. The SOSIP mutations, including I559P, A501C, T605C and R6, are shown in red. The Foldon (Fd) domains are shown in grey, with the His-tags in red. The predicted glycans of the NL4-3 Env protein are also shown in sketch map above the sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. Glycan profiling of gp140 proteins

The N-linked glycosylation profiles of purified Env proteins were determined by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) performed on a PA800 Plus system (Beckman Coulter, Fullerton, CA) [43]. Briefly, 1 mg gp140 proteins were denatured using the Fast Glycan Labeling and Analysis Kit (SCIEX, Brea, CA) using a denaturation buffer containing three internal glucose unit (GU) standards DP2, DP3, DP15. The glycan was enzymatically released using PNGase F (NEB) and fluorophore labeling was performed using 8-aminopyrene-1,3,6-trisulfonate (APTS), with excess (unreacted) APTS removed. For sample separations, a 20-cm (effective length) NCHO capillary (30 cm total length, 50 μm diameter) was filled with 1% PEO (MW 900,000; Sigma-Aldrich) solution in 25 mM lithium acetate buffer, pH 4.75, at 25 °C. The separation voltage was 30 kV in reverse polarity mode (cathode at the injection side and anode at the detection side). Samples were injected hydrodynamically at 3 psi (20.68 kPa) for 3 s, and separations were accomplished at an applied field strength of 1000 V/cm. The 32 Karat software package (SCIEX, Brea, CA), including the GU Value Software Component or the database website (https://lendulet.uni-pannon.hu/index.php/gudatabase), was used for data acquisition and automated glycan identification.

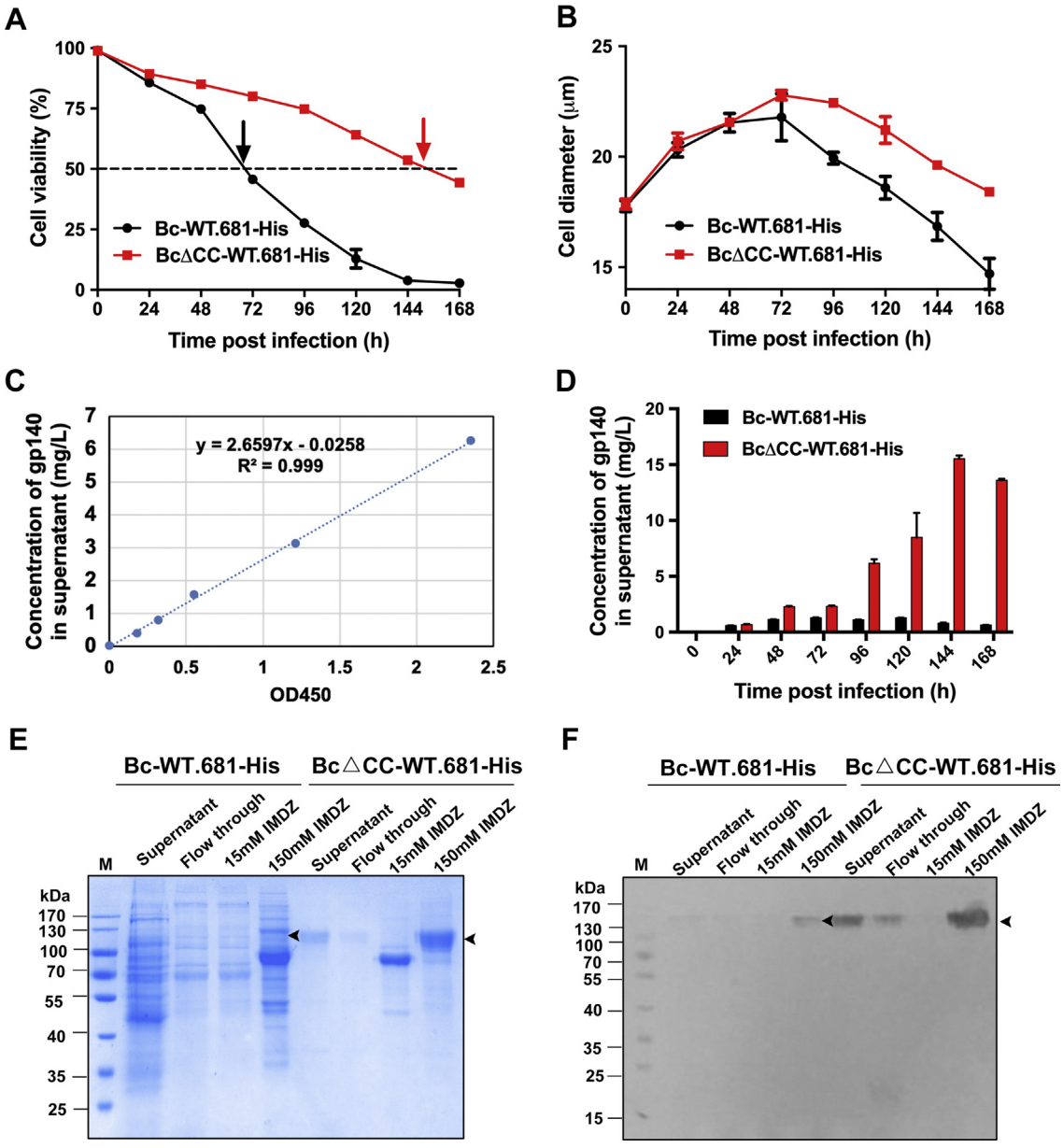


Fig. 2. Expression and purification of Bc-WT.681-His and BcΔCC-WT.681-His proteins. (A, B) Viability and diameter of cultured cells after infection with recombinant baculovirus of Bc-WT.681-His and BcΔCC-WT.681-His. The expression endpoint (arrow) is close to 50% viability. The Bc-WT.681-His cells were harvested at 72 h post-infection, whereas the BcΔCC-WT.681-his cells were harvested at 144 h. (C) Calibration of the quantification of gp140 protein using double-antibodies Sandwich ELISA, 293 cell-derived gp140 served as the standard calibrator. (D) The concentrations of Bc-WT.681-His and BcΔCC-WT.681-His in supernatant were measured every 24 h post infection according to quantification assay established from (C). (E, F) SDS-PAGE and western blotting (WB) analysis of WT.681-His protein purification, detected using HIV-1 linear mouse antibody 3A7. There was low secretion and poor purification (Co-NTA column) of Bc-WT.681-His (E). Expression of BcΔCC-WT.681-His using the *v-cath*/*chiA* knock-out baculovirus led to successful elution of gp140 protein prepared using the same expression and purification conditions.

3. Results

3.1. Improved gp140 production using the modified expression vector

We used the pAcgp67B vector, in which the original gp160 signal sequence is replaced by the gp67 signal sequence (Fig. 1A). The Env constructs were designed according to the HIV-1 strain pNL4-3 (clade B) *env* gene sequence (Fig. 1B). The unmodified gp140, designated WT.681-His, included the C-terminal region of the gp41 ectodomain ($gp41_{ECTO}$) and a 10-his tag for purification (Fig. 1B). The SOSIP.664-His construct was designed according to a previous study [20,44] but with a longer, 10-His tag. A third construct, designated SOSIP.681-Fd-His, contained the MPER moiety of gp160 and the Foldon sequence from T4 phage to stabilize trimerization.

Garnered from the previously reported low expression of Env in insect cells [35], we first tried to optimize gp140 yield. The WT.681-His construct plasmid was co-transfected with classical baculovirus DNA or without *v-cath/chiA* gene baculovirus DNA, referred to as Bc-WT.681-His and BcΔCC-WT.681-His, respectively. The amplified recombinant viruses were used to infect Hive Five cells. During the expression process, we used insect cell diameter expansion to mark infection efficiency, and monitored culture cell viability and diameter after infection every 24 h (Fig. 2A, B). We also monitored the gp140 contents every 24 h during the cell culture by a double-antibody sandwich ELISA with 293 cell-derived gp140 as a quantification calibrator. The results demonstrated that the production yields of gp140 generated in both expression vectors had a peak value at 72 hpi and 144 hpi time points for Bc-WT.681-His and BcΔCC-WT.681-His, respectively, and the cell viabilities of both cell culture were ~50% (Fig. 2C, D). At 72 h, BcΔCC-WT.681-His showed only approximately two fold of yield than that of Bc-WT.681-His (2.2 vs. 1.2 mg/L), and the yield further dramatically increased to the peak value of 15.8 mg/L at 144 h, as comparison, Bc-WT.681-His maintained at low yield level, which corroborated the extended expression time up to 50% cell viability benefits for expression efficiency in the *v-cath/chiA* gene knock-out system (Fig. 2D). In sum, the *v-cath/chiA* gene knock-out system confers 15-fold expression yield than that of the classical system in the case of HIV Env gp140. (Fig. 2A).

Soluble Env proteins were purified by metal affinity chromatography using Co-NTA. The eluted fractions were analyzed by SDS-PAGE, with purified WT.681-His gp140 mainly eluting at ~120 kDa in 150 mM imidazole (Fig. 2E, F). Western blotting indicated a much higher expression of BcΔCC-WT.681-His in the supernatant compared with Bc-WT.681-His (Fig. 2E, F). As shown in Fig. 2E, the amount of secreted Bc-WT.681-His in the supernatant was too low to obtain purified gp140 proteins (0.1 mg/L of protein concentration and only ~10% purity). Remarkably, we obtained approximately 13–15 mg BcΔCC-WT.681-His proteins per liter of culture medium, with about 90% purity after purification (Fig. 2E). These results demonstrated that deletion of the *v-cath/chiA* genes in the modified baculovirus could effectively prolong cell viability to promote the secretion, expression, and purification of soluble gp140 proteins.

Using the same protocol, BcΔCC-SOSIP.664-His and BcΔCC-SOSIP.681-Fd-His recombinant baculoviruses were constructed and produced in insect cells. The viability and diameter of the cultured cells were monitored and used to determine the harvest time

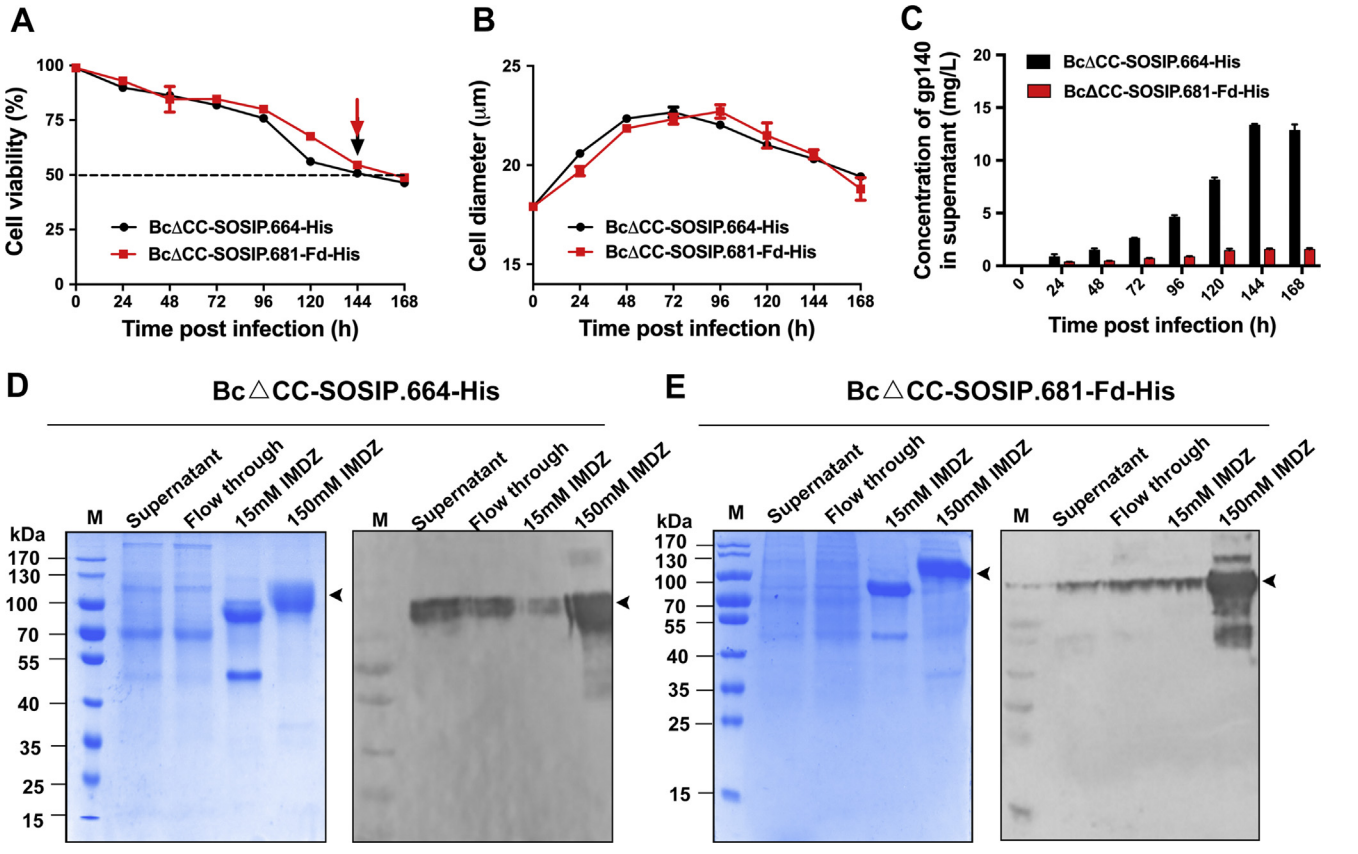


Fig. 3. Expression and purification of BcΔCC-SOSIP.664-His and BcΔCC-SOSIP.681-Fd-His proteins. (A, B) Viability and diameter of cultured cells after infection with recombinant baculovirus of Bc-WT.681-His and BcΔCC-WT.681-His. The expression endpoint (arrow) is close to 50% viability. (C) The concentration of BcΔCC-SOSIP.664-His and BcΔCC-SOSIP.681-Fd-His in supernatant were measured every 24 h post infection according to quantification assay established from Fig. 2C. (D, E) SDS-PAGE and western blotting (WB) analysis of BcΔCC-SOSIP.664-His and BcΔCC-SOSIP.681-Fd-His protein purification, detected using HIV-1 linear mouse antibody 3A7.

point (Fig. 3A, B). The Sandwich ELISA showed that the production yields of gp140 generated in konck-out *v-cath/chiA* genes vectors had a peak value both at about 144 hpi time points for BcΔCC-SOSIP.664-His and BcΔCC-SOSIP.681-Fd-His (Fig. 3C). The purified BcΔCC-SOSIP.664-His and BcΔCC-SOSIP.681-Fd-His proteins were analyzed by SDS-PAGE and western blot, which showed high purity in the eluted fractions (Fig. 3D, E), with 8–10 mg of BcΔCC-SOSIP.664-His and 2–4 mg of BcΔCC-SOSIP.681-Fd-His proteins obtained from the 1 L culture medium.

3.2. Trimerization of g140

Stable trimerization of Env is essential for eliciting NAbs [1]. Therefore, we next sought to characterize the trimerization of gp140 proteins purified from the insect cell system. High pressure size-exclusion chromatography (HPSEC) and sedimentation velocity analytical ultracentrifugation (SV-AUC) analyses were used to further evaluate oligomerization of the purified gp140 proteins in solution against standard molecular weight markers. HPSEC profiles showed a trimer fraction about 72% for SOSIP.681-Fd-His (Fig. 4A). However, WT.681-His and SOSIP.664-His do not show defined peaks for trimers, as the absorbance at the corresponded elution time might include the absorbance tail from the aggregates. In the SV-AUC analysis, the three purified gp140 samples presented with a more defined ratio of trimers (~10S, equated to ~410 kD) in solution: 42% for WT.681-His, 42% for SOSIP.664-His, and 61% for SOSIP.681-Fd-His (Fig. 4B). There appeared to be more trimer for gp140 from SOSIP.681-Fd-His than the other two samples; we surmise that Foldon and MPER may help to facilitate this better trimerization. Collectively, SOSIP.681-Fd-His mainly exists as a trimer in solutions, and this may be ascribed to the involvement of MPER and Foldon in the construct design (Fig. 1B).

3.3. Glycan composition of gp140

To date, the glycan composition of the glycosylated gp140 protein from insect cells has not been elucidated. We used capillary electrophoresis to analyze fluorescently labeled, N-linked glycans cleaved from gp140 proteins, as described in other studies [43,45]. In this assay, three peaks ascribable to internal reference materials, DP2, DP3 and DP15, were used to calibrate migration time (MT) of the glycan peaks in question, which were, in turn, aligned to the MT profile in a glycan composition database for glycosylation analysis. Overall, the major N-glycan type post-translationally modified in the insect cell contained both fucosylated and nonfucosylated paucimannose structures [Man_x-$GlcNAc_2$(±Fuc)] (Fig. 5), with a diverse glycosylation profile detected among the different constructs: 42% of glycans had

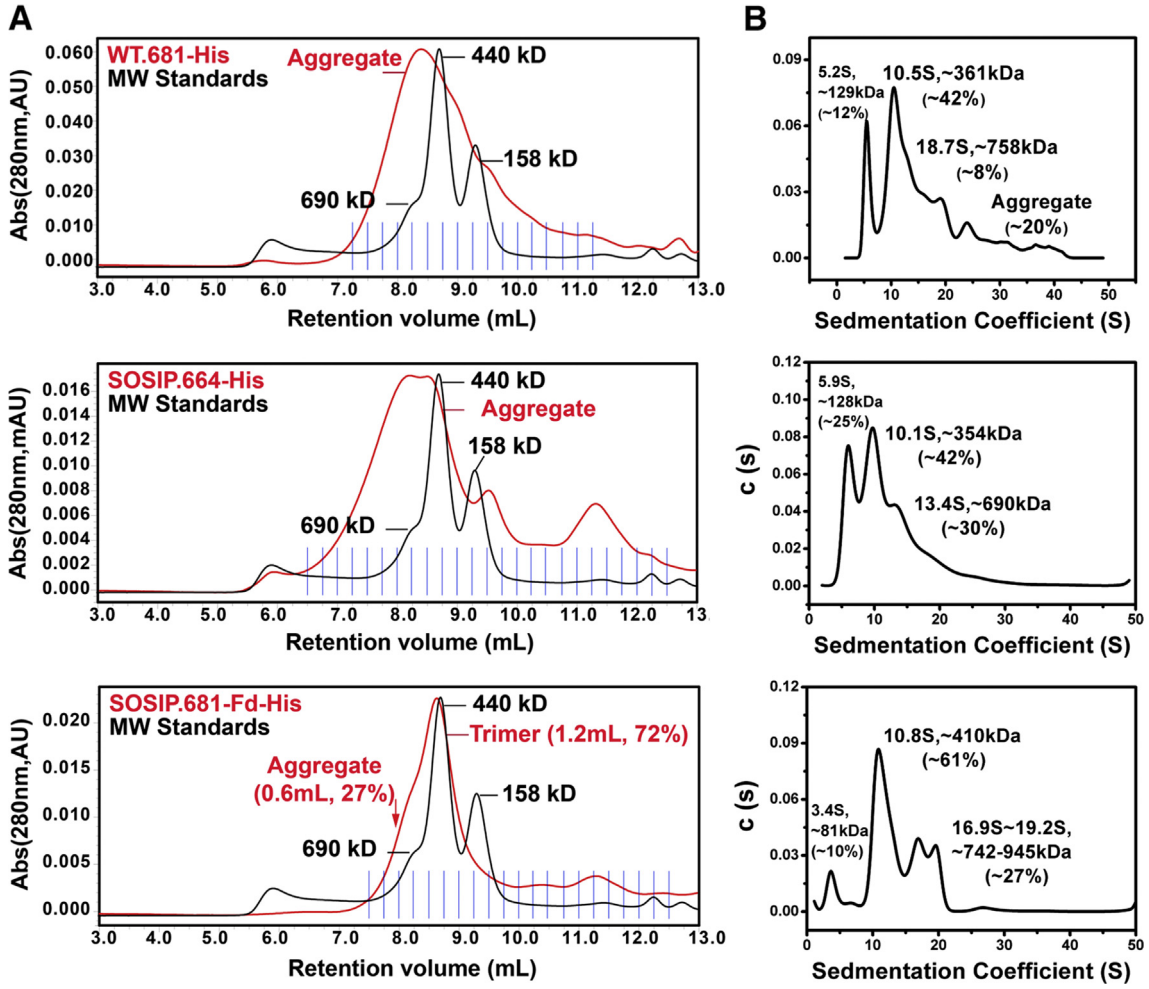
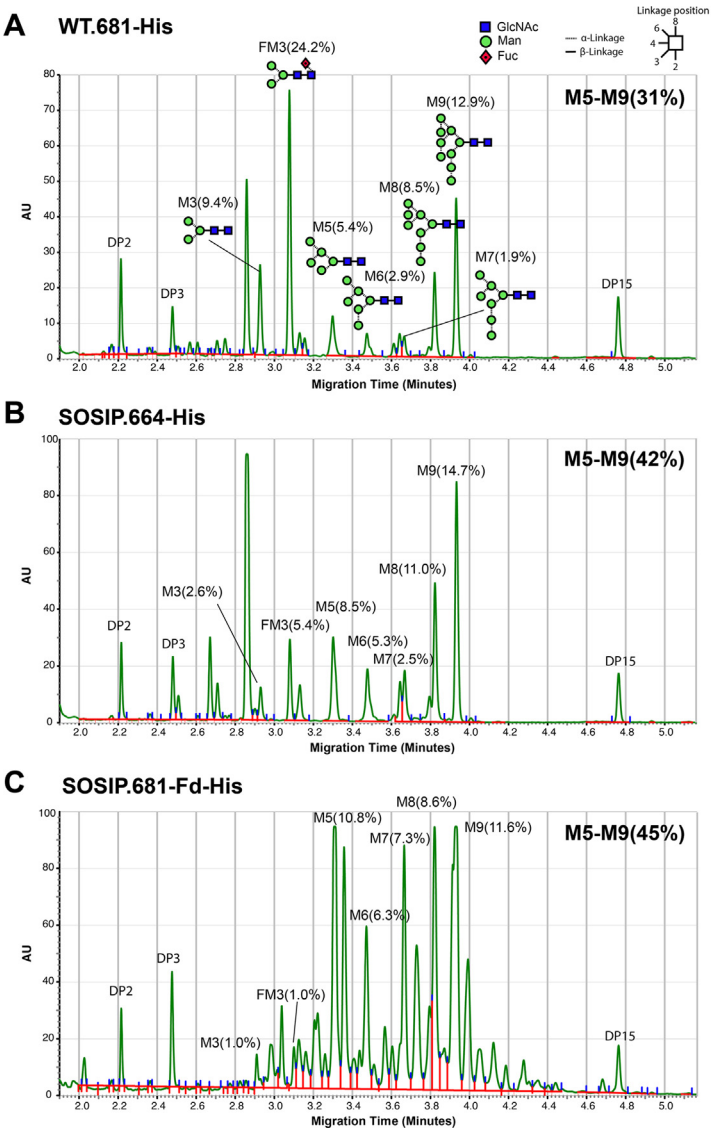


Fig. 4. Characterization of gp140 proteins. (A) HPSEC profiles of the purified WT.681-His, SOSIP.664-His, SOSIP.681-Fd-His. The black curves represent the elution profile of the molecular weight calibration standards. The percentage of aggregates, trimers, monomers or others in the fractions are indicated. (B) Sedimentation velocity (SV) analysis of the gp140 proteins. The main peak showed the component with a sedimentation coefficient (S) of ~10.5S, ~360–410 kDa, corresponding to trimer formation. Tetramers or aggregates showed a higher sedimentation coefficient (S) of 13.4S or higher.



Glycan compositions

Migration Time(Min)	Glucose Unit (GU)	Proposed glycan	GU of proposed glycan	Occupancy%
2.214	2.000	DP2	2.07	0.000
2.478	3.000	DP3	3.00	0.000
2.927	4.944	M3	4.96	9.348
3.077	5.686	FM3	5.69	24.161
3.298	6.849	M5	6.91	5.467
3.474	7.835	M6	7.81	2.981
3.642	8.787	M7[D3]	8.74	1.862
3.821	9.761	M8[D1D3]	9.73	8.446
3.931	10.357	M9	10.35	12.940
4.764	15.000	DP15	15.00	0.000

Migration Time(Min)	Glucose Unit (GU)	Proposed glycan	GU of proposed glycan	Occupancy%
2.216	2.000	DP2	2.07	0.000
2.480	3.000	DP3	3.00	0.000
2.930	4.944	M3	4.96	2.625
3.080	5.686	FM3	5.69	5.415
3.301	6.849	M5	6.91	8.490
3.477	7.835	M6	7.81	5.327
3.644	8.787	M7[D3]	8.74	2.541
3.823	9.761	M8[D1D3]	9.73	11.100
3.933	10.357	M9	10.35	14.704
4.764	15.000	DP15	15.00	0.000

Migration Time(Min)	Glucose Unit (GU)	Proposed glycan	GU of proposed glycan	Occupancy%
2.216	2.000	DP2	2.07	0.000
2.476	3.000	DP3	3.00	0.000
2.910	4.944	M3	4.96	1.001
3.039	5.686	FM3	5.69	1.012
3.313	6.849	M5	6.91	10.381
3.474	7.835	M6	7.81	6.322
3.666	8.787	M7[D3]	8.74	7.321
3.821	9.761	M8[D1D3]	9.73	8.598
3.933	10.357	M9	10.35	11.602
4.764	15.000	DP15	15.00	0.000

Fig. 5. Glycan composition of gp140 proteins. Capillary electrophoresis (CE) analysis of fluorescently labeled N-linked glycans released from WT.681-His, SOSIP.664-His, and SOSIP.681-Fd-His proteins. The gp140 proteins were treated with PNGase F to release glycans. Peaks corresponding to oligomannose glycans are labeled, and the ratios in these peak areas (as a percentage of total glycans) were calculated to obtain a quantitative estimation of glycan content in these three proteins. Glycan structures are depicted according to the color scheme, and bound to the Consortium for Functional Genomics.

high-mannose content ($Man_{5-9}GlcaNAc_2$) in SOSIP.664-His, 45% in SOSIP.681-Fd-His, and 31% in WT.681-His; whereas 8%, 2%, and 33%, respectively, had a total occupancy of FM3 ($Man_3GlcNAc_2Fuc$) and M3 ($Man_3GlcNAc_2$) (Fig. 5). These variations in glycosylation between the wild type and modified gp140 constructs might be associated with steric restrictions for glycosylase processing after gp140 translation in the insect cell; the closed native-like env spike show more high oligomannose population, a similar phenomenon has been identified for mature env spike in mammalian cells, where the SOSIP gp140 design modulates glycan processing to resemble the native-like glycosylation [15,46].

3.4. Antigenicity and immunogenicity of gp140

We employed three representative neutralizing human mAbs, VRC01, VRC03, and b12, which target the CD4 binding site (CD4bs) and cannot discriminate the oligomeric states of Env protein [25], and another neutralizing mouse mAb, 10G6, which recognizes a region involving the V3 loop without knowledge of glycan binding relatedness, to quantify the binding activity of the purified gp140 proteins, expressed in terms of the as 50% maximal effective concentration (EC_{50}) using ELISA. The SOSIP.664-Fd-His_293 produced in 293 cells in our previous study [41] served as a control. SOSIP.664-His showed the highest antigenicity with the three CD4bs mAbs, as indicated by the lowest EC_{50} values of 6.2, 42.7, and 0.87 ng/mL for VRC01, VRC03, and b12, respectively (Fig. 5A). In contrast, WT.681-His, SOSIP.664-His, and SOSIP.664-Fd_293 showed nearly equal binding activity to the mouse mAb 10G6, whereas SOSIP.681-Fd-his showed significantly lower reactivity, with a 2-log increase in the EC_{50}. These reaction profiles suggested variation in the antigenicity of gp140 from different sources, which, to some extent, may be due to the different glycosylation patterns and construct design. Collectively, this highlights immunogen design as a critical aspect of HIV vaccine development.

We next evaluated the immunogenicity of gp140 proteins in BALB/c mice. Three groups of BALB/c mice were immunized with WT.681-His, SOSIP.664-His, or SOSIP.681-Fd-His proteins, formulated with Freund's adjuvant at weeks 0, 2, and 4. The Env-

specific antibody titers of the three gp140 constructs were assessed by ELISA. As shown in Fig. 5B, the antibody response profiles indicated that all three gp140 proteins elicited high antibody titers of approximately 6-log. Notably, the serum from the mice immunized with SOSIP.681-Fd-His showed significantly better reactivity against the three gp140 proteins than those immunized with WT.681-His or SOSIP.664-His.

Finally, we assessed the neutralizing antibody responses elicited by the three gp140 proteins in a classical neutralization assay [47] using two HIV-1 strains, NL4-3 (clade B) and 94 (clade D). As shown in Fig. 6C, the serum samples from the three proteins at 6-weeks post-immunization still had neutralizing activities (>50% HIV inhibition rate) against the two viruses at 20-fold dilution, indicating that the gp140 proteins produced in insect cells con-

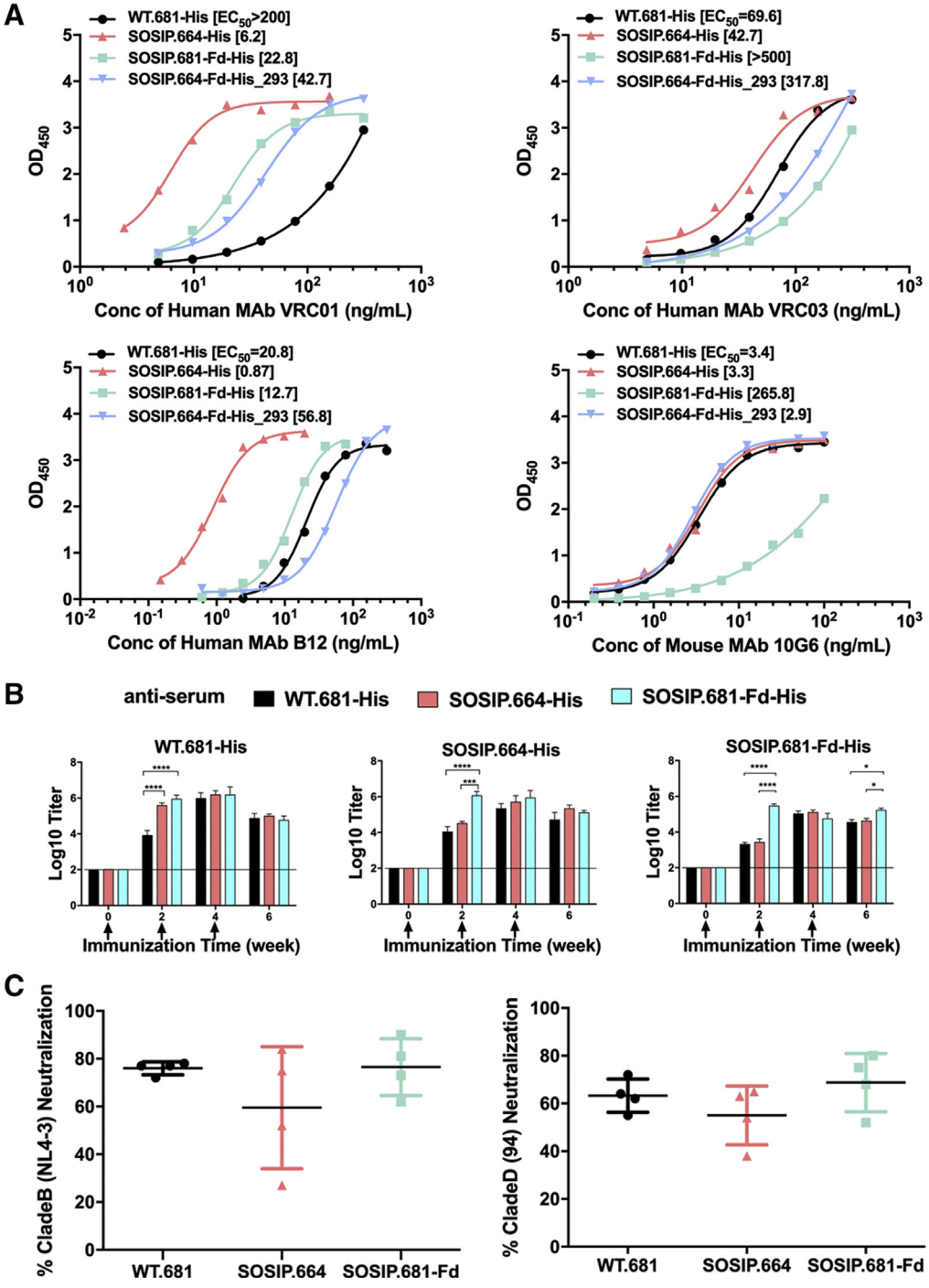


Fig. 6. Antigenicity and immunogenicity of gp140 proteins. (A) The reactivity of gp140 proteins with four mAbs (VRC01, VRC03, b12 and 10G6) tested by ELISA. mAbs were serial diluted and reacted with coated gp140 proteins, with the reaction detected using HRP-labeled anti-human antibody. The EC_{50} was calculated by sigmoid trend fitting using GraphPad Prism software. (B) Antibody titers from the immunized serum were tested by ELISA. Data at each time point is presented as geometric mean titers with standard deviations. The horizontal lines represent the cutoff value of ELISA test. All the data were analyzed by one-way analysis of variance (ANOVA), *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. (C) Neutralization assay of serum against two HIV-1 strains, representing clade B and clade D. The neutralization percentage was plotted as 20 dilutions of immunized serum.

ferred good immunogenicity of the neutralizing antibody in BALB/c mice.

4. Discussion

The production of a stable and homogeneous HIV-1 native-like Env trimer is crucial in the development of a HIV vaccine candidate. The previously reported SIV or HIV Env proteins [35,36] were constructed mainly using conventional Bac-to-Bac baculovirus vector systems, which lack *polh* but have an otherwise complete genome. The baculovirus expression system is cost-effective and easy to optimize for the deletion and insertion of the target gene. In this study, we employed the BestBac system, different from the Bac-to-Bac system in that it lacks the *v-cath/chiA* genes.

There is a difference in the amount of proteins being present in the supernatant between the four constructs. To our knowledge, the reasons for the substantial differences among the four constructs in our study would be: (1) In the case of Bc-WT.681-His vs. BcΔCC-WT.681-His, longer time sustained till the viability dropping to 50% for the latter (72 hpi vs. 144 hpi, Fig. 2B) may allow the protein to be expressed in longer time and lead to higher yield eventually. Furthermore, nearly few Bc-WT.681-His exists at the supernatant of the culture, which might due to undergo a degradation at the presence of cathepsin, a serine protease encoded by the v-cath gene of classical baculovirus [38]. (2) In spite of using the same virus backbone, we surmised that the different expression outcome may ensue from distinct construct design, i.e. BcΔCC-WT.681-His containing MPER region, SOSIP.664-His bearing several point mutations but no MPER and SOSIP.681-Fd-His including a Foldon sequence in addition to point mutations and MPER (Fig. 1B). Different construct design facilitating higher protein expression has been often seen in the literature [15]. The results suggested that the BestBac system enhanced Env production with a 100-fold increase in purified gp140 yield. This increase in yield is presumably because deletion of the *v-cath/chiA* genes prolongs cell viability, thereby increasing the time for gp140 protein accumulation in the culture medium. Deletion of v-*cath/chiA* removes a potentially detrimental protease and may reduce the competition for resources for protein synthesis during late gene expression, thereby improving the integrity of the secreted gp140 proteins and enhancing yield. Of note, another study has reported that the deletion of other non-essential genes, such as p10, p26 and p74, can further increase the yield in recombinant expression [48]. Thus, we could further optimize Env production by applying several strategies in future projects.

In the literature, Ni-NTA and affinity chromatography have been used for the successful purification of gp140 trimers from mammalian cells using either the 2G12 column or a Galanthus Nivalis (GN) lectin column, followed by size-exclusion chromatography [49,50]. However, we found that neither Ni-NTA nor the GN lectin column was suitable for the purification of gp140 proteins (data not shown), but that Co-NTA gave gp140 proteins of high purity and yield; we hypothesize that this might be associated with the specific affinity of gp140 for the Co cation. The failure of the purification of the WT.681-His might be caused by two-fold factor, one is lower start amount of target protein for purification that may encumber the efficient affinity absorbance under law of mass action, the other is the relatively more contaminants that could non-specifically bind to resin and consequently intervene the gp140 from efficient binding and being eluted, as manifested different elution profiles as compared to that of other constructs (Fig. 2E, 3D and E). As to the construct design, SOSIP.681-Fd-His showed the highest percentage of trimerization, suggesting that both MPER and Foldon enhanced Env trimer assembly.

The glycan composition analysis in our study implicated that all three gp140 protein surfaces were modified with a high proportion of mannose-type glycans. The terminal mannose made in wild-type insect cells is different to that from mammalian cells (with sialyl-glycan) due to a deficiency in an N-acetylglucosaminyltransferase, a galactosyltransferase and a sialyltransferase. In addition to that insect cells are not capable of synthesizing cmp-sialic acid, the substrate of the sialyltransferase [51,52]. A previous study also reported that the non-sialic-acid gp120 immunogens showed higher antigenicity and immunogenicity in terms of having a more solvent-accessible protein surface and electrostatic charge distribution [53]. These results might inspire an alternative strategy to improve the antigenicity of insect cell-derived gp140. We also assessed the immunogenicity of three gp140 proteins produced from insect cells in the BALB/c mice, and our preliminary results indicated a cross-clade neutralizing antibody response. In previous studies, it has been suggested that it is difficult to induce broadly-neutralizing antibodies for gp140 in a mouse model [54–56]. Now, researchers have moved to immune focusing to try and remove unwanted antibody response and increase the protective bnAbs response. To confirm the production of bNAbs seen with the insect cell-derived gp140, future studies should employ different immunization strategies and experimental animal models, such as guinea pigs and rabbits.

5. Conclusions

In conclusion, this study used the modified baculovirus system to promote HIV-1 NL4-3 gp140 protein expression in insect cells. Characterization of these proteins indicated one of three constructs, SOSIP.681-Fd-His, expresses a higher fraction of trimer with high oligomannose population, good antigenicity and good immunogenicity. These results offer insight for the further development of HIV-1 vaccine candidate using insect cells.

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Author Contributions

S.L. and Y.G. designed the study. T.L., Zhe.Z., Zhi.Z., J.Q., R.R., Y.Z., Q.Y., Z.L., H.S., F.H., W.X., S.G., and S.L. performed experiments., T.L., Zhe.Z., Zhi.Z., Q.Z., H.Y., J.Z, Y.G., S.L. and N.X. analysed data. T.L., Zhi.Z., Y.G. and S.L. wrote the manuscript. T.L., Zhe.Z., Zhi.Z., Q.Z., H.Y., J.Z., Y.G., S.L. and N.X. participated in discussion and interpretation of the results. All authors contributed to experimental design.

Competing interest

The authors have declared that no competing interests exist.

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